



**Industria y Comercio**  
**SUPERINTENDENCIA**

**PCT**  
SOLICITUD

**PATENTE DE INVENCION**

**03-38171**

21 EXPEDIENTE Nº

54 TÍTULO

METODO PARA LA PRODUCCION A GRAN ESCALA DE UN tPA QUE SE DERIVA  
DE UN ADN RECOMBINANTE, O DE MOLECULAS DE K2S

51 CLASIFICACIÓN INTERNACIONAL C12N 9/00

71 SOLICITANTE BOEHRINGER INGELHEIM INTERNATIONAL GMBH

DOMICILIO Ingelheim/Rhein, República Federal de Alemania.

74 APODERADO EMILIO FERRERO

Código No. 1092

22 BOGOTÁ, D. C.

QF

**Industria y Comercio**

03 038171 00

SUPERINTENDENCIA

**PETITORIO**

SUPERINTENDENCIA Y COMERCIO

Indicaciones: 0338171 00000000

Clase: 142

Fecha (GTM): 2003-05-07 15:29:54

Título: 011 PCT-PETITOR 372 FACE NACI 411 PRESENTAC

Deposición: 2003 27/11/03 DE NUEVAS CREACIONES

**FORMULARIO UNICO DE SOLICITUD DE PATENTE  
PCT  
ENTRADA EN FASE NACIONAL**

1

**SOLICITUD DE**

Patente de Invención



Patente de Modelo de Utilidad



Capítulo I



Capítulo II

2

**SOLICITANTE  
(71)****Nombre BOEHRINGER INGELHEIM  
INTERNATIONAL GMBH**sociedad constituida y existente bajo las  
leyes de la República Federal de  
AlemaniaDirección 55216 Ingelheim/Rhein,  
República Federal de Alemania

Teléfono 546 09 70

Fax 249 40 70

E-mail [clientes@camyp.com](mailto:clientes@camyp.com)

Domicilio Ingelheim/Rhein,

República Federal de Alemania

**IDENTIFICACION**

C C



NIT



C E



Otro



Cual \_\_\_\_\_

Número \_\_\_\_\_

3

**REPRESENTANTE  
O APODERADO****Nombre EMILIO FERRERO**

Dirección Carrera 4ª No 72-35

Teléfono 347 36 11

Fax 217 92 11

E-mail [clientes@camyp.com](mailto:clientes@camyp.com)

Domicilio Bogotá, Colombia

**IDENTIFICACION**

C C



NIT



C E



Otro



Cual \_\_\_\_\_

Número \_\_\_\_\_

T P. \_\_\_\_\_

4

**INVENTOR (ES)  
(72)**1- Rolf Günther WERNER ✓  
Hugo-Häring-Strasse 72,  
88400 Biberach an der Riss

República Federal Alemania

3- Chatchai TAYAPIWATANA ✓  
369/73 Soi Pradoo 1, Janava Rd ,  
Bangkok 10120,

Tailandia

5- Aranya MANOSROI ✓  
234/171 Siriwatana Nives Mahidol Road,  
Nong Hol, Muang Chiang Mai 50000  
Tailandia2- Friedrich GOETZ ✓  
Belm Herbsstenhof 31,  
72076 Tübingen,  
República Federal de Alemania4- Jiradej MANOSROI ✓  
234/171 Siriwatana Nives Mahidol Road,  
Nong Hol, Muang Chiang Mai 50000,  
Tailandia

5

**PCT (86)**Solicitud Internacional No- PCT/EP01/12857Fecha 7 de noviembre de 2001Publicación Internacional No WO 02/40650 A2Fecha 23 de mayo de 2002

6

**Título (54) METODO PARA LA PRODUCCIÓN A GRAN ESCALA DE UN tPA QUE SE DERIVA  
DE UN ADN RECOMBINANTE, O DE M LÉCULAS DE K2S**

7

**Clasificación Internacional (51) C12N 9/00 C12N 15/62**

|              |                                                                    |                     |                         |                          |
|--------------|--------------------------------------------------------------------|---------------------|-------------------------|--------------------------|
| <div>8</div> | Prioridad                                                          | (33) País de Origen | (32) Fecha              | (31) Número de Solicitud |
|              | SI <input checked="" type="checkbox"/> NO <input type="checkbox"/> | GB                  | 14 de noviembre de 2000 | 0027779 8                |

9

Comprobante de pago No. 03-27.856 Fecha 22 de abril de 2003

10

Anexos:

☒

Comprobante de pago de la tasa de presentación de la solicitud por \$ 400 000 oo

☐

Comprobante de pago por reivindicación de prioridad

☒

Documento que acredita la existencia y representación legal cuando el solicitante sea persona jurídica

☒

Poderes, si fuere el caso☐☐☒☒☒☐☐☐☐☐☐☐

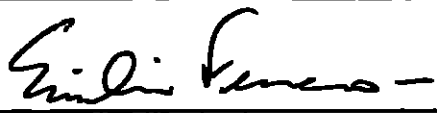
11

FIGURA CARACTERISTICA

12

Solicito la concesión de la patente,

NOMBRE EMILIO FERRERO

FIRMA   
C C 438 019 de Usaquén T P 31 929

For patents, utility models, industrial designs, trademarks, commercial names, trade emblems, slogans.

Para patentes, modelos de utilidad, diseños industriales, marcas de fábrica, nombres comerciales, enseñas, lemas comerciales

REPRESENTACION Y PODER

REPRESENTATION AND  
POWER OF ATTORNEY

9253  
0569

I (we), the undersigned / Yo (nosotros), abajo firmado (s)  
**BOEHRINGER INGELHEIM INTERNATIONAL GMBH**

of/domiciliado (s) en D-55216 Ingelheim am Rhein,  
GERMANY

por el presente nombramos a GERMAN CAVELIER, tpa 832, GONZALO PERDOMO, tpa 831, EMILIO FERRERO, tpa 31929, y LUZ CLEMENCIA DE PAEZ, tpa 23555, domiciliados en la Carrera 4ª Nº 72-35, Bogotá, Colombia, en obediencia a lo dispuesto en el parágrafo 1º del Artículo 543 del Código de Comercio, el primero como principal y los demás como suplentes, en su orden, como mis (nuestros) representantes en todos los asuntos de propiedad industrial con facultad para recibir notificaciones y nombrar apoderados judiciales o extrajudiciales. El primer representante suplente reemplazará al principal en caso de ausencia o impedimento temporal o definitivo de aquél. La misma regla se aplicará cuando el segundo representante suplente haya de reemplazar al primero, o el tercero al segundo. En tales casos, el representante principal, o el primero, o segundo suplente, en su caso, o ambos, declararán su intención de no ejercer la representación mediante declaración autenticada por Notario Público en Colombia, o por Notario u otro Funcionario Público, o Cónsul de Colombia, en el extranjero, y si se tratare de impedimento, con el certificado respectivo. Si alguno de los nombrados faltare definitivamente, el siguiente tomará su lugar. Cuando cesare la ausencia o impedimento del representante principal, o de su primero o segundo suplentes, en su caso, podrán ejercer la representación, en su orden, uno u otros según el caso, asumiéndola por el solo hecho de ejercerla, cesando en ese momento el ejercicio de la representación por el primero, segundo o tercer suplente en su caso.

Además, confiero (conferimos) poder a los abogados arriba nombrados, para obtener la protección de mi (nuestra) propiedad industrial y contra la competencia desleal, a cuyo efecto autorizo (amos) a los dichos apoderados para que me (nos) representen ante cualesquiera entidades o personas, ejerzan o no jurisdicción, especialmente ante las del orden administrativo, contencioso-administrativo y judicial, así como los tribunales de arbitramento, en todo lo concerniente a los siguientes asuntos: a) Obtención de patentes de invención, modelos de utilidad, y diseños industriales, el registro de marcas de fábrica, colectivas, defensivas, de servicio, lemas comerciales, nombres comerciales, enseñas, variedades vegetales y denominaciones de origen, su renovación, traspaso, cambio de nombre o domicilio, cancelación voluntaria, y el registro de licencias de uso de esos derechos; b) Las acciones de competencia desleal, protección del consumidor, oposiciones u observaciones, cancelación administrativa y judicial, nulidad, medidas cautelares, concesión de licencias, la tutela, la protección de secretos industriales, y en general los juicios civiles, penales, administrativos o contencioso-administrativos relacionados con estos derechos, acciones y procesos que promovamos o se nos promuevan. Y para que en todos los asuntos arriba determinados, puedan sustituir este mandato, transigir, conciliar, admitir los hechos del proceso, desistirse, cancelar, recibir, renunciar, y ratificar los actos de agentes oficiosos.

In due compliance with the terms of paragraph 1st of article 543 of the Commercial Code, do hereby appoint GERMAN CAVELIER, tpa 832, GONZALO PERDOMO, tpa 831, EMILIO FERRERO, tpa 31929, and LUZ CLEMENCIA DE PAEZ, tpa 23555, domiciled at Carrera 4ª Nº 72-35, Bogotá, Colombia, the first as principal and the others as alternates, in the order of their appointment, as our representatives for all industrial property matters with faculty to receive notifications and to appoint attorneys in and out of Court. The first alternate shall replace the principal in case of his absence or of temporal or definitive impediment. The same rule will apply when the second alternate representative is to replace the first one, or when the third is to replace the first or second alternates in their turn, or both of them, second. In such cases, the principal representative, or the first or second alternates in their turn, or both of them, shall state their intention not to act as representatives through a declaration given before a Notary Public in Colombia, or before a Notary or another Public Official or a Colombian Consul abroad, and in case of impediment, the appropriate certificate shall suffice. If any of the appointees were to be definitely absent, the following alternate shall take his place. When the absence or impediment of the principal representative, or of the first or second alternate in their turn ceases, the representation can be taken again in their sequence, by the principal, the first or second alternate depending on case, and the mere fact of its exercise will suffice. The exercise of the representation by the first, second or third alternates in their turn shall end at such time.

In addition, we grant a Power of Attorney in favor of the above-named attorneys-at-law so that they may obtain the protection of my (our) industrial property and against the unfair competition, for which purpose I (we) authorize the said attorneys to represent me (us) before any authorities or persons, with or without jurisdiction, specially those administrative, administrative contentious and judicial, as well as before Arbitration Court, in all matters concerning the following: a) Obtainment of patents utility models and industrial designs, the registration of trademarks, collective, defensive and service marks, slogans, commercial names, trade emblems, vegetal varieties and denominations of origin, its renewal, assignment, change of name or domicile, voluntary cancellation, and the registration of licenses of use of those rights; b) Actions of unfair competition, protection of the consumer, oppositions or observations, administrative or court cancellation, nullity, infringement, granting of licenses, the action for writ mandamus, the protection of trade secrets and in general the civil, penal, and administrative suits relating to those rights, actions and suits instituted by me (us) or against me (us). And that in all the above matters they may substitute this Power of Attorney, compromise, conciliate, admit the facts of the case, desist, cancel, receive, resign, and ratify acts done by representatives without a power of attorney.

Given and signed this/Dado y firmado hoy

Ingelheim am Rhein, 12.12.1995

BOEHRINGER INGELHEIM

In/en INTERNATIONAL G.M.B.H.

Dr. Dieter Laudien

Dr. Gerhard Huber

Signature

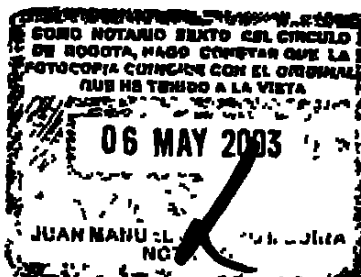
Firma

## LEGALIZACION

El Señor Cónsul de Colombia debe expedir un *certificado consular* sobre (1) la existencia legal de la compañía que otorga el poder, (2) que ésta ejerce su objeto social, y (3) que las personas que otorgan el poder están autorizadas para dárselo. A este efecto les solicitamos que muestren al Señor Cónsul las pruebas que acreditan esos hechos (1), (2) y (3).

## LEGALIZATION

The Colombia Consul must issue a *consular certificate*, on (1) the legal existence of the company which grants Power-of-Attorney, (2) that it is in actual exercise of company purposes, and (3) that the persons who execute the Power of Attorney is authorized to do so. To this effect, please show the Consul the evidence to prove the above facts (1), (2) and (3).



NOTA PARA EL SEÑOR CONSUL DE COLOMBIA Según los artículos 65 del C de P C y 480 del C de Co , si el poderdante es una sociedad, el Señor Cónsul debe certificar que tuvo a la vista las pruebas de su existencia, que ejerce su objeto social y que quien confiere el poder es su representante

due a consider... on 1/1  
company which grants  
it is in actual exercise of  
the persons who execute the  
do so. To this effect please  
note the above facts (1), (2)

24  
230 007  
3

UR Nr. 2659 / 95 V

Vorseitige, vor mir gefertigte Namensunterschriften der Herren

- 1 Dr Dieter L a u d i e n, Dipl -Chemiker, wohnhaft in 55127 Mainz,
- 2 Dr Gerhard H u b e r, Rechtsanwalt, wohnhaft in 55411 Bingen,

- mir bekannt -

beglaubige ich hiermit

Gleichzeitig bescheinige ich aufgrund heutiger Einsichtnahme in das Handelsregister des Amtsgerichts Bingen, daß die Firma Boehringer Ingelheim International GmbH mit Sitz in Ingelheim am Rhein im Handelsregister des Amtsgerichts Bingen unter HR B 1063 eingetragen ist und daß die Herren Dr Laudien und Dr Huber als Prokuristen gemeinschaftlich zur Vertretung der Gesellschaft berechtigt sind

Ingelheim am Rhein, den 12 Dezember 1995/hun

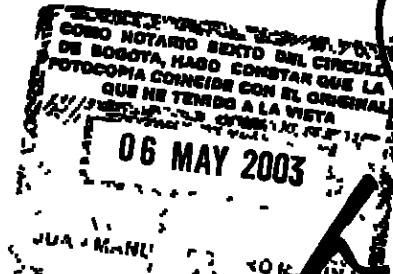


*cc. ...*

Notariatsverweser  
anstelle des Notars  
Prof Dr Weirich in Ingelheim

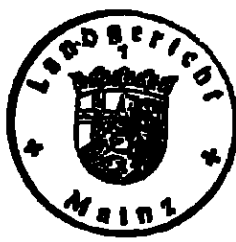


- 910 E 1 -



Die Echtheit vorstehender Unterschrift des Notariatsverwesers Dr. Peter Wirth - Notariatsverweser anstelle des Notars Prof. Dr. Weirich in Ingelheim und die Echtheit des beigedruckten Dienst - ~~scheins~~ - siegels werden hierdurch bestätigt. Zugleich wird bescheinigt, daß der Vorgenannte zur Vornahme der Amlshandlung gesetzlich befugt war.

Kostenberechnung  
Gebühr für Legalisation  
(Wert DM 500,-)  
JV KostO v 28 7 1957  
BGBl I S 661  
DM 30,-  
13. DEZ. 1995  
Mainz, den  
Amtsinspektor



Mainz, den dreizehnten Dezember neunzehnhundertfünfundneunzig  
Der Präsident des Landgerichts

*Dr. Hans Paul Tillerberg*  
(Dr. Hans Paul Tillerberg)



# CONSULADO GENERAL DE COLOMBIA

## MUNICH

Nr. 169

El Cónsul General de Colombia

### C E R T I F I C A

Que el Dr. Hanns Paul Tüttenberg -----, quién autentica la firma del signatario en el presente documento, ejercía en la fecha allí indicada las funciones de Presidente del Tribunal Regional  
-en Mainz.

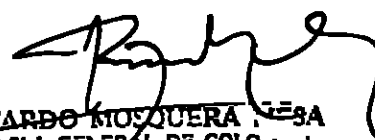
Que la firma BOEHRINGER INGELHEIM INTERNATIONAL GmbH, domiciliada en 55216 Ingelheim am Rhein, Registro Mercantil Nr. HR B 1063 del Juzgado Municipal de Bingen -----  
está constituida y ejerce su objeto social de conformidad con las leyes de la República Federal de Alemania.

Que los señores Dr. DIETER LAUDIN y Dr. GERHARD HUBER -----  
-----  
-----

quien (es) firman el presente documento tiene (n) facultad legal suficiente para representar a la mencionada firma y otorgar poderes en su nombre.

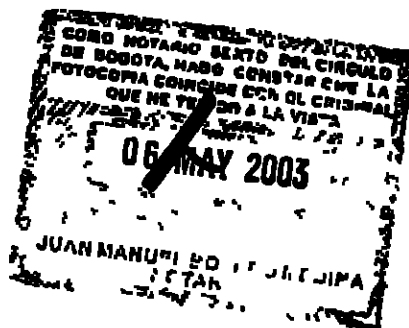
Que ha tenido a la vista la documentación que prueba lo anterior.

Munich, 18 de Diciembre de 1995

  
RICARDO MOSQUERA MESA  
CONSUL GENERAL DE COLOMBIA



|                         |              |                  |
|-------------------------|--------------|------------------|
| Derechos para la Nación | US \$        | 6.-              |
| Fondo Rotatorio         | US \$        | 100.-            |
| Tasa consular           | US \$ 1 x DM | 1,60 = DM 169.60 |





INSTRUMENTO DE RELACIONES  
SECRETARÍA

*P. Medina*

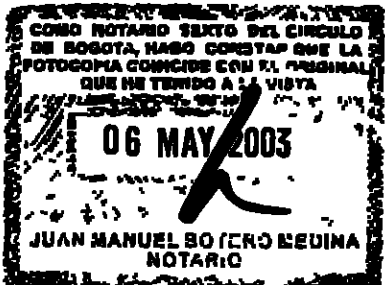
*Medina*

41 SE - 15  
SECRETARÍA

1998-11-11 A. 11 411

SECRETARÍA

7-004 5133 A. 11 411



7

TRADUCCION OFICIAL No. 5133

de un documento escrito en Alemán, el cual para su identificación se sella con el sello de la traductora, lo mismo que la presente.-

-----

(A continuación del poder conferido a GERMAN CAVELIER, tpa. 332; GONZALO PERDOMO, tpa. 831; EMILIO FERRERO tpa, 31929; y LUZ CLEMENCIA DE PAEZ, tpa 23555; domiciliados en la Carrera 4 No. 72-35, Bogotá, Colombia, por la BOEHRINGER INGELHEIM INTERNATIONAL GMBH, domiciliada en D-55216 Ingelheim am Rhein, RFA, a través de sus representantes los Srs. Dr. Dieter Laudien y Dr. Gerhard Huber:)

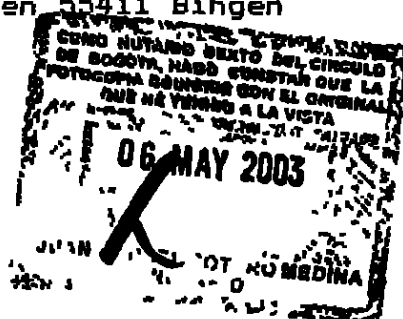
Legalización

Protocolo notarial No. 2659/95 V

Certifico la autenticidad de las firmas antecedentes puestas ante mí por los señores:

1. Dr. Dieter Laudien, Dipl. Chim., domiciliado en 55127 Mainz,
2. Dr. Gerhard Huber, abogado, domiciliado en 55411 Bingen

a quienes conozco en persona.



Igualmente certifico, por haber examinado en la fecha el Registro de Comercio del Juzgado Municipal de Bingen, HR B 1063, que la firma Boehringer Ingelheim International GmbH, domiciliada en Ingelheim am Rhein, está inscrita bajo el No. HK B 1063 en el Registro de Comercio del Juzgado Municipal de Bingen y que los señores Dr. Laudien y Dr. Huber, en su calidad de apoderados, están facultados para representar conjuntamente a la sociedad.

Ingelheim am Rhein, 12 de diciembre de 1995/hun

Fdo.: firma ilegible

(Hilo y oblea roja en relieve:)

El notario suplente

El notario suplente - Ingelheim

del notario de Ingelheim

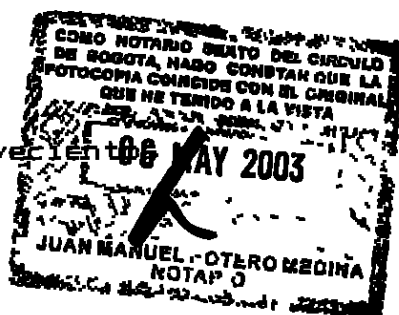
Prof. Dr. Weirich

(dos sellos redondos en tinta del Tribunal Regional de Mainz)

910 E 1

Se certifica la autenticidad de la firma antecedente del Dr. Peter Wirth, como notario suplente del Profr. Dr. Weirich, notario de Ingelheim, y la del sello oficial que la acompaña. Igualmente se certifica que el mencionado notario estaba facultado para realizar ese acto oficial.

Mainz, trece de diciembre de mil novecientos  
noventa y cinco



El Presidente del Tribunal Regional

(E) Fdo.: firma ilegible

(Dr. Hanns Paul Luttenberg)

Costas

Derechos de legalización  
(Valor:DM 5.000,-)  
JVKostO del 26 de julio 1957  
Gacetas Oficial I Pag. 861  
DM 20,--  
Mainz, 13 de diciembre de 1995  
Fdo.: firma ilegible  
Inspector

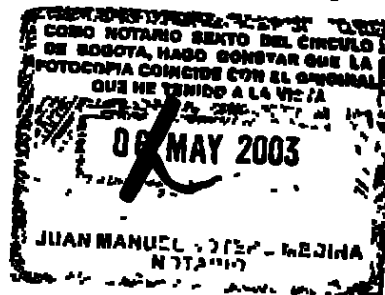
-----  
EN ESPAÑOL: La legalización de las firmas antecedentes por el  
Consulado de Colombia en Munich bajo el No. 169 del 18 de  
diciembre de 1995, y por el Ministerio de Relaciones Exteriores  
de Colombia bajo el No. 051606 del 4 de enero de 1996.

Pagados derechos y Fondo Rotatorio US\$ 106,--

-----  
ES TRADUCCION FIEL Y COMPLETA, de la cual se deja copia en los  
archivos de estas oficinas para su confrontación.

Santafé de Bogotá, 6 de enero de 1996.

*[Firma manuscrita]*



COMO NOTARIO SEXTO ENCARGADO DE SANTAFE  
DE BOGOTA HAGO CONSTAR QUE LA FIRMA DE

*Juan Carlos*  
*de la Torre*

C. C. \_\_\_\_\_  
CONCIDE CON LA REGISTRADA POR EL (LLENAR)  
EN ESTA NOTARIA

09 ENE 1996

SANTAFE DE BOGOTA, D.C.



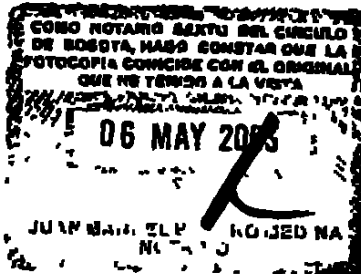
NO SE ASUME  
RESPONSABILIDAD DEL TEXTO

1496 ENE 10 A 11 33

JEFE DE AGENCIACIONES  
SANTAFE DE BOGOTA D.C.

MINISTERIO DE  
EXTERIORES

*U. G. 1911*  
*Prin*  
CITA FIRMADA  
RECIBIDA  
LAS FUNCIONES



TELEFAX (57) (1) 32-4850  
TELE 43479 CAVE CO  
1231740 7325 CAVE UC

**CAVELIER**  
ABOGADOS  
EDIFICIO SISKI  
CARRERA 4ª No 72 35  
BOGOTA 8 COLOMBIA

10  
CORREO ELECTRONICO MUNDIAL CAVE  
TELEFONO (57) (1) 2170100  
CABLES CAVELEYES

Señor Jefe de la  
DIVISION DE NUEVAS CREACIONES  
Superintendencia de Industria y Comercio  
E. S D

Poderdante: BOEHRINGER INGELHEIM INTERNATIONAL GMBH

Poder conferido el 12-12-95

Yo, GERMAN CAVELIER, obrando en mi calidad de apoderado principal del poderdante arriba mencionado atentamente manifiesto a Usted que sustituyo el poder que me ha sido conferido en la persona del doctor EMILIO FERRERO, Abogado con Tarjeta Profesional No 31 929, con todas las facultades que me fueron conferidas

Hago la anterior sustitución conforme al artículo 66 del Código de Procedimiento Civil ya que los otros sustitutos faltan en este momento.

Señor Jefe,

GERMAN CAVELIER

T P A No 832

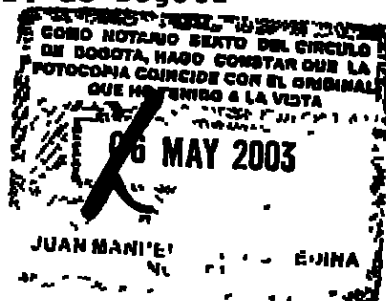
C C. No 2 877.924 de Bogotá

Acepto

EMILIO FERRERO

T P A No 31 929

C C No. 438 019 de Usaquén



2e-780-2

NOTARIA SEXTA DE BOGOTÁ

RECONOCIMIENTO Y PRESENTACION PERSONAL

Bogotá, D. C. 11 ENE 1996

Ante M. PABLO XIENDEZ BAZUJAS

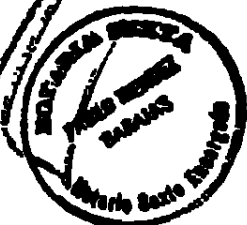
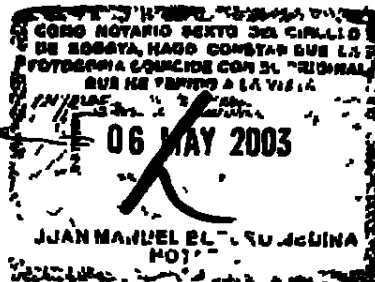
NOTARIO SEXTO ENCARGADO DEL CIRCULO DE BOGOTÁ.

Comparec.ó(eron) \_\_\_\_\_

quien(es) compareció(n) a, (n) \_\_\_\_\_

y la(s) Licrada(s) (n) \_\_\_\_\_

domicilada(s) en Bogotá, declararon que la(s) firma(s) que  
aparecen(n) en el presente documento es(son) la(s) suya(s) y  
que el contenido es cierto  
En constancia se firmó esta Declaración.



(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
23 May 2002 (23 05 2002)

PCT

(10) International Publication Number  
WO 02/40650 A2

- (31) International Patent Classification<sup>7</sup>. C12N 9/00 Aranya [TH/TH], 234/171 Siwatana Nives Mahadol Road, Nong Hoi, Muang Chiang Mai 50000 (TH).
- (21) International Application Number. PCT/TH01/12857
- (22) International Filing Date:  
7 November 2001 (07 11.2001)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data  
0027779 8 14 November 2000 (14 11.2000) GB
- (71) Applicant (for all designated States except US)  
BOEHRINGER INGELHEIM INTERNATIONAL  
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(54) Title METHODS FOR LARGE SCALE PRODUCTION OF RECOMBINANT DNA-DERIVED TPA OR K2S MOLECULES

(57) Abstract: The invention belongs to the field of thrombolysis and of tissue plasminogen activator (tPA) derivative production in prokaryotic cells. The invention relates to methods for the production of a recombinant DNA-derived tPA, a variant thereof or a (Kringle 2 Serine) K2S molecule or a variant thereof in prokaryotic cells, wherein said tPA or K2S or variant is secreted extracellularly as an active and correctly folded protein, and the prokaryotic cell contains and expresses a vector comprising the DNA coding for said tPA or K2S or variant operably linked to the DNA coding for the signal peptide OmpA. The invention further relates to specific K2S derivatives obtainable by said method. The invention further relates to said DNA molecules and the use of said DNA molecules in said methods.

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## Methods for large scale production of recombinant DNA-derived tPA or K2S molecules

The invention belongs to the field of thrombolysis and of tissue plasminogen activator (tPA) derivative production in prokaryotic cells.

The invention relates to methods for the production of a recombinant DNA-derived tPA, a variant thereof or a (Kringle 2 Serine) K2S molecule or a variant thereof in prokaryotic cells, wherein said tPA or K2S or variant is secreted extracellularly as an active and correctly folded protein, and the prokaryotic cell contains and expresses a vector comprising the DNA coding for said tPA or K2S or variant operably linked to the DNA coding for the signal peptide OmpA. The invention further relates to specific K2S derivatives obtainable by said method. The invention further relates to said DNA molecules and the use of said DNA molecules in said methods.

### Background art

Tissue plasminogen activator (tPA) is a polypeptide containing 527 amino acid residues (27) with a molecular mass of 72 kDa. The molecule is divided into five structural domains. Nearby the N-terminal region is a looped finger domain, which is followed by a growth factor domain. Two similar domains, kringle 1 and kringle 2, are following. Both finger and kringle 2 domains bind specifically to the fibrin clots thereby accelerating tPA protein activation of bound plasminogen. Downstream of kringle 2 is the serine protease, with its catalytic site located at the C-terminus. The serine protease is responsible for converting plasminogen to plasmin, a reaction important in the homeostasis of fibrin formation and clot dissolution. The correct folding of tPA requires the correct pairing of 17 disulfide bridges in the molecule (1).

Clinically, tPA is a thrombolytic agent of choice for the treatment of acute myocardial infarction, pulmonary embolism, stroke, peripheral arterial occlusions, and other thromboembolic diseases. It has the advantage of causing no side effects on systemic haemorrhaging and fibrinogen depletion (7). Bowes melanoma cells were first used as a source in tPA production for therapeutic purposes (12). Since a consistent process with efficient production of highly purified protein in good yield is required for clinical use, the construction of full-length recombinant-tPA (r-tPA) progressed to mammalian cells. Chinese hamster ovary cells were transfected with the tPA gene to synthesize the r-tPA (8, 22). The recombinant DNA derived product produced by a mammalian cell culture fermentation system is harvested and purified from the culture medium.

Attracted by simplicity and economy of production, a number of efforts in producing r-tPA from microorganisms, especially bacteria, and more especially from *Escherichia coli*, were investigated (10, 13, 30). Regarding the low yield and the formation of inclusion bodies, which resulted in misfolding and in an inactive enzyme, numerous strategies have been proposed to overcome these problems.

Several deletion-mutant variants including kringle 2 plus serine protease (K2S) were considered. However, the enzymatic activity of the recombinant-K2S (r-K2S) was obtained only when refolding processes of purified inclusion bodies from cytoplasmic compartment were achieved (16, 29). In order to avoid the cumbersome refolding processes, impurities of misfolded proteins, and periplasmic protein delivery, special bacterial expression systems were exploited (6, 31). Despite periplasmic expression of tPA, overexpression led to inactive aggregates, even in the relatively high oxidizing condition in the periplasm.

In the prior art, there are a few descriptions of methods for the preparation of recombinant K2S in *E. coli*. However, there is no disclosure of a method leading to a cost effective method for large scale production of biologically active K2S.

Obukowicz et al (25) expressed and purified r-K2S from periplasmic space. The obvious disadvantage of this method was an extra periplasmic extraction step, which is not suitable for large scale production.

Saito et al (29) disclose the cytoplasmic expression of r-K2S. The authors used an *in vivo* renaturation processes for the expressed r-K2S, which was purified from the cytoplasmic space of *E. coli* as inclusion body. Boehringer Mannheim use a similar cumbersome denaturing/refolding process involving the steps of cell digestion, solubilization under denaturing and reducing conditions and reactivation under oxidizing conditions in the presence of GSH/GSSG which is not cost effective (24) and requires mutation of the amino acid sequence with possibly antigenic potential.

In 1991, Waldenström et al (34) constructed a vector (pBZZK2P) for the secretion of kringle 2 plus serine protease domain to *E. coli* culture supernatant. Hydroxylamine was used to remove the ZZ fusion peptide from IgG-Sepharose purified fraction. The cleavage agent hydroxylamine required modification of the cleavage sites of kringle 2 plus serine protease (Asn<sup>177</sup> → Ser and Asn<sup>144</sup> → Gln) thus to protect it from hydroxylamine digestion. However, the resulting non-native, not properly folded K2S molecule is not suitable for therapeutic purposes. No enzymatic

activity regarding fibrin binding/protease activity was disclosed. The unusual sequence may even activate the human immune system

The problem underlying the present invention was thus to provide a commercially applicable method for large scale production of tPA molecules and derivatives thereof, e.g. K2S, wherein the K2S molecule is secreted in its biologically active form into the culture supernatant.

#### Description of the invention

The problem was solved within the scope of the claims and specification of the present invention

The use of the singular or plural in the claims or specification is in no way intended to be limiting and also includes the other form

The invention relates to a method for the production of a recombinant DNA-derived tissue plasminogen activator (tPA), a tPA variant, a Kringle 2 Serine protease molecule (K2S) or a K2S variant in prokaryotic cells, wherein said tPA, tPA variant, K2S molecule or K2S variant is secreted extracellularly as an active and correctly folded protein, characterized in that the prokaryotic cell contains and expresses a vector comprising the DNA coding for said tPA, tPA variant, K2S molecule or K2S variant operably linked to the DNA coding for the signal peptide OmpA or a functional derivative thereof.

Surprisingly, the use of the signal peptide OmpA alone and/ or in combination with the N-terminal amino acids SEGN (SEQ ID NO:9) / SEGNSD (SEQ ID NO 10) translocate the recombinant DNA-derived tPA, tPA variant, K2S molecule or K2S variant to the outer surface and facilitates the release of the functional and active molecule into the culture medium to a greater extent than any other method in the prior art. Before crossing the outer membrane, the recombinant DNA-derived protein is correctly folded according to the method of the present invention. The signal peptide is cleaved off to produce a mature molecule. Surprisingly, the efficiency of signal peptide removal is very high and leads to correct folding of the recombinant DNA-derived protein.

Said signal peptide OmpA interacts with SecE and is delivered across the inner membrane by energy generated by SecA, which binds to Sec components (SecE-SecY). SecY forms a secretion pore to dispatch the recombinant DNA-derived protein according to the invention. The space between the outer membrane and inner membrane of Gram-negative bacteria, periplasm, has higher oxidative condition in comparison to the cytoplasmic space. This supports the formation

of disulfide bonds and properly folding of the recombinant DNA-derived protein (e.g. K2S) in the periplasm to yield an active molecule. According to the present invention, the signal peptide will be cleaved off to produce a mature molecule. The complex of GspD secretin and GspS lipoprotein on the outer membrane serves as gate channel for secreting the recombinant DNA-derived protein according to the invention to the extracellular medium. This secretion process requires energy, which is generated in cytoplasm by GspE nucleotide-binding protein then transferred to the inner membrane protein (Gsp G-J, F and K-N). GspC transfers the energy to GspD by forming a cross-linker between a set of inner membrane protein (Gsp G-J, F and K-N) and GspD. Before crossing the outer membrane successfully, the recombinant DNA-derived protein is correctly folded.

Operably linked according to the invention means that the DNA encoding the tPA, tPA variant, K2S molecule or K2S variant (preferably comprising the nucleic acid encoding SEGN or SEGNSD at its N-terminal portion) is cloned in close proximity to the OmpA DNA into the vector in order to achieve expression of the OmpA-tPA, tPA variant, K2S molecule or K2S variant-fusion protein and to direct secretion outside the prokaryotic host cell. Typically, the majority of the tPA, tPA variant, K2S molecule or K2S variant is secreted and can then be purified by appropriate methods such as ammonium sulfate precipitation and/or affinity chromatography and further purification steps. The invention also includes the use of inducers such as IPTG or IPTG in combination with glycerol, the improvement of the incubation condition and harvesting period to maximize the amount of active protein.

In a preferred embodiment, said DNA encoding the OmpA signal peptide may be fused to a short peptide characterized by the amino acid sequence SEGN or SEGNSD or the coding nucleic acid sequence TCTGAGGGAAAC (SEQ ID NO 20) or TCTGAGGGAAACAGTGAC (SEQ ID NO 1) and located in the N-terminal portion or at the N-terminal portion of the tPA, tPA variant, K2S molecule or K2S variant. Thus, preferably, said fusion protein comprises OmpA-SEGN-SD-tPA, -tPA-variant, -K2S-molecule or -K2S-variant. Even more preferred, said amino acids characterized by SEGN or SEGNSD may carry a point mutation or may be substituted by a non-natural amino acid. Even more preferred, there may be an amino acid or non-amino acid spacer between OmpA and SEGN or SEGNSD and the tPA, tPA variant, K2S molecule or K2S variant.

Thus, in a preferred method according to the invention said the prokaryotic cell contains and expresses a vector comprising the DNA coding for said tPA, tPA variant, K2S molecule or K2S

variant operably linked to the DNA coding for the signal peptide OmpA which is operably linked to the nucleic acid molecule defined by the sequence TCTGAGGGAAACAGTGAC or a functional derivative thereof.

The method according to the invention comprises prokaryotic host cells such as, but not limited to *Escherichia coli* (*E. coli*), *Bacillus subtilis*, *Streptomyces*, *Pseudomonas*, e.g. *Pseudomonas putida*, *Proteus mirabilis*, *Saccharomyces*, *Pichia* or *Staphylococcus*, e.g. *Staphylococcus carnosus*. Preferably said host cells according to the invention are Gram-negative bacteria.

Preferably, a method according to the invention is also characterised in that the prokaryotic cell is *E. coli*. Suitable strains include, but are not limited to *E. coli* XL-1 blue, BL21(DE3), JM109, DH series, TOP10 and HB101.

Preferably, a method according to the invention is also characterised in that the following steps are carried out

a) the DNA encoding the tPA, tPA variant, K2S molecule or K2S variant is amplified by PCR,

b) the PCR product is purified;

c) said PCR product is inserted into a vector comprising the DNA coding for OmpA signal peptide and the DNA coding for gpIII in such a way that said PCR product is operably linked upstream to the DNA coding for the OmpA signal sequence and linked downstream to the DNA coding for gpIII of said vector;

d) that a stop codon is inserted between said tPA, tPA variant, K2S molecule or K2S variant and gpIII,

e) said vector is expressed by the prokaryotic cell

f) the tPA, tPA variant, K2S molecule or K2S variant is purified.

For step a) according to the invention the choice / design of the primers is important to clone the DNA in the right location and direction of the expression vector (see example 1). Thus, the primers as exemplified in example 1 and figure 4 comprise an important aspect of the present invention. With gp III of step c) gene protein III is meant which is present mainly in phagemid vectors. The stop codon is inserted to avoid transcription of gp III thus eventually leading to secretion of the tPA, tPA variant, K2S molecule or K2S variant of interest. Any suitable method for insertion of the stop codon may be employed such as site-directed mutagenesis (e.g. Weiner MP, Costa GL (1994) PCR Methods Appl 4(3):S131-136, Weiner MP, Costa GL, Schoettlin W, Clme J, Mathur E, Bauer JC (1994) Gene 151(1-2) 119-123; see also example 1).

Any vector may be used in the method according to the invention, preferably said vector is a phagemid vector (see below)

Preferably, a method according to the invention is also characterised in that the tPA, tPA variant, K2S molecule or K2S variant is selected from human tissue plasminogen activator (tPA, figure 16) or a fragment, a functional variant, an allelic variant, a subunit, a chemical derivative, a fusion protein or a glycosylation variant thereof. Such fragments, allelic variants, functional variants, variants based on the degenerative nucleic acid code, fusion proteins with an tPA protein according to the invention, chemical derivatives or a glycosylation variant of the tPA proteins according to the invention may include one, several or all of the following domains or subunits or variants thereof

- 1 Finger domain (4-50)
- 2 Growth factor domain (50-87)
- 3 Kringle 1 domain (87-176)
4. Kringle 2 domain (176-262)
5. Protease domain (276-527)

The numbering/naming of the domains is according to Genbank accession number GI 137119 or Nature 301 (5897), 214-221 (1983)

More preferably, a method according to the invention is also characterised in that the tPA, tPA variant, K2S molecule or K2S variant is selected from the Kringle 2 (4) plus Serine protease (5) K2S variant of human tissue plasminogen activator or a fragment, a functional variant, an allelic variant, a subunit, a chemical derivative, a fusion protein or a glycosylation variant thereof

More preferably, a method according to the invention is also characterised in that the vector is a phagemid vector comprising the DNA coding for OmpA signal peptide and the DNA coding for gpIII.

More preferably, a method according to the invention is also characterised in that the vector is the pComb3HSS phagemid (see also example 1)

More preferably, a method according to the invention is also characterised in that the DNA sequence comprises or consists of the following DNA sequence encoding OmpA and K2S or a functional variant thereof or a variant due to the degenerate nucleotide code

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTGGCC  
CAGGCGGCCTCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTACCG  
TGGCAGGCACAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGAATTCCATGAT  
CCTGATAGGCAAGGTTTACACAGCACAGAACCCCAGTGCCCAGGCACTGGGCCTGG

GCAAACATAATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGTGCCACGTG  
 CTGAAGAACCGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGC  
 GGCCTGAGACAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCTCTTCGCCGA  
 CATCGCCTCCCACCCCTGGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTCGCCCCGG  
 AGAGCGGTTCCTGTGCGGGGGCATACTCATCAGCTCCTGCTGGATTCTCTCTGCCGC  
 CCACTGCTTCCAGGAGAGGTTTCCGCCCCACCACCTGACGGTGATCTTGGGCAGAAC  
 ATACCGGGTGGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGTCGAAAAATACATTG  
 TCCATAAGGAATTCGATGATGACACTTACGACAATGACATTGCGCTGCTGCAGCTGA  
 AATCGGATTCTGTCCTCGTGTGCCAGGAGAGCAGCGTGGTCCGCACTGTGTGCCTTC  
 CCCC GGCGGACCTGCAGCTGCCGGACTGGACGGAGTGTGAGCTCTCCGGCTACGGC  
 AAGCATGAGGCCTTGTCTCCTTTCTATTCTGGAGCGGCTGAAGGAGGCTCATGTCAGA  
 CTGTACCCATCCAGCCGCTGCACATCACAACATTTACTTAACAGAACAGTCACCGAC  
 AACATGCTGTGTGCTGGAGACACTCGGAGCGGCGGGCCCCAGGCCAACTTGCACGA  
 CGCCTGCCAGGGCGATTTCGGGAGGCCCCCTGGTGTGTCTGAACGATGGCCGCATGA  
 CTTTGGTGGGCATCATCAGCTGGGGCCTGGGCTGTGGACAGAAGGATGTCCCGGGT  
 GTGTACACAAAGGTTACCAACTACCTAGACTGGATTCTGTGACAACATGCGACCG  
 (SEQ ID NO 2)

More preferably, a method according to the invention is also characterised in that the DNA  
 Sequence of OmpA comprises or consists of the following sequence or a functional variant  
 thereof or a variant due to the degenerate nucleotide code

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTGGCC  
 CAGGCGGCC (SEQ ID NO 3)

Said DNA encodes the following amino acid sequence of OmpA. OmpA thus comprises or  
 consists of a protein characterized by the following amino acid sequence or a fragment, a  
 functional variant, an allelic variant, a subunit, a chemical derivative or a glycosylation variant  
 thereof as part of the invention

MKKTAIAIAVALAGFATVAQAA (SEQ ID NO 21)

The untranslated region may contain a regulatory element, such as e.g. a transcription initiation  
 unit (promoter) or enhancer. Said promoter may, for example, be a constitutive, inducible or  
 development-controlled promoter. Preferably, without ruling out other known promoters, the  
 constitutive promoters of the human Cytomegalovirus (CMV) and Rous sarcoma virus (RSV),  
 as well as the Simian virus 40 (SV40) and Herpes simplex promoter. Inducible promoters  
 according to the invention comprise antibiotic-resistant promoters, heat-shock promoters,  
 hormone-inducible „Mammary tumour virus promoter“ and the metallothioneine promoter.

Preferred promoters include T3 promoter, T7 promoter, Lac/ara1 and Ltet0-1.

More preferably, a method according to the invention is also characterised in that the DNA of the  
 tPA, tPA variant, K2S molecule or K2S variant is preceded by a lac promoter and/or a  
 ribosomal binding site such as the Shine-Dalgarno sequence (see also example)

More preferably, a method according to the invention is also characterised in that the DNA coding for the tPA, tPA variant, K2S molecule or K2S variant is selected from the group of DNA molecules coding for at least 90% of the amino acids 87 – 527, 174 – 527, 180 – 527 or 220 – 527 of the human tissue plasminogen activator protein.

More preferably, a method according to the invention is also characterised in that the DNA Sequence of K2S comprises or consists of the following sequence

TCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTACCGTGGCACGCA  
CAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAATTCCATGATCCTGATAGG  
CAAGGTTTACACAGCACAGAACCCAGTGCCCAGGCACTGGGCCTGGGCAAACATA  
ATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGTGCCACGTGCTGAAGAAC  
CGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGCGGCCTGAGA  
CAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCTCTTCGCCGACATCGCCTCC  
CACCCCTGGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTGCCCCGGAGAGCGGTT  
CCTGTGCGGGGGCATACTCATCAGCTCCTGCTGGATTCTCTCTGCCGCCCACTGCTTC  
CAGGAGAGGTTTCCGCCCCACCACTGACGGTGATCTTGGGCAGAACATACCGGGT  
GGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGTCGAAAAATACATTGTCCATAAGG  
AATTCGATGATGACACTTACGACAATGACATTGCGCTGCTGCAGCTGAAATCGGATT  
CGTCCCGCTGTGCCAGGAGAGCAGCGTGGTCCGCACTGTGTGCCTTCCCCGGCGG  
ACCTGCAGCTGCCGGACTGGACGGAGTGTGAGCTCTCCGGCTACGGCAAGCATGAG  
GCCTTGTCTCCTTTCTATTTCGGAGCGGCTGAAGGAGGCTCATGTCAGACTGTACCCA  
TCCAGCCGCTGCACATCACAACATTTACTTAACAGAACAGTCACCGACAACATGCTG  
TGTGCTGGAGACACTCGGAGCGGCGGGCCCCAGGCAAACCTTGACGACGCCTGCCA  
GGGCGATTCTGGGAGGCCCCCTGGTGTGTCTGAACGATGGCCGCATGACTTTGGTGGG  
CATCATCAGCTGGGGCCTGGGCTGTGGACAGAAGGATGTCCCGGGTGTGTACACAA  
AGGTTACCAACTACCTAGACTGGATTCGTGACAACATGCGACCGTGA (SEQ ID NO 4)

The present invention also relates to variants of the before-mentioned nucleic acid molecules due to the degenerate code or to fragments thereof, nucleic acids which hybridize to said nucleic acids under stringent conditions, allelic or functional variants. The invention also relates to nucleic acids comprising said K2S nucleic acid fused to the nucleic acid encoding another protein molecule

Stringent conditions as understood by the skilled person are conditions which select for more than 85 %, preferred more than 90 % homology (Sambrook et al. 1989, Molecular Cloning: A

Laboratory Manual, 2<sup>nd</sup> ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York) The hybridisation will be carried out e.g. in 6x SSC/ 5x Denhardt's solution/ 0,1 % SDS (SDS: sodium dodecylsulfate) at 65 °C The degree of stringency is decided in the washing step. Thus, for example for a selection of DNA-sequences with approx. 85 % or more homology, the conditions 0,2 x SSC/ 0,01 % SDS/ 65 °C and for a selection of DNA-sequences of approx. 90 % or more homology the conditions 0,1x SSC/ 0,01 % SDS/ 65 °C are suitable. The composition of said reagents is described in Sambrook et al (1989, supra)

Another important part of the present invention is a variant of human tissue plasminogen activator comprising of or consisting of the Kringle 2 (4) plus Serine protease (5) (abbreviated K2S) protein or a variant or a fragment, a functional variant, an allelic variant, a subunit, a chemical derivative, a fusion protein or a glycosylation variant thereof.

The numbering/naming of the domains is according to Genbank accession number GI 137119 or Nature 301 (5897), 214-221 (1983), wherein the Kringle 2 domain extends from amino acid 176-262 and the protease domain from 276-527. Thus, according to the invention, a preferred K2S molecule may include amino acids 176-527 including the amino acids between Kringle 2 and the protease (amino acids 263 to 275; exemplified in fig. (structure A)). A K2S molecule according to the invention comprises the minimal part of the Kringle 2 domain and the protease domain still retaining protease activity and fibrin binding activity (measured as exemplified in the description/example). Said K2S molecule according to the invention comprises the amino acids SEGN or SEGNSD in its N-terminal portion (see infra). A preferred K2S molecule does not include amino acids 1 to 3 or 1 to 5 of the tPA molecule. Preferably, a K2S molecule according to the invention has the amino acid Asn at positions 177 and 184, i.e. it does not require the modifications as disclosed in Waldenström for improved producibility with a method according to the invention. Thus, a preferred K2S molecule according to the invention has the native amino acid sequence (no mutation) as opposed to the molecules known from the prior art. Most preferred, said K2S molecule according to the invention is a molecule characterized by the native amino acid sequence or parts thereof, does neither have amino acids 1 to 3 nor 1 to 5 of tPA and comprises N-terminally the amino acids SEGN or SEGNSD for improved producibility and/or correct folding of the molecule.

It is essential that the K2S protein according to the invention comprises in its N-terminal portion a peptide characterized by the amino acid sequence SEGN which advantageously allows commercial production with a method as described supra leading to a correctly folded, secreted

K2S protein Said 4 amino acids characterized by SEGN may have one or several amino acids more N-terminal, however said amino acids have to be located in the N-terminal portion as opposed to the C-terminal portion. Most preferably, said amino acids are located at the N-terminal portion. Preferably, the amino acids characterized by SEGN may be carry a point mutation or may be substituted by a non-natural amino acid.

Thus, in another important embodiment the invention relates to a K2S protein characterized in that it comprises the amino acids defined by the sequence SEGN or a variant or a fragment, a functional variant, an allelic variant, a subunit, a chemical derivative, a fusion protein or a glycosylation variant thereof

Such fragments are exemplified e.g. in figure 10 (Structure B-1) and figure 11 (Structure B-2) extending from amino acids 193-527. Structure B-1 has the native amino acid Cys in position 261, wherein in B-2 the amino acid is substituted by Ser. Further fragments according to the invention comprising the amino acids 220-527 (fig. 14, structure C) or comprising the amino acids 260-527 (fig. 15, structure D) may be modified according to the invention by addition of the amino acids SEGN and/or substitution of Cys-261 by Ser. The artisan can determine the minimal length of a K2S molecule according to the invention in order to retain its biological function and generate a K2S molecule with improved producibility and/or correct folding by adding the amino acids SEGN in the N-terminal portion. Thus, another preferred embodiment is said minimal K2S molecule with SEGN at its N-terminal portion.

In another important embodiment the invention relates to a K2S protein characterized in that it comprises the amino acids defined by the sequence SEGNSD or a variant or a fragment, a functional variant, an allelic variant, a subunit, a chemical derivative, a fusion protein or a glycosylation variant thereof. Such fragments are exemplified e.g. in figure 12 (Structure B-3) and figure 13 (Structure B-4) extending from amino acids 191-527. Structure B-3 has the native amino acid Cys in position 261, wherein in B-4 the amino acid is substituted by Ser. Further fragments according to the invention comprising the amino acids 220-527 (fig. 14, structure C) or comprising the amino acids 260-527 (fig. 15, structure D) may be modified according to the invention by addition of the amino acids SEGNSD and/or substitution of Cys-261 by Ser. The artisan can determine the minimal length of a K2S molecule according to the invention in order to retain its biological function and generate a K2S molecule with improved producibility and/or correct folding by adding the amino acids SEGNSD in the N-terminal portion. Thus, another preferred embodiment is said minimal K2S molecule with SEGNSD at its N-terminal portion.

Another more preferred embodiment of the present invention relates to a K2S protein comprising a protein characterized by the following amino acid sequence or a variant or a fragment, a functional variant, an allelic variant, a subunit, a chemical derivative or a glycosylation variant thereof

SEGNSDCYFGNGSAYRGTHSLTESGASCLPWNSMILIGKVYTAQNPSAQUALGLGKHNY  
CRNPDGDAKPWCHVLKNRRLTWEYCDVPSCSTCGLRQYSQPQFRIKGGLFADIASHPW  
QAAIFAKHRRSPGERFLCGGILISSCWILSAAHCFQERFPPHLLTVILGRITYRVVPGEEEQ  
KFEVEKYIVHKEFDDDDTYDNDIALQLKSDSSRCAQESSVVRTVCLPPADLQLPDWTEC  
ELSGYGKHEALSPFYSERLKEAHVRLYPSSRCTSQHLLNRTVTDNMLCAGDTRSGGPQA  
NLHDACQGDSGGPLVCLNDGRMTLVGIISWGLGCGQKDVPGVYTKVTNYLDWIRDNM  
RP\* (SEQ ID NO 11).

According to the invention, \* means STOP (i.e. encoded by a stop codon). This K2S molecule is exemplified in figure 8

One variant of the K2S molecule according to the invention relates to a fusion protein of K2S being fused to another protein molecule.

Another more preferred embodiment of the present invention relates to a K2S protein consisting of a protein characterized by the following amino acid sequence.

SEGNSDCYFGNGSAYRGTHSLTESGASCLPWNSMILIGKVYTAQNPSAQUALGLGKHNY  
CRNPDGDAKPWCHVLKNRRLTWEYCDVPSCSTCGLRQYSQPQFRIKGGLFADIASHPW  
QAAIFAKHRRSPGERFLCGGILISSCWILSAAHCFQERFPPHLLTVILGRITYRVVPGEEEQ  
KFEVEKYIVHKEFDDDDTYDNDIALQLKSDSSRCAQESSVVRTVCLPPADLQLPDWTEC  
ELSGYGKHEALSPFYSERLKEAHVRLYPSSRCTSQHLLNRTVTDNMLCAGDTRSGGPQA  
NLHDACQGDSGGPLVCLNDGRMTLVGIISWGLGCGQKDVPGVYTKVTNYLDWIRDNM  
RP\* (SEQ ID NO 11)

Said K2S molecules may be encoded by a DNA molecule as described supra.

Another important aspect of the invention relates to a DNA molecule characterized in that it is coding for

a) the OmpA protein or a functional derivative thereof operably linked to

b) a DNA molecule coding for a polypeptide containing the kringle 2 domain and the serine

protease domain of tissue plasminogen activator protein

More preferably, a DNA molecule according to the invention is also characterised in that the DNA sequence comprises or consists of the following DNA sequence encoding OmpA and K2S or a functional variant thereof or a variant due to the degenerate nucleotide code

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTGGCC  
 CAGGCGGCCTCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTGAGCCTACCG  
 TGGCACGCACAGCCTCACCGAGTCGGGTGCTCCTGCCTCCCGTGGAATTCATGAT  
 CCTGATAGGCAAGGTTTACACAGCACAGAACCCAGTGCCAGGCACTGGGCCTGG  
 GCAAACATAATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGTGCCACGTG  
 CTGAAGAACCGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGC  
 GGCCTGAGACAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCTCTTCGCCGA  
 CATCGCCTCCCAACCCTGGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTCGCCCGG  
 AGAGCGGTTCTGTGCGGGGGCATACTCATCAGCTCCTGCTGGATTCTCTCTGCCGC  
 CCACTGCTTCCAGGAGAGGTTTCCGCCCCACCACCTGACGGTGATCTTGGGCAGAAC  
 ATACCGGGTGGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGTCGAAAAATACATTG  
 TCCATAAGGAATTCGATGATGACACTTACGACAATGACATTGCGCTGCTGCAGCTGA  
 AATCGGATTCGTCCCGCTGTGCCAGGAGAGCAGCGTGGTCCGCACTGTGTGCCCTC  
 CCGCGGCGACCTGCAGCTGCCGACTGGACGGAGTGTGAGCTCTCCGGCTACGGC  
 AAGCATGAGGCCTTGTCTCCTTTCTATTTCGGAGCGGCTGAAGGAGGCTCA<sup>1</sup>TGTCAGA  
 CTGTACCCATCCAGCCGCTGCACATCACAACATTTACTTAACAGAACAGTCACCGAC  
 AACATGCTGTGTGCTGGAGACACTCGGAGCGGCGGGCCCCAGGCAA<sup>2</sup>ACTTGACGA  
 CGCCTGCCAGGGCGATTTCGGGAGGGCCCCCTGGTGTGTCTGAACGATGGCCGCATGA  
 CTTTGGTGGGCATCATCAGCTGGGGCCTGGGCTGTGGACAGAAGGATGTCCCGGGT  
 GTGTACACAAAGGTTACCAACTACCTAGACTGGATTCTGTGACAACATGCGACCG  
 (SEQ ID No 2)

Said DNA molecule encodes the following fusion protein of OmpA and K2S Said fusion protein of OmpA and K2S characterised in that it comprises or consists of a protein characterized by the following amino acid sequence or a fragment, a functional variant, an allelic variant, a subunit, a chemical derivative or a glycosylation variant thereof forms an important part of the present invention

MKKTAIAIAVALAGFATVAQAASEGNSDCYFGNGSAYRGTHSLTESGASCLPWNSMILI  
 GKVYTAQNPSAQALGLGKHNYCRNPDGDAKPWCHVLKNRRLTWEYCDVPSCSTCGLR  
 QYSQPQFRIKGGLFADIASHPWQAAIFAKHRRSPGERFLCGGILISSCWILSAAHCFQERF  
 PPHHLTVILGR<sup>3</sup>TYRVVPGE<sup>4</sup>EEQKFEVEKYIVHKEFDDDTYDNDIAL<sup>5</sup>LQLKSDSSRCAQES  
 SVVRTVCLPPADLQLPDWTECELSGYGKHEALSPFYSERLKEAHVRLYPSSRCTSQHLL  
 NRTVTDNMLCAGDTRSGGPQANLHDACQGDSGGPLVCLNDGRMTLVGIISWGLGCGQ  
 KDVPGVYTKVTNYLDWIRDNMRPG (SEQ ID NO 8)

Another preferred aspect of the invention relates to a DNA molecule according to the invention, characterized in that said DNA sequence b) is coding for at least 90% of the amino acids 87 –

527 of the human tissue plasminogen activator protein (numbering used herem as GI 137119 or Nature 301 (5897), 214-221 (1983).

Another preferred aspect of the invention relates to a DNA molecule according to the invention, characterized in that said DNA sequence b) is coding for at least 90% of the amino acids 174 –

527 of the human tissue plasminogen activator protein

Another preferred aspect of the invention relates to a DNA molecule according to the invention, characterized in that said DNA sequence b) is coding for at least 90% of the amino acids 180 – 527 of the human tissue plasminogen activator protein.

Another preferred aspect of the invention relates to a DNA molecule according to the invention, characterized in that said DNA sequence b) is coding for at least 90% of the amino acids 220 – 527 of the human tissue plasminogen activator protein

Another preferred aspect of the invention relates to a DNA molecule according to the invention, characterized in that said DNA sequence a) is hybridizing under stringent conditions to the following sequence

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTGGCC  
CAGGCGGCC (SEQ ID NO 3)

Another preferred aspect of the invention relates to a DNA molecule according to the invention, characterized in that said DNA sequence a) consists of the following sequence

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTGGCC  
CAGGCGGCC (SEQ ID NO 3).

Another preferred aspect of the invention relates to a DNA molecule according to the invention, characterized in that said DNA sequence b) is hybridizing under stringent conditions to the following sequence

TCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTACCGTGGC<sup>ˆ</sup>ACGCA  
CAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAAATCCATGATCCTGATAGG  
CAAGGTTTACACAGCACAGAACCCAGTGCCCAGGCACTGGGCCTGGGCAAACATA  
ATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGTGCCACGTGCTGAAGAAC  
CGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGCGGCCTGAGA  
CAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCTCTTCGCCGACATCGCCTCC  
CACCCCTGGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTGCCCCGGAGAGCGGTT  
CCTGTGCGGGGGCATACTCATCAGCTCCTGCTGGATTCTCTCTGCCGCCCACTGCTTC  
CAGGAGAGGTTTCCGCCCCACCACCTGACGGTGATCTTGGGCAGAACATACCGGGT

GGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGTCGAAAAATACATTGTCCATAAGG  
AATTCGATGATGACACTTACGACAATGACATTGCGCTGCTGCAGCTGAAATCGGATT  
CGTCCCGCTGTGCCCAGGAGAGCAGCGTGGTCCGCACTGTGTGCCTTCCCCCGGCGG  
ACCTGCAGCTGCCGGACTGGACGGAGTGTGAGCTCTCCGGCTACGGCAAGCATGAG  
GCCTTGTCTCCTTTCTATTTCGGAGCGGCTGAAGGAGGCTCATGTCAGACTGTACCCA  
TCCAGCCGCTGCACATCACAACATTTACTTAACAGAACAGTCACCGACAACATGCTG  
TGTGCTGGAGACACTCGGAGCGGCGGGCCCCAGGCAAACCTTGCACGACGCCTGCCA  
GGGCGATTTCGGGAGGCCCCCTGGTGTGTCTGAACGATGGCCGCATGACTTTGGTGGG  
CATCATCAGCTGGGGCCTGGGCTGTGGACAGAAGGATGTCCCGGGTGTGTACACAA  
AGGTTACCAACTACCTAGACTGGATTTCGTGACAACATGCGACCGTGA (SEQ ID NO 4)

Another preferred aspect of the invention relates to a DNA molecule according to the invention, characterized in that said DNA sequence b) consists of the following sequence

TCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTACCGTGGCACGCA  
CAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAATTCCATGATCCTGATAGG  
CAAGGTTTACACAGCACAGAACCCAGTGCCAGGCACTGGGCCTGGGCAAACATA  
ATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGTGCCACGTGCTGAAGAAC  
CGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGCGGCCTGAGA  
CAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCTCTTCGCCGACATCGCCTCC  
CACCCCTGGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTCGCCCGGAGAGCGGTT  
CCTGTGCGGGGGCATACTCATCAGCTCCTGCTGGATTCTCTCTGCCGCCCACTGCTTC  
CAGGAGAGGTTTCCGCCCCACCACCTGACGGTGATCTTGGGCAGAACATACCGGGT  
GGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGTCGAAAAATACATTGTCCATAAGG  
AATTCGATGATGACACTTACGACAATGACATTGCGCTGCTGCAGCTGAAATCGGATT  
CGTCCCGCTGTGCCCAGGAGAGCAGCGTGGTCCGCACTGTGTGCCTTCCCCCGGCGG  
ACCTGCAGCTGCCGGACTGGACGGAGTGTGAGCTCTCCGGCTACGGCAAGCATGAG  
GCCTTGTCTCCTTTCTATTTCGGAGCGGCTGAAGGAGGCTCATGTCAGACTGTACCCA  
TCCAGCCGCTGCACATCACAACATTTACTTAACAGAACAGTCACCGACAACATGCTG  
TGTGCTGGAGACACTCGGAGCGGCGGGCCCCAGGCAAACCTTGCACGACGCCTGCCA  
GGGCGATTTCGGGAGGCCCCCTGGTGTGTCTGAACGATGGCCGCATGACTTTGGTGGG  
CATCATCAGCTGGGGCCTGGGCTGTGGACAGAAGGATGTCCCGGGTGTGTACACAA  
AGGTTACCAACTACCTAGACTGGATTTCGTGACAACATGCGACCGTGA (SEQ ID NO 4)

Another preferred embodiment of the invention relates to a vector containing a DNA sequence according to the invention

Another preferred embodiment of the invention relates to a vector according to the invention, wherein said DNA sequence is preceded by a lac promoter and a ribosomal binding site

Suitable vectors according to the invention include, but are not limited to viral vectors such as e.g. Vaccinia, Semliki-Forest-Virus and Adenovirus, phagemid vectors and the like. Preferred are vectors which can be advantageously used in *E. coli*, but also in any other prokaryotic host such as pPROTet.E, pPROLar.A, members of the pBAD family, pSE family, pQE family and pCAL

Another preferred embodiment of the invention relates to the vector pComb3HSS containing a DNA according to the invention, wherein the expression of the gp III protein is suppressed or inhibited by deleting the DNA molecule encoding said gp III protein or by a stop codon between the gene coding for a polypeptide containing the kringle 2 domain and the serine protease domain of tissue plasminogen activator protein and the protein III gene.

Another important aspect of the present invention relates to a prokaryotic host cell comprising a DNA molecule according to the invention

Another important aspect of the present invention relates to a prokaryotic host cell comprising a vector according to the invention.

Another important aspect of the present invention relates to an *E. coli* host cell comprising a DNA molecule according to the invention

Another important aspect of the present invention relates to a *E. coli* host cell comprising a vector according to the invention

Yet another important aspect of the present invention is the use of a DNA molecule according to the invention or of a vector according to the invention or a host cell according to the invention in a method for the production of a polypeptide having the activity of tissue plasminogen activator

Yet another important aspect of the present invention is the use according the invention as described above, wherein said method is a method according to the invention.

Another very important aspect is a pharmaceutical composition comprising a substance obtainable by a method according to the invention and pharmaceutically acceptable excipients and carriers. An example for said substance is the K2S molecule described supra. The term "pharmaceutically acceptable carrier" as used herein refers to conventional pharmaceutical excipients or additives used in the pharmaceutical manufacturing art. Such physiologically acceptable compounds include, for example, carbohydrates, such as glucose, sucrose or dextrans,

antioxidants, such as ascorbic acid or glutathione, chelating agents, low molecular weight proteins or other stabilizers or excipients (see also e.g. Remington's Pharmaceutical Sciences (1990, 18th ed. Mack Publ, Easton.)) Said pharmaceutical composition according to the invention can be advantageously administered intravenously as a bolus, e.g. as a single bolus for 5 to 10 seconds intravenously

The invention further relates to the use of substances obtainable by a method according to the invention in the manufacture of a medicament in the treatment of stroke, cardiac infarction, acute myocardial infarction, pulmonary embolism, any artery occlusion such as coronary artery occlusion, intracranial artery occlusion (e.g. arteries supplying the brain), peripherally occluded arteries, deep vein thrombosis or related diseases associated with unwanted blood clotting

The following example is intended to aid the understanding of the invention and should in no way be regarded as limiting the scope of the invention

### Example 1

#### MATERIALS AND METHODS

**Primer design** In order to amplify a specific part of tPA gene, a pair of primers SK2/174 [5' GAGGAGGAGGTGCCCCAGGCGGCCTCTGAGGGAAACAGTGAC 3'] (SEQ ID NO 22) and ASSP

[5' GAGGAGGAGCTTGGCCGGCCTGGCCCGGTCGCATGTTGTCACG 3'] (SEQ ID NO-23) were synthesized (Life Technologies, Grand Island, NY) These primers were designed based on the human tPA gene retrieved from NCBI databases (g137119) They were synthesized with Sfi I end cloning sites (underlined) in such a way that the reading frame from the ATG of the gpIII gene in phagemid vector, pComb3HSS, will be maintained throughout the inserted sequence

Another primer set for site-directed mutagenesis was designed to anneal at the sequence situated between K2S gene and gene III in pComb3H-K2S The sequence of primers with mutation bases (underlined) for generating a new stop codon were MSTPA

[5' ACATGCGACCGTGACAGGCCGCCAG 3'] (SEQ ID NO 24) and MASTPA

[5' CTGGCCGGCCTGTCACGGTCGCATGT 3'] (SEQ ID NO 25).

Amplification of K2S gene by PCR. One µg SK2/174 and ASSP primers together with 50 ng of p51-3 template (obtained from Dr. Hiroshi Sasaki, Fujisawa Pharmaceutical, Japan) were suspended in 100 µl PCR mixture. An amount of 2.5 U Taq polymerase (Roche Molecular Biochemicals, Indianapolis, IN) was finally added to the solution. The titrated amplification condition was initiated with jump start at 85°C for 4 min, then denaturation at 95°C for 50 sec, annealing at 42°C for 50 sec, extension at 72°C for 1.5 min. Thirty five rounds were repeatedly performed. The mixture was further incubated at 72°C for 10 min. The amplified product of 1110 bp was subsequently purified by QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany). The correctness of purified product was confirmed by restriction enzymes.

Construction of phagemid expressing K2S. The purified PCR product of K2S and pComb3HSS phagemid (kindly provided by Dr. Carlos F. Barbas, Scripps Institute, USA) were digested with Sfi I (Roche Molecular Biochemicals, Indianapolis, IN) to prepare specific cohesive cloning sites. Four µg of the purified PCR product was digested with 60 U of Sfi I at 50°C for 18 h. For pComb3HSS, 20 µg of phagemid vectors were treated with 100 U of Sfi I. Digested products of purified PCR product of K2S and pComb3HSS (~3300 bp) were subsequently gel-purified by the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). T4 ligase (Roche Molecular Biochemicals, Indianapolis, IN) of 5 U were introduced to the mixture of 0.7 µg of purified Sfi I-digested pComb3HSS and 0.9 µg of purified Sfi I-digested PCR product. Ligation reaction was incubated at 30°C for 18 h. The newly constructed phagemid was named pComb3H-K2S.

Transformation of E. coli XL-1 Blue. Two hundred µl of CaCl<sub>2</sub> competent E. coli XL-1 Blue (Stratagene, La Jolla, CA) were transformed with 70 ng of ligated or mutated product. The transformed cells were propagated by spreading on LB agar containing 100 µg/ml ampicillin and 10 µg/ml tetracycline (Sigma, Saint Louis, MO). After cultivation at 37°C for 18 h, several antibiotic resistant colonies were selected for plasmid minipreps by using the alkaline lysis method. Each purified plasmid was subjected to Sfi I restriction site analysis. A transformant harboring plasmid with the correct Sfi I restriction site(s) was subsequently propagated for 18 h at 37°C in 100 ml LB broth with ampicillin 100 µg/ml and tetracycline 10 µg/ml. A plasmid maxiprep was performed using the QIAGEN Plasmid Maxi Kit (QIAGEN, Hilden, Germany). The purified plasmid was reexamined for specific restriction sites by Sfi I and sequenced by

AmpliTaq DNA Polymerase Terminator Cycle Sequencing Kit (The Perkin-Elmer Corporation, Forster City, CA)

Site-directed mutagenesis of pComb3H-K2S 10 ng of pComb3H-K2S template were mixed with 125 ng of MSTPA and MASTPA primers PfuTurbo DNA polymerase (Stratagene, LA Jolla, CA) of 2.5 U was added to the mixture for cycle amplification. The reaction started with one round of 95°C for 30 sec. Then it was followed by 16 rounds consisting of 95°C for 30 sec, 55°C for 1 min, and 68°C for 9 min. The reaction tube was subsequently placed on ice for 2 min. In order to destroy the template strands, 10 U of Dpn I restriction enzyme (Stratagene, LA Jolla, CA) were added to the amplification reaction and incubated for 1 h at 37°C. This synthesized product (MpComb3H-K2S) was further used to transform E. coli XL-1 Blue.

Preparation of phage-display recombinant-K2S After pComb3H-K2S was transformed to XL-1 Blue, the phage display technique was performed. A clone of pComb3H-K2S transformed E. coli XL-1 Blue was propagated in 10 ml super broth containing ampicillin 100 µg/ml and tetracycline 10 µg/ml at 37°C until the O.D. [600 nm] of 1.5 was reached. The bacterial culture was subsequently propagated in 100 ml of the same medium and culture for 2 h. An amount of 10<sup>12</sup> pfu of VCSM13 helper phage (Stratagene, La Jolla, CA) was used to infect the transformed E. coli XL-1 Blue. After 3 h incubation, kanamycin at a final concentration of 70 µg/ml final concentration was added to culture. The culture was left shaking (200 RPM) for 18 h at 37°C. Bacteriophages which harbored K2S on gp3 (K2S-φ) were then harvested by adding 4% w/v PEG MW 8000 (Sigma, Saint Louis, MO) and 3% w/v NaCl. Finally, the harvested phage was resuspended in 2 ml PBS pH 7.4. The phage number was determined by infecting E. coli XL-1 Blue. The colony-forming unit per milliliter (cfu/ml) was calculated as described previously (21).

Expression of recombinant-K2S in shaker flasks MpComb3H-K2S transformed E. coli XL-1 Blue was cultivated in 100 ml super broth (3% w/v tryptone, 2% w/v yeast extract and 1% w/v MOPS) at pH 7.0 in the presence of ampicillin (100 µg/ml) at 37°C until an O.D. [600 nm] of 0.8 was reached. Subsequently, the protein synthesis was induced by 1 mM of IPTG (Promega, Madison, WI). The bacteria were further cultured shaking (200 RPM) for 6 h at 30°C. The culture supernatant was collected and precipitated with 55% saturated ammonium sulfate (32).

The precipitate was reconstituted with PBS, pH 7.2, and dialysed in the same buffer solution at 4°C for 18 h. Periplasmic proteins from bacterial cells were extracted by using a chloroform shock as previously described by Ames et al. (2)

Immunoassay quantification of recombinant-K2S. In order to detect r-K2S, solid phase was coated with monoclonal anti-kringle 2 domain (16/B) (generously provided by Dr Ute Zacharias, Central Institute of Molecular Biology, Berlin-Buch, Germany) The standard ELISA washing and blocking processes were preformed. Fifty µl of  $10^{11}$  cfu/ml of K2S-φ or secretory r-K2S were added into each anti-kringle 2 coated well. Antigen-antibody detection was carried out as follows. Either sheep anti-M13 conjugated HRP (Pharmacia Biotech, Uppsala, Sweden) or sheep anti-tPA conjugated HRP (Cedarlane, Ontario, Canada), was added to each reaction well after the washing step. The substrate TMB was subjected to every well and the reaction was finally ceased with  $H_2SO_4$  solution after 30 min incubation. The standard melanoma tPA 86/670 (National Institute for Biological Standards and Control, Hertfordshire, UK) was used as positive control.

Amidolytic activity assay. A test kit for the detection of tPA amidolytic activity was purchased from Chromogenix (Molndal, Sweden). The substrate mixture containing plasminogen and S-2251 was used to determine serine protease enzymatic activity. The dilution of  $10^{-3}$  of each ammonium precipitated sample was assayed with and without stimulator, human fibrinogen fragments. The assay procedure was according to the COASET t-PA manual.

SDS-PAGE and immunoblotting. The dialysed precipitate-product from culture supernatant was further concentrated 10 folds with centricon 10 (AMICON, Beverly, MA). The concentrated sample was subjected to protein separation by SDS-PAGE, 15% resolving gel, in the reducing buffer followed by electroblotting to nitrocellulose. The nitrocellulose was then blocked with 4% skimmed milk for 2 hr. In order to detect r-K2S, a proper dilution of sheep anti-tPA conjugated HRP was applied to the nitrocellulose. The immunoreactive band was visualized by a sensitive detection system, Amplified Opti-4CN kit (BIORAD, Hercules, CA).

Copolymerized plasminogen polyacrylamide gel electrophoresis. An 11% resolving polyacrylamide gel was copolymerized with plasminogen and gelatin as previously described by

Heussen et al (14) The stacking gel was prepared as 4 % concentration without plasminogen and gelatin. Electrophoresis was performed at 4°C at a constant current of 8 mA. The residual SDS in gel slab was removed after gentle shaking at room temperature for 1h in 2.5% Triton X-100. Then the gel slab was incubated in 0.1 M glycine-NaOH, pH 8.3, for 5 h at 37°C. Finally, the gel slab was stained and destained by standard Coomassie brilliant blue (R-250) dyeing system. The location of the peptide harboring enzymatic activity was not stained by dye in contrast to blue-paint background.

## RESULTS

Construction of K2S gene carrying vector. From the vector p51-3 we amplified the kringle 2 plus the serine protease portion of tPA (Ser<sup>174</sup> in kringle 2 domain to Pro<sup>527</sup> in the serine protease) using primers sK2/174 and ASSP. The amplified 1110 bp product was demonstrated by agarose gel electrophoresis (Fig 1, lane 2) and was inserted into pComb3HSS phagemid by double Sfi I cleavage sites on 5' and 3' ends in the correct reading frame. Thus a new vector, pComb3H-K2S, harboring the K2S was generated. In this vector K2S is flanked upstream by the OmpA signal sequence and downstream by gp3. The correct insertion of K2S was verified both by restriction analysis with Sfi I (Fig 2, lane 3), PCR-analysis (demonstration of a single band at 1110 bp), and DNA sequencing. The schematic diagram of pComb3H-K2S map is given in Fig 3.

Phage-displayed r-K2S. VCSM13 filamentous phage was used to infect pComb3H-K2S transformed E. coli XL-1 Blue, X[K2S]. VCSM13 was propagated and incorporated the K2S-gp3 fusion protein during the viral packaging processes. The harvested recombinant phage (K2S-φ) gave a concentration of  $5.4 \times 10^{11}$  cfu/ml determined by re-infecting E. coli XL-1 Blue with PEG-precipitated phages. These recombinant phage particles were verified for the expression of r-K2S by sandwich ELISA. The phage-bound heterologous K2S protein was recognized by the monoclonal anti-kringle 2 antibody (16/B) by using sheep anti-tPA conjugated HRP antibody detection system. The absorbance of this assay was  $1.12 \pm 0.03$  (Table 1). The amount of K2S detectable on  $10^{12}$  phage particles is equal to 336 ng of protein in relation to the standard melanoma tPA. In order to corroborate that K2S-gp3 fusion protein was associated with phage particles, sheep anti-tPA conjugated HRP antibody was substituted by sheep anti-M13 antibody conjugated HRP. This immuno-reaction exhibited an absorbance of  $1.89 \pm 0.07$  (Table 1). In

contrast, if the capture antibody was sheep anti-M13 antibody, extremely low K2S was observed with sheep anti-tPA antibody conjugated HRP, the absorbance was only  $0.17 \pm 0.01$  (Table 1). This suggested that only a minority of purified phage particles carried K2S-gp3 fusion protein. VCSM13 prepared from non-transformed XL-1 Blue was used as a negative control.

**Construction of MpComb3H-K2S** We generated a stop codon between K2S and gp3 in pComb3H-K2S with the aid of the mutagenic primers (MSTPA and MASTPA) (Fig. 4). In order to enrich the newly synthesized and mutated MpComb3H-K2S, the cycle amplification mixture was thoroughly digested with Dpn I to degrade the old dam methylated pComb3H-K2S template (Dpn I prefers dam methylated DNA). After transforming of *E. coli* XL-1 Blue with MpComb3H-K2S, a transformant XM[K2S] was selected for further study. As a consequence of bp substitution, one Sfi I cleavage site close to the 3' end of K2S gene was lost after site-directed mutagenesis. A linear version of Sfi I cleaved MpComb3H-K2S was observed at 4319 bp without the appearance of inserted K2S gene fragment (Fig. 5, lane 3). Thus, the K2S gene encoding by MpComb3H-K2S was expressed in non-gp3 fusion form in XM[K2S].

**Expression and purification of K2S** K2S expression in XM[K2S] was induced by IPTG. r-K2S was detectable by using ELISA both in the periplasmic space and in the culture supernatant.

The amount of the heterologous protein in each preparation was determined by sandwich ELISA and related to the standard tPA. From 100 ml of the bacterial culture in shaker flask with the O.D. [600 nm] of 50, the periplasmic fraction yielded 1.38 µg of r-K2S (approximately 32%) whereas 2.96 µg of r-K2S (approximately 68%) was obtained in the ammonium precipitated culture supernatant. Sandwich ELISA was used to verify the PEG precipitated phage from VCSM13 infected XM[K2S]. No r-K2S captured by monoclonal anti-kringle 2 antibody was detected by anti-M13 conjugated HRP, indicating that K2S is not presented on the phage particles if gp3 is missing.

**Amidolytic activity measurement.** If serine protease domain is present in the sample, plasminogen will be converted to plasmin. The produced plasmin will further digest the S-2251 substrate to a colour product, p-nitroaniline, which has a maximum absorbance at 405 nm. The specific activity of the recombinant product is in accordance with the absorbance. The fibrinogen-dependent enzymatic activity of each sample i.e. K2S-φ, periplasmic r-K2S or culture

22  
supernatant r-K2S, was evaluated and compared. Both K2S- $\phi$  and periplasmic r-K2S illustrated notably low enzymatic activity, which was below the sensitivity of the test (0.25 IU/ml). The culture supernatant r-K2S gave the fibrinogen-dependent enzymatic activity of 7 IU/ml. Thus, from 100 ml culture we obtained a total of 700 IU enzymatic activity. Without fibrinogen no enzymatic activity of the r-K2S purified from culture supernatant was observed - whereas standard melanoma tPA showed some activity.

Demonstration of recombinant protein by immunoblotting. Partially purified K2S from culture supernatant of XM[K2S] revealed a molecular mass of 39 kDa by using sheep anti-tPA antibodies (Fig. 6). The negative control, partially purified culture supernatant of non-transformed XL1-Blue, contained no reactive band with a similar size.

Localization of active enzyme by PAGE. The plasminogen has been copolymerized and immobilized with gelatin in the polyacrylamide gel prior to electrophoresis. The ammonium sulfate precipitated culture supernatants of E. coli XL-1 Blue, E. coli XL-1 Blue transformed with pComb3HSS and XM[K2S] were analyzed (Fig. 7). All samples were processed in non-reducing condition to preserve the correct conformation and activity of the serine protease domain. Transparent areas of serine protease digested plasminogen were observed only in the ammonium sulfate precipitated culture supernatants of XM[K2S] at 34 and 37 kDa positions. The other samples gave no clearing zones. The positive control lane of standard melanoma tPA also demonstrated enzymatic activity at 66 and 72 kDa positions.

## REFERENCES

- 1 Allen, S., H. Y. Nam, and N. J. Bulleid. 1995. Intracellular folding of tissue-type plasminogen activator. Effects of disulfide bond formation on N-linked glycosylation and secretion. *J Biol. Chem.* 270:4797-4804.
- 2 Ames, G. F., C. Prody, and S. Kustu. 1984. Simple, rapid, and quantitative release of periplasmic proteins by chloroform. *J Bacteriol.* 160:1181-1183.
3. Barbas, C. F. III, A. S. Kang, R. A. Lerner, and S. J. Benkovic. 1991. Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. *Proc Natl Acad. Sci U S A.* 88:7978-7982.

- 4 Barbas, C F III, and J Wagner 1995 Synthetic human antibodies selecting and evolving functional proteins *A Companion to Methods in Enzymology* 8. 94-103
- 5 Bennett, W F, N F. Paoni, B A Keyt, D Botstein, A. J Jones, L. Presta, F M. Wurm, and M. J Zoller. 1991. High resolution analysis of functional determinants on human tissue-type plasminogen activator. *J Biol Chem.* 266:5191-5201
6. Betton, J M., N. Sassoon, M. Hofnung, and M. Laurent. 1998. Degradation versus aggregation of misfolded maltose-binding protein in the periplasm of *Escherichia coli* *J Biol. Chem.* 273 8897-8902
- 7 Camiolo, S M., S Thorsen, and T Astrup. 1971 Fibrinogenolysis and fibrinolysis with tissue plasminogen activator, urokinase, streptokinase-activated human globulin and plasmin. *Proc. Soc Exp Biol Med.* 38 277-280
- 8 Cartwright, T. 1992 Production of t-PA from animal cell culture, p 217-245 In R. E. Spier, and J B. Griffiths (ed.), *Animal Cell Biotechnology*, Vol 5. Academic Press, N Y
- 9 Curry, K. A., A. W. Yem, M R. Deibel, Jr, N T. Hatzenbuehler, J. G Hoogerheide, and C S Tomich. 1990 *Escherichia coli* expression and processing of human interleukin-1 beta fused to signal peptides *DNA Cell Biol* 9 167-175
- 10 Datar, R. V, T. Cartwright, and C-G Rosen. 1993 Process economics of animal cell and bacterial fermentations a case study analysis of tissue plasminogen activator *Biotechnology* 11:349-357
- 11 Deneffe, P, S Kovarik, T. Ciora, N Gosselet, J C Benichou, M. Latta, F. Gunet, A. Ryter, and J. F Mayaux. 1989 Heterologous protein export in *Escherichia coli* influence of bacterial signal peptides on the export of human interleukin 1 beta. *Gene* 85:499-510
- 12 Griffiths, J. B, A. Electricwala 1987 Production of tissue plasminogen activators from animal cells. *Adv. Biochem Eng Biotechnol* 34:147-166
- 13 Harris, T J, T Patel, F A. Marston, S Little, J S Emtage, G Opdenakker, G Volckaert, W Rombauts, A. Billiau, and P. De Somer 1986 Cloning of cDNA coding for human tissue-type plasminogen activator and its expression in *Escherichia coli*. *Mol Biol Med.* 3 279-292
- 14 Heussen, C, and E. B. Dowdle. 1980. Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. *Anal Biochem.* 102 196-202
- 15 Heussen, C., F. Joubert, and E B. Dowdle. 1984 Purification of human tissue plasminogen activator with Erythrina trypsin inhibitor. *J Biol Chem* 259 11635-11638

- 16 Hu, C K., U Kohnert, O Wilhelm, S. Fischer, and M Llinas 1994 Tissue-type plasminogen activator domain-deletion mutant BM 06 022 modular stability, inhibitor binding, and activation cleavage *Biochemistry* 33 11760-11766
- 17 Kipriyanov, S M., G. Moldenhauer, and M. Little 1997. High level production of soluble single chain antibodies in small-scale *Escherichia coli* cultures *J Immunol Methods* 200 69-77
18. Ko, J H., D K. Park, I C Kim, S H Lee, and S. M Byun 1995 High-level expression and secretion of streptokinase in *Escherichia coli*. *Biotechnol Lett* 17 1019-1024
19. Kouzuma, Y , N Yamasaki, and M. Kimura. 1997 The tissue-type plasminogen activator inhibitor ETIa from *Erythrina variegata*. structural basis for the inhibitory activity by cloning, expression, and mutagenesis of the cDNA encoding ETIa. *J Biochem. (Tokyo)* 121:456-463.
20. Lasters, L, N Van Herzele, H. R. Lijnen, D Collen, and L. Jespers 1997 Enzymatic properties of phage-displayed fragments of human plasminogen *Eur J Biochem* 244 946-952
- 21 Lobel, L I, P. Rausch, I Trakht, S Pollak, and J W Lustbader 1997 Filamentous phage displaying the extracellular domain of the hLH/CG receptor bind hCG specifically. *Endocrinology* 138 1232-1239.
- 22 Lubiniecki, A., R. Arathoon, G Polastri, J Thomas, M. Wiebe, R. Garnick, A Jones, R. van Reis, and S Builder. Selected strategies for manufacture and control of recombinant tissue plasminogen activator prepared from cell culture, p 442-451. In R. E Spier, J B Griffiths, J Stephenne, and P J Crooy (ed.), *Advances in animal cell biology and technology for bioprocesses* Butterworths, London
23. Lucic, M R., B. E. Forbes, S. E Grosvenor, J. M. Carr, J C. Wallace, and G Forsberg. 1998 Secretion in *Escherichia coli* and phage-display of recombinant insulin-like growth factor binding protein-2 *J. Biotechnol* 61.95-108
24. Martin, U., S Fischer, U. Kohnert, H. Lill, R. Rudolph, G Sponer, A Stern, and K. Strem. 1990. Properties of a novel plasminogen activator (BM 06 022) produced in *Escherichia coli*. *Z. Kardiol* 79 167-170
25. Obukowicz, M G , M. E. Gustafson, K. D. Junger, R. M. Lemgruber, A. J Wittwer, T C. Wun, T G Warren, B F Bishop, K. J. Mathis, D. T McPherson, N. R. Siegel, M G Jennings, B B Brightwell, J A Diaz-Cuellar, L D Bell, C S Craik, and W C Tacon. 1990 Secretion of active kringle-2-serine protease in *Escherichia coli*. *Biochemistry* 29 9737-9745.
- 26 Parmley, S F , and G P Smith 1988 Antibody-selectable filamentous fd phage vectors: affinity purification of target genes *Gene* 73 305-318

- 27 Pennica, D , W E. Holmes, W. J. Kohr, R. N Harkins, G A. Vehar, C A. Ward, W F Bennett, E. Yelverton, P. H. Seeburg, H. I Heyneker, D V. Goeddel, and D Collen 1983 Cloning and expression of human tissue-type plasminogen activator cDNA in E coli Nature 301:214-221
- 28 Rippmann, J F , M. Klein, C Hoischen, B. Brocks, W J. Rettig, J Gumpert, K. Pfizenmaier, R. Mattes, and D Moosmayer 1998. Prokaryotic expression of single-chain variable-fragment (scFv) antibodies: secretion in L-form cells of *Proteus mirabilis* leads to active product and overcomes the limitations of periplasmic expression in *Escherichia coli*. Appl Environ Microbiol. 64 4862-4869
- 29 Saito, Y , Y Ishii, H Sasaki, M. Hayashi, T Fujimura, Y. Imai, S Nakamura, S Suzuki, J Notani, T Asada, H. Horai, K. Masakazu, and N. Mineo. 1994 Production and characterization of a novel tissue-type plasminogen activator derivative in *Escherichia coli* Biotechnol Prog. 10 472-479
- 30 Sarmientos, P , M. Duchesne, P Denèfle, J. Boiziau, N. Fromage, N Delporte, F Parker, Y Lelièvre, J-F Mayaux, and T Cartwright. 1989 Synthesis and purification of active human tissue plasminogen activator from *Escherichia coli*. Biotechnology 7 495-501
31. Scherrer, S , N. Robas, H. Zouheiry, G Branlant, and C. Branlant. 1994. Periplasmic aggregation limits the proteolytic maturation of the *Escherichia coli* penicillin G amidase precursor polypeptide. Appl. Microbiol. Biotechnol 42:85-89
- 32 Soeda, S , M Kakiki, H. Shumeno, and A Nagamatsu 1986 Rapid and high-yield purification of porcine heart tissue-type plasminogen activator by heparin-sepharose chromatography. Life Sci 39 1317-1324
- 33 Szarka, S J., E G Sihota, H R. Habibi, and S-L. Wong 1999 Staphylokinase as a plasminogen activator component in recombinant fusion proteins Appl Environ Microbiol 65:506-513
- 34 Waldenström, M., E Holmgren, A. Attersand, C Kalderen, B. Lowenadler, B Raden, L. Hansson, and G. Pohl 1991 Synthesis and secretion of a fibrinolytically active tissue-type plasminogen activator variant in *Escherichia coli* Gene 99 243-248.
35. Wan, E. W -M., and F. Baneyx 1998 TolAIII Co-overexpression Facilitates the Recovery of Periplasmic Recombinant Proteins into the Growth Medium of *Escherichia coli* Protein Expr Purif. 14 13-22

24

36 Zacharias, U, B Fischer, F. Noll, and H. Will 1992 Characterization of human tissue-type plasminogen activator with monoclonal antibodies mapping of epitopes and binding sites for fibrin and lysine. *Thromb Haemost.* 67:88-94

#### FIGURE LEGENDS

FIG 1 Validation of PCR amplification product of the K2S gene from the p51-3 vector by using sK2/174 and ASSP primers. Lane 1 shows 1 kb marker (Roche Molecular Biochemicals, Indianapolis, IN) Lane 2 was loaded with 1 µl of amplified product. A single band at 1110 bp is depicted. The electrophoresis was performed on a 1% agarose gel.

FIG 2 Identification of inserted K2S gene at 1110 bp (\*) after Sfi I digested pComb3H-K2S was demonstrated in lane 3. Lane 1 shows 1 kb marker Lane 2 was loaded with uncut pComb3H-K2S The electrophoresis was performed on a 1% agarose gel

FIG. 3. Scheme of pComb3H-K2S showing two Sfi I cloning sites into which the K2S gene was inserted. Signal sequence (OmpA), ribosome binding site (RIBS), lac promotor, and gpIII gene are also depicted.

FIG. 4 Schematic diagram of the mutation site at the junction between the K2S and gpIII genes on pComb3H-K2S The annealing site of pComb3H-K2S is bound with a set of mutation primers (MSTPA and MASTPA) containing modified oligonucleosides (underlined). After performing the cycle amplification, the Sfi I site 1 (in bold) is modified and lost in the newly synthesized strand

FIG 5 Characterization of newly synthesized MpComb3H-K2S by the Sfi I restriction enzyme. A single band at 4319 bp that refers to a single cleavage site of MpComb3H-K2S is observed in lane 3 No inserted K2S band at 1110 bp can be visualized. Lane 1 shows 1 kb marker. Lane 2 was loaded with uncut MpComb3H-K2S. The electrophoresis was performed on a 1% agarose gel

FIG 6 Identification of immunological reactive band with of recombinant DNA-derived protein purified from XM[K2S] culture supernatant with sheep anti-tPA conjugated HRP. Lane 1 was loaded with 40 ng of standard melanoma tPA (86/670), which showed the reactive band at 70 kDa The partially purified and concentrated culture supernatants from non-transformed E. coli

XL1- Blue and XM[K2S] were applied to lane 2 and 3 respectively. The distinct reactive band was particularly demonstrated in lane 3 at 39 kDa.

FIG. 7. Molecular weight determination of extracellular r-K2S harboring active serine protease domain by copolymerized plasminogen polyacrylamide gel electrophoresis. Lane 1 contained the indicated molecular weight standards ( $\times 10^{-3}$ ), SDS-6H (Sigma, Saint Louis, MO). Fifty  $\mu$ g of the 55% saturated ammonium sulfate precipitated culture supernatant of XL-1 Blue, XL-1 Blue transformed with pComb3HSS, and XM[K2S] were loaded in lane 2, 3, and 4 respectively. Lane 5 contained 50 mIU of standard melanoma tPA (86/670). Transparent zones of digested plasminogen in polyacrylamide gel are visible only in lane 4 at molecular weight of 34 and 37 kDa (B) and lane 5 at molecular weight of 66 and 72 kDa (A).

FIG. 8 Structure A (SEQ ID NO 11)

Native K2S molecule from amino acids 174-527 without modification.

FIG. 9. Structure B-0 (SEQ ID NO 12)

Native K2S molecule from amino acids 197-527 without modification

FIG. 10 Structure B-1 (SEQ ID NO 13)

K2S molecule from amino acids 193-527, wherein to Structure B-0 of Fig. 9 the amino acids SEGK were added at the N-terminal portion.

FIG. 11 Structure B-2 (SEQ ID NO 14)

K2S molecule from amino acids 193-527, as in Fig. 10, wherein Cys-261 was exchanged for Ser

FIG. 12 Structure B-3 (SEQ ID NO 15)

K2S molecule from amino acids 191-527, wherein to Structure B-0 of Fig. 9 the amino acids SEGNSD were added at the N-terminal portion

FIG. 13 Structure B-4 (SEQ ID NO 16)

K2S molecule from amino acids 191-527, as in Fig. 12, wherein Cys-261 was exchanged for Ser

FIG 14 Structure C (SEQ ID NO.17)

- Native K2S molecule from amino acids 220-527 without modification. This molecule may be further modified in a similar manner as disclosed for structure B in figures 10-13.

FIG 15 Structure D (SEQ ID NO 18)

Native K2S molecule from amino acids 260-527 without modification. This molecule may be further modified in a similar manner as disclosed for structure B in figures 10-13

FIG 16 tPA molecule (SEQ ID NO 19)

TABLE 1. Detection of r-K2S molecule in phage preparation by sandwich ELISA

| Capture antibody            | Tracer antibody (conjugated HRP) |                     |             |             |
|-----------------------------|----------------------------------|---------------------|-------------|-------------|
|                             | Anti-tPA                         |                     | Anti-M13    |             |
|                             | K2S-φ                            | VCSM13 <sup>a</sup> | K2S-φ       | VCSM13      |
| Anti-kringle 2 <sup>b</sup> | 1 12 ± 0 04 <sup>c</sup>         | 0 12 ± 0 03         | 1 89 ± 0 02 | 0 16 ± 0 02 |
| Anti-M13                    | 0 17 ± 0 01                      | 0 14 ± 0.05         | 1 91 ± 0 02 | 1 88 ± 0.03 |

<sup>a</sup> VCSM13 was harvested from XL-1 Blue transformed with pComb3HSS

<sup>b</sup> Mouse monoclonal anti-kringle 2 (16/B) was used. The other antibodies were prepared from sheep immunoglobulin.

<sup>c</sup> Value is mean of absorbance of each sample which was assayed in triplicate

**Claims**

1 Method for the production of recombinant DNA-derived tissue plasminogen activator (tPA),  
a tPA variant, a Kringle 2 Serine protease molecule (K2S) or a K2S variant in prokaryotic cells,  
wherein said tPA, tPA variant, K2S molecule or K2S variant is secreted extracellularly as an  
active and correctly folded protein, characterized in that the prokaryotic cell contains and  
expresses a vector comprising the DNA coding for said tPA, tPA variant, K2S molecule or K2S  
variant operably linked to the DNA coding for the signal peptide OmpA or a functional  
derivative thereof.

2 Method according to claim 1, characterised in that said the prokaryotic cell contains and  
expresses a vector comprising the DNA coding for said tPA, tPA variant, K2S molecule or K2S  
variant operably linked to the DNA coding for the signal peptide OmpA which is operably linked  
to the nucleic acid molecule defined by the sequence TCTGAGGGAAACAGTGAC (SEQ ID  
NO.1) or a functional derivative thereof.

3 Method according to claim 1 or 2, characterised in that the prokaryotic cell is E. coli.

4 Method according to one of claims 1 to 3, characterised in that the the following steps are  
carried out:

a) the DNA encoding the tPA, tPA variant, K2S molecule or K2S variant is amplified by PCR,

b) the PCR product is purified,

c) said PCR product is inserted into a vector comprising the DNA coding for OmpA signal  
peptide and the DNA coding for gpIII in such a way that said PCR product is operably linked  
upstream to the DNA coding for the OmpA signal sequence and linked downstream to the DNA  
coding for gpIII of said vector;

d) that a stop codon is inserted between said tPA, tPA variant, K2S molecule or K2S variant and  
gpIII,

e) said vector is expressed by the prokaryotic cell;

f) the tPA, tPA variant, K2S molecule or K2S variant is purified.

5. Method according to one of claims 1 to 4, characterised in that the vector is a phagemid  
vector comprising the DNA coding for OmpA signal peptide and the DNA coding for gpIII.

6 Method according to one of claims 1 to 5, characterised in that the vector is the pComb3HSS  
phagemid

7 Method according to one of claims 1 to 6, characterised in that the DNA Sequence of OmpA linked upstream to K2S comprises the following sequence or a functional variant thereof or a variant due to the degenerate nucleotide code

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTGGCC  
CAGGCGGCCTCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTACCG  
TGGCACGCACAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAATTCCATGAT  
CCTGATAGGCAAGGTTTACACAGCACAGAACCCCAAGTGCCCAGGCACTGGGCCTGG  
GCAAACATAATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGTGCCACGTG  
CTGAAGAACCGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGC  
GGCCTGAGACAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCTCTTCGCCGA  
CATCGCCTCCCACCCCTGGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTCGCCCGG  
AGAGCGGTTCTGTGCGGGGGCATACTCATCAGCTCCTGCTGGATTCTCTCTGCCGC  
CCACTGCTTCCAGGAGAGGTTTCCGCCCCACCACCTGACGGTGATCTTGGGCAGAAC  
ATACCGGGTGGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGTCGAAAAATACATTG  
TCCATAAGGAATTCGATGATGACACTTACGACAATGACATTGCGCTGCTGCAGCTGA  
AATCGGATTTCGTCCCGCTGTGCCCAGGAGAGCAGCGTGGTCCGCACTGTGTGCCTTC  
CCCCGGCGGACCTGCAGCTGCCGGACTGGACGGAGTGTGAGCTCTCCGGCTACGGC  
AAGCATGAGGCCTTGTCTCCTTTCTATTTCGGAGCGGCTGAAGGAGGCTCATGTCAGA  
CTGTACCCATCCAGCCGCTGCACATCACAACTTTACTTAACAGAACAGTCACCGAC  
AACATGCTGTGTGCTGGAGACACTCGGAGCGGCGGGCCCCAGGCCAACTTGACGA  
CGCCTGCCAGGGCGATTTCGGGAGGCCCCCTGGTGTGTCTGAACGATGGCCGCATGA  
CTTTGGTGGGCATCATCAGCTGGGGCCTGGGCTGTGGACAGAAGGATGTCCCGGGT  
GTGTACACAAAGGTTACCAACTACCTAGACTGGATTTCGTGACAACATGCGACCG  
(SEQ ID NO 2)

8 Method according to one of claims 1 to 7, characterised in that the DNA Sequence of OmpA comprises the following sequence.

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTGGCC  
CAGGCGGCC (SEQ ID NO 3)

9 Method according to one of claims 1 to 8, characterised in that the DNA Sequence of OmpA consists of the following sequence

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTGGCC  
CAGGCGGCC (SEQ ID NO 3)

10 Method according to one of claims 1 to 9, characterised in that the DNA of the tPA, tPA variant, K2S molecule or K2S variant is preceded by a lac promotor and/or a ribosomal binding site.

11 Method according to one of claims 1 to 10, characterised in that the DNA coding for the tPA, tPA variant, K2S molecule or K2S variant is selected from the group of DNA molecules coding for at least 90% of the amino acids 87 – 527, 174 – 527, 180 – 527 or 220 – 527 of the human tissue plasminogen activator protein.

12 Method according to one of claims 5 to 11, characterised in that the DNA Sequence of K2S comprises the following sequence or a functional variant thereof or a variant due to the degenerate nucleotide code

TCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTACCGTGGCACGCA  
CAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAATTCCATGATCCTGATAGG  
CAAGGTTTACACAGCACAGAACCCCAAGTGCCAGGCACTGGGCCTGGGCAAACATA  
ATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGTGCCACGTGCTGAAGAAC  
CGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGCGGCCTGAGA  
CAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCTCTTCGCCGACATCGCCTCC  
CACCCCTGGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTGCCCCGGAGAGCGGTT  
CCTGTGCGGGGGCATACTCATCAGCTCCTGCTGGATTCTCTCTGCCGCCCACTGCTTC  
CAGGAGAGGTTTCCGCCCCACCACCTGACGGTGATCTTGGGCAGAACATAACCGGGT  
GGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGTCGAAAAATACATTGTCCATAAGG  
AATTCGATGATGACACTTACGACAATGACATTGCGCTGCTGCAGCTGAAATCGGATT  
CGTCCCGCTGTGCCAGGAGAGCAGCGTGGTCCGCACTGTGTGCCTTCCCCGGCGG  
ACCTGCAGCTGCCGGACTGGACGGAGTGTGAGCTCTCCGGCTACGGCAAGCATGAG  
GCCTTGTCTCCTTTCTATTCCGAGCGGCTGAAGGAGGCTCATGTCAGACTGTACCCA  
TCCAGCCGCTGCACATCACAACATTTACTTAACAGAACAGTCACCGACAACATGCTG  
TGTGCTGGAGACACTCGGAGCGGCGGGCCCCAGGCAAACCTGCACGACGCCTGCCA  
GGGCGATTCCGGAGGCCCCCTGGTGTGTCTGAACGATGGCCGCATGACTTTGGTGGG  
CATCATCAGCTGGGGCCTGGGCTGTGGACAGAAGGATGTCCCGGGTGTGTACACAA  
AGGTTACCAACTACCTAGACTGGATTCTGTGACAACATGCGACCGTGA (SEQ ID NO 4)

13. Method according to one of claims 5 to 12, characterised in that the DNA Sequence of K2S consists of the following sequence

TCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTACCGTGGCACGCA  
 CAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAATTCCATGATCCTGATAGG  
 5 CAAGGTTTACACAGCACAGAACCCAGTGCCCAGGCACTGGGCCTGGGCAAACATA  
 ATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGTGCCACGTGCTGAAGAAC  
 CGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGCGGCCTGAGA  
 CAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCTCTTCGCCGACATCGCCTCC  
 CACCCCTGGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTGCCCCGGAGAGCGGTT  
 10 CCTGTGCGGGGGCATACTCATCAGCTCCTGCTGGATTCTCTCTGCCGCCCACTGCTTC  
 CAGGAGAGGTTTCCGCCCCACCACCTGACGGTGATCTTGGGCAGAACATACCGGGT  
 GGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGTCGAAAAATACATTGTCCATAAGG  
 AATTCGATGATGACACTTACGACAATGACATTGCGCTGCTGCAGCTGAAATCGGATT  
 CGTCCCGCTGTGCCCAGGAGAGCAGCGTGGTCCGCACTGTGTGCCTTCCCCCGGCGG  
 15 ACCTGCAGCTGCCGGACTGGACGGAGTGTGAGCTCTCCGGCTACGGCAAGCATGAG  
 GCCTTGTCTCCTTTCTATTTCGGAGCGGCTGAAGGAGGCTCATGTCAGACTGTACCCA  
 TCCAGCCGCTGCACATCACAACATTTACTTAACAGAACAGTCACCGACAACATGCTG  
 TGTGCTGGAGACACTCGGAGCGGGCGGGCCCCAGGCCAACTTGACAGACGCCTGCCA  
 GGGCGATTTCGGGAGGCCCCCTGGTGTGTCTGAACGATGGCCGCATGACTTTGGTGGG  
 20 CATCATCAGCTGGGGCCTGGGCTGTGGACAGAAGGATGTCCCGGGTGTGTACACAA  
 AGGTTACCAACTACCTAGACTGGATTCTGTGACAACATGCGACCGTGA (SEQ ID NO 4)

14 DNA molecule characterized in that it is coding for

- a) the OmpA protein or a functional derivative thereof operably linked to
- b) a DNA molecule coding for a polypeptide containing the kringle 2 domain and the serine
- 25 protease domain of tissue plasminogen activator protein.

15. DNA molecule according to claim 14, characterized in that said DNA sequence comprises the following sequence or a functional variant thereof or a variant due to the degenerate nucleotide code

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTGGCC  
 30 CAGGCGGCCTCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTACCG  
 TGGCACGCACAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAATTCCATGAT  
 CCTGATAGGCAAGGTTTACACAGCACAGAACCCAGTGCCCAGGCACTGGGCCTGG  
 GCAAACATAATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGTGCCACGTG  
 CTGAAGAACCGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGC

GGCCTGAGACAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCTCTTCGCCGA  
CATCGCCTCCCACCCCTGGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTGCCCCGG  
AGAGCGGTTCTGTGCGGGGGGCATACTCATCAGCTCCTGCTGGATTCTCTCTGCCGC  
CCACTGCTTCCAGGAGAGGTTTCCGCCCCACCACCTGACGGTGATCTTGGGCAGAAC  
1 ATACCGGGTGGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGTCGAAAAATACATTG  
TCCATAAGGAATTCGATGATGACACTTACGACAATGACATTGCGCTGCTGCAGCTGA  
AATCGGATTCTGTCCTCGCTGTGCCAGGAGAGCAGCGTGGTCCGCACTGTGTGCCTTC  
CCCCGGCGGACCTGCAGCTGCCGGACTGGACGGAGTGTGAGCTCTCCGGCTACGGC  
AAGCATGAGGCCTTGTCTCCTTTCTATTTCGGAGCGGCTGAAGGAGGCTCATGTCAGA  
2 CTGTACCCATCCAGCCGCTGCACATCACAACATTTACTTAACAGAACAGTCACCGAC  
AACATGCTGTGTGCTGGAGACACTCGGAGCGGCGGGCCCCAGGCAAACCTTGACGA  
CGCCTGCCAGGGCGATTTCGGGAGGCCCTTGGTGTGTCTGAACGATGGCCGCATGA  
CTTTGGTGGGCATCATCAGCTGGGGCCTGGGCTGTGGACAGAAGGATGTCCCGGGT  
GTGTACACAAAGGTTACCAACTACCTAGACTGGATTCTGTGACAACATGCGACCG  
15 (SEQ ID NO 5)

16 DNA molecule according to claim 14 or 15, characterized in that said DNA sequence consists of the following sequence:

ATGAAAAAGACAGCTATCGCGATTGCACTGGCACTGGCTGGTTTCGCTACCGTGGCC  
20 CAGGCGGCCTCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTGAGCCTACCG  
TGGCACGCACAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAATTCATGAT  
CCTGATAGGCAAGGTTTACACAGCACAGAACCCAGTGCCCAGGCACTGGGCCTGG  
GCAAACATAATTACTGCCGAATCCTGATGGGGATGCAAGCCCTGGTGCCACGTG  
CTGAAGAACCGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGC  
25 GGCCTGAGACAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCTCTTCGCCGA  
CATCGCCTCCCACCCCTGGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTGCCCCGG  
AGAGCGGTTCTGTGCGGGGGGCATACTCATCAGCTCCTGCTGGATTCTCTCTGCCGC  
CCACTGCTTCCAGGAGAGGTTTCCGCCCCACCACCTGACGGTGATCTTGGGCAGAAC  
ATACCGGGTGGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGTCGAAAAATACATTG  
30 TCCATAAGGAATTCGATGATGACACTTACGACAATGACATTGCGCTGCTGCAGCTGA  
AATCGGATTCTGTCCTCGCTGTGCCAGGAGAGCAGCGTGGTCCGCACTGTGTGCCTTC  
CCCCGGCGGACCTGCAGCTGCCGGACTGGACGGAGTGTGAGCTCTCCGGCTACGGC  
AAGCATGAGGCCTTGTCTCCTTTCTATTTCGGAGCGGCTGAAGGAGGCTCATGTCAGA  
CTGTACCCATCCAGCCGCTGCACATCACAACATTTACTTAACAGAACAGTCACCGAC  
35 AACATGCTGTGTGCTGGAGACACTCGGAGCGGCGGGCCCCAGGCAAACCTTGACGA  
CGCCTGCCAGGGCGATTTCGGGAGGCCCTTGGTGTGTCTGAACGATGGCCGCATGA  
CTTTGGTGGGCATCATCAGCTGGGGCCTGGGCTGTGGACAGAAGGATGTCCCGGGT  
GTGTACACAAAGGTTACCAACTACCTAGACTGGATTCTGTGACAACATGCGACCG  
(SEQ ID NO 5)

17. DNA molecule according to one of claims 14 to 16, characterized in that said DNA sequence  
b) is coding for at least 90% of the amino acids 87 – 527 of the human tissue plasminogen  
activator protein.

18 DNA molecule according to one of claims 14 to 17, characterized in that said DNA sequence b) is coding for at least 90% of the amino acids 174 – 527 of the human tissue plasminogen activator protein.

19 DNA molecule according to any one of claims 14 to 18, characterized in that said DNA sequence b) is coding for at least 90% of the amino acids 180 – 527 of the human tissue plasminogen activator protein.

20 DNA molecule according to any one of claims 14 to 19, characterized in that said DNA sequence b) is coding for at least 90% of the amino acids 220 – 527 of the human tissue plasminogen activator protein

21 DNA molecule according to any one of claims 14 to 20, characterized in that said DNA sequence a) is hybridizing under stringent conditions to the following sequence

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTGGCC  
CAGGCGGCC (SEQ ID NO 6)

22 DNA molecule according to any one of claims 14 to 21, characterized in that said DNA sequence a) consists of the following sequence

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTGGCC  
CAGGCGGCC (SEQ ID NO 6)

23 DNA molecule according to any one of claims 14 to 22, characterized in that said DNA sequence b) is hybridizing under stringent conditions to the following sequence.

TCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTACCGTGGCACGCA  
CAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAAATTCATGATCCTGATAGG  
CAAGGTTTACACAGCACAGAACCCAGTGCCAGGCACTGGGCCTGGGCAAACATA  
ATTACTGCCGAATCCTGATGGGGATGCCAAGCCCTGGTGCCACGTGCTGAAGAAC  
CGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGCGGCCTGAGA  
CAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCTCTTCGCCGACATCGCCTCC  
CACCCCTGGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTGCCCCGGAGAGCGGTT  
CCTGTGCGGGGGCATACTCATCAGCTCCTGCTGGATTCTCTCTGCCGCCCACTGCTTC  
CAGGAGAGGTTTCCGCCCCACCACCTGACGGTGATCTTGGGCAGAACATAACCGGT  
GGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGTCGAAAAATACATTGTCCATAAGG  
AATTCGATGATGACACTTACGACAATGACATTGCGCTGCTGCAGCTGAAATCGGATT  
CGTCCCCTGTGCCCAGGAGAGCAGCGTGGTCCGCACTGTGTGCCTTCCCCGGGCGG  
ACCTGCAGCTGCCGGACTGGACGGAGTGTGAGCTCTCCGGCTACGGCAAGCATGAG

GCCTTGTCTCCTTTCTATTTCGGAGCGGCTGAAGGAGGCTCATGTCAGACTGTACCCA  
TCCAGCCGCTGCACATCACAAACATTTACTTAAACAGAACAGTCACCGACAACATGCTG  
TGTGCTGGAGACACTCGGAGCGGCGGGCCCCAGGCAAACCTTGACGACGCCTGCCA  
GGGCGATTTCGGGAGGCCCCCTGGTGTGTCTGAACGATGGCCGCATGACTTTGGTGGG  
CATCATCAGCTGGGGCCTGGGCTGTGGACAGAAGGATGTCCCGGGTGTGTACACAA  
AGGTTACCAACTACCTAGACTGGATTTCGTGACAACATGCGACCGTGA (SEQ ID NO 7).

24 DNA molecule according to any one of claims 14 to 23, characterized in that said DNA sequence b) consists of the following sequence

TCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTACCGTGGCAGCA  
CAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAATTCCATGATCCTGATAGG  
CAAGGTTTACACAGCACAGAACCCCAAGTGCCAGGCACTGGGCCTGGGCAAACATA  
ATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGTGCCACGTGCTGAAGAAC  
CGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGCGGCCTGAGA  
CAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCTCTTCGCCGACATCGCCTCC  
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25. Fusion protein of OmpA and K2S, characterised in that it comprises a protein characterized by the following amino acid sequence or a fragment, a functional variant, an allelic variant, a subunit, a chemical derivative or a glycosylation variant thereof:

MKKTAIAIAVALAGFATVAQAASEGNSDCYFGNGSAYRGTHSLTESGASCLPWNSMILI  
GKVYTAQNPSAQAALGLGKHNYCRNPDGDAKPWCHVLKNRRLTWBYCDVPSCSTCGLR

QYSQPQFRIKGGLFADIASHPWQAAIFAKHRRSPGERFLCGGILISSCWILSAAHCFQERF  
 PPHHLTVILGR TYRVVPGE EEQKFEVEKYIVHKEFDDDTYDNDIAL LQLKSDSSRCAQES  
 SVVRTVCLPPADLQLPDWTECELSGYGKHEALSPFYSERLKEAHVRLYPSSRCTSQHLL  
 NRTVTDNMLCAGDTRSGGPQANLHDACQGDSGGPLVCLNDGRMTLVGIISWGLGCGQ  
 KDVPGVYTKVTNYLDWIRDNM RPG (SEQ ID NO 8).

26 Fusion protein of OmpA and K2S according to claim 25, characterised in that it consists of a protein characterized by the following amino acid sequence

MKKTAIAIAVALAGFATVAQAASEGNSDCYFGNGSAYRGTHSLTESGASCLPWNSMILI  
 GKVYTAQNPSAQALGLGKHNYCRNPDGDAKPWCHVLKNRRLTWEYCDVPSCSTCGLR  
 QYSQPQFRIKGGLFADIASHPWQAAIFAKHRRSPGERFLCGGILISSCWILSAAHCFQERF  
 PPHHLTVILGR TYRVVPGE EEQKFEVEKYIVHKEFDDDTYDNDIAL LQLKSDSSRCAQES  
 SVVRTVCLPPADLQLPDWTECELSGYGKHEALSPFYSERLKEAHVRLYPSSRCTSQHLL  
 NRTVTDNMLCAGDTRSGGPQANLHDACQGDSGGPLVCLNDGRMTLVGIISWGLGCGQ  
 KDVPGVYTKVTNYLDWIRDNM RPG (SEQ ID NO 8)

27 K2S protein, characterised in that it comprises a protein defined by the sequence SEGN (SEQ ID NO 9) and a or a variant or a fragment, a functional variant, an allelic variant, a subunit, a chemical derivative, a fusion protein or a glycosylation variant thereof.

28. K2S protein according to claim 27, characterised in that it comprises a protein defined by the sequence SEGNSD (SEQ ID NO 10) and a or a variant or a fragment, a functional variant, an allelic variant, a subunit, a chemical derivative, a fusion protein or a glycosylation variant thereof.

29 K2S protein according to claim 28 or 29, characterised in that it comprises a protein characterized by the following amino acid sequence or a fragment, a functional variant, an allelic variant, a subunit, a chemical derivative or a glycosylation variant thereof.

SEGNSDCYFGNGSAYRGTHSLTESGASCLPWNSMILIGKVYTAQNPSAQALGLGKHNY  
 CRNPDGDAKPWCHVLKNRRLTWEYCDVPSCSTCGLRQYSQPQFRIKGGLFADIASHPW  
 QAAIFAKHRRSPGERFLCGGILISSCWILSAAHCFQERFPPHHLTVILGR TYRVVPGE EEQ  
 KFEVEKYIVHKEFDDDTYDNDIAL LQLKSDSSRCAQESSVVRTVCLPPADLQLPDWTEC  
 ELSGYGKHEALSPFYSERLKEAHVRLYPSSRCTSQHLLNRTVTDNMLCAGDTRSGGPQA  
 NLHDACQGDSGGPLVCLNDGRMTLVGIISWGLGCGQKDVPGVYTKVTNYLDWIRDNM  
 RP\* (SEQ ID NO-11)

30. K2S according to any one of claims 27 to 30, characterised in that it consists of a protein characterized by the following amino acid sequence

SEGNSDCYFGNGSAYRGTHSLTESGASCLPWNSMILIGKVYTAQNPSAQALGLGKHNY  
CRNPDGDAKFWCHVLKNRRLTWEYCDVPSCSTCGLRQYSQPQFRIKGGLFADIASHPW  
QAAIFAKHRRSPGERFLCGGILISSCWILSAAHCFQERFPPHFLTIVILGRTYRVVPGEEEQ  
KFEVEKYIVHKEFDDDTYDNDIALQLKSDSSRCAQESSVVRTVCLPPADLQLPDWTEC  
ELSGYGKHEALSPFYSERLKEAHVRLYPSSRCTSQHLLNRTVTDNMLCAGDTRSGGPQA  
NLHDACQGDSGGPLVCLNDGRMTLVGHISWGLGCGQKDVPGVYTKVTNYLDWIRDNM  
RP\* (SEQ ID NO.11).

31. A vector containing a DNA sequence according to any one of claims 14 to 24

32. A vector according to claim 31, wherein said DNA sequence is preceded by a lac promoter and a ribosomal binding site

33. The vector pComb3HSS containing a DNA according to any one of claims 14 to 24, wherein the expression of the gp III protein is suppressed or inhibited by deleting the DNA molecule encoding said gp III protein or by a stop codon between the gene coding for a polypeptide containing the kringle 2 domain and the serine protease domain of tissue plasminogen activator protein and the protein III gene

34. A prokaryotic host cell comprising a DNA molecule according to any one of claims 14 to 24

35. A prokaryotic host cell comprising a vector according to any one of claims 31 to 33

36. An E. coli host cell comprising a DNA molecule according to any one of claims 14 to 24

37. An E. coli host cell comprising a vector according to any one of claims 31 to 33

38. Use of a DNA molecule according to any one of claims 14 to 24 or of a vector according to any one of claims 31 to 33 or a host cell according to any one of claims 34 to 37 in a method for the production of a polypeptide having the activity of tissue plasminogen activator

39. Use according to claim 38, wherein said method is a method according to any one of claims 1 to 13

1 / 15

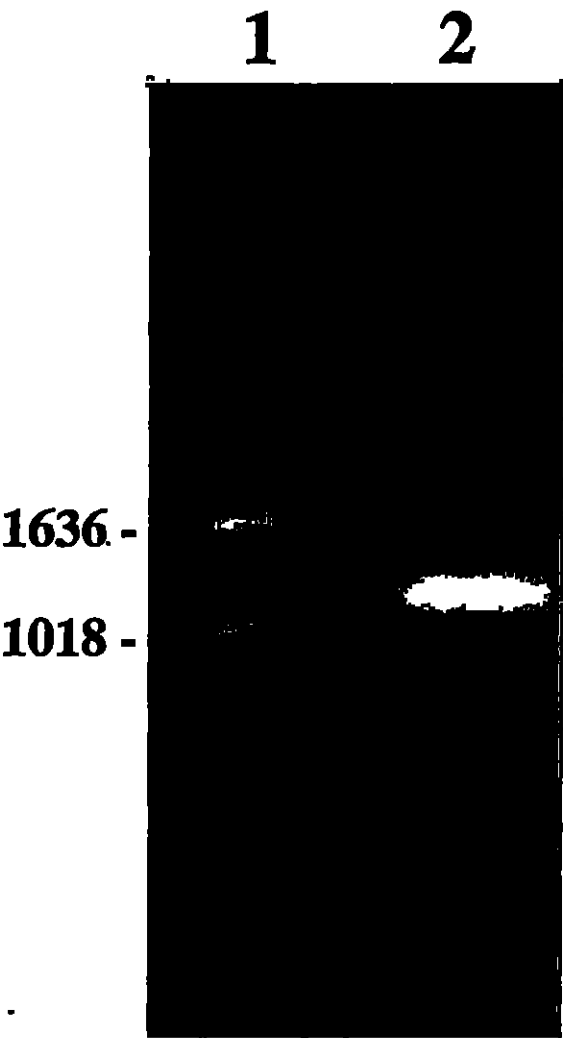


Fig. 1

2 / 15

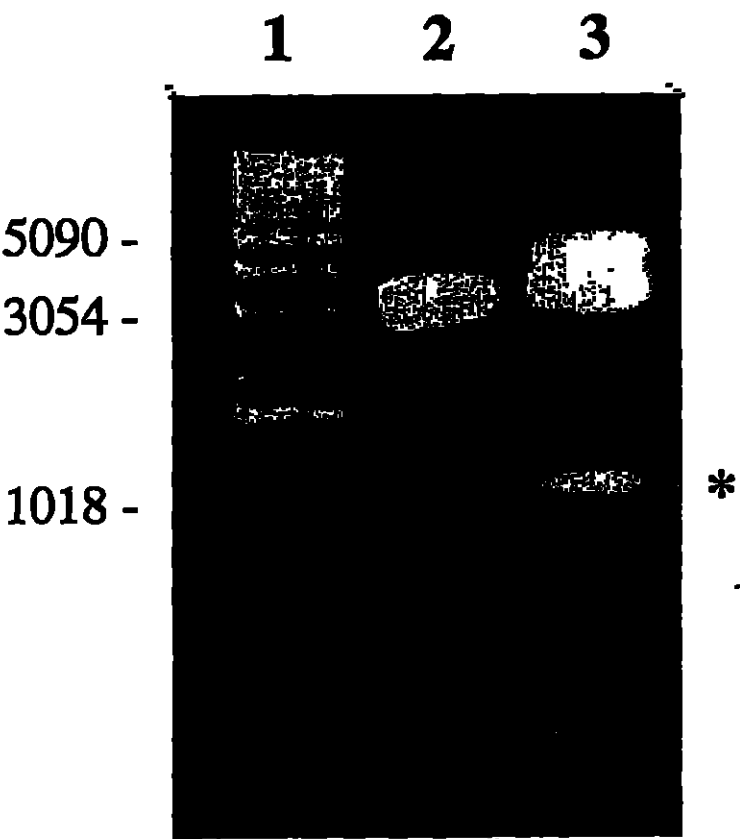


Fig. 2

3/15

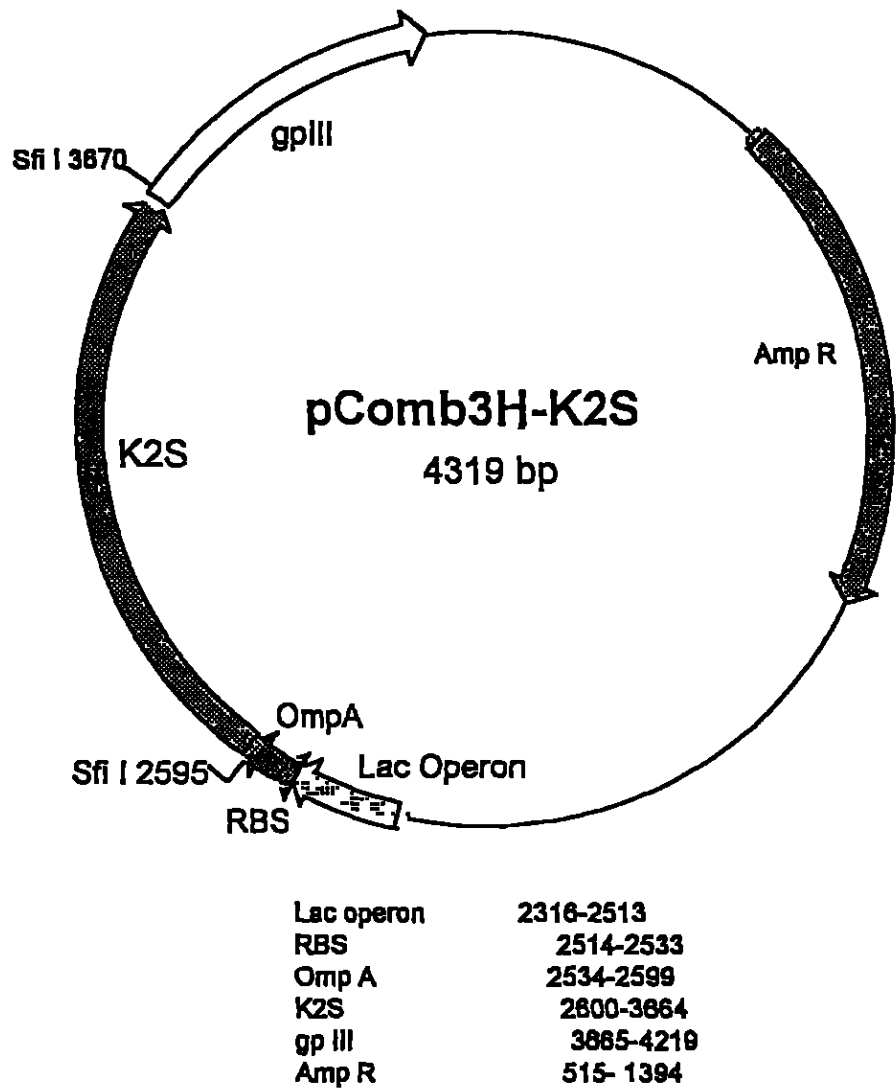


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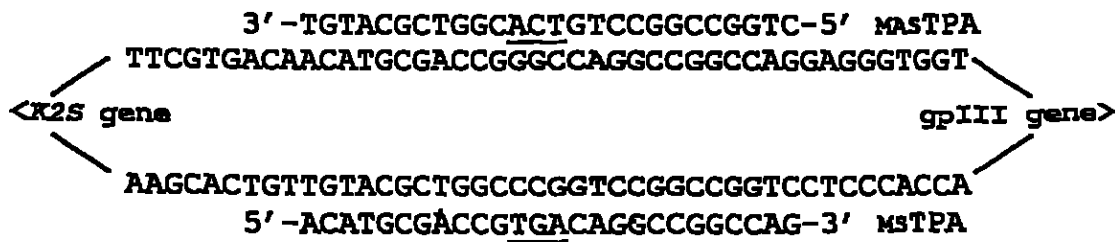


Figure 4

4/15

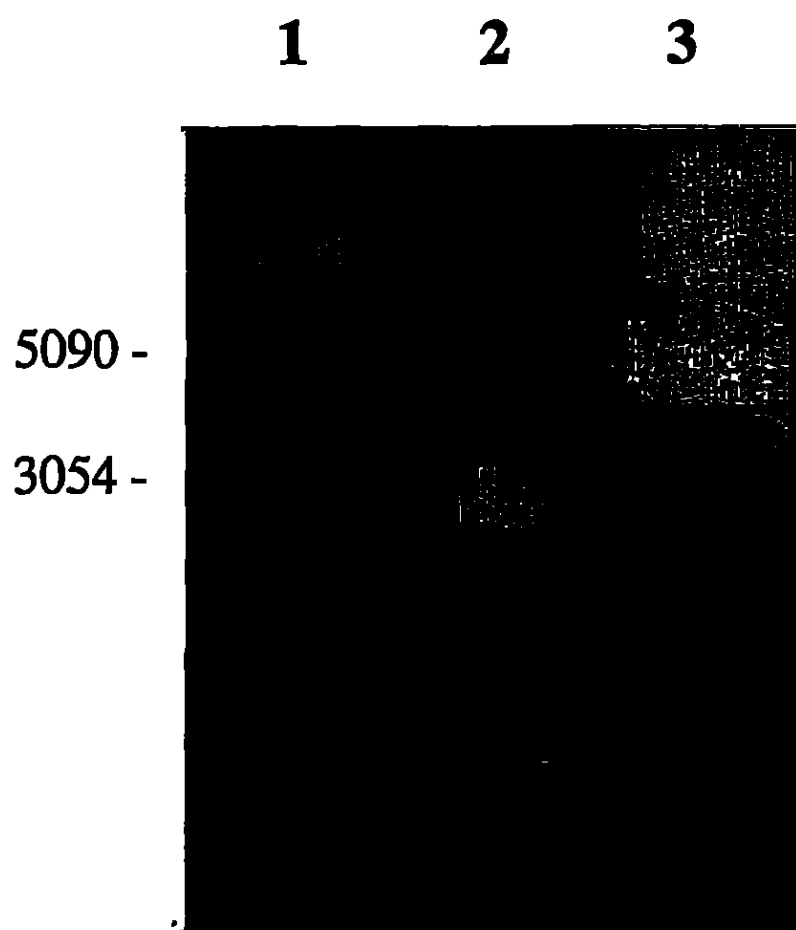


**Fig. 5**

32

32

5/15

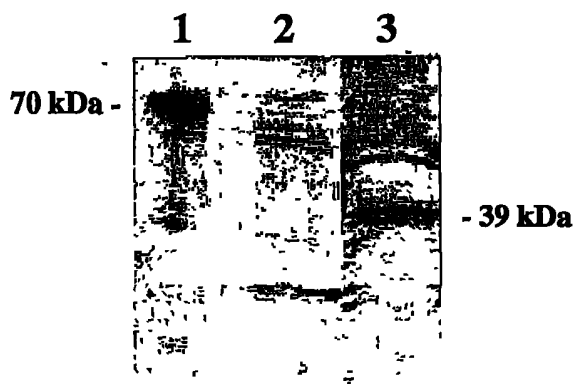


Fig. 6

6/15

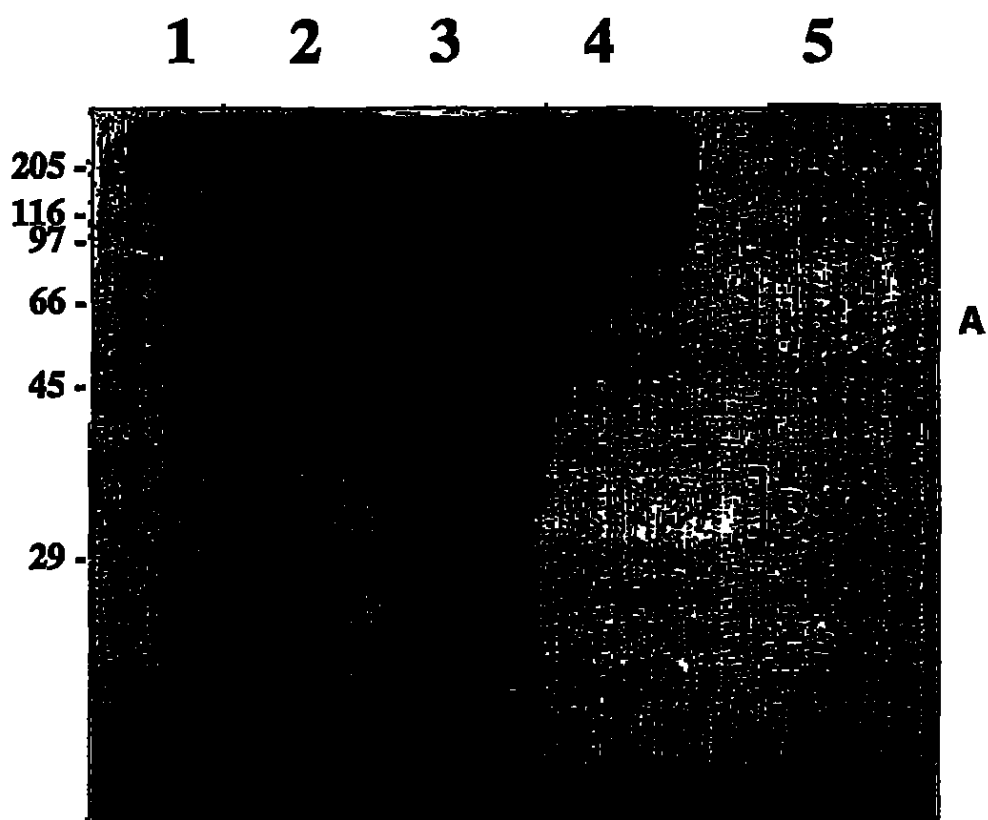


**Fig. 7**

23

23

7/15

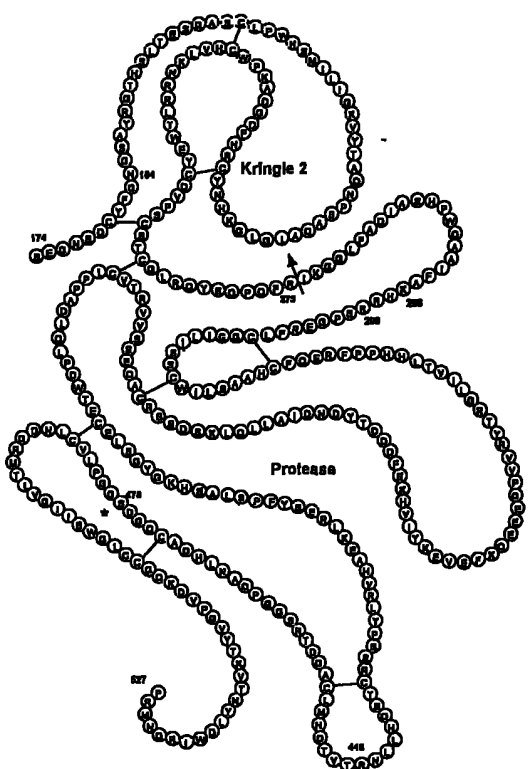


Fig. 8

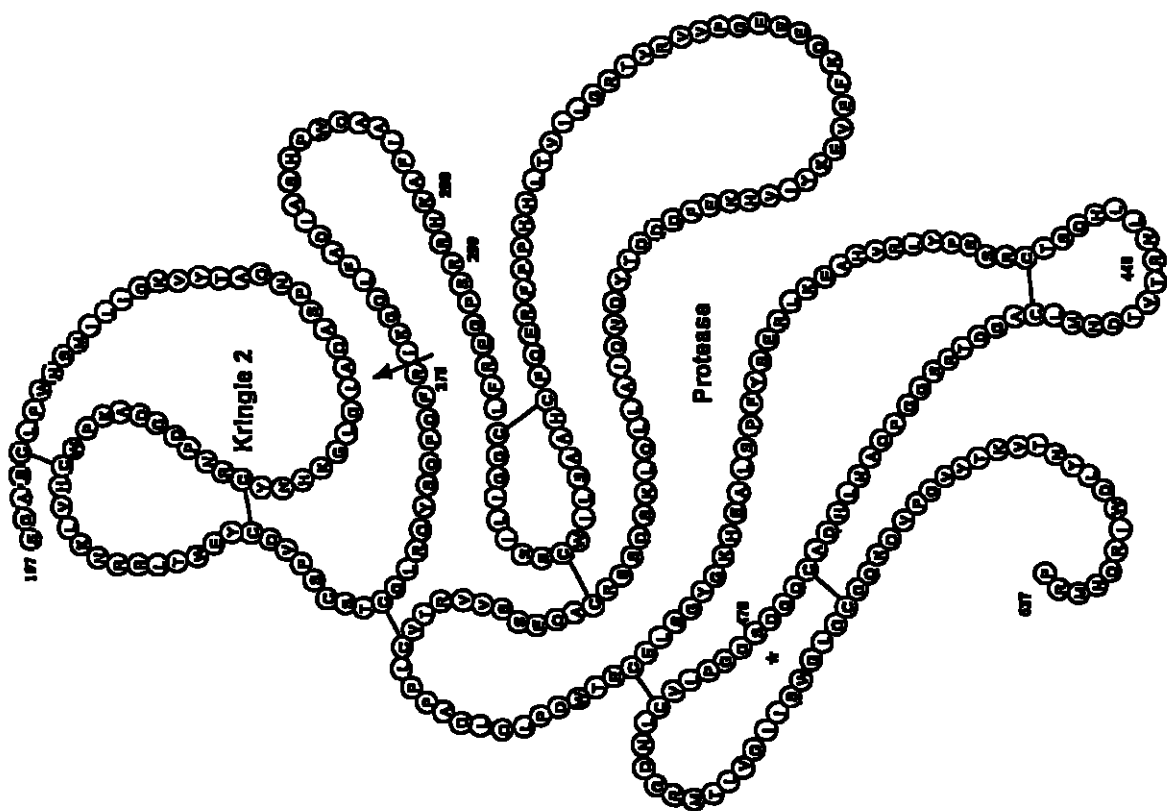


Fig. 9

34

9/15

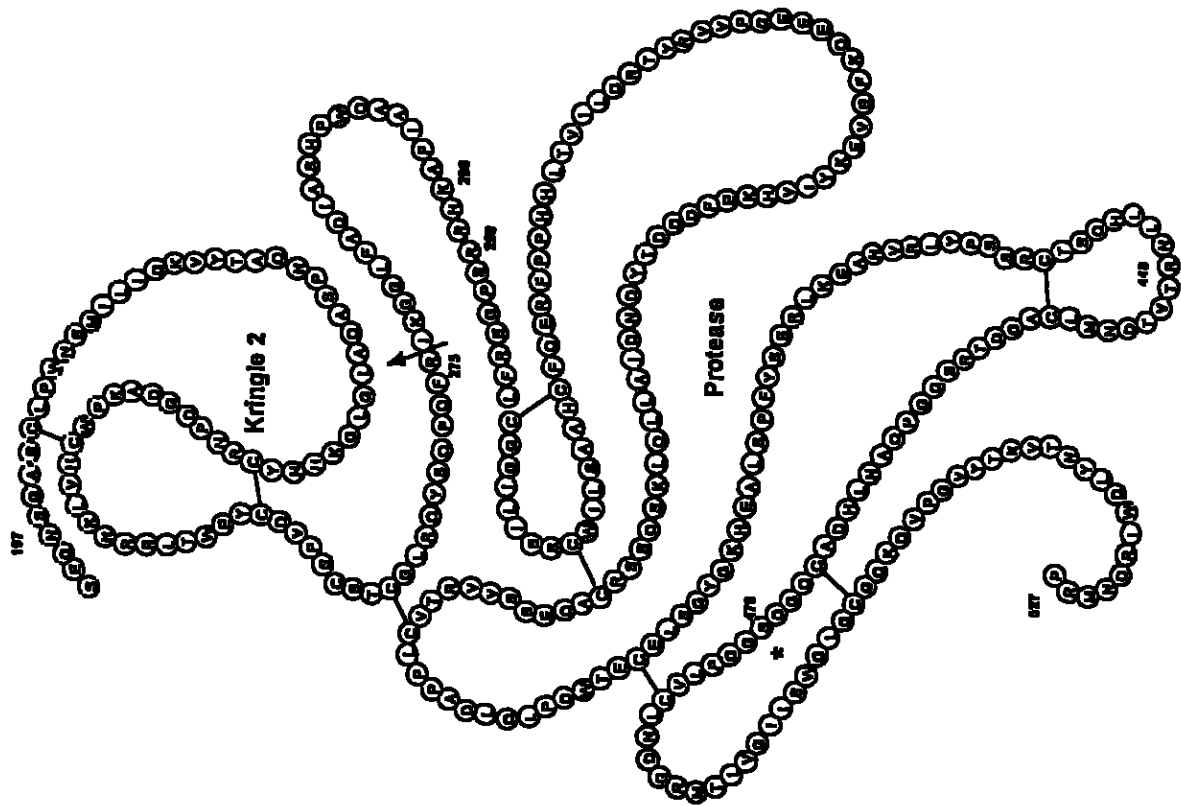


Fig. 10

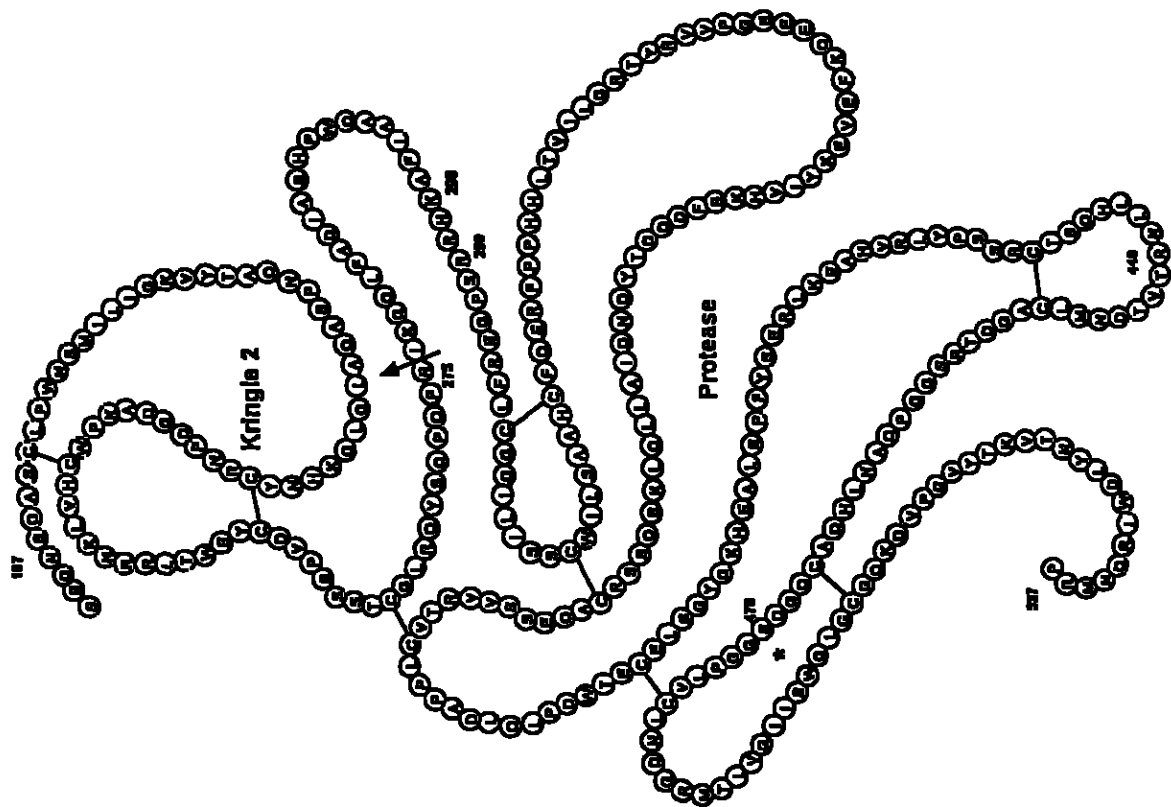


Fig 11

35

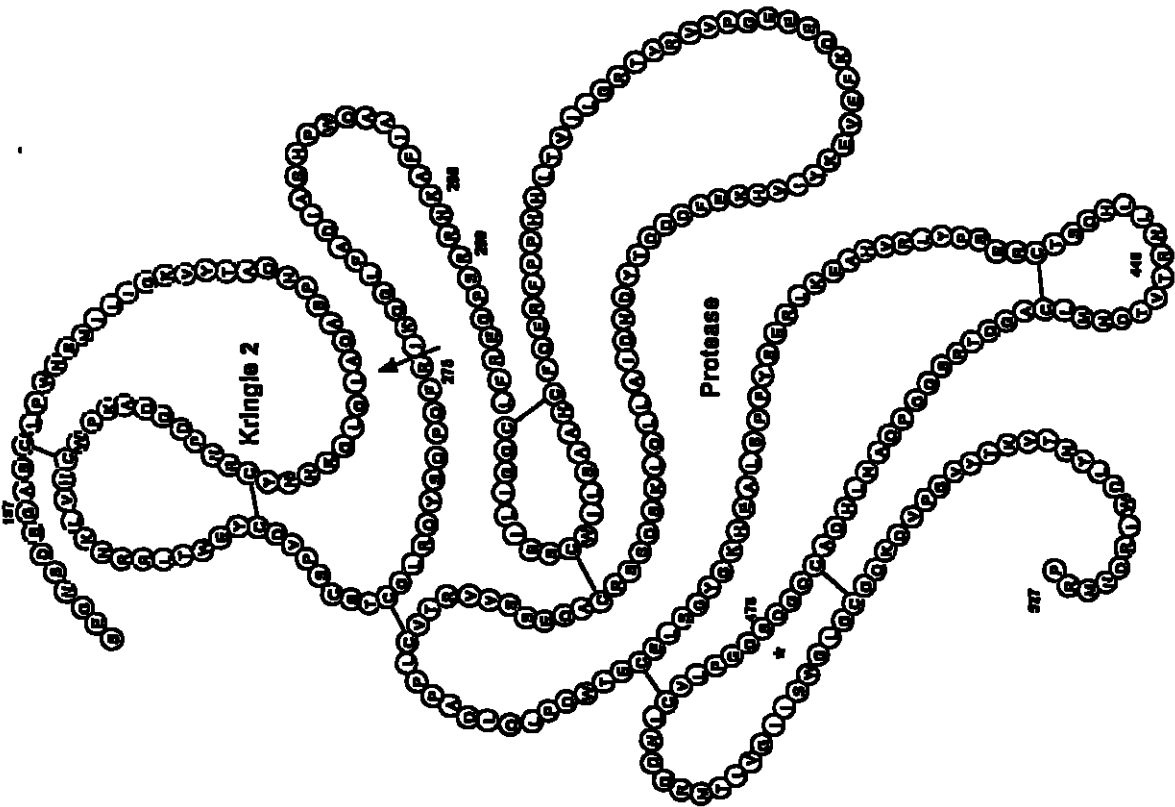


Fig. 12

12/15

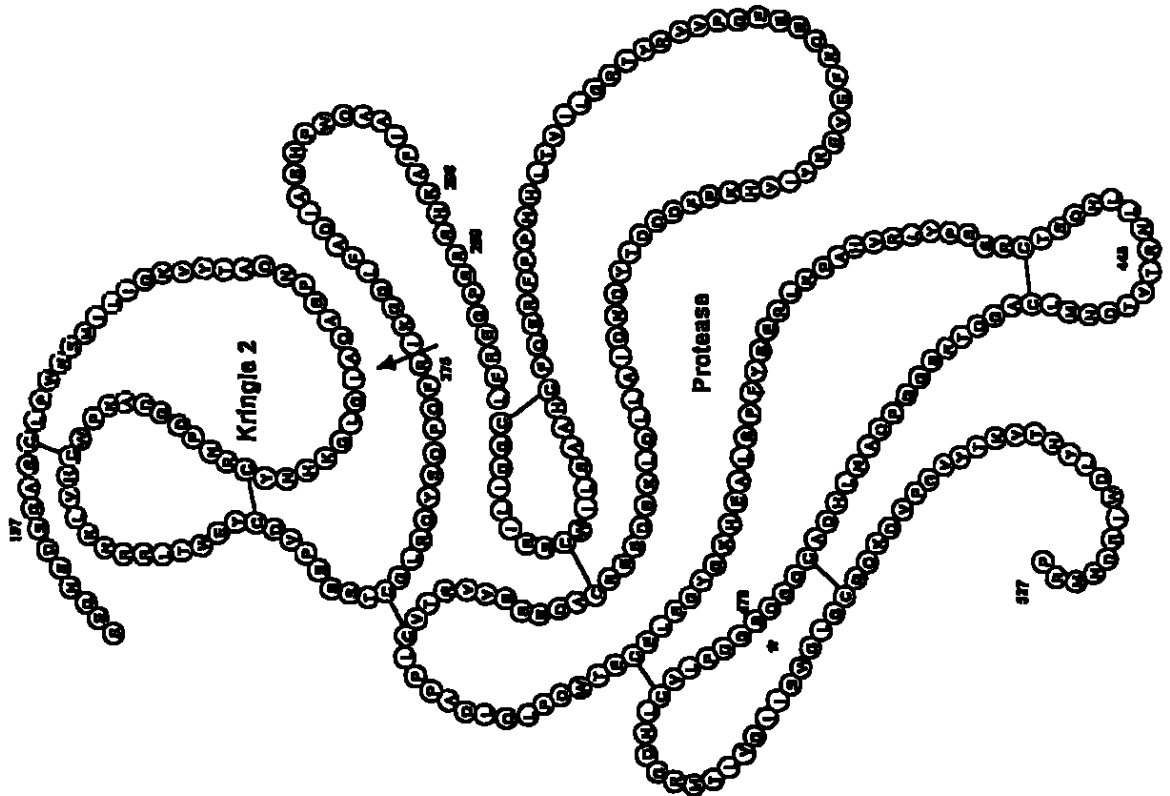


**Fig. 13**

36  
36

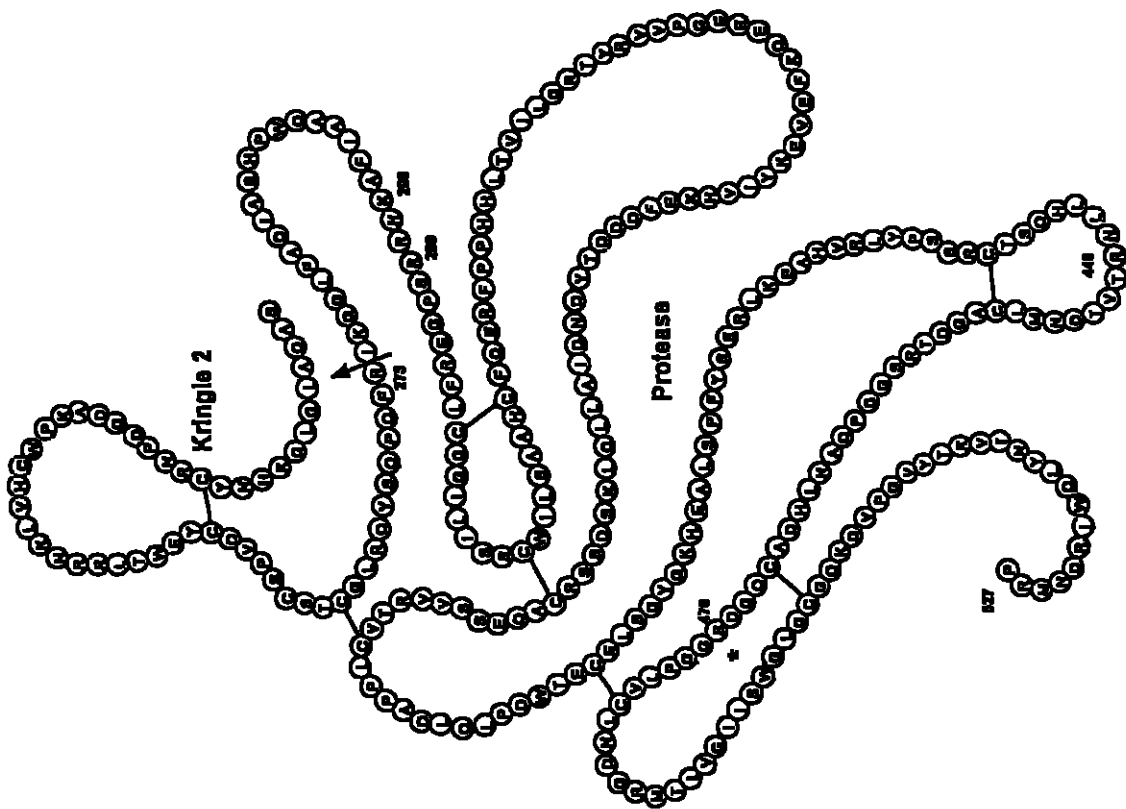


Fig. 14

14/15

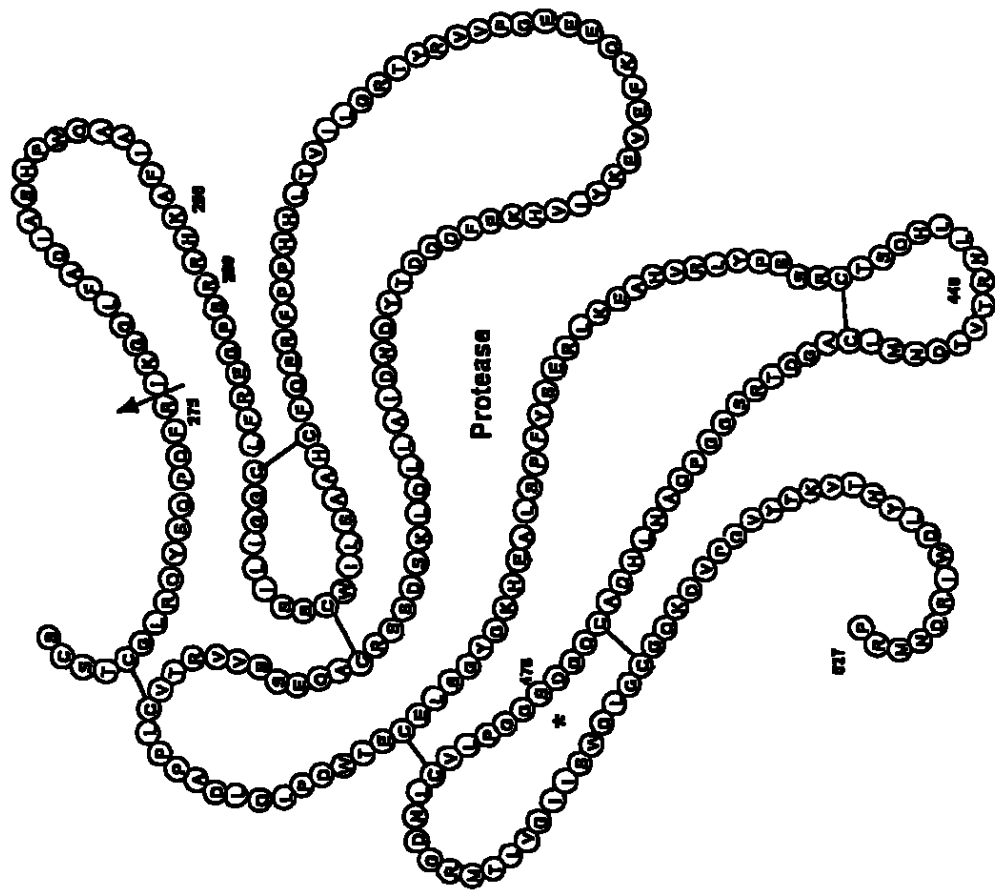


**Fig. 15**

37

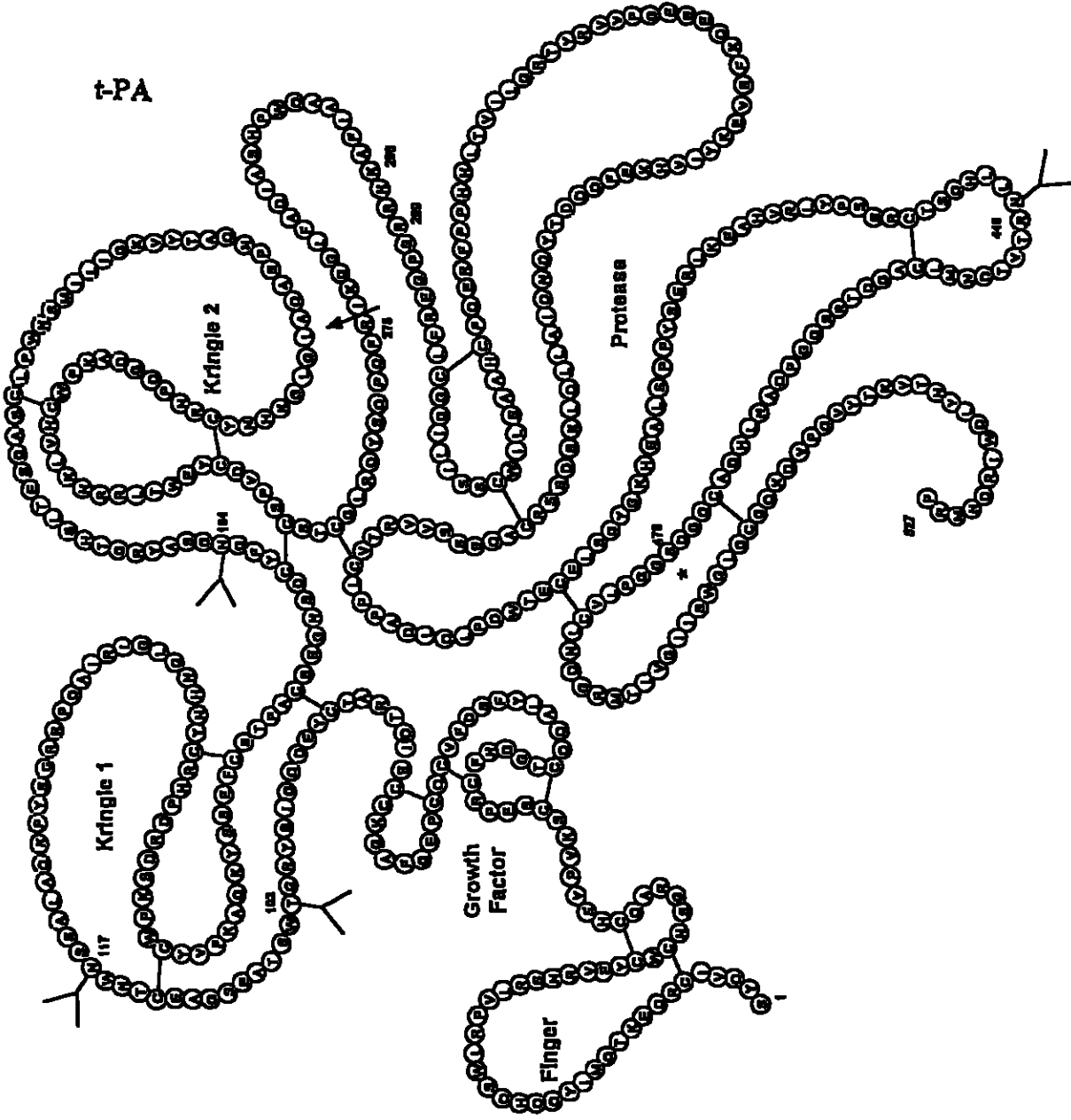


Fig. 16

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<211> 1065

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Ser Cys Leu Pro Trp Asn Ser Met Ile Leu Ile Gly Lys Val Tyr Thr  
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Ala Gln Asn Pro Ser Ala Gln Ala Leu Gly Leu Gly Lys His Asn Tyr  
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Cys Arg Asn Pro Asp Gly Asp Ala Lys Pro Trp Cys His Val Leu Lys  
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Leu Phe Ala Asp Ile Ala Ser His Pro Trp Gln Ala Ala Ile Phe Ala  
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Lys His Arg Arg Ser Pro Gly Glu Arg Phe Leu Cys Gly Gly Ile Leu  
145 150 155 160

Ile Ser Ser Cys Trp Ile Leu Ser Ala Ala His Cys Phe Gln Glu Arg  
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Phe Pro Pro His His Leu Thr Val Ile Leu Gly Arg Thr Tyr Arg Val  
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Val Pro Gly Glu Glu Glu Gln Lys Phe Glu Val Glu Lys Tyr Ile Val  
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His Lys Glu Phe Asp Asp Asp Thr Tyr Asp Asn Asp Ile Ala Leu Leu  
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Gln Leu Lys Ser Asp Ser Ser Arg Cys Ala Gln Glu Ser Ser Val Val  
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Arg Thr Val Cys Leu Pro Pro Ala Asp Leu Gln Leu Pro Asp Trp Thr  
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Glu Cys Glu Leu Ser Gly Tyr Gly Lys His Glu Ala Leu Ser Pro Phe  
260 265 270

Tyr Ser Glu Arg Leu Lys Glu Ala His Val Arg Leu Tyr Pro Ser Ser  
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Arg Cys Thr Ser Gln His Leu Leu Asn Arg Thr Val Thr Asp Asn Met  
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Asp Ala Cys Gln Gly Asp Ser Gly Gly Pro Leu Val Cys Leu Asn Asp  
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**<212> PRT**

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**Ser Glu Gly Asn**

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Ser Met Ile Leu Ile Gly Lys Val Tyr Thr Ala Gln Asn Pro Ser Ala

35

40

45

Gln Ala Leu Gly Leu Gly Lys His Asn Tyr Cys Arg Asn Pro Asp Gly

50

55

60

Asp Ala Lys Pro Trp Cys His Val Leu Lys Asn Arg Arg Leu Thr Trp

65

70

75

80

Glu Tyr Cys Asp Val Pro Ser Cys Ser Thr Cys Gly Leu Arg Gln Tyr

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|   | 115                                                             | 120 | 125     |
|   | Gly Glu Arg Phe Leu Cys Gly Gly Ile Leu Ile Ser Ser Cys Trp Ile |     |         |
| " | 130                                                             | 135 | 140     |
|   | Leu Ser Ala Ala His Cys Phe Gln Glu Arg Phe Pro Pro His His Leu |     |         |
|   | 145                                                             | 150 | 155 160 |
| " | Thr Val Ile Leu Gly Arg Thr Tyr Arg Val Val Pro Gly Glu Glu Glu |     |         |
|   | 165                                                             | 170 | 175     |
|   | Gln Lys Phe Glu Val Glu Lys Tyr Ile Val His Lys Glu Phe Asp Asp |     |         |
|   | 180                                                             | 185 | 190     |
| " | Asp Thr Tyr Asp Asn Asp Ile Ala Leu Leu Gln Leu Lys Ser Asp Ser |     |         |
|   | 195                                                             | 200 | 205     |
|   | Ser Arg Cys Ala Gln Glu Ser Ser Val Val Arg Thr Val Cys Leu Pro |     |         |
| " | 210                                                             | 215 | 220     |
|   | Pro Ala Asp Leu Gln Leu Pro Asp Trp Thr Glu Cys Glu Leu Ser Gly |     |         |
|   | 225                                                             | 230 | 235 240 |
| " | Tyr Gly Lys His Glu Ala Leu Ser Pro Phe Tyr Ser Glu Arg Leu Lys |     |         |
|   | 245                                                             | 250 | 255     |
|   | Glu Ala His Val Arg Leu Tyr Pro Ser Ser Arg Cys Thr Ser Gln His |     |         |
|   | 260                                                             | 265 | 270     |
| " | Leu Leu Asn Arg Thr Val Thr Asp Asn Met Leu Cys Ala Gly Asp Thr |     |         |
|   | 275                                                             | 280 | 285     |
|   | Arg Ser Gly Gly Pro Gln Ala Asn Leu His Asp Ala Cys Gln Gly Asp |     |         |

290

295

300

Ser Gly Gly Pro Leu Val Cys Leu Asn Asp Gly Arg Met Thr Leu Val  
305 310 315 320

Gly Ile Ile Ser Trp Gly Leu Gly Cys Gly Gln Lys Asp Val Pro Gly  
325 330 335

Val Tyr Thr Lys Val Thr Asn Tyr Leu Asp Trp Ile Arg Asp Asn Met  
340 345 350

Arg Pro

&lt;210&gt; 12

&lt;211&gt; 331

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: K2S 197-527

&lt;400&gt; 12

Ser Gly Ala Ser Cys Leu Pro Trp Asn Ser Met Ile Leu Ile Gly Lys  
1 5 10 15

Val Tyr Thr Ala Gln Asn Pro Ser Ala Gln Ala Leu Gly Leu Gly Lys  
20 25 30

His Asn Tyr Cys Arg Asn Pro Asp Gly Asp Ala Lys Pro Trp Cys His  
35 40 45

Val Leu Lys Asn Arg Arg Leu Thr Trp Glu Tyr Cys Asp Val Pro Ser  
50 55 60

Cys Ser Thr Cys Gly Leu Arg Gln Tyr Ser Gln Pro Gln Phe Arg Ile  
65 70 75 80

Lys Gly Gly Leu Phe Ala Asp Ile Ala Ser His Pro Trp Gln Ala Ala  
85 90 95

Ile Phe Ala Lys His Arg Arg Ser Pro Gly Glu Arg Phe Leu Cys Gly  
100 105 110

Gly Ile Leu Ile Ser Ser Cys Trp Ile Leu Ser Ala Ala His Cys Phe  
115 120 125

Gln Glu Arg Phe Pro Pro His His Leu Thr Val Ile Leu Gly Arg Thr  
130 135 140

Tyr Arg Val Val Pro Gly Glu Glu Glu Gln Lys Phe Glu Val Glu Lys  
145 150 155 160

Tyr Ile Val His Lys Glu Phe Asp Asp Asp Thr Tyr Asp Asn Asp Ile  
165 170 175

Ala Leu Leu Gln Leu Lys Ser Asp Ser Ser Arg Cys Ala Gln Glu Ser  
180 185 190

Ser Val Val Arg Thr Val Cys Leu Pro Pro Ala Asp Leu Gln Leu Pro  
195 200 205

Asp Trp Thr Glu Cys Glu Leu Ser Gly Tyr Gly Lys His Glu Ala Leu  
210 215 220

Ser Pro Phe Tyr Ser Glu Arg Leu Lys Glu Ala His Val Arg Leu Tyr  
225 230 235 240

Pro Ser Ser Arg Cys Thr Ser Gln His Leu Leu Asn Arg Thr Val Thr  
245 250 255

Asp Asn Met Leu Cys Ala Gly Asp Thr Arg Ser Gly Gly Pro Gln Ala  
260 265 270

Asn Leu His Asp Ala Cys Gln Gly Asp Ser Gly Gly Pro Leu Val Cys  
275 280 285

Leu Asn Asp Gly Arg Met Thr Leu Val Gly Ile Ile Ser Trp Gly Leu  
290 295 300

Gly Cys Gly Gln Lys Asp Val Pro Gly Val Tyr Thr Lys Val Thr Asn  
305 310 315 320

Tyr Leu Asp Trp Ile Arg Asp Asn Met Arg Pro  
325 330

<210> 13

<211> 339

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence. K2S 193-527,  
modified

<400> 13

Ser Glu Gly Asn Ser Leu Thr Glu Ser Gly Ala Ser Cys Leu Pro Trp  
1 5 10 15

Asn Ser Met Ile Leu Ile Gly Lys Val Tyr Thr Ala Gln Asn Pro Ser  
20 25 30

Ala Gln Ala Leu Gly Leu Gly Lys His Asn Tyr Cys Arg Asn Pro Asp  
35 40 45

Gly Asp Ala Lys Pro Trp Cys His Val Leu Lys Asn Arg Arg Leu Thr  
50 55 60

Trp Glu Tyr Cys Asp Val Pro Ser Cys Ser Thr Cys Gly Leu Arg Gln  
65 70 75 80

Tyr Ser Gln Pro Gln Phe Arg Ile Lys Gly Gly Leu Phe Ala Asp Ile  
85 90 95

Ala Ser His Pro Trp Gln Ala Ala Ile Phe Ala Lys His Arg Arg Ser

100 105 110

Pro Gly Glu Arg Phe Leu Cys Gly Gly Ile Leu Ile Ser Ser Cys Trp  
115 120 125

Ile Leu Ser Ala Ala His Cys Phe Gln Glu Arg Phe Pro Pro His His  
130 135 140

Leu Thr Val Ile Leu Gly Arg Thr Tyr Arg Val Val Pro Gly Glu Glu  
145 150 155 160

Glu Gln Lys Phe Glu Val Glu Lys Tyr Ile Val His Lys Glu Phe Asp  
165 170 175

Asp Asp Thr Tyr Asp Asn Asp Ile Ala Leu Leu Gln Leu Lys Ser Asp  
180 185 190

Ser Ser Arg Cys Ala Gln Glu Ser Ser Val Val Arg Thr Val Cys Leu  
195 200 205

Pro Pro Ala Asp Leu Gln Leu Pro Asp Trp Thr Glu Cys Glu Leu Ser  
210 215 220

Gly Tyr Gly Lys His Glu Ala Leu Ser Pro Phe Tyr Ser Glu Arg Leu  
225 230 235 240

Lys Glu Ala His Val Arg Leu Tyr Pro Ser Ser Arg Cys Thr Ser Gln  
245 250 255

His Leu Leu Asn Arg Thr Val Thr Asp Asn Met Leu Cys Ala Gly Asp  
260 265 270

Thr Arg Ser Gly Gly Pro Gln Ala Asn Leu His Asp Ala Cys Gln Gly  
275 280 285

Asp Ser Gly Gly Pro Leu Val Cys Leu Asn Asp Gly Arg Met Thr Leu  
290 295 300

Val Gly Ile Ile Ser Trp Gly Leu Gly Cys Gly Gln Lys Asp Val Pro

24

305                      310                      315                      320

Gly Val Tyr Thr Lys Val Thr Asn Tyr Leu Asp Trp Ile Arg Asp Asn

                         325                      330                      335

Met Arg Pro

<210> 14  
<211> 335  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: K2S 193-527,  
modified

<400> 14

Ser Leu Thr Glu Ser Gly Ala Ser Cys Leu Pro Trp Asn Ser Met Ile

1                      5                      10                      15

Leu Ile Gly Lys Val Tyr Thr Ala Gln Asn Pro Ser Ala Gln Ala Leu

20                      25                      30

Gly Leu Gly Lys His Asn Tyr Cys Arg Asn Pro Asp Gly Asp Ala Lys

35                      40                      45

Pro Trp Cys His Val Leu Lys Asn Arg Arg Leu Thr Trp Glu Tyr Cys

50                      55                      60

Asp Val Pro Ser Ser Ser Thr Cys Gly Leu Arg Gln Tyr Ser Gln Pro

65                      70                      75                      80

Gln Phe Arg Ile Lys Gly Gly Leu Phe Ala Asp Ile Ala Ser His Pro

85                      90                      95

Trp Gln Ala Ala Ile Phe Ala Lys His Arg Arg Ser Pro Gly Glu Arg

100                      105                      110

- Phe Leu Cys Gly Gly Ile Leu Ile Ser Ser Cys Trp Ile Leu Ser Ala  
115 120 125

, Ala His Cys Phe Gln Glu Arg Phe Pro Pro His His Leu Thr Val Ile  
130 135 140

Leu Gly Arg Thr Tyr Arg Val Val Pro Gly Glu Glu Glu Gln Lys Phe  
145 150 155 160

" Glu Val Glu Lys Tyr Ile Val His Lys Glu Phe Asp Asp Asp Thr Tyr  
165 170 175

Asp Asn Asp Ile Ala Leu Leu Gln Leu Lys Ser Asp Ser Ser Arg Cys  
" 180 185 190

Ala Gln Glu Ser Ser Val Val Arg Thr Val Cys Leu Pro Pro Ala Asp  
195 200 205

" Leu Gln Leu Pro Asp Trp Thr Glu Cys Glu Leu Ser Gly Tyr Gly Lys  
210 215 220

His Glu Ala Leu Ser Pro Phe Tyr Ser Glu Arg Leu Lys Glu Ala His  
225 230 235 240

" Val Arg Leu Tyr Pro Ser Ser Arg Cys Thr Ser Gln His Leu Leu Asn  
245 250 255

Arg Thr Val Thr Asp Asn Met Leu Cys Ala Gly Asp Thr Arg Ser Gly  
" 260 265 270

Gly Pro Gln Ala Asn Leu His Asp Ala Cys Gln Gly Asp Ser Gly Gly  
275 280 285

" Pro Leu Val Cys Leu Asn Asp Gly Arg Met Thr Leu Val Gly Ile Ile  
290 295 300

Ser Trp Gly Leu Gly Cys Gly Gln Lys Asp Val Pro Gly Val Tyr Thr  
305 310 315 320

45

- Lys Val Thr Asn Tyr Leu Asp Trp Ile Arg Asp Asn Met Arg Pro  
325 330 335

<210> 15

<211> 343

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence. K2S 191-527,  
modified

<400> 15

Ser Glu Gly Asn Ser Asp Thr His Ser Leu Thr Glu Ser Gly Ala Ser  
1 5 10 15

Cys Leu Pro Trp Asn Ser Met Ile Leu Ile Gly Lys Val Tyr Thr Ala  
20 25 30

Gln Asn Pro Ser Ala Gln Ala Leu Gly Leu Gly Lys His Asn Tyr Cys  
35 40 45

Arg Asn Pro Asp Gly Asp Ala Lys Pro Trp Cys His Val Leu Lys Asn  
50 55 60

Arg Arg Leu Thr Trp Glu Tyr Cys Asp Val Pro Ser Cys Ser Thr Cys  
65 70 75 80

Gly Leu Arg Gln Tyr Ser Gln Pro Gln Phe Arg Ile Lys Gly Gly Leu  
85 90 95

Phe Ala Asp Ile Ala Ser His Pro Trp Gln Ala Ala Ile Phe Ala Lys  
100 105 110

His Arg Arg Ser Pro Gly Glu Arg Phe Leu Cys Gly Gly Ile Leu Ile  
115 120 125

```

Ser Ser Cys Trp Ile Leu Ser Ala Ala His Cys Phe Gln Glu Arg Phe
-   130                               135                   140

Pro Pro His His Leu Thr Val Ile Leu Gly Arg Thr Tyr Arg Val Val
,   145                               150                   155                   160

Pro Gly Glu Glu Glu Gln Lys Phe Glu Val Glu Lys Tyr Ile Val His
                                165                   170                   175

"   Lys Glu Phe Asp Asp Asp Thr Tyr Asp Asn Asp Ile Ala Leu Leu Gln
                                180                   185                   190

Leu Lys Ser Asp Ser Ser Arg Cys Ala Gln Glu Ser Ser Val Val Arg
                                195                   200                   205

"   Thr Val Cys Leu Pro Pro Ala Asp Leu Gln Leu Pro Asp Trp Thr Glu
                                210                   215                   220

Cys Glu Leu Ser Gly Tyr Gly Lys His Glu Ala Leu Ser Pro Phe Tyr
"   225                               230                   235                   240

Ser Glu Arg Leu Lys Glu Ala His Val Arg Leu Tyr Pro Ser Ser Arg
                                245                   250                   255

"   Cys Thr Ser Gln His Leu Leu Asn Arg Thr Val Thr Asp Asn Met Leu
                                260                   265                   270

Cys Ala Gly Asp Thr Arg Ser Gly Gly Pro Gln Ala Asn Leu His Asp
                                275                   280                   285

"   Ala Cys Gln Gly Asp Ser Gly Gly Pro Leu Val Cys Leu Asn Asp Gly
                                290                   295                   300

Arg Met Thr Leu Val Gly Ile Ile Ser Trp Gly Leu Gly Cys Gly Gln
"   305                               310                   315                   320

Lys Asp Val Pro Gly Val Tyr Thr Lys Val Thr Asn Tyr Leu Asp Trp
                                325                   330                   335

```

Ile Arg Asp Asn Met Arg Pro  
340

<210> 16

<211> 343

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence K2S 191-527,  
modified

<400> 16

Ser Glu Gly Asn Ser Asp Thr His Ser Leu Thr Glu Ser Gly Ala Ser  
1 5 10 15

Cys Leu Pro Trp Asn Ser Met Ile Leu Ile Gly Lys Val Tyr Thr Ala  
20 25 30

35

Gln Asn Pro Ser Ala Gln Ala Leu Gly Leu Gly Lys His Asn Tyr Cys  
40 45

Arg Asn Pro Asp Gly Asp Ala Lys Pro Trp Cys His Val Leu Lys Asn  
50 55 60

Arg Arg Leu Thr Trp Glu Tyr Cys Asp Val Pro Ser Ser Ser Thr Cys  
65 70 75 80

Gly Leu Arg Gln Tyr Ser Gln Pro Gln Phe Arg Ile Lys Gly Gly Leu  
85 90 95

Phe Ala Asp Ile Ala Ser His Pro Trp Gln Ala Ala Ile Phe Ala Lys  
100 105 110

115

His Arg Arg Ser Pro Gly Glu Arg Phe Leu Cys Gly Gly Ile Leu Ile  
120 125

Ser Ser Cys Trp Ile Leu Ser Ala Ala His Cys Phe Gln Glu Arg Phe

130

135

140

Pro Pro His His Leu Thr Val Ile Leu Gly Arg Thr Tyr Arg Val Val  
 145 150 155 160

Pro Gly Glu Glu Glu Gln Lys Phe Glu Val Glu Lys Tyr Ile Val His  
 165 170 175

Lys Glu Phe Asp Asp Asp Thr Tyr Asp Asn Asp Ile Ala Leu Leu Gln  
 180 185 190

Leu Lys Ser Asp Ser Ser Arg Cys Ala Gln Glu Ser Ser Val Val Arg  
 195 200 205

Thr Val Cys Leu Pro Pro Ala Asp Leu Gln Leu Pro Asp Trp Thr Glu  
 210 215 220

Cys Glu Leu Ser Gly Tyr Gly Lys His Glu Ala Leu Ser Pro Phe Tyr  
 225 230 235 240

Ser Glu Arg Leu Lys Glu Ala His Val Arg Leu Tyr Pro Ser Ser Arg  
 245 250 255

Cys Thr Ser Gln His Leu Leu Asn Arg Thr Val Thr Asp Asn Met Leu  
 260 265 270

Cys Ala Gly Asp Thr Arg Ser Gly Gly Pro Gln Ala Asn Leu His Asp  
 275 280 285

Ala Cys Gln Gly Asp Ser Gly Gly Pro Leu Val Cys Leu Asn Asp Gly  
 290 295 300

Arg Met Thr Leu Val Gly Ile Ile Ser Trp Gly Leu Gly Cys Gly Gln  
 305 310 315 320

Lys Asp Val Pro Gly Val Tyr Thr Lys Val Thr Asn Tyr Leu Asp Trp  
 325 330 335

Ile Arg Asp Asn Met Arg Pro

340

&lt;210&gt; 17

&lt;211&gt; 308

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: K2S 220-527

&lt;400&gt; 17

Ser Ala Gln Ala Leu Gly Leu Gly Lys His Asn Tyr Cys Arg Asn Pro

1

5

10

15

Asp Gly Asp Ala Lys Pro Trp Cys His Val Leu Lys Asn Arg Arg Leu

20

25

30

Thr Trp Glu Tyr Cys Asp Val Pro Ser Cys Ser Thr Cys Gly Leu Arg

35

40

45

Gln Tyr Ser Gln Pro Gln Phe Arg Ile Lys Gly Gly Leu Phe Ala Asp

50

55

60

Ile Ala Ser His Pro Trp Gln Ala Ala Ile Phe Ala Lys His Arg Arg

65

70

75

80

Ser Pro Gly Glu Arg Phe Leu Cys Gly Gly Ile Leu Ile Ser Ser Cys

85

90

95

Trp Ile Leu Ser Ala Ala His Cys Phe Gln Glu Arg Phe Pro Pro His

100

105

110

His Leu Thr Val Ile Leu Gly Arg Thr Tyr Arg Val Val Pro Gly Glu

115

120

125

Glu Glu Gln Lys Phe Glu Val Glu Lys Tyr Ile Val His Lys Glu Phe

130

135

140

- Asp Asp Asp Thr Tyr Asp Asn Asp Ile Ala Leu Leu Gln Leu Lys Ser  
145 150 155 160

' Asp Ser Ser Arg Cys Ala Gln Glu Ser Ser Val Val Arg Thr Val Cys  
165 170 175

Leu Pro Pro Ala Asp Leu Gln Leu Pro Asp Trp Thr Glu Cys Glu Leu  
180 185 190

" Ser Gly Tyr Gly Lys His Glu Ala Leu Ser Pro Phe Tyr Ser Glu Arg  
195 200 205

Leu Lys Glu Ala His Val Arg Leu Tyr Pro Ser Ser Arg Cys Thr Ser  
210 215 220

" Gln His Leu Leu Asn Arg Thr Val Thr Asp Asn Met Leu Cys Ala Gly  
225 230 235 240

Asp Thr Arg Ser Gly Gly Pro Gln Ala Asn Leu His Asp Ala Cys Gln  
" 245 250 255

Gly Asp Ser Gly Gly Pro Leu Val Cys Leu Asn Asp Gly Arg Met Thr  
260 265 270

" Leu Val Gly Ile Ile Ser Trp Gly Leu Gly Cys Gly Gln Lys Asp Val  
275 280 285

Pro Gly Val Tyr Thr Lys Val Thr Asn Tyr Leu Asp Trp Ile Arg Asp  
290 295 300

" Asn Met Arg Pro  
305

" <210> 18  
<211> 268  
<212> PRT  
<213> Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence. K2S 260-527

&lt;400&gt; 18

, Ser Cys Ser Thr Cys Gly Leu Arg Gln Tyr Ser Gln Pro Gln Phe Arg  
1 5 10 15

Ile Lys Gly Gly Leu Phe Ala Asp Ile Ala Ser His Pro Trp Gln Ala  
20 25 30

"

Ala Ile Phe Ala Lys His Arg Arg Ser Pro Gly Glu Arg Phe Leu Cys  
35 40 45

"

Gly Gly Ile Leu Ile Ser Ser Cys Trp Ile Leu Ser Ala Ala His Cys  
50 55 60

Phe Gln Glu Arg Phe Pro Pro His His Leu Thr Val Ile Leu Gly Arg  
65 70 75 80

"

Thr Tyr Arg Val Val Pro Gly Glu Glu Glu Gln Lys Phe Glu Val Glu  
85 90 95

Lys Tyr Ile Val His Lys Glu Phe Asp Asp Asp Thr Tyr Asp Asn Asp  
100 105 110

"

Ile Ala Leu Leu Gln Leu Lys Ser Asp Ser Ser Arg Cys Ala Gln Glu  
115 120 125

"

Ser Ser Val Val Arg Thr Val Cys Leu Pro Pro Ala Asp Leu Gln Leu  
130 135 140

Pro Asp Trp Thr Glu Cys Glu Leu Ser Gly Tyr Gly Lys His Glu Ala  
145 150 155 160

"

Leu Ser Pro Phe Tyr Ser Glu Arg Leu Lys Glu Ala His Val Arg Leu  
165 170 175

Tyr Pro Ser Ser Arg Cys Thr Ser Gln His Leu Leu Asn Arg Thr Val  
180 185 190

- Thr Asp Asn Met Leu Cys Ala Gly Asp Thr Arg Ser Gly Gly Pro Gln  
195 200 205

, Ala Asn Leu His Asp Ala Cys Gln Gly Asp Ser Gly Gly Pro Leu Val  
210 215 220

Cys Leu Asn Asp Gly Arg Met Thr Leu Val Gly Ile Ile Ser Trp Gly  
225 230 235 240

"

Leu Gly Cys Gly Gln Lys Asp Val Pro Gly Val Tyr Thr Lys Val Thr  
245 250 255

Asn Tyr Leu Asp Trp Ile Arg Asp Asn Met Arg Pro

"

260 265

<210> 19

<211> 527

"

<212> PRT

<213> Homo sapiens

<400> 19

Ser Tyr Gln Val Ile Cys Arg Asp Glu Lys Thr Gln Met Ile Tyr Gln

"

1 5 10 15

Gln His Gln Ser Trp Leu Arg Pro Val Leu Arg Ser Asn Arg Val Glu

20 25 30

"

Tyr Cys Trp Cys Asn Ser Gly Arg Ala Gln Cys His Ser Val Pro Val

35 40 45

Lys Ser Cys Ser Glu Pro Arg Cys Phe Asn Gly Gly Thr Cys Gln Gln

50 55 60

"

Ala Leu Tyr Phe Ser Asp Phe Val Cys Gln Cys Pro Glu Gly Phe Ala

65 70 75 80

Gly Lys Cys Cys Glu Ile Asp Thr Arg Ala Thr Cys Tyr Glu Asp Gln

18

85

90

95

Gly Ile Ser Tyr Arg Gly Thr Trp Ser Thr Ala Glu Ser Gly Ala Glu  
100 105 110

Cys Thr Asn Trp Asn Ser Ser Ala Leu Ala Gln Lys Pro Tyr Ser Gly  
115 120 125

Arg Arg Pro Asp Ala Ile Arg Leu Gly Leu Gly Asn His Asn Tyr Cys  
130 135 140

Arg Asn Pro Asp Arg Asp Ser Lys Pro Trp Cys Tyr Val Phe Lys Ala  
145 150 155 160

Gly Lys Tyr Ser Ser Glu Phe Cys Ser Thr Pro Ala Cys Ser Glu Gly  
165 170 175

Asn Ser Asp Cys Tyr Phe Gly Asn Gly Ser Ala Tyr Arg Gly Thr His  
180 185 190

Ser Leu Thr Glu Ser Gly Ala Ser Cys Leu Pro Trp Asn Ser Met Ile  
195 200 205

Leu Ile Gly Lys Val Tyr Thr Ala Gln Asn Pro Ser Ala Gln Ala Leu  
210 215 220

Gly Leu Gly Lys His Asn Tyr Cys Arg Asn Pro Asp Gly Asp Ala Lys  
225 230 235 240

Pro Trp Cys His Val Leu Lys Asn Arg Arg Leu Thr Trp Glu Tyr Cys  
245 250 255

Asp Val Pro Ser Cys Ser Thr Cys Gly Leu Arg Gln Tyr Ser Gln Pro  
260 265 270

Gln Phe Arg Ile Lys Gly Gly Leu Phe Ala Asp Ile Ala Ser His Pro  
275 280 285

Trp Gln Ala Ala Ile Phe Ala Lys His Arg Arg Ser Pro Gly Glu Arg

290

295

300

Phe Leu Cys Gly Gly Ile Leu Ile Ser Ser Cys Trp Ile Leu Ser Ala  
 305 310 315 320

Ala His Cys Phe Gln Glu Arg Phe Pro Pro His His Leu Thr Val Ile  
 325 330 335

Leu Gly Arg Thr Tyr Arg Val Val Pro Gly Glu Glu Glu Gln Lys Phe  
 340 345 350

Glu Val Glu Lys Tyr Ile Val His Lys Glu Phe Asp Asp Asp Thr Tyr  
 355 360 365

Asp Asn Asp Ile Ala Leu Leu Gln Leu Lys Ser Asp Ser Ser Arg Cys  
 370 375 380

Ala Gln Glu Ser Ser Val Val Arg Thr Val Cys Leu Pro Pro Ala Asp  
 385 390 395 400

Leu Gln Leu Pro Asp Trp Thr Glu Cys Glu Leu Ser Gly Tyr Gly Lys  
 405 410 415

His Glu Ala Leu Ser Pro Phe Tyr Ser Glu Arg Leu Lys Glu Ala His  
 420 425 430

Val Arg Leu Tyr Pro Ser Ser Arg Cys Thr Ser Gln His Leu Leu Asn  
 435 440 445

Arg Thr Val Thr Asp Asn Met Leu Cys Ala Gly Asp Thr Arg Ser Gly  
 450 455 460

Gly Pro Gln Ala Asn Leu His Asp Ala Cys Gln Gly Asp Ser Gly Gly  
 465 470 475 480

Pro Leu Val Cys Leu Asn Asp Gly Arg Met Thr Leu Val Gly Ile Ile  
 485 490 495

Ser Trp Gly Leu Gly Cys Gly Gln Lys Asp Val Pro Gly Val Tyr Thr

500

505

510

Lys Val Thr Asn Tyr Leu Asp Trp Ile Arg Asp Asn Met Arg Pro  
515 520 525

&lt;210&gt; 20

&lt;211&gt; 12

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: coding  
sequence for SEGN

&lt;400&gt; 20

tctgagggaa ac

12

&lt;210&gt; 21

&lt;211&gt; 22

&lt;212&gt; PRT

&lt;213&gt; Escherichia coli

&lt;400&gt; 21

Met Lys Lys Thr Ala Ile Ala Ile Ala Val Ala Leu Ala Gly Phe Ala  
1 5 10 15

Thr Val Ala Gln Ala Ala

20

&lt;210&gt; 22

&lt;211&gt; 42

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence. primer

<400> 22

gaggaggagg tggcccaggc ggcctctgag ggaaacagtg ac

42

<210> 23

<211> 42

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 23

gaggaggagc tggccggcct ggcccggtcg catgttgtca cg

42

<210> 24

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 24

acatgcgacc gtgacaggcc ggccag

26

<210> 25

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 25

ctggccggcc tgtcacggtc gcatgt

26

## PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

|                                                          |                                                                                                                                                        |                                                         |
|----------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------|
| Applicant's or agent's file reference<br>1-1170-PCT      | <b>FOR FURTHER ACTION</b> see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, Item 5 below |                                                         |
| International application No<br>PCT/EP 01/12857          | International filing date (day/month/year)<br>07/11/2001                                                                                               | (Earliest) Priority Date (day/month/year)<br>14/11/2000 |
| Applicant<br><br>BOEHRINGER INGELHEIM INTERNATIONAL GMBH |                                                                                                                                                        |                                                         |

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

**1 Basis of the report**

- a With regard to the language, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item

☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b))

- b With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of the sequence listing

☒ contained in the international application in written form

☒ filed together with the international application in computer readable form

☐ furnished subsequently to this Authority in written form

☐ furnished subsequently to this Authority in computer readable form

☐ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished

☐ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

- 2 ☐ Certain claims were found unsearchable (See Box I)

- 3 ☐ Unity of invention is lacking (see Box II)

**4 With regard to the title,**

☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows.

**5 With regard to the abstract,**

☒ the text is approved as submitted by the applicant

☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

**6 The figure of the drawings to be published with the abstract is Figure No**

☐ as suggested by the applicant.

☐ because the applicant failed to suggest a figure

☐ because this figure better characterizes the invention

☒ None of the figures

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/62 C12N9/72 C12N15/58 C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

WPI Data, PAJ, EPO-Internal, BIOSIS, MEDLINE, CHEM ABS Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                        | Relevant to claim No. |
|------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| X          | EP 1 048 732 A (HOFFMANN LA ROCHE)<br>2 November 2000 (2000-11-02)<br>the whole document, in particular<br>paragraphs 0019, 0026, Fig 7, "Beispiele<br>7-9", SEQ ID NO 9, 10<br>---       | 1-39                  |
| X          | EP 0 302 456 A (FUJISAWA PHARMACEUTICAL<br>CO) 8 February 1989 (1989-02-08)<br>the whole document, in particular Fig 29<br>---                                                            | 27-29                 |
| X          | WO 98 54199 A (HUMAN GENOME SCIENCES INC<br>, EBNER REINHARD (US), MOORE PAUL A (US),)<br>3 December 1998 (1998-12-03)<br>the whole document, in particular SEQ ID<br>NO 3<br>---<br>-/-- | 27-29                 |

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"Z" document member of the same patent family

Date of the actual completion of the international search

15 April 2002

Date of mailing of the international search report

10/05/2002

Name and mailing address of the ISA

European Patent Office P.B. 5618 Patentlaan 2  
NL - 2280 HV Rijswijk  
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Fax: (+31-70) 340-3018

Authorized officer

Bassias, I

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 01/12857

| C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT |                                                                                                                                                                                                                                                                                                                                |                       |
|------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| Category *                                           | Citation of document with indication where appropriate, of the relevant passages                                                                                                                                                                                                                                               | Relevant to claim No. |
| Y                                                    | HUA ZI-CHUN ET AL "Synthesis and expression of a gene from Kringle-2 domain of tissue plasminogen activator in E coli "<br>SCIENCE IN CHINA SERIES B CHEMISTRY LIFE SCIENCES & EARTH SCIENCES,<br>vol 37, no 6, 1994, pages 667-676,<br>XP002196158<br>ISSN 1001-652X<br>the whole document                                    | 1-39                  |
| Y                                                    | WO 97 38123 A (UNIV TEXAS)<br>16 October 1997 (1997-10-16)<br>the whole document                                                                                                                                                                                                                                               | 1-39                  |
| Y                                                    | US 6 083 715 A (BESSETTE PAUL ET AL)<br>4 July 2000 (2000-07-04)<br>the whole document                                                                                                                                                                                                                                         | 1-39                  |
| P,X                                                  | EP 1 077 263 A (HOFFMANN LA ROCHE)<br>21 February 2001 (2001-02-21)<br>the whole document, in particular<br>paragraphs 0015, 0022, Fig 7, "Beispiele 7, 8", SEQ ID NO: 9, 10                                                                                                                                                   | 1-39                  |
| P,X                                                  | MANOSROI JIRADEJ ET AL "Secretion of active recombinant human tissue plasminogen activator derivatives in Escherichia coli."<br>APPLIED AND ENVIRONMENTAL MICROBIOLOGY,<br>vol 67, no 6, June 2001 (2001-06), pages<br>2657-2664, XP002195206<br>ISSN 0099-2240<br>the whole document                                          | 1-39                  |
| A                                                    | SCOTT CLEARY ET AL "Purification and characterization of tissue plasminogen activator kringle-2 domain expressed in Escherichia coli"<br>BIOCHEMISTRY, AMERICAN CHEMICAL SOCIETY<br>EASTON, PA, US,<br>vol 28, no 4,<br>21 February 1989 (1989-02-21), pages<br>1884-1891, XP002101498<br>ISSN 0006-2960<br>the whole document |                       |
| A                                                    | QIU JI ET AL "Expression of active human tissue-type plasminogen activator in Escherichia coli "<br>APPLIED AND ENVIRONMENTAL MICROBIOLOGY,<br>vol 64, no 12, December 1998 (1998-12),<br>pages 4891-4896, XP002195207<br>ISSN 0099-2240<br>the whole document                                                                 |                       |
|                                                      | ---                                                                                                                                                                                                                                                                                                                            |                       |
|                                                      | -/--                                                                                                                                                                                                                                                                                                                           |                       |

57

| C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT |                                                                                                                                                                                                                                                                      |                       |
|------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| Category *                                           | Citation of document with indication where appropriate of the relevant passages                                                                                                                                                                                      | Relevant to claim No. |
| A                                                    | WALDENSTROM M ET AL "SYNTHESIS AND<br>SECRETION OF A FIBRINOLYTICALLY ACTIVE<br>TISSUE-TYPE PLASMINOGEN ACTIVATOR VARIANT<br>IN ESCHERICHIA-COLI"<br>GENE (AMSTERDAM),<br>vol. 99, no 2, 1991, pages 243-248,<br>XP001063188<br>ISSN 0378-1119<br>the whole document |                       |
| A                                                    | LASTERS IGNACE ET AL "Enzymatic<br>properties of phage-displayed fragments of<br>human plasminogen "<br>EUROPEAN JOURNAL OF BIOCHEMISTRY,<br>vol 244, no 3, 1997, pages 946-952,<br>XP001068905<br>ISSN 0014-2956<br>the whole document                              |                       |

## INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 01/12857

| Patent document<br>cited in search report |   | Publication<br>date | Patent family<br>member(s) | Publication<br>date |
|-------------------------------------------|---|---------------------|----------------------------|---------------------|
| EP 1048732                                | A | 02-11-2000          | EP 1048732 A1              | 02-11-2000          |
|                                           |   |                     | CA 2304437 A1              | 26-10-2000          |
|                                           |   |                     | CN 1272550 A               | 08-11-2000          |
|                                           |   |                     | EP 1054063 A2              | 22-11-2000          |
|                                           |   |                     | JP 2000316591 A            | 21-11-2000          |
|                                           |   |                     | US 6309861 B1              | 30-10-2001          |
| EP 0302456                                | A | 08-02-1989          | AT 108206 T                | 15-07-1994          |
|                                           |   |                     | AU 623340 B2               | 14-05-1992          |
|                                           |   |                     | AU 2036188 A               | 09-02-1989          |
|                                           |   |                     | DE 3850531 D1              | 11-08-1994          |
|                                           |   |                     | DE 3850531 T2              | 24-11-1994          |
|                                           |   |                     | DK 431988 A                | 04-02-1989          |
|                                           |   |                     | EP 0302456 A1              | 08-02-1989          |
|                                           |   |                     | ES 2056082 T3              | 01-10-1994          |
|                                           |   |                     | IL 87276 A                 | 31-07-1995          |
|                                           |   |                     | JP 2606620 B2              | 07-05-1997          |
|                                           |   |                     | JP 8228773 A               | 10-09-1996          |
|                                           |   |                     | JP 1104167 A               | 21-04-1989          |
|                                           |   |                     | JP 2561708 B2              | 11-12-1996          |
|                                           |   |                     | JP 2570633 B2              | 08-01-1997          |
|                                           |   |                     | JP 7163346 A               | 27-06-1995          |
|                                           |   |                     | KR 9701237 B1              | 04-02-1997          |
|                                           |   |                     | US 5648250 A               | 15-07-1997          |
|                                           |   |                     | US 5840533 A               | 24-11-1998          |
| WO 9854199                                | A | 03-12-1998          | AU 8053798 A               | 30-12-1998          |
|                                           |   |                     | EP 1001965 A1              | 24-05-2000          |
|                                           |   |                     | JP 2002502248 T            | 22-01-2002          |
|                                           |   |                     | WO 9854199 A1              | 03-12-1998          |
| WO 9738123                                | A | 16-10-1997          | AU 2660397 A               | 29-10-1997          |
|                                           |   |                     | WO 9738123 A1              | 16-10-1997          |
|                                           |   |                     | US 6027888 A               | 22-02-2000          |
| US 6083715                                | A | 04-07-2000          | AU 743030 B2               | 17-01-2002          |
|                                           |   |                     | AU 8140498 A               | 30-12-1998          |
|                                           |   |                     | EP 1002111 A2              | 24-05-2000          |
|                                           |   |                     | JP 2002504826 T            | 12-02-2002          |
|                                           |   |                     | WO 9856930 A2              | 17-12-1998          |
| EP 1077263                                | A | 21-02-2001          | EP 1077263 A1              | 21-02-2001          |
|                                           |   |                     | CN 1283702 A               | 14-02-2001          |
|                                           |   |                     | EP 1077262 A1              | 21-02-2001          |
|                                           |   |                     | JP 2001061487 A            | 13-03-2001          |

# PCT

## REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty

|                                                                                          |                 |
|------------------------------------------------------------------------------------------|-----------------|
| For receiving Office use only                                                            |                 |
| International Application No.                                                            | PCT/EP 01/12857 |
| International Filing Date                                                                | 07 NOV 2001     |
| EUROPEAN PATENT OFFICE<br>PCT INTERNATIONAL APPLICATION                                  |                 |
| Name of receiving Office and "PCT International Application"                             |                 |
| Applicant's or agent's file reference<br>(if desired) (12 characters maximum) 1-1170-PCT |                 |

|                                                                                                                                                                                                                                                                                                                                                                                                               |                                                                                                                                                                                                                                                                           |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Box No. I TITLE OF INVENTION</b><br>Methods for large scale production of recombinant DNA-derived tPA or K2S molecules                                                                                                                                                                                                                                                                                     |                                                                                                                                                                                                                                                                           |
| <b>Box No. II APPLICANT</b> <input type="checkbox"/> This person is also inventor                                                                                                                                                                                                                                                                                                                             |                                                                                                                                                                                                                                                                           |
| Name and address (Family name followed by given name, for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)<br><br>Boehringer Ingelheim International GmbH<br>55216 Ingelheim / Rhein<br>DE        | Telephone No<br>07351-54-5468<br><br>Facsimile No<br>07351-54-4377<br><br>Teleprinter No<br><br>Applicant's registration No with the Office                                                                                                                               |
| State (that is, country) of nationality<br>DE                                                                                                                                                                                                                                                                                                                                                                 | State (that is, country) of residence<br>DE                                                                                                                                                                                                                               |
| This person is applicant for the purposes of <input type="checkbox"/> all designated States <input checked="" type="checkbox"/> all designated States except the United States of America <input type="checkbox"/> the United States of America only <input type="checkbox"/> the States indicated in the Supplemental Box                                                                                    |                                                                                                                                                                                                                                                                           |
| <b>Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)</b>                                                                                                                                                                                                                                                                                                                                          |                                                                                                                                                                                                                                                                           |
| Name and address (Family name followed by given name, for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)<br><br>WERNER, Rolf-Gunther<br>Hugo-Haering-Str 72<br>88400 Biberach an der Riss<br>DE | This person is<br><input type="checkbox"/> applicant only<br><input checked="" type="checkbox"/> applicant and inventor<br><input type="checkbox"/> inventor only (if this check-box is marked, do not fill in below.)<br><br>Applicant's registration No with the Office |
| State (that is, country) of nationality<br>DE                                                                                                                                                                                                                                                                                                                                                                 | State (that is, country) of residence<br>DE                                                                                                                                                                                                                               |
| This person is applicant for the purposes of <input type="checkbox"/> all designated States <input type="checkbox"/> all designated States except the United States of America <input checked="" type="checkbox"/> the United States of America only <input type="checkbox"/> the States indicated in the Supplemental Box                                                                                    |                                                                                                                                                                                                                                                                           |
| <input checked="" type="checkbox"/> Further applicants and/or (further) inventors are indicated on a continuation sheet.                                                                                                                                                                                                                                                                                      |                                                                                                                                                                                                                                                                           |
| <b>Box No. IV AGENT OR COMMON REPRESENTATIVE, OR ADDRESS FOR CORRESPONDENCE</b>                                                                                                                                                                                                                                                                                                                               |                                                                                                                                                                                                                                                                           |
| The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as <input type="checkbox"/> agent <input type="checkbox"/> common representative                                                                                                                                                                                 |                                                                                                                                                                                                                                                                           |
| Name and address (Family name followed by given name, for a legal entity, full official designation. The address must include postal code and name of country.)                                                                                                                                                                                                                                               | Telephone No.<br><br>Facsimile No<br><br>Teleprinter No<br><br>Agent's registration No with the Office                                                                                                                                                                    |
| <input type="checkbox"/> Address for correspondence Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent                                                                                                                                                                |                                                                                                                                                                                                                                                                           |

| Continuation of Box No III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)                                                                                                                                                                                                                                                                                                                                                                    |  |                                                                                                                                                                                                                                                                                          |  |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--|
| <i>If none of the following sub-boxes is used, this sheet should not be included in the request</i>                                                                                                                                                                                                                                                                                                                                             |  |                                                                                                                                                                                                                                                                                          |  |
| <b>Name and address</b> <i>(Family name followed by given name, for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)</i><br><br>GOETZ, Friedrich<br>Beim Herbstenhof 31<br>72076 Tübingen<br>DE                                     |  | <b>This person is</b><br><input type="checkbox"/> applicant only<br><input checked="" type="checkbox"/> applicant and inventor<br><input type="checkbox"/> inventor only <i>(If this check-box is marked, do not fill in below.)</i><br><br>Applicant's registration No. with the Office |  |
| <b>State (that is, country) of nationality</b><br>DE                                                                                                                                                                                                                                                                                                                                                                                            |  | <b>State (that is, country) of residence</b><br>DE                                                                                                                                                                                                                                       |  |
| <b>This person is applicant for the purposes of</b> <input type="checkbox"/> all designated States <input type="checkbox"/> all designated States except the United States of America <input checked="" type="checkbox"/> the United States of America only <input type="checkbox"/> the States indicated in the Supplemental Box                                                                                                               |  |                                                                                                                                                                                                                                                                                          |  |
| <b>Name and address</b> <i>(Family name followed by given name, for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)</i><br><br>TAYAPIWATANA, Chatchai<br>369/73 Soi Pradoo 1<br>Janava Rd , BKK<br>TH                              |  | <b>This person is</b><br><input type="checkbox"/> applicant only<br><input checked="" type="checkbox"/> applicant and inventor<br><input type="checkbox"/> inventor only <i>(If this check-box is marked, do not fill in below.)</i><br><br>Applicant's registration No. with the Office |  |
| <b>State (that is, country) of nationality</b><br>TH                                                                                                                                                                                                                                                                                                                                                                                            |  | <b>State (that is, country) of residence:</b><br>TH                                                                                                                                                                                                                                      |  |
| <b>This person is applicant for the purposes of</b> <input type="checkbox"/> all designated States <input type="checkbox"/> all designated States except the United States of America <input checked="" type="checkbox"/> the United States of America only <input type="checkbox"/> the States indicated in the Supplemental Box                                                                                                               |  |                                                                                                                                                                                                                                                                                          |  |
| <b>Name and address</b> <i>(Family name followed by given name, for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)</i><br><br>MANOSROI, Jiradej<br>234/171 Sirwatana Nives Mahidol Road<br>Nong Hoi, Muang Chiang Mai 50000<br>TH |  | <b>This person is</b><br><input type="checkbox"/> applicant only<br><input checked="" type="checkbox"/> applicant and inventor<br><input type="checkbox"/> inventor only <i>(If this check-box is marked, do not fill in below.)</i><br><br>Applicant's registration No. with the Office |  |
| <b>State (that is, country) of nationality</b><br>TH                                                                                                                                                                                                                                                                                                                                                                                            |  | <b>State (that is, country) of residence.</b><br>TH                                                                                                                                                                                                                                      |  |
| <b>This person is applicant for the purposes of</b> <input type="checkbox"/> all designated States <input type="checkbox"/> all designated States except the United States of America <input checked="" type="checkbox"/> the United States of America only <input type="checkbox"/> the States indicated in the Supplemental Box                                                                                                               |  |                                                                                                                                                                                                                                                                                          |  |
| <b>Name and address</b> <i>(Family name followed by given name, for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)</i><br><br>MANOSROI, Aranya<br>234/171 Sirwatana Nives Mahidol Road<br>Nong Hoi, Muang Chiang Mai 50000<br>TH  |  | <b>This person is</b><br><input type="checkbox"/> applicant only<br><input checked="" type="checkbox"/> applicant and inventor<br><input type="checkbox"/> inventor only <i>(If this check-box is marked, do not fill in below.)</i><br><br>Applicant's registration No. with the Office |  |
| <b>State (that is, country) of nationality</b><br>TH                                                                                                                                                                                                                                                                                                                                                                                            |  | <b>State (that is, country) of residence</b><br>TH                                                                                                                                                                                                                                       |  |
| <b>This person is applicant for the purposes of</b> <input type="checkbox"/> all designated States <input type="checkbox"/> all designated States except the United States of America <input checked="" type="checkbox"/> the United States of America only <input type="checkbox"/> the States indicated in the Supplemental Box                                                                                                               |  |                                                                                                                                                                                                                                                                                          |  |
| <input type="checkbox"/> Further applicants and/or (further) inventors are indicated on another continuation sheet.                                                                                                                                                                                                                                                                                                                             |  |                                                                                                                                                                                                                                                                                          |  |

**Box No. V    DESIGNATION OF STATES**

**Mark the applicable check-boxes below at least one must be marked.**

**The following designations are hereby made under Rule 4.9(a).**

### Regional Patent

- AP** ARIPO Patent GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, MZ Mozambique, SD Sudan, SL Sierra Leone, SZ Swaziland, TZ United Republic of Tanzania, UG Uganda, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
- EA** Eurasian Patent AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
- EP** European Patent AT Austria, BE Belgium, CH & LI Switzerland and Liechtenstein, CY Cyprus, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, TR Turkey, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- OA** OAPI Patent BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, GW Guinea-Bissau, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT (if other kind of protection or treatment desired, specify on dotted line)
- GR - Equatorial Guinea

20/58

**National Patent** (if other kind of protection or treatment desired, specify on dotted line)

- |                                                                           |                                                                                  |                                                                    |
|---------------------------------------------------------------------------|----------------------------------------------------------------------------------|--------------------------------------------------------------------|
| <input checked="" type="checkbox"/> AE United Arab Emirates               | <input checked="" type="checkbox"/> GH Ghana                                     | <input checked="" type="checkbox"/> MX Mexico                      |
| <input checked="" type="checkbox"/> AG Antigua and Barbuda                | <input checked="" type="checkbox"/> GM Gambia                                    | <input checked="" type="checkbox"/> MZ Mozambique                  |
| <input checked="" type="checkbox"/> AL Albania                            | <input checked="" type="checkbox"/> HR Croatia                                   | <input checked="" type="checkbox"/> NO Norway                      |
| <input checked="" type="checkbox"/> AM Armenia                            | <input checked="" type="checkbox"/> HU Hungary                                   | <input checked="" type="checkbox"/> NZ New Zealand                 |
| <input checked="" type="checkbox"/> AT Austria                            | <input checked="" type="checkbox"/> ID Indonesia                                 | <input checked="" type="checkbox"/> PL Poland                      |
| <input checked="" type="checkbox"/> AU Australia                          | <input checked="" type="checkbox"/> IL Israel                                    | <input checked="" type="checkbox"/> PT Portugal                    |
| <input checked="" type="checkbox"/> AZ Azerbaijan                         | <input checked="" type="checkbox"/> IN India                                     | <input checked="" type="checkbox"/> RO Romania                     |
| <input checked="" type="checkbox"/> BA Bosnia and Herzegovina             | <input checked="" type="checkbox"/> IS Iceland                                   | <input checked="" type="checkbox"/> RU Russian Federation          |
| <input checked="" type="checkbox"/> BB Barbados                           | <input checked="" type="checkbox"/> JP Japan                                     |                                                                    |
| <input checked="" type="checkbox"/> BG Bulgaria                           | <input checked="" type="checkbox"/> KE Kenya                                     | <input checked="" type="checkbox"/> SD Sudan                       |
| <input checked="" type="checkbox"/> BR Brazil                             | <input checked="" type="checkbox"/> KG Kyrgyzstan                                | <input checked="" type="checkbox"/> SE Sweden                      |
| <input checked="" type="checkbox"/> BY Belarus                            | <input checked="" type="checkbox"/> KP Democratic People's Republic of Korea     | <input checked="" type="checkbox"/> SG Singapore                   |
| <input checked="" type="checkbox"/> BZ Belize                             | <input checked="" type="checkbox"/> KR Republic of Korea                         | <input checked="" type="checkbox"/> SI Slovenia                    |
| <input checked="" type="checkbox"/> CA Canada                             | <input checked="" type="checkbox"/> KZ Kazakhstan                                | <input checked="" type="checkbox"/> SK Slovakia                    |
| <input checked="" type="checkbox"/> CH & LI Switzerland and Liechtenstein | <input checked="" type="checkbox"/> LC Saint Lucia                               | <input checked="" type="checkbox"/> SL Sierra Leone                |
| <input checked="" type="checkbox"/> CN China                              | <input checked="" type="checkbox"/> LK Sri Lanka                                 | <input checked="" type="checkbox"/> TJ Tajikistan                  |
| <input checked="" type="checkbox"/> CO Colombia                           | <input checked="" type="checkbox"/> LR Liberia                                   | <input checked="" type="checkbox"/> TM Turkmenistan                |
| <input checked="" type="checkbox"/> CR Costa Rica                         | <input checked="" type="checkbox"/> LS Lesotho                                   | <input checked="" type="checkbox"/> TR Turkey                      |
| <input checked="" type="checkbox"/> CU Cuba                               | <input checked="" type="checkbox"/> LT Lithuania                                 | <input checked="" type="checkbox"/> TT Trinidad and Tobago         |
| <input checked="" type="checkbox"/> CZ Czech Republic                     | <input checked="" type="checkbox"/> LU Luxembourg                                |                                                                    |
| <input checked="" type="checkbox"/> DE Germany                            | <input checked="" type="checkbox"/> LV Latvia                                    | <input checked="" type="checkbox"/> TZ United Republic of Tanzania |
| <input checked="" type="checkbox"/> DK Denmark                            | <input checked="" type="checkbox"/> MA Morocco                                   | <input checked="" type="checkbox"/> UA Ukraine                     |
| <input checked="" type="checkbox"/> DM Dominica                           | <input checked="" type="checkbox"/> MD Republic of Moldova                       | <input checked="" type="checkbox"/> UG Uganda                      |
| <input checked="" type="checkbox"/> DZ Algeria                            |                                                                                  | <input checked="" type="checkbox"/> US United States of America    |
| <input checked="" type="checkbox"/> EC Ecuador                            | <input checked="" type="checkbox"/> MG Madagascar                                |                                                                    |
| <input checked="" type="checkbox"/> EE Estonia                            | <input checked="" type="checkbox"/> MK The former Yugoslav Republic of Macedonia | <input checked="" type="checkbox"/> UZ Uzbekistan                  |
| <input checked="" type="checkbox"/> ES Spain                              |                                                                                  | <input checked="" type="checkbox"/> VN Viet Nam                    |
| <input checked="" type="checkbox"/> FI Finland                            | <input checked="" type="checkbox"/> MN Mongolia                                  | <input checked="" type="checkbox"/> YU Yugoslavia                  |
| <input checked="" type="checkbox"/> GB United Kingdom                     | <input checked="" type="checkbox"/> MW Malawi                                    | <input checked="" type="checkbox"/> ZA South Africa                |
| <input checked="" type="checkbox"/> GD Grenada                            |                                                                                  | <input checked="" type="checkbox"/> ZW Zimbabwe                    |
| <input checked="" type="checkbox"/> GE Georgia                            |                                                                                  |                                                                    |

**Check-boxes below reserved for designating States which have become party to the PCT after issuance of this sheet**

- |                                                    |                          |                          |
|----------------------------------------------------|--------------------------|--------------------------|
| <input checked="" type="checkbox"/> PH Philippines | <input type="checkbox"/> | <input type="checkbox"/> |
| <input type="checkbox"/>                           | <input type="checkbox"/> | <input type="checkbox"/> |

**Precautionary Designation Statement** In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation (including fees) must reach the receiving Office within the 15-month time limit.)*

**Box No. VI PRIORITY CLAIM**

The priority of the following earlier application(s) is hereby claimed

| Filing date<br>of earlier application<br>(day/month/year) | Number<br>of earlier application | Where earlier application is    |                                           |                                               |
|-----------------------------------------------------------|----------------------------------|---------------------------------|-------------------------------------------|-----------------------------------------------|
|                                                           |                                  | national application<br>country | regional application *<br>regional Office | international application<br>receiving Office |
| item (1)<br>(14/11/2000)<br>At JPO 2000                   | GB 00 27 779 8                   | GB                              |                                           |                                               |
| item (2)                                                  |                                  |                                 |                                           |                                               |
| item (3)                                                  |                                  |                                 |                                           |                                               |
| item (4)                                                  |                                  |                                 |                                           |                                               |
| item (5)                                                  |                                  |                                 |                                           |                                               |

☐ Further priority claims are indicated in the Supplemental Box

The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) (only if the earlier application was filed with the Office which for the purposes of this international application is the receiving Office) identified above as

☐ all items ☐ item (1) ☐ item (2) ☐ item (3) ☐ item (4) ☐ item (5) ☐ other, see Supplemental Box

\* Where the earlier application is an ARIPO application, indicate at least one country party to the Paris Convention for the Protection of Industrial Property or one Member of the World Trade Organization for which that earlier application was filed (Rule 4.10(b)(ii))

**Box No. VII INTERNATIONAL SEARCHING AUTHORITY**

Choice of International Searching Authority (ISA) (if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen, the two-letter code may be used)

ISA / EP

Request to use results of earlier search, reference to that search (if an earlier search has been carried out by or requested from the International Searching Authority)

Date (day/month/year)

Number

Country (or regional Office)

**Box No. VIII DECLARATIONS**

The following declarations are contained in Boxes Nos. VIII (i) to (v) (mark the applicable check-boxes below and indicate in the right column the number of each type of declaration)

Number of  
declarations

- ☐ Box No. VIII (i) Declaration as to the identity of the inventor
- ☐ Box No. VIII (ii) Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent
- ☐ Box No. VIII (iii) Declaration as to the applicant's entitlement, as at the international filing date, to claim the priority of the earlier application
- ☐ Box No. VIII (iv) Declaration of inventorship (only for the purposes of the designation of the United States of America)
- ☐ Box No. VIII (v) Declaration as to non-prejudicial disclosures or exceptions to lack of novelty

| Box No IX CHECK LIST, LANGUAGE OF FILING                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |                        |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------|
| <p>This international application contains</p> <p>(a) the following number of sheets in paper form</p> <p>request (including declaration sheets) 5</p> <p>description (excluding sequence listing part) 28</p> <p>claims 9</p> <p>abstract 1</p> <p>drawings 15</p> <p>Sub-total number of sheets 58</p> <p>sequence listing part of description (<i>actual number of sheets if filed in paper form, whether or not also filed in computer readable form, see (b) below</i>) 27</p> <p>Total number of sheets 85</p> <p>(b) sequence listing part of description filed in computer readable form</p> <p>(i) <input type="checkbox"/> only (under Section 801(a)(i))</p> <p>(ii) <input type="checkbox"/> in addition to being filed in paper form (under Section 801(a)(ii))</p> <p>Type and number of carriers (diskette, CD-ROM, CD-R or other) on which the sequence listing part is contained (<i>additional copies to be indicated under item 9(iii), in right column</i>)</p> | <p>This international application is accompanied by the following item(s) (mark the applicable check-boxes below and indicate in right column the number of each item)</p> <p>1 <input checked="" type="checkbox"/> fee calculation sheet</p> <p>2 <input type="checkbox"/> original separate power of attorney</p> <p>3 <input type="checkbox"/> original general power of attorney</p> <p>4 <input type="checkbox"/> copy of general power of attorney; reference number, if any</p> <p>5 <input type="checkbox"/> statement explaining lack of signature</p> <p>6 <input checked="" type="checkbox"/> priority document(s) identified in Box No VI as item(s) 1</p> <p>7 <input type="checkbox"/> translation of international application into (language)</p> <p>8 <input type="checkbox"/> separate indications concerning deposited microorganism or other biological material</p> <p>9 <input checked="" type="checkbox"/> sequence listing in computer readable form (indicate also type and number of carriers (diskette, CD-ROM, CD-R or other))</p> <p>(i) <input checked="" type="checkbox"/> copy submitted for the purposes of international search under Rule 13ter only (and not as part of the international application)</p> <p>(ii) <input type="checkbox"/> (only where check-box (b)(i) or (b)(ii) is marked in left column) additional copies including, where applicable, the copy for the purposes of international search under Rule 13ter</p> <p>(iii) <input checked="" type="checkbox"/> together with relevant statement as to the identity of the copy or copies with the sequence listing part mentioned in left column</p> <p>10 <input type="checkbox"/> other (specify)</p> | <p>Number of items</p> |
| <p>Figure of the drawings which should accompany the abstract</p>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   | <p>Language of filing of the international application English</p>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |                        |

## Box No X SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

Boehringer Ingelheim International GmbH

DPA



Dr Volker Mahlbacher



Dr Markus Wolfram

| For receiving Office use only                                                                                                                    |                                                                                                                    |
|--------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------|
| <p>1 Date of actual receipt of the purported international application</p> <p>07 NOV 2001 (07.11.01)</p>                                         | <p>2 Drawings</p> <p><input checked="" type="checkbox"/> received</p> <p><input type="checkbox"/> not received</p> |
| <p>3 Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application</p> | <p>6 <input type="checkbox"/> Transmittal of search copy delayed until search fee is paid</p>                      |
| <p>4 Date of timely receipt of the required corrections under PCT Article 11(2)</p>                                                              |                                                                                                                    |
| <p>5 International Searching Authority (if two or more are competent) ISA /</p>                                                                  |                                                                                                                    |

Ro/EP

| For International Bureau use only                                     |
|-----------------------------------------------------------------------|
| <p>Date of receipt of the record copy by the International Bureau</p> |

# PATENT COOPERATION TREATY

PCT/EP01/12857

612

PCT

EINZELGANG

From the INTERNATIONAL BUREAU

NOTICE INFORMING THE APPLICANT OF THE  
COMMUNICATION OF THE INTERNATIONAL  
APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47 1(c), first sentence)

To BOEHRINGER INGELHEIM INTERNATIONAL  
GMBH  
55216 Ingelheim/Bhein  
ALLEMAGNE

31. Mai 2002

SR: gesehen erledigt  
25.06.02

Date of mailing (day/month/year)  
23 May 2002 (23 05 02)

Applicant's or agent's file reference  
1-1170-PCT

ERFASST

IMPORTANT NOTICE

International application No  
PCT/EP01/12857

International filing date (day/month/year)  
07 November 2001 (07 11 01)

Priority date (day/month/year)  
14 November 2000 (14 11 00)

Applicant

BOEHRINGER INGELHEIM INTERNATIONAL GMBH et al

1 Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this notice  
KP, KR, US

In accordance with Rule 47 1(c), third sentence, those Offices will accept the present notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s)

2 The following designated Offices have waived the requirement for such a communication at this time

AE, AG, AL, AM, AP, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EA, EC, EE, EP, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OA, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA,

The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49 1(a-bis))

3 Enclosed with this notice is a copy of the international application as published by the International Bureau on  
23 May 2002 (23 05 02) under No WO 02/40860

4. TIME LIMITS for filing a demand for international preliminary examination and for entry into national phase

The applicable time limit for entering the national phase will, subject to what is said in the following paragraph be 30 MONTHS from the priority date, not only in respect of any elected Office if a demand for international preliminary examination is filed before the expiration of 18 months from the priority date, but also in respect of any designated Office, in the absence of filing of such demand, where Article 22(1) as modified with effect from 1 April 2002 applies in respect of the designated Office. For further details, see PCT Gazette No. 44/2001 of 1 November 2001, pages 19926, 19932 and 19934, as well as the PCT News letter, October and November 2001 and February 2002 issues

In practice, time limits other than the 30-month time limit will continue to apply, for various periods of time, in respect of certain designated or elected Offices. For regular updates on the applicable time limits (20, 21, 30 or 31 months, or other time limit), Office by Office, refer to the PCT Gazette, the PCT Newsletter and the PCT Applicant's Guide, Volume II, National Chapters, all available from WIPO's Internet site, at <http://www.wipo.int/pct/en/index.html>

For filing a demand for international preliminary examination, see the PCT Applicant's Guide, Volume IA, Chapter IX. Only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination (at present, all PCT Contracting States are bound by Chapter II)

It is the applicant's sole responsibility to monitor all these limits.

The International Bureau of WIPO  
34, chemin des Colombettes  
1211 Geneva 20, Switzerland

Facsimile No (41-22) 740 14 35

Authorized officer

J Zahra

Telephone No. (41 22) 338 81 11

## Continuation of Form PCT/IB/308

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF  
THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

62

|                                                                                                                                                                                                                                                                                                                              |                                                |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------|
| Date of mailing (day/month/year)<br>23 May 2002 (23 05 02)                                                                                                                                                                                                                                                                   | IMPORTANT NOTICE                               |
| Applicant's or agent's file reference<br>1-1170-PCT                                                                                                                                                                                                                                                                          | International application No<br>PCT/EP01/12857 |
| <p>The applicant is hereby notified that, at the time of establishment of this Notice, the time limit under Rule 48.1 for making amendments under Article 19 has not yet expired and the International Bureau had received neither such amendments nor a declaration that the applicant does not wish to make amendments</p> |                                                |

## PATENT COOPERATION TREATY

63

PCT

INFORMATION CONCERNING ELECTED  
OFFICES NOTIFIED OF THEIR ELECTION

(PCT Rule 61.3)

From the INTERNATIONAL BUREAU

To

20 NOV. 2002

BOEHRINGER INGELHEIM INTERNATIONAL  
GMBH  
55216 Ingelheim/Rhein  
Germany

Date of mailing (day/month/year)

07 November 2002 (07 11 02)

Applicant's or agent's file reference

1-1170-PCT

## IMPORTANT INFORMATION

International application No.

PCT/EP01/12857

International filing date (day/month/year)

07 November 2001 (07 11 01)

Priority date (day/month/year)

14 November 2000 (14 11 00)

Applicant

BOEHRINGER INGELHEIM INTERNATIONAL GMBH et al

- 1 The applicant is hereby informed that the International Bureau has, according to Article 31(7), notified each of the following Offices of its election

EP AT,BE,CH,CY,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE,TR  
National AU,BG,CA,CN,DE,GB,IL,JP,KP,KR,MN,NO,PL,RO,RU,SK,US

- 2 The following Offices have waived the requirement for the notification of their election, the notification will be sent to them by the International Bureau only upon their request

AP GH,GM,KE,LS,MW,MZ,SD,SL,SZ,TZ,UG,ZW  
EA AM,AZ,BY,KG,KZ,MD,RU,TJ,TM  
OA BF,BJ,CF,CG,CI,CM,GA,GN,GQ,GW,ML,MR,NE,SN,TD,TG  
National AE,AG,AL,AM,AT,AZ,BA,BB,BR,BY,BZ,CH,CO,CR,CU,CZ,DK,DM,DZ,EC,EE,  
ES,FI,GD,GE,GH,GM,HR,HU,ID,IN,IS,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,MG,  
MK,MW,MX,MZ,NZ,PH,PT,SD,SE,SG,SI,SL,TJ,TM,TR,TT,TZ,UA,UG,UZ,VN,YU,ZA,ZW

- 3 The applicant is reminded that he must enter the "national phase" before the expiration of 30 months from the priority date before each of the Offices listed above. This must be done by paying the national fee(s) and furnishing, if prescribed, a translation of the international application (Article 39(1)(a)), as well as, where applicable, by furnishing a translation of any annexes of the international preliminary examination report (Article 38(3)(b) and Rule 74.1)

Some offices have fixed time limits expiring later than the above-mentioned time limit. For detailed information about the applicable time limits and the acts to be performed upon entry into the national phase before a particular Office, see Volume II of the PCT Applicant's Guide

The entry into the European regional phase is postponed until 31 months from the priority date for all States designated for the purposes of obtaining a European patent

The International Bureau of WIPO  
34, chemin des Colombettes  
1211 Geneva 20, Switzerland

Facsimile No (41 22) 740 14.35

Authorized officer

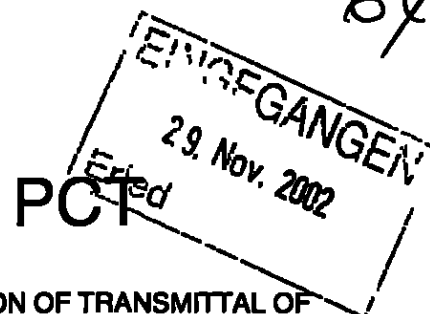
Farid ABBOU

Telephone No (41-22) 338 83.38

# PATENT COOPERATION TREATY

64

From the  
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY



|                                                                                 |         |           |
|---------------------------------------------------------------------------------|---------|-----------|
| To                                                                              |         |           |
| BOEHRINGER INGELHEIM INTERNATIONAL GMBH<br>55216 Ingelheim / Rhein<br>ALLEMAGNE |         |           |
| Eingang CD Patents<br>27. Nov. 2002                                             |         |           |
| SB                                                                              | g-senen | erleucht. |
| Wo                                                                              | 2.12.02 | Wo.       |

NOTIFICATION OF TRANSMITTAL OF  
THE INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT  
(PCT Rule 71.1)

|                                     |            |
|-------------------------------------|------------|
| Date of mailing<br>(day/month/year) | 26 11 2002 |
|-------------------------------------|------------|

|                                                     |                        |
|-----------------------------------------------------|------------------------|
| Applicant's or agent's file reference<br>1-1170-PCT | IMPORTANT NOTIFICATION |
|-----------------------------------------------------|------------------------|

|                                                |                                                          |                                              |
|------------------------------------------------|----------------------------------------------------------|----------------------------------------------|
| International application No<br>PCT/EP01/12857 | International filing date (day/month/year)<br>07/11/2001 | Priority date (day/month/year)<br>14/11/2000 |
|------------------------------------------------|----------------------------------------------------------|----------------------------------------------|

|                                                            |
|------------------------------------------------------------|
| Applicant<br>BOEHRINGER INGELHEIM INTERNATIONAL GMBH et al |
|------------------------------------------------------------|

1 The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application

2 A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices

3 Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices

4 REMINDER

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301)

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide

For the purpose of deciding whether the claimed invention is patentable or not, the elected Offices may apply criteria additional to or different from the criteria on which the international preliminary examination report is based (see Articles 27(5), 33(5)). Additional criteria may include e.g. exemptions from patentability and the requirements of enabling disclosure and of clarity and support of claims

|                                                                                                                                                                                                                                        |                                                      |                                                                                       |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------|---------------------------------------------------------------------------------------|
| Name and mailing address of the IPEA/<br><br>European Patent Office<br>D-80298 Munich<br>Tel +49 89 2399-0 Tx 523656 epmu d<br>Fax +49 89 2399-4485 | Authorized officer<br>COX, B<br>Tel +49 89 2399-8062 |  |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------|---------------------------------------------------------------------------------------|

## PATENT COOPERATION TREATY

## PCT

## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |                                                                                                                                                                    |                                              |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------|
| Applicant's or agent's file reference<br>1-1170-PCT                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | <b>FOR FURTHER ACTION</b> See Notification of Transmittal of International Preliminary Examination Report (Form PCT/PEA/418)                                       |                                              |
| International application No<br>PCT/EP01/12857                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     | International filing date (day/month/year)<br>07/11/2001                                                                                                           | Priority date (day/month/year)<br>14/11/2000 |
| International Patent Classification (IPC) or national classification and IPC<br>C12N15/82                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |                                                                                                                                                                    |                                              |
| Applicant<br>BOEHRINGER INGELHEIM INTERNATIONAL GMBH et al                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |                                                                                                                                                                    |                                              |
| <p>1 This International preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36</p> <p>2 This REPORT consists of a total of 4 sheets, including this cover sheet</p> <p><input checked="" type="checkbox"/> This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 807 of the Administrative Instructions under the PCT)</p> <p>These annexes consist of a total of 9 sheets</p>                                                                                                                                                                                                   |                                                                                                                                                                    |                                              |
| <p>3 This report contains indications relating to the following items.</p> <ul style="list-style-type: none"><li>I <input checked="" type="checkbox"/> Basis of the report</li><li>II <input type="checkbox"/> Priority</li><li>III <input type="checkbox"/> Non-establishment of opinion with regard to novelty, inventive step and industrial applicability</li><li>IV <input type="checkbox"/> Lack of unity of invention</li><li>V <input checked="" type="checkbox"/> Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement</li><li>VI <input type="checkbox"/> Certain documents cited</li><li>VII <input type="checkbox"/> Certain defects in the international application</li><li>VIII <input type="checkbox"/> Certain observations on the international application</li></ul> |                                                                                                                                                                    |                                              |
| Date of submission of the demand<br><br>29/05/2002                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 | Date of completion of this report<br><br>26.11.2002                                                                                                                |                                              |
| Name and mailing address of the International preliminary examining authority<br> European Patent Office<br>D-80298 Munich<br>Tel +49 89 2399 - 0 Tx 523856 epmu d<br>Fax +49 89 2399 - 4465                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    | Authorized officer<br><br>Bassias, I<br><br>Telephone No +49 89 2399 8108<br> |                                              |

# INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No PCT/EP01/12857

## I Basis of the report

- 1 With regard to the elements of the international application (*Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70 16 and 70 17)*)

Description, pages:

1-28 as originally filed

Claims, No :

1-34 as received on 12/11/2002 with letter of 12/11/2002

Drawings, sheets

1/15-15/15 as originally filed

Sequence listing part of the description, pages:

1-27, as originally filed

- 2 With regard to the language, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item

These elements were available or furnished to this Authority in the following language , which is

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23 1(b))
- ☐ the language of publication of the international application (under Rule 48 3(b))
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55 2 and/or 55 3)

- 3 With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing

- ☒ contained in the international application in written form
- ☒ filed together with the international application in computer readable form
- ☐ furnished subsequently to this Authority in written form
- ☐ furnished subsequently to this Authority in computer readable form
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

- 4 The amendments have resulted in the cancellation of

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No **PCT/EP01/12857**

- ☐ the description,        pages
- ☐ the claims,                Nos
- ☐ the drawings,            sheets
- 5 ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70 2(c))  
*(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report )*

6 Additional observations, if necessary

**V Reasoned statement under Article 35(2) with regard to novelty, inventive step or Industrial applicability, citations and explanations supporting such statement**

**1 Statement**

|                               |     |        |      |
|-------------------------------|-----|--------|------|
| Novelty (N)                   | Yes | Claims | 1-34 |
|                               | No  | Claims | -    |
| Inventive step (IS)           | Yes | Claims | 1-34 |
|                               | No  | Claims | -    |
| Industrial applicability (IA) | Yes | Claims | 1-34 |
|                               | No  | Claims | -    |

**2 Citations and explanations**  
**see separate sheet**

**Re Item V**

- 1 The application relates to methods for production of DNA-derived tissue plasminogen activator (tPA) or Kringle 2 Serine protease molecules (K2S)
- 2 The amended claims filed with the letter of 12 11 2002 appear to be allowable under Articles 19(2) and 34(2)(b) PCT
- 3 In the methods described in the present application, the DNA coding for the molecules to be produced is operably linked to the DNA coding for the signal peptide OmpA  
The use of OmpA as a signal peptide for the production of tPA or K2S is neither known nor considered obvious in the prior art  
The prior art used, refers to methods where PelB is used as a signal peptide  
However, it appears that OmpA-K2S fusions are superior to PelB-K2S fusion proteins, since the OmpA fusion leads to higher production yields  
Consequently, the subject-matter of the present application appears to meet the requirements of Article 33(2)-(4) PCT
- 4 Adaption of the description to the amended claims, including references to the prior art (D1-D3) needs to be done in the national phases

69

**Amended Claims (13.11.2002)**

- 1 Method for the production of recombinant DNA-derived tissue plasminogen activator (tPA), a tPA variant, a Kringle 2 Serine protease molecule (K2S) or a K2S variant in prokaryotic cells, wherein said tPA, tPA variant, K2S molecule or K2S variant is secreted extracellularly as an active and correctly folded protein, characterized in that the prokaryotic cell contains and expresses a vector comprising the DNA coding for said tPA, tPA variant, K2S molecule or K2S variant operably linked to the DNA coding for the signal peptide OmpA
2. Method according to claim 1, characterised in that said the prokaryotic cell contains and expresses a vector comprising the DNA coding for said tPA, tPA variant, K2S molecule or K2S variant operably linked to the DNA coding for the signal peptide OmpA which is operably linked to the nucleic acid molecule defined by the sequence TCTGAGGGAAACAGTGAC (SEQ ID NO-1) or a functional derivative thereof
3. Method according to claim 1 or 2, characterised in that the prokaryotic cell is E. coli.
4. Method according to one of claims 1 to 3, characterised in that the following steps are carried out:
  - a) the DNA encoding the tPA, tPA variant, K2S molecule or K2S variant is amplified by PCR;
  - b) the PCR product is purified;
  - c) said PCR product is inserted into a vector comprising the DNA coding for OmpA signal peptide and the DNA coding for gpIII in such a way that said PCR product is operably linked upstream to the DNA coding for the OmpA signal sequence and linked downstream to the DNA coding for gpIII of said vector;
  - d) that a stop codon is inserted between said tPA, tPA variant, K2S molecule or K2S variant and gpIII,
  - e) said vector is expressed by the prokaryotic cell,
  - f) the tPA, tPA variant, K2S molecule or K2S variant is purified.
- 5 Method according to one of claims 1 to 4, characterised in that the vector is a phagemid vector comprising the DNA coding for OmpA signal peptide and the DNA coding for gpIII
- 6 Method according to one of claims 1 to 5, characterised in that the vector is the pComb3HSS phagemid

70

7. Method according to one of claims 1 to 6, characterised in that the DNA Sequence of OmpA linked upstream to K2S comprises the following sequence or a variant due to the degenerate nucleotide code:

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTGG  
CCCAGGCGGCCTCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCT  
ACCGTGGCAGGCACAGCCTCACCAGTTCGGGTGCCTCCTGCCTCCCGTGGGAATTC  
CATGATCCTGATAGGCAAGGTTTACACAGCACAGAACCCCAGTGCCCAGGCACT  
GGGCCTGGGCAAACATAATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTG  
GTGCCACGTGCTGAAGAACCGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCC  
TGCTCCACCTGCGGCCTGAGACAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAG  
GGCTCTTCGCCGACATCGCCTCCCACCCCTGGCAGGCTGCCATCTTTGCCAAGCA  
CAGGAGGTGCGCCGGAGAGCGGTTCTGTGCGGGGGCATACTCATCAGCTCCTGC  
TGGATTCTCTCTGCCGCCCCTGCTTCCAGGAGAGGTTTCCGCCCCACCACCTGA  
CGGTGATCTTGGGCAGAACATACCGGGTGGTCCCTGGCGAGGAGGAGCAGAAAT  
TTGAAGTCGAAAAATACATTGTCCATAAGGAATTCGATGATGACACTTACGACAA  
TGACATTGCGCTGCTGCAGCTGAAATCGGATTTCGTCCTCGCTGTCCCCAGGAGAGC  
AGCGTGGTCCGCACTGTGTGCCTTCCCCCGGCGGACCTGCAGCTGCCGGACTGGA  
CGGAGTGTGAGCTCTCCGGCTACGGCAAGCATGAGGCCTTGTCTCCTTTCTATTC  
GGAGCGGCTGAAGGAGGCTCATGTCACTGTACCCATCCAGCCGCTGCACATC  
ACAACATTTACTTAACAGAACAGTCACCGACAACATGCTGTGTGCTGGAGACACT  
CGGAGCGGCGGGCCCCAGGCAAACCTTGACGACGCCTGCCAGGGCGATTCCGGGA  
GGCCCCCTGGTGTGTCTGAACGATGGCCGCATGACTTTGGTGGGCATCATCAGCT  
GGGGCCTGGGCTGTGGACAGAAGGATGTCCCGGGTGTGTACACAAAGGTTACCA  
ACTACCTAGACTGGATTTCGTGACAACATGCGACCG (SEQ ID NO 2)

- 8 Method according to one of claims 1 to 7, characterised in that the DNA Sequence of OmpA comprises the following sequence

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTGG  
CCCAGGCGGCC (SEQ ID NO 3)

9. Method according to one of claims 1 to 8, characterised in that the DNA Sequence of OmpA consists of the following sequence.

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTGG  
CCCAGGCGGCC (SEQ ID NO:3)

10. Method according to one of claims 1 to 9, characterised in that the DNA of the tPA, tPA variant, K2S molecule or K2S variant is preceded by a lac promotor and/or a ribosomal binding site.
11. Method according to one of claims 1 to 10, characterised in that the DNA coding for the tPA, tPA variant, K2S molecule or K2S variant is selected from the group of DNA molecules coding for at least 90% of the amino acids 87 – 527, 174 – 527, 180 – 527 or 220 – 527 of the human tissue plasminogen activator protein.
12. Method according to one of claims 5 to 11, characterised in that the DNA Sequence of K2S comprises the following sequence or a functional variant thereof or a variant due to the degenerate nucleotide code:

TCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTACCGTGGCACGC  
ACAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAATTCATGATCCTGAT  
AGGCAAGGTTTACACAGCACAGAACCCAGTGCCCAGGCACTGGGCCTGGGCAA  
ACATAATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGTGCCACGTGCTG  
AAGAACCGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGCG  
GCCTGAGACAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCTCTTCGCCGA  
CATCGCCTCCCACCCCTGGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTCGCCC  
GGAGAGCGGTTCTGTGCGGGGCATACTCATCAGCTCCTGCTGGATTCTCTCTG  
CCGCCCCACTGCTTCCAGGAGAGGTTTCCGCCCCACCACTGACGGTGATCTTGGG  
CAGAACATAACGGGTGGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGTCGAAAA  
ATACATTGTCCATAAGGAATTCGATGATGACACTTACGACAATGACATTGCGCTG  
CTGCAGCTGAAATCGGATTTCGTCCCGCTGTGCCAGGAGAGCAGCGTGGTCCGCA  
CTGTGTGCCTTCCCCCGGCGGACCTGCAGCTGCCGGA CTGGACGGAGTGTGAGCT  
CTCCGGCTACGGCAAGCATGAGGCCTTGTCTCCTTTCTATTTCGGAGCGGCTGAAG  
GAGGCTCATGTCAGACTGTACCCATCCAGCCGCTGCACATCACAACATTTACTTA  
ACAGAACAGTCACCGACAACATGCTGTGTGCTGGAGACACTCGGAGCGGCGGGC  
CCCAGGCCAACTTGCACGACGCCTGCCAGGGCGATTTCGGGAGGCCCCCTGGTGT  
GTCTGAACGATGGCCGCATGACTTTGGTGGGCATCATCAGCTGGGGCCTGGGCTG  
TGGACAGAAGGATGTCCCGGGTGTGTACACAAAGGTTACCAACTACCTAGACTG  
GATTCGTGACAACATGCGACCGTGA (SEQ ID NO:4).

13. Method according to one of claims 5 to 12, characterised in that the DNA Sequence of K2S consists of the following sequence:

TCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTACCGTGGCACGC  
ACAGCCTCACCQAGTCGGGTGCCTCCTGCCTCCCGTGGAATTCCATGATCCTGAT  
AGGCAAGGTTTACACAGCACAGAACCCAGTGCCCAGGCACTGGGCCTGGGCAA  
ACATAATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGTGCCACGTGCTG  
AAGAACCGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGCG  
GCCTGAGACAOTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCTCTTCGCCGA  
CATCGCCTCCCACCCCTGGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTGCCCC  
GGAGAGCGGTTTCTGTGCGGGGGCATACTCATCAGCTCCTGCTGGATTCTCTCTG  
CCGCCCCTGCTTCCAGGAGAGGTTTCCGCCCCACCACCTGACGGTGATCTTGGG  
CAGAACATAACCGGTGGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGTCGAAAA  
ATACATTGTCCATAAGGAATTCGATGATGACACTTACGACAATGACATTGCGCTG  
CTGCAGCTGAAATCGGATTCGTCCCGCTGTGCCCAGGAGAGCAGCGTGGTCCGCA  
CTGTGTGCCTTCCCCCGCGGAOCTGCAGCTGCCGGA CTGGACGGAGTGTGAGCT  
CTCCGGCTACGGCAAGCATGAGGCCCTGTCTCCTTTCTATTTCGGAGCGGCTGAAG  
GAGGCTCATGTGAGACTGTACCCATCCAGCCGCTGCACATCACAACATTTACTTA  
ACAGAACAGTCACCGACAACATGCTGTGTGCTGGAGACACTCGGAGCGGCGGGC  
CCCAGGCAAACCTTGACGACGCCTGCCAGGGCGATTTCGGGAGGCCCCCTGGTGT  
GTCTGAACGATGGCCGCGATGACTTTGGTGGGCATCATCAGCTGGGGCCTGGGCTG  
TGGACAGAAAGGATGTCCCGGGTGTGTACACAAAGGTTACCAACTACCTAGACTG  
GATTCGTGACAACATGCGACCGTGA (SEQ ID NO.4).

14. DNA molecule characterized in that it is coding for

- a) the OmpA protein operably linked to
- b) a DNA molecule coding for a polypeptide containing the kringle 2 domain and the serine protease domain of tissue plasminogen activator protein.

15. DNA molecule according to claim 14, characterized in that said DNA sequence comprises the following sequence or a variant due to the degenerate nucleotide code:

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTGG  
CCCAGGCGGCCTCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCT  
ACCGTGGCACGCACAGCCTCACCQAGTCGGGTGCCTCCTGCCTCCCGTGGAATTC  
CATGATCCTGATAGGCAAGGTTTACACAGCACAGAACCCAGTGCCCAGGCACT

GGGCCTGGGCAAACATAATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTG  
GTGCCACGTGCTGAAGAACCGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCC  
TGCTCCACCTGCGGCCTGAGACAGTACAGCCAGCCTCAGTTTCOCATCAAAGGAG  
GGCTCTTCGCCGACATCGCCTCCCACCCCTGGCAGGCTGCCATCTTTGCCAAGCA  
CAGGAGGTCGCCCCGAGAGCGGTTCTGTGCGGGGGCATACTCATCAGCTCCTGC  
TGGATTCTCTCTGCCGCCCCTGCTTCCAGGAGAGGTTTCCGCCCCACCACCTGA  
CGGTGATCTTGGGCAGAACATAACCGGGTGGTCCCTGGCGAGGAGGAGCAGAAAT  
TTGAAGTCGAAAAATACATTGTCCATAAGGAATTCGATGATGACACTTACGACAA  
TGACATTGCGCTGCTGCAGCTGAAATCGGATTTCGTCCCGCTGTGCCCAGGAGAGC  
AGCGTGGTCCGCACTGTGTGCCTTCCCCCGGCGGACCTGCAGCTGCCGGACTGGA  
CGGAGTGTGAGCTCTCCGGCTACGGCAAGCATGAGGCCTTGTCTCCTTTCTATTCT  
GGAGCGGCTGAAGGAGGCTCATGTCAGACTGTACCCATCCAGCCGCTGCACATC  
ACAACATTTACTTAACAGAACAGTCACCGACAACATGCTGTGTGCTGGAGACACT  
CGGAGCGGCGGGCCCCAGGCCAACTTGACAGACGCCTGCCAGGGCGATTTCGGA  
GGCCCCCTGGTGTGTCTGAACGATGGCCGCATGACTTTGGTGGGCATCATCAGCT  
GGGGCCTGGGCTGTGGACAGAAGGATGTCCCGGGTGTGTACACAAAGGTTACCA  
ACTACCTAGACTGGATTCTGTGACAACATGCGACCG (SEQ ID NO.5)

- 16 DNA molecule according to claim 14 or 15, characterized in that said DNA sequence consists of the following sequence:

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTGG  
CCCAGGCGGCCTCTGAGGGAAACAGTGAAGTACTTGGGAATGGGTCAGCCT  
ACCGTGGCACGCACAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGGAATTC  
CATGATCCTGATAGGCAAGGTTTACACAGCACAGAACCCAGTGCCCAGGCACT  
GGGCCTGGGCAAACATAATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTG  
GTGCCACGTGCTGAAGAACCGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCC  
TGCTCCACCTGCGGCCTGAGACAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAG  
GGCTCTTCGCCGACATCGCCTCCCACCCCTGGCAGGCTGCCATCTTTGCCAAGCA  
CAGGAGGTCGCCCCGAGAGCGGTTCTGTGCGGGGGCATACTCATCAGCTCCTGC  
TGGATTCTCTCTGCCGCCCCTGCTTCCAGGAGAGGTTTCCGCCCCACCACCTGA  
CGGTGATCTTGGGCAGAACATAACCGGGTGGTCCCTGGCGAGGAGGAGCAGAAAT  
TTGAAGTCGAAAAATACATTGTCCATAAGGAATTCGATGATGACACTTACGACAA

24

TGACATTGCGCTGCTGCAGCTGAAATCGGATTCGTCCCGCTGTGCCCAGGAGAGC  
AGCGTGGTCCGCACTGTGTGCCTTCCCCCGGCGGACCTGCAGCTGCCGGACTGGA  
CGGAGTGTGAGCTCTCCGGCTACGGCAAGCATGAGGCCTTGTCTCCTTTCTATTC  
GGAGCGGCTGAAGGAGGCTCATGTCAGACTGTACCCATCCAGCCGCTGCACATC  
ACAACATTTACTTAACAGAACAGTCACCGACAACATGCTGTGTGCTGGAGACACT  
CGGAGCGGCGGGCCCCAGGCAAACCTTGACGACGCCTGCCAGGGCGATTCCGGA  
GGCCCCCTGGTGTGTCTGAACGATGGCCGCATGACTTTGGTGGGCATCATCAGCT  
GGGGCCTGGGCTGTGGACAGAAGGATGTCCCGGGTGTGTACACAAAGGTTACCA  
ACTACCTAGACTGGATTCGTGACAACATGCGACCG (SEQ ID NO 5)

17. DNA molecule according to one of claims 14 to 16, characterized in that said DNA sequence b) is coding for at least 90% of the amino acids 87 – 527 of the human tissue plasminogen activator protein
18. DNA molecule according to one of claims 14 to 17, characterized in that said DNA sequence b) is coding for at least 90% of the amino acids 174 – 527 of the human tissue plasminogen activator protein.
19. DNA molecule according to any one of claims 14 to 18, characterized in that said DNA sequence b) is coding for at least 90% of the amino acids 180 – 527 of the human tissue plasminogen activator protein.
20. DNA molecule according to any one of claims 14 to 19, characterized in that said DNA sequence b) is coding for at least 90% of the amino acids 220 – 527 of the human tissue plasminogen activator protein.
21. DNA molecule according to any one of claims 14 to 20 characterized in that said DNA sequence b) is hybridizing under stringent conditions to the following sequence:

TCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTACCGTGGCACGC  
ACAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAATTCCATGATCCTGAT  
AGGCAAGGTTTACACAGCACAGAACCCAGTGCCCAGGCACTGGGCCTGGGCAA  
ACATAATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGTGCCACGTGCTG  
AAGAACCGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGCG  
GCCTGAGACAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCTCTTCGCCGA  
CATCGCCTCCCACCCCTGGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTGCCCC  
GGAGAGCGGTTCTGTGCGGGGGCATACTCATCAGCTCCTGCTGGATTCTCTCTG

CCGCCCACTGCTTCCAGGAGAGGTTTCCGCCCCACCACCTGACGGTGATCTTGGG  
CAGAACATACCGGGTGGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGTCGAAAA  
ATACATTGTCCATAAGGAATTCGATGATGACACTTACGACAATGACATTGCGCTG  
CTGCAGCTGAAATCGGATTCGTCCCGCTGTGCCAGGAGAGCAGCGTGGTCCGCA  
CTGTGTGCCTTCCCCCGGCGGACCTGCAGCTGCCGGAAGTGGACGGAGTGTGAGCT  
CTCCGGCTACGGCAAGCATGAGGCCTTGTCTCCTTTCTATTTCGGAGCGGCTGAAG  
GAGGCTCATGTCAGACTGTACCCATCCAGCCGCTGCACATCACAACATTTACTTA  
ACAGAACAGTCACCGACAACATGCTGTGTGCTGGAGACACTCGGAGCGGCGGGC  
CCCAGGCAAACCTTGACGACGCTGCCAGGGCGATTTCGGGAGGCCCCCTGGTGT  
GTCTGAACGATGGCCGCATGACTTTGGTGGGCATCATCAGCTGGGGCCTGGGCTG  
TGGACAGAAGGATGTCCCGGGTGTGTACACAAAGGTTACCAACTACCTAGACTG  
GATTCGTGACAACATGCGACCGTGA (SEQ ID NO:7)

22 DNA molecule according to any one of claims 14 to 21, characterized in that said DNA sequence b) consists of the following sequence:

TCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTACCGTGGCACGC  
ACAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAATTCCATGATCCTGAT  
AGGCAAGGTTTACACAGCACAGAACCCAGTGCCCAGGCACTGGGCCTGGGCAA  
ACATAATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGTGGCACGTGCTG  
AAGAACCGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGCG  
GCCTGAGACAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCTCTTCGCCGA  
CATCGCCTCCCACCCCTGGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTGCGCC  
GGAGAGCGGTTCTGTGCGGGGGCATACTCATCAGCTCCTGCTGGATTCTCTCTG  
CCGCCCACTGCTTCCAGGAGAGGTTTCCGCCCCACCACCTGACGGTGATCTTGGG  
CAGAACATACCGGGTGGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGTCGAAAA  
ATACATTGTCCATAAGGAATTCGATGATGACACTTACGACAATGACATTGCGCTG  
CTGCAGCTGAAATCGGATTCGTCCCGCTGTGCCAGGAGAGCAGCGTGGTCCGCA  
CTGTGTGCCTTCCCCCGGCGGACCTGCAGCTGCCGGAAGTGGACGGAGTGTGAGCT  
CTCCGGCTACGGCAAGCATGAGGCCTTGTCTCCTTTCTATTTCGGAGCGGCTGAAG  
GAGGCTCATGTCAGACTGTACCCATCCAGCCGCTGCACATCACAACATTTACTTA  
ACAGAACAGTCACCGACAACATGCTGTGTGCTGGAGACACTCGGAGCGGCGGGC  
CCCAGGCAAACCTTGACGACGCTGCCAGGGCGATTTCGGGAGGCCCCCTGGTGT  
GTCTGAACGATGGCCGCATGACTTTGGTGGGCATCATCAGCTGGGGCCTGGGCTG

TGGACAGAAGGATGTCCCGGGTGTGTACACAAAGGTTACCAACTACCTAGACTG  
GATTCGTGACAACATGCGACCGTGA (SEQ ID NO:7)

23. Fusion protein of OmpA and K2S, characterised in that it comprises a protein characterized by the following amino acid sequence:

MKKTAIAIAVALAGFATVAQAASEGNSDCYFGNGSAYRGTHSLTESGASCLPWNSM  
ILIGKVYTAQNPSAQALGLGKHNYCRNPDGDAKFWCHVLKNRRLTWEYCDVPSCST  
CGLRQYSQPQFRIKGGLFADIASHPWQAAIFAKHRRSPGERFLCGGILISSCWILSAAH  
CFQERFPPHLLTVILGRTYRVVPGEEEQKFEVEKYTVHKEFDDDDTYDNDIALLLQLKSD  
SSRCAQESSVVRTVCLPPADLQLPDWTECELSGYGKHEALSPFYSERLKEAHVRLYP  
SSRCTSQHLLNRTVTDNMLCAGDTRSGGPQANLHDACQGDSGGPLVCLNDGRMTL  
VGIIISWGLGCGQKDVPGVYTKVTNYLDWIRDNMIRPG (SEQ ID NO 8).

- 24 Fusion protein of OmpA and K2S according to claim 23, characterised in that it consists of a protein characterized by the following amino acid sequence:

MKKTAIAIAVALAGFATVAQAASEGNSDCYFGNGSAYRGTHSLTESGASCLPWNSM  
ILIGKVYTAQNPSAQALGLGKHNYCRNPDGDAKFWCHVLKNRRLTWEYCDVPSCST  
CGLRQYSQPQFRIKGGLFADIASHPWQAAIFAKHRRSPGERFLCGGILISSCWILSAAH  
CFQERFPPHLLTVILGRTYRVVPGEEEQKFEVEKYTVHKEFDDDDTYDNDIALLLQLKSD  
SSRCAQESSVVRTVCLPPADLQLPDWTECELSGYGKHEALSPFYSERLKEAHVRLYP  
SSRCTSQHLLNRTVTDNMLCAGDTRSGGPQANLHDACQGDSGGPLVCLNDGRMTL  
VGIIISWGLGCGQKDVPGVYTKVTNYLDWIRDNMIRPG (SEQ ID NO.8).

- 25 K2S protein characterized by the following amino acid sequence:

SEGNSDCYFGNGSAYRGTHSLTESGASCLPWNSMILIGKVYTAQNPSAQALGLGKH  
YCRNPDGDAKFWCHVLKNRRLTWEYCDVPSCSTCGLRQYSQPQFRIKGGLFADIAS  
HPWQAAIFAKHRRSPGERFLCGGILISSCWILSAAHCFQERFPPHLLTVILGRTYRVVP  
GEEEQKFEVEKYTVHKEFDDDDTYDNDIALLLQLKSDSSRCAQESSVVRTVCLPPADLQ  
LPDWTECELSGYGKHEALSPFYSERLKEAHVRLYPSSRCTSQHLLNRTVTDNMLCAG  
DTRSGGPQANLHDACQGDSGGPLVCLNDGRMTLVGIIISWGLGCGQKDVPGVYTKV  
TNYLDWIRDNMIRP\* (SEQ ID NO.11).

26. A vector containing a DNA sequence according to any one of claims 14 to 22

27. A vector according to claim 26, wherein said DNA sequence is preceded by a lac promoter and a ribosomal binding site.

- 28 The vector pComb3HSS containing a DNA according to any one of claims 14 to 22, wherein the expression of the gp III protein is suppressed or inhibited by deleting the DNA molecule encoding said gp III protein or by a stop codon between the gene coding for a polypeptide containing the kringle 2 domain and the serine protease domain of tissue plasminogen activator protein and the protein III gene.
- 29 A prokaryotic host cell comprising a DNA molecule according to any one of claims 14 to 22
30. A prokaryotic host cell comprising a vector according to any one of claims 26 to 28.
31. An E. coli host cell comprising a DNA molecule according to any one of claims 14 to 22
- 32 An E. coli host cell comprising a vector according to any one of claims 26 to 28.
- 33 Use of a DNA molecule according to any one of claims 14 to 22 or of a vector according to any one of claims 26 to 28 or a host cell according to any one of claims 29 to 32 in a method for the production of a polypeptide having the activity of tissue plasminogen activator.
- 34 Use according to claim 33, wherein said method is a method according to any one of claims 1 to 13

78

The demand must be filed directly with the competent International Preliminary Examining Authority or if two or more Authorities are competent, with the one chosen by the applicant. The full name or two-letter code of that Authority may be indicated by the applicant on the line below

IPEA/ EP

# PCT

## CHAPTER II

### DEMAND

under Article 31 of the Patent Cooperation Treaty  
The undersigned requests that the international application specified below be the subject of international preliminary examination according to the Patent Cooperation Treaty and hereby elects all eligible States (except where otherwise indicated)

|                                                                                                                                                                                                                                                          |                                                          |                                                         |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------|---------------------------------------------------------|
| For International Preliminary Examining Authority use only                                                                                                                                                                                               |                                                          |                                                         |
| Identification of IPEA                                                                                                                                                                                                                                   | Date of receipt of DEMAND                                |                                                         |
| <b>Box No. I IDENTIFICATION OF THE INTERNATIONAL APPLICATION</b>                                                                                                                                                                                         |                                                          |                                                         |
| Applicant's or agent's file reference<br>1-1170-PCT                                                                                                                                                                                                      |                                                          |                                                         |
| International application No.<br>PCT/EP01/12857                                                                                                                                                                                                          | International filing date (day/month/year)<br>07/11/2001 | (Earliest) Priority date (day/month/year)<br>14/11/2000 |
| Title of invention<br>Methods for large scale production of recombinant DNA-derived tPA or K2S molecules                                                                                                                                                 |                                                          |                                                         |
| <b>Box No. II APPLICANT(S)</b>                                                                                                                                                                                                                           |                                                          |                                                         |
| Name and address <small>(Family name followed by given name, for a legal entity, full official designation. The address must include postal code and name of country.)</small><br>Boehringer Ingelheim International GmbH<br>55216 Ingelheim/Rhein<br>DE |                                                          | Telephone No<br>07351/54-5468                           |
|                                                                                                                                                                                                                                                          |                                                          | Facsimile No<br>07351/54-4377                           |
|                                                                                                                                                                                                                                                          |                                                          | Teleprinter No                                          |
| Applicant's registration No with the Office                                                                                                                                                                                                              |                                                          |                                                         |
| State (that is, country) of nationality<br>DE                                                                                                                                                                                                            | State (that is, country) of residence<br>DE              |                                                         |
| Name and address <small>(Family name followed by given name, for a legal entity, full official designation. The address must include postal code and name of country.)</small><br>WERNER, Rolf-Guenther<br>Hugo-Haering-Str 72<br>88400 Biberach<br>DE   |                                                          |                                                         |
| State (that is, country) of nationality<br>DE                                                                                                                                                                                                            | State (that is, country) of residence<br>DE              |                                                         |
| Name and address <small>(Family name followed by given name, for a legal entity, full official designation. The address must include postal code and name of country.)</small><br>GOETZ, Friedrich<br>Beim Herbstenhof 31<br>72076 Tuebingen<br>DE       |                                                          |                                                         |
| State (that is, country) of nationality<br>DE                                                                                                                                                                                                            | State (that is, country) of residence<br>DE              |                                                         |
| <input checked="" type="checkbox"/> Further applicants are indicated on a continuation sheet.                                                                                                                                                            |                                                          |                                                         |

|                                                                                                                                                                                                                                                                                                                 |                                                               |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------|
| <p><b>Continuation of Box No II APPLICANT(S)</b></p> <p><i>If none of the following sub-boxes is used, this sheet should not be included in the demand.</i></p>                                                                                                                                                 |                                                               |
| <p><b>Name and address</b> <i>(Family name followed by given name, for a legal entity full official designation. The address must include postal code and name of country.)</i></p> <p>TAYAPIWATANA, Chatchai<br/>369/73 Soi Pradoo 1<br/>Janava Rd<br/>Bangkok 10120<br/>Thailand</p>                          |                                                               |
| <p><b>State (that is, country) of nationality</b></p> <p>TH</p>                                                                                                                                                                                                                                                 | <p><b>State (that is, country) of residence</b></p> <p>TH</p> |
| <p><b>Name and address</b> <i>(Family name followed by given name, for a legal entity full official designation. The address must include postal code and name of country.)</i></p> <p>MANOSROI, Jiradej<br/>234/171 Sirwatana Nives<br/>Mahidol Road<br/>Nong Hoi, Muang<br/>Chiang Mai 50000<br/>Thailand</p> |                                                               |
| <p><b>State (that is, country) of nationality</b></p> <p>TH</p>                                                                                                                                                                                                                                                 | <p><b>State (that is, country) of residence</b></p> <p>TH</p> |
| <p><b>Name and address</b> <i>(Family name followed by given name, for a legal entity full official designation. The address must include postal code and name of country.)</i></p> <p>MANOSROI, Aranya<br/>234/171 Sirwatana Nives<br/>Mahidol Road<br/>Nong Hoi, Muang<br/>Chiang Mai 50000<br/>Thailand</p>  |                                                               |
| <p><b>State (that is, country) of nationality</b></p> <p>TH</p>                                                                                                                                                                                                                                                 | <p><b>State (that is, country) of residence</b></p> <p>TH</p> |
| <p><b>Name and address</b> <i>(Family name followed by given name, for a legal entity full official designation. The address must include postal code and name of country.)</i></p>                                                                                                                             |                                                               |
| <p><b>State (that is, country) of nationality</b></p>                                                                                                                                                                                                                                                           | <p><b>State (that is, country) of residence</b></p>           |
| <p><input type="checkbox"/> Further applicants are indicated on another continuation sheet.</p>                                                                                                                                                                                                                 |                                                               |

**Box No III AGENT OR COMMON REPRESENTATIVE, OR ADDRESS FOR CORRESPONDENCE**The following person is ☐ agent ☐ common representativeand ☐ has been appointed earlier and represents the applicant(s) also for international preliminary examination☐ is hereby appointed and any earlier appointment of (an) agent(s)/common representative is hereby revoked☐ is hereby appointed, specifically for the procedure before the International Preliminary Examining Authority, in addition to the agent(s)/common representative appointed earlierName and address *(Family name followed by given name for a legal entity full official designation. The address must include postal code and name of country.)*

Telephone No

Facsimile No

Teleprinter No

Agent's registration No with the Office

☐ Address for correspondence Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.**Box No IV BASIS FOR INTERNATIONAL PRELIMINARY EXAMINATION****Statement concerning amendments \***

1 The applicant wishes the international preliminary examination to start on the basis of

☒ the international application as originally filedthe description ☒ as originally filed  
☐ as amended under Article 34the claims ☒ as originally filed  
☐ as amended under Article 19 (together with any accompanying statement)  
☐ as amended under Article 34the drawings ☒ as originally filed  
☐ as amended under Article 342 ☐ The applicant wishes any amendment to the claims under Article 19 to be considered as reversed.3 ☐ The applicant wishes the start of the international preliminary examination to be postponed until the expiration of 20 months from the priority date unless the International Preliminary Examining Authority receives a copy of any amendments made under Article 19 or a notice from the applicant that he does not wish to make such amendments (Rule 69 1(d)). *(This check-box may be marked only where the time limit under Article 19 has not yet expired.)*

\* Where no check-box is marked, international preliminary examination will start on the basis of the international application as originally filed or, where a copy of amendments to the claims under Article 19 and/or amendments of the international application under Article 34 are received by the International Preliminary Examining Authority before it has begun to draw up a written opinion or the international preliminary examination report, as so amended

Language for the purposes of international preliminary examination English☒ which is the language in which the international application was filed☐ which is the language of a translation furnished for the purposes of international search☐ which is the language of publication of the international application.☐ which is the language of the translation (to be) furnished for the purposes of international preliminary examination**Box No V ELECTION OF STATES**The applicant hereby elects all eligible States *(that is all States which have been designated and which are bound by Chapter II of the PCT)*

excluding the following States which the applicant wishes not to elect

## Box No. VI CHECK LIST

The demand is accompanied by the following elements, in the language referred to in Box No. IV, for the purposes of international preliminary examination

- |   |                                                                       |          |
|---|-----------------------------------------------------------------------|----------|
| 1 | translation of international application                              | sheets   |
| 2 | amendments under Article 34                                           | sheets   |
| 3 | copy (or, where required, translation) of amendments under Article 19 | sheets   |
| 4 | copy (or, where required, translation) of statement under Article 19  | sheets   |
| 5 | letter                                                                | 1 sheets |
| 6 | other (specify)                                                       | sheets   |

For International Preliminary  
Examining Authority use only

- |                          |                          |
|--------------------------|--------------------------|
| received                 | not received             |
| <input type="checkbox"/> | <input type="checkbox"/> |
| <input type="checkbox"/> | <input type="checkbox"/> |
| <input type="checkbox"/> | <input type="checkbox"/> |
| <input type="checkbox"/> | <input type="checkbox"/> |
| <input type="checkbox"/> | <input type="checkbox"/> |
| <input type="checkbox"/> | <input type="checkbox"/> |

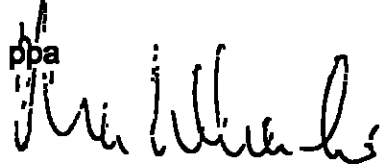
The demand is also accompanied by the item(s) marked below

- |                                                                                        |                                                                       |
|----------------------------------------------------------------------------------------|-----------------------------------------------------------------------|
| 1 <input checked="" type="checkbox"/> fee calculation sheet                            | 5 <input type="checkbox"/> statement explaining lack of signature     |
| 2 <input type="checkbox"/> original separate power of attorney                         | 6 <input type="checkbox"/> sequence listing in computer readable form |
| 3 <input type="checkbox"/> original general power of attorney                          | 7 <input type="checkbox"/> other (specify)                            |
| 4 <input type="checkbox"/> copy of general power of attorney; reference number, if any |                                                                       |

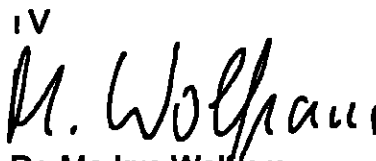
## Box No. VII SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the demand).

Boehringer Ingelheim International GmbH

ppa  


Dr Volker Mahlbacher

IV  


Dr Markus Wolfram

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3 ☐ The date of receipt of the demand is AFTER the expiration of 19 months from the priority date and item 4 or 5, below, does not apply

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## FEE CALCULATION SHEET

## Annex to the Demand

|                                                                                                                                                                                                                                                  |                                                                                |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------|
| International application No <b>PCT/EP01/12857</b>                                                                                                                                                                                               | For International Preliminary Examining Authority use only                     |
| Applicant's or agent's file reference <b>1-1170-PCT</b>                                                                                                                                                                                          | Date stamp of the IPEA                                                         |
| Applicant<br><b>Boehringer Ingelheim International GmbH</b>                                                                                                                                                                                      |                                                                                |
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|                                                                                                                                                                                                                                                  | Name <u>Boehringer Ingelheim GmbH</u>                                          |
|                                                                                                                                                                                                                                                  | Signature <u>1.V.</u> <u>1.V.</u>                                              |



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| 2                                                                                                                                                     |                                                  | -Request for detailed examination                                                                                         |
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**Métodos para la producción a gran escala de un tPA que se deriva de un ADN recombinante, o de moléculas de K2S**

5 El invento se refiere al sector de la trombolisis y de la producción de derivados del activador de plasminogeno tisular (tPA de *tissue plasminogen activator*) en células procaróticas

10 El invento se refiere a métodos para la producción de un tPA que se deriva de un ADN recombinante, una variante de este, o una molécula de K2S (Kningle 2-Senna) o una variante de ésta en células procaróticas, en que dicho tPA o dicha K2S o su variante se segrega extracelularmente como una proteína activa y correctamente plegada, y la célula procarótica contiene y expresa un vector que comprende el ADN que codifica dicho tPA o dicha K2S o su variante, unido operativamente al ADN que codifica el péptido de señal OmpA. El invento se refiere además a derivados específicos de K2S, obtenibles por dicho método. El invento se refiere además a dichas moléculas de ADN y al uso de dichas moléculas de ADN en dichos métodos

**Técnica de antecedentes**

20 Un activador de plasminógeno tisular (tPA) es un polipéptido que contiene 527 residuos de aminoácidos (27) con una masa molecular de 72 kDa. La molécula está dividida en cinco dominios estructurales. De modo aproximado, la región terminal de N es un dominio de dedo (finger) cerrado en bucle, que es seguido por un dominio de factor de crecimiento. Dos dominios similares, Kningle 1 y Kningle 2, siguen a continuación. Los dominios de dedo y Kningle 2 se fijan específicamente a los coágulos de fibrina acelerando con ello la activación por la proteína tPA del plasminógeno fijado. Secuencia 25 abajo del Kningle 2 está situada la proteasa de serina con su sitio catalítico situado en el extremo terminal de C. La proteasa de serina es responsable de convertir el plasminógeno en plasmina, una reacción que resulta importante en la homeostasis de la formación de fibrina y la disolución de coágulos. El correcto plegamiento del tPA requiere el correcto emparejamiento de 17 puentes de disulfuro en la molécula (1).

30 En el aspecto clínico, un tPA es un agente trombolítico selecto para el tratamiento de las enfermedades infarto de miocardio agudo, embolia pulmonar, ictus, oclusiones arteriales periféricas, y otras enfermedades tromboembólicas. Tiene la ventaja de no causar efectos colaterales en la formación de hemorragias sistémicas y el agotamiento de fibrinógeno (7). Células de melanoma de Bowes se usaron en primer término como 35 una fuente en la producción de tPA para finalidades terapéuticas (12). Puesto que se requiere para uso clínico un proceso compatible con la producción eficiente de una proteína purificada en alto grado con buen rendimiento, la construcción de un tPA

recombinante (r-tPA) de plena longitud progresó hasta llegar a células de mamíferos. Células de ovario de hámster chino (CHO) fueron transfectadas con el gen de tPA con el fin de sintetizar el r-tPA (8, 22). El producto que se deriva de un ADN recombinante, producido por un sistema de fermentación de un cultivo de células de mamífero, se cosecha y purifica a partir del medio de cultivo. Atraídos por la simplicidad y rentabilidad de la producción, se investigaron ciertos esfuerzos en la producción de r-tPA a partir de microorganismos, especialmente bacterias, y más especialmente a partir de *Escherichia coli* (10, 13, 30). Observando el bajo rendimiento y la formación de cuerpos de inclusión, que daban como resultado un plegamiento defectuoso y una enzima inactiva, se han propuesto numerosas estrategias para solventar estos problemas.

Se consideraron varias variantes mutantes por delección, inclusive la de Kringle 2 más proteasa de senna (K2S). Sin embargo, la actividad enzimática de la K2S recombinante (r-K2S) se obtuvo solamente cuando se consiguieron procesos de plegamiento renovado de cuerpos de inclusión purificados procedentes del compartimiento citoplásmico (16, 29). Con el fin de evitar los complicados procesos de plegamiento renovado, las impurezas procedentes de las proteínas defectuosamente plegadas y el suministro de proteínas periplásmicas, se explotaron sistemas especiales de expresión de bacterias (6, 31). A pesar de la expresión periplásmica del r-tPA, la expresión excesiva condujo a agregados inactivos, incluso en la condición oxidante de grado relativamente alto en el periplasma. En la técnica anterior, existen unas pocas descripciones de métodos para la preparación de una K2S recombinante en *E. coli*. Sin embargo, no existe ninguna descripción de un método que conduzca a un método barato para la producción a gran escala de una K2S biológicamente activa.

Obukowicz y colaboradores (25) expresaron y purificaron una r-K2S procedente del espacio periplásmico. La evidente desventaja de este método la constituía una operación suplementaria de extracción periplásmica, que no es apropiada para una producción a gran escala.

Saito y colaboradores (29) describen la expresión citoplásmica de una r-K2S. Los autores usaron unos procesos de renaturalización *in vivo* para la r-K2S expresada, que era purificada a partir del espacio citoplásmico de *E. coli* como cuerpo de inclusión. Boehringer Mannheim usa un similar y complicado proceso de desnaturalización y plegamiento renovado, que implica las operaciones de digestión de células, solubilización en condiciones desnaturalizantes y reductoras y reactivación en condiciones oxidantes en la presencia de GSH/GSSG, que no es rentable (24) y requiere una mutación de la secuencia de aminoácidos que tenga posiblemente un potencial antigénico.

En 1991, Waldenström y colaboradores (34) construyeron un vector (pEZZK2P) para la secreción del dominio de Kringle 2 más proteasa de senna a un material

- sobrenadante de cultivo de E coli. Se uso hidroxilamina para eliminar el peptido de fusión ZZ a partir de la fraccion purificada de IgG-Sepharose. El agente de disociacion, hidroxilamina, requería una modificacion de los sitios de disociacion del Kringle 2 mas proteasa de serina (Asn<sup>177</sup> - Ser y Asn<sup>184</sup> - Gln) para protegerlos de esta manera con respecto de la digestion por hidroxilamina. Sin embargo, la resultante molécula de K2S no nativa y no plegada apropiadamente, no es idonea para finalidades terapéuticas. No se describió ninguna actividad enzimática con respecto a la actividad de fijación de fibrina y de proteasa. La desacostumbrada secuencia puede incluso activar al sistema inmunitario humano.
- 10 El problema que subyace en el presente invento fue por consiguiente proporcionar un metodo aplicable comercialmente para la producción a gran escala de moléculas de tPA y derivados de ella, p ej la K2S, en el que la molécula de K2S se segrega en su forma biologicamente activa dentro del material sobrenadante de cultivo.
- 15 **Descripción del invento**
- Este problema se resolvió dentro del alcance de las reivindicaciones y de la memoria descriptiva del presente invento.
- El uso del singular o plural en las reivindicaciones o en la memoria descriptiva no se pretende de ninguna manera que sea limitativo e incluye también la otra forma.
- 20 El invento se refiere a un método para la producción de un activador de plasminógeno tisular (tPA) que se deriva de un ADN recombinante, una variante de tPA, una molécula de proteasa de Kringle 2 senna (K2S) o una variante de K2S en células procarinóticas, en el que dicho tPA, dicha variante de tPA, dicha molécula de K2S o dicha variante de K2S se segregan extracelularmente como una proteína activa y correctamente plegada, cuyo metodo está caracterizado porque la célula procarinótica contiene y expresa un vector que comprende el ADN que codifica dicho tPA, dicha variante de tPA, dicha molécula de K2S o dicha variante de K2S en union operativa con el ADN que codifica el péptido de señal OmpA o un derivado funcional de éste.
- 25 Sorprendentemente, el uso del peptido de señal OmpA a solas y/o en combinacion con los aminoácidos terminales de N, SEGN (SEQ ID NO 9) y SEGNSD (SEQ ID NO.10), traslada (transloca) al tPA que se deriva de un ADN recombinante, a la variante de tPA, a la molécula de K2S o a la variante de K2S hacia la superficie exterior y facilita la liberacion de la molécula funcional y activa dentro del medio de cultivo, en un grado mayor que cualquier otro metodo en la técnica anterior. Antes de
- 30 cruzar la membrana exterior, la proteína que se deriva de un ADN recombinante se pliega correctamente de acuerdo con el metodo del presente invento. El peptido de señal se separa por disociación para producir una molécula madura. Sorprendentemente, la
- 35

eficiencia de la eliminación de este péptido de señal es muy alta y conduce a un plegamiento correcto de la proteína que se deriva de un ADN recombinante

5 Dicho péptido de señal OmpA interactúa con la SecE y es suministrado a través de la membrana interior mediante energía generada por la SecA, que se fija a los componentes Sec (SecE-SecY) La SecY forma un poro de secreción para enviar la proteína que se deriva de un ADN recombinante de acuerdo con el invento El espacio existente entre la membrana exterior y la membrana interior de bacterias Gram-negativas, es decir el periplasma, tiene una condición de oxidación más alta en comparación con el espacio citoplásmico Esto sustenta la formación de enlaces de disulfuro y el plegamiento apropiado de la proteína que se deriva de un ADN recombinante, (p.ej., la K2S) en el periplasma para producir una molécula activa De acuerdo con el presente invento, el péptido de señal será separado por disociación para producir una molécula madura El complejo de secretina de GspD y lipoproteína de GspS en la membrana exterior sirve como canal de puerta para segregar la proteína que se deriva de un ADN recombinante de acuerdo con el invento hacia el medio extracelular Este proceso de secreción requiere energía, que se genera en el citoplasma por la proteína que fija nucleótidos, GspE, y luego se transfiere a la proteína de membrana interior (Gsp G-J, F y K-N) La GspC transfiere la energía a la GspD formando un enlazador cruzado entre un conjunto de proteínas de membrana interior (Gsp G-J, F y K-N) y la GspD Antes de cruzar con éxito la membrana exterior, la proteína que se deriva de un ADN recombinante se pliega correctamente

El hecho de estar unido operativamente de acuerdo con el invento significa que el ADN que codifica el tPA, la variante de tPA, la molécula de K2S o la variante de K2S (que preferiblemente comprende el ácido nucleico que codifica SEGN o SEGNSD en su porción terminal de N) se clona en íntima proximidad con el ADN de OmpA dentro del vector a fin de conseguir la expresión de la proteína de fusión del OmpA y el tPA, la variante de tPA, la molécula de K2S o la variante de K2S y dirigir la secreción en el exterior de la célula hospedante procarionótica. Típicamente, se segrega la mayoría del tPA, de la variante de tPA, de la molécula de K2S o de la variante de K2S, y luego se puede purificar por métodos apropiados tales como precipitación con sulfato de amonio y/o cromatografía de afinidad y operaciones de purificación ulteriores El invento incluye también el uso de inductores tales como IPTG, o IPTG en combinación con glicerol, el mejoramiento de la condición de incubación y del período de cosecha para hacer máxima la cantidad de proteína activa

35 En una realización preferida, dicho ADN que codifica el péptido de señal OmpA se puede fusionar a un péptido corto caracterizado por la secuencia de aminoácidos SEGN o SEGNSD o la secuencia de ácido nucleico de codificación TCTGAGGGAAAC

- (SEQ ID NO 20) o TCTGAGGGAAACAGTGAC (SEQ ID NO 1), y situado en la porción terminal de N o junto a la porción terminal de N del tPA, de la variante de tPA, de la molécula de K2S o de la variante de K2S. Así, preferiblemente, dicha proteína de fusión comprende OmpA-SEGN-SD -tPA, -variante de tPA, -molécula de K2S o -variante de K2S. Lo que es incluso más preferido, dichos aminoácidos caracterizados por SEGN o SEGN-SD pueden ser portadores de una mutación puntual o pueden estar reemplazados por un aminoácido no natural. Lo que es incluso más preferido, puede haber un espaciador aminoácido o que no sea aminoácido entre el OmpA y el SEGN o SEGN-SD y el tPA, la variante de tPA, la molécula de K2S o la variante de K2S. Por lo tanto, en un método preferido de acuerdo con el invento, dicha célula procarionótica contiene y expresa un vector que comprende el ADN que codifica dicho tPA, dicha variante de tPA, dicha molécula de K2S o dicha variante de K2S unida operativamente al ADN que codifica el péptido de señal OmpA que está unido operativamente a la molécula de ácido nucleico definida por la secuencia TCTGAGGGAAACAGTGAC o un derivado funcional de ella.
- El método de acuerdo con el invento comprende células hospederas procarionóticas tales como, pero sin limitarse a, *Escherichia coli* (*E. coli*), *Bacillus subtilis*, *Streptomyces*, *Pseudomonas*, p.ej. *Pseudomonas putida*, *Proteus mirabilis*, *Saccharomyces*, *Pichia* o *Staphylococcus*, p.ej. *Staphylococcus carnosus*. Preferiblemente, dichas células hospederas de acuerdo con el invento son bacterias Gram-negativas.
- Preferiblemente, un método de acuerdo con el invento está caracterizado también porque la célula procarionótica es *E. coli*. Cepas apropiadas incluyen, pero no se limitan a, *E. coli* XL-1 Blue, BL21(DE3), JM109, series de DH, TOP10 y HB101.
- Preferiblemente, un método de acuerdo con el invento está caracterizado también porque se llevan a cabo las siguientes operaciones:
- a) el ADN que codifica el tPA, la variante de tPA, la molécula de K2S o la variante de K2S se amplifica por una PCR (reacción en cadena de polimerasa),
  - b) el producto de PCR se purifica,
  - c) dicho producto de PCR se introduce dentro de un vector que comprende el ADN que codifica el péptido de señal OmpA y el ADN que codifica la gpIII de una manera tal que dicho producto de PCR se une operativamente secuencialmente con el ADN que codifica la secuencia de señal de OmpA y se une secuencialmente abajo con el ADN que codifica la gpIII de dicho vector,
  - d) se introduce un codón de detención entre dicho tPA, dicha variante de tPA, dicha molécula de K2S o dicha variante de K2S y dicha gpIII,
  - e) dicho vector se expresa por la célula procarionótica,
  - f) se purifica el tPA, la variante de tPA, la molécula de K2S o la variante de K2S.

Para la operación a) de acuerdo con el invento es importante la elección o el

diseño de los cebadores para clonar el ADN en la situación y la dirección correctas del vector de expresión (véase el Ejemplo 1) Por lo tanto, los cebadores que se ejemplifican en el Ejemplo 1 y en la Figura 4 constituyen un aspecto importante del presente invento Por la gpIII de la operación c) se entiende la proteína de gen III que está presente principalmente en vectores fagémidos El codón de detención se introduce para evitar la transcripción de la gpIII, conduciendo de esta manera finalmente a la secreción de la tPA, la variante de tPA, la molécula de K2S o la variante de K2S que interesa Se puede emplear cualquier método apropiado para la introducción del codón de detención, tal como una mutagénesis dirigida a un sitio (p.ej., Weiner MP, Costa GL (1994) PCR Methods Appl 4(3) páginas 131-136, Weiner MP, Costa GL, Schoettlin W, Cline J, Mathur E, Bauer JC (1994) Gene 151(1-2) 119-123, véase también el Ejemplo 1)

Se puede usar cualquier vector en el método de acuerdo con el invento, pero preferiblemente dicho vector es un vector fagémido (véase más adelante)

Preferiblemente, un método de acuerdo con el invento está caracterizado también porque el tPA, la variante de tPA, la molécula de K2S o la variante de K2S se selecciona a partir de un activador de plasminógeno tisular humano (tPA, Figura 16) o de un fragmento, de una variante funcional, de una variante alélica, de una subunidad, de un derivado químico, de una proteína de fusión o de una variante por glicosilación de este Dichos/as fragmentos, variantes alélicas, variantes funcionales, variantes basadas en el código degenerado de ácidos nucleicos, dichas proteínas de fusión con una proteína de tPA de acuerdo con el invento, derivados químicos o una variante por glicosilación de las proteínas de tPA de acuerdo con el invento pueden incluir uno/a, varios/as o la totalidad de los/las siguientes dominios o subunidades o variantes de ellos/as

- 1 Dominio de dedo (4-50)
- 2 Dominio de factor de crecimiento (50-87)
- 3 dominio de Kringle 1 (87-176)
- 4 dominio de Kringle 2 (176-262)
- 5 Dominio de proteasa (276-527)

La numeración y la denominación de los dominios son conformes al número de acceso en el Genbank (Banco Genético) GI 137119, o a Nature 301 (5897), 214-221 (1983)

Más preferiblemente, un método de acuerdo con el invento está caracterizado también porque el tPA, la variante de tPA, la molécula de K2S o la variante de K2S se selecciona entre la variante K2S Kringle 2 (4) más proteasa de serina (5) del activador de plasminógeno tisular humano, o un fragmento, una variante funcional, una variante alélica, una subunidad, un derivado químico, una proteína de fusión o una variante por glicosilación de éste

Más preferiblemente, un método de acuerdo con el invento está caracterizado también porque el vector es un vector fagemido que comprende el ADN y codifica el péptido de señal OmpA y el ADN que codifica la gpIII

5 Más preferiblemente, un método de acuerdo con el invento está caracterizado también porque el vector es el fagemido pComb3HSS (véase también el Ejemplo 1)

Más preferiblemente, un método de acuerdo con el invento está caracterizado también porque la secuencia de ADN comprende o consta de la siguiente secuencia de ADN que codifica OmpA y K2S o una variante funcional de ésta o una variante debida al código degenerado de nucleótidos

10 ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTG  
 GCCCAGGCGGCCTCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCT  
 ACCGTGGCACGCACAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAATT  
 CCATGATCCTGATAGGCAAGGTTTACACAGCACAGAACCCAGTGCCCAGGCACT  
 GGGCCTGGGCAACATAATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGG  
 15 TGCCACGTGCTGAAGAACCGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCC  
 TGCTCCACCTGCGGCCTGAGACAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGG  
 GCTCTTCGCCGACATCGCCTCCCACCCCTGGCAGGCTGCCATCTTTGCCAAGCAC  
 AGGAGGTGCGCCGGAGAGCGGTTCTGTGCGGGGGCATACTCATCAGCTCCTGCT  
 GGATTCTCTCTGCCGCCCACTGCTTCCAGGAGAGGTTTCCGCCCCACCACCTGACG  
 20 GTGATCTTGGGCAGAACATACCGGGTGGTCCCTGGCGAGGAGGAGCAGAAATTTGA  
 AGTCGAAAAATACATTGTCCATAAGGAATTCGATGATGACACTTACGACAATGACAT  
 TGGCGCTGCTGCAGCTGAAATCGGATTCTGTCCTCGCTGTGCCAGGAGAGCAGCG  
 TGGTCCGCACTGTGTGCCTTCCCCCGGCGGACCTGCAGCTGCCGGACTGGACG  
 GAGTGTGAGCTCTCCGGCTACGGCAAGCATGAGGCCTTGTCTCCTTTCTATTG  
 25 GAGCGGCTGAAGGAGGCTCATGTCAGACTGTACCCATCCAGCCGCTGCACATCA  
 CAACATTTACTTAACAGAACAGTCACCGACAACATGCTGTGTGCTGGAGACACTC  
 GGAGCGGCGGGCCCCAGGCAAACCTTGACGACGCCTGCCAGGGCGATTTCGGG  
 AGGCCCCCTGGTGTGTCTGAACGATGGCCGCATGACTTTGGTGGGCATCATCAG  
 CTGGGGCCTGGGCTGTGGACAGAAGGATGTCCCGGGTGTGTACACAAAGGTTA  
 30 CCAACTACCTAGACTGGATTCTGTGACAACATGCGACCG (SEQ ID NO 2)

Más preferiblemente, un método de acuerdo con el invento está caracterizado también porque la secuencia de ADN del OmpA comprende o consta de la siguiente secuencia, o una variante funcional de esta o una variante debida al código degenerado de nucleótidos

35 ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTG  
 GCCCAGGCGGCC (SEQ ID NO 3)

Dicho ADN codifica la siguiente secuencia de aminoácidos del OmpA Por lo

tanto, el OmpA comprende o consta de una proteína que esta caracterizada por la siguiente secuencia de aminoácidos o un fragmento, una variante funcional, una variante alelica, una subunidad, un derivado químico o una variante por glicosilación de este, como parte del invento

5 MKKTAIAIAVALAGFATVAQAA (SEQ ID NO 21)

La región no traducida puede contener un elemento regulador, tal como p.ej. una unidad de iniciación de la transcripción (promotor) o un intensificador. Dicho promotor puede ser, por ejemplo, un promotor constitutivo, inducible o de desarrollo controlado. Preferiblemente, sin descartar a otros promotores conocidos, se usan los promotores constitutivos del Cytomegalovirus (CMV) humano y del virus de sarcoma de Rous (RSV), así como los promotores del virus 40 de Simio (SV40) y del Herpes simple. Los promotores inducibles de acuerdo con el invento comprenden promotores resistentes a antibióticos, promotores de choque térmico, el "promotor del virus de tumor mamario" inducible por hormonas y el promotor de metalotioneína. Los promotores preferidos incluyen el promotor de T3, el promotor de T7, el Lac/ara1 y el Ltet0-1

Más preferiblemente, un método de acuerdo con el invento está caracterizado también porque el ADN del tPA, de la variante de tPA, de la molécula de K2S o de la variante de K2S es precedido por un promotor lac y/o por un sitio de fijación a ribosomas tal como la secuencia de Shine-Dalgarno (vease también el Ejemplo)

20 Más preferiblemente, un método de acuerdo con el invento está caracterizado también porque el ADN que codifica el tPA, la variante de tPA, la molécula de K2S o la variante de K2S se selecciona entre el grupo de moléculas de ADN que codifican por lo menos un 90% de los aminoácidos 87 - 527, 174 - 527, 180 - 527 ó 220 - 527 de la proteína del activador de plasminógeno tisular humano

25 Más preferiblemente, un método de acuerdo con el invento está caracterizado también porque la secuencia de ADN de la K2S comprende o consta de la siguiente secuencia

TCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTACCGTGGCACGC  
ACAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAATTCCATGATCCTGAT  
30 AGGCAAGGTTTACACAGCACAGAACCCCAAGTGCCCAGGCACTGGGCCTGGGCAA  
ACATAATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGTGCCACGTGCT  
GAAGAACCGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTG  
CGGCCTGAGACAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCTCTTCGC  
CGACATCGCCTCCCAACCCTGGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTC  
35 GCCCGGAGAGCGGTTCTGTGCGGGGGCATACTCATCAGCTCCTGCTGGATTCT  
CTCTGCCGCCCACTGCTTCCAGGAGAGGTTTCCGCCCCACCACCTGACGGTGAT  
CTTGGGCAGAACATACCGGGTGGTCCCTGGCGAGGAGGAGCAGAAATTTGAAG

TCGAAAATACATTGTCCATAAGGAATTCGATGATGACACTTACGACAATGACATT  
 GCGCTGCTGCAGCTGAAATCGGATTCGTCCCGCTGTGCCCAGGAGAGCAGCGT  
 GGTCCGCACTGTGTGCCTTCCCCCGGCGGACCTGCAGCTGCCGGACTGGACGG  
 AGTGTGAGCTCTCCGGCTACGGCAAGCATGAGGCCTTGTCTCCTTTCTATTCTCGG  
 5 AGCGGCTGAAGGAGGCTCATGTCAGACTGTACCCATCCAGCCGCTGCACATCAC  
 AACATTTACTTAACAGAACAGTCACCGACAACATGCTGTGTGCTGGAGACACTCG  
 GAGCGGGCGGGCCCCAGGCCAACTTGCACGACGCCTGCCAGGGCGATTCTGGGA  
 GGCCCCCTGGTGTGTCTGAACGATGGCCGCATGACTTTGGTGGGCATCATCAGC  
 TGGGCGCCTGGGCTGTGGACAGAAGGATGTCCCGGGTGTGTACACAAAGGTTAC  
 10 CAACTACCTAGACTGGATTCTGTGACAACATGCGACCGTGA (SEQ ID NO 4)

El presente invento se refiere también a variantes de las moléculas de ácidos nucleicos antes mencionadas debido al código degenerado o a fragmentos de este, a ácidos nucleicos que se hibndan con dichos ácidos nucleicos en condiciones rigurosas, y a variantes alélicas o funcionales. El invento se refiere también a ácidos nucleicos que comprenden dicho ácido nucleico de K2S, fusionado con el ácido nucleico que codifica otra molécula de proteína.

Condiciones rigurosas, tal como se entienden por una persona experta, son condiciones que seleccionan una homología de más de 85%, preferiblemente de más de 90% (Sambrook y colaboradores, 1989, Molecular Cloning A Laboratory Manual, 2ª edición, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, Nueva York). La hibndación se llevara a cabo p ej en un medio 6x SSC/5x solución de Denhardt/0,1% de SDS (SDS, dodecil-sulfato de sodio) a 65°C. El grado de rigurosidad se decide en la operación de lavado. Así, por ejemplo para una selección de secuencias de ADN con una homología de aproximadamente 85% o mas, son apropiadas las condiciones 0,2 x SSC/SDS al 0,01%/65°C y para una selección de secuencias de ADN con una homología de aproximadamente 90% o más, son apropiadas las condiciones 0,1 x SSC/SDS al 0,01%/65°C. La composición de dichos reactivos se describe en la cita de Sambrook y colaboradores (1989, véase *supra*).

Otra parte importante del presente invento es una variante del activador de plasminógeno tisular humano, que comprende o consta de la proteína de Kringle 2 (4) más proteasa de senna (5) (denominada abreviadamente K2S), o una variante o un fragmento, una variante funcional, una variante alélica, una subunidad, un derivado químico, una proteína de fusión o una variante por glicosilación de ella.

La numeración y la denominación de los dominios son conformes al número de acceso en el Genbank (Banco Genético) GI 137119, o a Nature 301 (5897), 214-221 (1983), en que el dominio de Kringle 2 se extiende desde el aminoácido 176-262 y el dominio de proteasa desde 276-527. Por lo tanto, de acuerdo con el invento, una

molécula de K2S preferida puede incluir los aminoácidos 176-527 inclusive los aminoácidos situados entre el Kringle 2 y la proteasa (aminoácidos 263 a 275, ejemplificados en la Figura (estructura A)) Una molécula de K2S de acuerdo con el invento comprende la parte mínima del dominio de Kringle 2 y el dominio de proteasa reteniendo todavía la actividad de proteasa y la actividad de fijación a fibrina (medida tal como se ejemplifica en la memoria descriptiva y en el ejemplo) Dicha molécula de K2S de acuerdo con el invento comprende los aminoácidos SEGN o SEGNSD en su porción terminal de N (véase *infra*) Una molécula de K2S preferida no incluye los aminoácidos 1 a 3 o bien 1 a 5 de la molécula de tPA Preferiblemente, una molécula de K2S de acuerdo con el invento tiene el aminoácido Asn en las posiciones 177 y 184, es decir que no requiere las modificaciones que se describen en la cita de Waldenström para presentar una posibilidad mejorada de producción con un método de acuerdo con el invento Por lo tanto, una molécula de K2S preferida de acuerdo con el invento tiene la secuencia nativa de aminoácidos (ninguna mutación) a diferencia de las moléculas conocidas por la técnica anterior Lo que es sumamente prefendo, dicha molécula de K2S de acuerdo con el invento es una molécula caracterizada por la secuencia nativa de aminoácidos o partes de ésta, que tampoco tiene los aminoácidos 1 a 3 o bien 1 a 5 del tPA y comprende en el extremo terminal de N los aminoácidos SEGN o SEGNSD para presentar una posibilidad mejorada de producción y/o un plegamiento correcto de la molécula

Es esencial que la proteína K2S de acuerdo con el invento comprenda en su porción terminal de N un péptido caracterizado por la secuencia de aminoácidos SEGN que permite ventajosamente una producción comercial con un método como se describe anteriormente y que conduce a una proteína K2S correctamente plegada y segregada Dichos 4 aminoácidos caracterizados por SEGN pueden tener uno o varios aminoácidos mas en el extremo terminal de N, pero dichos aminoácidos han de estar situados en la porción terminal de N en lugar de en la porción terminal de C Lo mas preferiblemente, dichos aminoácidos están situados en la porción terminal de N Preferiblemente, los aminoácidos caracterizados por SEGN pueden ser portadores de una mutación puntual o pueden estar reemplazados por un aminoácido no natural

Por lo tanto, en otra realización importante el invento se refiere a una proteína K2S, caracterizada porque comprende los aminoácidos definidos por la secuencia SEGN o una variante o un fragmento, una variante funcional, una variante alelica, una subunidad, un derivado químico, una proteína de fusión o una variante por glicosilación de ella

Dichos fragmentos se ejemplifican p ej en la Figura 10 (estructura B-1) y en la Figura 11 (estructura B-2) y se extienden entre los aminoácidos 193-527 La estructura

B-1 tiene el aminoácido nativo Cys en posición 261, mientras que en la B-2 el aminoácido está reemplazado por Ser. Fragmentos adicionales de acuerdo con el invento, que comprenden los aminoácidos 220-527 (Figura 14, estructura C) o que comprenden los aminoácidos 260-527 (Figura 15, estructura D) pueden ser modificados de acuerdo con el invento por adición de los aminoácidos SEGN y/o reemplazo del Cys en 261 por Ser. El experto puede determinar la longitud mínima de una molécula de K2S de acuerdo con el invento con el fin de retener su función biológica y generar una molécula de K2S con posibilidad mejorada de producción y/o correcto plegamiento por adición de los aminoácidos SEGN en la porción terminal de N. Por lo tanto, otra realización preferida es dicha molécula mínima de K2S con SEGN en su porción terminal de N.

En otra realización importante, el invento se refiere a una proteína K2S caracterizada porque comprende los aminoácidos definidos por la secuencia SEGNSD o una variante, o un fragmento, una variante funcional, una variante alélica, una subunidad, un derivado químico, una proteína de fusión o una variante por glicosilación de ésta. Dichos fragmentos son ejemplificados p.ej. en la Figura 12 (estructura B-3) y en la Figura 13 (estructura B-4) y se extienden entre los aminoácidos 191-527. La estructura B-3 tiene el aminoácido nativo Cys en posición 261, mientras que en la B-4 el aminoácido está reemplazado por Ser. Fragmentos adicionales de acuerdo con el invento, que comprenden los aminoácidos 220-527 (Figura 14, estructura C) o que comprenden los aminoácidos 260-527 (Figura 15, estructura D), se pueden modificar de acuerdo con el invento por adición de los aminoácidos SEGNSD y/o por reemplazo del Cys en 261 por Ser. El experto puede determinar la longitud mínima de una molécula de K2S de acuerdo con el invento con el fin de retener su función biológica y generar una molécula de K2S con posibilidad mejorada de producción y/o correcto plegamiento por adición de los aminoácidos SEGNSD en la porción terminal de N. Por lo tanto, otra realización preferida es dicha molécula mínima de K2S con SEGNSD en su porción terminal de N.

Otra realización más preferida del presente invento se refiere a una proteína K2S que comprende una proteína caracterizada por la siguiente secuencia de aminoácidos o una variante o un fragmento, una variante funcional, una variante alélica, una subunidad, un derivado químico o una variante por glicosilación de esta:

SEGNSDCYFGNGSAYRGTHSLTESGASCLPWNSMILIGKVYTAQNPSAQALGLGKH  
NYCRNPDGDAKPWCHVLKNRRLTWEYCDVPSCSTCGLRQYSQPQFRIKGGLFADIA  
SHPWQAAIFAKHRRSPGERFLCGGILISSCWILSAAHCFQERFPPHLLTVILGR TYRV  
VPGEEEQKFVEVEKYIVHKEFDDDTYDNDIALQLKSDSSRCAQESSVVRTVCLPPADL  
QLPDWTECELSGYGKHEALSPFYSERLKEAHVRLYPSSRCTSQHLLNRTVTDNMLC  
AGDTRSGGPQANLHDACQGDSGGPLVCLNDGRMTLVGIISWGLGCGQKDVPGVYT  
KVTNYLDWIRDNM RP\* (SEQ ID NO 11)

De acuerdo con el invento, \* significa STOP (es decir, codificado por un codón de detención) Esta molécula de K2S es ejemplificada en la Figura 8

Una variante de la molécula de K2S de acuerdo con el invento se refiere a una proteína de fusión de K2S que está fusionada con otra molécula de proteína

5 Otra realización más preferida del presente invento se refiere a una proteína K2S que consta de una proteína caracterizada por la siguiente secuencia de aminoácidos  
SEGNSDCYFGNGSAYRGTHSLTESGASCLPWNSMILIGKVYTAQNPSAQALGLGKH  
NYCRNPDGDAKPWCHVLKNRRLTWEYCDVPSCSTCGLRQYSQPQFRIKGGLFADIA  
SHPWQAAIFAKHRRSPGERFLCGGILISSCWILSAAHCFQERFPPHHLTIVILGRITYRV  
10 VPGEEEQKFEVEKYIVHKEFDDDTYDNDIALQLKSDSSRCAQESSVVRTVCLPPADL  
QLPDWTECELSGYGKHEALSPFYSERLKEAHVRLYPSSRCTSQHLLNRTVTDNMLC  
AGDTRSGGPQANLHDACQGDSGGPLVCLNDGRMTLVGIISWGLGCGQKDVPGVYT  
KVTNYLDWIRDNMRP\* (SEQ ID NO 11)

Dichas moléculas de K2S pueden ser codificadas por una molécula de ADN como se ha descrito *supra* Otro aspecto importante del invento se refiere a una molécula de ADN caracterizada porque codifica

- a) la proteína OmpA o un derivado funcional de ella unida/o operativamente a
- b) una molécula de ADN que codifica un polipéptido que contiene el dominio de Kringle 2 y el dominio de proteasa de serina de la proteína del activador de plasminógeno tisular

20 Más preferiblemente, una molécula de ADN de acuerdo con el invento está caracterizada también porque la secuencia de ADN comprende o consta de la siguiente secuencia de ADN que codifica OmpA y K2S o una variante funcional de esta o una variante debida al código degenerado de nucleótidos

ATGAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTGGC  
25 CCAGGCGGCCTCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTAC  
CGTGGCACGCACAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAATTCC  
ATGATCCTGATAGGCAAGGTTTACACAGCACAGAACCCAGTGCCCAGGCACTGG  
GCCTGGGCAAACATAATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGTGC  
CACGTGCTGAAGAACCGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCT  
30 CCACCTGCGGCCTGAGACAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCT  
CTTCGCCGACATCGCCTCCCACCCCTGGCAGGCTGCCATCTTTGCCAAGCACAG  
GAGGTGCCCCGAGAGCGGTTCTGTGCGGGGGCATACTCATCAGCTCCTGCTGG  
ATTCTCTCTGCCGCCCACTGCTTCCAGGAGAGGTTTCCGCCCCACCACCTGACG  
GTGATCTTGGGCAGAACATACCGGGTGGTCCCTGGCGAGGAGGAGCAGAAATTT  
35 GAAGTCGAAAATACATTGTCCATAAGGAATTCGATGATGACACTTACGACAATGA  
CATTGCGCTGCTGCAGCTGAAATCGGATTCGTCCCGCTGTGCCAGGAGAGCAG  
CGTGGTCCGCACTGTGTGCCTTCCCCCGGCGGACCTGCAGCTGCCGGACTGGA

CGGAGTGTGAGCTCTCCGGCTACGGCAAGCATGAGGCCTTGTCTCCTTTCTATT  
 CGGAGCGGCTGAAGGAGGCTCATGTCAGACTGTACCCATCCAGCCGCTGCACAT  
 CACAACATTTACTTAACAGAACAGTCACCGACAACATGCTGTGTGCTGGAGACAC  
 TCGGAGCGGCGGGCCCCAGGCAAACCTTGACGACGCCTGCCAGGGCGATTTCGG  
 5 GAGGCCCCCTGGTGTGTCTGAACGATGGCCGCATGACTTTGGTGGGCATCATCA  
 GCTGGGGCCTGGGCTGTGGACAGAAGGATGTCCCGGGTGTGTACACAAAGGTT  
 ACCAACTACCTAGACTGGATTCGTGACAACATGCGACCG (SEQ ID No 2)

Dicha molécula de ADN codifica la siguiente proteína de fusión de OmpA y K2S  
 Dicha proteína de fusión de OmpA y K2S esta caracterizada porque comprende o consta  
 10 de una proteína caracterizada por la siguiente secuencia de aminoácidos o un fragmento,  
 una variante funcional, una variante alelica, una subunidad, un derivado químico o una  
 variante por glicosilación de ella y constituye una parte importante del presente invento  
 MKKTAIAIAVALAGFATVAQAASEGNSDCYFGNGSAYRGTHSLTESGASCLPWNSMI  
 LIGKVYTAQNPSAQALGLGKHNYCRNPDGDAKPWCHVLKNRRLTWEYCDVPSCST  
 15 CGLRQYSQPQFRIKGGFLFADIASHPWQAAIFAKHRRSPGERFLCGGILISSCWILSAA  
 HCFQERFPPHHLTVILGRTYRVVPGEEEQKFEVEKYIVHKEFDDDTYDNDIALQLKS  
 DSSRCAQESSVVRTVCLPPADLQLPDWTECELSGYGKHEALSPFYSERLKEAHVRL  
 YPSSRCTSQHLLNRTVTDNMLCAGDTRSGGPQANLHDACQGDSSGGLVCLNDGRM  
 TLVGIISWGLGCGQKDVPGVYTKVTNYLDWIRDNMTPG (SEQ ID NO 8)

20 Otro aspecto prefendo del invento se refiere a una molécula de ADN de acuerdo  
 con el invento, caracterizada porque dicha secuencia de ADN b) codifica por lo menos  
 un 90% de los aminoácidos 87 - 527 de la proteína de un activador de plasminógeno  
 tisular humano (la numeración que se usa aquí es como en GI 137119 o Nature 301  
 (5897), 214-221 (1983) .

25 Otro aspecto prefendo del invento se refiere a una molécula de ADN de acuerdo  
 con el invento, caracterizada porque dicha secuencia de ADN b) codifica por lo menos  
 un 90% de los aminoácidos 174 - 527 de la proteína de un activador de plasminógeno  
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 con el invento, caracterizada porque dicha secuencia de ADN b) codifica por lo menos  
 un 90% de los aminoácidos 220 - 527 de la proteína de un activador de plasminógeno  
 tisular humano

Otro aspecto prefendo del invento se refiere a una molécula de ADN de acuerdo

con el invento, caracterizada porque dicha secuencia de ADN a) se hibrida en condiciones rigurosas con la siguiente secuencia

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTG  
GCCCAGGCGGCC (SEQ ID NO 3)

5 Otro aspecto preferido del invento se refiere a una molécula de ADN de acuerdo con el invento, caracterizada porque dicha secuencia de ADN a) consta de la siguiente secuencia

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTG  
GCCCAGGCGGCC (SEQ ID NO 3)

10 Otro aspecto preferido del invento se refiere a una molécula de ADN de acuerdo con el invento, caracterizada porque dicha secuencia de ADN b) se hibrida en condiciones rigurosas con la siguiente secuencia

TCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTACCGTGGCACGC  
ACAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAATTCCATGATCCTGATA  
15 GGCAAGGTTTACACAGCACAGAACCCAGTGCCCAGGCACTGGGCCTGGGCAAAC  
ATAATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGTGCCACGTGCTGA  
AGAACCGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGCG  
GCCTGAGACAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCTCTTCGCCG  
ACATCGCCTCCCACCCCTGGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTGCG  
20 CCGGAGAGCGGTTCTGTGCGGGGGCATACTCATCAGCTCCTGCTGGATTCTCT  
CTGCCGCCCCTGCTTCCAGGAGAGGTTTCCGCCCCACCACCTGACGGTGATCT  
TGGGCAGAACATACCGGGTGGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGTC  
GAAAAATACATTGTCCATAAGGAATTCGATGATGACACTTACGACAATGACATTGC  
GCTGCTGCAGCTGAAATCGGATTCTGTCCTGCTGTGCCAGGAGAGCAGCGTGG  
25 TCCGCACTGTGTGCCTTCCCCCGGCGGACCTGCAGCTGCCGGACTGGACGGAG  
TGTGAGCTCTCCGGCTACGGCAAGCATGAGGCCTTGTCTCCTTTCTATTCCGAG  
CGGCTGAAGGAGGCTCATGTCAGACTGTACCCATCCAGCCGCTGCACATCACAA  
CATTTACTTAACAGAACAGTCACCGACAACATGCTGTGTGCTGGAGACACTCGGA  
GCGGCGGGCCCCAGGCAAACCTTGACGACGCCTGCCAGGGCGATTCTGGGAGG  
30 CCCCCTGGTGTGTCTGAACGATGGCCGCATGACTTTGGTGGGCATCATCAGCTG  
GGGCCTGGGCTGTGGACAGAAGGATGTCCCGGGTGTGTACACAAAGGTTACCA  
ACTACCTAGACTGGATTCGTGACAACATGCGACCGTGA (SEQ ID NO 4)

35 Otro aspecto preferido del invento se refiere a una molécula de ADN de acuerdo con el invento, caracterizada porque dicha secuencia de ADN b) consta de la siguiente secuencia

TCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTACCGTGGCACGC  
ACAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAATTCCATGATCCTGATA

GGCAAGGTTTACACAGCACAGAACCCCAGTGCCCAGGCACTGGGCCTGGGCAAAC  
 ATAATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGTGCCACGTGCTGA  
 AGAACCGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGCG  
 GCCTGAGACAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCTCTTCGCCG  
 5 ACATCGCCTCCCACCCCTGGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTGCG  
 CCGGAGAGCGGTTCTGTGCGGGGGCATACTCATCAGCTCCTGCTGGATTCTCT  
 CTGCCGCCCACTGCTTCCAGGAGAGGTTTCCGCCCCACCACCTGACGGTGATCT  
 TGGGCAGAACATACCGGGTGGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGTC  
 GAAAAATACATTGTCCATAAGGAATTCGATGATGACACTTACGACAATGACATTGC  
 10 GCTGCTGCAGCTGAAATCGGATTTCGTCCCGCTGTGCCCAGGAGAGCAGCGTGG  
 TCCGCACTGTGTGCCTTCCCCCGGCGGACCTGCAGCTGCCGGACTGGACGGAG  
 TGTGAGCTCTCCGGCTACGGCAAGCATGAGGCCTTGTCTCCTTTCTATTGGAG  
 CGGCTGAAGGAGGCTCATGTCAGACTGTACCCATCCAGCCGCTGCACATCACAA  
 CATTACTTAACAGAACAGTCACCGACAACATGCTGTGTGCTGGAGACACTCGGA  
 15 GCGGCGGGCCCCAGGCAAACCTTGACGACGCCTGCCAGGGCGATTTCGGGAGG  
 CCCCCTGGTGTGTCTGAACGATGGCCGCATGACTTTGGTGGGCATCATCAGCTG  
 GGGCCTGGGCTGTGGACAGAAGGATGTCCCGGGTGTGTACACAAAGGTTACCA  
 ACTACCTAGACTGGATTTCGTGACAACATGCGACCGTGA (SEQ ID NO 4)

Otra realizacion preferida del invento se refiere a un vector que contiene una  
 20 secuencia de ADN de acuerdo con el invento

Otra realizacion preferida del invento se refiere a un vector de acuerdo con el  
 invento, en el que dicha secuencia de ADN esta precedida por un promotor lac y un sitio  
 de fijacion a ribosomas. Vectores apropiados de acuerdo con el invento incluyen, pero  
 no se limitan a, vectores de virus tales como p.ej. los virus de Vaccinia, Semliki-Forest  
 25 y Adenovirus, vectores fagémidos y similares. Se prefieren los vectores que se pueden  
 usar ventajosamente en E. coli, pero también en cualquier otra hospedante procariontica  
 tal como pPROTet E, pPROLar A, miembros de la familia pBAD, de la familia pSE, de  
 la familia pQE y pCAL.

Otra realizacion preferida del invento se refiere al vector pComb3HSS que  
 30 contiene un ADN de acuerdo con el invento, en el que la expresión de la proteína gpIII  
 es suprimida o inhibida por delección de la molecula de ADN que codifica dicha proteína  
 gpIII o por un codón de detención situado entre el gen que codifica un polipéptido que  
 contiene el dominio de Knirg 2 y el dominio de proteasa de serina de la proteína de un  
 activador de plasminógeno tisular, y el gen de proteína III.

35 Otro aspecto importante del presente invento se refiere a una célula hospedante  
 procariontica que comprende una molécula de ADN de acuerdo con el invento

Otro aspecto importante del presente invento se refiere a una célula hospedante

procanótica que comprende un vector de acuerdo con el invento

Otro aspecto importante del presente invento se refiere a una célula hospedante

E coli que comprende una molécula de ADN de acuerdo con el invento

Otro aspecto importante del presente invento se refiere a una célula hospedante

5 E coli que comprende un vector de acuerdo con el invento

Todavía otro aspecto importante del presente invento es el uso de una molécula de ADN de acuerdo con el invento o de un vector de acuerdo con el invento o de una célula hospedante de acuerdo con el invento en un método para la producción de un polipéptido que tiene la actividad del activador de plasminógeno tisular

10 Todavía otro aspecto importante del presente invento es el uso de acuerdo con el invento como antes se describe, en que dicho método es un método de acuerdo con el invento

Otro aspecto muy importante es una composición farmacéutica que comprende una sustancia obtenible por un método de acuerdo con el invento así como excipientes y vehículos farmacéuticamente aceptables. Un ejemplo de dicha sustancia la molécula de K2S descrita con anterioridad. La expresión "vehículo farmacéuticamente aceptable" como se usa en el presente contexto, se refiere a excipientes o aditivos farmacéuticos convencionales que se usan en la tecnología de fabricación de productos farmacéuticos. Dichos compuestos fisiológicamente aceptables incluyen, por ejemplo, hidratos de carbono, tales como glucosa, sacarosa o dextranos, antioxidantes, tales como ácido ascórbico o glutatión, agentes quelantes, proteínas de bajo peso molecular, u otros estabilizadores o excipientes (véase también p ej Remington's Pharmaceutical Sciences (1990, 18ª edición, Mack Publ, Easton )) Dicha composición farmacéutica de acuerdo con el invento se puede administrar por vía intravenosa como un bolo, p ej como un bolo único, durante 5 a 10 segundos por vía intravenosa

20 El invento se refiere además al uso de las sustancias obtenibles por un método de acuerdo con el invento en la producción de un medicamento destinado para el tratamiento de las enfermedades ictus, infarto de miocardio, infarto de miocardio agudo, embolia pulmonar y cualquier oclusión arterial tal como oclusión de la arteria coronaria, oclusión de la arteria intracraneal (p ej arterias que abastecen al cerebro), arterias ocluidas periféricamente, trombosis venosa profunda o enfermedades relacionadas que están asociadas con una indeseada coagulación de la sangre

30 Se pretende que el siguiente ejemplo ayude a comprender el invento y no deba ser considerado de ninguna de las maneras como que limite el alcance del invento

98

## Ejemplo 1

### MATERIALES Y MÉTODOS

Diseño del cebador Con el fin de amplificar una parte específica del gen de un tPA se sintetizaron un par de cebadores SK2/174

[5' GAGGAGGAGGTGGCCCGGCGCCTCTGAGGGAAACAGTGAC 3'] (SEQ ID NO 22) y ASSP

[5' GAGGAGGAGCTGGCCGGCCTGGCCCGGTTCGCATGTTGTCACG 3'] (SEQ ID NO 23) (Life Technologies, Grand Island, NY) Estos cebadores se diseñaron basándose

en el gen de un tPA humano recuperado a partir de bases de datos del NCBI (g137119) Éstos se sintetizaron con sitios de clonación en un extremo para Sfi I (subrayados) de tal manera que el cuadro de lectura procedente de la ATG del gen de gpIII en el vector fagémido, pComb3HSS, mantendrá a lo largo de la secuencia introducida

Otro conjunto de cebadores para una mutagénesis dirigida a un sitio se diseñó para reanillarlo junto a la secuencia situada entre el gen de K2S y el gen III en pComb3H-K2S

Las secuencias de cebadores con bases de mutación (subrayadas) para generar un nuevo codón de detención fueron MSTPA

[5' ACATGCGACCGTGACAGGCCGGCCAG 3'] (SEQ ID NO 24) y MASTPA

[5' CTGGCCGGCCTGTACAGGTTCGCATGT 3'] (SEQ ID NO 25)

Amplificación del gen de K2S por PCR Un microgramo (1 µg) de los cebadores SK2/174 y ASSP juntamente con 50 ng del molde de p51-3 (obtenido del Dr Hiroshi Sasaki, Fujisawa Pharmaceutical, Japón) se suspendieron en 100 µl de una mezcla para PCR Se añadió finalmente a la solución una cantidad de 2,5 U de polimerasa de Taq (Roche Molecular Biochemicals, Indianapolis, IN) La condición de amplificación valorada se inició con un comienzo de salto a 85°C durante 4 min, seguido por una desnaturalización a 95°C durante 50 s (segundos), un reanillamiento a 42°C durante 50 s, y una prolongación a 72°C durante 1,5 min Se realizaron repetidamente treinta y cinco rondas La mezcla se incubó adicionalmente a 72°C durante 10 min El producto amplificado de 1 110 pb (pares de bases) se purificó subsiguientemente mediante el Estuche de purificación para PCR QIAquick (QIAGEN, Hilden, Alemania) El carácter correcto del producto purificado se confirmó mediante enzimas de restricción

Construcción del fagémido que expresa la K2S El producto purificado de PCR de la K2S y el fagémido pComb3HSS (proporcionado amablemente por el Dr Carlos F Barbas, Scripps Institute, EE UU) se digirieron con Sfi I (Roche Molecular Biochemicals, Indianapolis, IN) para preparar sitios de clonación cohesivos específicos Cuatro

microgramos (4 µg) del producto purificado de PCR se digirieron con 60 U de Sfi I a 50°C durante 18 h. Para el pComb3HSS, 20 µg de vectores fagemidos se trataron con 100 U de Sfi I. Los productos digendos del producto purificado de PCR de la K2S y pComb-3HSS (-3 300 pb) se purificaron subsiguientemente en un gel mediante el Estuche de Extraccion en Gel QIAquick (QIAGEN, Hilden, Alemania). 5 U de ligasa de T4 (Roche Molecular Biochemicals, Indianapolis, IN) se introdujeron en la mezcla de 0,7 µg de pComb3HSS digiendo con Sfi I y purificado y de 0,9 µg del producto de PCR digiendo con Sfi I y purificado. La tanda de reacción de ligación se incubó a 30°C durante 18 h. El fagémido construido de nuevas se denominó pComb3H-K2S.

Transformación de E. coli XL-1 Blue. Doscientos microlitros (200 µl) de E. coli HL-1 Blue competente para CaCl<sub>2</sub> (Stratagene, La Jolla, CA) se transformaron con 70 ng de un producto ligado o mutado. Las células transformadas fueron propagadas diseminándolas sobre un agar LB que contenía 100 µg/ml de ampicilina y 10 µg/ml de tetraciclina (Sigma, Saint Louis, MO). Después de haber cultivado a 37°C durante 18 h, se seleccionaron vanas colonias resistentes a los antibioticos, para minipreparaciones de plásmidos usando el metodo de lisis en condiciones alcalinas. Cada plásmido purificado se sometió a un análisis en los sitios de restricción para Sfi I. Un transformante que albergaba un plásmido con el o los sitio(s) correcto(s) de restricción por Sfi I se propago subsiguientemente durante 18 h a 37°C en 100 ml de un caldo LB con 100 µg/ml de ampicilina y 10 µg/ml de tetraciclina. Una maxipreparación de plásmidos se realizó usando el estuche QIAGEN Plasmid Maxi Kit (QIAGEN, Hilden, Alemania). El plásmido purificado se volvió a examinar en cuanto a la existencia de sitios específicos de restricción por Sfi I y se amplificó por el Estuche de Secuenciación en Ciclos de Terminadores de Polimerasa de ADN AmpliTaq (The Perkin-Elmer Corporation, Foster City, CA).

Mutagénesis dirigida a un sitio de pComb3H-K2S. 10 ng de un molde de pComb3H-K2S se mezclaron con 125 ng de los cebadores MSTPA y MASTPA. Se añadieron a la mezcla 2,5 U de polimerasa de ADN PfuTurbo (Stratagene, La Jolla, CA) para la amplificación en ciclos. La reacción comenzó con una ronda a 95°C durante 30 s. Luego esta fue seguida por 16 rondas que consistían en 95°C durante 30 s, 55°C durante 1 min y 68°C durante 9 min. El tubo de reacción se colocó subsiguientemente sobre hielo durante 2 min. Con el fin de destruir las hebras del molde, se añadieron 10 U de la enzima de restricción Dpn I (Stratagene, La Jolla, CA) a la reacción de amplificación, y se incubó durante 1 h a 37°C. Este producto sintetizado (MpComb3H-K2S) se usó ultenormente para transformar a E. coli XL-1 Blue.

Preparación de K2S recombinante para su presentación a fagos Después de que el pComb3H-K2S se transformase dentro de XL-1 Blue, se realizó la técnica de presentación a fagos. Un clon de E. coli XL-1 Blue transformada por pComb3H-K2S se propagó en 10 ml de un caldo super que contenía 100 µg/ml de ampicilina y 10 µg/ml de tetraciclina a 37°C hasta que se alcanzase la densidad óptica D<sub>600</sub> [a 600 nm] de 1,5. El cultivo de bacterias se propagó subsiguientemente en 100 ml del mismo medio y se cultivó durante 2 h. Se usó una cantidad de 10<sup>12</sup> pfu del fago cooperante VCSM13 (Stratagene, La Jolla, CA) para infectar a la E. coli XL-1 Blue transformada. Después de incubación durante 3 h se añadió al cultivo kanamicina en una concentración final de 70 µg/ml. El cultivo se dejó en agitación (a 200 rpm) durante 18 h a 37°C. Luego se cosecharon los bacteriófagos que albergaban K2S en gpIII (K2S-Φ) añadiendo 4% p/v (peso/volumen) de PEG de PM 8 000 (Sigma, Saint Louis, MO) y 3% p/v de NaCl. Finalmente, el fago cosechado se volvió a suspender en 2 ml de PBS de pH 7,4. El número de fagos se determinó infectando la E. coli XL-1 Blue. Las unidades de formación de colonias por mililitro (cfu/ml) se calcularon como se ha descrito anteriormente (21).

Expresión de K2S recombinante en matraces con sacudimiento La E. coli XL-1 Blue transformada con MpComb3H-K2S se cultivó en 100 ml de caldo super (3% p/v de triptona, 2% p/v de extracto de levadura y 1% p/v de MOPS) a un pH de 7,0 en la presencia de ampicilina (100 µg/ml) a 37°C hasta que se alcanzase una D<sub>600</sub> [a 600 nm] de 0,8. Subsiguientemente, la síntesis de proteínas se indujo por 1 mM de IPTG (Promega, Madison, WI). Las bacterias se cultivaron adicionalmente con sacudimiento (a 200 rpm) durante 6 h a 30°C. El material sobrenadante de cultivo se recogió y precipitó con sulfato de amonio saturado al 55% (32). El material precipitado se reconstituyó con PBS, de pH 7,2, y se dializó en la misma solución tamponadora a 4°C durante 18 h. Proteínas periplásmicas procedentes de células bacterianas se extrajeron usando un choque con cloroformo como antes se ha descrito por Ames y colaboradores (2).

Cuantificación de K2S recombinante por análisis inmunológico Con el fin de detectar la r-K2S, la fase sólida se revistió con un dominio anti-kringle 2 monoclonal (16/B) (proporcionado generosamente por el Dr. Ute Zacharias, Instituto Central de Biología Molecular, Berlin-Buch, Alemania). Se realizaron procesos clásicos de lavado y bloqueo por ELISA. Se añadieron 50 µl de 10<sup>11</sup> cfu/ml de K2S-Φ o r-K2S secretado dentro de cada pocillo revestido con anti-kringle 2. La detección de antígenos y anticuerpos se llevó a cabo de la siguiente manera. O bien una HRP conjugada con anti-M13 de cordero (Pharmacia Biotech, Uppsala, Suecia) o una HRP conjugada con anti-tPA de cordero

(Cedarlane, Ontario, Canadá), se añadió a cada pocillo de reacción después de la operación de lavado. El sustrato TMB fue sometido a cada pocillo y la reacción se hizo cesar finalmente con una solución de  $H_2SO_4$  después de incubar durante 30 min. El patrón tPA 86/670 de melanoma (National Institute for Biological Standards and Control, Hertfordshire, Reino Unido) se usó como testigo positivo.

**Análisis de actividad amidolítica** Un estuche de ensayo para la detección de la actividad amidolítica de un tPA se adquirió de Chromogenix (Molndal, Suecia). La mezcla de sustratos que contenía plasminógeno y S-2251 se usó para determinar la actividad enzimática de la proteasa de serina. La dilución de  $10^{-2}$  de cada muestra precipitada con amonio se analizó con y sin fragmentos de fibrinógeno humano, estimuladores. El proceso de análisis estaba de acuerdo con el manual de tPA COASET.

**SDS-PAGE y transferencia inmunológica** El producto de precipitado dializado procedente del material sobrenadante de cultivo se concentró adicionalmente 10 veces con Centricon 10 (AMICON, Beverly, MA). La muestra concentrada se sometió a separación de proteínas por SDS-PAGE, 15% de gel para resolución, en el tampón de reducción seguido por transferencia por electroforesis a nitrocelulosa. Luego, la nitrocelulosa se bloqueó con leche desnatada al 4% durante 2 h. Con el fin de detectar la r-K2S, se aplicó a la nitrocelulosa una dilución apropiada de HRP de cordero conjugada con anti-tPA. La banda reactiva inmunológicamente se visualizó por un sensible sistema de detección, el Estuche Amplified Opti-4CN (BIORAD, Hercules, CA).

**Electroforesis en gel de poli(acrilamida) de un plasminógeno copolimerizado** Un gel de poli(acrilamida) para resolución al 11% se copolimerizó con un plasminógeno y gelatina tal como se ha descrito anteriormente por Heussen y colaboradores (14). El gel de apilamiento se preparó como una concentración al 4% sin plasminógeno ni gelatina. La electroforesis se realizó a 4°C con una intensidad de corriente constante de 8 mA. El SDS residual en la plancha de gel se retiró después de suave sacudimiento a la temperatura ambiente durante 1 h en Triton X-100 al 2,5%. Luego la plancha de gel se incubó en glicina-NaOH 0,1 M a pH 8,3 durante 5 h a 37°C. Finalmente, la plancha de gel se tiñó y destiñó por un sistema patrón de tinción con Coomassie brilliant Blue (R-250). El lugar donde estaba situado el péptido que albergaba la actividad enzimática no fue teñido por el colorante en contraste con el fondo pintado de azul.

## RESULTADOS

Construcción del vector portador del gen de K2S. A partir del vector p51-3 se amplificó la porción Kringle 2 más proteasa de senna de un tPA (Ser<sup>174</sup> en el dominio de Kringle 2 hasta Pro<sup>527</sup> en la proteasa de serina) usando los cebadores SK2/174 y ASSP. El producto de 1 110 pb amplificado se demostró por electroforesis en gel de agarosa (Figura 1, pista 2) y se introdujo en el fagemido pComb3HSS por sitios dobles de disociación con Sfi I en los extremos 5' y 3' en el cuadro de lectura correcto. Por lo tanto, se generó un nuevo vector, pComb3H-K2S, que albergaba a la K2S. En este vector, la K2S está flanqueada secuencia arriba por la secuencia de señal de OmpA y secuencia abajo por la gp3. La inserción correcta de la K2S se comprobó tanto mediante un análisis por restricción con Sfi I (Figura 2, pista 3) como por análisis por PCR (demostración de una única banda a 1 110 pb), y por secuenciación del ADN. El diagrama esquemático del mapa de pComb3H-K2S se presenta en la Figura 3.

r-K2S presentada a fagos. El fago filamentososo VCSM13 se usó para infectar a la *E. coli* XL-1 Blue transformada con pComb3H-K2S, X[K2S]. El VCSM13 se propagó e incorporó en la proteína de fusión K2S-gp3 durante los procesos de empaquetamiento de virus. El fago recombinante (K2S-Φ) cosechado dio una concentración de  $5,4 \times 10^{11}$  cfu/ml determinada volviendo a infectar a la *E. coli* XL-1 Blue con fagos precipitados por PEG. Estas partículas de fagos recombinantes se comprobaron en cuanto a la expresión de r-K2S por un ELISA en emparejado. La proteína K2S heteróloga fijada a un fago se reconoció por el anticuerpo monoclonal anti-kringle 2 (16/B) usando un sistema de detección de anticuerpos de HRP de cordero conjugados con anti-tPA. La absorbencia de este análisis fue de  $1,12 \pm 0,03$  (Tabla 1). La cantidad de K2S detectable en  $10^{12}$  partículas de fagos es igual a 336 ng de proteína en relación con el tPA de melanoma patrón. Con el fin de corroborar que la proteína de fusión K2S-gp3 estaba asociada con partículas de fagos, el anticuerpo de HRP de cordero conjugado con anti-tPA se reemplazó por el anticuerpo de HRP de cordero conjugado con anti-M13. Esta reacción inmunológica exhibió una absorbencia de  $1,89 \pm 0,07$  (Tabla 1). En contraste, si el anticuerpo para captura era el anticuerpo de cordero anti-M13, se observó una cantidad extremadamente baja de K2S con la HRP conjugada con el anticuerpo de cordero anti-tPA, la absorbencia fue solamente de  $0,17 \pm 0,01$  (Tabla 1). Esto sugiere que solamente una minoría de las partículas de fagos purificados eran portadoras de la proteína de fusión K2S-gp3. Se usó como un testigo negativo el VCSM13 preparado a partir de la *E. coli* XL-1 Blue no transformada.

- Construcción de MpComb3H-K2S Se generó un codón de detención entre K2S y gp3 en el pComb3H-K2S con la ayuda de los cebadores mutagenicos (MSTPA y MASTPA) (Figura 4) Con el fin de enriquecer al MpComb3H-K2S sintetizado de nuevas y mutado, la mezcla de amplificación en ciclo se digirió a fondo con Dpn I a fin de degradar al antiguo molde de pComb3H-K2S metilado en dique (la Dpn I prefiere al ADN metilado en dique) Después de haber transformado la E coli XL-1 Blue con el MpComb3H-K2S, se seleccionó para estudio ulterior un transformante XM[K2S] Como consecuencia del reemplazo de pares de bases, se perdió un sitio de disociación con Sfi I próximo al extremo 3' del gen de K2S después de la mutagenesis dirigida a un sitio Se observó en 4 319 pb una versión lineal del MpComb3H-K2S disociado con Sfi I sin la aparición de un fragmento insertado del gen de K2S (Figura 5, pista 3) Por lo tanto, el gen de K2S codificado por MpComb3H-K2S fue expresado en una forma sin fusión con gp3 en XM[K2S]
- Expresión y purificación de K2S La expresión de K2S en XM[K2S] se indujo mediante IPTG La r-K2S era detectable usando un ELISA tanto en el espacio periplásmico como en el material sobrenadante de cultivo La cantidad de la proteína heteróloga en cada preparación se determinó usando un ELISA en emparejado y se puso en relación con el tPA patrón A partir de 100 ml del cultivo bacteriano en un matraz con sacudimiento con una D O [a 600 nm] de 50, la fracción periplásmica proporcionó 1,38 µg de r-K2S (aproximadamente 32%) mientras que se obtuvieron 2,96 µg de r-K2S (aproximadamente 68%) en el material sobrenadante de cultivo precipitado con amonio Se usó un ELISA en emparejado para comprobar el fago precipitado por PEG procedente del XM[K2S] infectado con VCSM13 No se detectó r-K2S capturada por el anticuerpo monoclonal anti-kningle 2 mediante la HRP conjugada con anti-M13, indicando que la K2S no es presentada en las partículas de fago si falta la gp3
- Medición de la actividad amidolítica Si el dominio de proteasa de serina está presente en la muestra, el plasminogeno será convertido en plasmina La plasmina producida digiere adicionalmente el sustrato S-2251 para dar un producto coloreado, p-nitro-anilina, que tiene una absorbencia máxima a 405 nm La actividad específica del producto recombinante está en concordancia con la absorbencia La actividad enzimática dependiente del fibrinógeno que tiene cada muestra, es decir K2S-Φ, r-K2S periplásmico o r-K2S de material sobrenadante de cultivo, se evaluó y comparó Tanto la K2S-Φ como la r-KRS periplásmica ilustraron una actividad enzimática notablemente baja, que estaba por debajo de la sensibilidad del ensayo (0,25 UI/ml) La r-K2S en el material sobrenadante de cultivo dio la actividad enzimática dependiente de fibrinógeno de 7 UI/ml Así,

a partir de 100 ml de un cultivo se obtuvo un total de 700 UI (Unidades Internacionales) de actividad enzimática. Sin fibrinógeno no se observó ninguna actividad enzimática de la r-K2S purificada a partir del material sobrenadante de cultivo, mientras que el tPA de melanoma patrón mostró una cierta actividad.

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Demostración de la proteína recombinante por transferencia inmunológica. La K2S parcialmente purificada procedente del material sobrenadante de cultivo de XM[K2S] reveló una masa molecular de 39 kDa usando anticuerpos de cordero anti-tPA (Figura 6). El testigo negativo, un material sobrenadante de cultivo parcialmente purificado de la XL-1 Blue no transformada, no contenía una banda reactiva que tuviese un tamaño similar.

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Localización de la enzima activa mediante PAGE. El plasminógeno se ha copolimerizado e inmovilizado con gelatina en el gel de poli(acrilamida) antes de la electroforesis. Se analizaron los materiales sobrenadantes de cultivo, precipitados con sulfato de amonio, de E. coli XL-1 Blue, E. coli XL-1 Blue transformada con pComb3HSS y XM[K2S] (Figura 7). Todas las muestras se sometieron a tratamiento en una condición no reductora para conservar la conformación y la actividad correctas del dominio de proteasa de serina. Se observaron regiones transparentes de plasminógeno digerido con proteasa de serina solamente en los materiales sobrenadantes de cultivo, precipitados con sulfato de amonio de XM[K2S], en las posiciones de 34 y 37 kDa. Las otras muestras no dieron zonas de aclaramiento. La pista testigo positivo del tPA de melanoma patrón también demostró actividad enzimática en las posiciones de 66 y 72 kDa.

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## REFERENCIAS

- 1 Allen, S., H. Y. Naim, y N. J. Bulleid. 1995. Plegamiento intracelular del activador de plasminógeno de tipo tisular. Efectos de la formación de enlaces de disulfuro sobre la glicosilación enlazada a N y la secreción. *J. Biol. Chem.* 270: 4797-4804.
- 2 Ames, G. F., C. Prody, y S. Kustu. 1984. Liberación sencilla, rápida y cuantitativa de proteínas periplásmicas por cloroformo. *J. Bacteriol.* 160: 1181-1183.
- 3 Barbas, C. F. III, A. S. Kang, R. A. Lerner, y S. J. Benkovic. 1991. Ensamble de bibliotecas combinatorias de anticuerpos sobre superficies de fagos: el sitio del gen III. *Proc. Natl. Acad. Sci. U.S.A.* 88: 7978-7982.
- 4 Barbas, C. F. III, y J. Wagner. 1995. Anticuerpos humanos sintéticos: selección y desarrollo de proteínas funcionales. *A Companion to Methods in Enzymology* 8: 94-103.
- 5 Bennett, W. F., N. F. Paoni, B. A. Keyt, D. Botstein, A. J. Jones, L. Presta, F. M. Wurm, y M. J. Zoller. 1991. Análisis con alta resolución de determinantes funcionales en

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35

- un activador de plasminogeno de tipo tisular humano J Biol Chem 266 5191-5201
- 6 Betton, J M , N Sassoon, M Hofnung, y M Laurent 1988 Degradacion frente a agregacion de la proteina de fijacion a maltosa defectuosamente plegada en el periplasma de Escherichia coli J Biol Chem 273 8897-8902
- 5 7 Camiolo, S M , S Thorsen, y T Astrup 1971 Fibrinogenolisis y fibrinolisis con el activador de plasminógeno tisular, urocinasa, globulina humana activada por estreptocinasa y plasmina Proc. Soc. Exp Biol Med 38 277-280
- 8 Cartwright, T 1992 Produccion de t-PA a partir de un cultivo de células de animales, p 217-245, In R E Spier, y J B Griffiths (coordinadores de edición), Animal Cell Biotechnology, Volumen 5 Academic Press, N Y
- 10 9 Curry, K A , A W Yem, M R Deibel, Jr , N T Hatzenbuehler, J G Hoogerheide, y C S Tomich 1990 Expresion en Escherichia coli y tratamiento de interleucina 1 beta humana fusionada con péptidos de señal DNA Cell Biol 9 167-175
- 10 Datar, R V , T Cartwright, y C-G Rosen 1993 Condiciones económicas de tratamiento de fermentaciones de celulas de animales y bacterias un análisis de estudio por casos del activador de plasminógeno tisular Biotechnology 11 349-357
- 15 11 Deneffe, P , S Kovank, T Ciora, N Gosselet, J C Benichou, M Latta, F Guinet, A Ryter, y J F Mayaux 1989 Exportacion de proteínas heterólogas en Escherichia coli influencia de los péptidos de señal de bacteria sobre la exportación de interleucina 1 beta humana Gene 85 499-510
- 20 12 Griffiths, J B , A Electrowala 1987 Producción de activadores de plasminogeno tisulares a partir de celulas de animales Adv Biochem Eng Biotechnol 34 147-166
- 13 Harris, T J , T Patel, F A Marston, S Little, J S Emtage, G Opdenakker, G Volckaert, W Rombauts, A Billiau, y P De Somer 1986 Clonación de un ADNc que codifica un activador de plasminógeno de tipo tisular humano y su expresión en Escherichia coli Mol Biol Med 3 279-292
- 25 14 Heussen, C , y E B Dowdle 1980 Análisis por electroforesis de activadores de plasminogeno en geles de poli(acrilamida) que contienen dodecil-sulfato de sodio y substratos copolimerizados Anal Biochem 102 196-202
- 30 15 Heussen, C , F Joubert, y E B Dowdle 1984 Purificacion de un activador de plasminógeno tisular humano con el inhibidor de tripsina Erythrina J Biol Chem 259 - 11635-11638
- 16 Hu, C K , U Kohnert, O Wilhelm, S Fischer, y M Llinas 1994 Mutante BM 06 022 de supresión del dominio de un activador de plasminogeno de tipo tisular estabilidad modular, fijación de un inhibidor y disociación por activación Biochemistry 33 11760-11766
- 35 17 Kipryanov, S M , G Moldenhauer, y M Little 1997 Producción en alto nivel de

- anticuerpos solubles de cadena unica en cultivos a pequeña escala de *Escherichia coli*  
J Immunol Methods 200 69-77
- 18 Ko, J H , D K Park, I C Kim, S H Lee, y S M Byun 1995 Expresión en alto nivel  
y secreción de estreptocinasa en *Escherichia coli* Biotechnol Lett 17 1019-1024
- 5 19 Kouzuma, Y , N Yamasaki, y M Kimura 1997 El inhibidor ETIa procedente de  
*Erythrina vanegata* de un activador de plasminógeno de tipo tisular base estructural para  
la actividad inhibidora por clonación, expresión y mutagénesis del ADNc que codifica el  
ETIa J Biochem (Tokio) 121 456-463
- 20 Lasters, I , N Van Herzele, H R Lijnen, D Collen, y L Jespers 1997 Propiedades  
enzimáticas de fragmentos de plasminogeno humano presentados a fagos Eur J  
10 Biochem 244 946-952
- 21 Lobel, L I , P Rausch, I Trakht, S Pollak, y J W Lustbader 1997 El fago  
filamentoso que presenta el dominio extracelular del receptor de hLH/CG se fija  
específicamente a hCG Endocrinology 138 1232-1239
- 15 22 Lubiniecki, A , R Arathoon, G Polastn, J Thomas, M Wiebe, R Garnick, A Jones,  
R van Reis, y S Builder Estrategias seleccionadas para la producción y el control de un  
activador de plasminógeno tisular recombinante preparado a partir de un cultivo celular,  
p 442-451 En R E Spier, J B Griffiths, J Stephenne, y P J Crooy (coordinadores de  
edición), Advances in animal cell biology and technology for bioprocesses Butterworths,  
20 Londres
- 23 Lucic, M R , B E Forbes, S E Grosvenor, J M Carr, J C Wallace, y G Forsberg  
1998 Secrecion en *Escherichia coli* y presentación a fagos de la proteína 2 de fijación  
a un factor de crecimiento similar a insulina recombinante J Biotechnol 61 95-108
- 24 Martin U , S Fischer, U Kohnert, H Lill, R Rudolph, G Sponer, A Stem, y K Strein  
25 1990 Propiedades de un nuevo activador de plasminógeno (BM 06 022) producido en  
*Escherichia coli* Z Kardiol 79 167-170
- 25 Obukowicz, M G , M E Gustafson, K D Junger, R M Leimgruber, A J Wittwer,  
T C Wun, T G Warren, B F Bishop, K J Mathis, D T McPherson, N R Siegel, M  
G Jennings, B B Bnghtwell, J A Diaz-Collier, L D Bell, C S Craik, y W C Tacon  
30 1990 Secreción de Kningle-2-proteasa de serina activa en *Escherichia coli* Biochemistry  
29 9737-9745
- 26 Parmley, S F , y G P Smith 1988 Vectores de fagos fd filamentosos seleccionables  
por anticuerpos purificación por afinidad de genes dianas Gene 73 305-318
- 27 Pennica, D , W E Holmes, W J Kohr, R N Harkins, G A Vehar, C A Ward, W  
35 F Bennett, E Yelverton, P H Seeburg, H I Heyneker, D V Goeddel, y D Collen  
1983 Clonación y expresión del ADNc de un activador de plasminogeno de tipo tisular  
humano en *E. coli* Nature 301 214-221

- 28 Rippmann, J F , M Klein, C Hoischen, B Brocks, W J Rettig, J Gumpert, K Pfizenmaier, R Mattes, y D Moosmayer 1998 Expresión procarionótica de anticuerpos de fragmentos variables de cadena única (scFv) la secreción en células con forma de L de *Proteus mirabilis* conduce a un producto activo y supera las limitaciones de la expresión periplásmica en *Escherichia coli* Appl Environ Microbiol 64 4862-4869
- 5 29 Sato, Y , Y Ishii, H, Sasaki, M Hayashi, T Fujimura, Y Imai, S Nakamura, S Suzuki, J Notani, T Asada, H Honai, K Masakazu, y N Mineo 1994 Producción y caracterización de un nuevo derivado del activador de plasminógeno de tipo tisular en *Escherichia coli* Biotechnol Prog 10 472-479
- 10 30 Sarmientos, P , M Duchesne, P Denéfle, J Boziau, N Fromage, N Delporte, F Parker, Y Lelièvre, J -F Mayaux, y T Cartwright 1989 Síntesis y purificación de un activador de plasminógeno tisular humano activo a partir de *Escherichia coli* Biotechnology 7 495-501
- 31 Scherrer, S , N Robas, H Zouheiry, G Branlant, y C Branlant 1994 La agregación periplásmica limita la maduración proteolítica del polipeptido precursor de amidasa de penicilina G en *Escherichia coli* Appl Microbiol Biotechnol 42 85-89
- 15 32 Soeda, S , M Kakiki, H Shimeno, y A Nagamatsu 1986 Purificación rápida y con alto rendimiento de un activador de plasminógeno de tipo tisular de corazón de porcino por cromatografía con heparina-Sepharose Life Sci 39 1317-1324
- 20 33 Szarka, S J , E G Sihota, H R Habibi, y S -L Wong 1999 La estafilocinasa como un componente de un activador de plasminógeno en proteínas de fusión recombinantes Appl Environ Microbiol 65 506-513
- 34 Waldenström, M , E Holmgren, A Attersand, C Kalderen, B Lowenadler, B Raden, L Hansson, y G Pohl 1991 Síntesis y secreción de una variante de un activador de plasminógeno de tipo tisular activo con fibrinólisis en *Escherichia coli* Gene 99 243-248
- 25 35 Wan, E W -M, y F Baneyx 1998 La co-sobreexpresión de TolAIII facilita la recuperación de proteínas recombinantes periplásmicas dentro del medio de crecimiento en *Escherichia coli* Protein Expr Purif 14 13-22
- 36 Zachanas, U , B Fischer, F Noll, y H Will 1992 Caracterización de un activador de plasminógeno de tipo tisular humano con anticuerpos monoclonales cartografiado de epítopos y sitios de fijación para fibrina y lisina Thromb Haemost 67 88-94
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## LEYENDAS DE LAS FIGURAS

- FIGURA 1 Validacion del producto de amplificacion por PCR del gen de K2S a partir del vector p51-3 por uso de los cebadores SK2/174 y ASSP La pista 1 muestra un marcador de 1 kb (Roche Molecular Biochemicals, Indianapolis, IN) La pista 2 se cargo con 1 µl de un producto amplificado Se representa una unica banda a 1 110 pb La electroforesis se realizó sobre un gel de agarosa al 1%
- FIGURA 2 Identificación del gen insertado de K2S a 1 110 pb (\*) despues de que se hubo demostrado en la pista 3 la existencia de pComb3H-K2S digiendo con Sfi I La pista 1 muestra un marcador de 1 kb La pista 2 se cargo con pComb3H-K2S sin cortar La electroforesis se realizó en un gel de agarosa al 1%
- FIGURA 3 Esquema del pComb3H-K2S que muestra dos sitios de clonacion con Sfi I en los que se había insertado el gen de K2S Se describen tambien la secuencia de señal (OmpA), el sitio de fijacion a ribosomas (RIBS), el promotor lac y el gen de gpIII
- FIGURA 4 Diagrama esquemático del sitio de mutación en la unión entre los genes de K2S y de gpIII en el pComb3H-K2S El sitio de reanillamiento del pComb3H-K2S está unido con un conjunto de cebadores de mutación (MSTPA y MASTPA) que contienen oligonucleosidos modificados (subrayados) Después de haber realizado la amplificación en ciclo, el sitio 1 para Sfi I (en negrita) es modificado y se pierde en la hebra sintetizada de nuevas
- FIGURA 5 Caracterización del MpComb3H-K2S sintetizado de nuevas por la enzima de restriccion Sfi I Se observa en la pista 3 una unica banda de 4 319 pb que se refiere a un unico sitio de disociacion de MpComb3H-K2S No se puede visualizar ninguna banda de K2S insertada a 1 110 pb La pista 1 muestra un marcador de 1 kb La pista 2 se cargo con MpComb3H-K2S sin cortar La electroforesis se realizó en un gel de agarosa al 1%
- FIGURA 6 Identificacion de una banda reactiva inmunologica con un material sobrenadante de cultivo de una proteina que se deriva de un ADN recombinante purificada a partir de XM[K2S] con HRP de cordero conjugado con anti-tPA La pista 1 se cargo con 40 ng de un tPA de melanoma patrón (86/670), que mostró la banda reactiva a 70 kDa Los materiales sobrenadantes de cultivo parcialmente purificados y concentrados procedentes de E coli XL-1 Blue y XM[K2S] no transformados se aplicaron

a las pistas 2 y 3 respectivamente. La banda reactiva distinguible se demostró particularmente en la pista 3 a 39 kDa.

5      **FIGURA 7** Determinación del peso molecular de una r-KRS extracelular que alberga un dominio de proteasa de senna activa mediante electroforesis en gel de poli(acrilamida) de un plasminógeno copolimerizado. La pista 1 contenía los patrones de peso molecular indicados ( $\times 10^{-3}$ ), SDS-6H (Sigma, Saint Louis, MO). Se cargaron 50  $\mu$ g del material sobrenadante de cultivo precipitado con sulfato de amonio saturado al 55% de XL-1 Blue, de XL-1 Blue transformado con pComb3HSS y de XM[K2S], en las pistas 2, 3 y 4 respectivamente. La pista 5 contenía 50 mUI de tPA de melanoma patrón (86/670). Las zonas transparentes de plasminógeno digiendo en gel de poli(acrilamida) son visibles solamente en la pista 4 con unos pesos moleculares de 34 y 37 kDa (B) y en la pista 5 con unos pesos moleculares de 66 y 72 kDa (A).

15      **FIGURA 8** Estructura A (SEQ ID NO 11)  
Molécula de K2S nativa de los aminoácidos 174-527 sin modificación

20      **FIGURA 9** Estructura B-0 (SEQ ID NO 12)  
Molécula de K2S nativa de los aminoácidos 197-527 sin modificación

**FIGURA 10** Estructura B-1 (SEQ ID NO 13)  
Molécula de K2S de los aminoácidos 193-527, en que a la estructura B-0 de la FIGURA 9 se le añadieron los aminoácidos SEGN en la porción terminal de N

25      **FIGURA 11** Estructura B-2 (SEQ ID NO 14)  
Molécula de K2S de los aminoácidos 193-527, como en la FIGURA 10, en que el Cys en 261 se había cambiado por Ser

**FIGURA 12** Estructura B-3 (SEQ ID NO 15)  
30      Molécula de K2S de los aminoácidos 197-527, en que a la estructura B-0 de la FIGURA 9 se le añadieron los aminoácidos SEGNSD en la porción terminal de N

**FIGURA 13** Estructura B-4 (SEQ ID NO 16)  
Molécula de K2S de los aminoácidos 191-527, como en la FIGURA 12, en que el Cys en  
35      261 se había cambiado por Ser

FIGURA 14 Estructura C (SEQ ID NO 17)

Molécula de K2S nativa de los aminoácidos 220-527 sin modificación Esta molécula se puede modificar adicionalmente de una manera similar a como se describe para la estructura B en las FIGURAS 10-13

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FIGURA 15 Estructura D (SEQ ID NO 18)

Molécula de K2S nativa de los aminoácidos 260-527 sin modificación Esta molécula se puede modificar adicionalmente de una manera similar a como se describe para la estructura B en las FIGURAS 10-13

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FIGURA 16 Molécula de tPA (SEQ ID NO 19)

TABLA 1 Detección de una molécula de r-K2S en la preparación de fagos por un ELISA en emparejado

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| Anticuerpo de captura      | Anticuerpo trazador (HRP conjugado) |                     |             |                     |
|----------------------------|-------------------------------------|---------------------|-------------|---------------------|
|                            | Anti-tPA                            |                     | Anti-M13    |                     |
|                            | K2S-Φ                               | VCSM13 <sup>a</sup> | K2S-Φ       | VCSM13 <sup>a</sup> |
| Anti-knngle 2 <sup>b</sup> | 1,12 ± 0,04 <sup>c</sup>            | 0,12 ± 0,03         | 1,89 ± 0,02 | 0,16 ± 0,02         |
| Anti-M13                   | 0,17 ± 0,01                         | 0,14 ± 0,05         | 1,91 ± 0,02 | 1,88 ± 0,03         |

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<sup>a</sup> El VCSM13 se cosechó a partir de HL-1 Blue transformada con pComb3HSS

<sup>b</sup> Se usó un anticuerpo monoclonal de ratón anti-knngle 2 (16/B) Los otros anticuerpos se prepararon a partir de una inmunoglobulina de cordero

<sup>c</sup> El valor es la media de la absorbencia de cada una de las muestras que se analizó en triplicado

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LISTA DE SECUENCIAS

- <110> Boehringer Ingelheim International GmbH
- 5 <120> Métodos para la producción a gran escala de un tPA que se deriva de un ADN o de moléculas de K2S
- <130> case 1-1170
- 10 <140>  
<141>
- <150> GB 0027779 8  
<151> 2000-11-14
- 15 <160> 25
- <170> PatentIn Ver. 2.1
- 20 <210> 1  
<211> 18  
<212> ADN  
<213> Secuencia Artificial
- 25 <220>  
<223> Descripción de la Secuencia Artificial.  
Secuencia codificadora de la parte terminal de N de la proteína K2S
- 30 <400> 1  
tctgagggaa acagtgac  
18
- 35 <210> 2

<211> 1128

<212> ADN

<213> Secuencia Artificial

5 <220>

<223> Descripción de la Secuencia Artificial

Secuencia codificadora de la proteína de fusión OmpA-K2S

<400> 2

10 atgaaaaaga cagctatcgc gattgcagtg gcactggctg gtttcgctac  
cgtggcccag 60  
gcggcctctg agggaaacag tgactgctac tttgggaatg ggtcagccta  
ccgtggcacg 120  
cacagcctca ccgagtcggg tgctctctgc ctcccgtgga attccatgat  
15 cctgataggc 180  
aaggttttaca cagcacagaa cccagtgcc caggcactgg gcctgggcaa  
acataattac 240  
tgccggaatc ctgatgggga tgccaagccc tgggtgccacg tgctgaagaa  
ccgcaggctg 300  
20 acgtgggagt actgtgatgt gccctcctgc tccacctgcg gcctgagaca  
gtacagccag 360  
cctcagtttc gcatcaaagg agggctcttc gccgacatcg cctcccaccc  
ctggcaggct 420  
gccatctttg ccaagcacag gaggtcgccc ggagagcggg tcctgtgcgg  
25 gggcatactc 480  
atcagctcct gctggattct ctctgccgcc cactgcttcc aggagagggt  
tccgccccac 540  
cacctgacgg tgatcttggg cagaacatac cgggtgggtc ctggcgagga  
ggagcagaaa 600  
30 tttgaagtcg aaaaatacat tgtccataag gaattcgatg atgacactta  
cgacaatgac 660  
attgcgctgc tgcagctgaa atcggattcg tcccgtgtg cccaggagag  
cagcgtggtc 720  
cgcactgtgt gccttcccc ggccggacctg cagctgccgg actggacgga  
35 gtgtgagctc 780

tccggctacg gcaagcatga ggccttgtct cctttctatt cggagcggct  
gaaggaggct 840  
catgtcagac tgtaccatc cagccgctgc acatcacaac atttacttaa  
cagaacagtc 900  
5 accgacaaca tgctgtgtgc tggagacact cggagcggcg ggccccaggc  
aaacttgca 960  
gacgcctgcc agggcgatc gggaggcccc ctggtgtgtc tgaacgatgg  
cgcgatgact 1020  
ttggtgggca tcatcagctg gggcctgggc tgtggacaga aggatgtccc  
10 gggtgtgtac 1080  
acaaaggta ccaactacct agactggatt cgtgacaaca tgcgaccg  
1128  
  
15 <210> 3  
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<212> ADN  
<213> Escherichia coli  
  
20 <400> 3  
atgaaaaaga cagctatcgc gattgcagtg gcactggctg gtttcgctac  
cgtggcccag 60  
gcggcc  
66  
25  
  
<210> 4  
<211> 1065  
<212> ADN  
30 <213> Secuencia Artificial  
  
<220>  
<223> Descripción de la Secuencia Artificial  
Secuencia codificadora de la proteína K2S  
35

&lt;400&gt; 4

tctgagggaa acagtgactg ctactttggg aatgggtcag cctaccgtgg  
cacgcacagc 60

5 ctcaccgagt cgggtgcctc ctgcctcccg tggattcca tgatcctgat  
aggcaaggtt 120

tacacagcac agaaccacag tgcccaggca ctgggcctgg gcaaacataa  
ttactgccgg 180

aatcctgatg gggatgccaa gccctgggtg cacgtgctga agaacogcag  
gctgacgtgg 240

10 gagtactgtg atgtgccctc ctgctccacc tggggcctga gacagtacag  
ccagcctcag 300

tttcgcatca aaggagggct ctccgccgac atcgccctcc acccctggca  
ggctgccatc 360

15 tttgccaaagc acaggagggtc gcccgagag cggttcctgt gggggggcat  
actcatcagc 420

tcctgctgga ttctctctgc cgccactgc ttccaggaga ggtttcgcc  
ccaccacctg 480

acgggtgatct tgggcagaac ataccgggtg gtccctggcg aggaggagca  
gaaatttgaa 540

20 gtcgaaaaat acattgtcca taaggattc gatgatgaca ctacgacaa  
tgacattgog 600

ctgctgcagc tgaantcgga ttcgtcccg tgtgccagg agagcagcgt  
ggtccgcaat 660

25 gtgtgccttc ccccgccgga cctgcagctg ccggactgga cggagtgtga  
gctctccggc 720

taoggcaagc atgaggcctt gtctcctttc tattcggagc ggctgaagga  
ggctcatgtc 780

agactgtacc catccagccg ctgcacatca caacatttac ttaacagaac  
agtcaccgac 840

30 aacatgctgt gtgctggaga cactcggagc ggcgggcccc aggcaaactt  
gcacgacgcc 900

tgccagggcg attcgggagg cccctgggtg tgtctgaacg atggccgcat  
gactttggtg 960

ggcatcatca gctggggcct gggctgtgga cagaaggatg tcccgggtgt

35 gtacacaaag 1020

gttaccaact acctagactg gattcgtgac aacatgacgac cgtga  
1065

5 <210> 5  
<211> 1128  
<212> ADN  
<213> Secuencia Artificial

10 <220>  
<223> Descripción de la Secuencia Artificial:  
Secuencia codificadora de la proteína de fusión OmpA-K2S

<400> 5  
15 atgaaaaaga cagctatcgc gattgcagtg gcactggctg gtttcgctac  
cgtggcccag 60  
gcggcctctg agggaaacag tgactgctac tttgggaatg ggtcagccta  
ccgtggcacg 120  
cacagcctca ccgagtcggg tgccctcctgc ctcccgctga attccatgat  
20 cctgataggc 180  
aaggttttaca cagcacagaa cccagtgcc caggcactgg gcctgggcaa  
acataattac 240  
tgccggaatc ctgatgggga tgccaagccc tgggtgccacg tgctgaagaa  
ccgcaggctg 300  
25 acgtgggagt actgtgatgt gccctcctgc tccacctgcg gcctgagaca  
gtacagcccag 360  
cctcagtttc gcatcaaagg agggctcttc gccgacatcg cctcccaccc  
ctggcaggct 420  
gccatctttg ccaagcacag gaggtcgccc ggagagcggg tcctgtgcgg  
30 gggcatactc 480  
atcagctcct gctggattct ctctgcgcgc cactgcttcc aggagagggt  
tccgccccac 540  
cacctgacgg tgatcttggg cagaacatac cgggtgggtcc ctggcgagga  
ggagcagaaa 600  
35 tttgaagtcg aaaaatacat tgtccataag gaattcgatg atgacactta

cgacaatgac 660  
attgcgctgc tgcagctgaa atcggattcg tcccgtgtg cccaggagag  
cagcgtggtc 720  
cgcactgtgt gccttcccc ggcggacctg cagctgccgg actggacgga  
5 gtgtgagctc 780  
tccggctacg gcaagcatga ggccttgtct cctttctatt cggagcggct  
gaaggaggct 840  
catgtcagac tgtaccatc cagccgctgc acatcacaac atttacttaa  
cagaacagtc 900  
10 accgacaaca tgctgtgtgc tggagacact cggagcggcg ggccccaggc  
aaacttgcac 960  
gacgcctgcc agggcgattc gggaggcccc ctggtgtgtc tgaacgatgg  
ccgcatgact 1020  
ttggtgggca tcatcagctg gggcctgggc tgtggacaga aggatgtccc  
15 ggggtgtgtac 1080  
acaaaggtta ccaactacct agactggatt cgtgacaaca tgcgaccg  
1128  
  
20 <210> 6  
<211> 66  
<212> ADN  
<213> Escherichia coli  
  
25 <400> 6  
atgaaaaaga cagctatcgc gattgcagtg gcactggctg gtttcgctac  
cgtggccacag 60  
gcggcc  
66  
30  
  
<210> 7  
<211> 1065  
<212> ADN  
35 <213> Secuencia Artificial

<220>

<223> Descripción de la Secuencia Artificial.

Secuencia codificadora de la proteína K2S

5 <400> 7

tctgagggaa acagtgactg ctactttggg aatgggtcag cctaccgtgg  
cacgcacagc 60

ctcacogagt cgggtgcctc ctgcctcccg tggaattcca tgatcctgat  
aggcaaggtt 120

10 tacacagcac agaaccccag tgcccaggca ctgggcctgg gcaaacataa  
ttactgcogg 180

aatcctgatg gggatgcaa gccctggtgc cacgtgctga agaaccgcag  
gctgacgtgg 240

15 gagtactgtg atgtgccctc ctgctccacc tgccggcctga gacagtacag  
ccagcctcag 300

tttcgcatca aaggagggtc cttcgccgac atcgccctccc acccctggca  
ggctgccatc 360

tttgccaagc acaggaggtc gcccgagag cggttcctgt gcgggggcat  
actcatcagc 420

20 tcctgctgga ttctctctgc cgcccactgc ttccaggaga ggtttccgcc  
ccaccacctg 480

acggtgatct tgggcagaac ataccgggtg gtccctggcg aggaggagca  
gaaatttgaa 540

25 gtcgaaaaat acattgtcca taaggaaatc gatgatgaca cttacgacaa  
tgacattgog 600

ctgctgcagc tgaaatcgga ttctgtcccgc tgtgcccagg agagcagcgt  
ggtccgcact 660

gtgtgccttc ccccgggcga cctgcagctg ccggactgga cggagtgtga  
gctctccggc 720

30 tacggcaagc atgaggcctt gtctcctttc tattcggagc ggctgaagga  
ggctcatgtc 780

agactgtacc catccagccg ctgcacatca caacatttac ttaacagaac  
agtcaccgac 840

35 aacatgctgt gtgctggaga cactcggagc ggcggggccc aggcaaacctt  
gcacgacgcc 900

tgccagggcg attcgggagg cccctgtgtg tgtctgaacg atggccgcat  
gacttttggtg 960

ggcatcatca gctggggcct gggctgtgga cagaaggatg tcccgggtgt  
gtacacaaaag 1020

5 gttaccaact acctagactg gattcgtgac aacatgcgac cgtga  
1065

<210> 8

10 <211> 377

<212> PROTEÍNA

<213> Secuencia Artificial

<220>

15 <223> Descripción de la Secuencia Artificial.  
Proteína de fusión OmpA-K2S

<400> 8

Met Lys Lys Thr Ala Ile Ala Ile Ala Val Ala Leu Ala Gly Phe Ala  
20 1 5 10 15

Thr Val Ala Gln Ala Ala Ser Glu Gly Asn Ser Asp Cys Tyr Phe Gly  
20 25 30

25 Asn Gly Ser Ala Tyr Arg Gly Thr His Ser Leu Thr Glu Ser Gly Ala  
35 40 45

Ser Cys Leu Pro Trp Asn Ser Met Ile Leu Ile Gly Lys Val Tyr Thr  
50 55 60

30 Ala Gln Asn Pro Ser Ala Gln Ala Leu Gly Leu Gly Lys His Asn Tyr  
65 70 75 80

Cys Arg Asn Pro Asp Gly Asp Ala Lys Pro Trp Cys His Val Leu Lys  
35 85 90 95

|    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |  |
|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--|
|    | Asn | Arg | Arg | Leu | Thr | Trp | Glu | Tyr | Cys | Asp | Val | Pro | Ser | Cys | Ser | Thr |  |
|    |     |     |     | 100 |     |     |     |     | 105 |     |     |     |     |     | 110 |     |  |
| 5  | Cys | Gly | Leu | Arg | Gln | Tyr | Ser | Gln | Pro | Gln | Phe | Arg | Ile | Lys | Gly | Gly |  |
|    |     |     | 115 |     |     |     |     | 120 |     |     |     |     | 125 |     |     |     |  |
|    | Leu | Phe | Ala | Asp | Ile | Ala | Ser | His | Pro | Trp | Gln | Ala | Ala | Ile | Phe | Ala |  |
|    |     |     | 130 |     |     |     |     | 135 |     |     |     |     | 140 |     |     |     |  |
| 10 | Lys | His | Arg | Arg | Ser | Pro | Gly | Glu | Arg | Phe | Leu | Cys | Gly | Gly | Ile | Leu |  |
|    | 145 |     |     |     |     | 150 |     |     |     |     | 155 |     |     |     |     | 160 |  |
|    | Ile | Ser | Ser | Cys | Trp | Ile | Leu | Ser | Ala | Ala | His | Cys | Phe | Gln | Glu | Arg |  |
|    |     |     |     | 165 |     |     |     |     |     | 170 |     |     |     |     | 175 |     |  |
| 15 | Phe | Pro | Pro | His | His | Leu | Thr | Val | Ile | Leu | Gly | Arg | Thr | Tyr | Arg | Val |  |
|    |     |     |     | 180 |     |     |     |     |     | 185 |     |     |     |     | 190 |     |  |
|    | Val | Pro | Gly | Glu | Glu | Glu | Gln | Lys | Phe | Glu | Val | Glu | Lys | Tyr | Ile | Val |  |
| 20 |     |     | 195 |     |     |     |     | 200 |     |     |     |     | 205 |     |     |     |  |
|    | His | Lys | Glu | Phe | Asp | Asp | Asp | Thr | Tyr | Asp | Asn | Asp | Ile | Ala | Leu | Leu |  |
|    |     |     | 210 |     |     |     | 215 |     |     |     |     | 220 |     |     |     |     |  |
| 25 | Gln | Leu | Lys | Ser | Asp | Ser | Ser | Arg | Cys | Ala | Gln | Glu | Ser | Ser | Val | Val |  |
|    | 225 |     |     |     |     | 230 |     |     |     |     | 235 |     |     |     |     | 240 |  |
|    | Arg | Thr | Val | Cys | Leu | Pro | Pro | Ala | Asp | Leu | Gln | Leu | Pro | Asp | Trp | Thr |  |
|    |     |     |     | 245 |     |     |     |     |     | 250 |     |     |     |     | 255 |     |  |
| 30 | Glu | Cys | Glu | Leu | Ser | Gly | Tyr | Gly | Lys | His | Glu | Ala | Leu | Ser | Pro | Phe |  |
|    |     |     | 260 |     |     |     |     |     | 265 |     |     |     |     | 270 |     |     |  |
|    | Tyr | Ser | Glu | Arg | Leu | Lys | Glu | Ala | His | Val | Arg | Leu | Tyr | Pro | Ser | Ser |  |
| 35 |     |     | 275 |     |     |     |     | 280 |     |     |     |     | 285 |     |     |     |  |

Leu Cys Ala Gly Asp Thr Arg Ser Gly Gly Pro Gln Ala Asn Leu His  
305 310 315 320

10 Gly Arg Met Thr Leu Val Gly Ile Ile Ser Trp Gly Leu Gly Cys Gly  
340 345 350

15

20

### **<213> Secuencia Artificial**

25

### Secuencia de péptidos

**<400> 9**

30

**1**

**<210> 10**

35

**<211> 6**

<212> PROTEÍNA

<213> Secuencia Artificial

<220>

5 <223> Descripción de la Secuencia Artificial.

Secuencia de péptidos

<400> 10

Ser Glu Gly Asn Ser Asp

10 1 5

<210> 11

<211> 354

15 <212> PROTEÍNA

<213> Secuencia Artificial

<220>

20 <223> Descripción de la Secuencia Artificial K2S 174-527

<400> 11

Ser Glu Gly Asn Ser Asp Cys Tyr Phe Gly Asn Gly Ser Ala Tyr Arg

1 5 10 15

25 Gly Thr His Ser Leu Thr Glu Ser Gly Ala Ser Cys Leu Pro Trp Asn

20 25 30

Ser Met Ile Leu Ile Gly Lys Val Tyr Thr Ala Gln Asn Pro Ser Ala

35 40 45

30

Gln Ala Leu Gly Leu Gly Lys His Asn Tyr Cys Arg Asn Pro Asp Gly

50 55 60

Asp Ala Lys Pro Trp Cys His Val Leu Lys Asn Arg Arg Leu Thr Trp

35 65 70 75 80

- 41 -

|    |                                                                 |             |
|----|-----------------------------------------------------------------|-------------|
|    | Glu Tyr Cys Asp Val Pro Ser Cys Ser Thr Cys Gly Leu Arg Gln Tyr |             |
|    | 85                                                              | 90 95       |
| 5  | Ser Gln Pro Gln Phe Arg Ile Lys Gly Gly Leu Phe Ala Asp Ile Ala |             |
|    | 100                                                             | 105 110     |
|    | Ser His Pro Trp Gln Ala Ala Ile Phe Ala Lys His Arg Arg Ser Pro |             |
|    | 115                                                             | 120 125     |
| 10 | Gly Glu Arg Phe Leu Cys Gly Gly Ile Leu Ile Ser Ser Cys Trp Ile |             |
|    | 130                                                             | 135 140     |
|    | Leu Ser Ala Ala His Cys Phe Gln Glu Arg Phe Pro Pro His His Leu |             |
| 15 | 145                                                             | 150 155 160 |
|    | Thr Val Ile Leu Gly Arg Thr Tyr Arg Val Val Pro Gly Glu Glu Glu |             |
|    | 165                                                             | 170 175     |
|    | Gln Lys Phe Glu Val Glu Lys Tyr Ile Val His Lys Glu Phe Asp Asp |             |
| 20 | 180                                                             | 185 190     |
|    | Asp Thr Tyr Asp Asn Asp Ile Ala Leu Leu Gln Leu Lys Ser Asp Ser |             |
|    | 195                                                             | 200 205     |
| 25 | Ser Arg Cys Ala Gln Glu Ser Ser Val Val Arg Thr Val Cys Leu Pro |             |
|    | 210                                                             | 215 220     |
|    | Pro Ala Asp Leu Gln Leu Pro Asp Trp Thr Glu Cys Glu Leu Ser Gly |             |
| 30 | 225                                                             | 230 235 240 |
|    | Tyr Gly Lys His Glu Ala Leu Ser Pro Phe Tyr Ser Glu Arg Leu Lys |             |
|    | 245                                                             | 250 255     |
|    | Glu Ala His Val Arg Leu Tyr Pro Ser Ser Arg Cys Thr Ser Gln His |             |
| 35 | 260                                                             | 265 270     |

|    |                                                           |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |  |
|----|-----------------------------------------------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--|
|    | Leu                                                       | Leu | Asn | Arg | Thr | Val | Thr | Asp | Asn | Met | Leu | Cys | Ala | Gly | Asp | Thr |     |  |
|    | 275                                                       |     |     |     |     |     | 280 |     |     |     |     |     | 285 |     |     |     |     |  |
| 5  | Arg                                                       | Ser | Gly | Gly | Pro | Gln | Ala | Asn | Leu | His | Asp | Ala | Cys | Gln | Gly | Asp |     |  |
|    | 290                                                       |     |     |     |     |     | 295 |     |     |     |     |     | 300 |     |     |     |     |  |
|    | Ser                                                       | Gly | Gly | Pro | Leu | Val | Cys | Leu | Asn | Asp | Gly | Arg | Met | Thr | Leu | Val |     |  |
|    | 305                                                       |     |     |     |     |     | 310 |     |     |     |     |     | 315 |     |     |     | 320 |  |
| 10 | Gly                                                       | Ile | Ile | Ser | Trp | Gly | Leu | Gly | Cys | Gly | Gln | Lys | Asp | Val | Pro | Gly |     |  |
|    | 325                                                       |     |     |     |     |     | 330 |     |     |     |     |     | 335 |     |     |     |     |  |
|    | Val                                                       | Tyr | Thr | Lys | Val | Thr | Asn | Tyr | Leu | Asp | Trp | Ile | Arg | Asp | Asn | Met |     |  |
| 15 | 340                                                       |     |     |     |     |     | 345 |     |     |     |     |     | 350 |     |     |     |     |  |
|    | Arg Pro                                                   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |  |
|    | <210> 12                                                  |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |  |
| 20 | <211> 331                                                 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |  |
|    | <212> PROTEÍNA                                            |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |  |
|    | <213> Secuencia Artificial                                |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |  |
|    | <220>                                                     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |  |
| 25 | <223> Descripción de la Secuencia Artificial: K2S 197-527 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |  |
|    | <400> 12                                                  |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |  |
|    | Ser                                                       | Gly | Ala | Ser | Cys | Leu | Pro | Trp | Asn | Ser | Met | Ile | Leu | Ile | Gly | Lys |     |  |
|    | 1                                                         |     | 5   |     |     |     | 10  |     |     |     | 15  |     |     |     |     |     |     |  |
| 30 | Val                                                       | Tyr | Thr | Ala | Gln | Asn | Pro | Ser | Ala | Gln | Ala | Leu | Gly | Leu | Gly | Lys |     |  |
|    | 20                                                        |     |     |     |     |     | 25  |     |     |     |     |     | 30  |     |     |     |     |  |
|    | His                                                       | Asn | Tyr | Cys | Arg | Asn | Pro | Asp | Gly | Asp | Ala | Lys | Pro | Trp | Cys | His |     |  |
| 35 | 35                                                        |     |     |     |     |     | 40  |     |     |     |     |     | 45  |     |     |     |     |  |

|    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |  |
|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--|
|    | Val | Leu | Lys | Asn | Arg | Arg | Leu | Thr | Trp | Glu | Tyr | Cys | Asp | Val | Pro | Ser |  |
|    | 50  |     |     |     |     |     | 55  |     |     |     |     | 60  |     |     |     |     |  |
| 5  | Cys | Ser | Thr | Cys | Gly | Leu | Arg | Gln | Tyr | Ser | Gln | Pro | Gln | Phe | Arg | Ile |  |
|    | 65  |     |     |     |     | 70  |     |     |     |     | 75  |     |     |     |     | 80  |  |
|    | Lys | Gly | Gly | Leu | Phe | Ala | Asp | Ile | Ala | Ser | His | Pro | Trp | Gln | Ala | Ala |  |
|    |     |     |     |     | 85  |     |     |     |     | 90  |     |     |     |     | 95  |     |  |
| 10 | Ile | Phe | Ala | Lys | His | Arg | Arg | Ser | Pro | Gly | Glu | Arg | Phe | Leu | Cys | Gly |  |
|    |     |     |     | 100 |     |     |     |     | 105 |     |     |     |     | 110 |     |     |  |
|    | Gly | Ile | Leu | Ile | Ser | Ser | Cys | Trp | Ile | Leu | Ser | Ala | Ala | His | Cys | Phe |  |
|    |     | 115 |     |     |     |     |     | 120 |     |     |     |     | 125 |     |     |     |  |
| 15 | Gln | Glu | Arg | Phe | Pro | Pro | His | His | Leu | Thr | Val | Ile | Leu | Gly | Arg | Thr |  |
|    |     | 130 |     |     |     |     | 135 |     |     |     |     | 140 |     |     |     |     |  |
|    | Tyr | Arg | Val | Val | Pro | Gly | Glu | Glu | Glu | Gln | Lys | Phe | Glu | Val | Glu | Lys |  |
| 20 | 145 |     |     |     |     | 150 |     |     |     |     | 155 |     |     |     |     | 160 |  |
|    | Tyr | Ile | Val | His | Lys | Glu | Phe | Asp | Asp | Asp | Thr | Tyr | Asp | Asn | Asp | Ile |  |
|    |     |     |     |     | 165 |     |     |     |     | 170 |     |     |     |     | 175 |     |  |
| 25 | Ala | Leu | Leu | Gln | Leu | Lys | Ser | Asp | Ser | Ser | Arg | Cys | Ala | Gln | Glu | Ser |  |
|    |     |     |     | 180 |     |     |     |     | 185 |     |     |     |     | 190 |     |     |  |
|    | Ser | Val | Val | Arg | Thr | Val | Cys | Leu | Pro | Pro | Ala | Asp | Leu | Gln | Leu | Pro |  |
|    |     | 195 |     |     |     |     |     | 200 |     |     |     |     | 205 |     |     |     |  |
| 30 | Asp | Trp | Thr | Glu | Cys | Glu | Leu | Ser | Gly | Tyr | Gly | Lys | His | Glu | Ala | Leu |  |
|    |     | 210 |     |     |     |     | 215 |     |     |     |     | 220 |     |     |     |     |  |
|    | Ser | Pro | Phe | Tyr | Ser | Glu | Arg | Leu | Lys | Glu | Ala | His | Val | Arg | Leu | Tyr |  |
| 35 | 225 |     |     |     |     | 230 |     |     |     |     | 235 |     |     |     |     | 240 |  |

108

Pro Ser Ser Arg Cys Thr Ser Gln His Leu Leu Asn Arg Thr Val Thr  
245 250 255

5 Asp Asn Met Leu Cys Ala Gly Asp Thr Arg Ser Gly Gly Pro Gln Ala  
260 265 270

Asn Leu His Asp Ala Cys Gln Gly Asp Ser Gly Gly Pro Leu Val Cys  
275 280 285

10 Leu Asn Asp Gly Arg Met Thr Leu Val Gly Ile Ile Ser Trp Gly Leu  
290 295 300

Gly Cys Gly Gln Lys Asp Val Pro Gly Val Tyr Thr Lys Val Thr Asn  
305 310 315 320

15 Tyr Leu Asp Trp Ile Arg Asp Asn Met Arg Pro  
325 330

20 <210> 13  
<211> 339  
<212> PROTEÍNA  
<213> Secuencia Artificial

25 <220>  
<223> Descripción de la Secuencia Artificial: K2S 193-527,  
modificada

<400> 13

30 Ser Glu Gly Asn Ser Leu Thr Glu Ser Gly Ala Ser Cys Leu Pro Trp  
1 5 10 15

Asn Ser Met Ile Leu Ile Gly Lys Val Tyr Thr Ala Gln Asn Pro Ser  
20 25 30

35

|    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |  |
|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--|
|    | Ala | Gln | Ala | Leu | Gly | Leu | Gly | Lys | His | Asn | Tyr | Cys | Arg | Asn | Pro | Asp |  |
|    |     |     |     | 35  |     |     |     |     | 40  |     |     |     |     | 45  |     |     |  |
| 5  | Gly | Asp | Ala | Lys | Pro | Trp | Cys | His | Val | Leu | Lys | Asn | Arg | Arg | Leu | Thr |  |
|    |     | 50  |     |     |     |     | 55  |     |     |     |     | 60  |     |     |     |     |  |
|    | Trp | Glu | Tyr | Cys | Asp | Val | Pro | Ser | Cys | Ser | Thr | Cys | Gly | Leu | Arg | Gln |  |
|    | 65  |     |     |     |     | 70  |     |     |     |     | 75  |     |     |     |     | 80  |  |
| 10 | Tyr | Ser | Gln | Pro | Gln | Phe | Arg | Ile | Lys | Gly | Gly | Leu | Phe | Ala | Asp | Ile |  |
|    |     |     |     |     | 85  |     |     |     |     | 90  |     |     |     |     | 95  |     |  |
|    | Ala | Ser | His | Pro | Trp | Gln | Ala | Ala | Ile | Phe | Ala | Lys | His | Arg | Arg | Ser |  |
| 15 |     |     |     | 100 |     |     |     |     | 105 |     |     |     |     | 110 |     |     |  |
|    | Pro | Gly | Glu | Arg | Phe | Leu | Cys | Gly | Gly | Ile | Leu | Ile | Ser | Ser | Cys | Trp |  |
|    |     | 115 |     |     |     |     |     | 120 |     |     |     |     | 125 |     |     |     |  |
|    | Ile | Leu | Ser | Ala | Ala | His | Cys | Phe | Gln | Glu | Arg | Phe | Pro | Pro | His | His |  |
| 20 |     | 130 |     |     |     |     | 135 |     |     |     |     | 140 |     |     |     |     |  |
|    | Leu | Thr | Val | Ile | Leu | Gly | Arg | Thr | Tyr | Arg | Val | Val | Pro | Gly | Glu | Glu |  |
|    | 145 |     |     |     | 150 |     |     |     |     | 155 |     |     |     |     |     | 160 |  |
| 25 | Glu | Gln | Lys | Phe | Glu | Val | Glu | Lys | Tyr | Ile | Val | His | Lys | Glu | Phe | Asp |  |
|    |     |     |     | 165 |     |     |     |     | 170 |     |     |     |     | 175 |     |     |  |
|    | Asp | Asp | Thr | Tyr | Asp | Asn | Asp | Ile | Ala | Leu | Leu | Gln | Leu | Lys | Ser | Asp |  |
| 30 |     |     |     | 180 |     |     |     | 185 |     |     |     |     | 190 |     |     |     |  |
|    | Ser | Ser | Arg | Cys | Ala | Gln | Glu | Ser | Ser | Val | Val | Arg | Thr | Val | Cys | Leu |  |
|    |     | 195 |     |     |     |     |     | 200 |     |     |     | 205 |     |     |     |     |  |
|    | Pro | Pro | Ala | Asp | Leu | Gln | Leu | Pro | Asp | Trp | Thr | Glu | Cys | Glu | Leu | Ser |  |
| 35 |     | 210 |     |     |     |     | 215 |     |     |     |     | 220 |     |     |     |     |  |

|    |       |                                                      |     |     |     |     |     |     |     |     |     |     |     |     |     |     |  |
|----|-------|------------------------------------------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--|
|    | Gly   | Tyr                                                  | Gly | Lys | His | Glu | Ala | Leu | Ser | Pro | Phe | Tyr | Ser | Glu | Arg | Leu |  |
|    | 225   |                                                      |     |     |     | 230 |     |     |     |     | 235 |     |     |     |     | 240 |  |
| 5  | Lys   | Glu                                                  | Ala | His | Val | Arg | Leu | Tyr | Pro | Ser | Ser | Arg | Cys | Thr | Ser | Gln |  |
|    |       |                                                      |     |     | 245 |     |     |     |     | 250 |     |     |     |     |     | 255 |  |
|    | His   | Leu                                                  | Leu | Asn | Arg | Thr | Val | Thr | Asp | Asn | Met | Leu | Cys | Ala | Gly | Asp |  |
|    |       |                                                      |     | 260 |     |     |     |     | 265 |     |     |     |     |     | 270 |     |  |
| 10 | Thr   | Arg                                                  | Ser | Gly | Gly | Pro | Gln | Ala | Asn | Leu | His | Asp | Ala | Cys | Gln | Gly |  |
|    |       | 275                                                  |     |     |     |     | 280 |     |     |     |     |     | 285 |     |     |     |  |
|    | Asp   | Ser                                                  | Gly | Gly | Pro | Leu | Val | Cys | Leu | Asn | Asp | Gly | Arg | Met | Thr | Leu |  |
|    | 290   |                                                      |     |     |     | 295 |     |     |     |     |     | 300 |     |     |     |     |  |
| 15 | Val   | Gly                                                  | Ile | Ile | Ser | Trp | Gly | Leu | Gly | Cys | Gly | Gln | Lys | Asp | Val | Pro |  |
|    | 305   |                                                      |     |     |     | 310 |     |     |     |     | 315 |     |     |     |     | 320 |  |
|    | Gly   | Val                                                  | Tyr | Thr | Lys | Val | Thr | Asn | Tyr | Leu | Asp | Trp | Ile | Arg | Asp | Asn |  |
| 20 |       |                                                      |     | 325 |     |     |     |     |     | 330 |     |     |     |     | 335 |     |  |
|    | Met   | Arg                                                  | Pro |     |     |     |     |     |     |     |     |     |     |     |     |     |  |
|    | <210> | 14                                                   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |  |
| 25 | <211> | 335                                                  |     |     |     |     |     |     |     |     |     |     |     |     |     |     |  |
|    | <212> | PROTEÍNA                                             |     |     |     |     |     |     |     |     |     |     |     |     |     |     |  |
|    | <213> | Secuencia Artificial                                 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |  |
|    | <220> |                                                      |     |     |     |     |     |     |     |     |     |     |     |     |     |     |  |
| 30 | <223> | Descripción de la Secuencia Artificial. K2S 193-527, |     |     |     |     |     |     |     |     |     |     |     |     |     |     |  |
|    |       | modificada                                           |     |     |     |     |     |     |     |     |     |     |     |     |     |     |  |
|    | <400> | 14                                                   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |  |
|    | Ser   | Leu                                                  | Thr | Glu | Ser | Gly | Ala | Ser | Cys | Leu | Pro | Trp | Asn | Ser | Met | Ile |  |
| 35 | 1     |                                                      |     |     | 5   |     |     |     |     | 10  |     |     |     |     | 15  |     |  |

|    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |  |
|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--|
|    | Leu | Ile | Gly | Lys | Val | Tyr | Thr | Ala | Gln | Asn | Pro | Ser | Ala | Gln | Ala | Leu |  |
|    |     |     |     |     | 20  |     |     |     |     | 25  |     |     |     |     |     | 30  |  |
|    | Gly | Leu | Gly | Lys | His | Asn | Tyr | Cys | Arg | Asn | Pro | Asp | Gly | Asp | Ala | Lys |  |
| 5  |     |     |     | 35  |     |     |     |     | 40  |     |     |     |     | 45  |     |     |  |
|    | Pro | Trp | Cys | His | Val | Leu | Lys | Asn | Arg | Arg | Leu | Thr | Trp | Glu | Tyr | Cys |  |
|    |     |     |     | 50  |     |     |     | 55  |     |     |     |     | 60  |     |     |     |  |
| 10 | Asp | Val | Pro | Ser | Ser | Ser | Thr | Cys | Gly | Leu | Arg | Gln | Tyr | Ser | Gln | Pro |  |
|    |     | 65  |     |     |     |     | 70  |     |     |     |     | 75  |     |     |     | 80  |  |
|    | Gln | Phe | Arg | Ile | Lys | Gly | Gly | Leu | Phe | Ala | Asp | Ile | Ala | Ser | His | Pro |  |
|    |     |     |     |     | 85  |     |     |     |     | 90  |     |     |     |     |     | 95  |  |
| 15 | Trp | Gln | Ala | Ala | Ile | Phe | Ala | Lys | His | Arg | Arg | Ser | Pro | Gly | Glu | Arg |  |
|    |     |     |     | 100 |     |     |     |     |     | 105 |     |     |     |     | 110 |     |  |
|    | Phe | Leu | Cys | Gly | Gly | Ile | Leu | Ile | Ser | Ser | Cys | Trp | Ile | Leu | Ser | Ala |  |
| 20 |     |     | 115 |     |     |     |     | 120 |     |     |     |     | 125 |     |     |     |  |
|    | Ala | His | Cys | Phe | Gln | Glu | Arg | Phe | Pro | Pro | His | His | Leu | Thr | Val | Ile |  |
|    |     |     | 130 |     |     |     |     | 135 |     |     |     |     | 140 |     |     |     |  |
| 25 | Leu | Gly | Arg | Thr | Tyr | Arg | Val | Val | Pro | Gly | Glu | Glu | Glu | Gln | Lys | Phe |  |
|    |     | 145 |     |     |     |     | 150 |     |     |     |     | 155 |     |     |     | 160 |  |
|    | Glu | Val | Glu | Lys | Tyr | Ile | Val | His | Lys | Glu | Phe | Asp | Asp | Asp | Thr | Tyr |  |
|    |     |     |     |     | 165 |     |     |     |     | 170 |     |     |     |     |     | 175 |  |
| 30 | Asp | Asn | Asp | Ile | Ala | Leu | Leu | Gln | Leu | Lys | Ser | Asp | Ser | Ser | Arg | Cys |  |
|    |     |     |     |     | 180 |     |     |     |     | 185 |     |     |     |     | 190 |     |  |
|    | Ala | Gln | Glu | Ser | Ser | Val | Val | Arg | Thr | Val | Cys | Leu | Pro | Pro | Ala | Asp |  |
| 35 |     |     |     | 195 |     |     |     | 200 |     |     |     |     |     |     | 205 |     |  |

35

<400> 15

Ser Glu Gly Asn Ser Asp Thr His Ser Leu Thr Glu Ser Gly Ala Ser  
1 5 10 15

5 Cys Leu Pro Trp Asn Ser Met Ile Leu Ile Gly Lys Val Tyr Thr Ala  
20 25 30

Gln Asn Pro Ser Ala Gln Ala Leu Gly Leu Gly Lys His Asn Tyr Cys  
35 40 45

10

Arg Asn Pro Asp Gly Asp Ala Lys Pro Trp Cys His Val Leu Lys Asn  
50 55 60

15 Arg Arg Leu Thr Trp Glu Tyr Cys Asp Val Pro Ser Cys Ser Thr Cys  
65 70 75 80

Gly Leu Arg Gln Tyr Ser Gln Pro Gln Phe Arg Ile Lys Gly Gly Leu  
85 90 95

20 Phe Ala Asp Ile Ala Ser His Pro Trp Gln Ala Ala Ile Phe Ala Lys  
100 105 110

His Arg Arg Ser Pro Gly Glu Arg Phe Leu Cys Gly Gly Ile Leu Ile  
115 120 125

25

Ser Ser Cys Trp Ile Leu Ser Ala Ala His Cys Phe Gln Glu Arg Phe  
130 135 140

30 Pro Pro His His Leu Thr Val Ile Leu Gly Arg Thr Tyr Arg Val Val  
145 150 155 160

Pro Gly Glu Glu Glu Gln Lys Phe Glu Val Glu Lys Tyr Ile Val His  
165 170 175

35

111

|    |       |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |  |
|----|-------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--|
|    | Lys   | Glu | Phe | Asp | Asp | Asp | Thr | Tyr | Asp | Asn | Asp | Ile | Ala | Leu | Leu | Gln |  |
|    |       |     |     | 180 |     |     |     |     | 185 |     |     |     |     | 190 |     |     |  |
|    | Leu   | Lys | Ser | Asp | Ser | Ser | Arg | Cys | Ala | Gln | Glu | Ser | Ser | Val | Val | Arg |  |
| 5  |       |     | 195 |     |     |     |     | 200 |     |     |     |     | 205 |     |     |     |  |
|    | Thr   | Val | Cys | Leu | Pro | Pro | Ala | Asp | Leu | Gln | Leu | Pro | Asp | Trp | Thr | Glu |  |
|    |       | 210 |     |     |     |     | 215 |     |     |     |     |     | 220 |     |     |     |  |
| 10 | Cys   | Glu | Leu | Ser | Gly | Tyr | Gly | Lys | His | Glu | Ala | Leu | Ser | Pro | Phe | Tyr |  |
|    | 225   |     |     |     |     | 230 |     |     |     |     | 235 |     |     |     |     | 240 |  |
|    | Ser   | Glu | Arg | Leu | Lys | Glu | Ala | His | Val | Arg | Leu | Tyr | Pro | Ser | Ser | Arg |  |
|    |       |     |     | 245 |     |     |     |     |     | 250 |     |     |     |     |     | 255 |  |
| 15 | Cys   | Thr | Ser | Gln | His | Leu | Leu | Asn | Arg | Thr | Val | Thr | Asp | Asn | Met | Leu |  |
|    |       |     |     | 260 |     |     |     |     | 265 |     |     |     |     |     | 270 |     |  |
|    | Cys   | Ala | Gly | Asp | Thr | Arg | Ser | Gly | Gly | Pro | Gln | Ala | Asn | Leu | His | Asp |  |
| 20 |       |     | 275 |     |     |     |     |     | 280 |     |     |     |     | 285 |     |     |  |
|    | Ala   | Cys | Gln | Gly | Asp | Ser | Gly | Gly | Pro | Leu | Val | Cys | Leu | Asn | Asp | Gly |  |
|    |       | 290 |     |     |     |     | 295 |     |     |     |     |     | 300 |     |     |     |  |
| 25 | Arg   | Met | Thr | Leu | Val | Gly | Ile | Ile | Ser | Trp | Gly | Leu | Gly | Cys | Gly | Gln |  |
|    | 305   |     |     |     |     |     | 310 |     |     |     |     | 315 |     |     |     | 320 |  |
|    | Lys   | Asp | Val | Pro | Gly | Val | Tyr | Thr | Lys | Val | Thr | Asn | Tyr | Leu | Asp | Trp |  |
|    |       |     |     |     | 325 |     |     |     |     |     | 330 |     |     |     |     | 335 |  |
| 30 | Ile   | Arg | Asp | Asn | Met | Arg | Pro |     |     |     |     |     |     |     |     |     |  |
|    |       |     |     |     | 340 |     |     |     |     |     |     |     |     |     |     |     |  |
| 35 | <210> | 16  |     |     |     |     |     |     |     |     |     |     |     |     |     |     |  |
|    | <211> | 343 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |  |

<212> PROTEÍNA

<213> Secuencia Artificial

<220>

5 <223> Descripción de la Secuencia Artificial: K2S 191-527,  
modificada

<400> 16

Ser Glu Gly Asn Ser Asp Thr His Ser Leu Thr Glu Ser Gly Ala Ser  
10 1 5 10 15

Cys Leu Pro Trp Asn Ser Met Ile Leu Ile Gly Lys Val Tyr Thr Ala  
20 25 30

15 Gln Asn Pro Ser Ala Gln Ala Leu Gly Leu Gly Lys His Asn Tyr Cys  
35 40 45

Arg Asn Pro Asp Gly Asp Ala Lys Pro Trp Cys His Val Leu Lys Asn  
50 55 60

20 Arg Arg Leu Thr Trp Glu Tyr Cys Asp Val Pro Ser Ser Ser Thr Cys  
65 70 75 80

Gly Leu Arg Gln Tyr Ser Gln Pro Gln Phe Arg Ile Lys Gly Gly Leu  
25 85 90 95

Phe Ala Asp Ile Ala Ser His Pro Trp Gln Ala Ala Ile Phe Ala Lys  
100 105 110

30 His Arg Arg Ser Pro Gly Glu Arg Phe Leu Cys Gly Gly Ile Leu Ile  
115 120 125

Ser Ser Cys Trp Ile Leu Ser Ala Ala His Cys Phe Gln Glu Arg Phe  
130 135 140

35

|    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|    | Pro | Pro | His | His | Leu | Thr | Val | Ile | Leu | Gly | Arg | Thr | Tyr | Arg | Val | Val |
|    | 145 |     |     |     |     | 150 |     |     |     |     | 155 |     |     |     |     | 160 |
| 5  | Pro | Gly | Glu | Glu | Glu | Gln | Lys | Phe | Glu | Val | Glu | Lys | Tyr | Ile | Val | His |
|    |     |     |     |     | 165 |     |     |     |     | 170 |     |     |     |     | 175 |     |
|    | Lys | Glu | Phe | Asp | Asp | Asp | Thr | Tyr | Asp | Asn | Asp | Ile | Ala | Leu | Leu | Gln |
|    |     |     |     | 180 |     |     |     |     | 185 |     |     |     |     | 190 |     |     |
| 10 | Leu | Lys | Ser | Asp | Ser | Ser | Arg | Cys | Ala | Gln | Glu | Ser | Ser | Val | Val | Arg |
|    |     | 195 |     |     |     |     |     | 200 |     |     |     |     |     | 205 |     |     |
|    | Thr | Val | Cys | Leu | Pro | Pro | Ala | Asp | Leu | Gln | Leu | Pro | Asp | Trp | Thr | Glu |
| 15 |     | 210 |     |     |     |     | 215 |     |     |     |     | 220 |     |     |     |     |
|    | Cys | Glu | Leu | Ser | Gly | Tyr | Gly | Lys | His | Glu | Ala | Leu | Ser | Pro | Phe | Tyr |
|    | 225 |     |     |     |     | 230 |     |     |     |     | 235 |     |     |     |     | 240 |
|    | Ser | Glu | Arg | Leu | Lys | Glu | Ala | His | Val | Arg | Leu | Tyr | Pro | Ser | Ser | Arg |
| 20 |     |     |     | 245 |     |     |     |     |     | 250 |     |     |     |     | 255 |     |
|    | Cys | Thr | Ser | Gln | His | Leu | Leu | Asn | Arg | Thr | Val | Thr | Asp | Asn | Met | Leu |
|    |     |     |     | 260 |     |     |     |     | 265 |     |     |     |     | 270 |     |     |
| 25 | Cys | Ala | Gly | Asp | Thr | Arg | Ser | Gly | Gly | Pro | Gln | Ala | Asn | Leu | His | Asp |
|    |     | 275 |     |     |     |     |     | 280 |     |     |     |     | 285 |     |     |     |
|    | Ala | Cys | Gln | Gly | Asp | Ser | Gly | Gly | Pro | Leu | Val | Cys | Leu | Asn | Asp | Gly |
| 30 |     | 290 |     |     |     |     | 295 |     |     |     |     | 300 |     |     |     |     |
|    | Arg | Met | Thr | Leu | Val | Gly | Ile | Ile | Ser | Trp | Gly | Leu | Gly | Cys | Gly | Gln |
|    | 305 |     |     |     |     | 310 |     |     |     |     | 315 |     |     |     |     | 320 |
|    | Lys | Asp | Val | Pro | Gly | Val | Tyr | Thr | Lys | Val | Thr | Asn | Tyr | Leu | Asp | Trp |
| 35 |     |     |     |     | 325 |     |     |     |     | 330 |     |     |     |     | 335 |     |

Ile Arg Asp Asn Met Arg Pro  
340

5   <210> 17  
     <211> 308  
     <212> PROTEÍNA  
     <213> Secuencia Artificial

10   <220>  
     <223> Descripción de la Secuencia Artificial: K25 220-527

     <400> 17  
     Ser Ala Gln Ala Leu Gly Leu Gly Lys His Asn Tyr Cys Arg Asn Pro  
15       1                   5                   10                   15

     Asp Gly Asp Ala Lys Pro Trp Cys His Val Leu Lys Asn Arg Arg Leu  
                 20                   25                   30

20   Thr Trp Glu Tyr Cys Asp Val Pro Ser Cys Ser Thr Cys Gly Leu Arg  
         35                   40                   45

     Gln Tyr Ser Gln Pro Gln Phe Arg Ile Lys Gly Gly Leu Phe Ala Asp  
         50                   55                   60

25   Ile Ala Ser His Pro Trp Gln Ala Ala Ile Phe Ala Lys His Arg Arg  
     65                   70                   75                   80

     Ser Pro Gly Glu Arg Phe Leu Cys Gly Gly Ile Leu Ile Ser Ser Cys  
30                   85                   90                   95

     Trp Ile Leu Ser Ala Ala His Cys Phe Gln Glu Arg Phe Pro Pro His  
         100                   105                   110

35

- 54 -

|    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|    | His | Leu | Thr | Val | Ile | Leu | Gly | Arg | Thr | Tyr | Arg | Val | Val | Pro | Gly | Glu |
|    | 115 |     |     |     | 120 |     |     |     | 125 |     |     |     |     |     |     |     |
| 5  | Glu | Glu | Gln | Lys | Phe | Glu | Val | Glu | Lys | Tyr | Ile | Val | His | Lys | Glu | Phe |
|    | 130 |     |     |     | 135 |     |     |     | 140 |     |     |     |     |     |     |     |
|    | Asp | Asp | Asp | Thr | Tyr | Asp | Asn | Asp | Ile | Ala | Leu | Leu | Gln | Leu | Lys | Ser |
|    | 145 |     |     |     | 150 |     |     |     | 155 |     |     |     | 160 |     |     |     |
| 10 | Asp | Ser | Ser | Arg | Cys | Ala | Gln | Glu | Ser | Ser | Val | Val | Arg | Thr | Val | Cys |
|    | 165 |     |     |     | 170 |     |     |     | 175 |     |     |     |     |     |     |     |
|    | Leu | Pro | Pro | Ala | Asp | Leu | Gln | Leu | Pro | Asp | Trp | Thr | Glu | Cys | Glu | Leu |
| 15 | 180 |     |     |     | 185 |     |     |     | 190 |     |     |     |     |     |     |     |
|    | Ser | Gly | Tyr | Gly | Lys | His | Glu | Ala | Leu | Ser | Pro | Phe | Tyr | Ser | Glu | Arg |
|    | 195 |     |     |     | 200 |     |     |     | 205 |     |     |     |     |     |     |     |
|    | Leu | Lys | Glu | Ala | His | Val | Arg | Leu | Tyr | Pro | Ser | Ser | Arg | Cys | Thr | Ser |
| 20 | 210 |     |     |     | 215 |     |     |     | 220 |     |     |     |     |     |     |     |
|    | Gln | His | Leu | Leu | Asn | Arg | Thr | Val | Thr | Asp | Asn | Met | Leu | Cys | Ala | Gly |
|    | 225 |     |     |     | 230 |     |     |     | 235 |     |     |     | 240 |     |     |     |
| 25 | Asp | Thr | Arg | Ser | Gly | Gly | Pro | Gln | Ala | Asn | Leu | His | Asp | Ala | Cys | Gln |
|    | 245 |     |     |     | 250 |     |     |     | 255 |     |     |     |     |     |     |     |
|    | Gly | Asp | Ser | Gly | Gly | Pro | Leu | Val | Cys | Leu | Asn | Asp | Gly | Arg | Met | Thr |
| 30 | 260 |     |     |     | 265 |     |     |     | 270 |     |     |     |     |     |     |     |
|    | Leu | Val | Gly | Ile | Ile | Ser | Trp | Gly | Leu | Gly | Cys | Gly | Gln | Lys | Asp | Val |
|    | 275 |     |     |     | 280 |     |     |     | 285 |     |     |     |     |     |     |     |
|    | Pro | Gly | Val | Tyr | Thr | Lys | Val | Thr | Asn | Tyr | Leu | Asp | Trp | Ile | Arg | Asp |
| 35 | 290 |     |     |     | 295 |     |     |     | 300 |     |     |     |     |     |     |     |

Asn Met Arg Pro

305

5 <210> 18

<211> 268

<212> PROTEÍNA

<213> Secuencia Artificial

10 <220>

<223> Descripción de la Secuencia Artificial: K2S 260-527

<400> 18

Ser Cys Ser Thr Cys Gly Leu Arg Gln Tyr Ser Gln Pro Gln Phe Arg

15 1 5 10 15

Ile Lys Gly Gly Leu Phe Ala Asp Ile Ala Ser His Pro Trp Gln Ala

20 25 30

20 Ala Ile Phe Ala Lys His Arg Arg Ser Pro Gly Glu Arg Phe Leu Cys

35 40 45

Gly Gly Ile Leu Ile Ser Ser Cys Trp Ile Leu Ser Ala Ala His Cys

50 55 60

25

Phe Gln Glu Arg Phe Pro Pro His His Leu Thr Val Ile Leu Gly Arg

65 70 75 80

Thr Tyr Arg Val Val Pro Gly Glu Glu Glu Gln Lys Phe Glu Val Glu

30 85 90 95

Lys Tyr Ile Val His Lys Glu Phe Asp Asp Asp Thr Tyr Asp Asn Asp

100 105 110

35 Ile Ala Leu Leu Gln Leu Lys Ser Asp Ser Ser Arg Cys Ala Gln Glu

114

|    | 115                                                             |     | 120 |     | 125 |
|----|-----------------------------------------------------------------|-----|-----|-----|-----|
|    | Ser Ser Val Val Arg Thr Val Cys Leu Pro Pro Ala Asp Leu Gln Leu |     |     |     |     |
|    | 130                                                             |     | 135 |     | 140 |
| 5  | Pro Asp Trp Thr Glu Cys Glu Leu Ser Gly Tyr Gly Lys His Glu Ala |     |     |     |     |
|    | 145                                                             |     | 150 |     | 155 |
|    |                                                                 |     |     |     | 160 |
|    | Leu Ser Pro Phe Tyr Ser Glu Arg Leu Lys Glu Ala His Val Arg Leu |     |     |     |     |
| 10 |                                                                 | 165 |     | 170 | 175 |
|    | Tyr Pro Ser Ser Arg Cys Thr Ser Gln His Leu Leu Asn Arg Thr Val |     |     |     |     |
|    |                                                                 | 180 |     | 185 | 190 |
| 15 | Thr Asp Asn Met Leu Cys Ala Gly Asp Thr Arg Ser Gly Gly Pro Gln |     |     |     |     |
|    | 195                                                             |     | 200 |     | 205 |
|    | Ala Asn Leu His Asp Ala Cys Gln Gly Asp Ser Gly Gly Pro Leu Val |     |     |     |     |
|    | 210                                                             |     | 215 |     | 220 |
| 20 | Cys Leu Asn Asp Gly Arg Met Thr Leu Val Gly Ile Ile Ser Trp Gly |     |     |     |     |
|    | 225                                                             |     | 230 |     | 235 |
|    |                                                                 |     |     |     | 240 |
|    | Leu Gly Cys Gly Gln Lys Asp Val Pro Gly Val Tyr Thr Lys Val Thr |     |     |     |     |
| 25 |                                                                 | 245 |     | 250 | 255 |
|    | Asn Tyr Leu Asp Trp Ile Arg Asp Asn Met Arg Pro                 |     |     |     |     |
|    |                                                                 | 260 |     | 265 |     |
| 30 | <210> 19                                                        |     |     |     |     |
|    | <211> 527                                                       |     |     |     |     |
|    | <212> PROTEÍNA                                                  |     |     |     |     |
|    | <213> Homo sapiens                                              |     |     |     |     |
| 35 |                                                                 |     |     |     |     |

**5**    Gln His Gln Ser Trp Leu Arg Pro Val Leu Arg Ser Asn Arg Val Glu  
                    20                    25                    30

10

|    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|    | Ala | Leu | Tyr | Phe | Ser | Asp | Phe | Val | Cys | Gln | Cys | Pro | Glu | Gly | Phe | Ala |
| 15 | 65  |     |     |     |     | 70  |     |     |     |     | 75  |     |     |     |     | 80  |

20 Gly Ile Ser Tyr Arg Gly Thr Trp Ser Thr Ala Glu Ser Gly Ala Glu  
100 105 110

25

|    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|    | Arg | Asn | Pro | Asp | Arg | Asp | Ser | Lys | Pro | Trp | Cys | Tyr | Val | Phe | Lys | Ala |
| 30 | 145 |     |     |     |     | 150 |     |     |     |     | 155 |     |     |     |     | 160 |

35

115

|    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|    | Asn | Ser | Asp | Cys | Tyr | Phe | Gly | Asn | Gly | Ser | Ala | Tyr | Arg | Gly | Thr | His |
|    |     |     |     | 180 |     |     |     |     | 185 |     |     |     |     | 190 |     |     |
| 5  | Ser | Leu | Thr | Glu | Ser | Gly | Ala | Ser | Cys | Leu | Pro | Trp | Asn | Ser | Met | Ile |
|    |     |     | 195 |     |     |     |     | 200 |     |     |     |     | 205 |     |     |     |
|    | Leu | Ile | Gly | Lys | Val | Tyr | Thr | Ala | Gln | Asn | Pro | Ser | Ala | Gln | Ala | Leu |
|    |     | 210 |     |     |     |     | 215 |     |     |     | 220 |     |     |     |     |     |
| 10 | Gly | Leu | Gly | Lys | His | Asn | Tyr | Cys | Arg | Asn | Pro | Asp | Gly | Asp | Ala | Lys |
|    | 225 |     |     |     |     | 230 |     |     |     | 235 |     |     |     |     | 240 |     |
|    | Pro | Trp | Cys | His | Val | Leu | Lys | Asn | Arg | Arg | Leu | Thr | Trp | Glu | Tyr | Cys |
| 15 |     |     |     | 245 |     |     |     |     | 250 |     |     |     |     | 255 |     |     |
|    | Asp | Val | Pro | Ser | Cys | Ser | Thr | Cys | Gly | Leu | Arg | Gln | Tyr | Ser | Gln | Pro |
|    |     |     | 260 |     |     |     |     |     | 265 |     |     |     | 270 |     |     |     |
|    | Gln | Phe | Arg | Ile | Lys | Gly | Gly | Leu | Phe | Ala | Asp | Ile | Ala | Ser | His | Pro |
| 20 |     | 275 |     |     |     |     |     | 280 |     |     |     |     | 285 |     |     |     |
|    | Trp | Gln | Ala | Ala | Ile | Phe | Ala | Lys | His | Arg | Arg | Ser | Pro | Gly | Glu | Arg |
|    |     | 290 |     |     |     | 295 |     |     |     | 300 |     |     |     |     |     |     |
| 25 | Phe | Leu | Cys | Gly | Gly | Ile | Leu | Ile | Ser | Ser | Cys | Trp | Ile | Leu | Ser | Ala |
|    | 305 |     |     |     |     | 310 |     |     |     | 315 |     |     |     |     | 320 |     |
|    | Ala | His | Cys | Phe | Gln | Glu | Arg | Phe | Pro | Pro | His | His | Leu | Thr | Val | Ile |
| 30 |     |     |     | 325 |     |     |     |     | 330 |     |     |     |     | 335 |     |     |
|    | Leu | Gly | Arg | Thr | Tyr | Arg | Val | Val | Pro | Gly | Glu | Glu | Glu | Gln | Lys | Phe |
|    |     |     | 340 |     |     |     |     |     | 345 |     |     |     |     | 350 |     |     |
|    | Glu | Val | Glu | Lys | Tyr | Ile | Val | His | Lys | Glu | Phe | Asp | Asp | Asp | Thr | Tyr |
| 35 |     | 355 |     |     |     |     |     | 360 |     |     |     | 365 |     |     |     |     |



1

1

<220>

<223> Descripción de la Secuencia Artificial:

Secuencia codificadora de SEGN

5 <400> 20

tctgagggaa ac

12

10 <210> 21

<211> 22

<212> PROTEÍNA

<213> Escherichia coli

15 <400> 21

Met Lys Lys Thr Ala Ile Ala Ile Ala Val Ala Leu Ala Gly Phe Ala

1

5

10

15

Thr Val Ala Gln Ala Ala

20

20

<210> 22

<211> 42

25 <212> ADN

<213> Secuencia Artificial

<220>

<223> Descripción de la Secuencia Artificial. Cebador

30

<400> 22

gaggaggagg tggcccaggc ggcctctgag ggaaacagtg ac

42

35

<210> 23

<211> 42

<212> ADN

<213> Secuencia Artificial

5

<220>

<223> Descripción de la Secuencia Artificial Cebador

<400> 23

10 gaggaggagc tggccggcct ggcccgggtcg catgttgtca cg  
42

<210> 24

15 <211> 26

<212> ADN

<213> Secuencia Artificial

<220>

20 <223> Descripción de la Secuencia Artificial. Cebador

<400> 24

acatgcgacc gtgacaggcc ggccag  
26  
25

<210> 25

<211> 26

<212> ADN

30 <213> Secuencia Artificial

<220>

<223> Descripción de la Secuencia Artificial. Cebador

35

11/7

- 62 -

<400> 25

ctggccggcc tgtcacggtc gcatgt

26

### **REIVINDICACIONES**

- 1        Método para la producción de un activador de plasminogeno tisular (tPA) que se deriva de un ADN recombinante, de una variante de tPA, de una molécula de Knirgle 2-proteasa de senna (K2S) o de una variante de K2S en células procarionóticas, en el que dicho tPA, dicha variante de tPA, dicha molécula de K2S o dicha variante de K2S se segrega extracelularmente como una proteína activa y correctamente plegada, caracterizado porque la célula procarionótica contiene y expresa un vector que comprende el ADN que codifica dicho tPA, dicha variante de tPA, dicha molécula de K2S o dicha variante de K2S, unido operativamente con el ADN que codifica el péptido de señal OmpA o un derivado funcional de éste
- 5        2        Método de acuerdo con la reivindicación 1, caracterizado porque dicha célula procarionótica contiene y expresa un vector que comprende el ADN que codifica dicho tPA, dicha variante de tPA, dicha molécula de K2S o dicha variante de K2S, unido operativamente con el ADN que codifica el péptido de señal OmpA, que está unido operativamente con la molécula de ácido nucleico que es definida por la secuencia TCTGAGGGAAACAGTGAC (SEQ ID NO 1) o un derivado funcional de ésta
- 10        3        Método de acuerdo con la reivindicación 1 ó 2, caracterizado porque la célula procarionótica es E coli
- 15        4        Método de acuerdo con una de las reivindicaciones 1 a 3, caracterizado porque se llevan a cabo las siguientes operaciones
- 20        a) el ADN que codifica el tPA, la variante de tPA, la molécula de K2S o la variante de K2S se amplifica por una PCR,
- 25        b) el producto de la PCR se purifica,
- 30        c) dicho producto de PCR se introduce en un vector que comprende el ADN que codifica el péptido de señal OmpA y el ADN que codifica la gpIII de una manera tal que dicho producto de PCR está unido operativamente secuencia arriba con el ADN que codifica la secuencia de señal OmpA y está unido secuencia abajo con el ADN que codifica la gpIII de dicho vector,
- 35        d) se introduce un codón de detención entre dicho tPA, dicha variante de tPA, dicha molécula de K2S o dicha variante de K2S y la gpIII,
- 40        e) dicho vector se expresa por la célula procarionótica,
- 45        f) el tPA, la variante de tPA, la molécula de K2S o la variante de K2S se purifica
- 50        5        Método de acuerdo con una de las reivindicaciones 1 a 4, caracterizado porque el vector es un vector fagémico que comprende el ADN que codifica el péptido de señal OmpA y el ADN que codifica la gpIII
- 55        6        Método de acuerdo con una cualquiera de las reivindicaciones 1 a 5, caracterizado porque el vector es el fagémico pComb3HSS

- 7 Método de acuerdo con una cualquiera de las reivindicaciones 1 a 6, caracterizado porque la secuencia de ADN del OmpA unido secuencia arriba con la K2S comprende la siguiente secuencia o una variante funcional de ella o una variante debida al código degenerado de nucleótidos
- 5 ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTGG  
CCCAGGCGGCCTCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTA  
CCGTGGCACGCACAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAATTC  
CATGATCCTGATAGGCAAGGTTTACACAGCACAGAACCCCAGTGCCCAGGCACTG  
GGCCTGGGCAAACATAATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGT  
10 GCCACGTGCTGAAGAACCGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCT  
GCTCCACCTGCGGCCTGAGACAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAG  
GGCTCTTCGCCGACATCGCCTCCCACCCCTGGCAGGCTGCCATCTTTGCCAAGC  
ACAGGAGGTGCCCCGGAGAGCGGTTCTGTGCGGGGGCATACTCATCAGCTCC  
TGCTGGATTCTCTCTGCCGCCCACTGCTTCCAGGAGAGGTTTCCGCCCCACCACC  
15 TGACGGTGATCTTGGGCAGAACATACCGGGTGGTCCCTGGCGAGGAGGAGCAGA  
AATTTGAAGTCGAAAAATACATTGTCCATAAGGAATTCGATGATGACACTTACGAC  
AATGACATTGCGCTGCTGCAGCTGAAATCGGATTTCGTCGCCGCTGTGCCAGGAG  
AGCAGCGTGGTCCGCACTGTGTGCCTTCCCCCGGCGGACCTGCAGCTGCCGGA  
CTGGACGGAGTGTGAGCTCTCCGGCTACGGCAAGCATGAGGCCTTGTCTCCTTT  
20 CTATTCGGAGCGGCTGAAGGAGGCTCATGTCAGACTGTACCCATCCAGCCGCTG  
CACATCACAACATTTACTTAACAGAACAGTCACCGACAACATGCTGTGTGCTGGA  
GACACTCGGAGCGGCGGGCCCCAGGCAAACCTTGACGACGCCTGCCAGGGCGA  
TTCGGGAGGCCCCCTGGTGTGTCTGAACGATGGCCGCATGACTTTGGTGGGCA  
TCATCAGCTGGGGCCTGGGCTGTGGACAGAAGGATGTCCCGGGTGTGTACACA  
25 AAGGTTACCAACTACCTAGACTGGATTTCGTGACAACATGCGACCG (SEQ ID NO 2)
- 8 Método de acuerdo con una cualquiera de las reivindicaciones 1 a 7, caracterizado porque la secuencia de ADN del OmpA comprende la siguiente secuencia  
ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTG  
GCCCAGGCGGCC (SEQ ID NO 3)
- 30 9 Método de acuerdo con una de las reivindicaciones 1 a 8, caracterizado porque la secuencia de ADN del OmpA consta de la siguiente secuencia  
ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTG  
GCCCAGGCGGCC (SEQ ID NO 3)
10. Método de acuerdo con una de las reivindicaciones 1 a 9, caracterizado porque el ADN del tPA, de la variante de tPA, de la molécula de K2S o de la variante de K2S está precedido por un promotor lac y/o por un sitio de fijación a ribosomas
- 35

11 Método de acuerdo con una de las reivindicaciones 1 a 10, caracterizado porque el ADN que codifica el tPA, la variante de tPA, la molécula de K2S o la variante de K2S se selecciona entre el grupo de moléculas de ADN que codifican por lo menos un 90% de los aminoácidos 87 - 527, 174 - 527, 180 - 527 o 220 - 527 de la proteína de un activador de plasminógeno tisular humano

12 Método de acuerdo con una de las reivindicaciones 5 a 11, caracterizado porque la secuencia de ADN de la K2S comprende la siguiente secuencia o una variante funcional de ésta o una variante debida al código degenerado de nucleótidos

10 TCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTACCGTGGCACG  
CACAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAATTCCATGATCCTGA  
TAGGCAAGGTTTACACAGCACAGAACCCAGTGCCCAGGCACTGGGCCTGGGCA  
AACATAATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGTGCCACGTGCTG  
AAGAACCGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGC  
GGCCTGAGACAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCTCTTCGCC  
15 GACATCGCCTCCCACCCCTGGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTCG  
CCCGGAGAGCGGTTCTGTGCGGGGGCATACTCATCAGCTCCTGCTGGATTCTC  
TCTGCCGCCCACTGCTTCCAGGAGAGGTTTCCGCCCCACCACCTGACGGTGATC  
TTGGGCAGAACATACCGGGTGGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGT  
CGAAAAATACATTGTCCATAAGGAATTCGATGATGACACTTACGACAATGACATTG  
20 CGCTGCTGCAGCTGAAATCGGATTCTGTCCTCCGCTGTGCCCAGGAGAGCAGCGTG  
GTCCGCACTGTGTGCCTTCCCCCGGCGGACCTGCAGCTGCCGGACTGGACGGA  
GTGTGAGCTCTCCGGCTACGGCAAGCATGAGGCCTTGTCTCCTTTCTATTGGA  
GCGGCTGAAGGAGGCTCATGTGCACTGTACCCATCCAGCCGCTGCACATCACA  
ACATTTACTTAACAGAACAGTCACCGACAACATGCTGTGTGCTGGAGACACTCGG  
25 AGCGGCGGGCCCCAGGCAAACCTTGACGACGCCTGCCAGGGCGATTGCGGAG  
GCCCCCTGGTGTGTCTGAACGATGGCCGCATGACTTTGGTGGGCATCATCAGCT  
GGGGCCTGGGCTGTGGACAGAAGGATGTCCCGGGTGTGTACACAAAGGTTACC  
AACTACCTAGACTGGATTCGTGACAACATGCGACCGTGA (SEQ ID NO 4)

13 Método de acuerdo con una de las reivindicaciones 5 a 12, caracterizado porque la secuencia de ADN de la K2S consta de la siguiente secuencia

30 TCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTACCGTGGCACG  
CACAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAATTCCATGATCCTGA  
TAGGCAAGGTTTACACAGCACAGAACCCAGTGCCCAGGCACTGGGCCTGGGCA  
AACATAATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGTGCCACGTGCTG  
35 AAGAACCGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGC  
GGCCTGAGACAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCTCTTCGCC  
GACATCGCCTCCCACCCCTGGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTCG

CCCGGAGAGCGGTTCTGTGCGGGGGCATACTCATCAGCTCCTGCTGGATTCTC  
TCTGCCGCCCCACTGCTTCCAGGAGAGGTTTCCGCCCCACCACCTGACGGTGATC  
TTGGGCAGAACATACCGGGTGGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGT  
CGAAAAATACATTGTCCATAAGGAATTCGATGATGACACTTACGACAATGACATTG  
5 CGCTGCTGCAGCTGAAATCGGATTTCGTCCCGCTGTGCCCAGGAGAGCAGCGTG  
GTCCGCACTGTGTGCCTTCCCCCGGCGGACCTGCAGCTGCCGGACTGGACGGA  
GTGTGAGCTCTCCGGCTACGGCAAGCATGAGGCCTTGTCTCCTTTCTATTGCGA  
GCGGCTGAAGGAGGCTCATGTCAGACTGTACCCATCCAGCCGCTGCACATCACA  
ACATTTACTTAACAGAACAGTCACCGACAACATGCTGTGTGCTGGAGACACTCGG  
10 AGCGGCGGGCCCCAGGCAAACCTTGACGACGCCTGCCAGGGCGATTTCGGGAG  
GCCCCCTGGTGTGTCTGAACGATGGCCGCATGACTTTGGTGGGCATCATCAGCT  
GGGGCCTGGGCTGTGGACAGAAGGATGTCCCGGGTGTGTACACAAAGGTTACC  
AACTACCTAGACTGGATTCGTGACAACATGCGACCGTGA (SEQ ID NO 4)

14 Molécula de ADN caracterizada porque codifica

- 15 a) la proteína OmpA o un derivado funcional de ésta, unida/o operativamente a  
b) una molécula de ADN que codifica un polipéptido que contiene el dominio de Kringle  
2 y el dominio de proteasa de serina de la proteína de un activador de plasminogeno  
tisular

15 Molécula de ADN de acuerdo con la reivindicación 14, caracterizada

- 20 porque dicha secuencia de ADN comprende la siguiente secuencia o una variante  
funcional de ésta o una variante debida al código degenerado de nucleótidos

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTGG  
CCCAGGCGGCCTCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTA  
CCGTGGCACGCACAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAAATTCC  
25 ATGATCCTGATAGGCAAGGTTTACACAGCACAGAACCCAGTGCCCAGGCACTGGG  
CCTGGGCAAACATAATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGTGCC  
ACGTGCTGAAGAACCGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCT  
CCACCTGCGGCCTGAGACAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGC  
TCTTCGCCGACATCGCCTCCACCCCTGGCAGGCTGCCATCTTTGCCAAGCACA  
30 GGAGGTCGCCCCGGAGAGCGGTTCTGTGCGGGGGCATACTCATCAGCTCCTGC  
TGGATTCTCTCTGCCGCCCCACTGCTTCCAGGAGAGGTTTCCGCCCCACCACCTG  
ACGGTGATCTTGGGCAGAACATACCGGGTGGTCCCTGGCGAGGAGGAGCAGAA  
ATTTGAAGTCGAAAAATACATTGTCCATAAGGAATTCGATGATGACACTTACGACA  
ATGACATTGCGCTGCTGCAGCTGAAATCGGATTTCGTCCCGCTGTGCCCAGGAGA  
35 GCAGCGTGGTCCGCACTGTGTGCCTTCCCCCGGCGGACCTGCAGCTGCCGGAC  
TGGACGGAGTGTGAGCTCTCCGGCTACGGCAAGCATGAGGCCTTGTCTCCTTTC  
TATTCGGAGCGGCTGAAGGAGGCTCATGTCAGACTGTACCCATCCAGCCGCTGC

ACATCACAACATTTACTTTAACAGAACAGTCACCGACAACATGCTGTGTGCTGGAG  
ACACTCGGAGCGGCGGGCCCCAGGCAAACCTTGCACGACGCCTGCCAGGGCGAT  
TCGGGAGGGCCCCCTGGTGTGTCTGAACGATGGCCGCATGACTTTGGTGGGCAT  
CATCAGCTGGGGCCTGGGCTGTGGACAGAAGGATGTCCCGGGTGTGTACACAA  
5 AGGTTACCAACTACCTAGACTGGATTCGTGACAACATGCGACCG (SEQ ID NO 5)

16 Molécula de ADN de acuerdo con la reivindicación 14 ó 15, caracterizada  
porque dicha secuencia de ADN consta de la siguiente secuencia  
ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTGG  
CCCAGGCGGCCTCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTGAGCCTA  
10 CCGTGGCACGCACAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAATTCC  
ATGATCCTGATAGGCAAGGTTTACACAGCACAGAACCCCAGTGCCCAGGCACTGGG  
CCTGGGCAAACATAATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGTGCC  
ACGTGCTGAAGAACCGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCT  
CCACCTGCGGCCTGAGACAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGC  
15 TCTTCGCCGACATCGCCTCCCACCCCTGGCAGGCTGCCATCTTTGCCAAGCACA  
GGAGGTCGCCCCGAGAGCGGTTCTGTGCGGGGGCATACTCATCAGCTCCTGC  
TGGATTCTCTCTGCCGCCCACTGCTTCCAGGAGAGGTTTCCGCCCCACCACCTG  
ACGGTGATCTTGGGCAGAACATACCGGGTGGTCCCTGGCGAGGAGGAGCAGAA  
ATTTGAAGTCGAAAAATACATTGTCCATAAGGAATTCGATGATGACACTTACGACA  
20 ATGACATTGCGCTGCTGCAGCTGAAATCGGATTCGTCCCGCTGTGCCCAGGAGA  
GCAGCGTGGTCCGCACTGTGTGCCTTCCCCCGGCGGACCTGCAGCTGCCGGAC  
TGGACGGAGTGTGAGCTCTCCGGCTACGGCAAGCATGAGGCCTTGTCTCCTTTC  
TATTCGGAGCGGCTGAAGGAGGCTCATGTGAGACTGTACCCATCCAGCCGCTGC  
ACATCACAACATTTACTTTAACAGAACAGTCACCGACAACATGCTGTGTGCTGGAG  
25 ACACTCGGAGCGGCGGGCCCCAGGCAAACCTTGCACGACGCCTGCCAGGGCGAT  
TCGGGAGGGCCCCCTGGTGTGTCTGAACGATGGCCGCATGACTTTGGTGGGCAT  
CATCAGCTGGGGCCTGGGCTGTGGACAGAAGGATGTCCCGGGTGTGTACACAA  
AGGTTACCAACTACCTAGACTGGATTCGTGACAACATGCGACCG (SEQ ID NO 5)

17 Molécula de ADN de acuerdo con una de las reivindicaciones 14 a 16,  
30 caracterizada porque dicha secuencia de ADN b) codifica por lo menos un 90% de los  
aminoácidos 87 - 527 de la proteína de un activador de plasminógeno tisular humano.

18 Molécula de ADN de acuerdo con una de las reivindicaciones 14 a 17,  
caracterizada porque dicha secuencia de ADN b) codifica por lo menos un 90% de los  
aminoácidos 174 - 527 de la proteína de un activador de plasminógeno tisular humano

35 19 Molécula de ADN de acuerdo con una de las reivindicaciones 14 a 18,  
caracterizada porque dicha secuencia de ADN b) codifica por lo menos un 90% de los  
aminoácidos 180 - 527 de la proteína de un activador de plasminógeno tisular humano

20 Molécula de ADN de acuerdo con una de las reivindicaciones 14 a 19, caracterizada porque dicha secuencia de ADN b) codifica por lo menos un 90% de los aminoácidos 220 - 527 de la proteína de un activador de plasminógeno tisular humano

5 21 Molécula de ADN de acuerdo con una cualquiera de las reivindicaciones 14 a 20, caracterizada porque dicha secuencia de ADN

a) se hibnda en condiciones ngurosas con la siguiente secuencia

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTG  
GCCCAGGCGGCC (SEQ ID NO 6)

10 22 Molécula de ADN de acuerdo con una cualquiera de las reivindicaciones 14 a 21, caracterizada porque dicha secuencia de ADN a) consta de la siguiente secuencia

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTG  
GCCCAGGCGGCC (SEQ ID NO 6)

15 23 Molécula de ADN de acuerdo con una cualquiera de las reivindicaciones 14 a 22, caracterizada porque dicha secuencia de ADN b) se hibnda en condiciones ngurosas con la siguiente secuencia

20 TCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTACCGTGGCACG  
CACAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAATTCCATGATCCTG  
ATAGGCAAGGTTTACACAGCACAGAACCCAGTGCCCAGGCACTGGGCCTGGGC  
20 AACATAATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGTGCCACGTGCT  
GAAGAACCGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTG  
CGGCCTGAGACAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCTCTTCGCC  
GACATCGCCTCCCACCCCTGGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTGCG  
CCGGAGAGCGGTTCTGTGCGGGGGCATACTCATCAGCTCCTGCTGGATTCTCT  
25 CTGCCGCCCCACTGCTTCCAGGAGAGGTTTCCGCCCCACCACCTGACGGTGATCT  
TGGGCAGAACATACCGGGTGGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGTC  
GAAAAATACATTGTCCATAAGGAATTCGATGATGACACTTACGACAATGACATTGC  
GCTGCTGCAGCTGAAATCGGATTTCGTCCCGCTGTGCCCAGGAGAGCAGCGTGG  
TCCGCACTGTGTGCCTTCCCCCGGCGGACCTGCAGCTGCCGGACTGGACGGAG  
30 TGTGAGCTCTCCGGCTACGGCAAGCATGAGGCCTTGTCTCCTTTCTATTCCGAG  
CGGCTGAAGGAGGCTCATGTCAGACTGTACCCATCCAGCCGCTGCACATCACAA  
CATTTACTTAACAGAACAGTCACCGACAACATGCTGTGTGCTGGAGACACTCGGA  
GCGGCGGGCCCCAGGCAAACCTTGACGACGCCTGCCAGGGCGATTTCGGGAGG  
CCCCCTGGTGTGTCTGAACGATGGCCGCATGACTTTGGTGGGCATCATCAGCTG  
35 GGGCCTGGGCTGTGGACAGAAGGATGTCCCGGGTGTGTACACAAAGGTTACCA  
ACTACCTAGACTGGATTTCGTGACAACATGCGACCGTGA (SEQ ID NO 7)

24 Molécula de ADN de acuerdo con una cualquiera de las reivindicaciones

14 a 23, caracterizada porque dicha secuencia de ADN b) consta de la siguiente secuencia

TCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTACCGTGGCACG  
CACAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAATTCATGATCCTG  
5 ATAGGCAAGGTTTACACAGCACAGAACCCCAAGTGGCCAGGCACTGGGCCTGGGC  
AAACATAATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGTGCCACGTGCT  
GAAGAACCGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTG  
CGGCCTGAGACAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGGCTCTTCGCC  
GACATCGCCTCCCACCCCTGGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTGCG  
10 CCGGAGAGCGGTTTCCTGTGCGGGGGCATACTCATCAGCTCCTGCTGGATTCTCT  
CTGCCGCCCACTGCTTCCAGGAGAGGTTTCCGCCCCACCACCTGACGGTGATCT  
TGGGCAGAACATACCGGGTGGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGTC  
GAAAAATACATTGTCCATAAGGAATTCGATGATGACACTTACGACAATGACATTGC  
GCTGCTGCAGCTGAAATCGGATTCGTCCCGCTGTGCCCAGGAGAGCAGCGTGG  
15 TCCGCACTGTGTGCCTTCCCCCGGCGGACCTGCAGCTGCCGGACTGGACGGAG  
TGTGAGCTCTCCGGCTACGGCAAGCATGAGGCCTTGTCTCCTTTCTATTCCGAG  
CGGCTGAAGGAGGCTCATGTCAGACTGTACCCATCCAGCCGCTGCACATCACAA  
CATTTACTTAACAGAACAGTCACCGACAACATGCTGTGTGCTGGAGACACTCGGA  
GCGGCGGGCCCCAGGCAAACCTTGACGACGCCTGCCAGGGCGATTCTGGGAGG  
20 CCCCCTGGTGTGTCTGAACGATGGCCGCATGACTTTGGTGGGCATCATCAGCTG  
GGGCCTGGGCTGTGGACAGAAGGATGTCCCGGTGTGTACACAAAGGTTACCA  
ACTACCTAGACTGGATTCTGTGACAACATGCGACCGTGA (SEQ ID NO 7)

25 Proteína de fusión de OmpA y K2S, caracterizada porque comprende una proteína que se caracteriza por la siguiente secuencia de aminoácidos o un fragmento, una variante funcional, una variante alelica, una subunidad, un derivado químico o una variante por glicosilación de ésta

MKKTAIAIAVALAGFATVAQAASEGNSDCYFGNGSAYRGTHSLTESGASCLPWNSMI  
LIGKVYTAQNPSAQALGLGKHNYCRNPDGDAKPWCHVLKNRRLTWEYCDVPSCST  
CGLRQYSQPQFRIKGGLFADIASHPWQAAIFAKHRRSPGERFLCGGILISSCWILSAA  
30 HCFQERFPPHLLTVILGRTYRVVPGEEEQKFEVEKYIVHKEFDDDTYDNDIALQLKS  
DSSRCAQESSVVRTVCLPPADLQLPDWTECELSGYGKHEALSPFYSERLKEAHVRL  
YPSSRCTSQHLLNRTVTDNMLCAGDTRSGGPQANLHDACQGDSSGGLVCLNDGRM  
TLVGIIISWGLGCGQKDVPGVYTKVTNYLDWIRDNMTPG (SEQ ID NO 8)

26 Proteína de fusión de OmpA y K2S de acuerdo con la reivindicación 25, caracterizada porque consta de una proteína que se caracteriza por la siguiente secuencia de aminoácidos

MKKTAIAIAVALAGFATVAQAASEGNSDCYFGNGSAYRGTHSLTESGASCLPWNSMI

LIGKVYTAQNPSAQAALGLGKHNYCRNPDGDAKPWCHVLKNRRLTWEYCDVPSCST  
 CGLRQYSQPQFRIKGGLFADIASHPWQAAIFAKHRRSPGERFLCGGILISSCWILSAA  
 HCFQERFPPHHLTVILGRTRYRVVPGEEEQKFEVEKYIVHKEFDDDDTYDNDIALQLKS  
 DSSRCAQESSVVRTVCLPPADLQLPDWTECELSGYGKHEALSPFYSERLKEAHVRL  
 5 YPSSRCTSQHLLNRTVTDNMLCAGDTRSGGPQANLHDACQGDSGGPLVCLNDGRM  
 TLVGIIISWGLGCGQKDVPGVYTKVTNYLDWIRDNMIRPG (SEQ ID NO 8)

27 Proteína K2S, caracterizada porque comprende una proteína definida por la secuencia SEGN (SEQ ID NO 9) y/o una variante o un fragmento, una variante funcional, una variante alélica, una subunidad, un derivado químico, una proteína de fusión o una variante por glicosilación de ésta  
 10

28 Proteína K2S de acuerdo con la reivindicación 27, caracterizada porque comprende una proteína definida por la secuencia SEGNSD (SEQ ID NO 10) y/o una variante o un fragmento, una variante funcional, una variante alélica, una subunidad, un derivado químico, una proteína de fusión o una variante por glicosilación de esta

15 29 Proteína K2S de acuerdo con la reivindicación 28 ó 29, caracterizada porque comprende una proteína que se caracteriza por la siguiente secuencia de aminoácidos o un fragmento, una variante funcional, una variante alélica, una subunidad, un derivado químico o una variante por glicosilación de esta

SEGNSDCYFGNGSAYRGTHSLTESGASCLPWNSMILIGKVYTAQNPSAQAALGLGKH  
 20 NYCRNPDGDAKPWCHVLKNRRLTWEYCDVPSCSTCGLRQYSQPQFRIKGGLFADIA  
 SHPWQAAIFAKHRRSPGERFLCGGILISSCWILSAAHCFQERFPPHHLTVILGRTRYRV  
 VPGEEEQKFEVEKYIVHKEFDDDDTYDNDIALQLKSDSSRCAQESSVVRTVCLPPADL  
 QLPDWTECELSGYGKHEALSPFYSERLKEAHVRLYPSSRCTSQHLLNRTVTDNMLC  
 AGDTRSGGPQANLHDACQGDSGGPLVCLNDGRMTLVGIIISWGLGCGQKDVPGVYT  
 25 KVTNYLDWIRDNMIRP\* (SEQ ID NO 11)

30 K2S de acuerdo con una cualquiera de las reivindicaciones 27 a 30, caracterizada porque consta de una proteína que se caracteriza por la siguiente secuencia de aminoácidos

SEGNSDCYFGNGSAYRGTHSLTESGASCLPWNSMILIGKVYTAQNPSAQAALGLGKH  
 30 NYCRNPDGDAKPWCHVLKNRRLTWEYCDVPSCSTCGLRQYSQPQFRIKGGLFADIA  
 SHPWQAAIFAKHRRSPGERFLCGGILISSCWILSAAHCFQERFPPHHLTVILGRTRYRV  
 VPGEEEQKFEVEKYIVHKEFDDDDTYDNDIALQLKSDSSRCAQESSVVRTVCLPPADL  
 QLPDWTECELSGYGKHEALSPFYSERLKEAHVRLYPSSRCTSQHLLNRTVTDNMLC  
 AGDTRSGGPQANLHDACQGDSGGPLVCLNDGRMTLVGIIISWGLGCGQKDVPGVYT  
 35 KVTNYLDWIRDNMIRP\* (SEQ ID NO 11)

31 Un vector que contiene una secuencia de ADN de acuerdo con una cualquiera de las reivindicaciones 14 a 24

32 Un vector de acuerdo con la reivindicación 31, en el que dicha secuencia de ADN está precedida por un promotor de lac y un sitio de fijación a ribosomas

5 33 El vector pComb3HSS que contiene un ADN de acuerdo con una cualquiera de las reivindicaciones 14 a 24 en el que la expresión de la proteína gpIII está suprimida o inhibida por delección de la molécula de ADN que codifica dicha proteína gpIII o por un codón de detención situado entre el gen que codifica un polipéptido que contiene el dominio de Knirase 2 y el dominio de proteasa de serina de la proteína de un activador de plasminógeno tisular y el gen de la proteína III

10 34 Una célula hospedante procariótica que comprende una molécula de ADN de acuerdo con una cualquiera de las reivindicaciones 14 a 24

35 Una célula hospedante procariótica que comprende un vector de acuerdo con una cualquiera de las reivindicaciones 31 a 33

36 Una célula hospedante E. coli que comprende una molécula de ADN de acuerdo con una cualquiera de las reivindicaciones 14 a 24

15 37 Una célula hospedante E. coli que comprende un vector de acuerdo con una cualquiera de las reivindicaciones 31 a 33

20 38 Uso de una molécula de ADN de acuerdo con una cualquiera de las reivindicaciones 14 a 24 o de un vector de acuerdo con una cualquiera de las reivindicaciones 31 a 33 o de una célula hospedante de acuerdo con una cualquiera de las reivindicaciones 34 a 37, en un método para la producción de un polipéptido que tiene la actividad del activador de plasminógeno tisular

39 Uso de acuerdo con la reivindicación 38, en el que dicho método es un método de acuerdo con una cualquiera de las reivindicaciones 1 a 13

**RESUMEN**

El invento pertenece al sector de la trombolisis y de la produccion de derivados del activador de plasminógeno tisular (tPA) en celulas procarioticas El invento se refiere a métodos para la producción de un tPA que se deriva de un ADN recombinante, una variante de éste o una molecula de K2S (Kringle 2 Senna) o una variante de ésta en células procanóticas, en el que dicho tPA o dicha K2S o dicha variante se segrega extracelularmente como una proteina activa y correctamente plegada, y la celula procanótica contiene y expresa un vector que comprende el ADN que codifica dicho tPA o dicha K2S o dicha variante, unido operativamente con el ADN que codifica el peptido de señal OmpA. El invento se refiere además a derivados específicos de K2S obtenibles por dicho método El invento se refiere además a dichas moléculas de ADN y al uso de dichas moléculas de ADN en dichos métodos

1636 >

1018 >

Fig 1

1

2

3

5090 3

3054 3

1018 3

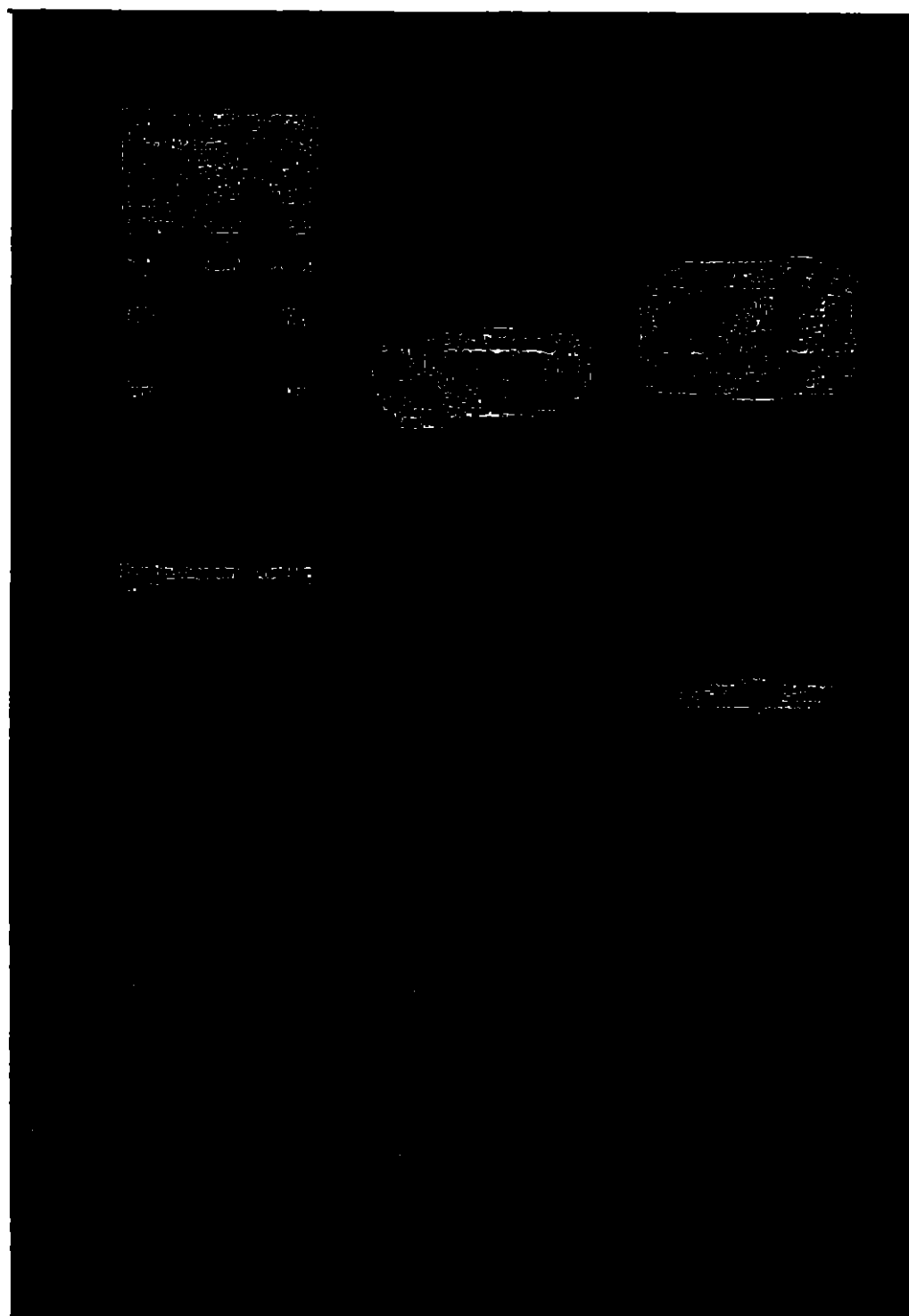
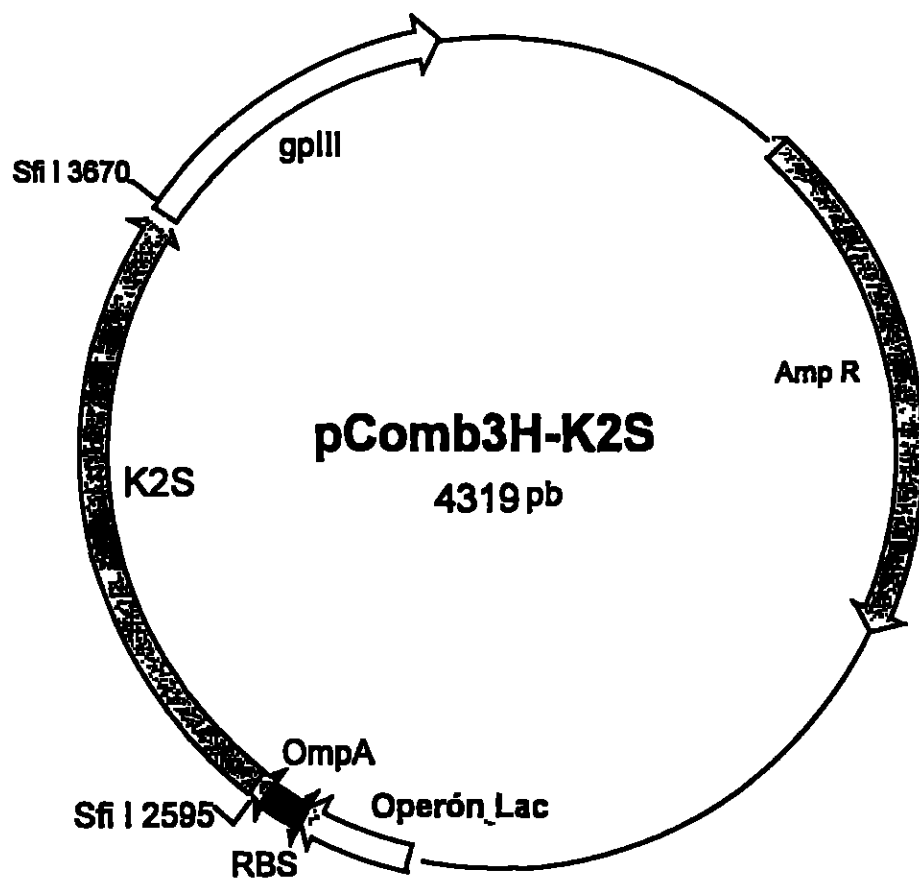


Fig 2



|            |           |
|------------|-----------|
| operón Lac | 2318-2513 |
| RBS        | 2514-2533 |
| Omp A      | 2534-2599 |
| K2S        | 2800-3684 |
| gp III     | 3685-4219 |
| Amp R      | 515- 1394 |

Figura 3

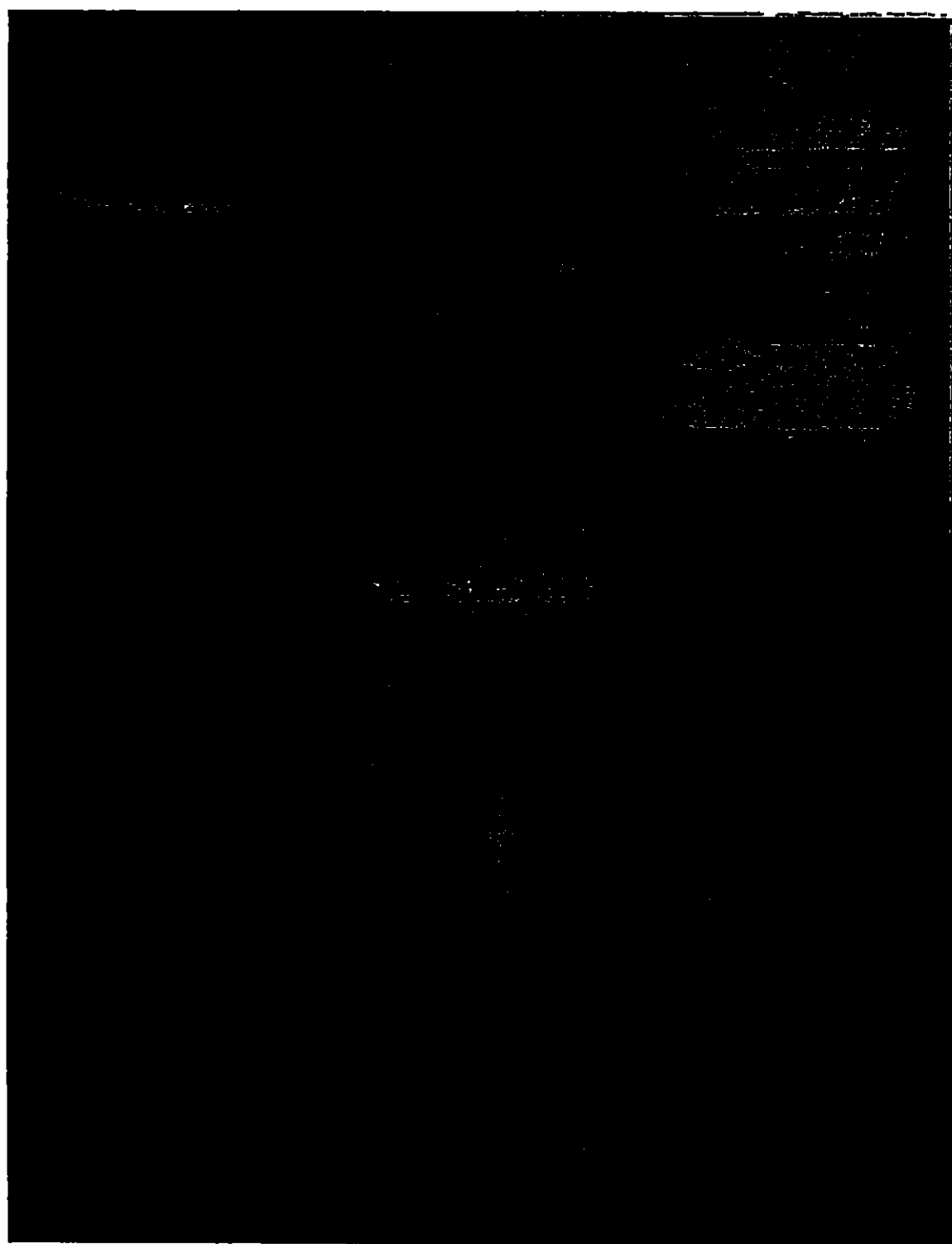


Figura 4

1

2

3



5090 &gt;

3054 &gt;

Fig 5

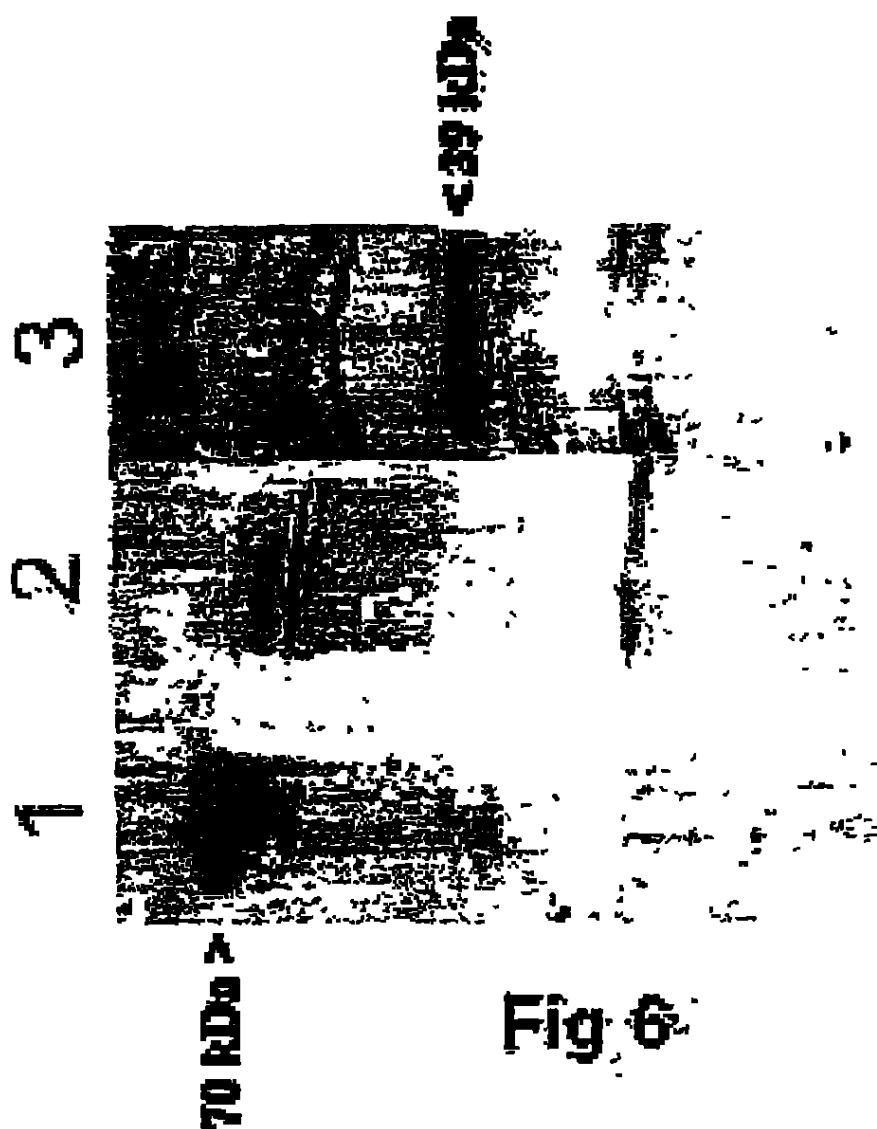


Fig 6

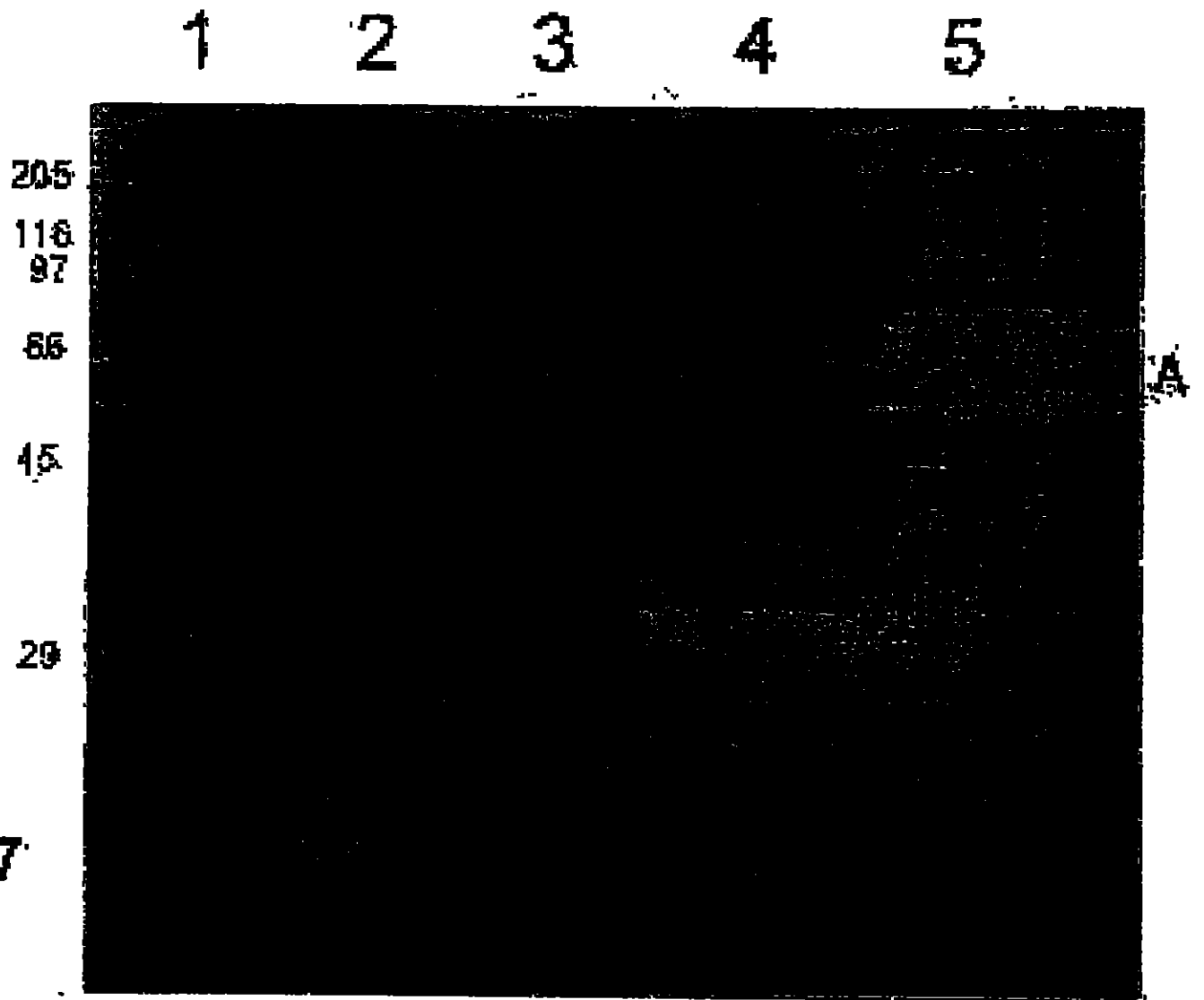


Fig 7

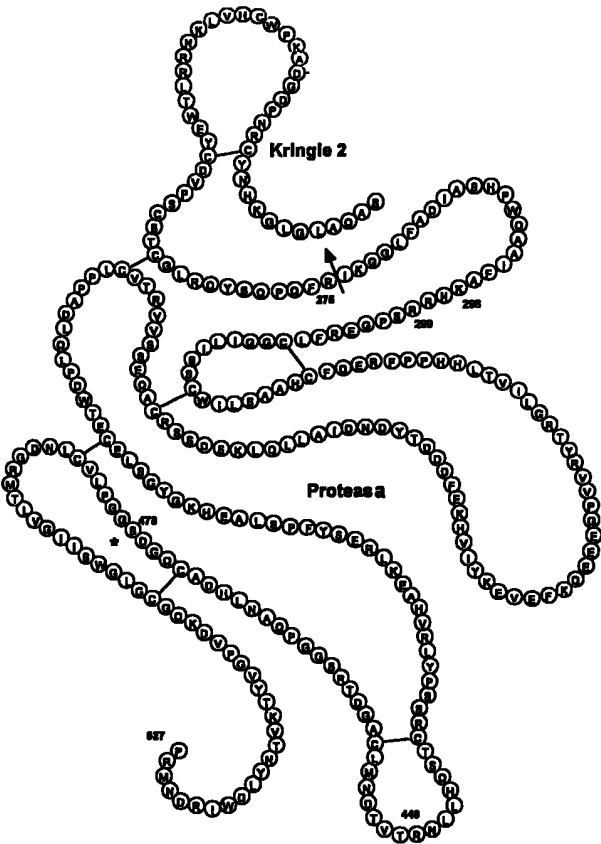


Fig 8

Estructura C

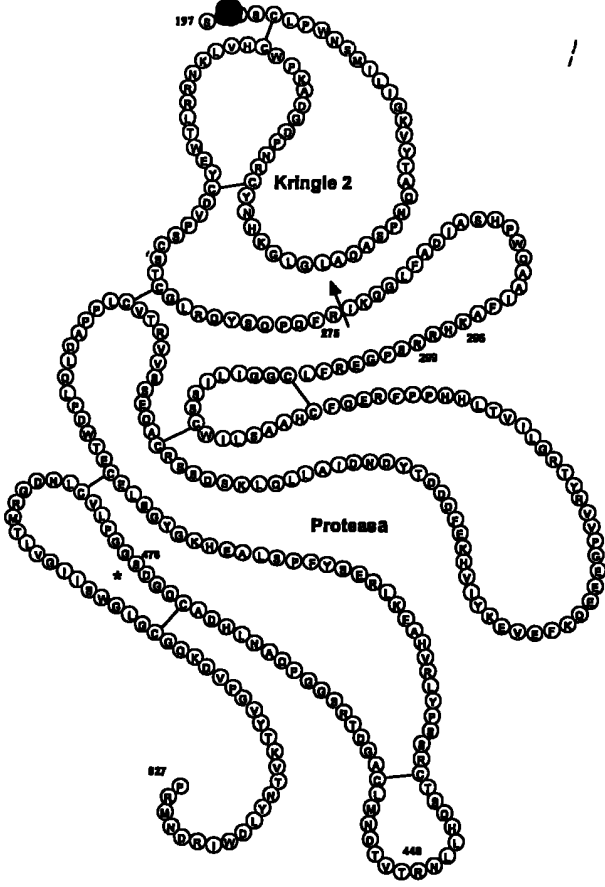


Fig. 9

Estructura B-0

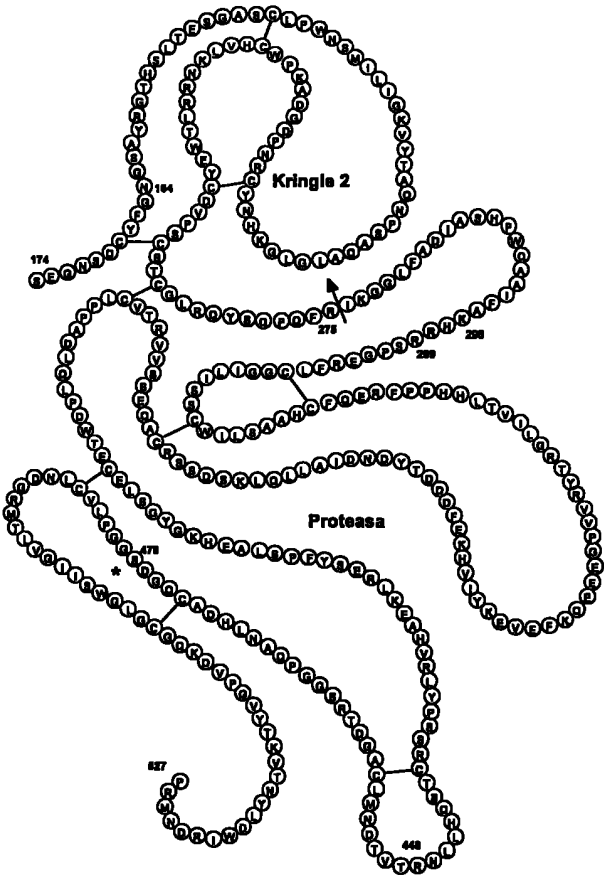


Fig. 10

Estructura A

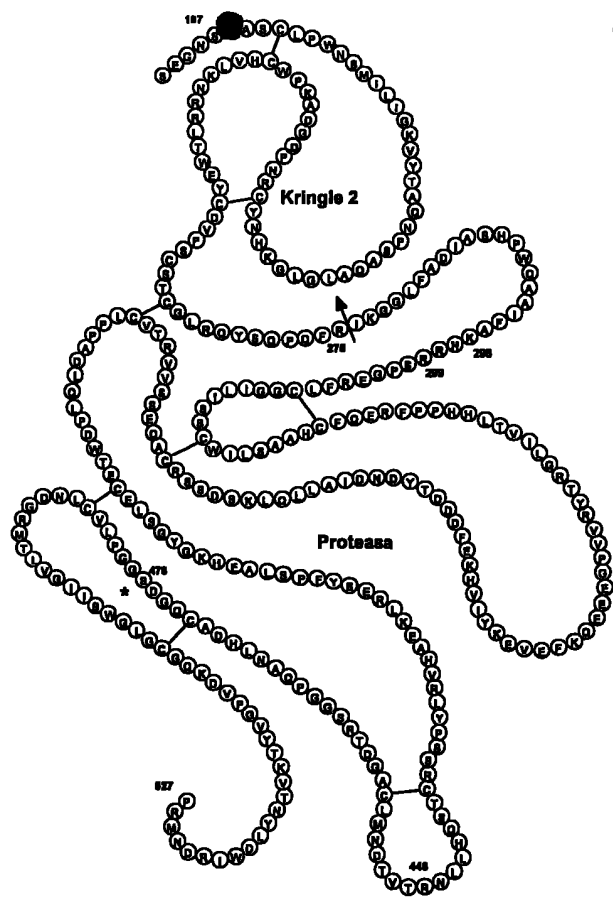


Fig. 11

Estructura B-1

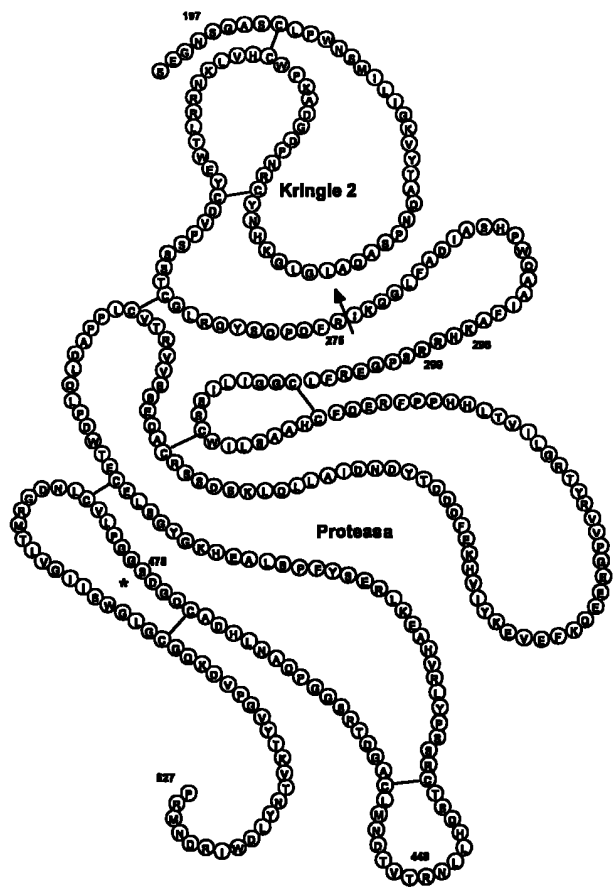


Fig. 12

Estructura B-2

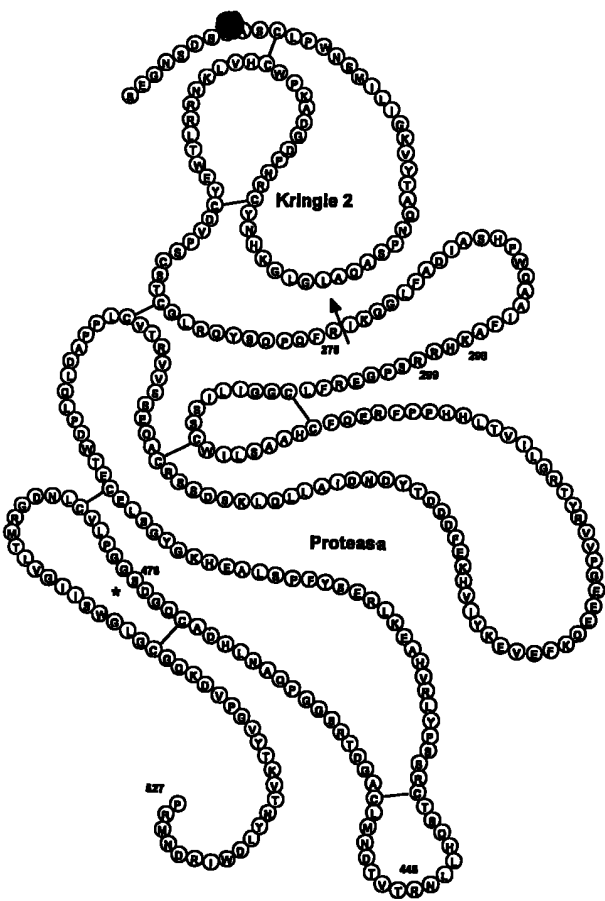


Fig. 13

Estructura B-3

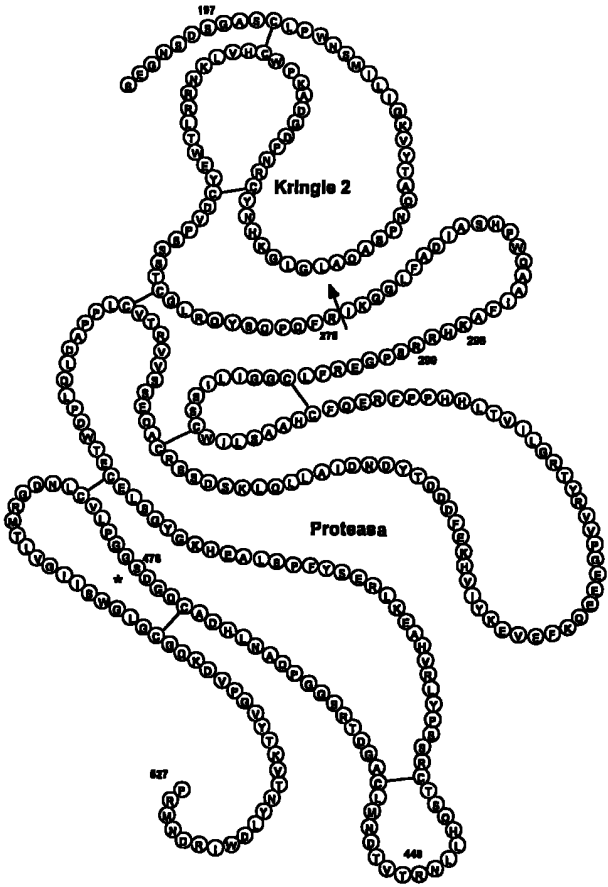


Fig. 14

Estructura B-4

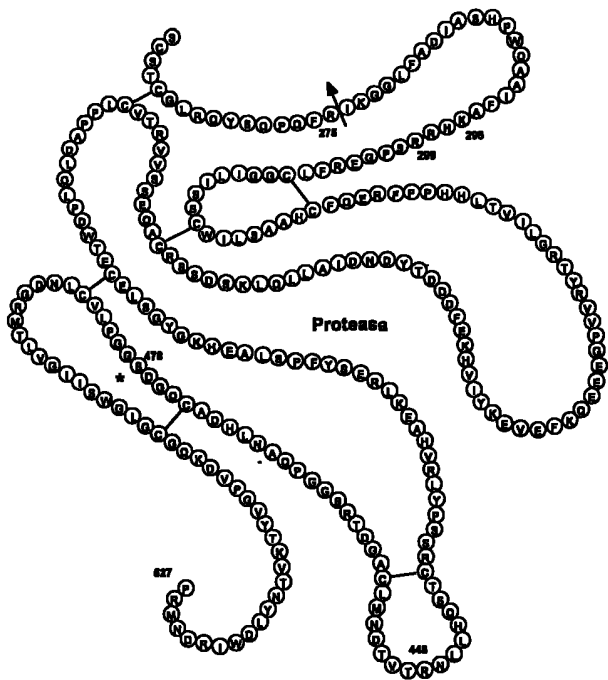
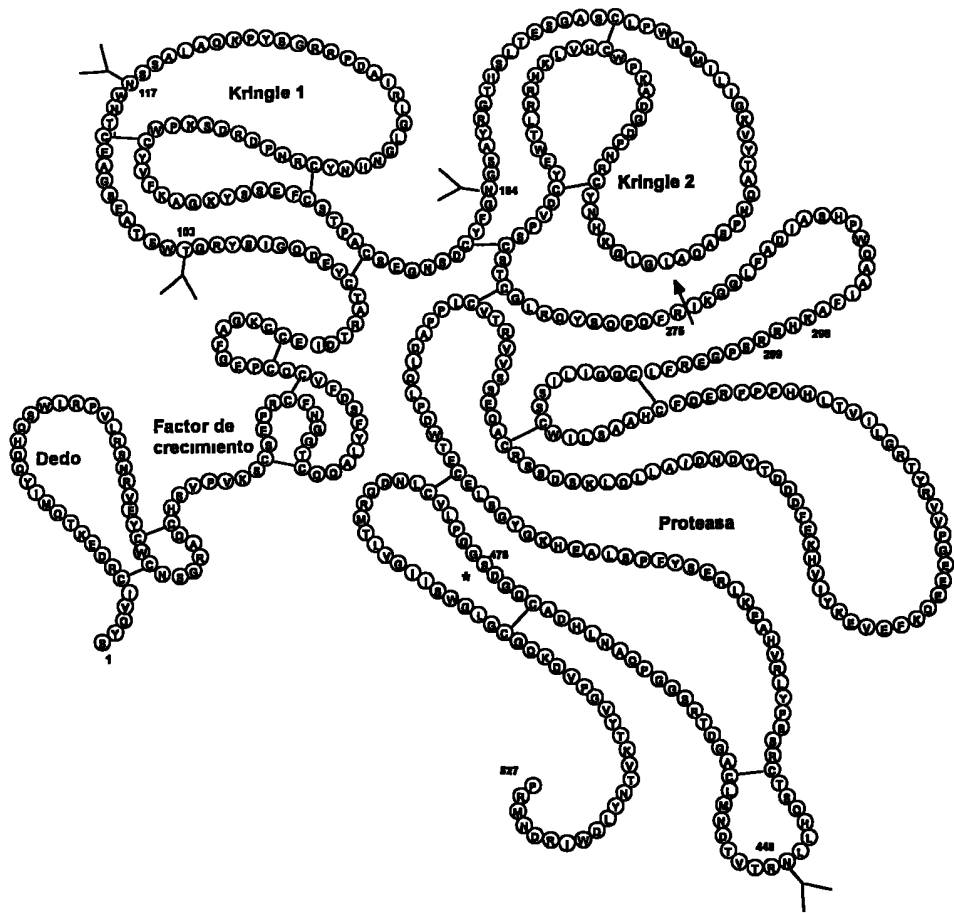


Fig. 16



**Reivindicaciones modificadas (13.11.2002)**

1       **Método para la producción de un activador de plasminógeno tisular (tPA) que se deriva de un un ADN recombinante, de una variante de tPA, de una molécula de Kningle 2-proteasa de serina (K2S) o de una variante de K2S en células procarióticas, en el que dicho tPA, dicha variante de tPA, dicha molécula de K2S o dicha variante de K2S se segrega extracelularmente como una proteína activa y correctamente plegada, caracterizado porque la célula procar-**  
5       **iótica contiene y expresa un vector que comprende el ADN que codifica dicho tPA, dicha variante de tPA, dicha molécula de K2S o dicha variante de K2S, unido operativamente con el ADN que codifica el péptido de señal OmpA**  
10

2       **Método de acuerdo con la reivindicación 1, caracterizado porque dicha célula procarionótica contiene y expresa un vector que comprende el ADN**  
15       **que codifica dicho tPA, dicha variante de tPA, dicha molécula de K2S o dicha variante de K2S, unido operativamente con el ADN que codifica el péptido de señal OmpA, que está unido operativamente con la molécula de ácido nucleico que es definida por la secuencia TCTGAGGGAAACAGTGAC (SEQ ID NO 1) o un derivado funcional de ésta**

20       **3       Método de acuerdo con la reivindicación 1 ó 2, caracterizado porque la célula procarionótica es E. coli**

4       **Método de acuerdo con una de las reivindicaciones 1 a 3, caracterizado porque se llevan a cabo las siguientes operaciones**

a) el ADN que codifica el tPA, la variante de tPA, la molécula de K2S o la variante de K2S se amplifica por una PCR,  
25

b) el producto de la PCR se purifica,

c) dicho producto de PCR se introduce en un vector que comprende el ADN que codifica el péptido de señal OmpA y el ADN que codifica la gpIII de una manera tal que dicho producto de PCR está unido operativamente secuencia  
30       **ambas con el ADN que codifica la secuencia de señal OmpA y está unido secuencia abajo con el ADN que codifica la gpIII de dicho vector,**

d) se introduce un codón de detención entre dicho tPA, dicha variante de tPA, dicha molécula de K2S o dicha variante de K2S y la gpIII,

e) dicho vector se expresa por la célula procariótica,

f) el tPA, la variante de tPA, la molécula de K2S o la variante de K2S se purifica

5           5       Método de acuerdo con una de las reivindicaciones 1 a 4, caracterizado porque el vector es un vector fagémido que comprende el ADN que codifica el péptido de señal OmpA y el ADN que codifica la gpIII

6           6       Método de acuerdo con una cualquiera de las reivindicaciones 1 a 5, caracterizado porque el vector es el fagémido pComb3HSS

7           7       Método de acuerdo con una cualquiera de las reivindicaciones 1 a 6, caracterizado porque la secuencia de ADN del OmpA unido secuencia ambia con la K2S comprende la siguiente secuencia o una debida al código degenerado de nucleótidos

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTAC  
CGTGGCCCAGGCGGCCTCTGAGGGAAACAGTGACTGCTACTTTGGGAAT  
GGGTCAGCCTACCGTGGCACGCACAGCCTCACCGAGTCGGGTGCCTCCT  
15 GCCTCCCGTGGAATTCCATGATCCTGATAGGCAAGGTTTACACAGCACAG  
AACCCAGTGCCCAGGCACTGGGCCTGGGCAAACATAATTACTGCCGGAA  
TCCTGATGGGGATGCCAAGCCCTGGTGCCACGTGCTGAAGAACCGCAGG  
CTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGCGGCCTGAG  
ACAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCTCTTCGCCGACA  
20 TCGCCTCCCACCCCTGGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTGCG  
CCCGGAGAGCGGTTCTGTGCGGGGGCATACTCATCAGCTCCTGCTGGA  
TTCTCTCTGCCGCCCACTGCTTCCAGGAGAGGTTTCCGCCCCACCACCTG  
ACGGTGATCTTGGGCAGAACATACCGGGTGGTCCCTGGCGAGGAGGAGC  
AGAAATTTGAAGTCGAAAAATACATTGTCCATAAGGAATTCGATGATGACA  
25 CTTACGACAATGACATTGCGCTGCTGCAGCTGAAATCGGATTGTCCTCCGC  
TGTGCCCAGGAGAGCAGCGTGGTCCGCACTGTGTGCCTTCCCCCGGCGG  
ACCTGCAGCTGCCGGACTGGACGGAGTGTGAGCTCTCCGGCTACGGCAA  
GCATGAGGCCTTGTCTCCTTTCTATTCCGAGCGGCTGAAGGAGGCTCATG  
TCAGACTGTACCCATCCAGCCGCTGCACATCACAACATTTACTTAACAGAA  
30 CAGTCACCGACAACATGCTGTGTGCTGGAGACACTCGGAGCGGCGGGGCC  
CCAGGCAAACCTTGACGACGCCTGCCAGGGCGATTCCGGGAGGCCCCCTG  
GTGTGTCTGAACGATGGCCGCATGACTTTGGTGGGCATCATCAGCTGGGG

CCTGGGCTGTGGACAGAAGGATGTCCCGGGTGTGTACACAAAGGTTACCA  
ACTACCTAGACTGGATTCTGTGACAACATGCGACCG (SEQ ID NO 2)

8 Método de acuerdo con una cualquiera de las reivindicaciones 1 a  
7, caracterizado porque la secuencia de ADN del OmpA comprende la siguien-  
5 te secuencia

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTAC  
CGTGGCCCAGGCGGCC (SEQ ID NO 3)

9 Método de acuerdo con una de las reivindicaciones 1 a 8, caracte-  
rizado porque la secuencia de ADN del OmpA consta de la siguiente secuen-  
10 cia

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTAC  
CGTGGCCCAGGCGGCC (SEQ ID NO 3)

10 Método de acuerdo con una de las reivindicaciones 1 a 9, caracte-  
rizado porque el ADN del tPA, de la variante de tPA, de la molécula de K2S o  
15 de la variante de K2S está precedido por un promotor lac y/o por un sitio de  
fijación a ribosomas

11 Método de acuerdo con una de las reivindicaciones 1 a 10, caracte-  
rizado porque el ADN que codifica el tPA, la variante de tPA, la molécula de K2S  
o la variante de K2S se selecciona entre el grupo de moléculas de ADN que  
20 codifican por lo menos un 90% de los aminoácidos 87 - 527, 174 - 527, 180 -  
527 ó 220 - 527 de la proteína de un activador de plasminógeno tisular huma-  
no

12 Método de acuerdo con una de las reivindicaciones 5 a 11, caracte-  
rizado porque la secuencia de ADN de la K2S comprende la siguiente se-  
25 cuencia o una variante funcional de ésta o una variante debida al código dege-  
nerado de nucleótidos

TCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTACCGTGG  
CACGCACAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAATTCCA  
TGATCCTGATAGGCAAGGTTTACACAGCACAGAACCCAGTGCCCAGGCA  
30 CTGGGCCTGGGCAAACATAATTACTGCCGGAATCCTGATGGGGATGCCAA  
GCCCTGGTGCCACGTGCTGAAGAACCGCAGGCTGACGTGGGAGTACTGT  
GATGTGCCCTCCTGCTCCACCTGCGGCCTGAGACAGTACAGCCAGCCTCA  
GTTTCGCATCAAAGGAGGGCTCTTCGCCGACATCGCCTCCACCCCTGGC  
AGGCTGCCATCTTTGCCAAGCACAGGAGGTGCCCCGAGAGCGGTTCT

GTGCGGGGGGCATACTCATCAGCTCCTGCTGGATTCTCTCTGCCGCCCCACT  
 GCTTCCAGGAGAGGTTTCCGCCCCACCACCTGACGGTGATCTTGGGCAGA  
 ACATACCGGGTGGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGTCGAAAA  
 ATACATTGTCCATAAGGAATTCGATGATGACACTTACGACAATGACATTGC  
 5 GCTGCTGCAGCTGAAATCGGATTCGTCCCGCTGTGCCCAGGAGAGCAGC  
 GTGGTCCGCACTGTGTGCCTTCCCCCGGCGGACCTGCAGCTGCCGGACT  
 GGACGGAGTGTGAGCTCTCCGGCTACGGCAAGCATGAGGCCTTGTCTCCT  
 TTCTATTCGGAGCGGCTGAAGGAGGCTCATGTCAGACTGTACCCATCCAG  
 CCGCTGCACATCACAACTTTACTTAACAGAACAGTCACCGACAACATGCT  
 10 GTGTGCTGGAGACACTCGGAGCGGCGGGCCCCAGGCAAACCTTGCACGAC  
 GCCTGCCAGGGCGATTCTGGGAGGCCCCCTGGTGTGTCTGAACGATGGCC  
 GCATGACTTTGGTGGGCATCATCAGCTGGGGCCTGGGCTGTGGACAGAA  
 GGATGTCCCGGGTGTGTACACAAAGGTTACCAACTACCTAGACTGGATTC  
 GTGACAACATGCGACCGTGA (SEQ ID NO 4)

15           13    Método de acuerdo con una de las reivindicaciones 5 a 12, carac-  
 tenizado porque la secuencia de ADN de la K2S consta de la siguiente secuen-  
 cia

TCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTGAGCCTACCGTGG  
 CACGCACAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAATTCCA  
 20 TGATCCTGATAGGCAAGGTTTACACAGCACAGAACCCCAGTGCCCAGGCA  
 CTGGGCCTGGGCAAACATAATTACTGCCGGAATCCTGATGGGGATGCCAA  
 GCCCTGGTGCCACGTGCTGAAGAACCGCAGGCTGACGTGGGAGTACTGT  
 GATGTGCCCTCCTGCTCCACCTGCGGCCTGAGACAGTACAGCCAGCCTCA  
 GTTTCGCATCAAAGGAGGGCTCTTCGCCGACATCGCCTCCCACCCCTGGC  
 25 AGGCTGCCATCTTTGCCAAGCACAGGAGGTCGCCCCGGAGAGCGGTTCT  
 GTGCGGGGGGCATACTCATCAGCTCCTGCTGGATTCTCTCTGCCGCCCCACT  
 GCTTCCAGGAGAGGTTTCCGCCCCACCACCTGACGGTGATCTTGGGCAGA  
 ACATACCGGGTGGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGTCGAAAA  
 ATACATTGTCCATAAGGAATTCGATGATGACACTTACGACAATGACATTGC  
 30 GCTGCTGCAGCTGAAATCGGATTCGTCCCGCTGTGCCCAGGAGAGCAGC  
 GTGGTCCGCACTGTGTGCCTTCCCCCGGCGGACCTGCAGCTGCCGGACT  
 GGACGGAGTGTGAGCTCTCCGGCTACGGCAAGCATGAGGCCTTGTCTCCT  
 TTCTATTCGGAGCGGCTGAAGGAGGCTCATGTCAGACTGTACCCATCCAG  
 CCGCTGCACATCACAACTTTACTTAACAGAACAGTCACCGACAACATGCT  
 35 GTGTGCTGGAGACACTCGGAGCGGCGGGCCCCAGGCAAACCTTGCACGAC

GCCTGCCAGGGCGATTCTGGGAGGCCCCCTGGTGTGTCTGAACGATGGCC  
GCATGACTTTGGTGGGCATCATCAGCTGGGGCCTGGGCTGTGGACAGAA  
GGATGTCCCGGGTGTGTACACAAAGGTTACCAACTACCTAGACTGGATTC  
GTGACAACATGCGACCGTGA (SEQ ID NO 4)

5           14    Molécula de ADN caracterizada porque codifica

a) la proteína OmpA unida operativamente a

b) una molécula de ADN que codifica un polipéptido que contiene el dominio de Kringle 2 y el dominio de proteasa de senna de la proteína de un activador de plasminógeno tisular

10           15    Molécula de ADN de acuerdo con la reivindicación 14, caracterizada porque dicha secuencia de ADN comprende la siguiente secuencia o una variante funcional de ésta o una variante debida al código degenerado de nucleótidos

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTAC  
15 CGTGGCCCAGGCGGCCTCTGAGGGAAACAGTGACTGCTACTTTGGGAAT  
GGGTCAGCCTACCGTGGCAGGCACAGCCTCACCGAGTCGGGTGCCTCCT  
GCCTCCCGTGGAATTCCATGATCCTGATAGGCAAGGTTTACACAGCACAG  
AACCCAGTGCCCAGGCACTGGGCCTGGGCAAACATAATTACTGCCGGAA  
TCCTGATGGGGATGCCAAGCCCTGGTGCCACGTGCTGAAGAACCGCAGG  
20 CTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGCGGCCTGAG  
ACAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCTCTTCGCCGACA  
TCGCCTCCCACCCCTGGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTGCG  
CCCGGAGAGCGGTTCTGTGCGGGGGCATACTCATCAGCTCCTGCTGGA  
TTCTCTCTGCCGCCCACTGCTTCCAGGAGAGGTTTCCGCCCCACCACCTG  
25 ACGGTGATCTTGGGCAGAACATACCGGGTGGTCCCTGGCGAGGAGGAGC  
AGAAATTTGAAGTCGAAAAATACATTGTCCATAAGGAATTCGATGATGACA  
CTTACGACAATGACATTGCGCTGCTGCAGCTGAAATCGGATTTCGTCCTCGC  
TGTGCCCAGGAGAGCAGCGTGGTCCGCACTGTGTGCCTTCCCCGGCGG  
ACCTGCAGCTGCCGGACTGGACGGAGTGTGAGCTCTCCGGCTACGGCAA  
30 GCATGAGGCCTTGTCTCCTTTCTATTCCGAGCGGCTGAAGGAGGCTCATG  
TCAGACTGTACCCATCCAGCCGCTGCACATCACAACATTTACTTAACAGAA  
CAGTCACCGACAACATGCTGTGTGCTGGAGACACTCGGAGCGGCGGGCC  
CCAGGCAAACCTTGACGACGCCTGCCAGGGCGATTCTGGGAGGCCCCCTG  
GTGTGTCTGAACGATGGCCGCATGACTTTGGTGGGCATCATCAGCTGGGG

CCTGGGCTGTGGACAGAAGGATGTCCCGGGTGTGTACACAAAGGTTACCA  
ACTACCTAGACTGGATTCGTGACAACATGCGACCG (SEQ ID NO 5)

16 Molécula de ADN de acuerdo con la reivindicación 14 ó 15, caracte-  
nizada porque dicha secuencia de ADN consta de la siguiente secuencia

5 ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTAC  
CGTGGCCCAGGCGGCCTCTGAGGGAAACAGTGA CTGCTACTTTGGGAAT  
GGGTCAGCCTACCGTGGCACGCACAGCCTCACCGAGTCGGGTGCCTCCT  
GCCTCCCGTGGAATTCCATGATCCTGATAGGCAAGGTTTACACAGCACAG  
AACCCCAGTGCCCAGGCACTGGGCCTGGGCAAACATAATTACTGCCGGAA  
10 TCCTGATGGGGATGCCAAGCCCTGGTGCCACGTGCTGAAGAACCGCAGG  
CTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGCGGCCTGAG  
ACAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCTCTTCGCCGACA  
TCGCCTCCCACCCCTGGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTGC  
CCCGGAGAGCGGTTCTGTGCGGGGGCATACTCATCAGCTCCTGCTGGA  
15 TTCTCTCTGCCGCCCCTGCTTCCAGGAGAGGTTTCCGCCCCACCACCTG  
ACGGTGATCTTGGGCAGAACATACCGGGTGGTCCCTGGCGAGGAGGAGC  
AGAAATTTGAAGTCGAAAAATACATTGTCCATAAGGAATTCGATGATGACA  
CTTACGACAATGACATTGCGCTGCTGCAGCTGAAATCGGATTTCGTCCCGC  
TGTGCCCAGGAGAGCAGCGTGGTCCGCACTGTGTGCCTTCCCCCGGCGG  
20 ACCTGCAGCTGCCGGACTGGACGGAGTGTGAGCTCTCCGGCTACGGCAA  
GCATGAGGCCTTGTCTCCTTTCTATTCCGAGCGGCTGAAGGAGGCTCATG  
TCAGACTGTACCCATCCAGCCGCTGCACATCACAACATTTACTTAACAGAA  
CAGTCACCGACAACATGCTGTGTGCTGGAGACACTCGGAGCGGCGGGCC  
CCAGGCAAACCTTGACACGACGCCTGCCAGGGCGATTTCGGGAGGCCCCCTG  
25 GTGTGTCTGAACGATGGCCGCATGACTTTGGTGGGCATCATCAGCTGGGG  
CCTGGGCTGTGGACAGAAGGATGTCCCGGGTGTGTACACAAAGGTTACCA  
ACTACCTAGACTGGATTCGTGACAACATGCGACCG (SEQ ID NO 5)

17 Molécula de ADN de acuerdo con una de las reivindicaciones 14 a  
16, caracterizada porque dicha secuencia de ADN b) codifica por lo menos un  
30 90% de los aminoácidos 87 - 527 de la proteína de un activador de plasminó-  
geno tisular humano

18 Molécula de ADN de acuerdo con una de las reivindicaciones 14 a  
17, caracterizada porque dicha secuencia de ADN b) codifica por lo menos un  
90% de los aminoácidos 174 - 527 de la proteína de un activador de plasminó-  
35 geno tisular humano

19 Molécula de ADN de acuerdo con una cualquiera de las reivindicaciones 14 a 18, caracterizada porque dicha secuencia de ADN b) codifica por lo menos un 90% de los aminoácidos 180 - 527 de la proteína de un activador de plasminógeno tisular humano

5 20 Molécula de ADN de acuerdo con una cualquiera de las reivindicaciones 14 a 19, caracterizada porque dicha secuencia de ADN b) codifica por lo menos un 90% de los aminoácidos 220 - 527 de la proteína de un activador de plasminógeno tisular humano

10 21 Molécula de ADN de acuerdo con una cualquiera de las reivindicaciones 14 a 20, caracterizada porque dicha secuencia de ADN b) se hibrida en condiciones rigurosas con la siguiente secuencia

TCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTACCGTGG  
CACGCACAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAATTCCA  
TGATCCTGATAGGCAAGGTTTACACAGCACAGAACCCCAGTGCCCAGGCA  
15 CTGGGCCTGGGCAAACATAATTACTGCCGGAATCCTGATGGGGATGCCAA  
GCCCTGGTGCCACGTGCTGAAGAACCGCAGGCTGACGTGGGAGTACTGT  
GATGTGCCCTCCTGCTCCACCTGCGGCCTGAGACAGTACAGCCAGCCTCA  
GTTTCGCATCAAAGGAGGGCTCTTCGCCGACATCGCCTCCCACCCCTGGC  
AGGCTGCCATCTTTGCCAAGCACAGGAGGTGCGCCGGAGAGCGGTTCTCT  
20 GTGCGGGGGCATACTCATCAGCTCCTGCTGGATTCTCTCTGCCGCCCACT  
GCTTCCAGGAGAGGTTTCCGCCCCACCACCTGACGGTGATCTTGGGCAGA  
ACATACCGGGTGGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGTCGAAAA  
ATACATTGTCCATAAGGAATTCGATGATGACACTTACGACAATGACATTGC  
GCTGCTGCAGCTGAAATCGGATTCGTCCCGCTGTGCCCAGGAGAGCAGC  
25 GTGGTCCGCACTGTGTGCCTTCCCCCGGCGGACCTGCAGCTGCCGGACT  
GGACGGAGTGTGAGCTCTCCGGCTACGGCAAGCATGAGGCCTTGTCTCCT  
TTCTATTCGGAGCGGCTGAAGGAGGCTCATGTGAGACTGTACCCATCCAG  
CCGCTGCACATCACAACATTTACTTAACAGAACAGTCACCGACAACATGCT  
GTGTGCTGGAGACACTCGGAGCGGGCGGGCCCCAGGCAAACTTGCACGAC  
30 GCCTGCCAGGGCGATTCGGGAGGCCCCCTGGTGTGTCTGAACGATGGCC  
GCATGACTTTGGTGGGCATCATCAGCTGGGGCCTGGGCTGTGGACAGAA  
GGATGTCCCGGGTGTGTACACAAAGGTTACCAACTACCTAGACTGGATTG  
GTGACAACATGCGACCGTGA (SEQ ID NO 7)

22 Molécula de ADN de acuerdo con una cualquiera de las reivindicaciones 14 a 21, caracterizada porque dicha secuencia de ADN b) consta de la siguiente secuencia

TCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTACCGTGG  
 5 CACGCACAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAATTCCA  
 TGATCCTGATAGGCAAGGTTTACACAGCACAGAACCCAGTGCCCAGGCA  
 CTGGGCCTGGGCAAACATAATTACTGCCGGAATCCTGATGGGGATGCCAA  
 GCCCTGGTGCCACGTGCTGAAGAACCGCAGGCTGACGTGGGAGTACTGT  
 GATGTGCCCTCCTGCTCCACCTGCGGCCTGAGACAGTACAGCCAGCCTCA  
 10 GTTTCGCATCAAAGGAGGGCTCTTCGCCGACATCGCCTCCCACCCCTGGC  
 AGGCTGCCATCTTTGCCAAGCACAGGAGGTGCCCCGGAGAGCGGTTCT  
 GTGCGGGGGCATACTCATCAGCTCCTGCTGGATTCTCTCTGCCGCCCCACT  
 GCTTCCAGGAGAGGTTTCCGCCCCACCACCTGACGGTGATCTTGGGCAGA  
 ACATACCGGGTGGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGTCGAAAA  
 15 ATACATTGTCCATAAGGAATTCGATGATGACACTTACGACAATGACATTGC  
 GCTGCTGCAGCTGAAATCGGATTTCGTCCCGCTGTGCCCAGGAGAGCAGC  
 GTGGTCCGCACTGTGTGCCTTCCCCCGGCGGACCTGCAGCTGCCGGACT  
 GGACGGAGTGTGAGCTCTCCGGCTACGGCAAGCATGAGGCCTTGTCTCCT  
 TTCTATTCCGAGCGGCTGAAGGAGGCTCATGTCAGACTGTACCCATCCAG  
 20 CCGCTGCACATCACAACATTTACTTAACAGAACAGTCACCGACAACATGCT  
 GTGTGCTGGAGACACTCGGAGCGGCGGGCCCCAGGCAAATTGCACGAC  
 GCCTGCCAGGGCGATTCCGGAGGCCCCCTGGTGTGTCTGAACGATGGCC  
 GCATGACTTTGGTGGGCATCATCAGCTGGGGCCTGGGCTGTGGACAGAA  
 GGATGTCCCGGGTGTGTACACAAAGGTTACCAACTACCTAGACTGGATTC  
 25 GTGACAACATGCGACCGTGA (SEQ ID NO 7)

23 Proteína de fusión de OmpA y K2S, caracterizada porque comprende una proteína que se caracteriza por la siguiente secuencia de aminoácidos

MKKTAIAIAVALAGFATVAQAASEGNSDCYFGNGSAYRGTHSLTESGASCLP  
 30 WNSMILIGKVYTAQNPSAQUALGLGKHNYCRNPDGDAKPWCHVLKNRRLTWE  
 YCDVPSCSTCGLRQYSQPQFRIKGGLFADIASHPWQAAIFAKHRRSPGERFL  
 CGGILISSCWILSAAHCFQERFPPHHLTVILGRTYRVVPGEESQKFEVEKYIVH  
 KEFDDDTYDNDIALQLKSDSSRCAQESSVVRTVCLPPADLQLPDWTECELS  
 GYGKHEALSPFYSERLKEAHVRLYPSSRCTSQHLLNRTVTDNMLCAGDTRSG

GPQANLHDACQGDSGGPLVCLNDGRMTLVGIISWGLGCGQKDVP-  
GYTKVTNYLDWIRDNMIRPG (SEQ ID NO 8)

24 Proteína de fusión de OmpA y K2S de acuerdo con la reivindicación 23, caracterizada porque consta de una proteína que se caracteriza por la siguiente secuencia de aminoácidos

5 MKKTAIAIAVALAGFATVAQAASEGNSDCYFGNGSAYRGTHSLTESGASCLP  
WNSMILIGKVYTAQNPSAQALGLGKHNYCRNPDGDAKPWCHVLKNRRLTWE  
YCDVPSCSTCGLRQYSQPQFRIKGGLFADIASHPWQAAIFAKHRRSPGERFL  
CGGILISSCWILSAAHCFQERFPPHLLTVILGRTYRVVPGEESQKFEVEKYIVH  
10 KEFDDDDTYDNDIALQLKSDSSRCAQESSVVRTVCLPPADLQLPDWTECELS  
GYGKHEALSPFYSERLKEAHVRLYPSSRCTSQHLLNRTVTDNMLCAGDTRSG  
GPQANLHDACQGDSGGPLVCLNDGRMTLVGIISWGLGCGQKDVP-  
GYTKVTNYLDWIRDNMIRPG (SEQ ID NO 8)

25 Proteína K2S, caracterizada por la siguiente secuencia de aminoácidos

15 SEGNSDCYFGNGSAYRGTHSLTESGASCLPWNSMILIGKVYTAQNPSAQALG  
LGKHNYCRNPDGDAKPWCHVLKNRRLTWEYCDVPSCSTCGLRQYSQPQFRI  
KGGLFADIASHPWQAAIFAKHRRSPGERFLCGGILISSCWILSAAHCFQERFPP  
HLLTVILGRTYRVVPGEESQKFEVEKYIVHKEFDDDDTYDNDIALQLKSDSSRC  
20 AQESSVVRTVCLPPADLQLPDWTECELSGYGKHEALSPFYSERLKEAHVRLY  
PSSRCTSQHLLNRTVTDNMLCAGDTRSGGPQANLHDACQGDSGGPLVCLND  
GRMTLVGIISWGLGCGQKDVPGYTKVTNYLDWIRDNMIRP\* (SEQ ID NO 11)

26 Un vector que contiene una secuencia de ADN de acuerdo con una cualquiera de las reivindicaciones 14 a 22

25 27 Un vector de acuerdo con la reivindicación 26, en el que dicha secuencia de ADN está precedida por un promotor de lac y un sitio de fijación a ribosomas

28 El vector pComb3HSS que contiene un ADN de acuerdo con una cualquiera de las reivindicaciones 14 a 22, en el que la expresión de la proteína gpIII está suprimida o inhibida por delección de la molécula de ADN que codifica dicha proteína gpIII o por un codón de detención situado entre el gen que codifica un polipéptido que contiene el dominio de Kringle 2 y el dominio de proteasa de serina de la proteína de un activador de plasminógeno tisular y el gen de la proteína III

29 Una célula hospedante procariótica que comprende una molécula de ADN de acuerdo con una cualquiera de las reivindicaciones 14 a 22

30 Una célula hospedante procariótica que comprende un vector de acuerdo con una cualquiera de las reivindicaciones 26 a 28

5 31 Una célula hospedante E coli que comprende una molécula de ADN de acuerdo con una cualquiera de las reivindicaciones 14 a 22

32 Una célula hospedante E coli que comprende un vector de acuerdo con una cualquiera de las reivindicaciones 26 a 28

10 33 Uso de una molécula de ADN de acuerdo con una cualquiera de las reivindicaciones 14 a 22 o de un vector de acuerdo con una cualquiera de las reivindicaciones 26 a 28 o de una célula hospedante de acuerdo con una cualquiera de las reivindicaciones 29 a 32, en un método para la producción de un polipéptido que tiene la actividad del activador de plasminógeno tisular.

15 34 Uso de acuerdo con la reivindicación 33, en el que dicho método es un método de acuerdo con una cualquiera de las reivindicaciones 1 a 13

SUPERINTENDENCIA Y COMERCIO  
Indicaciones : C3800171 C3800000 Folios: 140  
Fecha (UTC): 2003-05-07 16:52:34  
Licencia : 011 PAT-PALEN 379 FASE N201 411 FASEN202  
Dependencia: COM DIVISION DE NOMBRES COMERCIALES

NIT : 800176089-2

RECIBO OFICIAL DE CAJA : 03 - 27,656  
FECHA : ABRIL 22 DE 2003

\*\*\*\*\* CONSIGNACION \*\*\*\*\*

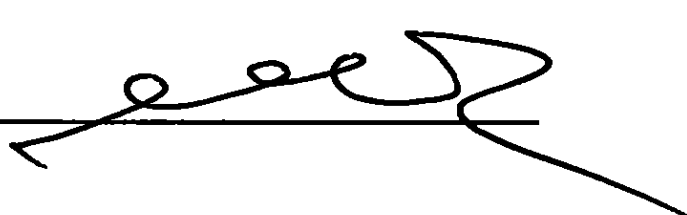
| DEPOSITANTE       | TIPO PAGO    | BANCO         | CUENTA      | No. PAGO | FECHA PAGO | Vr. PAGO      |
|-------------------|--------------|---------------|-------------|----------|------------|---------------|
| CAVELIER ABOGADOS | CONSIGNACION | BANCO POPULAR | 050-00110-6 | 9003691  | 22/04/2003 | 23,079,000.00 |

\*\*\*\*\* CONCEPTO \*\*\*\*\*

| CANT. CONTABILITICO       | CONCEPTO                            | TOTAL CONCEPTO |
|---------------------------|-------------------------------------|----------------|
| 1 50005-01-01 SOLICITUDES | 1 TRAMITES DE SOL. DE PATENTE DE IN | 400,000.00     |
|                           | TOTAL :                             | \$ 400,000.00  |

SON: CUATROCIENTOS MIL PESOS

RESPONSABLE :



RECIBO DE CAJA APLICADO AL EXPEDIENTE No. \_\_\_\_\_

Cdo 09255-841-004-6

REQUISITOS NECESARIOS PARA PRESENTACION Y AGILIZACION DEL TRAMITE

|                                                                                        | USUARIO | RADICADOR |
|----------------------------------------------------------------------------------------|---------|-----------|
| PATENTE DE INVENCION                                                                   | ( )     | ( )       |
| MODELO DE UTILIDAD                                                                     | ( )     | ( )       |
| - Indicación de que se solicita la concesión de una patente                            | ( )     | ( )       |
| - Datos de identificación del solicitante o de la persona que se presenta la solicitud | ( )     | ( )       |
| - Descripción de la invención.                                                         | ( )     | ( )       |
| - Dibujos de ser estos pertinentes                                                     | ( )     | ( )       |
| - Comprobante de Pago de las tasas establecidas                                        | ( )     | ( )       |

COMPLETA ( ) INCOMPLETA ( )

DISEÑO INDUSTRIAL ( )

|                                                                                                             |     |     |
|-------------------------------------------------------------------------------------------------------------|-----|-----|
| - Indicación de que se solicita el registro de Diseño Industrial                                            | ( ) | ( ) |
| - Datos de identificación del solicitante o de la persona que presenta la solicitud.                        | ( ) | ( ) |
| - Representación gráfica y fotográfica del Diseño Industrial o muestra del Material que incorpora el diseño | ( ) | ( ) |
| - Comprobante de pago de las tasas establecidas                                                             | ( ) | ( ) |

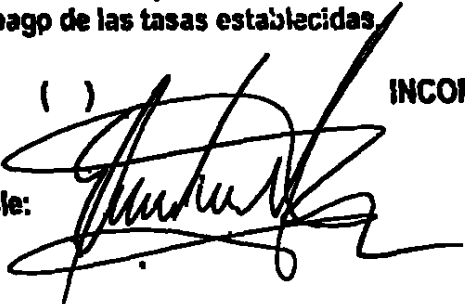
COMPLETA ( ) INCOMPLETA ( )

ESQUEMA DE TRAZADO ( )

|                                                                                      |     |     |
|--------------------------------------------------------------------------------------|-----|-----|
| - Indicación de que solicita el registro de un Esquema de trazado.                   | ( ) | ( ) |
| - Datos de identificación del solicitante o de la persona que presenta La solicitud. | ( ) | ( ) |
| - Representación gráfica del esquema de trazado                                      | ( ) | ( ) |
| - Comprobante de pago de las tasas establecidas                                      | ( ) | ( ) |

COMPLETA ( ) INCOMPLETA ( )

Firma Responsable:



Fecha: SUPERINDUSTRIA Y COMERCIO  
Calificación: 03030471 00000000 Folios: 140  
Fecha (INC): 2003-05-07 16:52:54  
Tramite : 011 PCT-PATENT 379 FASE NACI 411 PRESENTAC  
Dependencia: 2022 MINISTERIO DE NUEVAS COSEANCIAS



2020  
Bogotá, D.C.,

Folio 140

**Doctor:**  
Emilio Ferrero  
**Apoderado**  
Boehringer Ingelheim International GMBH

**Oficio N° 07278**

|                |                                   |                |
|----------------|-----------------------------------|----------------|
| <b>ASUNTO:</b> | <b>Radicación N°</b>              | 03-038171      |
|                | <b>Solicitud internacional</b>    | PCT/EP01/12857 |
|                | <b>Fecha inicio fase nacional</b> | 14/06/2003     |
|                | <b>Trámite</b>                    | 011            |
|                | <b>Evento</b>                     | 379            |
|                | <b>Actuación</b>                  | 430            |
|                | <b>Folios</b>                     | 001            |

Como resultado del examen de forma, realizado con fundamento en el artículo 38 de la Decisión 486 de la Comisión de la Comunidad Andina, artículo 27 del Tratado de Cooperación en Materia de Patentes (PCT), regla 51 bis del Reglamento y Circular Única de la Superintendencia de Industria y Comercio, se le comunica que:

- ☐ La solicitud no contiene la copia del documento en que consta la cesión del derecho a la patente del inventor al solicitante o a su causante. (Art. 26-k) Decisión 486).
- ☐ El solicitante debe aclarar si la solicitud corresponde al capítulo I o II del PCT por que la indicación en el petitorio no corresponde a los documentos del expediente, en caso necesario adjuntar los documentos que corresponden.

En cumplimiento de lo dispuesto por el artículo 39 de la Decisión 486, se le requiere para que dentro del término de dos meses siguientes a la fecha de notificación del presente oficio, complete los requisitos anteriormente enunciados. En caso de no dar cumplimiento al presente requerimiento, la solicitud se considerará abandonada y perderá su prelación.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única N° 10 de 2001.

**ALIX CARMENZA CESPEDES DE VERGEL**  
Jefe de la División de Nuevas Creaciones.

El anterior requerimiento fue notificado por fijación en lista de fecha, 15 SEI 2003