SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado
GERMAN CASTILLO GRAU

ASUNTO

Radicación	08-99914
Trámite	11
Evento	378
Actuación	519
Oficio	12749
Folios	10

Patente de Invención	☒			Patente de Modelo de Utilidad	☐
Vía Nacional	☐				
Vía PCT (Fase Nacional)	☒	Capítulo I	☒	Capítulo II	☐

No. Solicitud Internacional	PCT/GB2007/000814
Fecha de Presentación Internacional	9/Marzo/2007

Como resultado del Examen de Fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Folios 7 a 54	22/Septiembre/2008
Reivindicaciones:	Folios 55 a 61	22/Septiembre/2008
Dibujos:	Folios 62 a 102	22/Septiembre/2008
Prioridad:	GB 0604964.7	11/Marzo/2006

2. Análisis de la Prioridad

Se acepta la reivindicación de Prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486), y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

Título de la solicitud: "Plegamiento de proteína".

El problema planteado en esta solicitud consiste en la necesidad de suministrar un método para plegar o replegar un factor de crecimiento transformante beta.

Para resolver este problema técnico la solicitud en estudio proporciona un método para plegar un factor de crecimiento transformante beta que comprende agregar un factor de crecimiento monomérico no plegado solubilizado a una solución que contiene (i) ácido 2-(ciclohexilamino)-etansulfónico o un análogo funcional de este; y (ii) un sistema redox de sulfhidrilo/disulfuro de bajo peso molecular; e incubar el factor de crecimiento en la solución hasta que se forma el factor de crecimiento dimérico biológicamente activo.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): C07K 14/435.

4. De la Solicitud de Patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

La reivindicación 1 carece de concisión porque utiliza las expresiones "un factor de crecimiento transformante beta", "un análogo funcional", "un sistema redox de sulfidrilo/disulfuro", lo cual dificulta determinar la extensión del objeto que se busca proteger.

Para "un factor de crecimiento transformante beta" y "un análogo funcional", no se definen las características estructurales, como el tipo de factor de crecimiento beta o su secuencia de aminoácidos.

Para "un sistema redox de sulfidrilo/disulfuro", no se define el compuesto que da origen a las formas oxidadas y reducidas.

Para las reivindicaciones 4 – 7 existen múltiples posibilidades de sustitución a nivel del compuesto de fórmula I para los radicales especificados de acuerdo con los parámetros de sustitución señalados, lo cual dificulta determinar la extensión del objeto que se busca proteger. Se encuentran términos no concisos como "sustituidos o no sustituidos", "núcleo aromático", "sistema anular", porque:

Para los grupos "núcleo aromático" y "sistema anular" no se definen el número de átomos de carbono ni miembros que los conforman, tamaños de los anillos.

Para el término "sustituidos", no se definen los posibles sustituyentes que puede tener cada grupo.

La reivindicación 1 no es clara porque no se definen las condiciones de incubación del factor de crecimiento en la solución.

Las reivindicaciones 2, 3, 9, 11 y 15 no son claras porque utilizan el término "aproximadamente", el cual es impreciso y no permite distinguir la invención del estado de la técnica. Se sugiere sustituirlo por un término preciso o un rango concreto.

La reivindicación 25 no es clara porque si bien reclama un método, no se describen las condiciones de las etapas (B) de aislamiento y (D) de purificación. Así mismo, no se describe el tipo de organismo procariótico y la secuencia del vector de expresión del miembro de la familia del factor de crecimiento transformante beta.

La reivindicación 25 no es clara porque en la etapa (C) hace referencia a "(...) de acuerdo con el primer aspecto de la invención". Se requiere al solicitante hacer la aclaración respectiva.

La reivindicación 29 no es clara porque hace referencia a "la Figura 10". Se requiere al solicitante hacer la aclaración necesaria.

Se requiere al solicitante renumerar las reivindicaciones 28 y 29, porque se encuentran repetidas al final del capítulo reivindicatorio.

Se requiere al Solicitante hacer las aclaraciones necesarias.

5. Unidad de invención (Art 25 D 486)

La Solicitud no cumple con el Requisito de Unidad de Invención porque se refiere a 2 Grupos Inventivos.

Los Grupos Inventivos están definidos por las siguientes características **esenciales**:

1° Un método para plegar un factor de crecimiento transformante beta que comprende agregar un factor de crecimiento monomérico no plegado solubilizado a una solución que comprende CHES. Reivindicaciones 1 – 3 y 8 – 29, parcialmente.
2° Un método para plegar un factor de crecimiento transformante beta que comprende agregar un factor de crecimiento monomérico no plegado solubilizado a una solución que comprende análogos de CHES. Reivindicaciones 1, 4 – 15, 18 – 20 y 23 – 29, parcialmente.

No se guarda un concepto común inventivo porque métodos para plegar proteínas de la familia del factor de crecimiento transformante beta en presencia de un sistema redox de sulfhidrilo/disulfuro de bajo peso molecular y en la presencia de CHES o un análogo funcional ya había sido divulgado en los documentos D1 – D4, por lo que las características técnicas esenciales no se encuentran unidas por un concepto común inventivo.

Se sugiere presentar las Solicitudes Divisionales que considere necesarias, ó limitar la solicitud inicial a una sola invención, eliminando de ella las otras invenciones.

6. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: Marzo 11 de 2006, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	WO99/18196	Methods for refolding proteins by use of zwitterionic low molecular weight agents	15/Abril/1999
D2	WO9516034	Mutants of bone morphogenic proteins	15/Junio/1995
D3	Journal of Biotechnology, Vol. 94, Pág. 185-194.	Renaturation and purification of bone morphogenetic protein-2 produced as inclusion bodies in high-cell-density cultures of recombinant *Escherichia coli*	28/Marzo/2002
D4	EP0433225	Process for the production of biologically active protein (e.g. TGF)	19/Junio/1991
D5	EP0943690	Method for refolding human activin A	22/Septiembre/1999

D1 http://worldwide.espacenet.com/publicationDetails/biblio?CC=WO&NR=9918196&KC=&FT=E&locale=en_EP divulga Un método para plegar la proteína morfogénica de hueso en una forma dimérica biológicamente activa (Pág. 16, ejemplo 6).

D2 http://worldwide.espacenet.com/publicationDetails/biblio?CC=WO&NR=9516034&KC=&FT=E&locale=en_EP divulga un método para plegar la proteína morfogénica de hueso en una forma dimérica biológicamente activa (Pág. 18, ejemplo 5).

D3 http://www.sciencedirect.com/science/article/pii/S0168165601004254 divulga un método para plegar la proteína morfogénica de hueso (BMP-2) en una forma dimérica biológicamente activa (Resumen y Pág. 188, *Solubilization of inclusion bodies and in vitro refolding of rhBMP-2*).

D4 http://worldwide.espacenet.com/publicationDetails/biblio?CC=EP&NR=0433225&KC=&FT=E&locale=en_EP divulga un método para plegar miembros del factor de crecimiento transformante, como el TGF-β3 (Pág. 29, reivindicación 1 y Pág. 32, reivindicación 7).

D5 http://worldwide.espacenet.com/publicationDetails/biblio?CC=EP&NR=0943690&KC=&FT=E&locale=en_EP divulga

7. Examen de Patentabilidad (Art 14 D486)

Novedad (Art 16 D486)

La **reivindicación 1** consiste en un método para plegar un factor de crecimiento transformante beta, o un análogo funcional de este en una forma dimérica

biológicamente activa que comprende agregar un factor de crecimiento monomérico no plegado solubilizado a una solución que contiene:

(i) ácido 2-(ciclohexilamino)-etansulfónico o un análogo funcional de este; y

(ii) un sistema redox de sulfhidrilo/disulfuro de bajo peso molecular; e

incubar el factor de crecimiento en la solución hasta que se forma el factor de crecimiento dimérico biológicamente activo.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características **esenciales**	**D1**	**D2**	**D3**	**D4**
Un método para plegar un factor de crecimiento transformante beta, o un análogo funcional de este en una forma dimérica biológicamente activa que comprende agregar un factor de crecimiento monomérico no plegado solubilizado a una solución que contiene: (i) ácido 2-(ciclohexilamino)-etansulfónico o un análogo funcional de este; y	Un método para plegar la proteína morfogénica de hueso en una forma dimérica biológicamente activa que comprende agregar la proteína a una solución que contiene CHES; y (Pág. 16, ejemplo 6)	Un método para plegar la proteína morfogénica de hueso en una forma dimérica biológicamente activa que comprende agregar la proteína a una solución que contiene CHES; y (Pág. 18, ejemplo 5)	Un método para plegar la proteína morfogénica de hueso en una forma dimérica biológicamente activa que comprende agregar la proteína a una solución que contiene CHES; y (Resumen y Pág. 188, *Solubilization of inclusion bodies and in vitro refolding of rhBMP-2*)	Un método para plegar miembros del factor de crecimiento transformante en una forma dimérica biológicamente activa que comprende agregar el factor a una solución que contiene un análogo funcional deñ ácido 2-(ciclohexilamino)-etansulfónico (Pág. 29, reivindicación 1 y Pág. 5, línea 51)
(ii) un sistema redox de sulfhidrilo/disulfuro de bajo peso molecular; e	Glutatión oxidado y glutatión reducido (Pág. 16, ejemplo 6)	Glutatión oxidado y glutatión reducido (Pág. 18, ejemplo 5)	Glutatión oxidado y glutatión reducido (Resumen y Pág. 188, *Solubilization of inclusion bodies and in vitro refolding of rhBMP-2*)	un sistema redox de sulfhidrilo/disulfuro de bajo peso molecular; (Pág. 30, reivindicación 14)
incubar el factor de crecimiento en la solución hasta que se forma el factor de crecimiento dimérico biológicamente	incubar la proteína en la solución hasta que se forma el factor dimérico biológicamente activo.	incubar la proteína en la solución hasta que se forma el factor dimérico biológicamente activo.	incubar la proteína en la solución hasta que se forma el factor dimérico biológicamente activo.	incubar el factor de crecimiento en la solución hasta que se forma el factor de crecimiento dimérico biológicamente

activo.	(Pág. 16, ejemplo 6)	(Pág. 18, ejemplo 5)	(Resumen y Pág. 188, *Solubilization of inclusion bodies and in vitro refolding of rhBMP-2*)	activo. (Pág. 6, líneas 31-33)

Las características **esenciales** del método de la reivindicación 1 habían sido divulgadas previamente en los documentos D1, D2, D3 y D4, porque:
D1 divulga un método para plegar la proteína morfogénica de hueso en una forma dimérica biológicamente activa que comprende agregar la proteína a una solución que contiene CHES 0.7M y glutatión oxidado 1mM y glutatión reducido 2mM a pH 8.3 e incubar la proteína en la solución a temperatura ambiente hasta que se forma el factor dimérico biológicamente activo (Pág. 16, ejemplo 6).
D2 enseña un método para plegar la proteína morfogénica de hueso en una forma dimérica biológicamente activa que comprende agregar la proteína a una solución que contiene CHES 50-100mM y glutatión oxidado 1mM y glutatión reducido 2mM e incubar la proteína en la solución a pH 9.5 y una temperatura de 4°C hasta que se forma el factor dimérico biológicamente activo (Pág. 18, ejemplo 5).
D3 divulga un método para plegar la proteína morfogénica de hueso (BMP-2) en una forma dimérica biológicamente activa que comprende agregar la proteína a una solución que contiene CHES 0.75M y glutatión oxidado 1mM y glutatión reducido 2mM e incubar la proteína en la solución a pH 8.5 y una temperatura de 20°C hasta que se forma el factor dimérico biológicamente activo (Resumen y Pág. 188, *Solubilization of inclusion bodies and in vitro refolding of rhBMP-2*). D3 también enseña la producción de BMP-2 recombinante en células de E. coli y la recuperación de la proteína de cuerpos de inclusión (Pág. 186 – 189, *Materials and methods*).
D4 enseña un método para plegar miembros del factor de crecimiento transformante, como el TGF-β3 (Pág. 32, reivindicación 7) en una forma dimérica biológicamente activa que comprende agregar el factor a una solución que contiene un análogo funcional del ácido 2-(ciclohexilamino)-etansulfónico (Pág. 29, reivindicación 1 y Pág. 5, línea 51), un sistema redox de sulfhidrilo/disulfuro de bajo peso molecular 1-10 mM (Pág. 30, reivindicación 14) e incubar el factor de crecimiento en la solución a pH 6-10 y una temperatura 0-37°C hasta que se forma el factor de crecimiento dimérico biológicamente activo (Pág. 6, líneas 31-33).

En consecuencia, la reivindicación 1 carece de novedad, según lo divulgado en los documentos D1, D2, D3 o D4.

Las reivindicaciones dependientes 2 – 3 y 8 – 17 no mencionan alguna característica que haga nueva al método de la reivindicación 1, por lo cual se considera que no son nuevas.

Nivel Inventivo (Art18 D486)

La **reivindicación 18** consiste en el método de acuerdo con cualquiera de las reivindicaciones precedentes en donde también se agrega taurodesoxicolato a la solución.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	**D1**	**D2**	**D3**	**D4**	**D5**
El método de acuerdo con cualquiera de las reivindicaciones precedentes en donde también se agrega taurodesoxicolato a la solución.	Un método para plegar la proteína morfogénica de hueso en una forma dimérica biológicamente activa que comprende agregar la proteína a una solución que contiene CHES; y (Pág. 16, ejemplo 6) Glutatión oxidado y glutatión reducido (Pág. 16, ejemplo 6) e incubar la proteína en la solución hasta que se forma el factor dimérico biológicamente activo. (Pág. 16, ejemplo 6)	Un método para plegar la proteína morfogénica de hueso en una forma dimérica biológicamente activa que comprende agregar la proteína a una solución que contiene CHES; y (Pág. 18, ejemplo 5) Glutatión oxidado y glutatión reducido (Pág. 18, ejemplo 5) e incubar la proteína en la solución hasta que se forma el factor dimérico biológicamente activo. (Pág. 18, ejemplo 5)	Un método para plegar la proteína morfogénica de hueso en una forma dimérica biológicamente activa que comprende agregar la proteína a una solución que contiene CHES; y (Resumen y Pág. 188, *Solubilization of inclusion bodies and in vitro refolding of rhBMP-2*) Glutatión oxidado y glutatión reducido (Resumen y Pág. 188, *Solubilization of inclusion bodies and in vitro refolding of rhBMP-2*) e incubar la proteína en la solución hasta que se forma el factor dimérico biológicamente activo. (Resumen y Pág. 188, *Solubilization of inclusion bodies and in vitro refolding of rhBMP-2*)	Un método para plegar miembros del factor de crecimiento transformante en una forma dimérica biológicamente activa que comprende agregar el factor a una solución que contiene un análogo funcional deñ ácido 2-(ciclohexilamino)-etansulfónico (Pág. 29, reivindicación 1 y Pág. 5, línea 51) un sistema redox de sulfhidrilo/disulfuro de bajo peso molecular; (Pág. 30, reivindicación 14) e incubar el factor de crecimiento en la solución hasta que se forma el factor de crecimiento dimérico biológicamente activo. (Pág. 6, líneas 31-33)	Un método para plegar miembros del factor de crecimiento transformante en una forma dimérica biológicamente activa donde se agrega taurodesoxicolato a la solución. (Pág. 6, ejemplo 3)

Los documentos D1, D2, D3, D4 y D5 se consideran el estado de la técnica cercano a la invención definida en la reivindicación 18 porque:

D1 divulga un método para plegar la proteína morfogénica de hueso en una forma dimérica biológicamente activa que comprende agregar la proteína a una solución que contiene CHES 0.7M y glutatión oxidado 1mM y glutatión reducido 2mM a pH 8.3 e incubar la proteína en la solución a temperatura ambiente hasta que se forma el factor dimérico biológicamente activo (Pág. 16, ejemplo 6).

La diferencia entre la invención, definida en la reivindicación 18 y el método de D1 consiste en que también se agrega taurodesoxicolato a la solución.

D2 enseña un método para plegar la proteína morfogénica de hueso en una forma dimérica biológicamente activa que comprende agregar la proteína a una solución que contiene CHES 50-100mM y glutatión oxidado 1mM y glutatión reducido 2mM e incubar la proteína en la solución a pH 9.5 y una temperatura de 4°C hasta que se forma el factor dimérico biológicamente activo (Pág. 18, ejemplo 5).

La diferencia entre la invención, definida en la reivindicación 18 y el método de D2 consiste en que también se agrega taurodesoxicolato a la solución.

D3 divulga un método para plegar la proteína morfogénica de hueso (BMP-2) en una forma dimérica biológicamente activa que comprende agregar la proteína a una solución que contiene CHES 0.75M y glutatión oxidado 1mM y glutatión reducido 2mM e incubar la proteína en la solución a pH 8.5 y una temperatura de 20°C hasta que se forma el factor dimérico biológicamente activo (Resumen y Pág. 188, *Solubilization of inclusion bodies and in vitro refolding of rhBMP-2*). D3 también enseña la producción de BMP-2 recombinante en células de *E. coli* y la recuperación de la proteína de cuerpos de inclusión (Pág. 186 – 189, *Materials and methods*).

La diferencia entre la invención, definida en la reivindicación 18 y el método de D3 consiste en que también se agrega taurodesoxicolato a la solución.

D4 enseña un método para plegar miembros del factor de crecimiento transformante, como el TGF-β3 (Pág. 32, reivindicación 7) en una forma dimérica biológicamente activa que comprende agregar el factor a una solución que contiene un análogo funcional del ácido 2-(ciclohexilamino)-etansulfónico (Pág. 29, reivindicación 1 y Pág. 5, línea 51), un sistema redox de sulfhidrilo/disulfuro de bajo peso molecular 1-10 mM (Pág. 30, reivindicación 14) e incubar el factor de crecimiento en la solución a pH 6-10 y una temperatura 0-37°C hasta que se forma el factor de crecimiento dimérico biológicamente activo (Pág. 6, líneas 31-33).

La diferencia entre la invención, definida en la reivindicación 18 y el método de D4 consiste en que también se agrega taurodesoxicolato a la solución.

El efecto que logra el incluir taurodesoxicolato a la solución es que permite obtener un factor de crecimiento transformante dimérico replegado.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: "cómo modificar los métodos conocidos en los documentos D1, D2, D3 y D4 para obtener un factor de crecimiento transformante dimérico replegado".

Sin embargo, la utilización de taurodesoxicolato para plegar un factor de crecimiento transformante ya había sido divulgado en D5, el cual enseña un método para plegar activina humana A, el cual es un miembro de la superfamilia del factor de crecimiento transformante beta, en una forma dimérica biológicamente activa donde se agrega taurodesoxicolato a la solución (Pág. 6, ejemplo 3).

En consecuencia, la persona del oficio normalmente versada en la materia, incluiría taurodesoxicolato de D5 en los métodos de D1, D2, D3 o D4 para llegar así al objeto de la reivindicación 18 de la solicitud. Por lo cual se considera obvia. La persona del oficio normalmente versada en la materia se vería motivada a adicional taurodesoxicolato porque mejora la obtención de la forma dimérica del factor (Pág. 6, párrafo 33).

De manera que la solución propuesta por esta solicitud en la reivindicación 18 y en las reivindicaciones 19 – 29 es obvia y no tiene Nivel Inventivo.

8. Conclusión

Los requisitos de Patentabilidad de la presente Solicitud están afectados así:

Item	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	WO99/18196	1 – 29	Novedad y Nivel Inventivo
D2	WO9516034	1 – 29	Novedad y Nivel Inventivo
D3	Journal of Biotechnology, Vol. 94, Pág. 185-194.	1 – 29	Novedad y Nivel Inventivo
D4	EP0433225	1 – 29	Novedad y Nivel Inventivo
D5	EP0943690	1 – 29	Novedad y Nivel Inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante para dar respuesta a este requerimiento y presentarla por escrito

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 No. 10 de 2001.

JESÚS FERNEL GARCÍA GARCÍA
Director de Nuevas Creaciones (E)

El anterior requerimiento fue notificado por fijación en lista, de fecha 25 JUL. 2012

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ELSEVIER

Journal of Biotechnology 94 (2002) 185–194

JOURNAL OF Biotechnology

www.elsevier.com/locate/jbiotec

Renaturation and purification of bone morphogenetic protein-2 produced as inclusion bodies in high-cell-density cultures of recombinant *Escherichia coli*

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Received 15 June 2001; accepted 26 September 2001

Abstract

Escherichia coli was genetically engineered to produce recombinant human bone morphogenetic protein-2 (rhBMP-2) in a non-active aggregated form using a temperature-inducible expression system. High concentrations of both biomass (75 g cell dry weight per liter of culture broth) and inactive rhBMP-2 (8.6 g l^{-1}) were obtained by applying a high-cell-density cultivation procedure. After washing and solubilizing the inclusion bodies, rhBMP-2 was refolded and dimerized at concentrations up to 100 mg l^{-1} by means of a simple dilution method with yields exceeding 50%. Finally, a one-step purification procedure based on affinity chromatography was implemented to isolate the rhBMP-2 dimer. With the established renaturation and purification protocols, yields of more than 10 mg rhBMP-2 dimer per gram cell dry weight were obtained corresponding to 750 mg rhBMP-2 dimer per liter of culture broth. The purified rhBMP-2 dimer showed biological activity equivalent to CHO produced rhBMP-2 as tested by the induction of alkaline phosphatase activity in C2C12 cells.

Keywords: rhBMP-2; *E. coli*; In vitro refolding; Purification

1. Introduction

Bone morphogenetic proteins (BMPs) are a group of proteins involved in the development of many organs and tissues as well as in the establishment of the basic embryonic body plan (Hogan, 1996). Their crucial role in the latter sets the stage for their potential use in the regeneration of many types of tissues following injuries and diseases (Kirker-Head, 2000). For instance, some BMPs have the unique capacity of inducing bone regeneration and ectopic bone formation in adult

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0168-1656/02/$ - see front matter
PII: S0168-1656(01)00425-4

vertebrates. In fact, clinical trials performed recently have shown that, when delivered properly, recombinant BMPs are effective and safe alternatives to autologous bone grafting (Boden et al., 2000). As reviewed recently, further therapeutic applications of BMPs, including their application for osteointegration of metallic implants, tendon and ligaments reunion with bones, cartilage repair and even in non-skeletal diseases, are foreseen (Kirker-Head, 2000).

Biologically active BMPs are homo- or heterodimers, linked by a single disulfide bond (Kirker-Head, 2000). The monomers have three intrachain disulfide bridges, forming a so-called cystine knot (Griffith et al., 1996; Scheufler et al., 1999). In vivo, the monomer is synthesized as a large precursor, which after dimerization, is proteolytically cleaved releasing the smaller C-terminal fragment forming the mature dimer (Wang et al., 1990).

BMPs have been isolated directly from bones, however with low yields (1–3 $\mu g\,kg^{-1}$) and a complicated purification scheme (Wang et al., 1988; Bessho et al., 1999). Even more, the potential health risk associated with their isolation from allogeneic donor bone limits their clinical application (Kirker-Head, 2000). Alternatively, the active form of recombinant bovine and human BMPs has been obtained from mammalian cell cultures (Israel et al., 1996; Wang et al., 1990). However, post-translational problems (incomplete monomer processing) and low yields ($ng\,ml^{-1}$ range) are usual in these processes. Similar problems occur during the production of rhBMP-2 in virus infected insect cells (Maruoka et al., 1995). As an alternative, several patents and patent applications describe the production of biologically active rhBMPs through in vitro refolding of *Escherichia coli* produced inclusion bodies (Cerletti et al., 1991; Cerletti, 2000; Vicik, 1999; Wolfman and McCoy, 1998). However, a common disadvantage of these procedures is the requirement of several time-consuming purification and concentration steps before the refolding attempt. In addition, a low overall yield of 0.2 mg rhBMP-2 per gram cell wet weight has been reported for rhBMP-2 production through in vitro refolding (Ruppert et al., 1996).

The aim of the present work was to develop a highly productive and simplified process for rhBMP-2 production. For this purpose, we used a strong temperature-inducible expression vector in combination with a high-cell-density cultivation (HCDC) procedure. Afterwards, a simplified and high yielding method for renaturation of rhBMP-2 was established. Finally, a one-step purification scheme was implemented to isolate the biologically active rhBMP-2.

2. Material and methods

2.1. Strain and plasmid

The *E. coli* strain TG1 (DSM 6056) was used as a host for rhBMP-2 production. The mature rhBMP-2 cDNA sequence was amplified by PCR using the cDNA as template (Ahrens et al., 1993). At the same time, restriction sites for *Nde*I, and thus a start codon, and *Bam*HI, preceded by a termination codon, were inserted into the mature cDNA using the following primers: 5′-GCTACGCATATGCAAGCCAAACACAAACAGCGG. 5′-TCTAGAGGATCCCTAGCGACACCCACAACCCT. This construct was inserted into the temperature inducible expression vector pCYTEXP3 (Fig. 1(A)) (Schneppe et al., 1994). The plasmid obtained was named as pCYTEXP3-BMP-2 (Fig. 1(B)).

2.2. Shake flask cultivation

Unless otherwise stated all cultivation media contained 50 $\mu g\,ml^{-1}$ ampicillin and the ratio between medium and shake flask volumes was 1:10. Fresh colonies of recombinant *E. coli* were obtained after growth on Luria Bertani (LB) agar plates. A single colony was transferred to 10 ml of LB medium (250 ml shake flask) and incubated for 8 h at 120 rpm and 30 °C. From these cultures, 25 ml of either LB or the medium defined were inoculated to an OD_{600} of 0.1. The composition and preparation of the defined medium has been described (Seeger et al., 1995). After overnight incubation at the aforementioned conditions, the cultures were used to inoculate 50 ml

of each medium at an OD_{600} of 0.1. These shake flask cultures were incubated at the conditions mentioned above and sampled hourly for OD_{600} measurements. At an OD_{600} of 0.8, rhBMP-2 production was induced by temperature shift (42 °C). The samples after induction were pelleted (15 000 × *g*, room temperature, 5 min) and stored at −70 °C for assaying the content of rhBMP-2.

2.3. High-cell-density cultivation

The high-cell-density cultivation was accomplished, with minor changes, as already described (Korz et al., 1995; Seeger et al., 1995). A 5-l bioreactor (MD5 model, B. Braun Diessel Biotech GmbH, Melsungen, Germany) with 3 l of the defined medium was inoculated at an OD_{600} of 0.1. The cultivation was performed at 30 °C, pH 6.8 and dissolved oxygen concentration (pO_2) of 25%. Culture samples were taken for determining OD_{600}, glucose, rhBMP-2 and cell dry weight. In addition, pO_2, pH, off-gas concentrations of O_2 and CO_2, stirrer speed, base consumption and aeration rate, were measured on-line and recorded by an external data acquisition and control system (Ubicon, Hannover, Germany).

After the initial glucose in the bioreactor was consumed, the exponential addition of the feeding solution was started as already described (Korz et al., 1995). During the first part of the fed-batch cultivation, a growth rate of 0.12 h^{-1} was set (μ_{set}). When the culture reached an OD_{600} of 100, the temperature was shifted to 42 °C to induce the recombinant protein synthesis concomitant with a reduction in the exponential feeding rate to prevent the accumulation of acetic acid (μ_{set} = 0.08 h^{-1}: Seeger et al., 1995). The cultivation was continued for six additional hours. Thereafter, biomass produced was collected by centrifugation at 5500 × *g* and 4 °C for 30 min, resuspended to the original volume in 50 mmol l^{-1} sodium phosphate buffer (pH 7.0) and centrifuged again. The obtained pellet was stored in aliquots at −70 °C.

2.4. Inclusion bodies isolation

The frozen cell pellet was resuspended in 50 mmol l^{-1} sodium phosphate buffer (pH 7.0) at a wet weight concentration of 220 g l^{-1} (corresponding to a dry weight concentration of 55 g l^{-1}). The obtained slurry was pumped twice at 650 bar through a high-pressure homogenizer (Lab60, APV Gaulin, Lübeck, Germany) and cen-

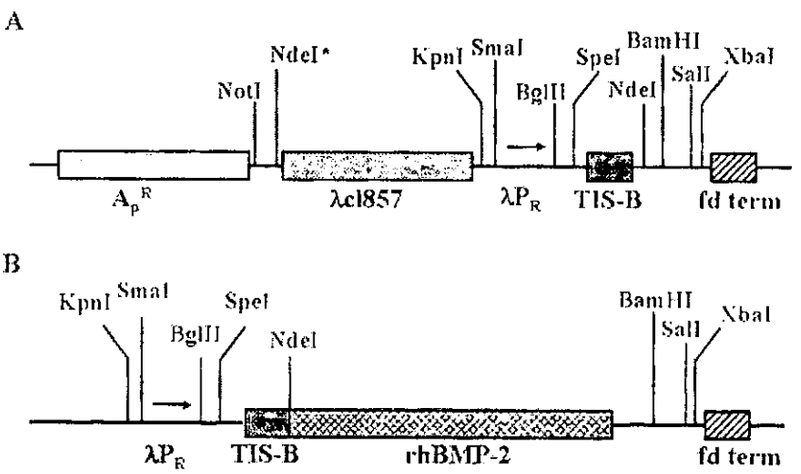

Fig. 1. (A) Original expression vector pCYTEXP3 (Schneppe et al., 1994). The expression cassettes comprised the bacteriophage λP_R promotor, a 5′ non-coding initiation sequence (TIS-B), the phage fd transcriptional terminator and the bacteriophage λcI857 repressor gene. (B) rhBMP-2 encoding expression vector pCYTEXP3-BMP-2. In order to obtain direct cytoplasmic synthesis of mature rhBMP-2 in *E. coli*, the gene was inserted via *Nde*I and *Bam*HI restriction sites.

trifuged for 45 min at 5500 × g and 4 °C. The resulting pellet was washed with the same volume of 50 mmol l^{-1} sodium phosphate buffer, centrifuged again and stored in aliquots at −70 °C for further purification. The frozen pellet was resuspended at a wet weight concentration of 25 mg ml^{-1} in 20 mmol l^{-1} Tris–HCl (pH 8.5), 0.5 mmol l^{-1} EDTA, 2% (v/v) Triton X-100 (Müller and Rinas, 1999). After vigorous vortexing, the suspension was centrifuged for 30 min at 4 °C and 26 000 × g. The last procedure was repeated once for the pellet obtained.

2.5. Solubilization of inclusion bodies and in vitro refolding of rhBMP-2

The washed inclusion bodies were resuspended in 6 mol l^{-1} Gnd–HCl, 0.1 mol l^{-1} Tris–HCl (pH 8.5), 0.1 mol l^{-1} dithiothreitol, 1 mmol l^{-1} EDTA at a wet weight concentration of 35 mg ml^{-1} (Müller and Rinas, 1999). This suspension was incubated overnight at room temperature (ca. 20 °C) with constant stirring and centrifuged later at 26 000 × g for 45 min.

The solubilized rhBMP-2 was diluted, at a final concentration of 20 or 100 μg ml^{-1}, in different renaturation buffers. These solutions consisted of a common buffer (5 mmol l^{-1} EDTA, 1 mmol l^{-1} oxidized glutathione, 2 mmol l^{-1} reduced glutathione, 1 mol l^{-1} NaCl, 50 mmol l^{-1} Tris–HCl, pH 8.5) and different concentrations of either 3-(1-pyridinio)-1-propanesulfonate (PPS), pyridine-3 sulfonic acid (PSA), 2-(cyclohexylamino)ethanesulfonic acid (CHES) or nicotinic acid (NA) (Vicik, 1999). The renaturation mixture was incubated for 4 days at 20 °C.

2.6. Purification of refolded rhBMP-2 by heparin affinity chromatography

Purification of the in vitro refolded rhBMP-2 dimer was performed by heparin affinity chromatography. The whole procedure was performed at room temperature (ca. 20 °C). Briefly, the final renaturation mixture was extensively dialyzed against 4 mol l^{-1} urea, 20 mmol l^{-1} Tris–HCl (pH 8.0) and later passed through a 0.45 μm filter (Minisart, Sartorious, Göttingen, Germany). Thereafter, 1.2 mg of the total protein were applied to a 1-ml HiTrap heparin–sepharose column (Pharmacia, Upsala, Sweden), equilibrated beforehand with 5 column volumes (CV) of dialysis buffer. The column was washed again with dialysis buffer (5 CV), and the monomeric and dimeric forms of rhBMP-2 were eluted in a two-step NaCl gradient: 350 and 500 mmol l^{-1}, respectively. Eluted fractions were concentrated to 1 mg ml^{-1} by ultrafiltration (10 kDa vivaspin, vivascience, Binbrook, UK) and stored at −70 °C.

2.7. Analytical methods and biological activity tests

Samples withdrawn during cultivation were analyzed for OD_{600}, glucose, cell dry weight and recombinant protein concentration as described earlier (Seeger et al., 1995). Electrophoresis was done using precast 8–16% SDS-PAGE gels following supplier guidelines (Criterion, Biorad, Hercules, USA). The gels were stained with Coomassie Brilliant Blue and subjected to densitometry analysis (Quantiscan, Biosoft, Ferguson, USA). Purified rhBMP-2 was used as a standard for densitometric quantification. The protein concentration of the standard was determined by UV280 with a calculated molar extinction coefficient of 18 860 l mol^{-1} cm^{-1} (Wetlaufer, 1962).

For analyzing the extent of dimerization, the renaturation mixture was centrifuged for 5 min at 38 000 × g, the resulting supernatant treated with trichloroacetic acid (Ozols, 1990), and the pellet obtained subjected to non-reducing SDS-PAGE.

Biological activity of the purified rhBMP-2 dimer and monomer was measured by two different methods. Initially, the capacity of both the dimer and monomer to induce alkaline phosphatase activity in C2C12 cells (ATCC-1772) was measured according to an established procedure (Kirsch et al., 2000). A commercially available CHO produced rhBMP-2 dimer was used as a standard for this test (R&D systems, Minneapolis, USA). In addition, the induction of the FGF-receptor 3 (FGFR3) by both rhBMP-2 dimer and monomer was determined. In this case, C3H10T1/2 cells (ATCC-226) were plated at a density of

5500 cells cm^{-2} in 25 cm^2 flasks. After 48 h cells were first washed twice with phosphate buffered saline and then the cells were starved for 24 h in Dulbecco's modified Eagle's medium without serum. Cells were then treated for 180 min with dimerized or monomeric rhBMP-2. For determining the induction of the FGFR3, total cellular RNA was prepared by TriReagent^{LS} according to manufacturer's protocol (Molecular Research Center Inc., Cincinnati, USA). Five micrograms of total RNA was reverse transcribed and cDNA aliquots were subjected to PCR. RT-PCR was normalized by the transcriptional levels of hypoxanthine-guanine phosphoribosyltransferase (HPRT). The HPRT-specific 5′ and 3′ primers were GCTGGTGAAAAGGACCTCT and AAGTAGATGGCCACAGGACT, respectively. The primer pairs for FGFR3 were: CCTGCGCAGTCCCCCAAAGAAG and CTGCAGGCATCAAAGGAGTAGT.

3. Results

3.1. Production of rhBMP-2 in shake flask and high-cell-density cultivation

Recombinant protein production at a laboratory scale is in general performed using complex medium such as the LB. However, for producing protein in high-cell-density cultures a defined medium is required. Therefore, production of rhBMP-2 was initially compared in recombinant *E. coli* shake flask cultures using either LB or defined medium. At an OD_{600} of 0.8, rhBMP-2 production was induced by temperature shift (42 °C) and monitored by SDS-PAGE analysis and densitometry. In shake flask cultures, rhBMP-2 accumulated during the first 2–3 h after induction to 25% of the total cell protein (Fig. 2). It was exclusively deposited as inclusion bodies and no significant difference in the final amount was obtained on either LB or defined medium (Fig. 2).

For maximizing the volumetric productivity of rhBMP-2, a robust high-cell-density cultivation procedure with a pre-determined exponential-feeding strategy was applied (Korz et al., 1995).

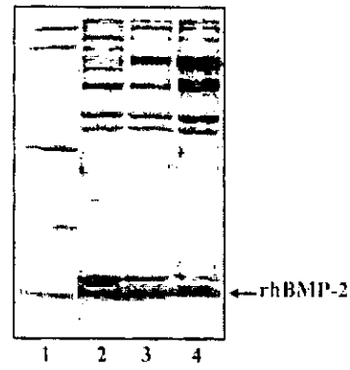

Fig. 2. Total cell protein content from 4 h post-induced *E. coli* cultures grown at different conditions. Lanes: 1, LMW (14, 20, 30, 43, 67, 94 kDa); 2, shake flask cultivation in LB medium; 3, shake flask cultivation in defined medium; 4, high-cell-density cultivation.

This strategy has been used successfully for the production of various heterologous proteins (e.g. Babu et al., 2000; Schmidt et al., 1999; Wilms et al., 2001).

Cultivation of recombinant *E. coli* TG1:pCYTEXP3-BMP-2 started as a batch process at 30 °C with a specific growth rate and a biomass to glucose yield of 0.42 h^{-1} and 0.5 g g^{-1}, respectively (Fig. 3). At a biomass concentration of 16 g l^{-1}, the end of the batch phase was noticed by a steep increase in the dissolved oxygen concentration (pO_2) and the absence of glucose in the medium (Fig. 3). At this moment, the exponential addition of concentrated feed solution was started aiming at a specific growth rate of μ_{set} = 0.12 h^{-1} (μ_{real} = 0.11 h^{-1}). After the culture reached an OD_{600} of 100, the temperature was shifted to 42 °C to induce the production of rhBMP-2 concomitant with a reduction of the feeding rate to prevent accumulation of acetic acid (μ_{set} = 0.08 h^{-1}). During the first 2 h after the temperature shift, the real specific growth rate matched the set value of 0.08 h^{-1}, but dropped significantly thereafter (μ_{real} = 0.04 h^{-1}) despite the continuous addition of feed medium. In addition, the majority of rhBMP-2 was produced during the first 2 h after induction and continued thereafter at lower rates (Fig. 3). Similar growth inhibitory effects have been observed during the temperature-induced production of other het-

erologous proteins in high-cell-density cultures (e.g. Babu et al., 2000; Schmidt et al., 1999). At the end of the cultivation, rhBMP-2 represented about 20% of the total cell protein (see also Fig. 2). The final biomass concentration was 75 g l^{-1} cell dry weight with a specific product concentration of 115 mg rhBMP-2 per gram cell dry weight. This corresponds to a volumetric yield of 8.6 g l^{-1} rhBMP-2.

3.2. Solubilization of inclusion bodies and in vitro refolding of rhBMP-2

The produced inclusion bodies were isolated and washed as described earlier (Müller and Rinas, 1999). The rhBMP-2 represented 85% of the total protein content of the isolated inclusion bodies. Solubilization of this material was attempted by two different methods. On one hand, treatment of inclusion bodies at low pH and in the presence of dithiothreitol did not efficiently solubilize the non-native rhBMP-2 monomer contradicting the already published results (Cerletti et al., 1991; Cerletti, 2000; Wolfman and McCoy, 1998). On the other hand, the use of high concentrations of denaturants, 6 mol l^{-1} Gnd–HCl, effectively solubilized the inclusion bodies. Additionally, this treatment incremented the purity of the solution obtained slightly due to a selective solubilization of rhBMP-2. Indeed, several cell protein contaminants remained insoluble under these conditions (data not shown). Even more, no protein loss was observed during the solubilization step as was reported when the denaturant used was 8 mol l^{-1} urea at pH 5.0 (Ruppert et al., 1996).

All the published procedures for rhBMP-2 renaturation include at least one purification step of the solubilized monomer followed by time-consuming steps such as dialysis or even lyophilization before the renaturation attempt (Ruppert et al., 1996; Vicik, 1999; Wolfman and McCoy, 1998). Therefore, by aiming to simplify the process, we attempted the refolding of solubilized rhBMP-2 by different methods without subsequent purification.

Initially, attempts were made to refold rhBMP-2 by using size exclusion chromatography according to a method applied successfully for the renaturation of platelet-derived growth factor, a structurally related dimeric protein containing the common cystine knot motif (Müller and Rinas, 1999). However, this procedure did not result in the formation of rhBMP-2 dimer. Alternatively,

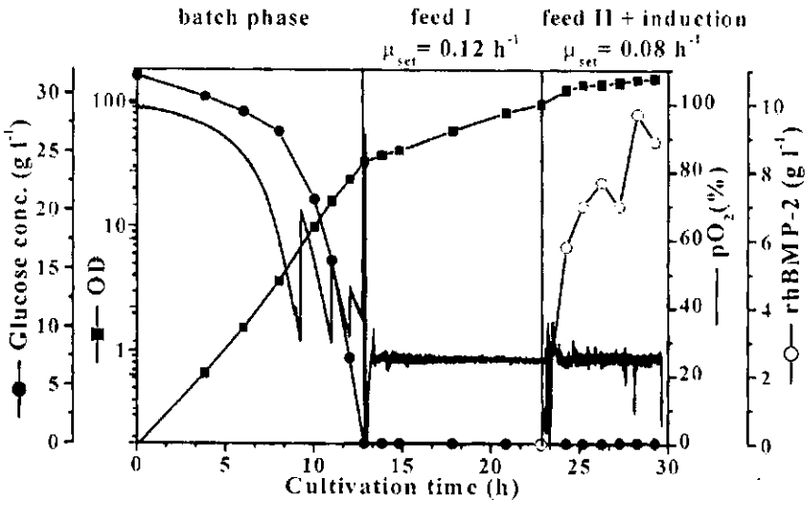

Fig. 3. Temperature-induced production of rhBMP-2 in high-cell-density cultivation of recombinant *E. coli*. After unlimited growth during batch mode, feeding of concentrated medium was performed at rates aiming at the indicated specific growth rates. Product formation was induced at an OD_{600} of 100 by a temperature shift from 30 to 42 °C.

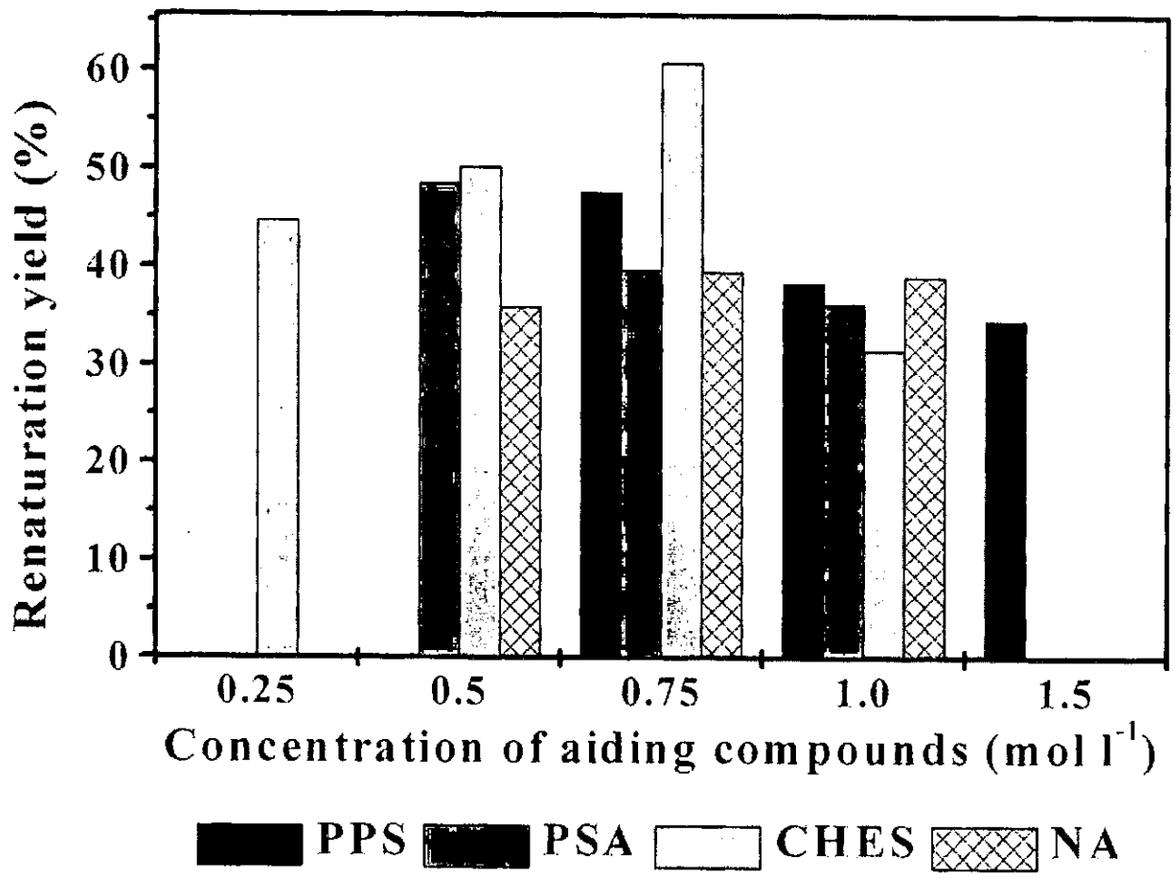

Fig. 4. Effect of different aiding compounds on the in vitro dimerization of rhBMP-2 by the dilution method. Twenty micrograms per milliliter of rhBMP-2 in the refolding buffer. Refolding solution contained 5 mmol l^{-1} EDTA, 1 mmol l^{-1} oxidized glutathione, 2 mmol l^{-1} reduced glutathione, 1 mol l^{-1} NaCl, 50 mmol l^{-1} Tris–HCl (pH 8.5) and different concentrations of 3-(1-pyridinio)-1-propanesulfonate (PPS), pyridine-3 sulfonic acid (PSA), 2-(cyclohexylamino)ethanesulfonic acid (CHES) and nicotinic acid (NA).

refolding of rhBMP-2 was attempted by direct dilution of solubilized inclusion bodies into buffers containing different concentrations of aiding compounds such as PPS, PSA, CHES and NA (Fig. 4) (Vicik, 1999). Specifically, renaturation was attempted at concentrations of 20 or 100 μg ml^{-1} rhBMP-2. At low protein concentrations, most of the conditions tested resulted in dimer formation, with dimerization yields up to 60% after a period of 4 days (Fig. 4). In the original patent application, though no renaturation yields were mentioned, the optimum concentrations of the aiding compounds for renaturation of rhBMP-2 at 50 μg ml^{-1} were given as: 0.4–0.6 mol l^{-1} PSA, 0.7 mol l^{-1} CHES, 1.0 mol l^{-1} NA and 1.0–1.7 mol l^{-1} PPS (Vicik, 1999). Our results are in agreement with the data mentioned at least for the first three compounds (Fig. 4). Remarkably, the presence of the aiding compounds was obligatory for renaturation to occur even at low protein concentrations. At high rhBMP-2 concentrations, aggregation was observed in most of the buffers tested. However, in the presence of more than 0.5 mol l^{-1} CHES, aggregation was not observed at rhBMP2 concentrations of 100 μg ml^{-1} while renaturation yields above 50% were still obtained. Therefore, routine refolding of rhBMP-2 was carried out in the presence of 0.75 mol l^{-1} CHES.

3.3. Purification

The biologically active rhBMP-2 dimer was purified in only one chromatographic step. Improving the procedures published earlier, the heparin-affinity chromatography purification process integrates the removal of both host cell protein contaminants and non-dimerized rhBMP-2 monomer. In fact, some host cell protein contaminants did not bind to heparin when the dialyzed renaturation mixture was applied. The monomer and the remaining residual host cell protein contaminants eluted at 0.3 mol l^{-1} NaCl. Later, at 0.5 mol l^{-1} NaCl the dimer eluted at high purity (> 95%) (Fig. 5).

In summary, yields above 10 mg rhBMP-2 dimer per gram cell dry weight were obtained by applying the proposed renaturation and purification procedures. This yield is more than 12 times higher than those reported earlier (Ruppert et al., 1996). The volumetric yield obtained corresponds to 750 mg biologically active rhBMP-2 dimer per liter of culture broth.

3.4. Biological activity test

The biological activity of the refolded and purified rhBMP-2 dimer was tested by two different in vitro assays. First, the purified rhBMP-2 dimer induced ALP activity in C2C12 mesenchymal cells in a dose dependent form (Fig. 6). This

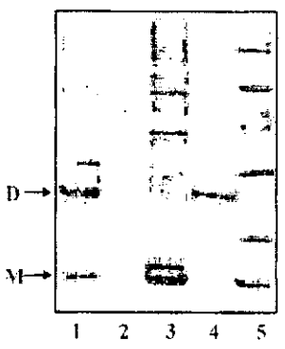

Fig. 5. rhBMP-2 dimer purification by heparin affinity chromatography. Lanes: 1, dialyzed renaturation mixture; 2, flow through fraction; 3, monomer fraction; 4, dimer fraction; 5, LMW (14, 20, 30, 43, 67, 94 kDa).

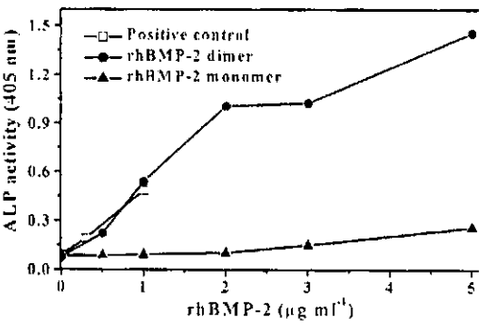

Fig. 6. Biological activity of in vitro refolded and heparin-purified rhBMP-2 monomer and dimer, measured by the induction of alkaline phosphatase activity (ALP) in C2C12 cells. As positive control, commercially available CHO produced rhBMP-2 was used.

level of activity was comparable to the activity of the CHO produced rhBMP-2 used as a standard and in agreement with the data reported in the literature (Kirsch et al., 2000). The purified monomer did not show any significant biological activity even at concentrations six times above the reported median effective dose.

In addition, the capacity of both the rhBMP-2 dimer and monomer to induce the FGF-receptor 3 (FGFR3) was determined. It has been demonstrated recently that FGFR3 is upregulated in murine mesenchymal progenitor cells C3H10T1/2 by rhBMP-2 treatment (Hoffmann et al., in press). Here, it is shown by RT-PCR analysis that rhBMP-2-dependent upregulation of FGFR3 was more sensitive to renatured dimeric rhBMP-2 than to the monomeric form (Fig. 7). FGFR3-activation is detected in the presence of 10 ng ml^{-1} dimeric rhBMP-2 while an order of magnitude more rhBMP-2 (100 ng ml^{-1}) was needed to elicit a similar level of FGFR3 induction.

4. Conclusion

We have developed a simple process for the production of biologically active rhBMP-2 from *E. coli* produced inclusion bodies. Initially, we successfully introduced the gene of the mature sequence of rhBMP-2 into one of the strongest expression vectors for *E. coli*. Subsequently, the

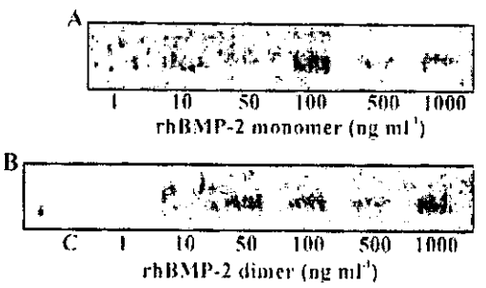

Fig. 7. Induction of FGF-receptor 3 (FGFR3) by monomeric or dimeric rhBMP-2. Treatment of murine mesenchymal progenitor C3H10T1/2 cells with monomeric or dimerized recombinant BMP-2 was as described in Materials and Methods. Cells were mock-treated (C) or were treated with varying amounts of monomeric (A) or dimeric (B) rhBMP-2 for 180 min. RT-PCR analyses of FGFR3 mRNA levels were as described in Section 2 (the picture presented is the negative image of the original one).

recombinant *E. coli* that hosted the plasmid obtained was grown in a complex and defined medium using shake flasks. After temperature induction, rhBMP-2 was produced in the form of inclusion bodies representing 25% of the total cell protein independent of the cultivation medium. Taking advantage of the high volumetric yields obtainable by high cell density cultivation, 8.6 g rhBMP-2 per liter of culture broth was produced.

Although renaturation of rhBMP-2 has been described before, all the available methods contain complicated and time-consuming steps for purifying the inactive rhBMP-2 before the refolding attempt. Here, the purity of rhBMP-2 was incremented to more than 85% by a simple procedure of inclusion bodies' washing and Gnd HCl solubilization. The low content of remaining impurities did not interfere with the refolding process. Indeed, high renaturation yields were obtained by diluting the solubilized material into a series of buffers. Specially, the presence of CHES at concentrations above 0.5 mol l^{-1} prevented protein precipitation and aided dimer formation during the renaturation process. Finally, a one-step purification process was established to isolate the biologically active rhBMP-2 dimer from both the remaining rhBMP-2 monomer and host cell protein contaminants. The purified rhBMP-2 dimer showed biological activity similar to CHO produced rhBMP-2. To our knowledge, the procedures described give the highest overall yield reported so far.

Acknowledgements

L.F. Vallejo acknowledges a scholarship from COLCIENCIAS (Colombian Institute for Science and Technology). J. Cabrera-Crespo thanks FAPESP (The State S. Paulo Research Foundation, No. 99/05554-5) and the Foundation Institute Butantan (S. Paulo, Brasil) for a post-doctoral scholarship. This work was supported in part by the SFB 578 and DFG grants We 1211/4-1. We are also thankful to J. van den Heuvel for advice concerning primer selection and expression vector construction.

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