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10/17

Señor

SUPERINTENDENTE DE INDUSTRIA Y COMERCIO

DIVISION DE NUEVAS CREACIONES

E. S. D.

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

Rad: 03-009311-00009-0000 Fac: 2006-06-10 17:23:06
Dep. 2020 NUECREACIONES
Tte 11 PATENTEYS Eje 379 FASENACIONALI
A/E 293 IMPULSOPROCESAL Fases: 1

Re: P1.-GLAXOSMITHKLINE BIOLOGICALS S.A.-Solicitud de patente de
Invención en Fase Nacional PCT para "PURIFICACION DE
ANTIGENOS HBV PARA EL TRATAMIENTO USO EN VACUNAS".-
Clase CO7K 14/02.- Expediente número 03-009,311.-

1. Me refiero a mi memorial de 5 de Noviembre de 2004.
2. Por medio del presente atentamente ruego a usted, se sirva pronunciarse en relación con la solicitud arriba nombrada, ya que desde el 1 de Septiembre de 2004, esto hace ya mas de dos (2) años, que fue publicado el extracto de la presente solicitud en la Gaceta de la Propiedad Industrial No. 543, y el examen de patentabilidad correspondiente fue solicitado en su debido momento mediante memorial de 5 de Noviembre de 2004, sin que haya sido adelantado ningún otro trámite.
3. Cabe anotar que esta demora en el trámite acarrea grave perjuicio para mi representado.

Señor Superintendente,


ALICIA LLOREDA RICAURTE

T.P. 53.215

AMCW/fegv

DIVISION DE NUEVAS CREACIONES

RADICACIÓN EXPEDIENTE No. 03-009311

EL EXTRACTO DE LA PRESENTE SOLICITUD DE PATENTE DE INVENCION

PCT FUE PUBLICADO EN LA GACETA DE PROPIEDAD INDUSTRIAL No 543

DE FECHA 01 DE SEPTIEMBRE DEL 2004, EL TERMINO HABIL SEÑALADO

POR LA LEY EXPIRA EL 29 DE NOVIEMBRE DEL 2004

NOTA: Dentro del plazo de los seis (6) meses siguientes a partir de la fecha de publicación en la gaceta de Propiedad Industrial, deberá solicitar y cancelar el examen de patentabilidad so pena de que la solicitud caiga en abandono, de acuerdo con lo establecido en el Artículo 44 de la Decisión 486/2000.

SE PRESENTARON OPOSICIONES POR PARTE DE TERCEROS.

SI ☐ NO ☒

Bogotá D.C., Diciembre de 2006

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152

**SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIVISIÓN DE NUEVAS CREACIONES**

2020
Bogotá, D.C.,

Doctor
ALICIA LLOREDA
Smithkline Beecham Biologicals
(Apoderado)

ASUNTO	Radicación	03-9311
	Trámite	002
	Evento	001
	Actuación	430
	Oficio	.. - - 3888
	Folios	

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 le comunica:

Documentos estudiados:

Los textos estudiados fueron la descripción (Folios: 28 a 88) y las reivindicaciones 1 a 27(folios: 89 a 95).

La presente solicitud reivindica prioridad británica N° GB0019728.5 de 10 de agosto de 2000 y cumple con lo establecido en los artículos 9, 10 y 11 de la Decisión 486 de la Comisión de la Comunidad Andina.

Objeto de invención:

El objeto de la presente solicitud se refiere a un método para producir una vacuna de hepatitis B purificado, la cual comprende un antígeno superficial de hepatitis purificado, el antígeno comprende menos de 0.25µg de Hg, donde el antígeno se purifica en presencia de un agente reductor que tiene un grupo SH libre y un excipiente farmacéuticamente aceptable. En el anterior método el antígeno de hepatitis crudo a) está sujeto a cromatografía de permeación en gel, b)cromatografía de intercambio de iones; opcionalmente se purifica en presencia de tiomersal y c) se mezcla con un agente reductor que tiene un grupo-SH libre, tal como cisteína. De igual forma se reclama una composición de vacuna que comprende un antígeno de hepatitis B purificado comprendiendo menos de 0.25µg de Hg/20µg de proteína y que es obtenido por el método descrito anteriormente y es mezclado con un adyuvante de aluminio o un inductor Th1.

El objeto de la solicitud definido anteriormente se clasificó en la IPC A61K 31/29, C07K 14/02.

Claridad de la solicitud:

El artículo 28 de la Decisión 486 de la Comisión de la Comunidad Andina establece "La descripción deberá divulgar la invención de manera suficientemente clara y completa para su comprensión y para

que una persona capacitada en la materia técnica correspondiente pueda ejecutarla."

El artículo 30 de la Decisión 486 de la Comisión de la Comunidad Andina establece que: "Las reivindicaciones definirán la materia que se desea proteger mediante la patente. Deben ser claras y concisas y estar enteramente sustentadas por la descripción."

Las reivindicaciones podrán ser independientes o dependientes. Una reivindicación será independiente cuando defina la materia que se desea proteger sin referencia a otra reivindicación anterior. Una reivindicación será dependiente cuando defina la materia que se desea proteger refiriéndose a una reivindicación anterior. Una reivindicación que se refiera a dos o más reivindicaciones anteriores se considerará una reivindicación dependiente múltiple."

En general el capítulo reivindicatorio carece de concisión porque comprende varias cláusulas independientes que tienen el mismo alcance; por ejemplo las cláusulas 21 y 22 comprenden materia previamente reclamada en 1, la 23 es prácticamente igual a la 5, la 24 igual a la 6, entre otras. Lo anterior impide que un tercero pueda determinar con exactitud cual es el verdadero alcance de la protección solicitada.

Las reivindicaciones 1 a 4, 11, 12 reclaman un método para producir una vacuna, caracterizándolo por el producto a obtener, más no por sus características técnicas propias. Un método debe definirse mediante la secuencia lógica de etapas que lo conforman y por las condiciones a las cuales se éstas se llevan a cabo, mas no por el resultado a alcanzar.

En la reivindicación 5 no es completamente claro si los literales a), b) y c) constituyen etapas consecutivas o simplemente son opciones de los métodos de purificación a los que puede ser sometido el antígeno.

La reivindicación 25 reclama un antígeno de hepatitis B inmunogénico, que comprende menos de 0.25 µg de Hg por 20 µg de proteína que se obtiene por purificación en presencia de tiomersal antes del tratamiento con un agente reductor con un grupo tiol libre. En este caso se intenta reclamar una molécula por su proceso de purificación y no a través de sus características técnicas propias (estructura).

De acuerdo con lo anterior, las cláusulas en mención no son claras a la luz del artículo 30 de la D. 486

Reivindicaciones de uso:

El artículo 14 de la Decisión 486 de la Comisión de la Comunidad Andina establece: "Los Países Miembros otorgarán patentes para las invenciones, sean de producto o procedimiento, en todos los campos de la tecnología, siempre que sean nuevas, tengan nivel inventivo y sean susceptibles de aplicación industrial".

La reivindicación 20 reclama expresamente el uso de un antígeno superficial de HBV en la preparación de un medicamento. Considerando que la D.486 solamente considera protección para invenciones de producto o procedimiento, pero no contempla tal privilegio para los usos, se conceptúa que la materia reclamada se encuentra fuera del campo de la patentabilidad nombrado en el artículo 14.

Determinación del estado de la técnica:

El artículo 16 de la Decisión 486 de la Comisión de la Comunidad Andina establece que "El estado de la técnica comprenderá todo lo que haya sido accesible al público por una descripción escrita u oral,

utilización, comercialización o cualquier otro medio antes de la fecha de presentación de la solicitud de Patente o, en su caso, de la prioridad reconocida.

Sólo para el efecto de la determinación de la novedad, también se considerará dentro del estado de la técnica, el contenido de una solicitud de patente en trámite ante la oficina nacional competente, cuya fecha de presentación o de prioridad fuese anterior a la fecha de presentación o de prioridad de la solicitud de patente que se estuviese examinando, siempre que dicho contenido esté incluido en la solicitud de fecha anterior cuando ella se publique o hubiese transcurrido el plazo previsto en el artículo 40°.

Considerando que la fecha de presentación de la primera solicitud de patente de la cual se reivindica prioridad es: 10 de agosto de 2000 y 18 de enero de 2001, se buscaron documentos del estado de la técnica relacionados, con fecha de publicación anterior a esta.

Se hizo la búsqueda de documentos relevantes en el estado de la técnica, entendiendo como estado de la técnica la definición provista en el artículo 16 de la Decisión 486 de la Comisión de la Comunidad Andina y las excepciones fijadas en el artículo 17 de la misma.

Se encontró como anterioridades (documentos cuya fecha de publicación es anterior a la fecha de presentación de la primera solicitud de patente sobre la cual se reivindica prioridad o de la fecha de presentación de esta solicitud) que pueden afectar la patentabilidad del objeto de la presente solicitud:

Nº	Documento	Título	Fecha de Publicación
1	WO994557	Combined vaccine compositions	19-09-1999
2	EP314240	Process for purifying recombinant hepatitis antigens	03-05-89
3	US4649192	Method for the isolation and purification of hepatitis B surface antigen ..	10-03-1987

Evaluación de los requisitos de Patentabilidad:

El Artículo 14 de la Decisión 486 de la Comisión de la Comunidad Andina el cual establece: "Los Países Miembros otorgarán Patentes para las invenciones, sean de producto o de procedimiento en todos los campos de la tecnología siempre que sean nuevas, tengan nivel inventivo y sean susceptibles de aplicación industrial".

Se procedió a evaluar los requisitos de novedad, nivel inventivo y aplicación industrial con el fin de establecer la patentabilidad de la invención reivindicada en la solicitud en estudio.

Evaluación de Novedad:

El artículo 16 de la Decisión 486 de la Comisión de la Comunidad Andina establece que "Una invención se considerará nueva cuando no esté comprendida en el estado de la técnica". "El estado de la técnica comprenderá todo lo que haya sido accesible al público por una descripción escrita u oral, utilización, comercialización o cualquier otro medio antes de la fecha de presentación de la solicitud de patente o, en su caso, de la prioridad reconocida.

Sólo para el efecto de la determinación de la novedad, también se considerará dentro del estado de la técnica, el contenido de una solicitud de patente en trámite ante la oficina nacional competente, cuya fecha de presentación o de prioridad fuese anterior a la fecha de presentación o de prioridad de la solicitud de patente que se estuviese examinando, siempre que dicho contenido esté incluido en la

solicitud de fecha anterior cuando ella se publique o hubiese transcurrido el plazo previsto en el artículo 40°.

Esta se evaluó por comparación entre lo solicitado y los documentos encontrados en el estado de la técnica y que se relacionan con el objeto reivindicado y de acuerdo con la definición de novedad del Artículo 16 de la Decisión 486 de la Comisión de la Comunidad Andina.

Comparando elemento por elemento el objeto de esta solicitud con lo mencionado en las anterioridades, tenemos que:

Las reivindicaciones 13,19,25, 26 reclaman una composición de vacuna conteniendo un antígeno de HBV, o reivindican expresamente dicho antígeno HBV, que se caracteriza porque contiene menos de 0.025µg de Hg por 20 µg de proteína, y es obtenido mediante purificación en presencia de tiomersal y tratamiento con agente reductor con un grupo SH. Los antígenos pueden estar en combinación con otros de bacterias patógenas conocidas.

La anterioridad D1 revela composiciones de vacuna que comprenden la combinación de antígenos de hepatitis B (especialmente HBsAg), con antígenos de HSV y un adyuvante inductor Th1. Algunos de los antígenos mencionados en D1, pueden contener tiomersal (y a su vez Hg), pero otros antígenos que han sido purificados mediante métodos en los cuales el mercurio no está presente, como los referenciados en la página 8 de D1, afectan la novedad de la materia reivindicada en las cláusulas en mención; pues el indicar que el antígeno esta obtenido por un método de purificación determinado (tiomersal/reductor-SH) no necesariamente lo distingue de la materia previamente divulgada en algunas realizaciones de D1. Las vacunas divulgadas en esta anterioridad (y de hecho muchas otras vacunas del estado de la técnica) que no contienen Hg caen dentro del alcance de la materia reclamada en la presente solicitud y por ende afectan su novedad.

De igual manera el documento D3 divulga la purificación de un antígeno de HBsAg obtenido a partir de una levadura modificada, así como formulaciones de vacunas que lo contienen. En este proceso para la producción y formulación de vacunas de HBsAg, no se divulga el uso de tiomersal (conteniendo Hg) luego las composiciones, los procesos y las vacunas reveladas aquí, también serían perjudiciales para la novedad de los antígenos y composiciones reclamados en las cláusulas 13, 19,25y 26. Lo anterior teniendo en cuenta que aún cuando en las anterioridades no se divulga el empleo de un agente reductor con -SH, para reducir los niveles de mercurio, no implica que las vacunas obtenidas por tal método sean diferentes a las composiciones de vacuna de HBV del estado del arte manufacturadas por diferentes procesos.

Como se observa, el objeto de las cláusulas anteriormente mencionadas puede ser idéntico a lo comprendido en estado de la técnica, con lo cual se considera que podría no cumplir con el requisito de Novedad.

Evaluación de Nivel Inventivo:

El artículo 18 de la Decisión 486 de la Comisión de la Comunidad Andina establece que "Se considerará que una invención tiene nivel inventivo, si para una persona del oficio normalmente versada en la materia técnica correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica".

El Nivel Inventivo se evaluó por comparación entre lo solicitado y encontrado en el estado de la técnica y que se relaciona con el objeto reivindicado y de acuerdo con

la definición de nivel inventivo del Artículo 18 de la Decisión 486 de la Comisión de la Comunidad Andina.

Las cláusulas 1 a 12, 21 a 24 reclaman un método para producir una vacuna de hepatitis B purificado, la cual comprende un antígeno superficial de hepatitis purificado, conteniendo menos de 0.25µg de Hg, donde el antígeno se purifica en presencia de un agente reductor que tiene un grupo SH libre y un excipiente farmacéuticamente aceptable. En el anterior método el antígeno de hepatitis crudo a) está sujeto a cromatografía de permeación en gel, b) cromatografía de intercambio de iones; opcionalmente se purifica en presencia de tiomersal y c) se mezcla con un agente reductor que tiene un grupo-SH libre, tal como cisteína.

La anterioridad D2 se refiere a una técnica simple y rápida para la producción y purificación de HBsAg con alto rendimiento y pureza, donde los antígenos son estables y potentes. El proceso abarca varias etapas de purificación, junto a un paso de solubilización en el que pueden emplearse varios agentes reductores como urea DTT(SH libre), cisteína y homocisteína.

Las cromatografías de permeación en gel y de intercambio de iones; son conocidas por cualquier persona versada en la materia como métodos de separación de distintos tipos de moléculas. Ahora bien, considerando que el uso de agentes reductores con grupos tiol libres para la purificación de antígenos de superficie de HBV ya había sido divulgado en D2, un técnico medio del oficio podría partir de las enseñanzas de ésta anterioridad y seleccionar otras metodologías de separación conocidas en el arte para ajustar el proceso de purificación, mediante ensayos sistemáticos propios de la actividad investigativa, hasta obtener el rendimiento deseado.

Como se observa, el objeto de esta solicitud no representa un salto cualitativo sobre la formulación de la regla técnica y por lo tanto no cumple con el requisito de Nivel Inventivo.

Evaluación de la Aplicación Industrial:

El artículo 19 de la Decisión 486 de la Comisión de la Comunidad Andina establece que "Se considerará que una invención es susceptible de aplicación industrial, cuando su objeto pueda ser producido o utilizado en cualquier tipo de industria, entendiéndose por industria la referida a cualquier actividad productiva, incluidos los servicios".

El objeto de la presente solicitud cumple con el requisito de Aplicación Industrial.

En conclusión los requisitos de patentabilidad de la presente solicitud se encuentran afectados así:

Nº	Documento	Reivindicaciones afectadas	Requisito que afecta
1	WO994557	13, 19,25y 26	Novedad
2	EP314240	1 a 12, 21 a 24	Nivel Inventivo
	US4649192	13, 19,25y 26	Novedad

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 días contados a partir de la fecha de la 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.

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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 CÉSPEDES DE VERGEL
Jefe de la División de Nuevas Creaciones

El anterior requerimiento fue notificado por fijación en lista, de fecha 30 MAR 2007

CONCEPTO TÉCNICO N°: 241	EXAMINADOR TÉCNICO Código N°: 202063	2007-02-09
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 39/295, 39/39	A2	(11) International Publication Number: WO 99/45957 (43) International Publication Date: 16 September 1999 (16.09.99)
(21) International Application Number: PCT/EP99/01406 (22) International Filing Date: 4 March 1999 (04.03.99) (30) Priority Data: 9805105.5 9 March 1998 (09.03.98) GB 9813561.9 23 June 1998 (23.06.98) GB (71) Applicant (for all designated States except US): SMITHKLINE BEECHAM BIOLOGICALS S.A. (BE/BE); Rue de l'Institut 89, B-1330 Rixensart (BE). (72) Inventors; and (73) Inventors/Applicants (for US only): STEPHENNE, Jean (BE/BE); SmithKline Beecham Biologicals S.A., Rue de l'Institut 89, B-1330 Rixensart (BE). WETTENDORFF, Martine, Anne, Cecile (BE/BE); SmithKline Beecham Biologicals s.a., Rue de l'Institut 89, B-1330 Rixensart (BE). (74) Agent: TYRRELL, Arthur, William, Russell; SmithKline Beecham Corporate Intellectual Property, Two New Horizons Court, Brentford, Middlesex TW8 9EP (GB).	(51) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>	
(54) Title: COMBINED VACCINE COMPOSITIONS (57) Abstract Novel combined vaccine compositions preferentially for administration to adolescents are provided, comprising a hepatitis B viral antigen and a herpes simplex viral antigen and optionally in addition one or more of the following: an EBV antigen, a hepatitis A antigen or inactivated attenuated virus, an HPV antigen, a VZV antigen, an HCMV antigen, a Toxoplasma gondii antigen. The vaccine compositions are formulated with an adjuvant which is a preferential stimulator of TH1 cell response such as 3D-MPL and QS21.		

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The present invention provides a vaccine composition comprising:

- (a) a hepatitis B viral (HBV) antigen; and
5 (b) a herpes simplex viral (HSV) antigen
in combination with an adjuvant which is a preferential stimulator of TH1 cell
response.

The vaccine composition of the invention is of great benefit for administration to
10 adolescents who may be particularly at risk of HBV, and/or HSV infection.

Optionally the vaccine composition of the invention additionally comprises one or
more of a number of other antigens as described below.

15 It has been found that the vaccine compositions according to the invention
surprisingly show no interference, that is to say that the immune response to each
antigen in the composition of the invention is essentially the same as that which is
obtained by each antigen given individually in conjunction with an adjuvant which
is a preferential stimulator of TH1 cell response.

20 The vaccine Havrix (Trade Mark), also from SmithKline Beecham Biologicals is an
example of a vaccine that can be used to prevent hepatitis A infections. It is
formulated with aluminium hydroxide as adjuvant. This vaccine comprises an
attenuated strain of the HM-175 Hepatitis A virus inactivated with formol
25 (formaldehyde); see Andre et. al. (Prog. med. Virol., vol. 37, p1-24).

As used herein, the term hepatitis A viral (HAV) antigen is used to refer to either a
protein derived from hepatitis A virus or an attenuated strain of HAV, optionally
inactivated, e.g. with formaldehyde. If the HAV antigen is a protein derived from
30 hepatitis A virus it may optionally be a recombinant protein:

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The hepatitis B viral (HBV) antigen in the composition of the invention is typically hepatitis B surface antigen.

- 5 The preparation of Hepatitis B surface antigen (HBsAg) is well documented. See for example, Harford et. al. in Develop. Biol. Standard 54, page 125 (1983), Gregg et. al. in Biotechnology, 5, page 479 (1987), EP-A- 0 226 846, EP-A-0 299 108 and references therein.
- 10 As used herein the expression 'Hepatitis B surface antigen', abbreviated herein to 'HBsAg' or 'HBS' includes any HBsAg antigen or fragment thereof displaying the antigenicity of HBV surface antigen. It will be understood that in addition to the 226 amino acid sequence of the HBsAg S antigen (see Tiollais et. al. Nature, 317, 489 (1985) and references therein) HBsAg as herein described may, if desired,
- 15 contain all or part of a pre-S sequence as described in the above references and in EP-A- 0 278 940. HBsAg as herein described can also refer to variants, for example the 'escape mutant' described in WO 91/14703. In a further aspect the HBsAg may comprise a protein described as L* in European Patent Application Number 0 414 374, that is to say a protein, the amino acid sequence of which
- 20 consists of parts of the amino acid sequence of the hepatitis B virus large (L) protein (ad or ay subtype), characterised in that the amino acid sequence of the protein consists of either:
- (a) residues 12 - 52, followed by residues 133 - 145, followed by residues 175 - 400 of the said L protein; or
 - 25 (b) residue 12, followed by residues 14 - 52, followed by residues 133 - 145, followed by residues 175 - 400 of the said L protein.
- HBsAg may also refer to polypeptides described in EP 0 198 474 or EP 0 304 578.

- Normally the HBsAg will be in particle form. It may comprise S protein
- 30 alone or may be as composite particles, for example (L*,S) wherein L* is as defined above and S denotes the S-protein of hepatitis B surface antigen.

The HBsAg may be adsorbed on aluminium phosphate as described in WO93/24148.

Preferably the hepatitis B (HBV) antigen used in the formulation of the invention is
5 HBsAg S-antigen as used in the commercial product Egerix-B (Trade Mark; SmithKline Beecham Biologicals).

A vaccine comprising hepatitis B surface antigen in conjunction with 3D-MPL was described in European Patent Application 0 633 784.

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Epstein Barr Virus (EBV), a member of the herpesvirus group, causes infectious mononucleosis as a primary disease in humans. Predominantly it affects children or young adults. More than 90% of the average adult population is infected by EBV that persists for lifetime in peripheral B-lymphocytes. The virus is lifelong
15 produced in the parotid gland and spread primarily by exchange of saliva from individuals who shed the virus. Children infected with EBV are largely asymptomatic or have very mild symptoms, while adolescents and adults who become infected develop typical infectious mononucleosis, characterised by fever, pharyngitis, and adenopathy. People who have been infected maintain anti-EBV
20 antibodies for the remainder of their lives, and are thus immune to further infection.

In addition to its infectious qualities, EBV has been shown to transform lymphocytes into rapidly dividing cells and has therefore been implicated in several different lymphomas, including African Burkitt's lymphoma (BL). EBV may also
25 be involved in causing nasopharyngeal carcinoma (NPC). Worldwide it is estimated that 80,000 cases of nasopharyngeal carcinoma occur and it is more prevalent in ethnic Chinese populations. Infectious mononucleosis is a consequence of primary infection by EBV. It is not a life-threatening disease if additional risk factors are absent.

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Four proteins of the EBV viral envelope constituting the so-called membrane antigen complex have been described. They are usually referred to as gp 220/350

Example 1: Immunogenicity study with gD + HBs combination

5 The objective of the study was to demonstrate the feasibility of the HSV
gD/HBV HBs combination in Al(OH)₃ / 3D-MPL formulation. Immune responses
induced in guinea pigs by immunisation with these antigens used alone or in
combination were compared. HBs is an abbreviation for Hepatitis B surface
antigen, specifically the S-protein as described hereinabove. gD is an abbreviation
for rgD₁ as described hereinabove.

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Experimental protocol

The experimental protocol was the following. Groups of 6 female Hartley
guinea pigs were injected intramuscularly on day 0 and day 28 with the following
15 formulations:

- group 1: HBs 5 µg / Al(OH)₃ 125 µg / 3D-MPL 12.5 µg
- group 2: gD 5 µg / Al(OH)₃ 125 µg / 3D-MPL 12.5 µg
- group 3: HBs 5 µg + gD 5 µg / Al(OH)₃ 125 µg / 3D-MPL 12.5 µg
- 20 - group 4: HBs 5 µg + gD 5 µg / Al(OH)₃ 125 µg / 3D-MPL* 12.5 µg

* different 3D-MPL batch

Animals were bled 14 and 31 days after the second immunization. The
25 humoral immune response against HSV gD and HBV HBs was evaluated at both
time points in ELISA.

Delayed type hypersensitivity (DTH) reactions were also evaluated for HBs.
They consisted in the intradermal injection of 10 µg HBs in duplicate. The
30 development of DTH reactions was monitored by measuring skin thickness at 0, 24
and 48 hours after injection.

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163**Results****1. Antibody responses**

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Anti-gD ELISA titers are shown in Fig. 1. Anti-gD titers in the group immunized with gD were comparable to those induced in animals immunized with the gD + HBs combination. The presence of HBs in the formulation did not affect the induction of anti-gD antibody responses.

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Similarly, anti-HBs antibody titers were compared in animals immunized with HBs alone or combined with gD. Fig. 2 shows that comparable anti-HBs titers were observed in animals immunized with HBs alone or with HBs + gD.

15 The results are shown in Figures 1-4 from which it may be concluded that in the tested formulation, the gD / HBs combination induces antibody responses comparable to those induced by the same antigens used alone and DTH responses to HBs comparable to those induced by HBs alone. Thus no significant differences in DTH responses to HBs are observed in HBs or HBs + gD vaccinated animals. The
20 presence of gD did not affect the DTH response to HBs.

Example 2: PRO30 Experiment HBV/HSV combination

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The objective of this study was to evaluate in the HSV guinea pig model the protective efficacy of an HSV gD + HBV HBs combination in a 3D-MPL/alum formulation as compared to gD alone in a 3D-MPL/alum formulation. The 3D-MPL/alum formulation comprises alum (10 parts by weight) to 3D-MPL (1 part by
30 weight).

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② **EUROPEAN PATENT APPLICATION**

⑦ Application number: 88202338.9

⑥ Int. Cl. C12P 21/00 , C07K 3/12 ,
//A61K39/29

⑧ Date of filing: 19.10.88

⑨ Priority: 28.10.87 US 113583

④ Date of publication of application:
03.05.89 Bulletin 89/18

③ Designated Contracting States:
CH DE FR GB IT LI NL

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⑤ Process for purifying recombinant hepatitis antigens.

⑬ Methods of solubilizing recombinant surface antigen of hepatitis B virus are disclosed. In one protocol, solubilization is achieved with buffer, urea and dithiothreitol.

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PROCESS FOR PURIFYING RECOMBINANT HEPATITIS ANTIGENS

INTRODUCTION

Hepatitis B surface antigen often occurs as a glycoprotein-protein complex that confers immunity against the pathological effects of subsequent infection by hepatitis B virus. Sources for safe, rapid and inexpensive manufacture of the antigen for vaccination purposes have been limited to serum or plasma from patients who synthesize large quantities of the requisite antigen, usually in the form of 22 nm particles. With the advent of recombinant DNA techniques, DNA coding for the surface antigen is inserted into yeast, *E. coli* and other cellular systems, then expressed. The resulting polypeptide product varies substantially with the DNA construction used as well as the host or cellular systems employed to express the inserted DNA.

The HB virion is composed of two groups of structural proteins, the core proteins and the envelope or surface ("S") proteins. In addition to being the major surface proteins of the virion, i.e., Dane particle, the "S" proteins are the sole constituents of Australia antigen, or 22 nm particles. The "S" proteins are the translational products of a large open reading frame (ORF) encoding 389-400 amino acids, depending upon serotype. This ORF is demarcated into three domains, each of which begins with an ATG codon that is capable of functioning as a translational initiation site *in vivo*. These domains are referred to as pre-S1 (108-119 amino acids), pre-S2 (55 amino acids), and S (226 amino acids) in their respective 5'-3' order in the gene. The six protein products derived from this ORF have the following compositions:

- 1) gp42 (42,000 dalton glycoprotein) = pre-S1/S2/S (meaning pre-S1 contiguous with pre-S2, contiguous with S),
- 2) p39 (p = protein) = pre-S1/S2/S,
- 3) gp36 = pre-S2/S (two glycosylation sites),
- 4) gp33 = pre-S2/S (one glycosylation site),
- 5) gp27 = S (one glycosylation site),
- 6) p24 = S.

All six proteins are present to an approximately equimolar extent in the HBV Dane particle. In the 22 nm particle, the 4 smaller proteins are present to an approximately equimolar extent, while gp42 and p39 are present in at most one or a few molecules per particle. The pre-S1 and pre-S2 regions may have functions of promoting secretion of the S region. For reviews of these fundamental properties of the protein, see Tiollais, P. et al., *Science* 213, 406 (1981) and Milich, D. R. et al., *Proc. Natl. Acad. Sci.* 82, 8168 (1985).

The pre-S2 region of the hepatitis B antigen comprises about 55AA (amino acid) residues. Its presence provides a dominant epitope that is more immunogenic *in vivo* than epitopes of the S protein, according to Neurath, A. R. et al., *Science* 224, 392 (1984), Neurath, A. R. et al., *Nature* 315, 164 (1985), and Milich, D. R. et al., *supra*. The pre-S2 polypeptide also has receptor-like properties for polymerized human serum albumin (pHSA), a trait also possessed by liver cells which are known to bind pHSA, Machida, A. et al., *Gastroenterology* 88, 910 (1984).

Since the presence of pre-S2 sequences in the surface antigen is a desirable property for the purposes of immunization, expression systems have been developed for the expression of pre-S1/S2/S protein and other variants. See, for example, U.S. application no. 824,405, filed January 31, 1986.

The methods of the present invention relate to solubilizing precipitates of recombinant surface antigen of hepatitis B virus (rHbsAg). Partially purified rHbsAg precipitates are solubilized by a combined and simultaneous treatment of urea and DTT. Precipitates or other insoluble entities frequently occur in the process of purifying rHbsAg from yeast and other expression systems. The present invention provides methods of rendering soluble such precipitates for the purposes of making a better and cheaper vaccine against hepatitis-associated diseases.

The technical advantages of solubilizing the partially purified recombinant surface antigen with denaturing agents in the presence of reducing agents include higher yield and greater purity. The solubilized product is also more stable. The technique of solubilization is rapid and simple.

Purification of recombinant proteins not infrequently requires new methods or novel combinations of old methods, since the composition of the source for recombinant forms e.g. yeast are different from conventional sources e.g. serum. One cannot predict what methods are useful for isolating proteins from recombinant cell cultures on the basis of the knowledge and know-how of methods useful for isolating proteins from classical or otherwise conventional sources. Purification processes designed for preparing vaccine require unusual purity in the product another indication of the unreliability in the art. For a

similar view, see U.S. Patent 4,824,818.

BRIEF DESCRIPTION OF THE INVENTION

In this invention, there is disclosed a method for solubilizing precipitates of recombinant hepatitis B surface antigen from a precipitated partially purified recombinant hepatitis antigen obtained from a recombinant expression system, comprising the steps of:

(a) treating a preparation of precipitated, partially purified recombinant hepatitis antigen, or portion of said antigen thereof, with a quantity of denaturing agent in the presence of a reducing agent effective to solubilize said preparation, and

(b) removing the denaturing agent and the reducing agent, yielding a preparation of a substantially soluble, partially purified recombinant hepatitis antigen, or portion of said antigen thereof.

DEFINITIONS AND ABBREVIATIONS

Specific activity: a weight-to-weight ratio of immunochemically reactive sAg to total protein.

AA	amino acid
DTT	dithiothreitol
ED ₅₀	50% effective dose
EDTA	ethylenediamine tetracetic acid trisodium salt
L	liter
Mr	molecular weight
MW	molecular weight
NMW	nominal molecular weight cutoff
pHSA	polymerized human serum albumin
PBS	phosphate buffered saline, 0.15M NaCl in 7mM sodium phosphate buffer, pH about 7.2
PMSF	phenylmethylsulfonyl fluoride
psi	pounds per square inch
rHbsAg	recombinant hepatitis B surface antigen
S	surface
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

DETAILED DESCRIPTION OF THE INVENTION

The processes of the present invention involve methods of solubilizing recombinant hepatitis B surface antigen (rHbsAg) from yeast extracts. These methods involve the combined treatment of rHbsAg with denaturing agent in the presence of reducing agent.

It will be understood that the novel purification processes of the present invention are applicable to a range of expressed rHbsAg including recombinant S protein or recombinant pre-S1/S2/S protein. One principle example is rHbsAg produced by yeast cells transformed by yeast vectors of Example 1. This system expresses an S amino acid sequence. Processes for the purification of other variant amino acid sequences of the S protein are encompassed by the present invention. The processes of the present invention are designed to provide rapid and efficient methods of purifying any rHbsAg, including S protein variants as well as pre-S1/S2/S protein variants, in accordance with the principles of the present invention. For example, conservative substitutions [defined as sets in Table 1 of Taylor, W.R., J. Mol. Biol. 186, 233 (1986)] in the pre-S1/S2/S amino acid sequence generally will not result in any substantial or novel modification of the principles and practice of the present invention. Conservative substitutions of hepatitis surface antigen are known; see Efessli, E. et al., J. Theor. Biol. 121, 371 (1988). Alternatively, deletions within the S, pre-S1 or pre-S2 regions will not in general require any modifications of the processes for purification discussed herein. It will be understood that rHbsAg or surface antigen or portion thereof in this application includes any such variations in the amino acid sequence, whether by conservative amino acid

- heparin
 hirudin
 4-hydroxymercuribenzoate
 iodoacetamide
 5 iodoacetic acid
 leupeptin
 α_2 -macroglobulin
 mercaptoethanol
 p-mercuribenzoate
 10 mercuric chloride
 α -microglobulin
 α -N-(p-nitrobenzyl-oxy-carbonyl)-L-arginylchloromethyl ketone
 oxalate
 pepstatin from a variety of sources, including Streptomyces
 15 1,10-phenanthroline
 2-phenanthroline
 phenothiazine-N-carbonyl chloride
 phosphoramidate
 PMSF
 20 pyrophosphate
 SH-blocking reagents
 silver nitrate
 soybean trypsin inhibitor
 p-toluene sulfonyl fluoride
 25 TLCK[L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone-hydrochloride]
 TRITON X-100 and other detergents at low concentrations
 TPCK[L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone]-
 trypsin inhibitor from hen egg
 ZPCK (benzyloxycarbonyl-L-phenylalanine)
 30 Buffered solutions containing one or more of the listed inhibitors may be adapted to one or more steps
 in the process of purifying surface antigen.

PURIFICATION

- 35 Yeast cells transformed with expression vectors coding for pre-S1/S2/S protein or variants thereof, are grown and harvested. If storage of the cells is desired, the cells are then washed in buffer, e.g. PBS, to form a cell paste. Storage of the cell paste is typically done by freezing in liquid N₂.
 40 Purification typically begins as follows. A batch of frozen cell paste is thawed and suspended in buffer containing proteolytic inhibitors, e.g. PBS in 2mM PMSF, or hypertonic phosphate buffers. Cells are then disrupted, preferably by mechanical means. The gentle bead breakage method for disruption has been found unsuitable for scale-up. Disruption by a cell disrupter is the most preferred because of its rapid operation.
 45 Yeast cell disruption results in a crude extract. It is necessary at this point to remove cell debris in the extract so that mechanical clogging in subsequent purification steps is avoided. Centrifugation at about 4,400 x g for 5 minutes has been found adequate, but it is readily understood that different centrifugation speeds for different times are also possible and easily rendered operable. The presence of pre-S polypeptide epitopes can be ascertained by the binding of these epitopes to polymerized human serum albumin (pHSA) in a sandwich RIA assay with labelled antibody specific to HBV surface antigen.
 50 Further steps in the purification process are performed on the 4,400 x g supernatant remaining after the cell debris is removed from the crude extract by centrifugation at 4,400 x g for 5 minutes, as described above.

SOLUBILIZATION

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(k) crystallization.

This list is by no means exhaustive. Its order is not an indication of the preferred order of purification. It will be understood that a successful purification of rHbsAg may be conducted with any or all of steps (a)-(k), and that in principle solubilization as described above may be inserted as an additional step at any point.

5 In the examples the following materials were purchased from commercial sources: Urea and guanidine-HCl, Schwartz/Mann Biotech; TRITON X-100, Fisher Scientific Company; dithiothreitol, Bio-Rad.

Throughout this application, glycoprotein was determined by the periodic acid-Schiff base procedure of Neville, D. M. et al., *Methods in Enzymology* 32, 82 (1974). Protein concentrations were determined by the SDS-Lowry assay, e.g. Lowry, O. H. et al., *J. Biol. Chem.* 193, 265 (1951); and the Coomassie blue-binding assay, e.g. Bradford, M.M., *Anal. Biochem.* 72, 248 (1976).

10 Immunochemical tests were performed on the lots purified according to the following procedures. Binding to polymerized human serum albumin (pHSA) was conducted according to Valenzuela et al., *Biotechnology*, 3, 317 (1985). Immunochemical determinants were assayed by a variety of conventional procedures, including AUSRIA® II-125 (Abbott Labs), and AUSAB® (Abbott Labs). Biological activity was tested by the mouse potency test.

EXAMPLE 1

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Preparation of partially purified rHbsAg

25 Samples of partially purified recombinant surface antigen were prepared substantially by the procedures of Wampler, D.E. et al., *Proc. Natl Acad. Sci.* 82, 6630 (1985) through the step involving hydrophobic chromatography on butyl-agarose, yielding butyl-agarose product. These procedures are as follows:

Two yeast strains were used as a source of rHbsAg. The first is that described by Valenzuela et al. *Nature* 288, 347 (1982). The second used a glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter instead of the alcohol dehydrogenase (ADH) promoter. Cells were grown as described by Carty et al., Eighty-Fourth ASM Meeting, March 12, 1984, St. Louis, MO, abstr 030, p. 194, and harvested. The cell paste was suspended in an equal volume of hypertonic phosphate buffer (0.1 M sodium phosphate, pH 7.2/0.5 M NaCl). Phenylmethylsulfonyl fluoride (0.2 M in isopropanol) was added to a final concentration of 2mM and the cells were disrupted by seven or nine passes through a laboratory homogenizer for 15 minutes at 10,000 psig yielding a crude extract. The crude extract (32-70 mg of protein per ml) was diluted with 4 vol of 0.01 M phosphate buffer (pH 7.5) containing 0.1% TRITON X-100 (Rohm and Haas). Cell debris was removed by continuous-flow centrifugation, and the supernatant solution was concentrated 5-fold in a hollow fiber unit and dialyzed with 5 vol of phosphate-buffered saline (7mM sodium phosphate, pH 7.2/0.15 M NaCl). TRITON X-100 was removed by using an XAD-4 beads cartridge [Rohm and Haas, see Cheetham, P.S.J. *Anal. Biochem.* 82, 447 (1979)]. HbsAg was partially purified by adsorption and elution with 80 mM sodium borate buffer, pH 8.7, from fused silica [Pillot, J. et al., *J. Clin. Microbiol.* 4, 205 (1978). The eluate was clarified by centrifugation for 35 minutes at 11,000 x g at 4° C, then routinely stored at 4° C. For removal of insoluble precipitates at this stage, centrifugation at 8000 x g for 4 minutes was performed and the resulting clarified product was further purified by adsorption on butyl-agarose, yielding the butyl-agarose product as eluate.

EXAMPLE 2

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Solubilization

35 The butyl-agarose product was treated with urea-DTT or KSCN, or both, as follows:

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(a) Urea-DDT treatment. A sample of the butyl-agarose product of Example 1 was incubated for 15-20 minutes at 4°C in 4M Urea and 5 mM DTT. The impurities dissociated from the antigen were separated from the antigen by dialfiltration over a hollow fiber membrane (100,000 NMW, 0.8 ft², 8 psi) with 5 vol. of 2M Urea in 2 mM DTT, followed by 10 volumes of PBS, yielding retentates treated with Urea-DDT.

(b) KSCN treatment. Butyl-agarose product samples of Example 1, or urea-DDT treated samples of step (a) of this Example, were incubated in 3M KSCN in PBS at 4°C for 18 hours. The impurities dissociated from the antigen were then removed by dialfiltration over a hollow fiber membrane (100,000 NMW, 0.8 ft²) with 5 volumes of 3M KSCN in PBS followed by 10 volumes of PBS, yielding samples treated with KSCN.

Samples treated with Urea-DDT or KSCN, or both, were examined under electron microscopy and found to be 20 nm particles of typical appearance. These samples were then analyzed for immunological potency by the mouse potency test.

Mouse potency was assessed by the procedures as follows. Antigen samples were prepared for testing by subjecting to sterile filtration with a 0.22 µm membrane, then treating a sample (20 µg/ml protein) with 1:4,000 formalin for 72 hours at 37°C. The formalin-treated sample was then adsorbed on alum by adding 0.086 ml of a sterile 5.44% solution of potassium aluminum sulfate, with stirring, to each ml of sample and adjusting the pH of the mixture to 8.75 with 1 N NaOH. After stirring for two hours, the suspension was centrifuged 10 minutes at 1000 x g. The supernatant liquid was decanted from the alum precipitate and discarded. The alum adsorbed rHBeAg was resuspended in physiological saline to the original volume and thimerosal was added to a concentration of 1:20,000. The preparation was tested in mice undiluted and diluted in alum placebo to contain 0.025, 0.008 and 0.0016 µg/ml. One milliliter of each preparation, or alum placebo, was injected intraperitoneally into each of 10 mice. The mice were individually bled 28 days later and antibody titers were measured by the AUSAB® radioimmuno assay (Abbott). Data were analyzed to determine seroconversion rates to the different doses of subunit HBeAg. A probit analysis was performed, plots of empirical probit versus dose were made, iterative maximum likelihood least squares performed on the data and the ED₅₀ values (µg) were obtained.

Results of the mouse potency tests are summarized in the table as follows. Treatment with urea-DDT substantially improved mouse potency.

TABLE
Mouse Potency Tests of rHBeAg

Lot No.	Starting Material	Treatment		Mouse Potency ^b ED ₅₀ (µg)
		Urea-DDT	KSCN ^a	
87706	Butyl agarose product	No	No	1.01
		No	Yes ^c	0.74
		Yes ^d	No	0.44
		Yes ^d	No	0.82
		Yes	Yes	0.57
87708	Butyl agarose product	Yes	Yes	0.36
87708	Pre-Sepharose 6B pooled (waste) fraction ^e	No	Yes	1.36
		Yes	No	1.11
		Yes	Yes	0.98

United States Patent [19]
Van Wijnsendaele et al.

[11] **Patent Number:** **4,649,192**
 [45] **Date of Patent:** **Mar. 10, 1987**

[34] **METHOD FOR THE ISOLATION AND PURIFICATION OF HEPATITIS B SURFACE ANTIGEN USING POLYSORBATE**

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[21] **Appl. No.:** 739,415

[22] **Filed:** May 30, 1985

[51] **Int. Cl.⁴** A61K 39/29; C07K 3/18; C07K 3/28

[52] **U.S. Cl.** 530/371; 424/89; 530/380; 530/415; 530/806; 530/824; 530/808

[58] **Field of Search** 424/89; 260/112 R, 112 B; 530/380, 371, 415, 806, 824, 808

[56] **References Cited**

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4,481,189	11/1984	Prince	434/101

4,554,157	11/1985	Shelly et al.	434/89
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[57] **ABSTRACT**

The supernatant of engineered yeast cells having produced hepatitis B surface antigen and disrupted in the presence of a polysorbate non-ionic detergent is clarified by addition of urea and adsorbed on colloidal silica from which the purified antigen is desorbed with a low ionic strength buffer supplemented with urea and a polysorbate non-ionic detergent.

9 Claims, No Drawings

METHOD FOR THE ISOLATION AND PURIFICATION OF HEPATITIS B SURFACE ANTIGEN USING POLYSORBATE

FIELD OF THE INVENTION

This invention relates to the preparation of an improved method for the isolation and purification of hepatitis B surface antigen (HBsAg) valuable for vaccinal purpose and to the preparation thereof.

The HBsAg antigen obtained by the process of the invention is in the form of 22 nm-like HBsAg particles. It is valuable for the preparation of vaccines.

BACKGROUND OF THE INVENTION

Hepatitis B is a widespread and potentially fatal viral disease. Although its etiological agent—i.e. the hepatitis B virus—has been isolated a long time ago from the blood of patients suffering of the disease, the development of a commercial hepatitis B vaccine has faced considerable drawback: hepatitis B virus is a 42 nm particle called the Dane particle consisting of (a) a core containing the viral genome bound to the core protein and containing the core antigen (HBcAg) which is not immunogenic and (b) an envelope containing the immunogenic surface antigen (HBsAg). HBsAg is a group of morphologically heterogeneous complex macromolecular structures; it contains proteins, carbohydrates, glycoproteins and lipids, the main constituents of which are phosphatidylcholine, cholesterol ester, cholesterol and triglycerides. Infection with hepatitis B virus (HBV) leads not only to the production of Dane particles but also to overproduction of 22 nm particles and filaments containing the elements of the surface envelope. It is known that these 22 nm particles are about 1000-fold more immunogenic than the monomer HBsAg protein but the replication and expression of HBV has been hampered for a long time by the lack of an appropriate cell culture system to grow the virus *in vitro*. The lack of *in vitro* culture systems in which the virus can be efficiently propagated led to the development of searches towards either synthesis of HBsAg-like molecules or expression of HBsAg in alternative host systems by using recombinant DNA technique. For instance, expression of HBsAg has been reported from yeast cells transformed with DNA vectors bearing the gene for HBsAg and it is known that the HBsAg synthesized in yeast is assembled into particles having properties similar to the 22 nm particles secreted by human cells (P. VALENZUELA et al. *Nature*, 298; 347-350; 1982 and R. A. HITZEMANN et al. *Nucl. Ac. Res.* 11; 2743-2763; 1983). The production of HBsAg by recombinant technique in engineered cell cultures, for instance engineered yeast strains cultures is well known to the art (see for instance European Patent application publication No. 0 106 828; W. J. McALEER et al. in *Nature* 307; 178-180, 1984 and C. E. CARTY et al. in Abstract 030 of the annual meeting of the American Society for Microbiology 1984, 84th meeting, Mar. 4-9 1984).

Numerous articles and patents describing processes of extracting and purifying HBsAg from plasma, serum or other blood products or biological fluids or engineered cell cultures have also appeared.

The process of the invention is applicable to engineered yeast cells producing HBsAg.

The extraction and purification of HBsAg from an engineered yeast cell culture which has been grown to

a satisfactory cell density generally requires 3 successive steps which are:

- (1) removing HBsAg from the cell interior;
- (2) enriching the medium in HBsAg;
- (3) eliminating substantially all contaminants from the medium.

In most procedures, step (1) is performed by mechanical forces such as shearing forces (for example X-press or French press) or shaking the glass beads, eventually with addition of a detergent. The use of a non ionic detergent (Triton X-100) has been reported by K. MURRAY et al. in *The EMBO J.* 3; 645-650; 1984 and by R. A. HITZEMANN et al. *loc. cit.*

It has also been reported that incubation of HBsAg with detergents breaks down the particle. With that view

GB Pat. No. 2 093 039 discloses treatment of HBsAg with a non ionic detergent (Triton X-100) to form a polypeptide mixture which is introduced on top of an aqueous buffer solution containing a sucrose gradient and recovering a fraction substantially free from detergent and containing micelles of the polypeptide mixture;

EP Pat. No. 0 005 864 discloses contacting antigenic mass containing hepatitis B surface antigen (HBsAg) and core antigen (HBcAg) of a particle size of at least 20 nm with a detergent (e.g. Tween 20 or 80) followed by treating the detergent containing antigenic composition with an aqueous aldehyde solution. The obtained product is a subparticulate mass having a particle size less than 20 nm especially less than 5 nm.

Japanese Patent application No. 53 104 724 (Derwent abstract 75324 A) discloses a process wherein HBsAg particles are heated in the presence of (1) a non ionic surfactant (i.e. polyoxyethylene alkylphenyl with 7-10 oxyethylene chains), (2) a protein-denaturing agent (i.e. urea or guanidine) and (3) optionally a reducing agent. The obtained product has a particle size comprised between 10 and 20 nm.

Japanese Patent application No. 50 160 420 (Derwent abstract 27420 A) discloses a process wherein HBsAg is treated with different surfactants to produce subunit products.

W. German Patent application No. 26 11 723 discloses a process wherein HBsAg spherical particles are obtained by warming the antigen in presence of a defatting surfactant.

U.S. Pat. No. 4,113,712 discloses HBsAg particles composed of single polypeptide subunits prepared by heating HBsAg in isotonic sodium chloride solution at about neutral pH containing a surfactant capable of delipidation.

U.S. Pat. No. 4,481,189 discloses a process wherein blood plasma or plasma derivative is sterilized by contact with a detergent (e.g. a non ionic detergent).

Steps (2) and (3) incorporate a variety of techniques, among which adsorption/desorption using colloidal silica, more particularly 'AEROSIL' (a product sold by DEGUSSA, Frankfurt, FRG.)

Such technique is described in the following references:

W. J. M. DUIMEL et al. in *Vox Sang.* 23; 249-255; 1972;

J. PILLOT et al. in *J. Clin. Microbiol.* 4; 205-207; 1976 and in *Molec. Immunol.* 21; 53-60; 1984.

F. BARIN et al. in *Ann. Microbiol. (Inst. Pasteur)* 129B; 87-100; 1978

which teach that at best only a fraction (i.e. about 60%) of the adsorbed HBaAg is recovered (the best results being obtained by elution with a low ionic strength buffer) unless some amount of deoxycholate be added to the eluant.

In another study (SIEBKE et al. in *Acta Path. Microbiol. Scand. Section B*; 80; 935-936; 1972), it has been pointed out that Australian antigen is partly eluted from Aerosil by 2% Tween 80 and, likewise, is poorly adsorbed in the presence of the detergent.

DESCRIPTION OF THE INVENTION

When the previously known technique involving adsorption to silica gel and desorption with deoxycholate buffer is applied to a supernatant resulting from the lysate of post-culture HBaAg producing cells obtained by addition of a non ionic detergent, it has been noticed that, not only it is not possible to obtain a clear supernatant of the disrupted cells but the use of deoxycholate buffer for the desorption from colloidal silica does only lead to partial desorption of HBaAg; on the contrary, there is a breakdown of the yeast particle as shown by chromatography on Sepharose 4B-C1 since no hepatitis antigen is then detected at the usual position (K_d : 0.25).

By gradient centrifugation in sucrose it has also been found that, when the disruption of the yeast cells is performed in the presence of 0.5% (w/v) Triton X-100, the structure of the HBaAg particle does not remain intact whereas when 0.5% (v/v) of a polysorbate (e.g. polysorbate 20) is substituted for Triton X-100, the yield of intact HBaAg is substantially improved.

Moreover, it has now also been found that, when the supernatant of yeast cells disrupted with a polysorbate non ionic detergent (e.g. polysorbate 20 or 80) is supplemented with urea—made up to a final concentration from 2 to 6M,—there is obtained (e.g. by stirring for 30 minutes at room temperature) a partially clarified solution.

Furthermore, it has also been found that, upon treatment of the clarified solution with Aerosil, the HBaAg can be completely desorbed therefrom with a low ionic strength buffer system when an urea/non ionic detergent additive is substituted for the previously used deoxycholate additive.

The completeness of HBaAg desorption performed by this way has been shown by the absence of a 22K band in desorbed colloidal silica and it has been confirmed that the isolated HBaAg is assembled into particles of about 22 nm which induce high level of HBaAg antibodies upon parenteral administration to laboratory animals.

Thus, it is a first object of the present invention to provide an improved method for preparing hepatitis B surface antigen valuable for vacccinal purpose from a culture of engineered yeast cells having expressed HBaAg which process comprises disrupting the cells in the presence of a non ionic detergent—more particularly a polysorbate (e.g. 0.3% v/v polysorbate 20 or 80)—and adding thereto urea up to final concentration 2-6M, to yield a clarified HBaAg solution (e.g. by stirring for 30 minutes at room temperature) adsorbing the HBaAg on colloidal silica (such as AEROSIL) (e.g. by stirring overnight at 4° C.), desorbing HBaAg at 37° C. at a pH comprised between 8 and 9.5 with a low ionic strength buffer (e.g. 10 mM) supplemented with urea at final concentration 8M and with a non ionic detergent, more particularly a polysorbate (e.g. polysorbate 20 or 80 at a final concentration 0.1-1% v/v).

The desorbate contains purified HBaAg particles which can be further processed according to any technique well known to the art, e.g. ultrafiltration, chromatography on agarose, precipitation with dextran sulfate/calcium chloride and dialysis.

The invention is illustrated by the following examples.

EXAMPLE 1

10 Pelleted cells (80 g wet cells weight) of an engineered yeast strain culture expressing HBaAg which have grown up to 30 g dry cells weight per liter of culture are suspended in 150 ml of Na_2HPO_4 aqueous solution (7.098 g/l). This suspension is supplemented with 3 ml 4% (w/v) EDTA solution, 0.9 ml polysorbate 20 and 90 ml isopropanol containing 60 mg phenylmethylsulfonyl fluoride (PMSF). The pH is adjusted to 8.1 with NaOH (10% w/v in water). The suspension is refrigerated in an ice bath and disrupted by 2 passages through a cooled glassbead homogenizer. The homogenate is then centrifuged for 30 minutes at 13,000 g.

20 The crude extract supernatant (160 ml) is made up to a final concentration of 4M urea and stirred for 30 minutes at room temperature to detach HBaAg particles from contaminating yeast cell membranes and AEROSIL @380 (2.5% w/v) prewashed in 50 mM phosphate buffer ($\text{NaH}_2\text{PO}_4/\text{NaOH}$) pH 7.2 containing 0.3% (v/v) polysorbate 20 and 4M urea is added thereto. The solution is stirred overnight at 4° C. to allow maximal adsorption of HBaAg particles on the silica particles.

35 The colloidal silica is then centrifuged for 15 minutes at 6300 g and washed twice with 250 ml portions of 10% (w/v) NaCl.

The washed silica is suspended in 80 ml of 10 mM borate buffer pH 9.0 ($\text{Na}_2\text{B}_4\text{O}_7/\text{HCl}$) supplemented with 0.5% (v/v) polysorbate 20 and urea (at final concentration 8M) and the suspension is stirred for 4 hours at 37°.

40 The desorbed colloidal silica is then centrifuged for 30 minutes at 27,000 g to spin down a maximal amount of colloidal silica particles.

Gradient centrifugation in a sucrose gradient as well as electron microscopy show that the antigen is present in particle form having a diameter of about 22 nm.

The desorbate is concentrated to 15 ml in an AMICON DC (an apparatus sold by AMICON CORP. DANVERS, MA, USA) equipped with a cartridge having a cut off of 100,000 daltons and applied to a column (82.5 cm $\times$ 100 cm) on SEPHAROSE 4B-C1 (an agarose gel manufactured and sold by PHARMACIA FINE CHEMICALS, Uppsala, Sweden) equilibrated in 10 mM trometamol/HCl buffer (pH 8.0) containing 0.3% (v/v) of polysorbate 20.

55 The antigen containing peak (K_d =0.25) is treated with dextran sulfate (MW 50,000) at a final concentration 0.1% (w/v) and with CaCl_2 at a final concentration of 40 mM.

Excess dextran sulfate is precipitated by dialysis against 40 mM BaCl_2 solution in 10 mM trometamol/HCl buffer (pH 8.0). The dialysate (30 ml) is submitted to CaCl_2 gradient centrifugation and the HBaAg containing peak (9 ml) is collected.

60 The HBaAg purification at the different stages of the hereabove process are summarized in Table II.

Scanning analysis by SDS-PAGE gel shows that the final product is at least 90% pure in HBaAg.

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TABLE I

Fraction	HBaAg (μ g)	Protein (mg)	Poly- saccharides (mg)	Nucleic acids (μ g)
Crude extract super- natant	1790	994	333	3300
Concentrated desor- bate	1000	49	21.5	700
Kd 0.25 peak	328	20	15	780
Dextran sulfate supernatant	432	2.8	32	724
BaCl ₂	432	2.2	17.5	790
CaCl gradient	270	0.306	0.378	108

(a) by RIA (Radio Immune Assay)

EXAMPLE 2

Pelleted cells (80 g wet cells weight) of an engineered yeast strain culture expressing HBaAg which have grown up to 30 g dry cells weight per liter of culture are suspended in 150 ml of Na₂HPO₄ aqueous solution (7.098 g/l). This suspension is supplemented with 3 ml 4% (w/v) EDTA solution, 0.9 ml polysorbate 20 and 9 ml isopropanol containing 60 mg phenylmethylsulfonyl fluoride (PMSF). The pH is adjusted to 8.1 with NaOH (10% w/v in water). The suspension is refrigerated in an ice bath and disrupted by 2 passages through a cooled glassbead homogenizer. The homogenate is then centrifuged for 30 minutes at 13,000 g.

The crude extract supernatant (160 ml) is made up to a final concentration of 4M urea and stirred for 30 minutes at room temperature to detach HBaAg particles from contaminating yeast cell membranes and AEROSIL®380 (2.5% w/v) prewashed in 50 mM phosphate buffer (NaH₂PO₄/NaOH) pH 7.2 containing 0.3% (v/v) polysorbate 20 and 4M urea is added thereto. The solution is stirred overnight at 4° C. to allow maximal adsorption of HBaAg particles on the silica particles.

The colloidal silica is then centrifuged for 15 minutes at 6300 g and washed twice with 250 ml portions of 10% (w/v) NaCl.

The washed silica is suspended in 80 ml of 10 mM borate buffer pH 9.0 (Na₂B₄O₇/HCl) supplemented with 0.5% (v/v) polysorbate 20 and urea (at final concentration 8M) and the suspension is stirred for 4 hours at 37°.

The desorbed colloidal silica is then centrifuged for 30 minutes at 27,000 g to spin down a maximal amount of colloidal silica particles.

The desorbate is dialyzed against 10 mM trometamol/HCl buffer (pH 7.5) in an AMICON DC equipped with a cartridge having a cut-off of 100,000 daltons in order to dialyze out urea and applied to a column of 25 ml of hexylagarose (a product manufactured and sold by PHARMACIA FINE CHEMICALS, Uppsala, Sweden) equilibrated in 10 mM trometamol/HCl buffer (pH 7.5) at a flow rate of 10 ml per hour.

The column is then washed with 10 mM trometamol/HCl buffer (pH 7.5) and eluted with a 50/50 (v/v) mixture of ethylene glycol and 10 mM trometamol/HCl buffer (pH 7.5) supplemented with NaCl at a final concentration 1M and thereafter with 10 mM trometamol/HCl buffer (pH 7.5) supplemented with urea at a final concentration 4M.

The HBaAg purification at the different stages of the hereinabove process are summarized in Table II.

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TABLE II

Fraction	HBaAg (μ g)	Protein (mg)	Poly- saccharides (mg)	Nucleic acids (μ g)
Crude extract	3.5	2234	517	17.9
Desorbate	2.1	86.4	36.1	2.1
Ethylene glycol/ NaCl eluate	1.4	1.10	0.05	ND (2)
Urea eluate	0.7	0.72	0	ND (2)

(1) by RIA (Radio Immune Assay)

(2) ND = non detectable

Scanning analysis by SDS-PAGE gel shows that the final product is at least 97% pure in HBaAg.

EXAMPLE 3

The technique is that of Example 2 but 25 ml of phenylagarose (a product manufactured by PHARMACIA FINE CHEMICALS, Uppsala, Sweden) is substituted for the hexyl agarose therein specified.

The HBaAg purification scheme and the HBaAg content of the final product are similar to those given in Example 2.

EXAMPLE 4

The solution of HBaAg obtained at the end of Example 1 was adjusted to a protein content of 10 μ g per ml by addition of NaCl, phosphate buffer (Na₂HPO₄/NaH₂PO₄) and ALHYDROGEL® (an aluminum hydronide gel manufactured and sold by SUPERPHOS Export Co., Copenhagen, Denmark) up to final concentration of 0.9% (w/v), 20 mM and 0.15% (w/v) of Al(OH)₃ respectively, the final pH being 6.9.

The preparation was sterilized and distributed into 2 ml glass vials, each containing a one ml dosage unit of vaccine.

EXAMPLE 5

Different dilutions of dosage units of the vaccine preparation of Example 4 were administered by intraperitoneal route to 5 groups of five-week old Swiss mice.

Twenty eight days after administration, the animals were bled and the serum anti-HBa titres were determined for each sample by the AUSAB® method (AUSAB is the registered trade mark of a test kit manufactured and sold by ABBOTT Diagnostic Products GmbH, Wiesbaden, FRG).

At the same time and following the same vaccination schedule, same dilutions of dosage units of a vaccine preparation obtained by the technique of Example 4 but with an antigen prepared by the technique of Example 1 without the colloidal silica step were also administered to 5 groups of five week old Swiss mice.

The results of this comparative trial are summarized in Table III wherein (A) refers to the vaccine obtained by the technique of the invention urea while (B) refers to the vaccine obtained by the process without colloidal silica step.

TABLE III

Vaccine preparation	Protein (μ g)	Number of mice	Seroconversion rate (%)	ED ₅₀ (μ g RIA)
A	.039	10	0	180
	.156	10	60	
	.625	10	90	
	2.5	10	90	
	10	6	83	
B	.039	10	10	

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TABLE III-continued

Vaccine preparation	Protein (mg)	Number of mice	Seroconversion rate (%)	ED ₅₀ (mg RIA)
	.156	10	10	1934
	.313	10	20	
	2.4	10	30	
	10	10	80	

ED₅₀: effective dose in 50% of the tested animals.
RIA: Radio Immune Assay

What we claim is:
1. A method for the isolation and purification of hepatitis B surface antigen by treatment with colloidal silica of the supernatant of engineered yeast cells having produced said antigen and disrupted in the presence of a non ionic detergent which is more particularly a polysorbate which comprises clarifying the supernatant by addition of urea, adsorbing the antigen on the colloidal silica, desorbing therefrom the antigen with a low ionic buffer (pH 8-9.5) supplemented with urea and with a non ionic detergent.

2. A method according to claim 1 wherein the clarification of the supernatant is performed by addition of urea up to a final concentration comprised between 2 and 6M.

3. A method according to claim 1 or 2 wherein the clarification step is performed at 4° C.

4. A method according to claim 1 or 2 wherein the low ionic strength buffer is 10 mM.

5. A method according to claim 2 wherein urea is supplemented to the low ionic buffer up to a final concentration 8M.

6. A method according to claim 2 or 5 wherein the desorption is performed at 37° C.

7. A method according to claim 2 wherein the non ionic detergent supplemented to the low ionic strength buffer is a polysorbate.

8. A method according to claim 7 wherein the polysorbate is polysorbate 20 or 80.

9. A method according to claim 7 wherein the final concentration in polysorbate is comprised between 0.1 and 1% (v/v).

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