Production of Antibodies in Transgenic Plants

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REPORTES

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Plant Bioreactors

The first transgenic plants were reported in 1983 [1, 2]. Since then, many recombinant proteins have been expressed in several important agronomic species of plants including tobacco, corn, tomato, potato, banana, alfalfa, and canola [3]. Recent work suggests that plants will be an economic bioreactor for large-scale production of industrial and pharmaceutical recombinant proteins [3-6]. Perhaps most important are the cost benefits of plant production. For example, [3] calculated the cost of producing a recombinant protein in various agricultural crops. Although crops with more protein content (*e.g*. soybeans 400%, versus potatoes, 2%) are more cost effective. These costs are 10- to 50-fold less than protein produced at high-level in *E. colí* (*i.e*. 20% of total protein). Depending upon the use of the protein and the requirements for purification for *in vivo* pharmaceutical use, purification costs will obviously augment final product costs; however, at the hundred kilogram to metric ton level plant produced proteins will provide obvious savings.

To date three immunotherapeutic products produced in plants have entered the clinic: two antibodies and an oral vaccine (Table).

Antibodies in plants: plantibodies

Although antibodies were first expressed in plants in the mid-1980s (Steiger, During) by two German graduate students, the first report was published in 1989 [7]. Since then a diverse group of "plantibody" types and forms have been prepared. Originally foreign antibody genes were introduced into plant cells by nonpathogenic strains of the natural plant pathogen *Agrobacterium tumefaciens* and regeneration in tissue culture resulted in the recovery of stable transgenic plants. Although this initial work to generate multichain proteins required crossing of plants expressing each chain, more recent studies have shown that multiple chains can be introduced via a single biolistic transformation event [8] (Wycoff *et al*., unpublished data), greatly reducing the time to final assembled plantibody.

SIgA: a novel antibody isotype

This laboratory has focused on the production of secretory IgA (SIgA) plantibodies [9]. At the present time plants offer the only large-scale commercially viable system for production of this unique form of antibody. SIgA is the most abundant antibody class produced by the body (>60% of total immunoglobulin). SIgA is secreted onto mucosal surfaces to provide local protection from toxins and pathogens. Dimeric IgA containing J chain derived from submucosal B cells binds to the epithelial cell polyimmunoglobulin receptor (PIG ^{R}) that transports the IgA to the mucosal surface. Binding triggers transcytosis to the mucosal surface where protease releases a portion of the PIG^{R} called secretory component conveniently used to bind the SlgA. The secretory component protects the dimeric IgA from proteases and denaturation on the mucosal surface. Previously it was not possible to obtain therapeutic quantities of this class of immunoglobulin. The recent availability of large amounts of secretory IgA plantibodies opens up a number of novel therapeutic opportunities for disorders of the mucosal immune system. These include therapies for intestinal pathogens such as hepatitis viruses, *Helicobacter pylori*, and enterotoxigeníc *E. coli*, cholera etc., respiratory pathogens such as rhinovirus and influenza, and genitourinary sexually transmitted diseases and contraception.

Table

Protein	Target	Company	Development stage
SIgA	Caries	PLANET	Phase II
IgG	Cancer	NeoRx/Monsanto	Phase II, dropped (unpub.)
E. coli	*E. coli* diarrhea	Boyce Thompson	Phase I/II

Clinical studies of CaroRx^{TM} anti-*S. mutans* SIgA to prevent dental caries

The most clinically advanced SlgA plantibody, called CaroRx^{TM}, recognizes and inhibits the binding of the major oral pathogen, *S. mutans* to teeth. In preliminary work, a series of *in vivo* passive immunization experiments was carried out in 84 human subjects using murine anti-*S. mutans* antibodies [4-6]. Topical application of anti-*S. mutans* antigen SA I/II MAbs prevented colonization of both artificially implanted exogenous strains of *S. mutans*, as well as natural recolonization by indigenous *S. mutans*. In these studies the pathogenic *S. mutans* was replaced by endogenous flora.

The presence of the complement-activating and phagocyte-binding sites on the Fc fragment of the MAb was not essential for activity, because the F(ab')$_2$ portion of the MAb was as protective as the intact IgG; however, the Fab fragment failed to prevent recolonization of *S. mutans*. Prevention of recolonization was specifically restricted to *S. mutans*, as the proportion of other organisms, such as *S. sanguis*, did not change significantly. The surprising feature of these experiments was that protection from recolonization by *S. mutans* lasted up to 2 years (Ma J, personal communication), although MAb was applied for only 3 weeks and functional MAb was detected on the teeth for only 3 days following the final application of MAb. All studies indicated that this form of immunotherapy appears to be safe and well tolerated. The long-term protection could therefore not be accounted for by a persistence of MAb on the teeth, but may be due to a shift in the microbial balance in which other bacteria

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occupy the ecological niche vacated by *S. mutans*, resulting in resistance to recolonization by *S. mutans*.

The antigen-binding V regions of the best murine MAb identified by Ma and Lehner, Guy's 13, has been used to create an SlgA plantibody produced in tobacco designated CaroRx™ [9, 14]. Levels of production of CaroRx™ in tobacco are up to 0.5 mg/gram fresh weight. Future plans call for production of CaroRx™ in corn and other cereal grains. CaroRx™ has been produced and purified from tobacco under GMP conditions for clinical testing in the UK and USA. CaroRx™ was engineered with an additional IgG CH2 domain to facilitate purification of the antibody by protein G affinity chromatography. By protein G affinity purification CaroRx™ can be recovered with a high purity from green plant tissue.

Clinical evaluation of CaroRx™ in a pilot Phase ll trial has been completed at Guy's Hospital, London, UK [14]. In this trial a functional comparison was made between CaroRx™ and the parent IgG monoclonal antibody Guy's 13. BIACORE analysis revealed that the affinity of the antibodies for purified *S. mutans* SA l/ll was similar (Kd=0.5-1.3 x 10^{-9} M); however CaroRx™ had 4-fold higher avidity (functional affinity), a not unexpected result given the tetravalent binding of the SlgA.

Using an experimental design similar to that used to demonstrate activity of the parent MAb, CaroRx™ gave specific protection against colonization by oral streptococci for over four months [14]. In addition to this therapeutic endpoint, pharmacokinetics studies showed that in the human oral cavity, CaroRx™ survived for >3 days versus 1 day for the IgG antibody and multiple serum antibody samples were negative for human anti-mouse (HAMA) or anti-rabbit antibodies. There was no evidence of local or systemic toxicity of the topically applied plantibody.

These initial clinical studies demonstrate that topically applied anti-*S. mutans* SlgA plantibody (CaroRx™) is safe (no HAMA, no local or systemic toxicity) and prevents colonization by *S. mutans*, the major cause of human dental caries [14]. Planet Biotechnology Inc. has submitted an IND (investigational new drug application) to the US FDA and Phase l/ll confirmatory clinical trials are underway at the School of Dentistry at the University of California in San Francisco.

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New Approaches to Quantitative Proteome Analysis

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With the completion of a rapidly increasing number of complete genomic sequences much attention is currently focused on the questions if and how the information contained in sequence databases can be interpreted in terms of the structure, function and control of biological systems. Quantitative proteome analysis, the global analysis of protein expression, has been proposed as a method to study genes at a steady state and after perturbation-induced changes. Here we discuss the justification for gene expression analysis at the protein level, highlight the limitations in the current standard proteome technology, and introduce a new experimental approach to quantitative proteome analysis.

The poor correlation between mRNA and protein levels in cells provides justification for quantitative proteome analysis. With recent technical advances including the development of differential display-PCR [1], cDNA microarray and DNA chip technology [2, 3], and serial gene analysis (SAGE) [4, 5], it is now feasible to establish global and quantitative mRNA expression maps of cells and tissues in species for which the sequence of all the genes is known. The discoveries of post-transcriptional mechanisms which control translation rate [6] and protein and mRNA half-lives [7] led us to predict that quantitative transcript expression measurements are insufficient for predicting the quantity of protein expression. To test this hypothesis we determined the correlation between the mRNA and protein levels for a group of genes expressed in exponentially growing cells of the yeast *Saccharomyces cerevisiae.* Protein expression levels were quantitated by metabolic labeling of the yeast proteins to a steady state, followed by 2D-gel electrophoresis and liquid scintillation counting of the selected, separated protein species. Separated proteins were identified by tryptic digestion of spots with subsequent analysis by microcapillary high performance liquid chromatography-tandem mass spectrometry (mLC-MS/MS) and sequence database searching [8-10]. The corresponding mRNA transcript levels were calculated from serial analysis of gene expression (SAGE) frequency tables [5].

The correlation between mRNA and protein levels was calculated for a data set consisting of more than 100 mRNA and protein products of selected genes. For the entire set of genes, there was a general trend of increased mRNA levels resulting in increased protein levels. The Pearson product moment correlation coefficient for the whole data set was 0.935. This number is highly biased by a relatively small number of genes with very large protein and message levels. A more representative subset of the was the group of genes for which message level was measured below 10 copies/cell. This subset included 70% of the data used in the study. The Pearson product moment correlation coefficient for this data set was 0.356. This weak correlation is further evident by the observation that levels of protein expression coded for by mRNA with comparable abundance varied by as much as 30 fold and that the mRNA levels coding for protein with comparable expression levels varied by as much as 20 fold. This study, for the first time, correlated the mRNA transcript and protein expression levels of a relatively large number of genes expressed in cells representing the same state. lt is apparent that the observed correlation is not sufficiently high to allow for protein levels to be predicted by mRNA levels.

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We therefore conclude that quantitative proteome analysis is an essential component of any comprehensive analysis of biological systems.

Current proteome technology is biased towards the analysis of high abundance proteins: the current standard approach to quantitative proteome analysis is based on the separation of proteins by 2D-gel electrophoresis (2DE) and the subsequent identification of individually separated and detected protein spots by mass spectrometry or tandem mass spectrometry followed by sequence database searching [9-11]. The method is sequential, labor intensive and difficult to automate. lt does, however, provide precise quantitation and is well suited to reveal relative changes in protein expression, clusters of concurrently regulated proteins and additional features which affect the electrophoretic mobility of proteins, including post-translational protein processing and modifications. As a true proteome technology, the 2DE/MS/MS method would be expected to display every protein in a protein mixture. To assess to what extent the 2DE protein pattern obtained from a total yeast lysate represented the proteome of this microorganism, we related protein expression levels from protein detected by silver staining to the predicted expression levels of all the open reading frames (ORF) in the yeast.

Prediction of the level of protein expression was based on the codon bias of the respective genes. The codon bias indicates the propensity for a gene to utilize the same codon to encode an amino acid even though other codons would insert the identical amino acid into the growing polyeptide chain. Its value varies between 960.3 and 1.0, and it has further been found empirically that highly expressed proteins have large codon bias values (>0.2) and proteins expressed at low levels have low codon bias values (>0.1) [12]. Comparison of the codon bias distributions for all the yeast ORFs with the distribution of all the proteins analyzed by 2DE, silver staining and tandem mass spectrometry indicated that the population of proteins analyzed by the standard 2DE/MS/MS proteome analysis technique was highly biased towards the most highly expressed proteins. No proteins with codon bias values <0.2 were detected, whereas the majority of ORF92s predicted from the yeast genome sequence have codon bias values <0.2. We therefore conclude that the current proteome technology, used without sample pre-enrichment is not a true proteome technology and that the construction of complete proteome maps will be very challenging, even for relatively simple, unicellular organisms.

A novel method for quantitative proteome analysis

To address the limitations inherent to the 2DE/MS/MS method to proteome analysis, we have developed a new experimental approach. lt is intended to retain relative quantitative information while still rapidly and conclusively identifying even the minor components of a mixture. This method is based on a class of new chemical reagents termed isotope coded affinity tags (ICAT) and MS/MS. ICAT reagents consist of three functional units, namely a chemical reactivity directed towards a functional group in proteins (e.g. SH, NH_2, COOH), a linker group synthesized in isotopically heavy and light forms, respectively, and an affinity tag (typically a biotin group) [13].

The ICAT strategy consists of the following steps. Proteins in protein mixtures 1 and 2 are treated after reduction with a sulfhydryl-specific ICAT reagent. The reagents exist in two forms: isotopically light (d0) and isotopically heavy (d8). The heavy and light forms are used to derivatize the proteins in samples 1 and 2, respectively. After treatment with the ICAT reagents the samples are mixed, At this point, any optional fractionation technique can be performed to enrich for low abundance proteins or to reduce the complexity of the mixture, while the relative quantities are maintained. The combined protein sample is then proteolyzed and the ICAT-tagged peptides are selectively enriched by avidin-biotin affinity chromatography. These peptides are separated and analyzed by microcapillary HPLC-ESI-MS/MS. The relative ion intensities of the two differentially isotopically tagged forms of a specific peptide indicate their relative abundance. Such pairs of tagged peptides are easily detected because they essentially co-elute from the column and because of the eight mass units difference encoded in the ICAT tag, which is detected in the mass spectrometer. Every other scan is devoted to fragmenting and then recording sequence information about an eluting peptide (MS/MS spectrum). The protein from which this peptide originated is then identified by searching a sequence database with the recorded MS/MS spectrum. The procedure thus provides the relative quantitation and identification of the components of protein mixtures in a single analysis.

In this manuscript, we argue that in the emerging post-genomic era technologies that can quantitatively, globally, and automatically measure gene expression at the protein level are essential for the comprehensive analysis of biological processes and systems. We indicate the limitations of the current standard method for large scale protein analysis with respect to the analysis of low abundance proteins and propose a new approach to quantitative proteome analysis. We anticipate that the new ICAT strategy will provide broadly applicable means for the quantitative cataloging and comparison of expressed proteins in a variety of normal, developmental, and disease states.

Acknowledgments

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Establecimiento a escala productiva de un proceso para la purificación de interferón alfa 2b humano recombinante que utiliza la cromatografía de afinidad por iones metálicos

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Introducción

El IFN alfa 2b humano recombinante se considera actualmente como un producto de gran demanda en el mercado biotecnológico debido al amplio espectro de aplicaciones en el tratamiento de diversas enfermedades humanas como agente antiviral y antiprotiferativo. Durante casi 20 años se han venido desarrollando diversas tecnologías para la obtención de un producto con una calidad óptima, y se han aplicado diferentes métodos de purificación en los cuales se incluyen específicamente la cromatografía de afinidad por anticuerpos monoclonales [1].

Actualmente, debido a los altos costos productivos que generan la producción de IFN Alfa 2b recombinante mediante la tecnología de purificación con anticuerpos monoclonales, se necesitan otras alternativas que posibiliten la obtención de un producto con niveles de pureza similares o superiores a la cromatografía de inmunoafinidad [2] y con niveles de recuperación elevados. El método de purificación por cromatigrafía de afindad por iones metalicos IMAC ha sido seleccionado como una de las alternativas para esta sustitución.

En este trabajo se reflejan los distintos resultados obtenidos a escala semipreparativa en el incremento del nivel de pureza del debris celular que posteriormente se solubiliza y se incorpora a las etapas de purificación, y los diferentes resultados que se obtuvieron en la IMAC que se establece en sustitución de la cromatografía de afinidad por anticuerpos monoclonales.

Materiales y Métodos

Equipamiento. Homogeneizador de Alta Presión APV Gaulin, Centrifuga Beckman J21, Homogeneizador T50, Detector UV, 280 nm (Pharmacia LKB), Sistema HPLC (Pharmacia LKB), Columna cromatográfica XK 16120 (Pharmacia), Los reactivos empleados fueron de calidad purísima, gel de quelato IDA-Sepharose CL-6B, gel Sephadex G-25 (Pharmacia, Suecia), gel de intercambio catiónico Fractogel EMD COO- 650 (Merck, Alemania), columna analítica C8, semipreparativa C4 y preparativa C4 de fase inversa (rp) VYDAC (USA).

Ruptura y lavado celular. La biomasa obtenida en el proceso de fermentación (conteniendo 10% de IFN respecto al total de proteínas), fue sometida a un proceso de ruptura celular y lavado, de acuerdo con las condiciones establecidas previamente [3].

Desnaturalización y renaturalización. El material lavado que contenía el IFN en forma insoluble fue solubilizado en una solución de GuHCI 6 M, Tris 50 mM, NACI 50 mM en condiciones reductoras (DTT 2 mM) a una concentración de proteínas de 3 mg/mL. La renaturalización del IFN se realizó por dilución lenta, usando una solución RE (Tris 50 mM, NACI 50 mM pH 7,2). Se utilizó como agente oxidante $CuSO_4$ 80 mM.

Purificación. A partir de resultados previos en cuanto a las condiciones de trabajo a escala analítica para la purificación de IFN alfa 2b humana recombinante por IMAC [4], se realizaron estudios a escala preparativa en cuanto a capacidad, velocidad lineal y presencia de impurezas. Con las mejores condiciones se realizaron tres lotes consecutivos a escala productiva, con lo que se demostró la eficacia de la nueva tecnología que se propone a usar en cuanto a calidad del producto final y disminución de costos.

Técnicas analíticas. La concentración de IFN fue determinada por ELISA, las proteínas totales por Lowry, la pureza por electroforesis y por RP-HPLC analítico, así como las técnicas específicas para la liberación de un lote de materia prima activa [5].

Resultados y Discusión

La combinación del lavado inicial con detergentes y a continuación con urea, ofreció resultados de interés en este trabajo por obtenerse el IFN con valores de pureza superiores a 50%, manteniendo un recobrado del IFN mayor de 70%. Esto permitió purificar este material por IMAC y se logró el producto con una pureza superior a 80%, y luego por RP-HPLC para obtener un producto de alta calidad (99% de pureza).

En los estudios realizados en cuanto a capacidad de IFN alfa 2b en la matriz de IMAC, se obtuvieron niveles de hasta 4 mg de IFN/mL de gel, valor considerado 8 veces superior al obtenido mediante cromatografía de inmunoafinidad. De las distintas velocidades lineales utilizadas, se seleccionó como la mejor variante la de 40 cm/h, obteniéndose una recuperación y pureza del material eluído (RP-HPLC) de 85% y 80%, respectivamente.

A partir de los resultados anteriores se diseñaron las nuevas etapas de lavado del debris celular y de IMAC, y se llevó a cabo la producción de un lote de materia prima activa a escala preparativa. El producto final obtenido se sometió a todos los análisis para la determinación de la identidad de la molécula. Finalmente, se procedió a elaborar tres lotes a escala productiva con el objetivo de obtener la aprobación por la entidad de control de medicamentos en el uso de esta nueva tecnología en la producción de IFN alfa 2b recombinante. Todos los lotes fueron liberados de manera satisfactoria, y la tecnología fue aprobada por la autoridad nacional regulatoria (CECMED).

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Tecnología más limpia por medio de la biotecnología

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La búsqueda de tecnología más limpia que permita un desarrollo sustentable a mediano y largo plazos, ha sido una preocupación generalizada desde hace varios años. Sin embargo, aún cuando se han generado programas y políticas a nivel nacional e internacional para fomentar su desarrollo, en realidad los avances han sido limitados y se han encontrado resistencias estructurales difíciles de vencer.

Una de ellas es a nivel educativo, ya que la concepción de tecnología más limpia es nueva y, en términos generales, en la mayoría de los cursos de ingeniería sólo se analiza y estudia la eliminación de la contaminación, con poco énfasis en la reducción de contaminantes a través de nuevos diseños de procesos o bien por el uso alternativo de subproductos. Otra restricción importante ha sido que muchos de los procesos presentes fueron diseñados y concebidos tecnológicamente hace muchos años, y, por lo tanto no ha sido fácil rediseñarlos sin que esto signifique grandes inversiones y volver a entrenar personal.

En esta presentación daré algunos ejemplos concretos de cómo la biotecnología, por sus características, puede ser una buena herramienta en la generación de tecnología más limpia. Han habido cuatro avances importantes en el conocimiento biológico que han permitido extender el uso y aplicación de la biotecnología en diversos sectores productivos: la ingeniería genética ofrece la oportunidad de obtener organismos transformados genéticamente con nuevas y mejores capacidades de transformación y además ha logrado que la producción de proteínas sea más abundante y barata; las reacciones biocatalíticas en fase no acuosa han ampliado las posibilidades de transformación, permitiendo la utilización de recursos naturales considerados hasta hace muy poco tiempo como inaccesibles a los seres vivos; la ingeniería de proteínas nos ha dado la capacidad de diseñar mejores biocatalizadores, más eficientes y con menor generación de subproductos, o bien de ampliar su especificidad y permitir que los sustratos sean más diversos y, por último, cabe destacar la selección reciente de organismos extremófilos, los cuales realizan reacciones biológicas en condiciones ambientales más cercanas a las de la industria tradicional.

En general, la especificidad con que se manejan los procesos biológicos disminuye la cantidad de subproductos y la mayoría de éstos son de tipo biodegradable. También las condiciones de operación de la biotecnología, a temperaturas cercanas a la ambiental, pH no extremos y presiones bajas, dan como resultado que el consumo energético por unidad de producto disminuya sustancialmente, lo que significa una reducción en las emisiones y en el consumo de energía.

Los casos que presentaré se refieren a industrias diversas como la petrolera, la·alimentarla, la agroindustrial y al sector agrícola.

Industria petrolera. En este sector el uso de la biotecnología está principalmente dirigido hacia la eliminación de contaminantes presentes en las aguas, o bien de suelos que se han contaminado con hidrocarburos durante la obtención y procesamiento del petróleo. A ella hay que añadir que desde principios de la década de 1990, se han iniciado importantes esfuerzos de investigación y desarrollo tecnológico dirigidos hacia la eliminación de compuestos azufrados y de metales mediante procedimientos biológicos. El proyecto de biodesulfuración por células viables ha alcanzado una escala comercial, y muestra claramente cómo esta nueva tecnología reduce el consumo de energía, disminuye la inversión requerida y, sobre todo, se alcanzan niveles de azufre compatibles con las políticas establecidas para la obtención de combustibles más limpios. Sobre la base de esta experiencia, se han iniciado trabajos muy interesantes relacionadas con la transformación de crudos pesados en crudos más ligeros por medio de microorganismos, proyecto que, en caso de tener éxito, será de gran trascendencia en esta industria.

Industria alimentaria. El uso de enzimas se ha incrementado, pues por una parte se pueden obtener nuevos productos con propiedades organolépticas diferentes, con consumos de energía mínimos y menor generación de contaminantes. Un ejemplo que se debe discutir en esta sesión será el uso de las lipasas y proteasas, las cuales además de su uso tradicional como degradadoras de biopolímeros, ahora tienen nuevos usos cuando son utilizadas en condiciones no acuosas. Se han desarrollado reacciones de alcohólisis muy interesantes y se han obtenido nuevas moléculas y polímeros.

Industria agroindustrial. La obtención de nuevas enzimas cada vez más baratas en este sector, capaces de operar en condiciones ambientales más variadas, ha permitido que los procesos microbiológicos sean económicamente rentables y competitivos en el uso de residuos agrícolas, principalmente lignocelulósicos, o bien que las grandes cantidades excedentes de almidón sean utilizadas para la obtención de energía, y/o de productos de fermentación de gran volumen.

Sector agrícola. El uso de plantas transgénicas está creciendo rápidamente en los países industrializados, y aún cuando esto ha encontrado reticencias en algunas comunidades, la realidad en términos ambientales ha sido que se ha reducido el consumo de insecticidas y herbicidas, con un aumento en la productividad agrícola y en el ingreso de los productores. En algunos otros casos, las nuevas propiedades de los productos vegetales transgénicos han disminuido el consumo energético en su procesamiento, o bien su nueva composición es tal que su rendimiento de transformación es mayor. Un área con crecimiento significativo es el uso de agrobiológicos. Tanto en la fertilización como en el control de plagas, estos productos son de carácter biodegradable, bastante específicos y su costo está disminuyendo rápidamente, lo que hará que su consumo aumente en el futuro.

Optimization of a Perfused Stirred Tank Bioreactor Process for rhEPO Production

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*I*ntroduction

Erythropoietin (EPO) is a sialoglycoprotein hormone that regulates red blood cell production. This hormone is mainly produced by the kidney in human adults and anemia associated with renal failure often results from decreased level of EPO. This observation has led to the recombinant expression and subsequent therapeutic use of EPO for patients with chronic renal failure. Since the sugar chains of EPO play an important role in the expression of its biological activity, animal cells are selected as host cells for the production of recombinant human EPO (rhEPO). For its commercial production several methods have been described. In the present study, recombinant BHK cells producing human EPO were adapted to suspension growth and culture conditions were optimized in order to carry out the scaling up without carriers in a perfused stirred tank bioreactor.

*M*aterials and Methods

Cell line. BHK 21 cells were manipulated by genetic methods to produce human EPO constitutively under the control of SV 40 promoter [1].

Media. Culture medium consisted of a 1:1 mixture of DMEM and Ham's F 12 (Gibco BRL, USA) supplemented as described for Medium 1 [2]. Other media used consisted of Medium 1 supplemented with different concentration of fetal calf serum (FCS) (Bioser, Argentina) and/or 100 ppm Antifoam (Dow Corning, USA).

Culture systems and conditions. Adherent culture was achieved in T flasks (Nunc, USA) in Medium 1 containing 1% FCS, in order to estimate the rhEPO specific productivity (q_{rhEPO}) and the monolayer performance during long term culture with medium changes every 2 days. Cells were adapted to suspension growth in spinner flasks (Techne, UK) by successive subcultures of the free growing cell-enriched fractions determined by measurement of the pellet's diameter. Once adapted to suspension growth, q_{rhEPO} was evaluated in spinner flask under different conditions: antifoam supplementation, volume/area ratio, FCS concentration and culture pH. Dense cell cultivation was carried out in a perfused 25 1 stirred tank bioreactor employing a spin sieve system (MBR, Switzerland). Oxygen supply was performed by a sparger provided with sintered stainless steel diffusers. The set point for dissolved oxygen was adjusted to 40% air saturation. This value corresponds to an oxygen concentration of 2.7 mg/L. The stirred speed was adjusted to 70 rpm.

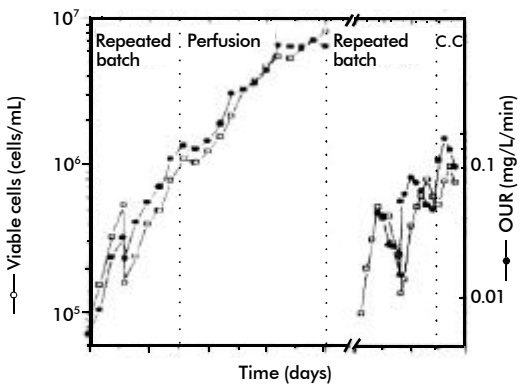
Analysis of samples. Glucose and lactate content were determined with YSI 2700 glucose and lactate analysers (Yellow Springs Instruments, OH, USA). The total cell number was determined by nuclei staining method. The proportion of dead cells was estimated by trypan blue exclusion. The rhEPO concentration was determined by DOT BLOT with own monoclonal antibodies [3].

*R*esults and Discussion

The q_{rhEPO} in adherent cultures was 0.2 pg/cell/d and remained constant over the whole 48 days culture period assayed. During the adaptation to suspension growth a gradual decrease in the pellet number and size was observed. Finally, after 29 subcultures (5 months) a suspension of majority single cells was obtained, with q_{rhEPO} of 0.5 pg/cell/d. This value represents a 2.5-fold enhancement in comparison to the adherent culture. Similar effects were informed for other recombinant protein [2]. q_{rhEPO} increased linearily with the pH of the culture, reaching a double value when the pH ranged from 6.4 to 7.4. Supplementation with antifoam up to 100 ppm had no effects on cell proliferation and on rhEPO productivity. A 2.7 times increase in the volume/area ratio showed no effects either on the specific growth rate ($\mu = 0.36\ d^{-1}$) or on the q_{rhEPO} value. The results also indicate that the dependence of m with FCS concentration follows a simple hyperbolic kinetic. As reported by other authors [4] q_{rhEPO} values increased parallely with the decrease of μ.

The figure shows the results obtained during 50 days of continuous fermentation with different operation modes: repeated batch, perfusion and continuous culture with cell removal (c.c.). In all cases, cell density varied in parallel to the oxygen uptake rate (OUR). Therefore, the culture state should be followed only by on-line oxygen concentration monitoring. Medium perfusion was adjusted according to on-line redox potential and off-line glucose and lactate measures. High cell densities up to 10^7cells/mL were achieved. Besides, q_{rhEPO} resulted directly proportional to the perfusion rate. As previously reported [5], maximal rhEPO production was 80 mg/d with a perfusion rate of 1 volume reactor/d.

In conclusion, despite the long time spent by the cell adaptation in the suspension growth mode, this procedure is convenient to diminish the costs and steps of the production process.



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Large-scale Production of CB.Hep-1 Monoclonal Antibody Using BALB/c Mice

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*I*ntroduction

Several reasons compel to the replacement of *in vivo* production technology by *in vitro* technology, for instance: using animals is not easy to scale-up, potential pathogen contamination will always be present, hard validation work should be done and *in vivo* production has relatively high costs compared to the *in vitro* [1] procedure. However, sometimes it turns out difficult to obtain high levels of antibody secretion in cell culture technologies. This article describes an *in vivo* large-scale production of the CB.Hep-1 monoclonal antibody, a pharmaceutical preparation, specific for the rHBsAg.

*M*aterials and Methods

Ascitic fluid production

10^6 cells, previously growing in spinner flasks, were inoculated in mice.

IgG purification

Ascitic fluid was filtrated and two saline precipitation steps with (NH4)2SO4 at 50% of saturation conditions were respectively carried out. The ascitis fluid was desalted by Gel Filtration Chromatography and purified by Protein A affinity chromatography.

Immunosorbent

Sepharose CL-4B was activated by the CNBR method and CB.Hep-1 MAb was covalently immobilized on the support. In order to calculate the total virus clearance factor, the validation of the purification system using five different model viruses was done.

*R*esults and Discussion

All batches were released under WHO regulations, thus guaranteeing safety [2]. Since we introduced the large scale-up process, we have kept a steady production, purity levels are higher than 90% mean, while the yield is higher than 1 mg/mL. Assays performed on the process showed the appropriate quality and purity. The immunosorbents were prepared at large scale with high yield. Consistent values of coupling efficiency greater than 95% were observed in all batches, the recovered rHBsAg from the immunosorbents, showed consistence in its purity above 85% (Figure), which demonstrated the high purification power of the designed immunoaffinity chromatography system; while the ligand leakage was below 0.5 ng MAb/pg rHBsAg.

The process validation study using viral models is showed in the Table. This result provides a high level of assurance that the final product will be free of contaminants.

In conclusion, the large-scale production process permits to obtain a great amount of the CB.Hep-1 monoclonal antibody, guaranteeing the biosafety of the product.

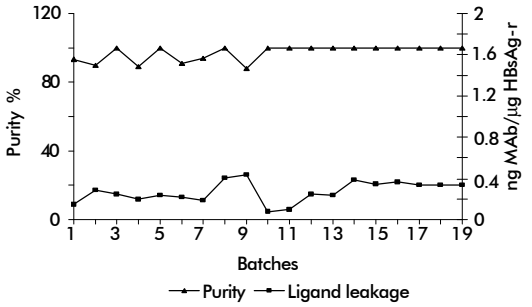

Table. Reduction factors (RF) when viral validation was carried out.

Viral model	Initial viral titer	RF for protein A chromatography	RF for pH inactivation	RF for inactivation heat	Total RF
Sendai	10^8	8.0	8.0	7.1	23.1
Herpesvirus	10^8	7.5	7.0	6.1	20.6
Polivirus	10^9	5.0	Not inactivated	8.6	13.6
Parvovirus	10^8	Not inactivated	3.4	6.3	9.7
HIV- 1	10^6	5.3	5.8	4.0	15.1

Large-scale Production of recombinant Hepatitis B Surface Antigen from Pichia pastoris

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*I*ntroduction

An downstream process for the purification of HBsAg produced in *Pichia pastoris* was established first at laboratory scale [1] and further scaled-up from about 250- to 500-fold [2]. However, the effectiveness of the key steps for the large-scale production of *P. pastoris*-derived HBsAg had not been described yet.

We analyze here the performance of our production technology in terms of its ability to both render a highly pure HBsAg and remove most of the intrinsic (yeast total proteins, nucleic acids, carbohydrates, lipids) and extrinsic (immunopurification released immunoglobulin [lg] G, endotoxin) contaminants. The results obtained verified that this technology satisfies most of the World Health Organization (WHO) requirements for the safe purification of yeast-derived, biologically-active HBsAg particles. Consequently, the vaccine (HEBERBIOVAC HB, Heberbiotec SA, Cuba), which is formulated with *P. pastoris*-derived HbsAg, has proven to be safe and efficacious, providing protection against hepatitis B infection [3]

*M*ethods

Ten independent industrial batches of HBsAg, five made in 1993 and five in 1998, were obtained as previously described [2 and citations therein]. Briefly, the recombinant *P. pastoris* yeast strain was kept as a master seed lot at –70 ºC to guarantee that each bioreaction run was started from the same original preparation. Under carefully controlled multiplication conditions, the HBsAg gene-containing yeast cells were passed from shake flasks into medium-scale bioreactors, and finally into a large-scale bioreaction unit. After harvesting, the yeast cells were disrupted to recover and purify HBsAg by a series of well-established steps [2 and citations therein] These included acid precipitation, adsorption/desorption from diatomaceous earth matrix and, finally, successive purification through immunoaffinity, ion-exchange and gel-filtration chromatographic procedures. Each step was currently checked to be within specifications of the WHO guidelines for quality and/or in-process control procedures approved by the National Control Authority. Also, the quality of the water and al buffer solutions were monitored for conductivity, pH and microbiological or pyrogenic contaminants, and were strictly controlled.

*R*esults and Discussion

The ability of the *P. pastoris*-based technology for large-scale production of recombinant hepatitis B virus surface antigen (HBsAg) and to both reproducibly purify HBsAg and remove most of the relevant contaminants was ascertained by evaluating 10 industrial production batches: 5 in 1993 and 5 in 1998. At an early stage, the clarification of mechanically disrupted yeast cells by acid precipitation rendered HBsAg with a purity as low as 3.8 ± 0.6%. However, by adsorption/desorption from diatomaceous earth matrix, the purity of HBsAg rapidly increased to 18.8 ± 5%, which is suitable for chromatographic processing. This step also eliminated non-particulated forms of HBsAg, significantly lowered the amount of carbohydrates and lipids, and concentrated the HBsAg 4.8-fold. Finally, a sequential purification procedure that included large-scale immunoaffinity, ion-exchange, and size-exclusion chromatographies further purified the preparation, resulting in a product (HBsAg at a concentration of 1.3 ± 0.2 g/L) with a purity of 95% or more. Furthermore, each of the other contaminants measured reached the following low levels per 20 mg HBsAg: host deoxyribonucleic acid (less than 10 pg), carbohydrates (1.2 ± 0.02 mg), lipids (14 ± 0.28 mg), immunopurification-released IgG (less than 100 ppm), and endotoxins (106.7 ± 19.3 pg). These values were below those specified for recombinant DNA hepatitis B vaccines according to WHO guidelines.

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The Conformational Stability of Proteins: Rational Stabilization by Protein Engineering

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*I*ntroduction

Proteins are linear polymers that fold into compact conformations in order to acquire the particular shape that confers them useful biological properties. The native conformations of proteins are stabilized by intra protein interactions (hydrogen bonds, van der Waals, charge/charge, charge/dipole, charge/p and p/p) and by the hydrophobic effect, and they are destabilized mainly by the huge conformational entropy change of folding. A delicate balance between all those factors determines that the average stability of proteins is low (5-15 kcal mol-1). Although 5 kcal mol is enough to ensure that more than 99% of the protein molecules are in the native conformation at any time, the equilibrium between native and denatured conformations forces all the molecules to be unfolded now and then. In the absence of side reactions this is not a problem, but the unfolded state and, perhaps even more, some conformations intermediate between the native and the unfolded state can experience aggregation and other reactions leading to an irreversibly inactivated protein. Irreversible inactivation from the folded conformation is also possible but usually less severe. Since the unfolded state has a greater tendency to irreversible inactivation, one way to minimize these reactions is to reduce the concentration of unfolded molecules. This can be easily done by increasing the conformational stability of proteins.

*M*aterials and Methods

The conformational stability of a protein can be measured by chemical denaturation (usually with urea or guanidinium hydrochoride) or by thermal denaturation [1]. Amino acid residues of a protein can be replaced at will if the gene is cloned. The rational replacement of residues requires knowledge of the tridimensional structure (by X-ray or NMR).

*R*esults and Discussion

There are plenty of possible strategies to rationally stabilize proteins and many have been tested and shown to work fine in many cases. Introduction ion of new disulfide bridges, engineering of charge/dipole interactions, decreasing the entropy of the denatured state by removing glicines or introducing prolines [2], removing hyperexposed hydrophobic residues, engineering metal binding sites, etc. As an example, we have recently shown that neutral hydrogen bonds may sometimes be more stable than charged ones [3]. Based on this, we have stabilized our model protein apoflavodoxin by neutralizing solvent exposed hydrogen bonds by site directed mutagenesis.

Stabilizing proteins may be important for biotechnological purposes as it may increase the operational life of the valuable protein. This is, however, not the only interesting application, as there is now growing evidence of many diseases being caused by protein misfolding, such as amyloid diseases and certain cancers [4]. The latter case could be a simple matter of insufficient stability of a protein that regulates the cell cycle leading to malfunction and cell proliferation.

Rational protein stabilization is thus an important, yet not very used strategy, that may have a great impact both in industry and in medicine.

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