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ScienceDirect
Journal of Chromatography B, 861 (2008) 186–195
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Strategies for improving production and purification of a recombinant protein: rP30 of *Toxoplasma gondii* expressed in the yeast *Schizosaccharomyces pombe*☆

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Received 3 April 2007; accepted 23 July 2007
Available online 2 August 2007

Abstract

Many problems concerned with the production and the purification of recombinant proteins must be addressed prior to launching an industrial production process. Among these problems, attention is focused on low-level expression that complicates the purification step and can jeopardise the process. The expression of a membrane protein, rP30, of *Toxoplasma gondii* in the yeast *Schizosaccharomyces pombe* led to a secretion of only 0.5 μg ml^{-1}. In order to obtain a sufficient quantity for biochemical characterization and evaluation in vitro diagnostic test development, strategies for both production and purification had to be optimized. First, the influence of four nitrogen sources (three peptones and yeast extract) on the growth rate, but also on the separation between the protein and the components of the fermentation broth was assessed. Second, batch and fed-batch fermentations were compared in terms of final biomass and rP30 concentrations. Third, three different protocols that included fixed and expanded bed ion exchange chromatography were compared for processing a large volume of feedstock. By using the most appropriate strategies, i.e. fed-batch fermentation, capture on EBA cation exchanger and affinity chromatography polishing, a purification factor of 1778 and a yield of 49% were achieved. These performances allowed a 12.5-fold increase for the overall rP30 process productivity.

Keywords: Production; Purification; Recombinant protein; Optimization; *Toxoplasma gondii*; *Schizosaccharomyces pombe*

1. Introduction

Since the 1980's, the advances in DNA technology have allowed the expression of numerous bio-products from various pathogens in appropriate prokaryotic or eukaryotic hosts. The therapeutic or diagnostic uses of these recombinant proteins require their extraction and purification with a sufficient degree of purity on one hand, and with an acceptable production cost on the other hand. Subsequent to obtaining adequate expression at bench scale, i.e. correct protein folding and biological activity, two principle tasks remain: (1) the production of sufficient biomass to obtain enough crude starting materials and (2) application of a purification strategy, providing an economic yield while preserving the biological activity. The use of short peptide affinity tags linked to the sequence to be expressed has become commonplace for the expression and purification of recombinant proteins for the primary isolation step of proteins, and could lead one to believe that protein purification thus becomes simpler, but this tool cannot be used in all cases and the tag must be removed with additional steps [1]. Even if the biotechnologist is able today to collect more analytical data via bioinformatics and can benefit from improvements in chromatographic matrices and materials, the downstream process is still the bottleneck of a project and the cost related to the number of steps to provide a given protein purity remains a decisive success factor.

Toxoplasma gondii is a widespread parasitic protozoan and humans and other mammals become infected by accidental

☆ This paper was presented at the Biochromatography and Nanotechnologies Conference, Vellore, Tamil Nadu, India, 12–15 February 2007.

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doi:10.1016/j.jchromb.2007.07.034

ingestion of cysts either excreted by cats or present in contaminated meat [2,3]. Generally asymptomatic in healthy individuals, toxoplasmosis may cause severe complications in pregnant women and immunocompromised patients [4]. For serodiagnosis, the antigen source comes from tachyzoites cultured on mice, and the main antigen is a membrane protein of molecular mass (M_r) 30,000 (P30), present all on the parasite surface [5–7]. There is a need for highly purified P30 antigen to perform an ELISA test for IgG and IgM detection with the sensitivity and specificity required [8–10].

John Boothroyd's team first described the P30 gene and protein purification in 1988 [11]. To replace the cumbersome culture and extraction of the native antigen by means of detergents and affinity chromatography [12,13], attempts to obtain heterologous expression of the P30 gene have been made in different hosts like *E. coli* [14,15], Cos and CHO cells [15,16], Sindbis virus [17], *Pichia pastoris* [15,18,19], as well as Adenovirus, baculovirus, and *Schizosaccharomyces pombe* (in house work). The latter was selected for optimizing expression with the correct protein folding and antigenicity, but the expression level remained low (0.5 μg/ml in batch culture). The need for approximately 20 mg of this protein for biological activity testing led us, inevitably, to consider a rational optimization of the purification stages. Rare cases of such expression level have already been reported, and are similar to monoclonal antibodies purification from mammalian cell culture [20]. In such conditions of large culture volume, the challenge is to recover the target protein from the numerous components of the medium and yeast metabolites, with the additional drawback of water being the largest component to eliminate.

Tutorial reviews stress the necessity to employ robust methodologies that allow up-scaling of the process, as well as to limit the number of steps for yield optimization [21]. The general procedures of downstream processing start with clarification of the feedstock from cell removal by centrifugation, or filtration [22]. Then, the volume may be reduced by ultrafiltration with the appropriate pore size to enable concentration of the target and elimination of low molecular weight contaminants simultaneously. The subsequent steps are usually adsorption chromatography with the appropriate selectivity to capture and concentrate the target from contaminants, depending on its surface properties. Generally, a polishing step achieves the desired degree of purity [23]. To improve this traditional multi-step combination of techniques, expanded bed adsorption chromatography has emerged as an alternative to achieve cell separation, concentration and capture step in a single operation [24,25].

Pumping the liquid upwards through a particle bed in a column allows the bed to fluidize when the flow rate balances the particle settling velocity, and generates particle classification depending on their size distribution. Such fluidized bed with a large void fraction allows cells and debris to pass through the particles and to be washed out of the column. A packed configuration is usually preferred for collection of the product in a small volume. This chromatographic mode using homogeneous size distribution particles is named expanded bed adsorption (EBA) chromatography [26]. Moreover, the hydrodynamic principle of EBA meets large volume processing requirements by high flow rate capability. Many studies have reported successful application of EBA with a direct connection between the fermenter and an expanded bed column [27–32].

In this work, the purification of a recombinant protein P30 of *Toxoplasma gondii* expressed in the yeast *S. pombe* is described. In order to improve yield and purity, optimization of both expression levels and the protein purification process has been achieved in an integrated fashion. A successful strategy is proposed to mutually adapt the upstream and downstream steps to obtain the purified protein in adequate amounts for diagnostic test development.

2. Experimental

2.1. Yeast strain and culture process

A *Schizosaccharomyces pombe* strain P3 carrying the plasmid pTG8630 (Transgene, Strasbourg, France) was used for the production of recombinant P30 (rP30) (antigen used by bioMérieux under exclusive licence from Stanford University USA, Inventors Dr. J. Boothroyd and Dr. L. Kasper). The predicted P30 amino acid sequence reveals one potential *N*-glycosylation, which is not glycosylated in the parasite. Accordingly, the glycosylation site Asparagine 211 was mutated to allow expression of a non-glycosylated protein as a mimic of the native parasitic form. In addition, the 20 C-terminal amino acids corresponding to a hydrophobic moiety linked to a glycosylphosphatidylinositol anchor were deleted to facilitate production of a soluble form.

The medium, culture and batch fermentation conditions have been previously described [33]. Additionally, four separate nitrogen sources were tested: (1) an acid digest of casein (Hy-Case SF, Sheffield Products, Norwich, NY, USA); (2) casein hydrochloric peptone (bio-Case, bioMérieux, Marcy l'Etoile, France); (3) casein tryptic peptone (bio-Trypcase, bioMérieux); and (4) yeast extract, yeast lysate (bioMérieux).

For the fed-batch mode, a peristaltic pump with adjustable flow rate was used to feed the culture with the same medium as batch fermentation supplemented with glucose 300 g l^{-1}. The flow rate was adjusted to maintain a final glucose concentration less than 1 g l^{-1}.

Samples were taken at 3 h intervals from each culture and the optical density at 600 nm was measured on a UV-VIS spectrophotometer (Cary 100 Varian, Les Ulis, France). The relationship of biomass DCW/$A_{600\,nm}$ was determined by filtration assays (DCW g l^{-1} = 0.32 × $A_{600\,nm}$). Samples were immediately pretreated as follows: 2 ml of sample was centrifuged at 10,000 × *g* for 5 min at 4 °C and then stored at 4 °C. Glucose analysis was performed by enzymatic analysis, using Glucose kit (bioMérieux).

2.2. Analytical chromatography

The chromatographic equipment was composed of a Waters™ 626 pump with 600S controller, and a Waters™ 996

Photodiode Array Detector set at 280 nm (Waters SAS, Guyancourt, France). Analytical chromatography experiments were carried out at 25 °C using a glass column (77 mm × 8.5 mm I.D.) filled with various ionic supports: DEAE-5PW (Waters, Ref. 11783), SP-5PW (Waters, Ref. 11784), SP-Sepharose FF (GE Healthcare, France), S-Hyper D (F) (Pall Biosepra, Cergy France Ref. 200173), SP-Cellthru BigBead (Sterogen Ref. 11024), SP Trisacryl (M) (Pall Biosepra Ref. 259101), SP-Spherodex (M) (Pall Biosepra Ref. 273441), Poros 50 HS (Applied Biosystems, Foster City CA Ref. 1-3359-06), SP-XL Streamline (GE Healthcare Orsay, France, Ref. 17-5076-01) of porosity 4×10^6 Da and particle diameter of 100–300 μm. The optimization of the buffer conditions of this cation exchange chromatography has been previously described [33].

The analytical chromatography reverse phase and gel filtration of peptones were performed, respectively, on a column reverse phase C18 (Novapack, Waters Ref. 086344) and a Superdex Peptide 10/30 (GE Healthcare Ref. 17-5176-01). The feedstock was centrifuged at 10,000 × *g* for 15 min (J2-25 Beckman Coulter SAS, Roissy, France), and the supernatant was then filtered through a 0.45 μm membrane filter (Millipore France Saint Quentin en Yvelines) before injection of 5 ml samples. Buffered pH values (from 3 to 7) were assayed and four nitrogen sources were compared.

2.3. Preparative chromatography

The chromatographic system consisted of a UV1 Detector (GE Healthcare) set at 280 nm with a preparative flow-cell of 5 mm path length, and two peristaltic pumps (Masterflex, Cole-Palmer Instrument Company, Vernon Hills, Illinois). For packed bed chromatography, a XK50/30 column and a Ø9 cm BioSepra column were used. In expanded bed mode a Streamline-50 column with an internal diameter of 50 mm was used for samples of 2–5 l; a Streamline-100 column with an internal diameter of 100 mm was used for samples of 20 l. The SP matrice used was Streamline-SP-XL (GE Healthcare, Ref. 17-5076-01). The void fraction of 300 ml of sedimented gel was determined by injection of 5 ml of Dextran Blue (GE Healthcare) eluted with PBS at 15.3 cm h^{-1}. The liquid was pumped upwards through a bed of adsorbent beads: the bed expands and spaces open between the beads. The particles can pass through the bed without becoming trapped. The feedstock was pumped upward on an equilibrated Streamline SP at a flow rate of 170 cm h^{-1} that corresponds to a two-fold bed height expansion. Then, all the feedstock was applied to the column; the column was washed with 20 mM sodium citrate buffer pH 3.3 containing 100 mM NaCl until the $A_{280\ nm}$ of the eluent was back at the baseline. Then, the bed was allowed to settle and the adapter was lowered to the top of the sedimented bed. The adsorbed product was eluted in downward mode with 20 mM sodium citrate pH 3.3 containing 0.5 M NaCl at 50 cm h^{-1}. The $A_{280\ nm}$ peak was collected. The cleaning step was performed in upstream mode with 0.1 M NaOH containing 1 M NaCl.

In order to evaluate the maximum flow velocity applicable for EBA and the consequent bed voidage obtained, the modified Stokes expression and the relationship of Richardson–Zaki [34], respectively, were used:

$$U_t = \frac{g d^2 (\rho_p - \rho_l)}{18\eta} \quad (1)$$

$$\varepsilon^n = \frac{U_p}{U_t} \quad (2)$$

The average bed voidage can be estimated from the following formula:

$$\frac{h}{h_0} = \frac{1-\varepsilon_0}{1-\varepsilon}, \quad \text{thus} \quad \varepsilon = 1 - (1-\varepsilon_0)\frac{h_0}{h} \quad (3)$$

2.4. Affinity chromatography

The chromatographic system was composed of a UV1 Detector (GE Healthcare) set at 280 nm with a flow-cell of 2.0 mm path length and a peristaltic pump P-1 (GE Healthcare). The internal diameter of the column was 6 mm and the length 6.5 cm for analytical purifications (1.8 ml of gel) and for preparative scale the internal diameter was 26 mm and the length 11 cm (60 ml of gel). The eluted fraction from cation exchange chromatography was adjusted to pH 7.4 with 1 M NaOH and then applied to an anti-P30 monoclonal antibody-conjugated CNBr-Sepharose 4B (GE Healthcare, Ref.17-0430-01) column. Three monoclonal antibodies (1E1E7, 4H11C8, 4F11E12) and three elution conditions were compared. The rP30 fraction was eluted using either 0.15 M NaCl, 0.1 M glycine/HCl, pH 2.8, or 0.1 M sodium carbonate, pH 11, or successively glycine/HCl, pH 2.8 and sodium carbonate, pH 11. The eluted fraction was finally neutralized to pH 7.0 using either Tris or HCl solutions.

2.5. Tangential flow filtration

The clarified feedstock was filtered on an 8 μm membrane (Millipore Ref. SCWP04700) before concentration on a Minisette Omega 10 membrane (Millipore Ref. OS010C01). Samples of 2 l were concentrated up to 200 ml.

2.6. Determination of rP30 concentration and bioactivity

The bioactivity of rP30 has been demonstrated by an inhibition immunoassay. The principle of the test was to incubate the sample with an alkaline phosphatase-labelled monoclonal antibody (MnAb 1E1E7), specific for native P30, and to allow the remaining free MnAb 1E1E7 to be captured onto a native P30 polystyrene solid phase. rP30 concentration was determined using this assay calibrated with the native purified P30.

Moreover, the antigenic property of rP30 was demonstrated by the serological analysis of 1798 sera (bioMerieux serum collection). Toxoplasmosis serological status of each sera was established using VIDAS® TXG (bioMérieux, Marcy l'Etoile, France), and in case of discrepancy, a dye test was performed for confirmation [35].

The rP30 purity was assessed as the ratio between the bioactive rP30 to the total protein concentration. Protein concentration was determined using the Bicinchoninic Acid Protein Assays enhanced method (Pierce Perbio Science, France Ref. 23225).

Table 1
Comparison of peptone sources in batch culture, erlenflasks

Peptones 10 g/l	Hy-Case	bio-Case	bio-Trypcase	Yeast extract
Growth rate $\mu(h^{-1})$	0.2 ± 0.05	0.32 ± 0.05	0.35 ± 0.05	0.33 ± 0.03
Biomasse DCW (g/l)	2.45	2.32	2.2	3.1
rP30 (μg/ml)	0.1 ± 0.05	0.22 ± 0.05	0.29 ± 0.07	0.55 ± 0.04

The growth rates were calculated during the exponential growth phase. The rP30 expression levels were assayed from the 30 h samples.

The purification factor was calculated as the ratio between the initial and the final rP30 purity.

3. Results

3.1. Culture media formulation and fermentation

In order to improve the expression level of rP30, four nitrogen sources were tested. The growth rate, final biomass and rP30 concentration were compared (Table 1). The enzymatically hydrolyzed peptones bio-Case and bio-Trypcase, and the yeast extract exhibited growth rate greater than $0.30\,h^{-1}$. As rP30 expression is associated with growth, the higher the growth rate, the greater the expression of the protein. The highest growth rate was obtained with the yeast extract modified medium with a maximum of $0.55\,\mu g\,ml^{-1}$ in flasks and this nitrogen source was therefore chosen for batch culture.

A further increase in protein concentration can be achieved at high cell densities by using a bioreactor with pH and dissolved oxygen controllers. After 24 h, the $A_{600\,nm}$ was about 20, but the concentration of rP30 was only $0.65\,\mu g\,ml^{-1}$. An increase in the protein concentration was obtained by using the fed-batch mode. In order to determine the optimal feed rate to be applied to a fed-batch, 2 l chemostat was performed by increasing the dilution rate from 0.05 to $0.5\,h^{-1}$. Up to $a = 0.24\,h^{-1}$, the medium did not show any limitation. Accordingly, the exponential feed rate of the fed-batch was set at: $Q_e = Q_{e0}\ \exp.(a\ t)$ with $Q_{e0} = 66\,ml\,h^{-1}$. The culture was stopped when the volume reached 4 l. Results are presented in Fig. 1. The biomass obtained at the end of this fed-batch reached an $A_{600\,nm}$ of 91, equivalent to about $29\,g\,l^{-1}$ dry cell weight. The final rP30 concentration was equal to $2.5\,\mu g\,ml^{-1}$. By using fed-batch culture, the production of rP30 was more than five-fold that obtained using the batch mode.

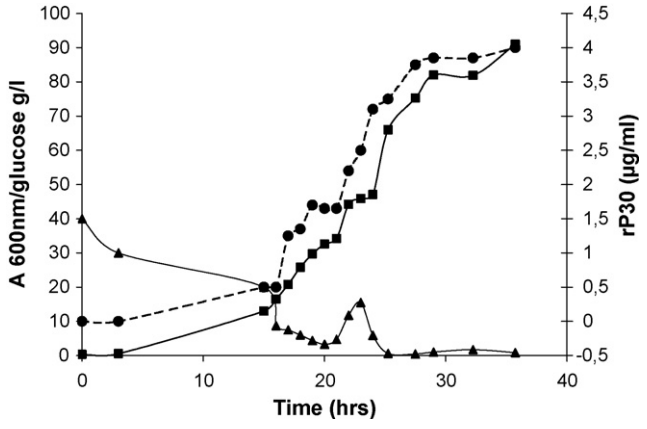

Fig. 1. Fed-batch fermentation of *S. pombe* at 4 l scale. Samples were taken every 3 h for biomass, glucose, rP30 measurements. Legend: (–■–) $A_{600\,nm}$, (- -▲- -) glucose g/h, (- -●- -) rP30.

3.2. Analytical chromatography

3.2.1. Analytical rP30 purification

Initial analytical assays were performed on anionic supports DEAE 5PW with 25 mM Tris buffer, pH 9 for adsorption and elution with a sodium chloride gradient up to 1 M. The rP30 could not be separated as one fraction (data not shown). Adversely, the use of a cation exchange chromatography SP 5PW support with 20 mM sodium citrate buffer at pH 3.3 favoured the rP30 adsorption. This protein is eluted with a sodium chloride gradient in the same buffer. As the culture medium has a final conductivity equivalent to 5–7 mS, this was appropriate for adsorption of rP30. The clarified medium was adjusted from pH 4 to 3.3 by addition of 1 M citrate buffer and 1 M NaCl was added up to 11 mS. To avoid binding of contaminants on the support and consequential reduction of the dynamic capacity, the adsorption step was performed with 0.1 M sodium chloride, and the rP30 elution by adding 0.5 M sodium chloride. In spite of these optimizations, the dynamic capacity remained at 20 ml of sample for 3 ml of gel. The chromatogram is shown in Fig. 2. These conditions were used for the preparative chromatography.

3.2.2. Chromatographic behaviour of peptones

The previous analytical approach on sulfopropyl support emphasized the problem of the poor dynamic capacity of the

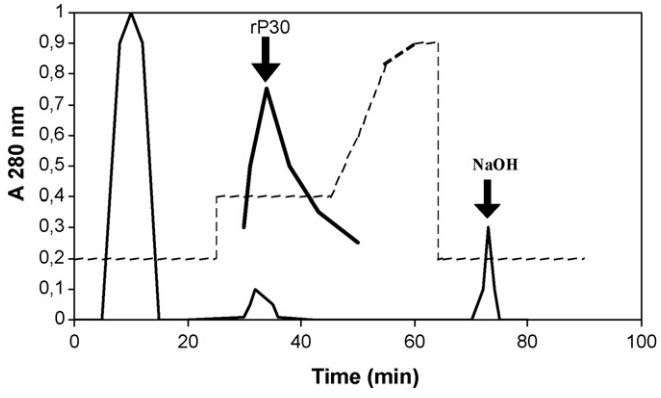

Fig. 2. Chromatography profile of batch culture supernatant on sulfopropyl SP Sepharose 5 ml samples was injected on 0.1 M sodium citrate buffer pH 3.3, 0.1 M NaCl and eluted with a 0.1 M sodium citrate buffer step pH 3.3, 0.5 M NaCl, 0.1 M NaOH was used for cleaning. Legend: (▬) profile, (- - - -) gradient.

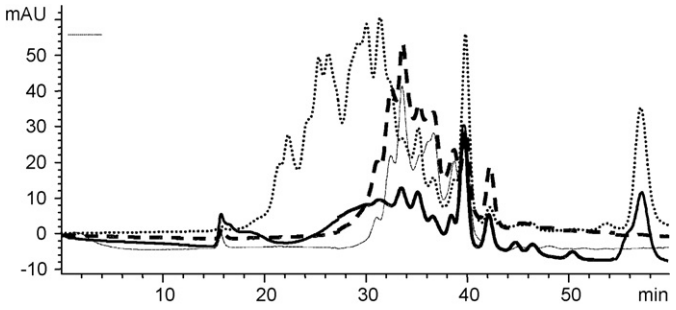

Fig. 3. Chromatography profile of four peptone from different source using gel filtration Superdex peptide 10/30. 1 ml of 5 mg/ml peptones was injected in PBS Buffer. Legend: (· · · ·) bio-Trypcase, (▬ ▬ ▬) bio-Case, (- - - -) Hy-Case, (▬▬▬) yeast extract.

support, which had to be solved before going to preparative scale. The primary purpose of peptones sources in a culture medium is to support the nitrogen and amino acids requirements of microorganisms for growth, but peptones are also peptide molecules with their intrinsic physical properties of mass, charge, and hydrophobicity. The chromatographic behaviour of the four peptones sources, such as Hy-Case, bio-Case, bio-Trypcase and yeast extract compared for growth previously were tested on gel filtration (Superdex peptide 10/30), reverse phase chromatography (C18), and cation exchange on sulfopropyl Streamline (Figs. 3–5, respectively). The chromatographic profiles of each peptone are different, which is due to the chemical or enzymatic hydrolysis of these peptones. Chemical hydrolysis leads to small-size hydrophobic peptides (Hy-Case, bio-Case) compared to enzymatic hydrolysis (bio-Trypcase and yeast extract). For cation exchange chromatography, the selected protocol was applied to peptones samples from various sources for comparison (Fig. 5). The peak areas of the flow-through correspond to non-retained materials, and the 0.5 M NaCl eluted fraction corresponds to contaminants co-eluting with rP30. The yeast extract binds least under adsorption conditions but co-elutes significant proteinaceous material absorbing at A_{280} under rP30 elution conditions. Hy-Case and bio-Case show opposite behaviour for these two fractions. As yeast extract enhances

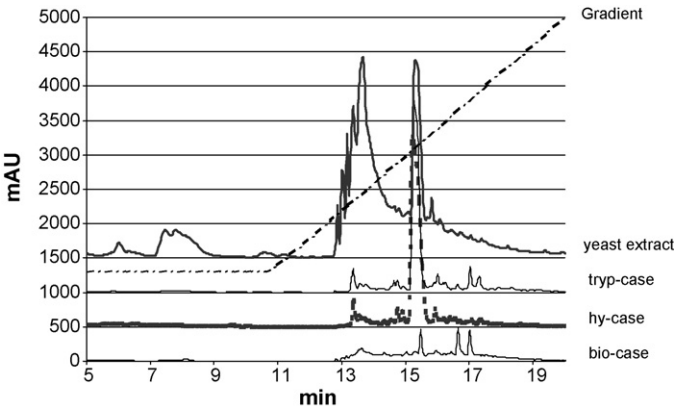

Fig. 4. Chromatography profile of four peptone from different sources on Novapack^{TM} C18 column. 1 ml of 5 mg/ml peptones was injected and eluted with a gradient water TFA 0.065%, acetonitrile (0–100%). Legend: (▬ ▬ ▬) Hy-Case, (· · · ·) tryp-case, (▬▬▬) yeast extract, (—) bio-Case.

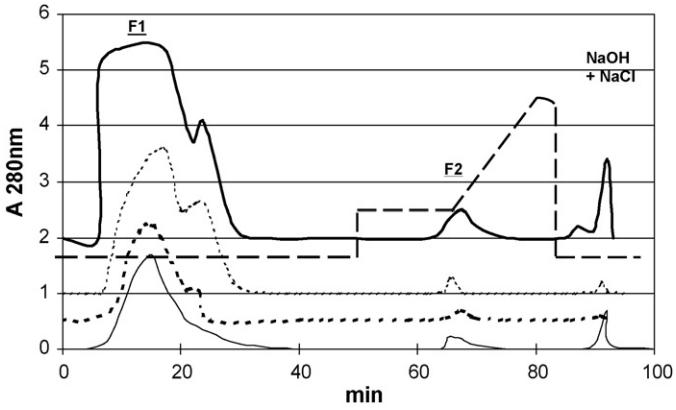

Fig. 5. Chromatography profile of four peptone from different sources on SP XL Streamline column. 5 ml of 5 mg/ml peptones was injected on 0.1 M sodium citrate buffer pH 3.3 0.1 M NaCl and eluted with a step gradient of 0.1 M sodium citrate buffer pH 3.3 0.5 M NaCl. 0.1 M NaOH was used for cleaning. Legend: (▬▬▬) yeast extract, (- - - -) bio-Trypcase, (▪▪▪▪▪) Hy-Case, (—) bio-Case, (▬ ▬) gradient.

growth and expression of rP30, it was selected for the yeast medium despite the drawback observed on the cation exchanger.

3.2.3. Non-specific binding: comparison of sulfopropyl matrices

To select the best support for large-scale purification among the sulfopropyl supports available from different suppliers, the most important criterion in this case is scalability and matrix inertness. The NaOH cleaning fractions obtained from cation exchange chromatography on seven different supports were challenged by SDS Page and Western Blott with a rabbit serum raised against yeast medium contaminants. The banding intensity of each lane (Fig. 6) enabled ranking of the support from low to high for non-specific binding. The polymeric matrices bind high molecular weight contaminants more than the agarose-based matrices, such as SP Sepharose Fast Flow and SP Streamline which can be selected for preparative scale.

3.2.4. Polishing step: affinity chromatography

As a polishing step was necessary to further purify the rP30 captured on the cation exchange, an affinity step was assayed by comparing three monoclonal antibodies raised against the membrane protein P30 of *Toxoplasma gondii*. Three modes of elution were compared for their efficiency to release rP30: (1) acidic buffer alone, (2) acidic buffer followed by alkaline buffer, and (3) alkaline buffer alone (Fig. 7). The alkaline condition was always more efficient than the acidic one for the three monoclonal antibodies. The best yield was obtained with the 4F11E12 monoclonal antibody but by using the two successive conditions, acidic and basic elution. As only one condition is easier to handle than two, the 1E1E7 monoclonal antibody was chosen with the alkaline buffer alone for elution.

3.3. Preparative chromatography

The scaling up of the analytically designed steps was necessary to meet the requirement for immunological testing which

	Matrices	Composition	Non-specific Binding
1	**SP-Streamline**	6% crosslinked agarose/crystalline quartz core	+
2	SP-Spherodex (M)	Silica microencapsulated by dextran	++++
3	Poros 50 HS	crosslinked poly(styrene divinylbenzene)	+++++
4	SP-Trisacryl (M)	Hydrophilic, copolymeric matrix	++++
5	SP-5PW	porous, hydrophilic resin on polymer base	+++
6	SP-Sepharose (FF)	6 % highly crosslinked agarose	+
7	SP-Cellthru Bigbead	Hardened 4 % agarose	++++
8	S-Hyper D (F)	rigid composite structure	++

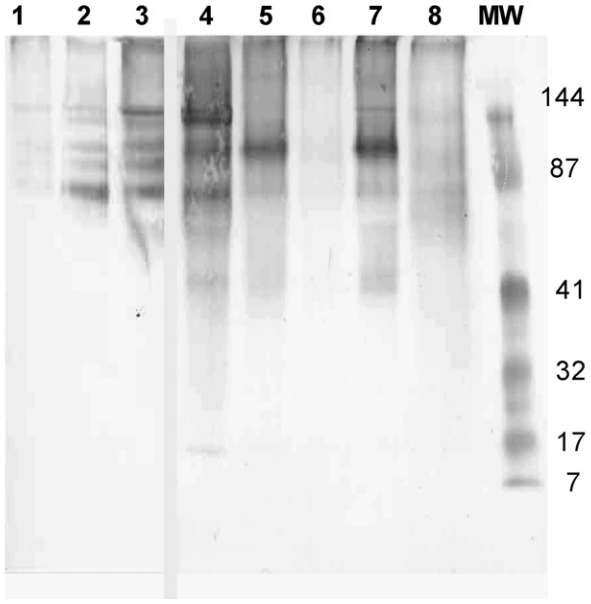

Fig. 6. Immunodetection of non-specific binding on different sulfopropyl matrices suppliers. Each column was injected with 5 ml of yeast *S. Pombe* supernatant. After SDS-PAGE and electrotransfer on PVDF membranes, the membranes was revealed with rabbit sera raised against yeast culture medium, followed by an anti-rabbit peroxydase-labelled goat conjugate and diamino benzidine as the substrate.

is about 20 mg. Therefore, the volume of the fed-batch step was increased up to 10 l. As the volume of feedstock to be processed increases, the conditioning step of clarification and concentration become difficult to handle and shortening the process arises as priority. In addition to the classical approach (1) corresponding to the scale-up of each separate step, two alternative approaches were performed: (2) without the concentration step but with a larger column for ion exchange, and (3) with only expanded bed chromatography as the capture step directly from the fermenter. These three approaches were compared for feasibility and productivity using 5 l samples from the same feedstock and sulfopropyl SP XL Streamline as the adsorbent. For adsorption chromatography in packed bed, the scale-up was based on an increase in column section while the length and the linear flow rate remained equivalent to the analytical scale. To establish a proper process, the characteristics of an expanded bed, flow velocity and homogenous bed expansion must satisfy the mass transfer conditions for adsorption of solutes on particles. In addition, the particle bed must have enough porosity to allow the washing out of the cells. Before starting EBA experiments, the maximum flow velocity acceptable with our conditions has been calculated by using the modified Stokes expression: SP XL Streamline particle size: 200×10^{-6} m, SP XL Streamline particle density: $1.2 \times 10^{3}\,\mathrm{kg\,m^{-3}}$, feedstock density: $1.008 \times 10^{3}\,\mathrm{kg\,m^{-3}}$. The feedstock concentration is 25 g/l DCW corresponding to $\eta = 5.0 \times 10^{-3}\,\mathrm{kg\,m^{-1}\,s^{-1}}$, then $U_t = 3\,\mathrm{m\,h^{-1}}$.

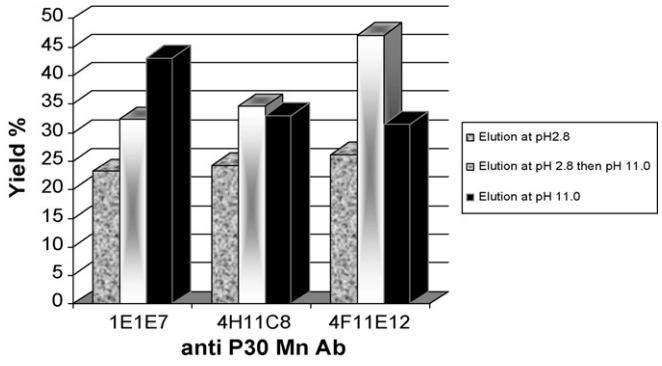

Fig. 7. Comparison of three monoclonal antibodies and elution conditions for rP30 affinity purification. Legend: (▨) elution at pH 2.8 then pH 11, (▩) elution at pH2.8, (■) elution at pH 11.

The recommended flow velocity for the Streamline column is $0.3\,\mathrm{m\,h^{-1}}$, which is rather under the theoretical maximum flow velocity.

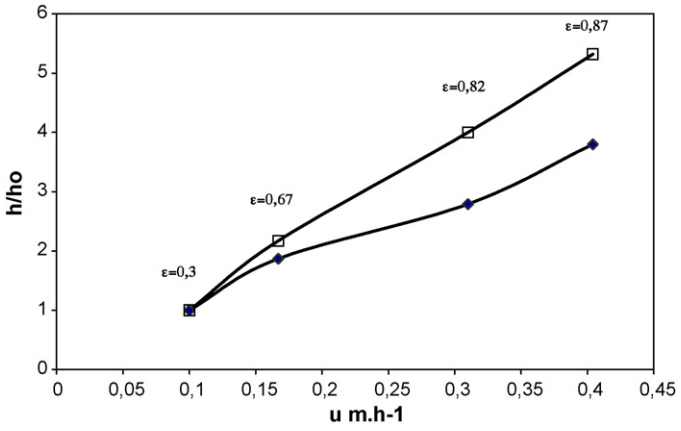

Fig. 8. Expansion characteristics of SP XL Streamline in a 50 mm column under buffered feedstock and conditions 0.1 M sodium citrate buffer, 0.1 M NaCl, pH 3.3. Legend: (-□-) feedstock, (—▰—) buffer.

From the first assay of support bed expansion with 0.1 M sodium citrate buffer pH 3.3 0.1 M NaCl and feedstock with yeast at 25 g/l DCW, the relation between the flow velocity and expansion characteristics presented in Fig. 8 obeys the Richardson–Zaki empirical relation. As ε_0 settled bed determined with dextran blue was 0.3, the bed voidage varied from 0.67 to 0.87 depending on the flow velocity (Fig. 8).

The impact of the flow velocity during adsorption on the purification factor and yield was evaluated with 5 l sample assays (Fig. 9). When the flow velocity increases from 0.1 to 0.4 m s^{-1}, there is a 10% decrease in the recovery, from 80 to 70%. Inversely, the purification factor increases from 4 to 4.5. The optimized flow velocity to be applied in our case for the next assays was 0.167 m s^{-1}, which corresponds to a bed expansion of 2.

As these preliminary assays determined the best conditions for using an EBA step in the process, the performance of the three proposed processes was compared with 5 l samples up to the polishing step (Table 2). As expected, the four-steps approach

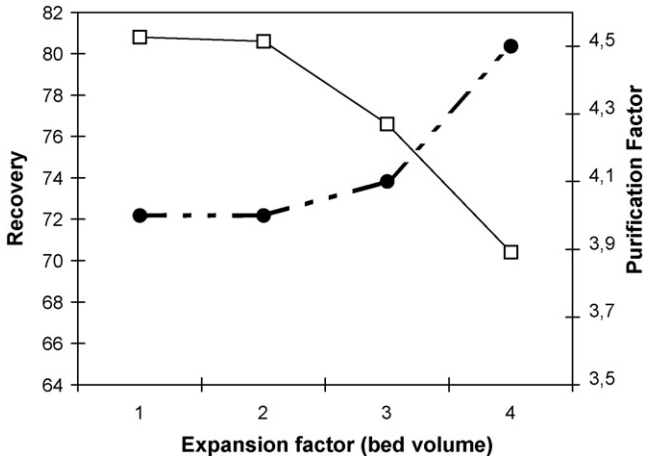

Fig. 9. Effect of feedstock flow velocity during adsorption stage. 2 l samples were purified on 300 ml SP XL Streamline. Legend: (-□-) recovery, (●) purification factor.

Table 2
Comparison of the three purification approaches

Purification process	Recovery (%)	Purification factor	Purity rP30/total proteins (%)
Clarification Concentration Packed Ion Exchange Affinity	20	843	25
Clarification Packed Ion Exchange Affinity	36	858	26
Expanded bed Ion Exchange Affinity	49	1778	53

5 l samples of the same crude feedstock were purified.

with clarification and concentration led to poor recovery and purity, 20 and 25%, respectively. When avoiding the concentration step, a better recovery of 36% was noted but purity remained at 26%. The two-step process with EBA brought an increase in both recovery (49%) and purity (53%), with a purification factor of 1778 against 850 for the other two alternatives. The purity was determined as the ratio of rP30 quantified by the specific Elisa protocol and the total protein quantitation (colorimetric assay).

For the treatment of a larger volume of feedstock, 10 l, this protocol was used with an EBA column 100 Streamline and 600 ml of resin. The results thus obtained confirm the efficiency of this two-step process for rP30 purification.

4. Discussion

In the field of health care and pharmaceuticals, there is a need for biomolecules which mimic native molecules for therapeutic, vaccine or diagnostic uses. Even though nucleic acid technology has today reached a mature level for obtaining heterologous expression of proteins, two indissociable issues remain of paramount importance for biomedical applications: the need to conserve bioactivity while establishing an economically viable industrial purification process for the target protein. The subject of this article concerns the production of the protein rP30, a major antigen for the serodiagnosis of toxoplasmosis, to replace the cumbersome culture of the pathogenic parasite in mice and membrane protein extraction [36]. Numerous expression attempts have been carried out in prokaryotic [14,37], viral [17] and eukaryotic hosts [15,17] including the methylotropic yeast *Pichia pastoris* [16,18,19] but problems of solubility, incomplete protein folding or *O*-glycosylation were found to be limiting for diagnostic or vaccine purposes.

The expression by a transformed *Schizosaccharomyces pombe* strain of a recombinant P30 was retained for correct protein folding and antigenicity was monitored with human sera. However, the very low-level of secretion in the medium, 0.5 μg/ml, made optimization for an industrial development critical. Moreover, the rP30 was expressed without an additional tag for the sequence integrity, so a traditional purification approach had to be designed. As initial purification trials with

clarified-concentrated samples on ionic exchangers and affinity chromatography led to low recovery (15%), it became obvious that improvements both upstream for productivity and downstream for processing were needed. Despite the use of a high capacity cation exchanger (70 mg lysozyme/ml gel, supplier data), the dynamic capacity of SP XL Streamline is only 7 ml sample for 1 ml of gel, due to the competitive adsorption among the medium components, contaminant proteins and salts and the target protein [38,39].

Improving the performance of culture media can be accomplished with numerous strategies [40]. Taking into account successive optimizations of the synthetic medium for yeast introduced by Fiechter et al. [41] and modified by Mendoza et al. [42] concerning the addition of casein hydrolysate as organic nitrogen, the influence of the source of nitrogen was explored. The addition of $10\,g\,l^{-1}$ of a complex nitrogen source obtained from chemical or enzymatic hydrolysate of casein and yeast extract brings a gain in growth rate, and consequently in the biomass and expression of rP30 especially for the bio-Trypcase and the yeast extract. A similar effect using complex nitrogen sources was also observed on the growth of industrial yeasts [43].

To carry out cultures with high cell density, the fermentation mode of choice is fed-batch, which supports the exponential phase of growth while bringing the source of carbon to the optimal dilution rate. As expected, fed-batch culture carried out with an initial dilution rate determined in continuous culture generated a four-fold increase in biomass and a five-fold increase in rP30 concentration. Analytical chromatography of the four sources of nitrogen provides information on their characteristics and their complex heterogenous contaminant distribution with respect to size, hydrophobicity and charge on the respective supports. Yeast extract contained the lowest amounts of $A_{280\,nm}$ absorbing material and was therefore chosen even though certain components co-elute with the rP30 on the cation exchanger. In addition, yeast extract is composed of rather low molecular weight molecules with weak hydrophobic characteristic which are favourable for ease of elimination. These observations led us to also investigate the contaminants which co-adsorb non-specifically in order to select the most favourable matrix. The chemical nature of the matrices can sometimes contribute in a positive way to the selectivity of the support, or on the contrary limit the quality of separation. As far as possible, the biotechnologist must know made up with these side effects [44,45]. The search for an optimal result for each stage of purification involves selecting the support which retains the undesirable molecules as little as possible, and this is the case for the hydrophilic matrices Sepharose and Streamline. For the polishing step, affinity chromatography using a monoclonal antibody raised against the native P30 protein was chosen, with an alkaline condition for elution to optimize the yield of this step.

For the scaling up of the various stages of a process, especially for adsorption chromatography, it is useful to increase the size of the columns and volumes of buffer by respecting the linear flow rates, so that the same purity profiles are achievable. However, before the primary stage of recovery, the pretreatments of clarification and concentration achievable on a laboratory scale become more difficult to handle in time and space with more important volumes. To address this problem, the technique of fluidized beds was proposed at the primary capture stage for the direct handling of fermentation mediums or cellular lysates. The development of specific supports with a dense core to increase the density favour its use in various applications according to ligand selectivity. The numerous reported examples show its interest and effectiveness in primary capture for the treatment of supernatants of bacteria [28,29,46], yeasts [47,48] and even of mammalian cells [49] and hybridoma [50–52]. For some of these examples, this chromatographic mode was challenged against the packed bed columns with the same support or equivalent, and the authors reported significant yield improvements from 15 to 65% [30], 5.5 to 9.6% [53] and time saving [28,53]. In our case, the gain obtained by EBA in this work was two-fold compared with the two other traditional approaches from 25 to 49%.

Both for perfect EBA implementation and for the interpretation of the results obtained, the guiding principles of chromatography must be verified. The creation of an expanded bed requires a sufficient velocity of the liquid phase to compensate for the terminal settling velocity of a particle. Taking into account the difference in density between the medium of fermentation and the saline buffers, the expansion level varies. This expansion creates a dead volume necessary and adequate to allow the free passage of the cells and debris between the particles of the support. These characteristics, described in the relationship of Stokes and Richardson–Zaki, were calculated under our conditions. The optimal flow velocity corresponding to the optimal yield is of $0.167\,m\,h^{-1}$ corresponding to a void fraction of 0.67 against 0.3 for the same support in packed mode. An increase in flow velocity greater than $0.3\,m\,h^{-1}$ led to a yield reduction, while the purification factor increases. The dynamic binding capacity decreases with increasing flow rate as with packed bed mode. These conditions reveal the limit where the mass transfer becomes unfavourable as much for the adsorption of target protein as for the contaminants.

The purification factor usually reported for an EBA stage range from 4 to 20 [29,30,53,54]. The theoretical approach carried out in EBA mode with known proteins added to cellular suspensions lead to the same conclusions concerning yield and purification factor [48], however our previous work [33] did not show a performance decrease with high cell density feedstock. The performance of this chromatographic mode lies more in the reduction of the number of purification steps than in the resolution. On the other hand, the objective of the capture of proteins of interest is achieved as well as volume reduction. The yield and purification factor of an EBA step in this work are about 80% and 4, respectively. The use of large buffer volumes in EBA mode, especially for the washing step, is largely compensated by the time saving and productivity benefit compared to the three previous steps of centrifugation, concentration, packed column. Affinity chromatography raises the factor of purification to a factor of 400 for each purification approach, while impacting the yield of approximately 50%.

The reproduction of these fed-batch protocols coupled with the purification scheme on a 10 l scale made it possible to obtain

Table 3
Serological performance of routine sera samples for purified rP30 expressed in *S. pombe*

	Sera toxoplasmosis status, IgG		
	Positive	Negative	Total
rP30			
Positive	828	5	833
Negative	9	956	965
Total	837	961	1798

The ELISA tests were performed using a VIDAS® system, with 1798 routine sera. The cut-off value was 7 UI/ml.

sufficient quantities of rP30 proteins to carry out the serologic tests. The rP30 sensitivity and specificity were, respectively, calculated to be 98.9 and 99.4%. By comparison, the performance of a native antigen immunoassay, such as VIDAS® TXG is, respectively, 99.8 and 99.9%. The comparison of the sensitivity and specificity obtained for the rP30 and the native antigen showed values slightly lower than the required quality standard, in particular the specificity, which is crucial to avoid false positive results (Table 3). Some posttranslational modifications, such as unwanted glycosylation could be responsible for this lack in specificity.

As for all bio-products, the cost of its production is inversely proportional to its concentration in the starting raw material. In the developed project, the increase in productivity via culture volume followed by optimization of the purification led to an 12.5-fold improvement in productivity, which signifies the same factor in Fed-Batch culture volume reduction. These results make it possible not only to reduce production costs by a factor of about 10, but also to consider an industrial production process.

5. Conclusions

This case study confronts the challenge of the purification of a recombinant protein poorly expressed in yeast, and outlines the experimental methodology implemented. The exploratory efforts carried out on the strategic parameters and the integration of the choices at upstream and downstream level made possible the objective of preparing 20 mg of protein which could be used for diagnostic test evaluation. Successive optimizations of peptone composition for growth, fermentation mode, choice of the support and matrix, and finally the comparison of three potential purification strategies made it possible to increase total productivity by a factor of 12.5 times. The process selected links fed-batch fermentation to an expanded bed chromatography mode to carry out the direct capture of rP30 in the crude feedstock. Affinity chromatography as a polishing step is critical to reach a level of purity higher than 80%. The reduced process time thus obtained decreases the economic costs by a factor 10. Although the immunoreactivity of rP30 compared to *Toxoplasma gondii* native antigen is quite good, it remains lower than the standards of quality required for the serodiagnosis of the toxoplasmosis and therefore has not been chosen for industrial use.

Nomenclature

a	exponential feed rate
d_p	particle diameter (m)
g	acceleration due to gravity ($m\,s^{-2}$)
h_0	Height of settled bed (m)
h	Height of expanded bed (m)
n	proportional exponent
Q_{e0}	initial inlet flow rate fermentor
$Q_{e\ Inlet}$	flow rate fermentor
U_p	true particle hindered velocity (m/s)
U_t	terminal velocity from Stokes Law (m/s)

Greek letters

ε	expanded bed voidage
ε_0	settled bed voidage
η	solution viscosity
ρ_p	particle density ($kg\,m^{-3}$)
ρ_l	liquid density ($kg\,m^{-3}$)

Acknowledgements

The authors wish to thank Professor E. Candolfi, Institut de Parasitologie et de Pathologie Tropicale, Strasbourg, Professor H. Pelloux, Parasitologie-Mycologie, CHU Grenoble, Dr. P. Thulliez, Institut de Périculture, Paris, for supplying the human sera for use in this study. We are grateful to Dr. Carolyn Roitsch for her scientific comments on the manuscript. We also would like to thank Christine Micolaud for her assistance with the translation of the manuscript and Johanna Dimino for technical assistance.

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