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Purification of a recombinant protein expressed in yeast: optimization of analytical and preparative chromatography

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Abstract

The industrial production of recombinant proteins requires control of both fermentation and purification steps. For the serodiagnosis of toxoplasmosis, the main antigen is a membrane protein of 30 kDa (P30). The P30 gene was cloned and expressed in *Schizosaccharomyces pombe* at 0.7 μg/ml in culture medium. Batch fermentation was optimized by the specific choice of peptones, which enabled optimum growth and protein expression without reducing the efficacy of the purification step. Analytical purification was then carried out using cation-exchange chromatography. For larger volumes, scaling up was performed on expanded mode by using a Streamline system (Pharmacia). This purification step allowed us to obtain a 67.5% recovery with a purification factor greater than 27-fold. Expanded bed adsorption technology is a convenient and effective technique for protein capture directly from feedstock, and the eluted fraction is ready for a second affinity chromatography step. This second step is performed with a yield of 40% and provides a final purification factor of 2000-fold. © 1998 Elsevier Science B.V.

Keywords: Proteins; Recombinant proteins; Expanded bed adsorption

1. Introduction

In the sector of public healthcare, toxoplasmosis is an on-going epidemiological disease for pregnant women and immunocompromized patients [1,2]. Since infection is not recognized clinically in about 90% of cases, detection of toxoplasmosis is based on serological screening. Assay kits, which are currently available on the market for the serodiagnosis of toxoplasmosis, use membrane extracts of *Toxoplasma gondii* [3,4]. Indeed, methods that use membrane antigens are recommended for the detection of acute acquired toxoplasmosis because they are more sensitive than those focusing on the detection of antibodies against various toxoplasma antigens [5]. Many authors have reported the importance of a 30-kDa surface protein (P30) for the detection of human immunoglobulins [6–8]. This protein is the major toxoplasma antigen that elicits a very early and intense antibody response. To use the P30 protein in assay kits, it was of great interest to obtain this protein via a genetically modified organism, free of virulent parasites, and without using mice, thereby satisfying animal protection legislation [9]. Therefore, the P30 gene was cloned [10] and expressed in the yeast *Schizosaccharomyces pombe*. This strain was selected after assays using *E. coli*, Chinese hamster ovary (CHO) cells, adenovirus or baculovirus failed to express rP30 with the right folding and antigenicity. When expressed in *S. pombe*, rP30 is secreted with a well folded structure, which is recognized by toxoplasmosis human sera. Batch

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fermentation of this strain enables the secretion of recombinant P30 (rP30) at a concentration of around 0.7 μg/ml in the culture medium.

The objective of this study was to investigate rP30 expression and to develop an efficient procedure for obtaining biologically active rP30 in high yields. First, we selected the peptone source in the medium that gives optimum expression of P30 protein and provides the most favourable conditions for purification. Indeed, purification of rP30 is problematic due to its low concentration in the medium (~170 n*M*), the presence of yeast cells, contaminants and cell nutrients, which interfere with successive purification steps. The first purification step was developed using analytical chromatography on a cation exchanger to determine the adsorption and elution conditions of rP30. To scale up this chromatographic step, processing traditionally includes clarification steps, such as centrifugation and/or filtration. The use of those clarification techniques leads to loss of product, an increase in the processing time and an increase in downstream processing expenditure. Consequently, it is of interest to use a technique for simplifying the purification of rP30 protein, which is in-line with a bioreactor. Expanded bed adsorption has this potential [11,12].

In this study, we compared pilot scale packed bed and expanded bed adsorption with clarified medium, reproducibility of assays, and the effect of increasing biomass on product recovery. Three cation-exchange supports were compared in expanded bed adsorption. The polishing step is performed using immunoaffinity chromatography.

2. Experimental

2.1. Yeast strain and fermentation process

A *S. pombe* strain P3, carrying the plasmid pTG8630 (Transgène, Strasbourg, France), has been used for the production of recombinant P30[1]. Preculture was performed on a non-selective YNBG modified medium and batch growth medium was modified from a chemically defined medium optimized for yeast and supplemented with peptones at 10 g l^{-1} [13]. Three peptones were tested; an acid digest of casein (Hy-Case SF, Sheffield Products, Norwich, NY, USA), casein hydrochloric peptone (bio-Case, bioMérieux, Marcy-l'Etoile, France) and casein tryptic peptone (bio-Trypcase, bioMérieux). A chemical antifoam (Struktol J673, Schill and Seilacher, Hamburg, Germany) was added to the medium at 0.0125% before autoclaving. A preculture was initiated with 100 μl from a strain-tube plated on YNBG solid medium and incubated for 96 h at 30°C. Many colonies were pooled in physiological serum to obtain a suspension with an absorbance of one at 600 nm ($A_{600}=1.0$). This solution was used to inoculate a 500-ml erlenmeyer flask (1:10, v/v), which was incubated at 30°C for 24 h using an orbital shaker at 300 rpm. At $A_{600}=1.0$, this preculture was used to inoculate the 4 l bioreactor. The cultivation temperature was maintained at 30±0.1°C, aeration at 0.9 vvm (volume air/volume medium/min), and the speed was varied so that the dissolved oxygen concentration remained at 30% saturation. The pH was regulated at 4.0±0.05 through the addition of 10% (v/v) NH_4OH. The total culture duration was around 24 h and the final A_{600} was approximately 25.0.

2.2. Analytical chromatography

The chromatographic equipment was composed of a Waters 626 pump with a 600S controller, a Waters 996 photodiode array detector, set at 280 nm. Cation-exchange chromatography experiments were carried out at 25°C using a glass column (77×8.5 mm I.D.) filled with SP-Streamline (Pharmacia, Uppsala, Sweden) of porosity $4\cdot10^6$ Da and with a particle diameter of 100–300 μm. The feedstock was centrifuged at 10 000 *g* for 15 min (Beckman J2-25) and the supernatant was then filtered through a 0.45-μm membrane filter (Millipore) before injection. Different buffer pH values (from three to seven) were assayed and three peptones sources were compared on a Biocad 600 system with the same analytical column as above.

[1]Antigen used by bioMérieux under exclusive licence from Stanford University (USA). (Letters Patent corresponding to US Patent Applications 166384). Inventors: Dr. J. Boothroyd and Dr. L. Kasper.

2.3. Preparative chromatography

The chromatographic system consisted of a Pharmacia UV1 detector, set at 280 nm, with a preparative flow-cell of 5 mm pathlength and two peristaltic pumps (Masterflex). In expanded bed mode, the liquid is 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 column was a Streamline-50 Pharmacia column with an internal diameter of 50 mm. The feedstock was pumped upwards on equilibrated Streamline SP at a flow-rate of 300 cm h^{-1}. When all the feedstock was applied to the column, the column was washed with 20 m*M* citrate buffer, pH 3.3, containing 100 m*M* NaCl until the A_{280} 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 packed column. The adsorbed product was eluted in the downward mode with 20 m*M* citrate buffer, pH 3.3, containing 0.5 *M* NaCl at 50 cm h^{-1}. The A_{280} peak was collected. The cleaning step was performed in an upstream mode with 0.1 *M* sodium hydroxide containing 1 *M* NaCl. The SP matrices tested were Streamline-SP-XL (Pharmacia, no. 17-5076-01), Streamline-SP (Pharmacia, no. 17-0993-01) and SP-Spherodex-LS (Biosepra, no. 264520).

2.4. Affinity chromatography

The chromatographic system was composed of a Pharmacia UV1 detector, set to 280 nm, with a flow-cell of 2.0 mm pathlength and a peristaltic pump P-1 (Pharmacia). The internal diameter of the column was 6 mm, the length was 6.5 mm for analytical purifications (1.8 ml of gel) and, for the preparative scale, the internal diameter was 26 mm and the length was 11 mm (60 ml of gel). The eluted fraction from cation-exchange chromatography was applied to an anti-P30 monoclonal antibody-conjugated CNBr–Sepharose 4B (Pharmacia, no. 17-0430-01) column. The bound fraction, rich in recombinant protein, was eluted with 0.1 *M* glycine–HCl, pH 2.8, containing 0.15 *M* NaCl and was then neutralized to pH 7.0 using Tris.

2.5. Determination of the concentration of the P30 recombinant

rP30 was titrated using an automated enzyme-linked immunosorbent assay (ELISA) with fluorescence detection on a Vidas immunoanalyser (bioMérieux). A standard of rP30 was coated on a polystyrene support. A standard curve was determined using rP30 in the concentration range from 0.0625 to 2 μg/ml. The standards and fraction were mixed with a diluted monoclonal antibody against native P30 labeled with alkaline phosphatase (bioMérieux) and dispensed on the support. The accuracy of this immunoassay was around 10%.

Purity was determined using reducing SDS–PAGE on a 10% acrylamide gel followed by silver staining using a PlusOne silver staining kit, Protein (Pharmacia, No. 71-1264-00-AB).

2.6. Protein determination

The protein concentration was determined using the Pierce BCA (no. 23225) enhanced method.

3. Results

3.1. Fermentation media

A fermentation protocol for the production of large quantities of recombinant P30 has been developed. We examined cell growth in batch fermentations at a 4-l scale with different peptone sources in the media (Fig. 1). These results suggested that the use of bio-Trypcase at 10 g l^{-1} gave the best growth rate, with the less contaminant peptones in the eluted fraction using cation-exchange. It lead to a growth of yeast cells to a concentration of at least 8.5 g l^{-1} dry weight biomass for a rP30 level of around 0.5–1 mg l^{-1}.

3.2. Analytical scale purification

The first purification step could be performed on an analytical scale with clarified medium to optimize conditions. rP30 would be expected to interact with the cation-exchange matrix by electrostatic attraction

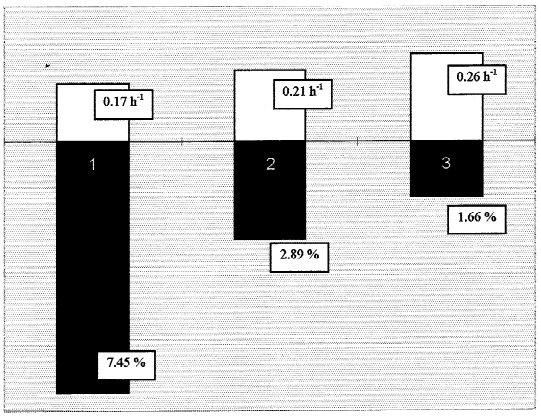

Fig. 1. Choice of peptones is determined according to their growth rate and chromatographic behavior. □, Growth rate (h^{-1}) was determined in batch culture. ■, Area (%) of eluted peak comprising peptone (1, bio-Case; 2, Hy-Case; 3, bio-Trypcase). Each peptone was diluted (10 g l^{-1}) in 20 m*M* citrate buffer with 0.1 *M* NaCl, pH 3.3, injected on SP-Streamline and submitted to the purification process for the fermentation supernatant. The peptone peak eluted at the same retention time as that of the rP30 peak when the supernatant was purified.

between the positively charged rP30 and the negatively charged sulfopropyl groups at a pH lower than the isoelectric point of the protein (~5.0). In this study, we achieved the purification of clarified feedstock at different loading pH values, between 3.0 and 7.0 (Fig. 2). The feedstock was adjusted to different pH values with citric acid or sodium hydroxide. Purification at pH 3.0 gave the best elution of rP30 in a gradient of sodium chloride. On a pilot scale with the Streamline system, we could not perform a gradient elution. Thus, we determined the sodium chloride molarity at the adsorption step to reduce the interaction of peptones with the matrix. Therefore, the yield and purity were compared with 100 or 175 m*M* NaCl in 20 m*M* citrate buffer, pH 3.3, at injection (Table 1). Loadability was also studied with these two molarities (Fig. 3). The results are in accordance with the previous table, i.e. more rP30 is recovered with 100 m*M* NaCl.

3.3. Purification on a pilot scale

Processing of larger volumes (2–4 l) could be performed on SP-Streamline in the expanded bed mode. An effective expanded bed operation was achieved with an upward flow-rate of 300 cm h^{-1} using 20 m*M* citrate buffer, pH 3.3, containing 100 m*M* NaCl. The yield in expanded and packed bed modes was compared first. For these tests, the crude feedstock was first centrifuged to 10 000 *g* for 10 min before being filtered through a 0.45-μm filter. The protein was loaded at the same linear velocity. Comparison between the two modes is summarized in Table 2. Results were similar and consequently reproducibility was tested in the expanded bed mode (Table 3). Then, to test the robustness of expanded bed adsorption, increasing biomass dry weights were added to the supernatant. The effect of biomass on product recovery is summarized in Fig. 4. Comparison of different cation-exchange supports in the expanded bed mode was performed with clarified supernatants (Table 4).

3.4. Polishing step

After the polishing step using immunoaffinity chromatography, the purity of rP30 was evaluated on SDS–PAGE (Fig. 5) by lazer densitometry. Purity is >80%.

4. Discussion

When purifying recombinant secreted proteins, the first step is their capture. The efficiency of this step will lead to a reduction in the volume [14] and the isolation of protein from the bulk. Direct processing between upstream and downstream limits loss of time and enhances protein stability and, therefore, the combination of clarification, concentration and capture in a single process remains the main issue. Three different approaches can meet this requirement, i.e., liquid–liquid extraction, tangential flow ultrafiltration and fluidized bed chromatography [15]. Cation-exchange chromatography is often chosen as a first step to capture proteins in fermentation broth as it offers a high binding capacity, good resolution, easy scaling-up and cleaning in place, it is relative cheap and leads to volume reductions [16,17]. The pioneering work of Chase and Draeger [18] on fluidized bed chromatography led to the design of a support with defined density and settling velocity, particle size and repartition to obtain a stable ex-

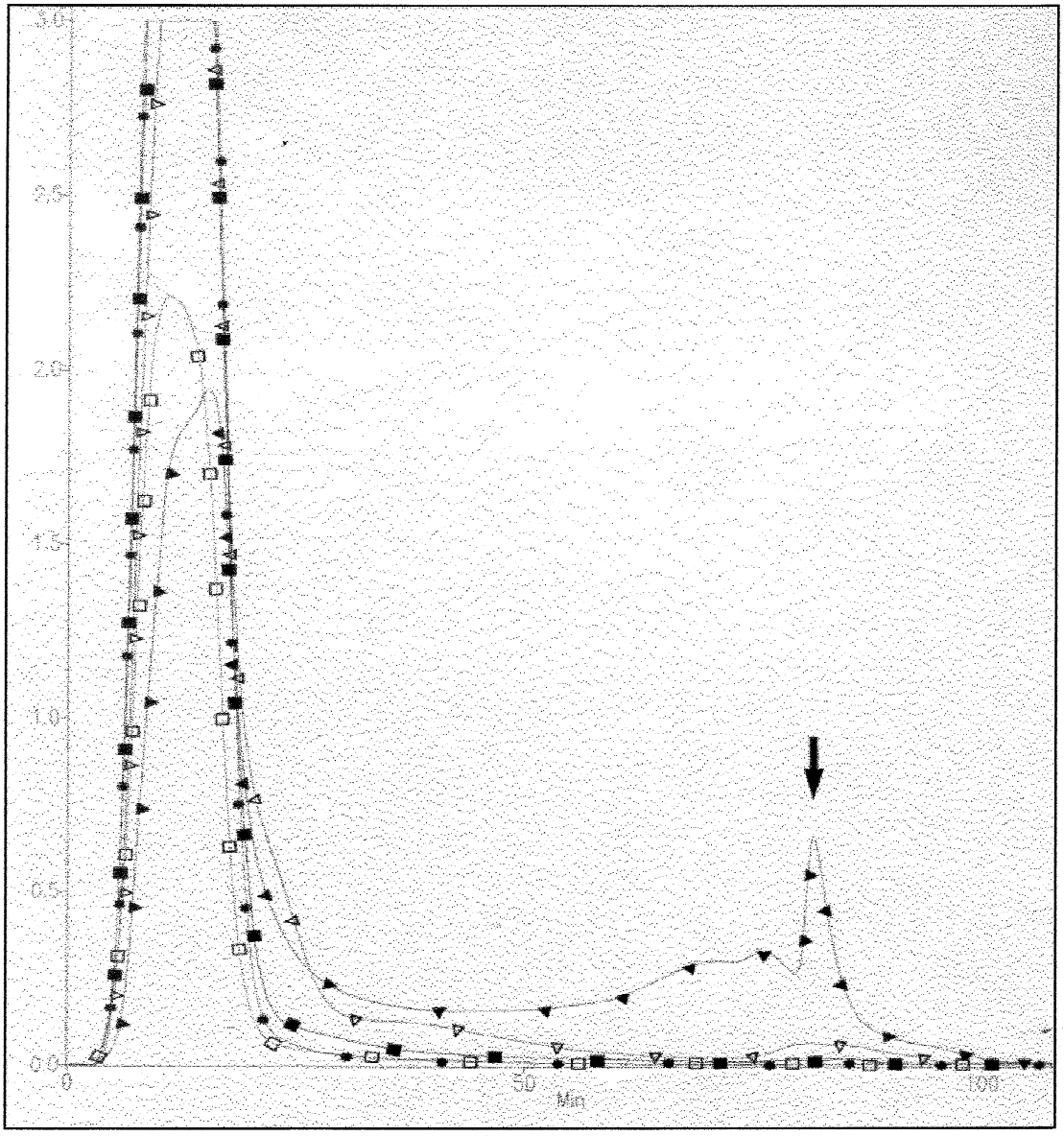

Fig. 2. Purification of clarified feedstock at different loading pH values (from 3.0 to 7.0). Elution was performed with a gradient of 1 *M* NaCl in 20 m*M* citrate buffer at different pH values. The position of rP30 is indicated by an arrow. (●) bio-Trypcase, pH 7.0; (□) bio-Trypcase, pH 6.0; (■) bio-Trypcase, pH 5.0; (▽) bio-Trypcase, pH 4.0; (▲) bio-Trypcase, pH 3.0.

panded bed. Expanded bed mode chromatography, which allows direct purification from feedstock without clarification or concentration steps, was recently developed. In previous work, expanded bed mode at a linear velocity of 300 cm h^{-1} and packed bed mode were compared [19,20]: our tests also show the equivalence of both techniques for the purification of clarified supernatant. Tests with crude feedstocks in increasing biomass concentrations, up to 25 g l^{-1}, were performed and confirmed previous results, i.e. that recovery and loadability are maintained in the presence of yeast. However, under our

Table 1
Effect of adsorption conditions on yield and purity

Fraction	Total protein (μg/ml)	Specific protein (μg/ml)	Specific protein /total protein (%)	Purification factor	Recovery (%)
Clarified supernatant	17800.0	0.98	0.0055	–	–
F2 (SP-Streamline) 100 m*M* NaCl	154.7	1.30	0.84	153	87.6
F2 (SP-Streamline) 175 m*M* NaCl	86.0	0.87	1.01	184	40.2

Clarified supernatant with 100 or 175 m*M* NaCl and adjusted to pH 3.3 with 1 *M* citric acid was purified using SP-Streamline in expanded bed mode.
Elution was performed in 20 m*M* citrate buffer containing 0.5 *M* NaCl, pH 3.15, and gave the peak labeled F2.

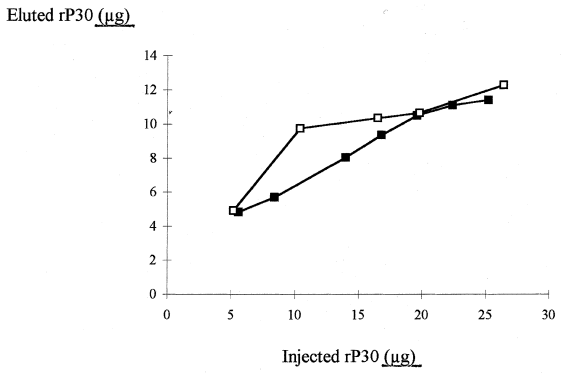

Fig. 3. Loadability is measured using SP-Streamline for two adsorption buffers: 100 and 175 m*M* NaCl in 20 m*M* citrate buffer, pH 3.3. (□) 100 m*M* NaCl; (■) 175 m*M* NaCl. Increasing volumes of clarified supernatant were injected using a constant volume (4.4 ml) of packed SP-Streamline. Then, eluted rP30 was titrated for each purification.

experimental conditions, using 25 g l^{-1} dry weight biomass, the gel cloggs under the upper adapter, which decreases the flow-rate. Flow has to be reversed sequentially to restore the correct conditions. The washing step has the function of removing particles retained within the whole adsorbent bed. To reduce the volume of the wash buffer, previous work has shown that it had to be as viscous as the feedstock [11]. The cleaning step has to be efficient and vigourous to keep expanded bed characteristics and enable the adsorbent gel to be reused in multiple cycles.

Yield of this cation-exchange step is rather low compared to the results generally obtained with fermentation feedstock purification (75–95%) [20–22]. We operated at the linear flow-rate recommended by the supplier (300 cm h^{-1}), but we can hypothesize that a lower flow-rate would increase the residence time in the column and, therefore, favor protein adsorption. Recovery for the three supports tested varied from 49 to 90%, which represents a high coefficient of variation (24%). As reported at the First International Conference on Expanded Bed Adsorption (Cambridge, UK, December 1996), Biosepra gel has good physical properties and the capacity to be used in expanded mode. Despite the coefficient of variation, the three gels show equiva-

Table 2
Comparison of packed bed mode and expanded bed adsorption: Effect on yield and purity

Fraction	Total protein (μg/ml)	Specific protein (μg/ml)	Specific protein /total protein (%)	Purification factor	Recovery (%)
Clarified supernatant	1378.0	0.76	0.055	–	–
F2 100 m*M* NaCl Expanded bed adsorption	71.8	1.06	1.5	27	48
F2 100 m*M* NaCl Packed bed mode	89.4	1.06	1.2	22	48

Clarified supernatants with 100 m*M* NaCl were injected onto SP-Streamline.
Elution was performed in 20 m*M* citrate buffer containing 0.5 *M* NaCl, pH 3.15, and gave the peak labeled F2.
rP30 was titrated in this fraction (F2).

Table 3
Reproducibility on Streamline-SP-XL in expanded bed adsorption

F2 Streamline-SP-XL	Total protein (μg/ml)	Specific protein (μg/ml)	Specific protein /total protein (%)	Purification factor	Recovery (%)
Test 1	353	1.26	0.36	90	90
Test 2	666	1.25	0.19	48	49
Test 3	533	1.20	0.23	58	49

A clarified supernatant was injected in three runs onto SP-Streamline and purified under the same conditions.
Fractions eluted using 20 m*M* citrate buffer containing 0.5 *M* NaCl were titrated with regard to total protein and rP30 before being compared.

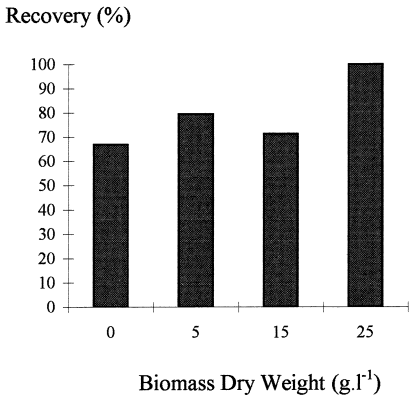

Fig. 4. Effects of increasing the biomass on product recovery. A feedstock overloaded with increasing centrifuged yeast biomass was purified using expanded bed adsorption according to the usual protocol. Recovery of rP30 was determined for each test.

lent behaviour in this application. The purification factor of our recombinant protein obtained from the different purification protocols on a Streamline col-

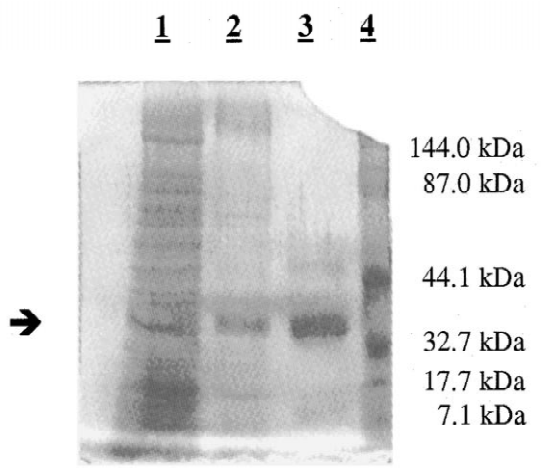

Fig. 5. 10% SDS–PAGE analysis of the rP30 purification process after silver staining. (1) Clarified supernatant; (2) rP30 after the first purification step using SP-Streamline; (3) rP30 after the second purification step using immunoaffinity chromatography. The three fractions were adjusted to the same level of rP30. (4) Molecular mass markers. The position of rP30 is indicated by an arrow.

Table 4
Comparison of three cation exchangers, Streamline-SP, Streamline-SP-XL and Spherodex-SP, for their ability to purify rP30 in clarified supernatant in expanded bed adsorption

Cation exchanger	Total protein (μg/ml)	Specific protein (μg/ml)	Specific protein /total protein (%)	Purification factor	Recovery (%)
Streamline-SP	800	1.30	0.16	40	57
Streamline-SP	1200	1.40	0.12	30	82
Streamline-SP-XL	353	1.26	0.36	90	90
Streamline-SP-XL	666	1.25	0.19	48	49
Streamline-SP-XL	533	1.20	0.23	58	49
Spherodex-SP	733	1.00	0.14	35	73
Spherodex-SP	454	1.25	0.28	70	75

Purification factor and recovery were compared.
The coefficient of variation was 24% for these tests.

umn varies from 27 to 184, depending on the total amount of protein remaining after fermentation. These results illustrate the influence of feedstock composition, which can vary from one fermentation to another. No difference was observed in capacity between the two gels SP and SP-XL, despite an improvement made on SP-XL with dextran links between the Streamline matrix and the sulfopropyl functions.

The protein expression level that is usually reported varies from 1 to 100 mg l^{-1}, depending on the nature of the host. In our study, the expression level in batch fermentation is low and purification of this protein is rather problematic. The rP30 has to be isolated from the supernatant culture, which influences purification, depending on its complexity. It was of great interest to test the behavior of the three peptone sources on the cation exchanger in parallel with their influence on growth during fermentation. Enzymatic peptones were chosen and it was found that bio-Trypcase gave the highest growth level and had the least adsorption during purifications using analytical SP-Streamline. However, some polypeptides coelute with the purified protein and a second purification step, using anti-P30 affinity chromatography, is required to obtain suitable purity. After these two purification steps, the purity of rP30 is approximately 80% and its immunological activity is maintained.

Development of the purification procedure on an analytical scale led us to choose adsorption on a cation exchanger at pH 3.3. This pH is easy to adjust because fermentation broths are set at pH 4.0 and it does not damage the yeast cells. To prevent adsorption of peptones or culture products on the support, we chose a salt concentration of 100 m*M* instead of 175 m*M* to favor protein adsorption, even though this decreased the purity slightly.

The theoretical capacity (70 mg lysozyme/ml) on this support is not obtained for our recombinant protein. Indeed, loadability tests performed led to a ratio of 1 ml of cation exchanger per 7 ml supernatant. Moreover, elution of the interesting fraction gives larger volumes than expected and leads to a low concentration on the Streamline column (volume reduction, 2). In our case, the low ratio of rP30 in broth (~170 n*M*) induced an unfavourable behavior of protein on the support, given the competition between peptones, amino acids, fermentation products and rP30 for adsorption. Nevertheless, this first capture step induced an average recovery of 67.5% in buffer conditions that are favourable for performing a second purification step using affinity chromatography with an anti-P30 monoclonal antibody.

Streamline technology provides a gain of time and yield and represents a real improvement for biotechnologists. Moreover, other chromatographic supports should be adapted in this expanded mode to offer a larger choice of chemistry for purification. However, it is of great interest to note the importance of the cleaning, collection steps, as well as the elimination of larger volumes of the fermentation broth ($\times$2.5).

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References

[1] U. Gross, Parasitol. Today 12 (1996) 1–4.
[2] J.P. Dubey, J. Am. Vet. Med. Assoc. 205 (1994) 1593–1598.
[3] M. Gorgievski-Hrisoho, D. Germann, L. Matter, J. Clin. Microbiol. 34 (1996) 1506–1511.
[4] V. Luyasu, A.R. Robert, L. Schaefer, J. Macioszek, Multicenter Study Group, Eur. J. Clin. Microbiol. Infect. Dis. 14 (1995) 787–793.
[5] A. Decoster, B. Lecolier, J. Clin. Microbiol. 34 (1996) 1606–1609.
[6] A. Decoster, P. Gontier, E. Dehecq, J.L. Demory, M. Duhamel, J. Clin. Microbiol. 33 (1995) 2206–2208.
[7] M.F. Letillois, V. Laigle, F. Santoro, M. Micoud, B.F.F. Chumpitazi, Eur. J. Clin. Microbiol. Infect. Dis. 14 (1995) 899–903.
[8] E. Kumolosasi, A. Bonhomme, A. Beorchia, H. Lepan, C. Marx, F. Foudrinier, M. Pluot, J.M. Pinon, Microsc. Res. Tech. 29 (1994) 231–239.
[9] A. Obwaller, A. Hassl, O. Picher, H. Aspöck, Parasitol. Res. 81 (1995) 361–364.
[10] J.L. Burg, D. Perelman, L.H. Kasper, P.L. Ware, J.C. Boothroyd, J. Immunol. 141 (1988) 3584–3591.
[11] H.A. Chase, Trends Biotechnol. 12 (1994) 296–303.
[12] Y.K. Chang, H.A. Chase, Biotechnol. Bioeng. 49 (1996) 204–216.

[13] O. Mendoza-Vega, C. Hebert, S.W. Brown, J. Biotechnol. 32 (1994) 249–259.
[14] Y.K. Chang, H. A Chase, Biotechnol. Bioeng. 49 (1996) 512–526.
[15] J. Thömmes, Adv. Biochem. Eng. Biotechnol. 58 (1997) 185–230.
[16] P.R. Levison, C. Mumford, M. Streater, A. Brandt-Nielsen, N.D. Pathirana, S.E. Badger, J. Chromatogr. A 760 (1997) 151–158.
[17] E. Karlsson, L. Rydén, J. Brewer, in J.C. Janson, L. Rydén (Editors), Protein Purification, VCH, New York, 1989, pp. 107–148.
[18] H.A. Chase, N.M. Draeger, J. Chromatogr. 597 (1992) 129.
[19] Y.K. Chang, H. A Chase, Sep. Biotechnol. 3 (1994) 106–112.
[20] Y.K. Chang, G.E. McCreath, H.A. Chase, Biotechnol. Bioeng. 48 (1995) 355–366.
[21] G. Pohlig, G. Fendrich, R. Knecht, B. Eder, G. Piechottka, C.P. Sommerhoffand, J. Heim, Eur. J. Biochem. 241 (1996) 619–626.
[22] C. Zurek, E. Kubis, P. Keup et al., Process Biochem. 31 (1996) 679–689.