

Development of production and purification processes of recombinant fragment of pneumococcal surface protein A in *Escherichia coli* using different carbon sources and chromatography sequences

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Abstract Pneumococcal surface protein A (PspA) is essential for *Streptococcus pneumoniae* virulence and its use either as a novel pneumococcal vaccine or as carrier in a conjugate vaccine would improve the protection and the coverage of the vaccine. Within this context, the development of scalable production and purification processes of His-tagged recombinant fragment of PspA from clade 3 (rfPspA3) in *Escherichia coli* BL21(DE3) was proposed. Fed-batch production was performed using chemically defined medium with glucose or glycerol as carbon source. Although the use of glycerol led to lower acetate production, the concentration of cells were similar at the end of both fed-batches, reaching high cell density of *E. coli* (62 g dry cell weight/L), and the rfPspA3 production was higher with glucose (3.48 g/L) than with glycerol (2.97 g/L). A study of downstream process was also carried out, including cell disruption and clarification steps. Normally, the first chromatography step for purification of His-tagged proteins is metal affinity. However, the purification design using anion exchange followed by metal affinity gave better results for rfPspA3 than the opposite sequence. Performing this new design of chromatography steps, rfPspA3 was obtained with 95.5% and 75.9% purity,

respectively, from glucose and glycerol culture. Finally, after cation exchange chromatography, rfPspA3 purity reached 96.5% and 90.6%, respectively, from glucose and glycerol culture, and the protein was shown to have the expected alpha-helix secondary structure.

Keywords *Streptococcus pneumoniae* · *Escherichia coli* · High cell density · PspA · Recombinant protein purification · Liquid chromatography

Introduction

Pneumococcal surface protein A (PspA) is an essential virulence factor of *S. pneumoniae*, a pathogenic bacterium responsible for pneumonia, meningitis, otitis media, sinusitis, bacteremia and sepsis, causing millions of death around the world, mainly of children and elderly. All current pneumococcal vaccines are based on the protection given by capsular polysaccharides (PS), alone or conjugated to a carrier protein. PS are T cell independent antigens which do not provide protection for children younger than 2 years old, nonetheless, the covalent linkage of PS to a carrier protein through chemical conjugation alters the immune response against PS, as a result, T cell-dependent response is achieved, booster effect is observed, and children are protected (Bogaert et al. 2004). More than 90 pneumococcal serotypes are known, each one corresponding to a different PS on bacterial capsule, and approximately 20 of them have been responsible for severe infections (Kamerling 2000). Due to the relatively low coverage of the available pneumococcal conjugate vaccines, serotype replacement has been observed in

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all countries that introduced these vaccines in their immunization programs (Mera et al. 2008; Ansaldi et al. 2010). Existing conjugate vaccines are being produced with proteins not related to the pneumococcal disease, such as nontoxic diphtheria protein CRM₁₉₇. An alternative to broaden the coverage of conjugate vaccines is to use a pneumococcal protein as carrier (Briles et al. 2000b).

PspA was shown to be indispensable for *S. pneumoniae* virulence (Abeyta et al. 2003; Miyaji et al. 2003; Silva et al. 2007) and to give high cover after immunization (Briles et al. 1997; Briles et al. 2000a), which led PspA to be considered a potential candidate for a protein vaccine as well as a good carrier candidate. Based on the alignment of amino acids sequence of PspA from distinct *S. pneumoniae* strains, this protein was classified in three families and six clades (Hollingshead et al. 2000). Antibody cross-recognition occurs into the same family and around 94% strains so far isolated from patients belong to families 1 and 2 (Brandileone et al. 2004; Miyaji et al. 2002; Silva et al. 2007), and a clinical trial with healthy adults immunized with a PspA from clade 2 showed cross-reactivity with PspA belonging to clades 1 to 5 from different strains (Nabors et al. 2000). If PspA were used as carrier, this new conjugate vaccine should reach much wider coverage than the current 13-valent conjugate pneumococcal vaccine. PspA has been also proposed as vaccine alone or together with other pneumococcal proteins (Baril et al. 2006; Ferreira et al. 2009; Ochs et al. 2008; Oliveira et al. 2010).

Escherichia coli has been one of the most used platform for producing recombinant proteins at large scale, since *E. coli* is a well-characterized prokaryote (Shiloach and Fass 2005) and able to reach high cell densities (Choi et al. 2006). Fed-batch culture is one of the most performed strategies to reach high cell densities of *E. coli* and consequently high recombinant protein productivities (Babaepour et al. 2008; Goyal et al. 2009; McDonald et al. 1996; Son et al. 2010). Chemically defined medium is commonly applied in this production strategy because the cultivation parameters can be easier controlled during the feeding phase and avoid inhibition by medium compounds, for instance, carbon source accumulation (Gnoth et al. 2008).

Although glucose is the most commonly used and preferential carbon source for *E. coli*, its use may produce high concentration of acetate, which can provoke negative effects on cell growth and protein production. Investigations on cultivation conditions, medium compositions, and genetic approaches have been used in order to minimize acetate formation (De Mey et al. 2007). The use of different carbon sources such as glycerol has been shown to reduce acetate formation (Hansen and Eriksen 2007; Martinez et al. 2008) and also improve the activity of the products (Hansen and Eriksen 2007).

In general, recombinant protein purification for vaccine purposes requires high purity and a process able to maintain bioactivity of the protein at the end of the downstream process; thereby, expensive and more complex purification technologies are required, which lead to increased costs of the process (Geng and Wang 2008). Liquid chromatography is a largely well-established tool in industrial scale purification (Noble 2001). In addition, a great number of possibilities for the purification strategy are available nowadays, and the choice of the strategy can influence time and cost of total purification process (Shukla et al. 2007). All these facts demonstrate the need of purification process optimization along with right process selection in order to achieve the highest efficiency with a minimum number of steps (Ansenjo and Andrews 2008).

Developing a whole production process of a specific product requires not only efficient but also economical up- and downstream processing, especially when this production is aimed to be scaled-up for mass vaccination (Hewitt and Nienow 2007). Studies of up- and downstream processing are usually performed separately (Hortsch and Weuster-Botz 2010; Goyal et al. 2009; Azevedo et al. 2010); however, if such studies were performed in an integrated manner, they can provide suitable results in order to develop more effective process (Balbas 2001; Gottschalk 2008; Roush and Lu 2008). Here, the influence of distinct carbon sources, glucose, and glycerol, at high cell density of *E. coli* BL21 (DE3) was compared for production and purification of the recombinant fragment of PspA3 in an attempt to obtain an effective process amenable to industrial scale-up.

Materials and methods

Microorganism and plasmid

The recombinant N-terminal fragment of *pspA* gene from clade 3 of family 2 (GenBank accession n. AY082389.1) was obtained from *S. pneumoniae* strain St 259/98, deposited at Nucleo de Colecao de Microrganismos, Instituto Adolfo Lutz (Sao Paulo, SP, Brazil). The fragment without the signal peptide, containing the amino acids 17 to 360, was cloned into pET37b+ plasmid vector (Department of Microbial Genetics, National Institute of Genetics Shizuoka-ken, Japan), with a polyhistidine tag in the amino terminal and inserted into *E. coli* BL21(DE3) (Invitrogen, Carlsbad, CA, USA). The clone was kindly provided by Dr. Eliane Miyaji from Centro de Biotecnologia, Instituto Butantan (São Paulo, Brazil).

E. coli cultivation for rfpSpA3 production

The cell production was performed in three distinct phases: inoculum, batch, and fed-batch. Two different carbon sources,

glycerol, or glucose were used to compare the growth and the protein production. The glycerol concentration was 20 g/L for inoculum preparation, 40, and 696 g/L in batch and feeding media, respectively, and the glucose concentration was 10 g/L for inoculum preparation, 20 and 600 g/L in batch and feeding media, respectively. The composition of chemically defined media was adapted from Seeger et al. (1995). For inoculum and batch cultivation, the medium contained 13.3 g/L KH_2PO_4 , 4.0 g/L $(\text{NH}_4)_2\text{HPO}_4$, 1.2 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.7 g/L citric acid, 14.1 mg/L EDTA, 2.5 mg/L $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 15.0 mg/L $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 1.5 mg/L $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 3.0 mg/L H_3BO_3 , 2.1 mg/L $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 33.8 mg/L Zn $(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$, 100.8 mg/L Fe(III)citrate, 4.5 mg/L thiamine hydrochloride, 25 mg/L kanamycin sulfate. The feeding medium was composed of 20.0 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 13.0 mg/L EDTA, 4.0 mg/L $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 23.5 mg/L $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 2.3 mg/L $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 4.7 mg/L H_3BO_3 , 4.0 mg/L $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 16.0 mg/L Zn $(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$, 40.0 mg/L Fe(III)citrate, 4.5 mg/L thiamine hydrochloride, 25 mg/L kanamycin sulfate.

Inoculum

Two stages of inoculum preparation were performed in flasks. One isolated colony from LB/kanamycin plate was inoculated into a flask of 100 mL containing 10 mL of chemically defined medium and, after growing to optical density at 600 nm ($\text{OD}_{600\text{nm}}$) of approximately 3.0, 2 mL aliquot from this culture was added to 200 mL of fresh medium in flasks of 2 L. An orbital shaker Innova 44 (New Brunswick Scientific, USA) was used to maintain both cultures at 200 rpm and 37°C. The second culture was grown until $\text{OD}_{600\text{nm}}=5.0$ and inoculated into the reactor obtaining an initial $\text{OD}_{600\text{nm}}$ of 0.1, i.e., 0.04 gDCW/L.

Batch cultivation

Five liters bioreactor Bioflo 2000 (New Brunswick Scientific, USA) was used to perform the cultivation. The automatic control of the culture was set at 30°C for temperature and 30% saturation of dissolved oxygen (DO) by agitation and adding pure oxygen gas. The pH was controlled at 6.7 by addition of ammonia solution (24% w/w). Polypropylene glycol (PPG) was used as antifoam agent when it was necessary. The cultivation started with 4.5 L of medium to which 0.5 L of 20 g/L lactose was added for induction.

Fed-batch cultivation

The initial volume was 3.5 L of medium and, after complete consumption of the carbon source of batch phase (same bioreactor and conditions as batch cultivation), shown by abrupt DO increase, the feeding was started using the fed-batch

medium. The pH was adjusted to 6.9, and the exponential feeding followed the equation proposed by Seeger et al. (1995). The yield coefficients were calculated from previous batches on glucose and glycerol. The specific cell growth rate set (μ_{set}) during the fed-batch phase was 0.12 and 0.10 h^{-1} , respectively, for glucose and glycerol, and the μ_{set} during the induction phase was 0.05 h^{-1} for both cultures.

Induction

The induction was performed by a mix of isopropyl- β -D-thiogalactopyranoside (IPTG, Sigma-Aldrich, USA) and 20 g/L of lactose for 4 h for both batch and fed-batch cultivations. The IPTG concentration was 0.1 mM for induction of batch culture and 0.5 mM for fed-batch culture. The rPspA3 was produced as soluble intracellular protein, and after the cultivation process, the cell separation was performed by centrifugation at $17,969 \times g$ for 30 min at 4°C. The supernatant was discarded, and the cells were frozen at -20°C.

Cell disruption

The suspension for cell disruption was prepared with 100 g of wet cell pellet resuspended in 1 L of lysis buffer, 25 mM Tris pH 8.0+0.1% of Triton X-100+1.0 mM of phenylmethanesulfonylfluoride (PMSF-protease inhibitor). A mixer X520 (CAT Ingenieurbüro, Germany) was used before lysing to homogenize the cell suspension. The homogenized suspension was disrupted using a continuous high-pressure APV 60 Homogenizer (APV Gaulin, MA, USA) in a close loop for 10 min at 600 bars. The homogenizer has a jacketed reservoir and a tube-and-shell heat exchanger in the inlet and outlet, respectively, to maintain the temperature during the disruption at about 12°C.

The efficiency of the homogenizer was tested with cell suspensions from both cultures, with glucose or glycerol as carbon source. Disruption samples were collected at intervals of 1 min from a total of 10 min. The samples were divided in three parts: (1) one aliquot was used for measurement of $\text{OD}_{600\text{nm}}$; (2) other aliquot was serially diluted and plated on LB/agar for measurement of colony forming-units (CFU); (3) 2.0 mL was centrifuged at $20,817 \times g$ for 60 min, and the supernatant was used to quantify total protein and release of rPspA3 by SDS-PAGE densitometry.

Clarification

Three different techniques were tested: centrifugation, micro-filtration with 0.1 μm of cut-off, and ultrafiltration with 750 kDa of cut-off.

The cell disruption fraction was centrifuged at $17,696 \times g$ for 2 h at 4°C (Avanti J-25i, Beckman, CA, USA). After that, the supernatant was separated from the pellet and filtered in 0.45 μm .

The filtration tests were performed with hollow fibers of polyestersulfone: (1) with a pore of 0.1 μm and 1,200 cm^2 of filter area (CFP-1-E-5A); 2) with 750 kDa of cut-off and 850 cm^2 of filter area (UFP-750-E-4X2MA), both membranes purchased from GE Health Care, USA. In both cases, the cell disruption fraction was concentrated to one third of the initial volume and then the concentrate was washed with six volumes of 25 mM acetate buffer pH 6.0. The transmembrane pressure was kept constant in 5 psi.

Chromatography steps

The chromatographic steps were operated by Äkta Explorer system and monitored and controlled by Unicorn 3.21 software. Metal affinity resin, 500 mL of IMAC-Sepharose, and 250 mL of anion exchange Q-Sepharose were packed into XK50 columns, while 75 mL of cation exchange SP-Sepharose was packed into XK26 column. A flow-rate of 150 cm^3/h was applied for all chromatography steps. All chromatography equipment and resins were purchased from GE Healthcare Life Sciences, USA.

Previous studies of adsorption and desorption were performed in test tubes to choose the best concentration of eluents during the process. The initial conditions were chosen based on the theoretical pI (4.74) of the rPspA3. After that, a study of the sequence of chromatography steps was performed using cells from production with glucose as carbon source. Finally, after the definition of the best sequence, the whole purification process was performed with cell disruption fractions from cultures with both carbon sources, and the results were compared to study the integration of production and purification processes. The chromatography steps were performed as described below.

IMAC-Sepharose was previously charged with 500 mM NiSO_4 , washed with water, and equilibrated with 25 mM Tris buffer pH 8.0. Protein adsorption was done with 5 mM imidazole in 25 mM Tris pH 8.0, and elution of the protein of interest was performed with 200 mM of imidazole in the same Tris buffer. In order to obtain higher purity, a washing step with 7 mM imidazole was introduced for the material from glycerol production and with 15 mM imidazole for the material from glucose production. The column was cleaned with 500 mM imidazole and 300 mM NaCl in 25 mM Tris buffer pH 8.0. Ethylenediaminetetraacetic acid (EDTA) (100 mM) was used to strip off the metal ion and prior to resin regeneration with NiSO_4 .

Q-Sepharose was equilibrated with acetate buffer (25 mM sodium acetate, pH 6.0) with 50 mM NaCl. The same buffer was used for washing and elution steps. The washing step was performed with 100 mM NaCl and the elution step with 300 mM NaCl. The cleaning of this resin was performed with 500 mM NaCl in 25 mM Tris buffer pH 8.0. The regeneration was performed with 100 mM NaOH.

SP-Sepharose was equilibrated with histidine buffer (20 mM L-histidine hydrochloride, pH 6.0), and the rPspA3 was eluted with 30 mM phosphate buffer pH 7.0. The cleaning was performed with 25 mM Tris buffer pH 8.0 with 500 mM NaCl.

Analytical methods

Biomass concentration

Sampling of the cultures was done every hour. The spectrophotometer U-1800 (Hitach High-Tech, Japan) was used at 600 nm of wavelength for OD reading, after appropriated sample dilution. To determine the dry cell weight, 10 mL of each sample were centrifuged in previously weighed tubes at 17,800 $\times g$ for 15 min, and the supernatant was discarded. The pellets were dried at 60°C until constant weight. A linear correlation was obtained between $\text{OD}_{600\text{nm}}$ and cell concentration in grams per liter.

Concentration of glucose, glycerol, and acetic acid

The concentration of glucose, glycerol, and acetic acid was analyzed by HPLC (Shimadzu, Japan). Aminex HPX-87H column (Biorad Life Sciences, USA) was used for this analysis. The mobile phase was 5 mM H_2SO_4 , and the running was performed at 60°C for 30 min at 0.6 mL/min. The refraction index detection (RID) was used for glycerol and glucose analysis and UV detection at 210 nm for acetic acid.

Plasmid stability

The vector stability test was performed in the inoculum, before the induction and at the end of the production process. A sample of cell suspension was serially diluted in 0.85% NaCl and 100 μL of each dilution were plated in LB agar. After 24 h, 70 colonies were replicated on two plates with and without 25 mg/L kanamycin. The stability was recorded as the percentage of kanamycin resistant colonies.

Protein quantification

Total protein was quantified by Lowry's method using Folin phenol reagent (Lowry et al. 1951) and bovine serum albumin as standard. Samples with interfering chemical compounds were previously dialyzed against 20 mM phosphate buffer pH 7.0 for 24 h.

Gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli et al.

(1970) with 12% acrylamide gel. Samples with high concentration of NaCl or other interfering chemicals were previously dialyzed for 24 h as described above. Samples from cell suspension were adjusted to $OD_{600nm}=5.0$ and heated at 100°C for 15 min in presence of SDS and beta-mercaptoethanol to disrupt the cells and 20 μ L was applied to the gel. Diluted samples from the purification process were first concentrated by centrifugal vacuum concentrator Centrивap (Labconco, USA). Gels were stained by Comassie Blue R-250.

Relative purity of rFPspA3

Two methods were used to determine the relative purity of rFPspA3: densitometry of SDS-PAGE bands using a densitometer GS-800 with Quantity One 4.6.3 software (Biorad Life Sciences, USA) and high performance size exclusion chromatography (HPSEC) in TKSgel G2000SWXL (Tosoh Bioscience, Japan) with UV detection at 280 nm. In the densitometry, the relative purity was calculated as the percentage of the intensity of rFPspA3 band against the sum of intensity of all other bands in the same lane and, in HPSEC, as the percentage of rFPspA3 peak area against the sum of all peaks areas in the chromatogram.

Circular dichroism

The method was performed according to Kelly et al. (2005). Sample (60 μ L) at approx. 8.5 μ M rFPspA3 was used for analysis. The polarimeter spectrum Jasco J-810 was used to perform the analysis with Software Spectra Manager (Jasco, Germany) in a wavelength range from 185 to 260 nm. The analysis was performed at room temperature.

Results

E. coli cultivation

Two carbon sources were used to produce rFPspA3 and evaluate their influence on *E. coli* growth, recombinant

protein production, and also on the purification of rFPspA3 along the process.

Kinetic parameters were similar in batch culture mode (data not shown) and during the batch phase of fed-batch mode performed with the same carbon source. The yield coefficient on biomass was the same (0.3 gDCW/g carbon source), while the maximum specific growth rate (μ_{max}) was higher with glucose ($\mu_{max}=0.54\text{ h}^{-1}$) than with glycerol ($\mu_{max}=0.41\text{ h}^{-1}$). The lower acid acetic production in the glycerol culture resulted in higher cell concentration at the end of batch. As the maximum cell concentration was higher with glycerol, but the cultivation time was longer due to slower growth rate, the same cell productivity was achieved in batch culture mode with both carbon sources (Table 1).

Comparing fed-batch phase of both cultures, the growth profiles were similar for them and cell concentration reached 62 g/L in both cases. During the process, higher acetate production was obtained using glucose as carbon source, it peaked at 4.5 g/L at the end of batch phase and its production was between 1.0 and 2.5 g/L during fed-batch phase, while using glycerol acetate production peaked at 0.58 g/L at the end of batch phase and its production was near zero during fed-batch phase. In spite of higher acetate production, the fed-batch strategy permitted to achieve the same final cell concentration with glucose and glycerol and, as the cell production was faster with glucose, slightly higher cell productivity was obtained when compared to that with glycerol (Table 1).

Similar cell productivities were obtained in batch mode using both carbon sources; however, it was achieved sixfold higher cell production and almost threefold higher cell productivity in fed-batch mode when compared to simple batch mode with both carbon sources (Table 1). Regarding rFPspA3 production, an increase of productivity was also obtained in fed-batch mode compared to simple batch, 1.6-fold higher rFPspA3 productivity was achieved with glucose, and 1.4-fold higher with glycerol (Table 1).

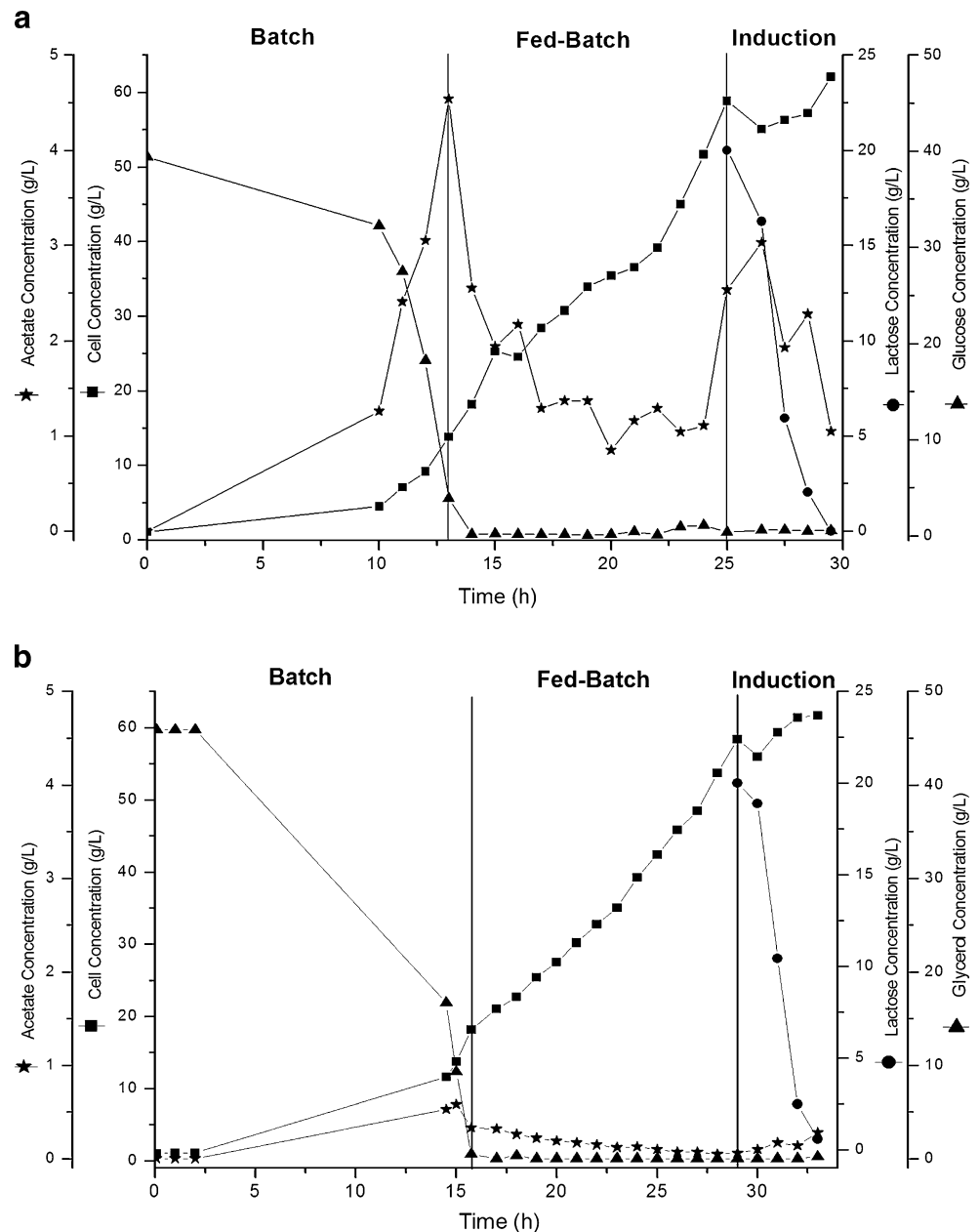
During the induction phase of fed-batch cultures, lactose was totally consumed by the microorganism after 4 h (Fig. 1) and the production of rFPspA3 increased with the time of induction (Fig. 2). It can be also observed in Fig. 2 that

Table 1 Comparison of cultivation parameters of batch and fed-batch cultures of *E. coli* BL21(DE3) with glucose or glycerol for rFPspA3 production

Cell production process	Carbon source	Maximum cell concentration (gDCW/L)	Cell productivity (gDCW/(L × h))	rFPspA3 production (g/L) ^a	rFPspA3 productivity (g/(L × h))
Batch	Glucose	10.7	0.7	1.12	0.075
	Glycerol	13.0	0.7	1.11	0.063
Fed-batch	Glucose	62.0	2.1	3.48	0.12
	Glycerol	61.7	1.9	2.97	0.09

^a Measured by densitometry of SDS-PAGE bands

Fig. 1 *E. coli* BL21(DE3) fed-batch culture for rPspA3 production using two different carbon sources, glucose (A) and glycerol (B), and combined induction with 0.5 mM of IPTG and 20 g/L of lactose. Acetate (stars), cell (squares), lactose (circles), glucose or glycerol (triangles)



besides rPspA3, many other proteins were produced in high cell density cultivation. These by-products can vary according to the environment that *E. coli* is exposed to and can also interfere in the downstream process; for this reason, it is important to integrate up- and downstream processes.

Cell disruption

Cells pellets, around 100 g from both cultures, were thawed for disruption, which was performed in a close loop using a high-pressure continuous homogenizer for 10 min. The profile of cell disruption and rPspA3 release showed that 5–6 min were enough for maximum disruption (Fig. 3).

According to Fig. 3, the values of CFU and OD_{600nm} were near zero after 6 min, indicating complete cell lysis. The cells from the glycerol culture resisted the freezing and thawing process better than cells from glucose. Initial cell viability of the culture was 9.8×10^4 CFU/mL with glycerol and 4×10^4 CFU/mL with glucose (Fig. 3, circles), while the initial concentration of rPspA3 in the supernatant was 1.0 mg/mL with glycerol and 1.6 mg/mL with glucose (Fig. 3, squares). After 10 min, the cell disruption yielded 2.9 ± 0.1 and 3.3 ± 0.1 mg/mL of rPspA3 from fed-batch cultures with glucose and glycerol as carbon source, respectively. The rPspA3 produced here was shown to be soluble: after 2 h of centrifugation at $20,817 \times g$, all rPspA3 was in the supernatant and none in the insoluble fraction (data not shown).

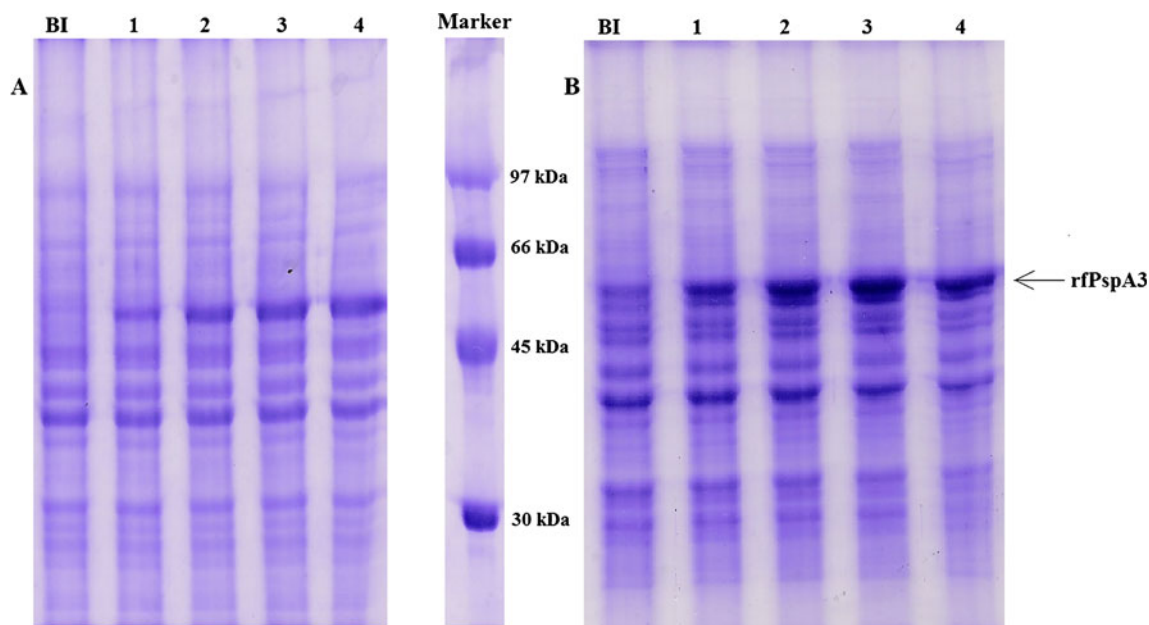


Fig. 2 SDS-PAGE of rFPspA3 production in fed-batch cultures with chemically defined medium using glucose (left side) or glycerol (right side) as carbon source. BI—before induction; 1—after 1 h of

induction; 2—after 2 h of induction; 3—after 3 h of induction; 4—after 4 h of induction. A cell suspension of each sample was adjusted to OD=5.0 and 20 μ L of the suspension was applied to all lanes

Efficiency of cell lysis was also compared to a control sample where cells were treated with 100 mM NaOH and a complete reduction in OD_{600nm} was observed, representing 100% efficiency of disruption. The efficiency of the disruption process by high-pressure continuous homogenizer, measured as reduction of OD_{600nm}, was 96.7±4.0% and 92.7±3.8% for culture with glucose and glycerol, respectively.

Although higher production of rFPspA3 was obtained in fed-batch with glucose, higher rFPspA3 concentration from the cultivation with glycerol was obtained during cell disruption (Fig. 3). This result cannot be attributed to the efficiency of the disruption based on OD_{600nm} reduction,

since it was similar for material from glucose and glycerol cultures, but it could be related to the cryoprotector effect of glycerol, which could also have a positive consequence avoiding rFPapA3 degradation by proteases released from *E. coli* during disruption. Regarding rFPspA3 released from cell disruption compared to the amount produced in fed-batch cultures (3.48 and 2.97 g/L, Table 1), the yield was higher for material from glycerol (about 3.3 mg/mL of rFPspA3 from glycerol and 2.9 mg/mL from glucose culture, Fig. 3).

Clarification

The performance of mechanical disruption provides different sizes of cell debris in the homogenate, which can be related to the choice of the clarification method in this work. No purification was observed after centrifugation and the tangential filtration resulted in 1.4-fold (micro-filtration) and 1.3-fold (ultrafiltration) of purification (results analyzed by HPSEC); however, they became unfeasible due to the lower yield of rFPspA3 obtained in both (43.9% and 62.5%, respectively) comparing to the centrifugation (100%). Probably, debris obstructed the pores of membranes forming secondary layers which retained the rFPspA3. The low recovery in these purification steps would mean a loss of 9.5 g rFPspA3 for microfiltration and 6.4 g for ultrafiltration of all protein produced in 5 L reactors (17 g). Thus, high value of recovery was prioritized in the clarification step, since other purification steps were included and provided the required purity.

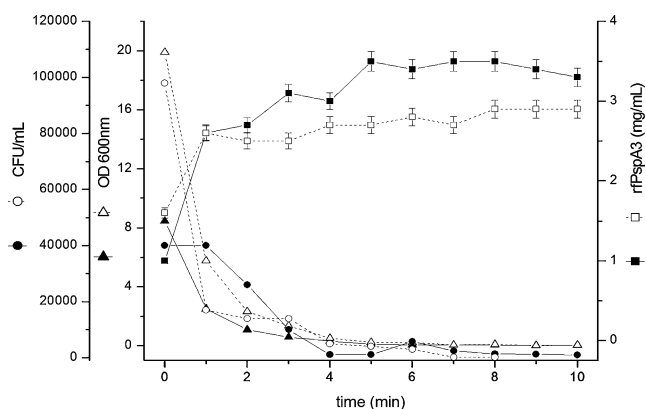


Fig. 3 Profile of cell disruption by high-pressure continuous homogenizer of *E. coli* BL21(DE3) produced with glucose (solid line and symbols) or glycerol (dashed line and open symbols). OD_{600nm} (triangles), CFU/mL (circles), concentration of rFPspA3 released into the supernatant (squares)

Table 2 Comparison between two chromatography sequences for purification of rPspA3 from *E. coli* BL21(DE3) produced in fed-batch with glucose

Purification steps		rPspA3 RP ^a (%)	rPspA3 recovery ^b (%)	PF ^c
Centrifugation		6.9	100	1.0
First sequence	Anion exchange	28.6	82.0	4.2
	Metal affinity	81.1	62.8 (76.5) ^d	11.8 (2.8) ^d
Second sequence	Metal affinity	46.6	96.6	6.8
	Anion exchange	75.8	54.1 (56.0) ^d	11.0 (1.6) ^d

^a Relative purity, value obtained by HPSEC, and 8.8 % of deviation was calculated for the results

^b The recovery was calculated based on total rPspA3 amount after centrifugation

^c Purification factor

^d Step recovery or step purification factor, both calculated based on previous input protein, in parentheses

Chromatography steps

Two chromatography steps were pre-selected based on physicochemical features of rPspA3: the anion exchange Q-Sepharose, and the metal affinity IMAC-Sepharose. Starting from the same centrifuged fraction, two sequences were tested: anion exchange followed by metal affinity and the opposite sequence. Both sequences resulted in similar outcomes (Table 2).

The first sequence was selected because it yielded a slightly better purification and recovery results. Besides, anion exchange resins are cheaper than metal affinity resins and can be loaded with high amounts of proteins from a very impure raw material. Hence, the first sequence should increase the life-time of metal affinity, which will be used

with a pre-purified fraction. Thereby, the centrifuged material from fed-batch culture with glycerol as carbon source was also purified using the sequence anion exchange followed by metal affinity chromatography (Table 3).

Another screening of liquid chromatography was performed with size exclusion, hydrophobic, and strong cation exchange chromatography. Only cation exchange chromatography (SP-Sepharose) yielded good purification factor. Despite the theoretical pI of rPspA3 is 4.74, the protein was adsorbed to the resin at pH 6.0, i.e., theoretically with the same charge of the cation exchange. As PspA has two well-defined regions with opposite charges (Jedrzejas et al. 2001), this allowed the protein to adsorb to the SP-Sepharose at pH 6.0.

The whole process was applied for purification of rPspA3 from fed-batch cultures with glucose and glycerol. As it can be seen in Table 3, the performance of each individual chromatography differed according to the carbon source used to produce the raw material, but the final results were relatively similar. The anion exchange worked better for the material from glucose culture, resulting in rPspA3 with higher purity and recovery than that produced with glycerol. This may be a consequence of higher amount (3.48 g/L, Table 1) and relative purity of rPspA3 in the centrifuged fraction from glucose culture (22.1%, Table 3) than from glycerol (2.97 g/L, Table 1 and 17.7%, Table 3, respectively for amount and purity). Besides, the conditions of anion exchange were established using material from fed-batch with glucose and after applied for purification of PspA from glycerol culture.

The metal affinity chromatography, on the opposite, seemed to work better for the fraction from glycerol culture, resulting in higher step recovery than that from glucose (70% against 39%, Table 3) and higher purification factor (2.55 against 1.45, Table 3). Finally, the purification factor of cation exchange was higher in the fraction from glycerol than that from glucose (1.19 against 1.01, Table 3), while it

Table 3 Purification of rPspA3 from *E. coli* BL21(DE3) produced in fed-batch high cell density cultivation with glucose or glycerol

Purification steps		Total protein (mg)	rPspA3 RP ^a (%)	rPspA3 (mg)	Recovery ^b (%)	PF ^c
Glucose	Centrifugation	12,771	22.1	2,822	100	1.0
	Anion exchange	3,963	65.8	2,608	92	2.98
	Metal affinity	1,068	95.5	1,020	36 (39) ^d	4.32 (1.45) ^d
	Cation exchange	654	96.5	631	22 (62) ^d	4.37 (1.01) ^d
Glycerol	Centrifugation	15,476	17.7	2,739	100	1.0
	Anion exchange	4,939.6	29.7	1,465	53	1.68
	Metal affinity	1,342	75.9	1,019	37 (70) ^d	4.29 (2.55) ^d
	Cation exchange	387	90.6	351	13 (34) ^d	5.12 (1.19) ^d

^a Relative purity, values obtained by densitometry

^b The recovery was calculated based on total rPspA3 amount after centrifugation

^c Purification factor

^d Step recovery or step purification factor, both calculated based on previous input protein, in parentheses

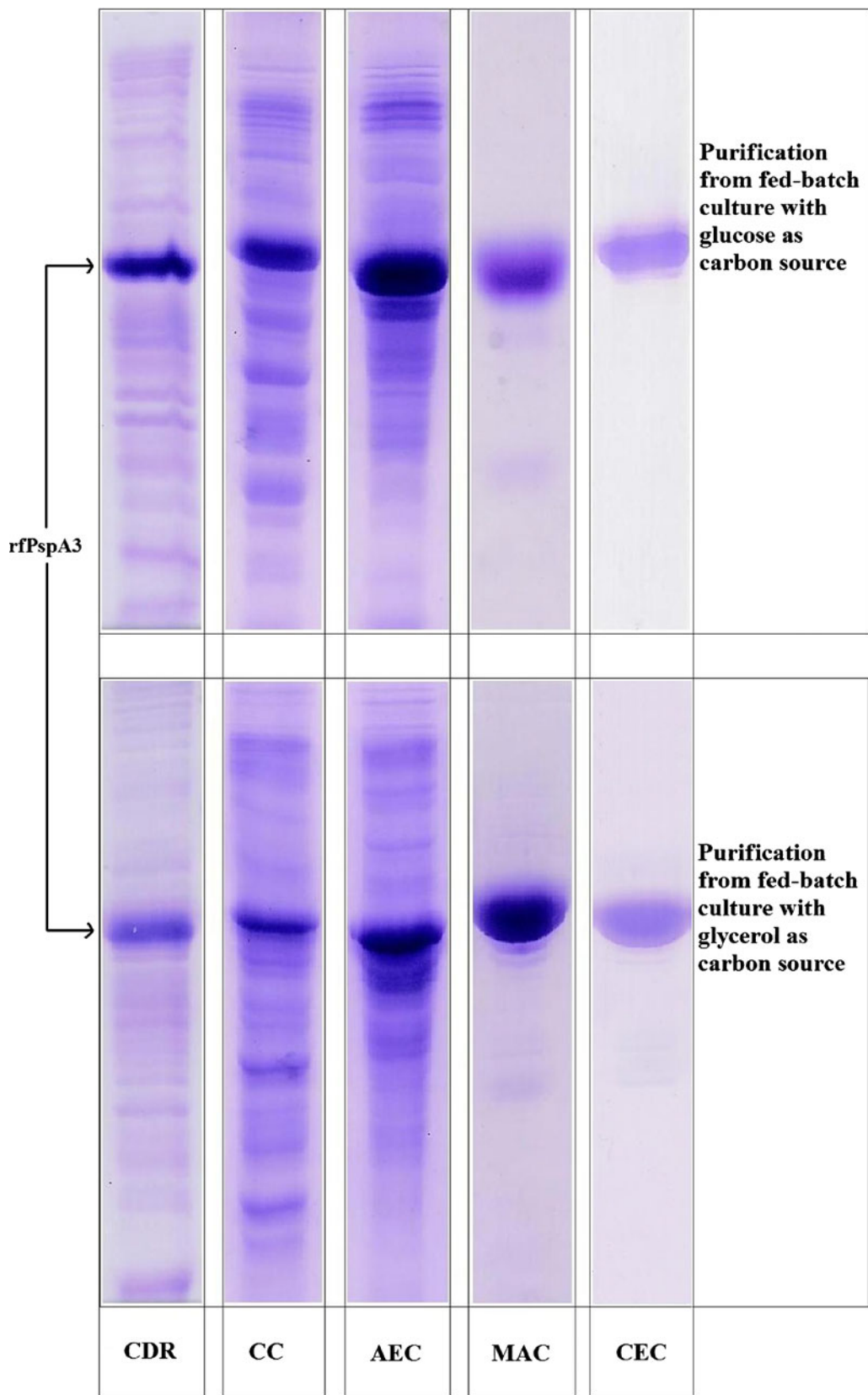


Fig. 4 SDS-PAGE of fractions from downstream steps. *CDR*—cell disruption, *CC*—clarification by centrifugation, *AEC*—anion exchange chromatography, *MAC*—metal affinity chromatography, *CEC*

—cation exchange chromatography. The following amount of total protein was applied to each lane: *CDR* and *CC*—15 μ g, *AEC* and *MAC*—5 μ g, *CEC*—3 μ g

occurred the opposite with the recovery, which reached 62% from glucose and only 34% from glycerol (Table 3).

In order to visualize the efficiency of downstream process, the Fig. 4 presents the fractions obtained in each purification step from fed-batch cultures with glucose or glycerol as carbon sources. The increase of purity can be clearly noted. A large amount of impurities were removed in the first and mainly in the second chromatography steps. The rfPspA3 purity after cation exchange was high in both process, but the recovery was low in the fraction obtained from glycerol culture, therefore an improvement of this last step in an attempt to obtain higher rfPspA3 recovery might be necessary.

Circular dichroism

At the end of the purification process, the purified rfPspA3 retained the secondary structure as shown by the circular dichroism (CD) spectrum (Fig. 5). The CD spectrum profile showed α -helical secondary structure without β -sheets (Kelly et al. 2005). This profile is similar to the N-terminal fragments of PspA from different strains obtained by Jedrzejewski et al. (2001). According to this author, all PspA constructions had α -helical secondary structure content, adopting a coiled-coil structure with an elongated rod-like shape, and no β -sheet was detected.

Discussion

Batch cultures were firstly performed in order to obtain the kinetic parameters to apply them later in the fed-batch cultures to reach high cell densities. The cell production was similar for both carbon sources at the end of the fed-batch, approximately 62 g/L, but the cell productivity was slightly lower in the glycerol culture, due to lower specific growth rate during the fed-batch phase and consequently longer cultivation time (Table 1).

The culture with glucose produced 4.5 g/L acetate at the end of batch phase, but only 0.58 g/L with glycerol. During the fed-batch phase, the acetate was consumed and a plateau of about 1.2 g/L was reached with glucose, while it remained near zero with glycerol. According to De Mey et al. (2007), the presence of acetate in concentrations above 1 g/L can be harmful to the cell growth and also to recombinant protein production. The comparison of these carbon sources has been reported by different authors (Martinez et al. 2008; Hansen and Eriksen 2007), and they have also found a decrease of acetate formation using glycerol. Hansen and Eriksen (2007) obtained 40% higher product activity using glycerol. However, in our case, the final cell concentration was higher only in simple batch culture mode with glycerol, but it did not lead to higher

protein production (Table 1). In fed-batch culture mode, the specific growth rate used during the fed-batch phase was more important to the final results than the acetate produced, certainly because the acetate level was below the critical value in both cases and the overflow mechanism did not occur. Maybe the lower protein production observed using glycerol was related to the higher maintenance coefficient on glycerol than on glucose (Neijssel et al. 1999), which means that more energy would be required for growth and protein synthesis, and the cultivation conditions have to be optimized for glycerol.

This higher protein production in fed-batch with glucose could be also explained by the *lac operon* repression system, which avoids the recombinant protein production before induction (Narang 2009). Glycerol does not exert carbon catabolite repression effect on *E. coli*, as can be observed in Fig. 2. Besides that, the plasmid stability was lower during production with glycerol (88%), while it was 100% stable during all process with glucose.

Concerning the sequence of chromatography steps, metal affinity certainly presented higher PF values than anion exchange chromatography, and both sequences yielded similar final results (Table 2). In almost all articles on recombinant protein purification, the sole chromatography step used is metal affinity and, when more than one step is necessary, metal affinity is the first step in the large majority of reports. Very few articles report the use of ion exchange before metal affinity and, in general, they did not discuss the reasons for adopting this sequence. Zhao et al. (2010) states that cation exchange is efficient for LPS removal and Schmidt et al. (2007) attributes the low recovery of His-tagged protein to a change in oxidation state or to a complex formation between the Ni^{2+} resin and yeast intracellular contents when large volumes of crude yeast cell lysate is applied to Ni^{2+} resin. Here, the sequence anion exchange followed by metal affinity was chosen since the binding capacity of IMAC-Sepharose was greatly diminished after five performances as first chromatography step (not shown);

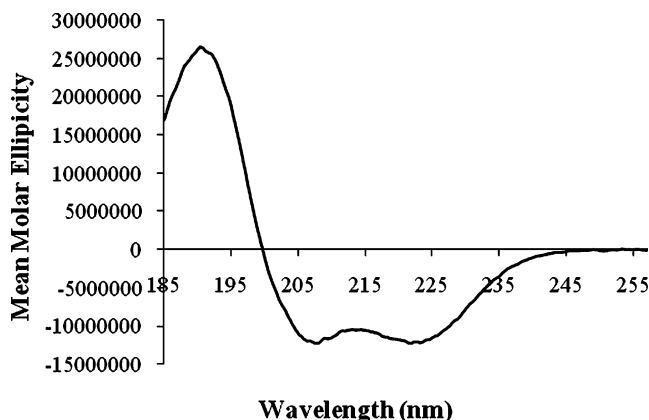


Fig. 5 CD spectrum of rfPspA3 purified from fed-batch culture

this probably could be caused by the high load of impurities into the column. Therefore, choosing the anion exchange as the first chromatography step could decrease the total cost of the process, since Q-Sepharose resin has about 70% lower prices than IMAC-Sepharose (supplier information) and longer life-time.

The highest final purity reached when glucose was used for the upstream processing could be explained by the difference between the rPspA3 relative purity in the initial fractions: 22.1% in centrifuged fraction from glucose culture and 17.7% from glycerol, which was a consequence of the higher specific production of rPspA3 on glucose than on glycerol, 56 and 48 mg/gDCW, respectively. Higher specific production was also reached using glucose in batch than using glycerol, 106 against 86 mg/gDCW.

Studying a large scale production and purification of recombinant fibroblast, three chromatography steps were necessary to reach 90.6% of purity (McDonald et al. 1996), but only two steps were necessary to purify recombinant thermostable chitinase up to 12.7 purification factor; however, non-homogeneous product with incorrectly folded proteins was obtained in this process (Christodoulou et al. 2001). Here, rPspA3 with the same CD profile as previous study (Jedrzejewski et al. 2001) was obtained after three chromatography steps with no damage to the secondary structure (Fig. 4). According to Table 3, the rPspA3 could be obtained with 36% of recovery and 95.5% of purity from the glucose fed-batch culture after only two chromatographic steps. As it is already a quite high purity, the feasibility of a procedure with two instead of three chromatography steps will depend on the criteria of purity that would be required by regulatory agencies for licensing the vaccine product.

In conclusion, the carbon sources used in fed-batch cultures exerted the expected influence on acetate production, although the higher acetate amount produced with glucose did not affect the rPspA3 production and the fed-batch permitted to reach the same final cell concentration of *E. coli* BL21(DE3) than that obtained with glycerol. The higher rPspA3 specific production on glucose culture seemed to influence downstream processing, since higher purity was obtained using the protein produced with glucose as carbon source. Finally, a scalable process for production of PspA with high yield in high cell density fed-batch culture was developed as well as a purification process consisting of cell disruption by high-pressure continuous homogenizer, centrifugation, and three steps of liquid chromatography, which allowed to obtain the rPspA3 with high purity and right conformation.

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