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PURIFICATION OF MBP-β-GALACTOSIDASE AND MBP-RUBREDOXIN THROUGH AFFINITY MEMBRANE SEPARATION

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ABSTRACT

A system based on the use of affinity membranes for the recovery and purification of a class of fusion proteins containing the Maltose Binding Protein (MPB) domain has been studied. An affinity support was obtained through a chemical modification protocol of microporous cellulosic membranes, and amylose was used as the specific ligand. A membrane module was realized in a column configuration, suitable for flat sheet membranes. The total membrane area available was arranged in a series of stages in order to benefit of the fluid-dynamic effects throughout the entire stack. The performance of the process was compared with the one offered by stationary phases based on porous beads or resins. Two different fusion proteins, MBP-β galactosidase and MBP-rubredoxin, characterized by a molecular weight of 160 and 51 kDa, respectively, were used. The feasibility of a single-step separation process of MBP fusions with amylose affinity

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membranes has been demonstrated, with good results both in terms of selectivity and purity of the recovered product. In comparison to the commercially available supports: (i) the binding capacity per unit volume of the membranes obtained is approximately the same; (ii) the process time is much shorter when affinity membranes are used; and (iii) protein concentration as well as purity of the resulting protein solutions are higher for the affinity membrane process.

INTRODUCTION

Downstream operations devoted to separations and purifications are most often the key-factor for the economics of biotechnology processes. When choosing a separation technique and its large-scale application, several variables need to be considered. Some of the methods proposed exhibit optimum purifying abilities but are too expensive to be applied on a large scale, while other methods are economical but with a poor purifying ability. Thus, the best choice depends on several factors like required product purity, market price, process cost, instrumentation, and engineering aspects (1).

One of the finest techniques available for the purification of biomolecules is affinity chromatography (2). The basic principle of such separation process exploits the affinity interaction between the desired product and a complementary substance called ligand, often immobilized into an insoluble matrix. In general, the affinity technology is exploited in several processes with the purpose of selective isolation of a target species rather than the chromatographic separation of all species. The development of affinity separation technology requires choices of the immobilization chemistry, support materials and affinity ligand design (3). Affinity separation processes are normally performed with polymeric matrices in the form of porous beads or resins, both with dead-end pores, in column or packed bed configurations. As a consequence, a series of fluid-dynamic limitations are present such as large pressure-drop in the column, diffusion mass transfer limitation encountered by the biomolecules to reach the active sites inside the dead-end pores, low convective flowrates, packing risks of the stationary phase.

The use of microporous membranes, in the form of flat sheets or hollow fibers, as affinity matrices is then proposed in order to overcome the major limitations encountered with affinity resins (4,5). The liquid protein solution is conveyed throughout the porous matrix, containing the ligand selective for a target protein. Diffusion limitations are reduced, all the unbound molecules can pass through and are collected in the permeate side. The main advantages for the use of affinity membranes are associated to the high efficiency of the washing

step, as well as to the possibility of processing large volumes of liquid solution in a much shorter time than by using packed beds of porous beads.

In affinity adsorption, the ligate solution is loaded to the system until the concentration exiting from the column reaches a predetermined threshold value. Once the working parameters have been optimized in a bench scale, the scale-up of preparative affinity adsorption processes is not so immediate, due to several difficulties and especially kinetic limitations. The kinetics of the sorption process plays a major role in determining the speed and efficiency of capture in such a process (6). An affinity separation process is the sequence of a series of physico-chemical steps such as diffusion in the liquid phase, diffusion inside the pores to the active binding site, adsorption reaction, desorption reaction, and diffusion of the target molecules back to the liquid phase. The efficiency of active site utilization depends strongly on the ratio between the residence time of the liquid phase in the column and the diffusion-reaction time. Thus, the effect of flowrate depends clearly on the particle size: the shorter is the diffusion distance the higher the feed flowrate can be; however, smaller beads lead to a stationary phase packed more closely and thus with higher pressure drops. To overcome this limitation, the effort is to arrange the column in a large cross-section and shallow configuration, the limit of which is just like a membrane. Nevertheless, this does not reduce substantially the diffusion path inside the single bead.

On the contrary, when a solution permeates through a membrane, the solutes are transported to the active sites by convection, and the diffusion resistances are much smaller than in the porous beads endowed with the dead-end pores. In addition, the use of affinity membranes also provides a pressure drop reduction with respect to traditional affinity columns (7,8). The production rate through a membrane stack in which the ligand loading is equal to that of a sorbent bed will be much faster than through a packed column.

In this work, the efficiency of microporous affinity membranes in separation processes of recombinant proteins is investigated. A membrane module, containing flat sheet membranes, has been designed and used to perform protein purification processes on a preparative scale.

EXPERIMENTAL

Growth Protocol for *Escherischia coli* Genetically Modified Strains

Genetically modified *E. coli* strains of the "Protein Fusion and Purification System®" from New England Biolabs Incorporation were used to produce the fusion protein. Two different strains were considered, each one modified with the plasmid vector able to express a specific MBP fusion protein, either MBP-β galactosidase or MBP-rubredoxin.

As reported in the technical bulletin of New England Biolabs (11), the plasmid vectors of the p-MAL-2 type provide a method to express and purify a protein produced from a cloned gene or an open reading frame. The cloned gene is inserted downstream from the *malE* gene of *E. coli*, which encodes MBP, resulting in the expression of a MBP fusion protein. The vectors express the *malE* gene fused to the *lacZα* gene. Restriction sites between *malE* and *lacZα* are available to insert the coding sequence of interest. The p-MAL-2 vectors exist in two different sequences, p-MAL-c2 and p-MAL-p2. The former has an exact deletion of the *malE* signal sequence resulting in cytoplasmatic expression of the fusion protein, the latter contains the normal *malE* signal sequence, which directs the fusion protein through the cytoplasmic membrane to the periplasm. The p-MAL-2 vectors also contain the sequence coding for the recognition site of the specific protease Factor Xa, located just 5′ to the polylinker insertion sites. The two protein domains are then separated by a sequence of 10 asparagine residues and for the specific sequence coding for the recognition site of the specific protease Factor Xa (Ile-Glu-Gly-Arg), thus recognized as the cleaving site between the two protein domains. The plasmid vector carries the lacI^q gene, coding for the repressor lac^o (9–11). Isopropyl-β-D-thiogalacto pyranoside (IPTG) is used as an inductor.

The β-galactosidase protein is one of the several oligosaccharides-splitting enzymes that have been purified and characterized. It has a molecular weight of about 110 kDa, and is often found in solution as a tetrameric aggregate (12). Rubredoxin is a small molecule with a molecular weight of about 6 kDa, characterized by the presence of iron–sulfur groups, fundamental for the role of electron carrier played by this protein (13).

The modified *E. coli* strain (TB1, with plasmid vector of the p-MAL-c2 type) was first grown on plates (solid medium, tryptone 20 g/L, NaCl 8 g/L, Na_2HPO_4 2 g/L, KH_2PO_4 1 g/L, agar 15 g/L, ampicillin 100 mg/L), at 37°C, then in liquid broth (LB, trypton 10 g/L, NaCl 5 g/L, yeast extract 5 g/L, glucose 2 g/L, ampicillin 200 mg/L), at 37°C. Isopropyl-β-D-thiogalacto pyranoside as inductor was added in the last growing stage (final concentration 1 m*M*) to stimulate the production of the recombinant protein inside the bacteria. The last stage of the growing procedure was performed either in a 3-L shake flask or in a fermentor. At the end of the last growing stage, the culture was harvested at 2×10^8 cell/mL (7500 rpm, $t = 20$ min, $T = 4$°C). The cells were resuspended in a volume of lysis β buffer (20 m*M* Tris–HCl, pH = 7.4, 10 m*M* $MgCl_2$, 1.6 *M* NaCl, 700 μL/L β-mercaptoethanol), then sonicated with a probe sonicator at $T = 4$°C; lysozyme (supplied by Sigma, Molecular Biology Grade) can be added to facilitate cell walls disruption. The solution containing the cell extract was then centrifuged using a bench-scale centrifugette at 14,000 rpm, for 20 min. The supernatant was collected and stored at −20°C.

Purification of MBP-β-galactosidase with Affinity Resin

The fusion protein of interest was first purified from the cell lysate through a classical affinity resin procedure (14). To that aim, the affinity resin (*p*-aminophenyl-β-D-thio-galactopyranoside, supplied by Sigma), specific for β-galactosidase, was used. Lactose was obtained from Carlo Erba. Other chemicals used to prepare buffer solutions were obtained from Sigma, Molecular Biology grade. The purification of MBP-β-galactosidase from the concentrated shock fluid protein was performed in a batch system as follows: 25 mL of resin with 25 mL of lysis β buffer and 20 mL of cell extract were loaded in a beaker, stirred at $T = 4°C$ for 70 min. The lysate resin slurry was filtered off to remove the unbound proteins. The resin was then washed four times with 25 mL of lysis β buffer, each time at $T = 4°C$ for 30 min. Finally, the resin was first eluted twice using 20 mL of elution buffer (NaCl 1 *M*, $MgCl_2$ 1 m*M*, lactose 7.5% (w/w)) at $T = 4°C$ for 60 min; then the elution continued with 20 mL of borate buffer (boric acid 100 m*M*, β-mercaptoethanol 10 m*M*, pH = 9.8) at $T = 4°C$ for 60 min. The fractions collected from the elution steps were dialyzed against standard buffer solution and stored at $T = 4°C$.

The composition of the protein solutions, recovered from the purification process performed with affinity resins, was determined with SDS–PAGE analysis. Acrylamide/bisacrylamide vertical gels (denaturing conditions) were prepared in order to have a 6% final acrylamide concentration in the separating gels (15). The gels were run at a constant current setting of 36 mA using an OWL cell. Protein bands were stained with Coomassie brilliant blue staining solution. Perfect Protein Marker 10–225 kDa (from Novagen) was used to recognize the position of the fusion protein MBP-β-galactosidase.

Protein Concentration Measurement

MBP-β-galactosidase

MBP-β-galactosidase concentration in the solution collected after the elution step was measured in terms of enzymatic activity of the β-galactosidase domain towards the substrate *o*-nitrophenyl-β-D-galactopyranoside (ONPG, supplied by Sigma) (16). The enzymatic assay was performed as follows: the protein sample was diluted in *Z*-buffer (60 m*M* $Na_2HPO_4 \cdot 7H_2O$, 40 m*M* $NaH_2PO_4 \cdot H_2O$, 10 m*M* KCl, 1 m*M* $MgSO_4 \cdot 7H_2O$, 50 m*M* β-mercaptoethanol, pH 7), added with 180 μL of ONPG stock solution (1.33 m*M* ONPG in *Z*-buffer) and stored at room temperature up to 1 min. Then 450 μL of 1 *M* Na_2CO_3 stock solution were added to block the reaction. The amount of *o*-nitrophenol liberated during the enzymatic reaction was determined by recording the absorbance at

420 nm (OD_{420}). A correction factor was applied equal to 1.75 OD_{550}. The final concentration was estimated using the following relation:

$$c_U = K\frac{V(OD_{420} - 1.75OD_{550})}{t\nu}, \quad (1)$$

where V is the final solution volume, t the reaction time and ν the protein sample volume. K indicates the extinction factor, here estimated to be equal to 0.869 U min/mL.

The amount of ONPG transformed in *o*-nitrophenol is directly related to the protein concentration, measured in terms of activity units. One unit of β-galactosidase activity was defined as the amount of enzyme catalyzing the hydrolysis of 1 μmol of the substrate ONPG in 1 min at 28°C. A calibration curve was constructed using pure β-galactosidase supplied by Sigma.

MBP-Rubredoxin

MBP-rubredoxin concentration was measured performing the Bradford assay based on the formation of a protein–dye complex with a characteristic absorbance peak at 595 nm (17,18). A calibration curve was prepared using BSA (supplied by Sigma, fraction *V*, 96–99% albumin) as standard protein. Bradford reagent was purchased from Sigma. The UV extinction coefficient used to determine protein concentration is 0.9973 mg/ABS_{595}.

Affinity Membrane Preparation

Microporous amylose affinity membranes were prepared through the chemical modification of native cellulose matrix (Whatman 541 membranes, d_p = 25 μm). The choice of the support is suggested by several factors such as chemical, mechanical and biological stability, porosity, hydrophilic behavior, low non-specific interactions, presence of reactive groups for chemical modification, as well as low costs, and availability. The choice of amylose (19) as the ligand is mainly due to its effectiveness and the feasibility of an immobilization reaction of the ligand itself onto the matrix. The chemical modification procedure is performed as a sequence of three subsequent reactions: (i) coupling of the matrix with a spacer, in this specific case 1,4-butanediol diglycidil ether (approximately 70% (w/w)); (ii) coupling reaction of the ligand with the free reactive groups on the spacer chain; (iii) blocking of the non-reacted free epoxy groups, in order to avoid interference during the subsequent adsorption process. The reaction sequence is schematically shown in Fig. 1 (20–22).

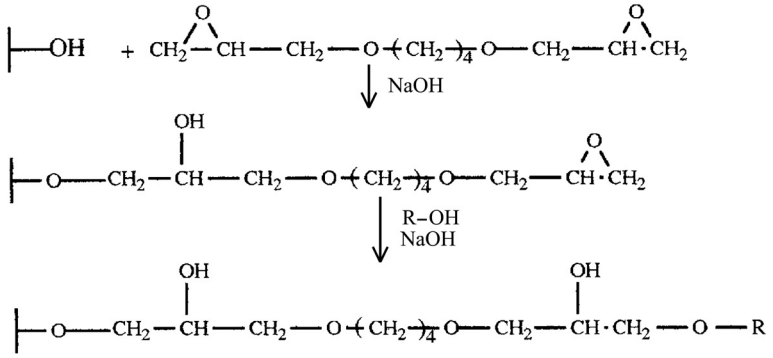

Figure 1. Scheme of matrix activation and ligand immobilization: 1,4 butanediol diglycidyl ether reacts with the —OH groups present on the polymer surface. Amylose (R—OH) is then immobilized onto the activated matrix.

In the first step, the coupling reaction occurs between the —OH groups of the cellulose matrix with one of the reactive epoxy groups of the spacer, in a basic environment, in the presence of a catalyst such as $NaBH_4$, at room temperature. The surface concentration of epoxy groups was monitored using titration of the free epoxy groups with sodium thiosulfate (20). The final reaction time was chosen in order to have the maximum activation degree of the membrane surface. At the end of this first step, the membranes are washed alternatively with de-ionized water and acetone. After activating the cellulose matrix through the introduction of the spacer arm, the coupling of the ligand with the reactive groups is performed. The reaction is conducted at pH $>$ 13, adding $NaBH_4$ at 37°C; the amylose used is approximately 70% pure (practical grade). The high concentration of amylose (50 mg/mL) results in an extreme viscous solution. After the second reaction step, the membranes are washed with a surfactant (LDAO, supplied by Fluka) and with an NaCl solution. In the last step, the membranes are finally soaked in a solution of 2-monoethanol amine, pH 9.5, at room temperature. At the end of the step, the membranes are rinsed with NaCl solution and de-ionized water alternatively. The amylose affinity membranes obtained are then stored in standard buffer solution at $T = 4$°C until use.

Membrane Modules

A Millipore® cell, 3.8 cm internal diameter, was used to perform adsorption experiments from pure protein solution, when a small membrane area was sufficient. In that case, the modified membranes were located between two stainless steel plates together with a flow distributor; the module can hold up to 10

membranes. The two plates were bolted together to form an upstream and a downstream chamber. The schematic representation of the experimental set-up is shown in Fig. 2.

A larger membrane module, shown in Fig. 3, was also set-up suitable for larger solution volumes; it contains 16 PVC discs, assembled in a column configuration, tight between two header plates. On the top plate, two holes allow the inlet of the feed solution and the exit of air bubbles, respectively. From the bottom plate the permeate solution was collected through an outlet section and recirculated to the feed vessel. Three affinity membranes are placed over each of the 16 internal discs as well as on the bottom plate, so that a total number of 51 membranes are accommodated in the entire module. In the module, the cross-section wetted by the liquid solution is 5.0 cm in diameter; the total membrane area available is $A = 1000\,\mathrm{cm}^2$. The void fraction of the membranes is 0.55 and has been estimated as follows: by measuring the increase in membrane weight after filling the pores with liquid water, the volume occupied by the voids was obtained; the total volume of the wetted membrane was also measured and thus the void fraction $\varepsilon_p = V_{void}/V_{tot}$ was calculated. The single discs are designed to maintain a uniform flow distribution over the cross section, all along the membrane stack. A support net was located on each plate under the three-membrane stacks, to avoid possible membrane damage due to pressure action on the filter surface.

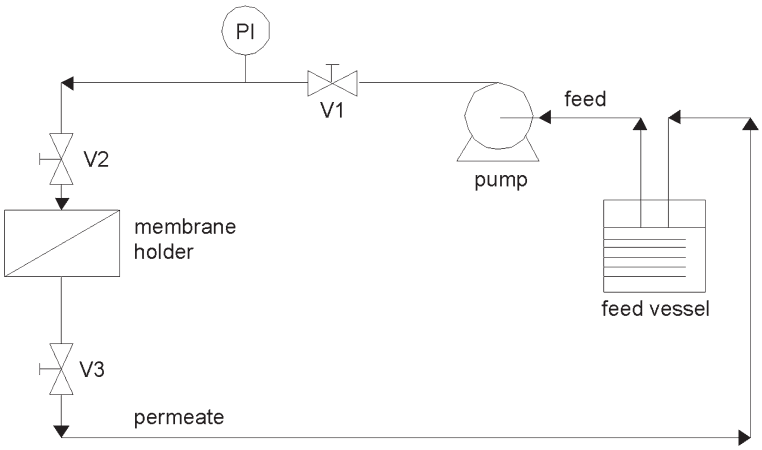

Figure 2. Scheme of the experimental equipment used to perform affinity membrane purification: the feed solution is pumped from the feed vessel to the membrane module, where the adsorption of the target protein takes place. The permeate is collected and recirculated to the feed vessel.

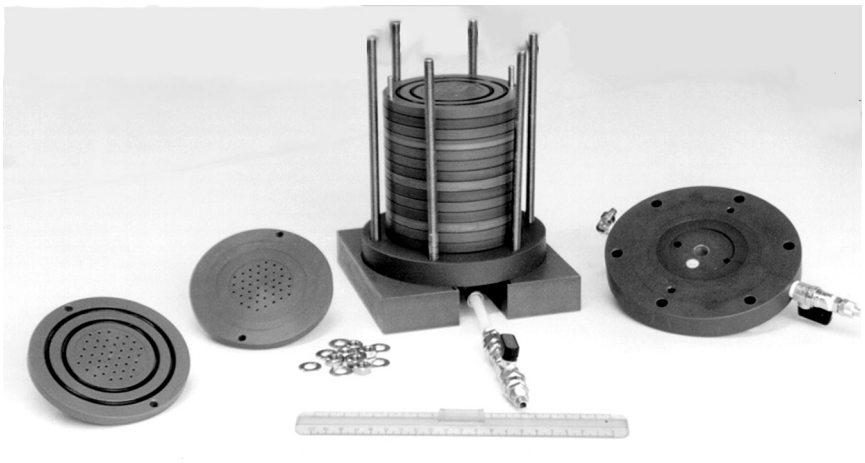

Figure 3. Picture of the large scale membrane holder realized suitable for flat sheet membranes. A series of discs are arranged in a column configuration. On each disc a stack of membrane is located.

Protein Purification Based on Affinity Membranes

As a first step of the feasibility analysis, the affinity membrane properties were tested by using pure protein solutions according to the following procedure. Five modified membranes were accommodated inside the Millipore module to form a single membrane stack. A conditioning step was performed using standard buffer solution in order to remove all possible impurities and solid fragments. For MBP-β-galactosidase, an adsorption step was performed by using a solution of the nominally pure protein, previously obtained through an affinity resin selective for β-galactosidase; to that aim *p*-aminophenyl-β-D-thio galactopyranoside was used.

The protein solution was fluxed through the affinity support and the permeate recirculated for 1 hr at the flowrate of 0.6 mL/min. The molecules not specifically adsorbed on the membranes were removed during the washing step. For a more effective removal of unadsorbed materials, the buffer solution was backflushed throughout the membrane stack. Samples were collected to monitor protein concentration in the exit stream. Finally, the elution of the target protein was obtained by applying the elution buffer (0.1 *M* maltose, 0.1 *M* NaCl in standard buffer solution) (23,24). The adsorption and elution steps were performed maintaining a flowrate equal to 0.6 mL/min. For the washing step, a flowrate of 1.2 mL/min was adopted. UV spectra between 240

and 320 nm were recorded for the samples obtained in each stage of the separation process.

The feasibility analysis then proceeds by using the cell lysate containing MBP-β-galactosidase, directly as a feed to the same system described above. The procedure and operating conditions are the same as for the pure protein solution, except that in this case no recirculation was applied.

The use of a larger module was then dictated by the necessity of purifying larger quantities of the protein of interest, directly from crude cell lysate. Due to the reduced number of membranes and the small cross-section, the above Millipore module did not allow to treat in a single purification step the total amount of protein required, of the order of a few milligrams; thus the membrane column previously described was used.

Affinity membranes, after rinsing with distilled water, were placed inside the membrane column, and conditioned with standard buffer solution. A volume of 25 mL of crude cell lysate was diluted with lysis β buffer solution up to a final volume of 100 mL. The protein mixture to be purified was loaded after removing the conditioning solution from the entire circuit. The feed solution was recirculated several times through the membrane stack using a peristaltic pump; a flowrate of about 12 mL/min was maintained. The overall pressure drop of approximately 1 bar was measured. The adsorption step lasted about 1 hr. Immediately after adsorption, the circuit was emptied from the protein solution and thoroughly washed with standard buffer solution. The washing step was performed until the protein concentration of the outcoming solution was no longer detectable (practically equal to zero). The elution step was performed using the elution buffer solution; several subsequent fractions of the eluted solution were collected separately. The same procedure was adopted for the recovery of both MBP-β-galactosidase and MBP-rubredoxin from their respective cell lysates.

SDS–PAGE analysis of the protein solutions was performed with electrophoresis equipment from Bio-Rad, using Ready Gel Precast Gels. Molecular mass markers were supplied by Bio-Rad (SDS–PAGE Standards, Broad Range) containing myosin (200 kDa), *E. coli* β-galactosidase (116.25 kDa), rabbit muscles phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg white ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), hen egg white lysozyme (14.4 kDa), bovine pancreas aprotinin (6.5 kDa). Protein bands were stained using the Blue Coomassie method.

Dialysis Procedure

The eluted fractions contain fusion proteins, the MBP active site of which is bound to maltose; in addition, an excess of pure maltose is present in the

solution eluted, together with NaCl. With the aim to remove both NaCl as well as virtually all the maltose present, the eluted fractions are then dialyzed using cellulose membranes with $d_p = 24$ Å (Medicell International Ltd.) and standard buffer solution. The conditions adopted are the same as in a previous work (25). Several stages were performed, each of 24 hr, for a total time of 5 days. The system was kept at $T = 4$°C, and mechanically stirred. Pure protein solutions are thus finally obtained, with the MBP active sites free of maltose.

RESULTS

MBP-β-galactosidase Purification with Affinity Resin *p*-Aminophenyl-β-D-thio-galactopyranoside

The recovery of MBP-β-galactosidase is based, in this case, on the specific interaction between the affinity matrix and the β-galactosidase domain of the fusion protein. The separation protocol is performed in batch configuration which requires long times for each stage of the separation procedure. In this configuration, the rate-controlling step is the diffusion of the solute from the liquid phase to the active binding sites immobilized on the internal pore surface.

The SDS–PAGE electrophoresis of the samples recovered from all the different stages of the purification process is shown in Fig. 4. Clearly, the fractions recovered at the end of each elution step are highly concentrated in the target protein, even though traces of impurities are detected, especially at low molecular weights. Due to their amount, the complete removal of small molecules from inside the pores would thus require much longer washing times. The purification process can be improved by increasing the duration of the washing step, although proper attention must be paid to avoid losses of product.

The UV spectra of the elution samples E1, E2 and E3 were acquired between 240 and 320 nm in order to verify the typical absorbance behavior of protein solutions. The observed trend shows significant deviation from the typical spectra of protein solutions, which is characterized by a maximum of absorbance near 280 nm and a minimum near 245 nm. On the contrary, the maximum in the absorbance curves shown in Fig. 5 is found around 250 nm. For these samples, the same results were obtained after an overnight dialysis step. Possible reasons for that behavior can be the presence of DNA–RNA fragments, often co-eluted with the desired product. A specific electrophoresis analysis for nucleic acids, albeit performed on a single fraction, lead us to exclude this possibility; no further investigations have been performed to determine the nature of the contaminants present, since that is not crucial for this work.

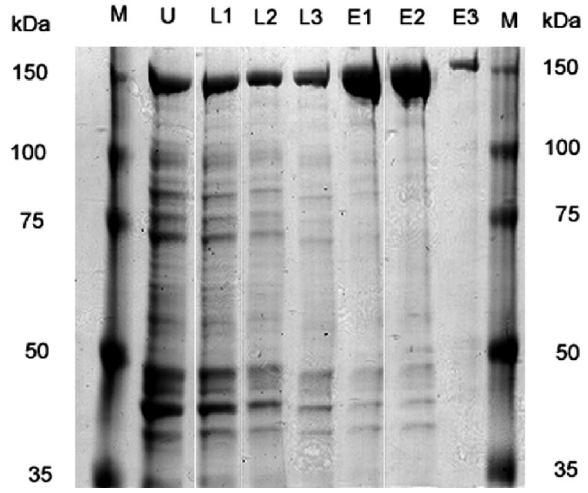

Figure 4. The SDS–PAGE analysis on a 6% polyacrylamide gel of the solutions recovered at the end of each step of the purification procedure carried out with affinity resin. U: unbound; L1: first washing; L2: second washing; L3: third washing; E1: first elution; E2: second elution; E3: third elution; M: Perfect Protein Markers. Working conditions: room temperature, constant current 36 mA.

Purification of MBP-β-galactosidase with Affinity Membranes

With the aim to test if the amylose affinity membranes obtained are suitable to recover MBP fusion proteins, we first considered the determination of their total active binding area, and the qualitative response during the separation steps, without any specific analysis of the fluid-dynamic aspects of the process.

The behavior of the amylose affinity membranes has been initially tested in the Millipore membrane cell, by loading a MBP-β-galactosidase solution, obtained from the purification process performed with affinity resins. In Fig. 6, the UV spectra of the different protein solutions are reported and clearly document the fine separation obtained. The UV spectrum of the sample recovered from the elution step after adsorption on affinity membranes, indeed shows the maximum absorbance between 275 and 280 nm, as expected for pure protein solutions, and is no longer at 255 nm as it was observed for the solution purified by the resins, before its purification with affinity membranes. This behavior is an indication that a sort of fractionation of the feed solution in two different fractions has been achieved: the proteins are bound to the solid surface and the

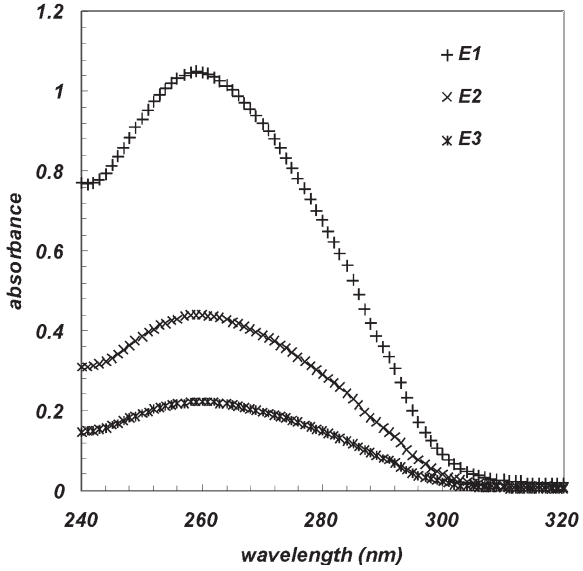

Figure 5. UV spectra of the subsequent elution fractions E1, E2, and E3 recovered during the purification procedure with affinity resin, after an overnight dialysis step.

contaminants, although not specifically detected, are removed during the washing stage. Only the protein with specific affinity for the binding ligand, in this specific case MBP-β-galactosidase, is immobilized onto the affinity sites of the matrix; the same does not happen for other particles and molecules which are thus completely removed during the washing step. The removal of undesired products is greatly facilitated by the open porous structure of the affinity support and is also favored by the fluid-dynamic effects of the washing solution which greatly enhance the overall mass transfer rate.

Completely analogous results were obtained also by loading directly the cell lysate, suitably diluted, to a stack of amylose modified affinity membranes in the Millipore membrane cell; the corresponding plots are here omitted for the sake of brevity. Remarkably, the protein of interest is recovered to a high degree of purity through a single step separation process, even from the complex mixture represented by direct cell lysate.

The above result clearly proves the feasibility of the use of the amylose affinity membranes for the purification process of MBP fusions directly from cell lysate also in a single step. The scale up necessary for the treatment of higher solution volumes, can be approached in the rather straightforward way, typical of membrane processes.

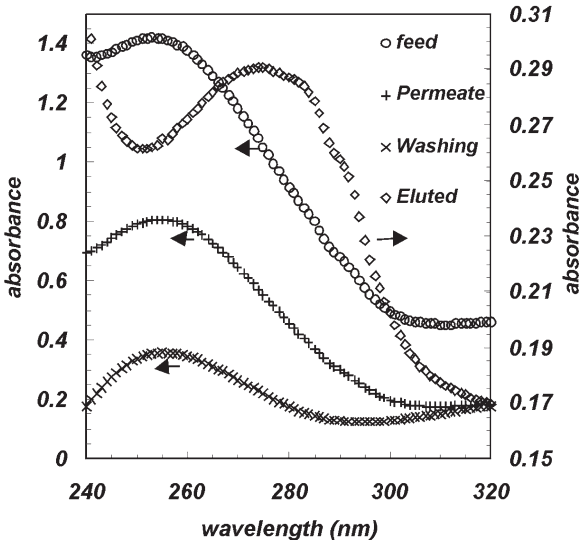

Figure 6. Spectra of protein solutions collected during the subsequent steps of a purification process with affinity membranes. The feed solution loaded during the adsorption step is a protein solution (E2) eluted from the purification process performed with *p*-aminophenyl-β-D-thio-galacto pyranoside affinity resin.

Large Scale Purification Process Results MPB-β-galactosidase and MBP-Rubredoxin

In the case of MBP-β-galactosidase, the feed solution was recirculated five times through the stack, leading to a final adsorption time of about 40 min, due to the high protein concentration in the cell lysate. For MBP-rubredoxin the adsorption time was extended to 10 recycles of the feed solution through the membrane stack for a total adsorption time of 80 min. The relatively short time required to perform the adsorption step is strictly connected to the high concentration of protein in the cell lysate. In fact, the amount of target product is in large excess in comparison with the active binding sites available on the surface of the stationary phase located in the membrane module.

The washing step involved a total of 2 L washing solution, forced through the membranes without recirculation. The effectiveness of washing was monitored by measuring the UV absorbance of the fractions collected at regular time intervals, as a measure of the amount of biomolecules removed. The overall duration of the washing step was of about 1.5 hr.

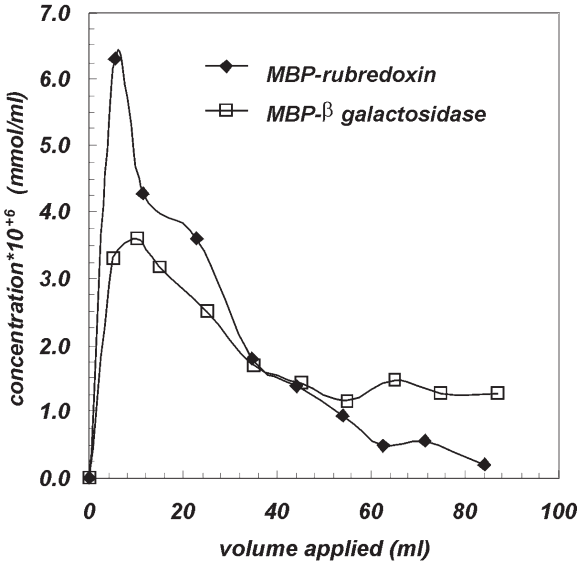

Figure 7. Concentration trend of MBP-β-galactosidase and MBP-rubredoxin during the elution step of the purification process.

Elution required a reduced time, in comparison with the adsorption step. The maltose buffer used is extremely efficient in recovering the adsorbed protein due to the combined effect of a significant concentration of maltose, as competing substrate, and of the high ionic strength of the elution solution. Fractions collected during subsequent elution steps, show a decreasing concentration of the target protein; as it is apparent from Fig. 7, the protein concentration in the first elution step is rather high, documenting the effectiveness of the elution buffer. For both cases in which the feed solution is either the cell lysate containing MBP-β-galactosidase or the cell lysate containing MBP-rubredoxin, the SDS–PAGE analysis has been performed by loading the solutions obtained from the washing and elution steps. The SDS–PAGE results shown in Figs. 8 and 9 show a unique protein band, indicating that the protein of interest is recovered in very pure solutions.

Clearly, the affinity membrane process is highly selective towards both fusion proteins considered and, in comparison with systems using resins or beads as stationary phases, requires purification times much shorter. In addition, the adsorption UV spectra of the elution samples, Fig. 10, show indeed the expected typical trend, proper of pure protein solutions, for the case of MBP-β-galactosidase as well as for MBP-rubredoxin. It is quite reasonable to conclude that by using affinity membranes almost all impurities were removed from the support before elution, which was not the case for affinity resins.

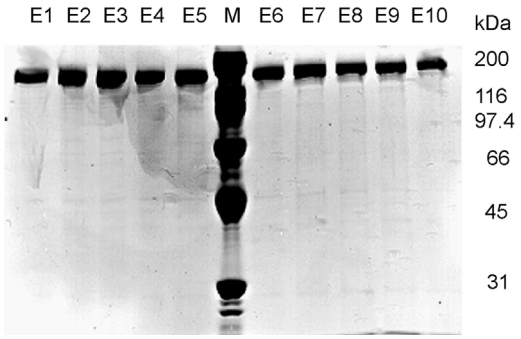

Figure 8. The SDS–PAGE analysis with 10% polyacrylamide gel of MBP-β-galactosidase purified with amylose modified affinity membranes. From left to right: subsequent elution fractions; M indicates reference markers.

Removal of Maltose from the Recovered Protein

The fusion protein recovered in the elution solutions is bound to the maltose used as the selective extractor agent; maltose removal is thus needed in order to clear the active binding sites of the MBP domains. Of course this step is not specifically associated to the use of affinity membranes, but is common to all affinity separations based on MBP selective ligands. It has been considered here as a necessary stage of the process, needed to obtain pure MBP fusions, with free MBP active sites.

A dialysis stage was thus considered for this, as described in the "Experimental" section. The species to be removed, i.e., maltose, is present either

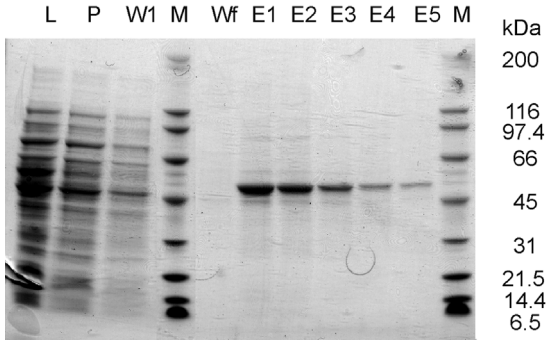

Figure 9. The SDS–PAGE analysis of samples from MBP-rubredoxin separation process with amylose modified affinity membranes. L indicates feed, P indicates permeate, W1 and Wf are the first and the last washing solutions, respectively, E1–E5 label elution fractions.

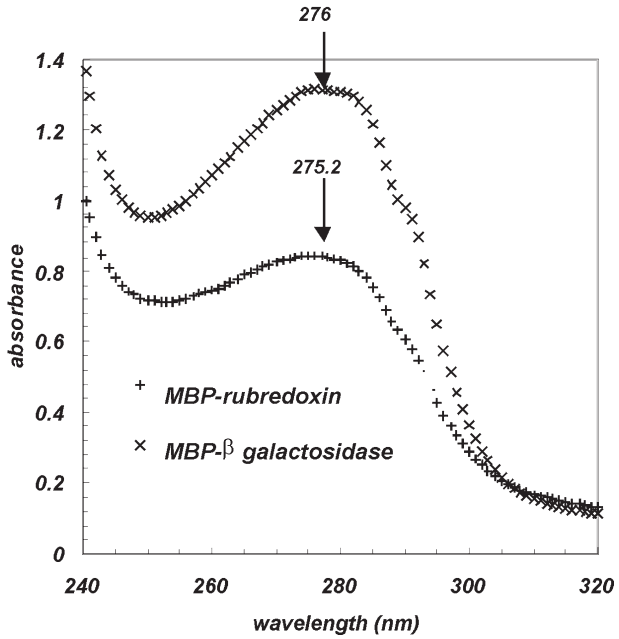

Figure 10. UV spectra of MBP-β galactosidase and MBP-rubredoxin samples after dialysis. The samples used were first collected from the elution step, then dialyzed against standard buffer solution for the removal of maltose.

as a free unbound species in solution, or as a species bound to the active sites of the MBP domain. That will slow down the process kinetics with respect to the usual dialysis conditions. The duration of the dialysis step, needed to assure complete removal of both bound and unbound maltose, was first theoretically estimated, in order to obtain reasonable guidelines for the experiments. The basic hypothesis considered are: (i) equilibrium is always attained between the species present in solutions such as maltose (L), protein (P) and the complex protein–ligand (PL); (ii) osmotic pressure is the same on both sides of the membrane, i.e., the net water flux is negligible. This assumption was supported by the experimental evidence indicating a constant volume of the solution dialyzed. The mathematical description of maltose concentration in the protein solution is therefore given by the following equations (25):

Maltose mass balance in the protein solution:

$$\frac{\mathrm{d}[L]}{\mathrm{d}t} = -\alpha([L_\mathrm{f}] - [L_\mathrm{e}]), \tag{2}$$

where $[L]$ indicates the total maltose concentration, $[L_f]$ is the concentration of free maltose in the protein solution and $[L_e]$ is the maltose concentration in the external solution; α is a coefficient directly proportional to the membrane area and to the mass transfer coefficient and inversely proportional to the protein solution volume.

Equilibrium between pure and bound maltose:

$$K_d = \frac{[P_f][L_f]}{[PL]}, \tag{3}$$

where K_d is the equilibrium constant for the reaction $P_f + L_f = PL$.

Overall maltose balance:

$$[L_e] = \frac{V_p}{V_e}([L_0] - [L]), \tag{4}$$

where subscript 0 indicates the initial value, V_p and V_e represent the volumes of protein and external solutions, respectively.

In order to estimate the duration of each dialysis step before renewing the dialysis solution, the differential equation (2) was integrated accounting for Eqs. (3) and (4) and considering that during each dialysis step the total amount of protein is constant in V_p, while the total amount of maltose is constant in the entire system $V_p + V_e$; fresh dialysis solution was considered as soon as $[L_e]/[L_f] = 0.75$. The value of the equilibrium constant K_d was taken equal to $1.25 \times 10^{-6} M$ (25); the value of the parameter α, depending on system geometry and membrane transport properties, was estimated to be equal to $2 \times 10^{-4}\,\text{sec}^{-1}$ from direct experimental data. The total protein concentration in the liquid solution, $[P]$, was measured using the analytical methods already described. For convenience, the parameters used in the simulation are listed in Table 1. The predicted trend of maltose concentration $[L]$ and of the free protein concentration $[P]$ in the protein solution are shown in Fig. 11(a) and (b). In Fig. 11(a) a slope discontinuity labels the times at which the external dialysis solution is renewed.

Table 1. Parameter Values Used as Input for the Prediction of Maltose and Protein Concentration During Dialysis

Ligand	Maltose
K_d (M)	1.25×10^{-6}
α (sec^{-1})	0.0002
Protein concentration, [P] (mol/L)	1×10^{-6}
Protein solution volume, V_0 (L)	0.02
Dialysis solution volume V_e (L)	2
Constraint on ligand concentration $R = [L_e]/[L_f]$	0.75

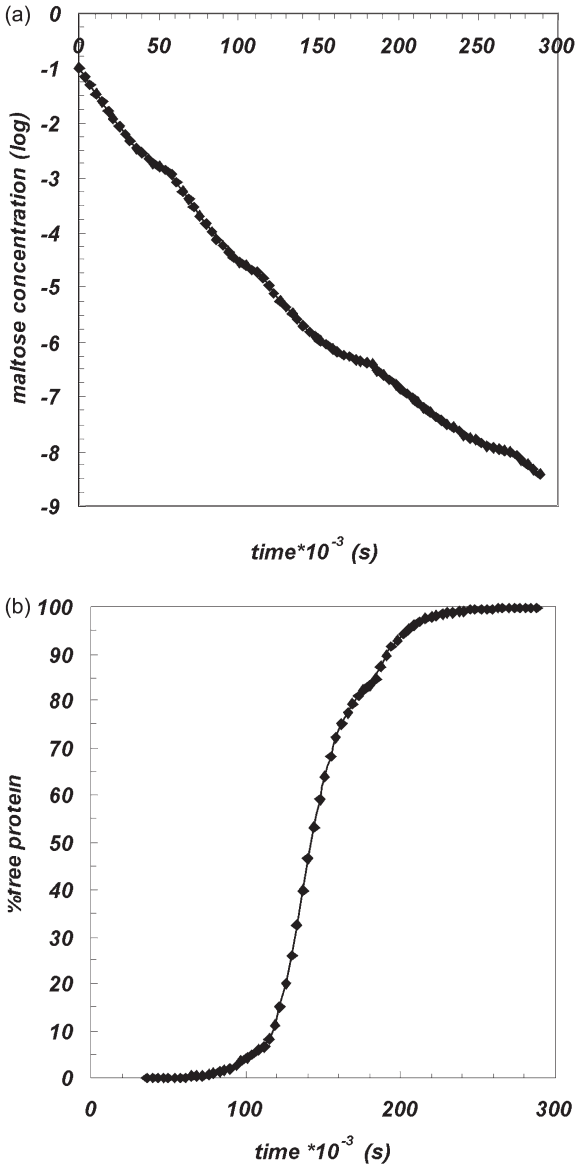

Figure 11. Estimated trends of maltose concentration (a) and free protein concentration (b) during the dialysis process. The dialysis solution was changed when $[L_e] = 0.75[L_f]$.

The dialysis steps were performed following the indications obtained by the simulation procedure. In particular, each 100 mL of protein solution were dialyzed against 4 L of dialysis buffer (in this case lysis buffer with EDTA) for at least 5 days for a total of 20 L of dialysis buffer used.

The effectiveness of the dialysis process to recover active and maltose-free protein was inspected by testing separately the two domains, β-galactosidase and MBP, of the fusion protein. For the first, an enzymatic assay specific for β-galactosidase has been considered. Experimental results clearly show that the stability of the protein is preserved during the dialysis step, indeed the specific activity of the protein is increased. The activity of the MBP domain can be assayed only by testing the binding with complementary substances such as the ligands used. The solution obtained from the dialysis step was thus used in a subsequent sorption step onto the affinity membranes, maintaining the operative conditions previously used in the case of sorption of the pure protein solutions obtained from resin purification. In particular, a total volume of 14 mL of pure MBP-β-galactosidase solution with a concentration of 401.5 U/mL (0.47 mg/mL) was recirculated for 1 hr in the Millipore module containing 6 membranes (total binding area $A = 68\ cm^2$), at a flowrate of 0.6 mL/min. At the end of the adsorption process, elution was performed and six elution fractions were collected, of 1.5 mL each; they were analyzed by reading the UV spectrum between 240 and 320 nm and measuring their respective protein concentration. It has been observed that indeed the MBP fusion protein obtained free of maltose from the dialysis step is again active to bind the affinity membrane ligand. In view of the use of the Millipore module instead of the membrane column, the total membrane capacity allowed to bind only a fraction of the total protein available after the dialysis step. The total amount removed during the elution step was in fact 1934 U, corresponding to the total membrane capacity of $30\ U/cm^2$; for MBP-β-galactosidase that is equivalent to the capacity of $0.017\ mg/cm^2$, very close to what was measured from cell lysate and discussed hereafter. In view of the absence of maltose, of the preserved activity of the β-galactosidase domain, and of the observed activity of the fraction of MBP domains tested, we can confidently assert that the fusion protein was recovered from dialysis in its active configuration.

The washing profile obtained in the affinity chromatography using the dialyzed protein solution is shown in Fig. 12, as well as the integral amount of product eluted as function of the elution volume applied. The peak of maximum concentration is observed for low applied volumes; more than the 80% of the final product is recovered with the first half of the elution volume. From the data reported in Table 2, it is possible to notice that the specific activity of the protein eluted is higher than the specific activity of the protein in the feed mixture; indeed the purification factor, i.e., the ratio between the above two quantities, is calculated equal to 2. Thus the purity of the product is increased as a consequence of the second purification step.

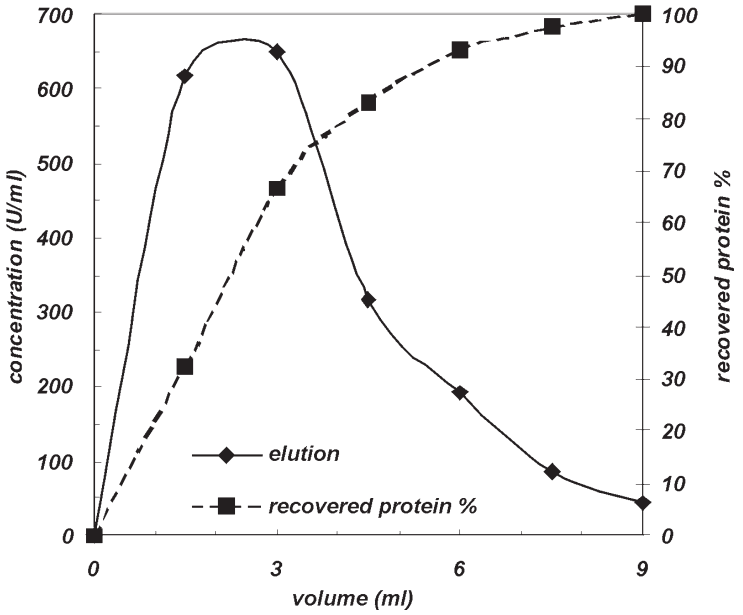

Figure 12. Elution profile for the MBP-β-galactosidase immobilized onto amylose affinity membranes after the removal of maltose through dialysis. The concentration is reported in terms of U/mL as estimated from enzymatic assay.

Membrane Binding Capacity

The binding capacity of the affinity membranes obtained in the present work was evaluated through a mass balance, after measuring the amount of eluted protein from the completely saturated membranes. The elution steps considered are obtained from membranes loaded to the maximum protein amount, achievable from cell lysate with a continuous recirculating flow, as described

Table 2. Recovery of MBP-β-galactosidase from Pure Solution

Fraction	Volume (mL)	Protein Concentration (mg/mL)	Specific Activity (U/mg)	Total Activity (U)	Purification Factor
Feed	14	0.48	842	5621	1
Eluted	9	0.13	1676	1903	2

Table 3. Binding Capacity for Amylose Affinity Membranes

Protein	Adsorption Time (min)	Binding Capacity (mmol/cm^2)	Binding Capacity (mg/cm^2)
MBP-β galactosidase	40	1.62×10^{-7}	0.026
MBP-rubredoxin	80	1.57×10^{-7}	0.008

previously. The values obtained are reported in Table 3, the binding capacity per unit area of the membrane sheet is calculated as 1.6×10^{-7} mmol/cm^2 for both fusion proteins inspected. Such property is significant for a direct comparison with the affinity resins commercially available.

In Fig. 13 the amount of eluted protein is reported on a molar basis, as a function of the elution volume, for both cases of MBP-β-galactosidase and MBP-rubredoxin. As it is clear, one obtains very close elution curves for the two different fusion proteins. The substantial similarity of the elution curves in Fig. 13 indicates that the elution step is essentially governed by the desorption reaction of the MBP domain

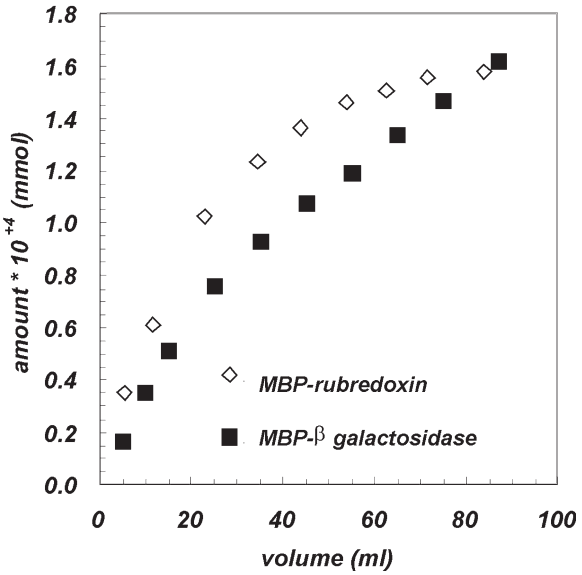

Figure 13. Amount of recovered protein during the elution step as a function of eluted volume. Comparison between experiments performed with different fusion proteins of the MBP class. Elution is performed with 0.1 *M* maltose, 0.1 *M* NaCl in standard buffer solution, flowrate 6 mL/min, at room temperature.

from the affinity active site on the membrane. Indeed, the amount of desorbed protein is essentially associated to the amount of maltose, the selective extractant, progressively fed during the elution step. Some differences between the curves of the two proteins are reasonably associated to the differences in the molecular weight (160 kDa for MBP-β-galactosidase and 51 kDa for MBP-rubredoxin). MBP-β-galactosidase is eluted in a somewhat lesser amount, with respect to MBP-rubredoxin, reasonably due to lower mobility and/or steric hindrance.

In conclusion, a further comparison of the affinity membranes here obtained with commercially available affinity supports is in order. The binding capacity, per bed volume, of the amylose affinity membranes results in 0.55 mg/mL (since the membrane thickness is 150 μm). On the other hand, amylose affinity resins manufactured by New England BioLabs, and used for the purification of MBP-fusion proteins, have a binding capacity of 1.3 mg/mL of bed volume (26), for a protein of 50.8 kDa; in addition, the binding capacity of a starch–cellulose matrix, suitable for the purification of the same class of fusion proteins, was found (26) equal to 0.19 mg/mL. Both values are indeed of the same order of the volumetric binding capacity measured for the affinity membranes under consideration.

CONCLUSIONS

A chemical modification protocol was set-up to produce amylose affinity membranes. The affinity membranes thus obtained have been tested in separation processes of MBP fusion proteins produced by genetically modified *E. coli* strains. Both pure protein solutions as well as the cell lysate itself have been fed to the affinity membrane apparatuses used.

The purified protein solution obtained after a single purification step from affinity resins specific for the β-galactosidase domain, showed a peak in the region around 255 nm of the absorbance spectrum, indicating the presence of some impurities, which were not washed out during the standard washing step. On the contrary, the use of affinity membranes directly with cell lysate allowed to obtain the desired MBP fusion protein with a higher degree of purity in a single step.

The results obtained from the separation process performed using affinity membranes, show: (i) the selective interaction of the target protein with the matrix; (ii) the effectiveness of the washing procedure in the removal of the non-specifically adsorbed materials; (iii) the efficiency of the maltose elution buffer in the removal of the affinity bound protein.

The quite complete removal of the entire undesired product during the washing step is crucial to obtain a pure protein solution from a single-step process; that appears one of the relevant advantages in the use of affinity membranes. In addition, the fluid-dynamics of the liquid solution through the stationary phase allows for a considerable increase in the mass transfer rate of the protein between the

pore surface and the bulk solution. As a consequence, the overall rates of each process step (adsorption, washing and elution) are much higher for affinity membranes than for the usual resins.

The feed flow velocity has typical values around 10 cm/hr, for both resins and porous beads; on the contrary, by using affinity membranes the feed flow rate can be largely increased, even in a single pass process. Indeed, we have successfully worked at 70 cm/hr in the affinity membrane column, obtaining a highly pure protein in a single pass. In addition, the corresponding pressure drop is much smaller than for the traditional affinity columns.

A large scale module was realized in order to process larger volumes of protein solutions and to obtain larger amounts of the required product, with high protein activity and purity. With this configuration, the selectivity of the affinity membranes toward different fusion proteins containing the MBP domain was verified. Significantly large amounts of MBP-β-galactosidase and MBP-rubredoxin were thus purified directly from cell lysate, and rather pure and concentrated solutions were finally obtained in both cases.

The binding capacity per bed volume of stationary phase was evaluated as 0.55 mg/mL, for MBP-rubredoxin and 1.8 mg/mL for MBP-β-galactosidase, at a flow rate of 70 cm/hr.

A straightforward comparison with other supports commonly applied, indicates that the use of amylose affinity membranes leads to appreciable reduction in process time. The overall pressure drop measured along the column is in the order of 0.6–1 bar at the flowrate considered. Such working conditions safely preserve the mechanical stability of the stationary phase.

Finally, the dialysis performed on the protein solutions recovered from the elution step was effective to remove maltose from the MBP active sites.

LIST OF SYMBOLS

c_U	protein concentration (U/mL)
K	constant defined in Eq. (1) (U min/mL)
V	total volume in Eq. (1) (mL)
ν	protein sample volume in Eq. (1) (mL)
t	time (min)
d_p	membrane pore size (μm)
ε_p	membrane void fraction
A	total membrane area (cm^2)
$[L]$	maltose concentration in protein solution (mol/L)
$[L_f]$	free maltose concentration in protein solution (mol/L)
$[L_e]$	free maltose concentration in dialysis solution (mol/L)
$[L_0]$	initial maltose concentration in protein solution (mol/L)

α	geometric parameter (sec^{-1})
$[P_f]$	free protein concentration (mol/L)
[PL]	ligand–protein complex concentration (mol/L)
K_d	equilibrium constant (mol/L)
V_p	protein solution volume (L)
V_e	dialysis solution volume (L)

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