Journal of Chromatography B, 879 (2011) 2741–2745

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Affinity adsorption of lysozyme on a macroligand prepared with Cibacron Blue 3GA attached to yeast cells

María del Pilar Ferraris*, Guillermo I. Barrera, A. Pérez Padilla, Jorge A. Rodríguez

Laboratory of Molecular Biology and Biotechnology, Department of Biochemistry, San Luis University, Ejército de los Andes 950, D5700HHW, San Luis, Argentina

ARTICLE INFO

Article history:
Received 1 April 2011
Accepted 29 July 2011
Available online 6 August 2011

Keywords:
Affinity macroligand
Yeast cells
Cibacron Blue 3GA
Lysozyme
Affinity ultrafiltration

ABSTRACT

The objective of this study was the development of affinity adsorbent particles with the appropriate characteristics to be applied in protein purification using the affinity ultrafiltration method. To prepare affinity macroligands Cibacron Blue 3GA, as a ligand molecule, was immobilized by covalent bonding onto yeast cell walls, the support material or matrix. The maximum attachment of the ligand to the matrix was 212 μmol/g (ligand dry weight/yeast dry weight). Lysozyme was selected as the protein model for the adsorption studies. Its adsorption onto the matrix without ligand and matrix with attached ligand were investigated batch-wise. The adsorption equilibrium isotherms appeared to follow a typical Langmuir isotherm. The maximum adsorption capacity (q_m) of the Cell-Cibacron macroligand for lysozyme was 110 mg/ml of wet macroligand. The adsorbent was also employed for the separation of lysozyme from hen egg white. High purity lysozyme was obtained.

1. Introduction

The preparation of an affinity adsorbent particle involves the use of a solid as a support matrix to attach the ligand molecule. Diverse support materials such as agarose, plant cellulose, dextran, silica, polyacrylamide gels, methacrylates and other synthetic polymers have been extensively used [1]. The appropriate selection of the matrix for the ligand immobilization is considered critically important for the efficiency and separative quality of an affinity chromatography system. The effect of different support materials on the adsorption of human seroalbumin (HSA) using the dye–ligand Cibacron Blue 3GA immobilized to these materials has been studied [2]. Results show a significant disparity regarding the HSA adsorption. The study concludes that the selection of the support matrix is particularly important in the affinity chromatography since it shows to have substantial effects on the adsorption. Therefore the two components, matrix and ligand, of the adsorbent or macroligand must jointly work to improve the adsorption efficiency. Likewise, it should be noted that the selection of the support matrix is fundamentally empiric.

On the other hand, the complete separation of two molecules through membranes can be achieved if their sizes differ at least by a factor of ten. Hence, in order to use micro or ultrafiltration for the separation of a target protein among a mixture containing proteins of similar size it is necessary to complex the protein before the purification step. The resulting complex must be considerably bigger than the rest of the proteins from the extract or homogenate. This aim may be achieved by binding the target protein through specific adsorption to a macroligand which can be a soluble polymer or an insoluble microparticle that is coupled to a ligand that show affinity for the target protein [3]. The principle of affinity ultrafiltration relies on the binding of the target protein to the macroligand by specific adsorption and the use of a membrane whose pore size is able to retain solely the protein–macroligand complex and allows the passage of other proteins from the mixture. The macroligand used in affinity filtration must fulfill several requirements: principally to have selective and reversible adsorption towards the target protein and a minimum of non-specific adsorption to the rest of the proteins present in the mixture.

The preparation of an affinity macroligand comprises the coupling or immobilization by covalent bond of the ligand molecule to the carrier matrix. The macroligands prepared from microparticles that are insoluble in water simplify the separation of the macroligand–protein complex from the mixture by micro or ultrafiltration. The particles used as support or carrier matrix are diverse and have a wide range of sizes. Literature describes macroligands prepared with other supports such as agarose granules, starch or silica nanoparticles [4,5].

Mattiasson B. and Ramstorp M., authors of the first studies conducted on affinity cross flow ultrafiltration used *Saccharomyces cerevisae* cells as an affinity macroligand. The paper describes the purification of concanavalin A from a crude extract of *Canavalia ensiforme* using the surface structures of heat-killed yeast cells as macromolecular ligands [6]. The carbohydrate molecules present

* Corresponding author. Tel.: +54 2652 520300; fax: +54 2652 520300.
E-mail address: ferraris@unsl.edu.ar (M. del Pilar Ferraris).

1570-0232/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2011.07.040

in the cellular wall act as ligands for the binding with the target protein.

Diverse molecules can be used as ligands for the design and preparation of an affinity adsorbent or macroligand. The production of extremely specific ligands, such as oligonucleotides, antibodies, oligopeptides, cofactors, coenzymes, etc., is expensive. An alternative to the biospecific natural ligands is the dye–ligand molecules, known to bind to several proteins, through the so-called pseudo-biospecific adsorption [7]. The binding between the dye–ligand and the protein is attributed to a combination of electrostatic, hydrophobic, charge-transfer and hydrogen bonds interactions. Dyes are commercially available at a low cost and can be easily immobilized by covalent bonds between the reactive groups on the support–matrix, mainly hydroxyl groups, and the triazinyl groups on the dyes molecules. One of these ligand molecules, the dye Cibacron Blue 3GA, shows affinity towards NAD^+-dependent enzymes. This is due to the conformational and charge-distribution resemblance between the dye and nucleotides. Previous studies described the use of Cibacron Blue coupled to different supports for protein purification such as lysozyme, bovine serum albumin (BSA), human serum albumin (HSA), alkaline phosphatase, lactate dehydrogenase, etc. [8–10].

The present study considered the potential that the methodology of affinity filtration exhibits for protein purification processes. On the other hand, the use of yeast cells as affinity macroligands demonstrated the cells suitability to be used as insoluble microsupports for the preparation of a macroligand. Furthermore, previous studies using membrane technology have been conducted by our investigation group. An experimental model with yeast biomass [11] was designed to determine the filtrate parameters involved in the use of an inorganic membrane to a separative process. Consequently, the present work is aimed to the development of affinity adsorbents with suitable characteristics to be used in purification of proteins through affinity ultrafiltration. Macroligand made of chemically modified yeast cells plus Cibacron Blue 3GA was prepared immobilizing the ligand molecule to the cell wall by covalent bond. The amount of ligand immobilized to the support-cells was determined by spectrophotometric methods. The binding of lysozyme on these microparticles were performed in batch and analyzed by adsorption isotherm assays. Finally, the separation of the enzyme from hen egg white, using the macroligands, is also investigated in this study.

2. Materials and methods

2.1. Materials and apparatus

Baker's yeast cells *Saccharomyces cerevisiae* (wet pressed) was purchased from Calsa Inc., Argentine. High purity chicken egg white lysozyme was kindly provided by Inovatech BioProducts Co. Cibacron Blue 3GA and *Micrococcus lysodeikticus* lyophilized cells were supplied by Sigma Chemical Co. All other chemicals were of analytical grade and used without further purification. Fresh hen eggs were purchased at a local market (San Luis) and egg white was obtained by mechanic separation from egg yolk and homogenized in Janke and Junkel D-7813.A Shimadzu 3101 UV/Vis Spectrophotometer was used for optical absorption measurements. The SEM analysis was carried out using a scanning electron microscope LEO 1450VP.

2.2. Assays

The amount of Cibacron Blue 3GA coupled to the yeast cells was determined spectrophotometrically at the wavelength of maximum absorption (610 nm). Lysozyme concentration was established by using a standard calibration curve of absorbance versus concentration and Michaelis–Menten kinetics application. Lysozyme activity was determined according to the method described by Shugar [12]. One unit of lysozyme activity will produce a ΔA_{450} of 0.001 per min at pH 6.24 and 25 °C, using a suspension of *M. lysodeikticus* as substrate in a 2.6 ml reaction mixture (1 cm light path). The protein concentration was analyzed by the Bradford method [13] using bovine serum albumin as standard. The purity of the lysozyme separated from hen egg white was assayed by gel electrophoresis (SDS–PAGE) at 13% as described by Laemmli [14]. Gel was stained with Coomassie Blue R250.

2.3. Preparation of the Cell-Cibacron Blue 3GA macroligand

The raw material used as insoluble microsupport for the preparation of the macroligand was *S. cerevisiae* cells (baker's yeast). Through the first stage of the preparation, 2 g of wet yeast were washed twice using 10 ml of ultra pure water to remove impurities. After each wash, cells were separated from the aqueous phase by centrifugation at $5000 \times g$ for 10 min (Sigma 4K centrifuge). Cells were subsequently suspended in 10 ml of 96% ethyl alcohol and heated at 80 °C in a thermostatized bath for 90 min in a beaker. Cells were recovered by centrifugation ($5000 \times g$ for 10 min) using falcon tubes. Two water washes were carried out to remove the remaining alcohol.

The covalent coupling of the dye–ligand Cibacron Blue 3GA was performed using a chemical protocol which has been specifically applied to Cibacron Blue 3GA and cellulose membranes [15], as well as Cibacron Blue F3GA and chitosan and chitin membranes [16]. Previous studies have not reported the immobilization of Cibacron dye to yeast or any other cells.

The procedure consisted of submerging wet yeast cells treated with alcohol in an aqueous solution of Cibacron Blue 3GA (25 ml, 1% w/v) at 80 °C with 30 min stirring. Afterwards, NaCl was added to the solution (60 g/l of the reaction solution) with agitation and kept for 1 h at 80 °C. Finally, Na_2CO_3 (20 g/l of the reaction solution) was added to the reactor maintaining the same stirring and temperature conditions for 1 h. The latter addition provided alkaline conditions that enabled the covalent bond between the group of the dye containing chloride and the hydroxyl group of yeast cell surface. Once the immobilization took place, the Cell-Cibacron affinity microparticle (or macroligand) were repeatedly washed using distilled water and centrifuged to remove the unbound dye until the washing solution became colorless. Finally, the blue yeast cells were washed with buffer (50 mM Tris–HCl/50 mM NaCl, pH 8.0) and placed in a vial for further lyophilization. A blank was made to analyze non-specific interactions between the proteins and the microsupport. The blank consisted of yeast cells treated with ethanol and undyed with Cibacron Blue (Cell-Blank microparticle).

2.4. Determination of the amount of Cibacron Blue 3GA immobilized on the yeast cells

The binding capacity of yeast cells for the Cibacron Blue 3GA ligand was evaluated using an acid hydrolysis technique. This technique has been described in the determination of the amount of Cibacron dye coupled to chitin and chitosan membranes [16]. Our work is the first report of this technique applied to yeast. The procedure applied to the Cell-Cibacron macroligand required the modification of temperature and time of the acid hydrolysis reaction that release and solubilize the dye–ligand attached to the yeast cell in aqueous media. The assay is described as follows. 50 mg of the lyophilized Cell-Cibacron macroligand and 50 mg of the lyophilized Cell-Blank (undyed cells, see Section 2.3) were weighted. Hydrogen chloride (HCl, 12 N) was added to both materials and the mixtures were heated at 90 °C for 1 h. After-

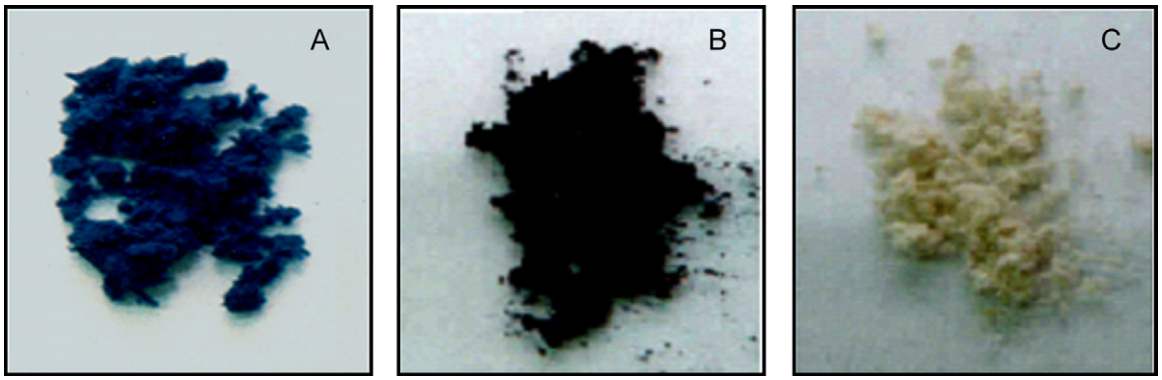

Fig. 1. Photographic images of the macroligand. (A) lyophilized yeast cells without previous chemical treatment and with immobilized Cibacron Blue dye; (B) lyophilized yeast cells treated with ethanol and immobilized Cibacron Blue dye (Cell-Cibacron macroligand); (C) lyophilized yeast cells treated with ethanol and without immobilized Cibacron Blue dye (Cell-Blank microparticle).

wards, the solution was diluted with ultrapure water to obtain a 6 N HCl solution which was neutralized with a sodium hydroxide solution (NaOH, 6 N). The suspension was then centrifuged at 5000 × *g* for 10 min and the absorbance at 610 nm was determined for the aqueous solution of the dye–ligand supernatant (25 ml of the total volume) by spectrophotometry. This data was used to calculate the Cibacron concentration through the comparison with standard solutions. The experiments were performed by triplicate.

2.5. Lysozyme adsorption at equilibrium

It follows a typical assay involving the adsorption of lysozyme with the Cell-Cibacron macroligand and Cell-Blank. The assay was carried out batchwise using 20 mg of lyophilized Cell-Cibacron macroligand and 20 mg of lyophilized Cell-Blank microparticle. Both adsorbents were washed with ultrapure water, centrifuged and equilibrated with 5 ml of adsorption buffer (50 mM Tris–HCl/50 mM NaCl, pH 8.0) for 30 min. The adsorption buffer was removed by centrifugation and 5 ml of the adsorption buffer containing lysozyme at concentrations ranging between 0.5 and 30 mg/ml was added. The solution was agitated for 12 h at 20 °C. Subsequently, the adsorbent particles were separated by centrifugation at 5000 × *g* for 10 min. The concentration of lysozyme in the supernatant was determined by measuring the initial and final concentration of lysozyme within the adsorption medium by UV spectrophotometry at 280 nm. The lysozyme concentration in the adsorbents was calculated using the mass balance method. Measurements of triplicate samples were performed and averaged.

2.6. Separation of lysozyme from hen egg white

The adsorption of egg white lysozyme was studied batchwise. 50 mg of Cell-Cibacron macroligand and 50 mg of Cell-Blank microparticle (see Section 2.3) were equilibrated with adsorption buffer (5 ml; 50 mM Tris–HCl/50 mM NaCl, pH 8) for 30 min. Egg white was homogenized for 3 min. After, 5 ml of homogenate was mixed with the Cell-Cibacron macroligand and the Cell-Blank microparticle (see Section 2.3) and left. The solution was agitated for 12 h at 20 °C. The adsorbents particles were recovered by 5 min centrifugation at 10,000 × *g* and washed with ultrapure water. Finally, for the elution of the adsorbed proteins, different eluants were assayed: 2 N NaCl, 1 N Acetic acid, 0.1 N NaSCN and 50 mM NaOH. The purity of lysozyme was assayed by gel electrophoresis and specific activity determination.

3. Results and discussion

3.1. Preparation of the Cell-Cibacron affinity macroligand

Immobilization preliminary assays between native yeast cells, without previous chemical treatment, and the Cibacron Blue ligand molecule were carried out. The results showed a weak blue coloring on the cells after the dyeing reaction which suggested a low content of Cibacron Blue immobilized (Fig. 1). Further assays were conducted to achieve a modification of the cell wall structure in order to get a higher concentration of bound ligand through the dyeing reaction. Prior to staining, the alcohol and temperature treatment should inactivate the cells and favor the removal of glycosylated mannoproteins present on the outer layer of the yeast cell wall [17,18]. The free hydroxyl groups of glucans on the subjacent layer would remain exposed to the nucleophilic attack on the carbon of the triazine ring of the dye. Consequently, a covalent bond between the Cibacron and the cell–support would be formed under reaction conditions.

After the cells treated with ethanol were washed they were put into contact with the ligand (Cibacron Blue) to produce the covalent binding reaction according to the described technique. The addition of NaCl increases the ionic strength of the solution causing the water molecules to be well arranged due to the solvatation of a large number of ions. As a result, the Cibacron Blue molecules showed to be less compatible with water favoring their attachment to the yeast cell surface and improving the coupling reaction. The aggregate of sodium carbonate increases the pH during the reaction time. Under mild alkalinity and high temperature conditions, the chemical reaction between the chlorine of the dye triazine group and the hydroxyl groups present in the polymers (glucans) of the cell wall, takes place. The HCl removal is the result of the covalent bond between the ligand Cibacron Blue and the microsupport (yeast cell).

3.2. Chemical–physical characteristics of the affinity macroligand

The amount of dye–ligand coupled to the microparticle constitutes an important parameter in protein adsorption. The results indicated that the ligand immobilization capacity of the yeast cell used as microsupport was 212 μmol of Cibacron dry/g of dry cell. This value is comparatively higher or similar to previous reported results on other support materials using Cibacron Blue as ligand molecule [19]. However high dye content does not necessarily mean high binding capacities because of steric hindrance.

The morphology of the Cell-Cibacron macroligand was studied using SEM micrographs and optical microscopy. The observations of the macroligand showed appropriate conservation of cellular

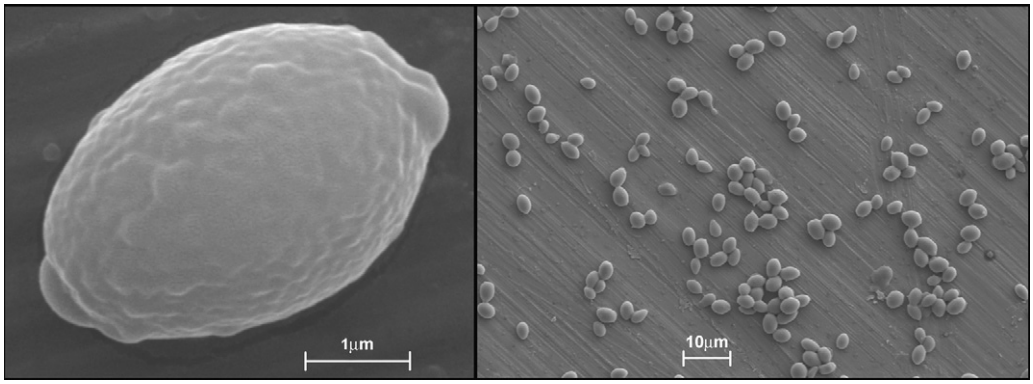

Fig. 2. SEM micrographs of lyophilized Cell-Cibacron macroligand.

morphology with a mean size of 5 μm (Fig. 2). Macroligand particles did not form aggregates when suspended in aqueous solutions or homogenized egg white. Centrifugation or frontal microfiltration (0.22 μm membrane pore) allowed a full recuperation of the microparticles. Moreover, a high sedimentation velocity was observed. The macroligand uniform size and rigidity are suitable characteristics to be used in affinity filtration. Small particles show larger surface to volume ratio which in turn allows higher adsorption capacities and faster adsorption rates.

The yield obtained of adsorbent from pressed wet yeast by the described procedure was approximately 10%. Hence, for each 10 g of wet yeast cells, 1 g of lyophilized Cell-Cibacron macroligand can be obtained.

3.3. Functional characteristics of the affinity macroligand

3.3.1. Adsorption of lysozyme at equilibrium

Due to the physical nature of the affinity macroligand the method to evaluate the capacity of the adsorbent is by means of the adsorption analysis of the protein to the solid surface using Langmuir isotherms. This method is widely accepted for integrating experimental data of protein adsorption onto different solid surfaces. The adsorption behavior is described by the Langmuir equation:

$$q = \frac{q_m \times C}{K_d + C} \quad (1)$$

where q (mg/ml) and C (mg/ml) are the protein concentrations at equilibrium in solid and liquid phase, respectively. The q_m (mg/ml) and K_d (mg/ml) constants are the main thermodynamic parameters to be determined. The q_m value represents the maximum adsorption capacity of the adsorbent and K_d is the apparent dissociation constant of the protein–ligand complex, which represents the affinity or binding strength between the protein and the ligand molecule under the specified operating conditions.

Eq. (1) can be rewritten in the form

$$\frac{C}{q} = \frac{C}{q_m} + \frac{K_d}{q_m} \quad (2)$$

The K_d and q_m values were calculated from the straight-line plot of C/q against C by linear regression analysis. The obtained Langmuir-type isotherms indicated a high affinity and adsorption capacity of the Cell-Cibacron macroligand for lysozyme (Fig. 3). The strong affinity is attributed to the structural similitude of the dye and the adenine nucleotides, giving rise mainly to ionic and hydrophobic interactions. The significant adsorption capacity showed by the Cell-Blank (Fig. 4) might be attributed to chitin, other component of the yeast cell wall. Chitin contains N-acetyl-D-glucosamine residues in its structure which can bind lysozyme. Diverse chitin

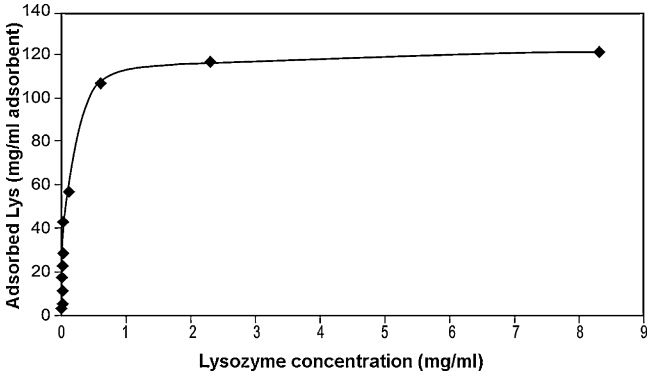

Fig. 3. Adsorption isotherm of the binding of lysozyme to the Cell-Cibacron macroligand from lysozyme solutions containing 50 mM Tris–HCl/50 mM NaCl, pH 8.0 and 20 °C after 12 h.

adsorbents for column separation of lysozyme from hen egg white have been reported [20]. In that study macroporous chitin membranes were prepared as alternative adsorbents for the affinity separation of lysozyme.

The obtained results of the adsorption parameters are presented in Table 1.

3.4. Selectivity: separation of lysozyme from hen egg white

Hen egg white is a major source of lysozyme and constitutes a suitable mixture of proteins to study the selectivity of macroligand. Competitive protein adsorption was carried out and the results are shown in (Fig. 5). The solutions containing the eluted proteins were analyzed by SDS–PAGE after the adsorption stage. The results

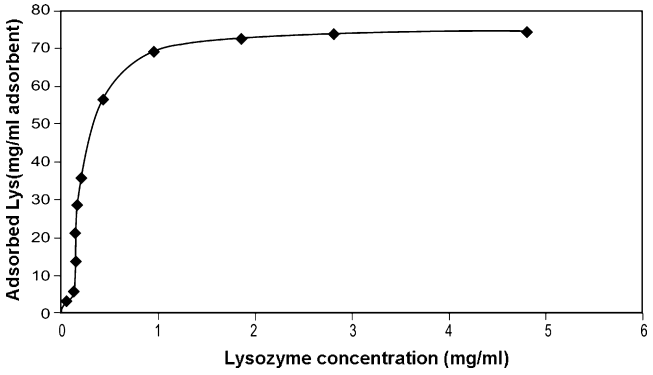

Fig. 4. Adsorption isotherm of the binding of lysozyme to the Cell-Blank microparticle from lysozyme solutions containing 50 mM Tris–HCl/50 mM NaCl, pH 8.0 and 20 °C after 12 h.

Table 1
Langmuir isotherms constants of lysozyme adsorption on Cell-Cibacron macroligand and Cell-Blank microparticle.

Adsorption parameters	
Cell-Cibacron macroligand	Cell-Blank microparticle
q_m = 110 mg lys/ml[a]	q_m = 70 mg lys/ml[a]
$K_d = 7.8 \times 10^{-6}$ M	$K_d = 1.8 \times 10^{-5}$ M

[a] 1 ml of wet adsorbent is equivalent to 0.350 g of dry adsorbent.

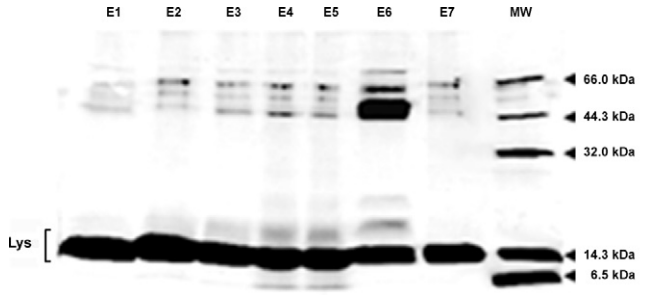

Fig. 5. SDS–PAGE assay of lysozyme recovered from the Cell-Blank and Cell-Cibacron macroligand by elution after adsorption in egg white. Polyacrylamide gel was performed (4% stacking gel and 13% separation gel). The gel was visualized using Coomassie blue solution (0.1% Coomassie blue R250, 40% methanol, 10% glacial acetic acid). Identification of lanes: E1: standard Lys (Inovatech); E2: Lys eluted with 2 N NaCl from Cell-Blank; E3: Lys eluted with 1 N Acetic Acid from Cell-Blank; E4: Lys eluted with 0.1 N NaSCN from Cell-Blank; E5: Lys eluted with 50 mM NaOH; E6: hen egg white proteins (1:50 dilution) with addition of Lys; E7: Lys eluted with 1 N NaSCN from Cell-Cibacron; last lane: molecular weight marker.

indicate that lysozyme was eluted from the Cell-Blank microparticle with a similar efficiency for all employed eluants. Regarding the active lysozyme recovered, the elution results obtained from Cell-Blank was considerably improved by using 2 N NaCl. High lysozyme desorption from Cell-Cibacron macroligand was solely observed when eluted using the chaotropic agent NaSCN. These results demonstrate that the Cell-Cibacron macroligand has a high adsorption capacity and selectivity towards lysozyme. However, due to the strong interaction with the ligand the elution step showed difficulties. Comparatively, the Cell-Blank microparticle has a lower adsorption capacity, easier elution, similar selectivity and an efficient recovery of active lysozyme.

The values of the adsorption parameters obtained with the Cell-Cibacron adsorbent are comparable to the experimental results reported by Suen at al. [21]. In that article the adsorption isotherms for the batch equilibrium binding of lysozyme to Cibacron Blue 3GA (immobilized onto gel beads, regenerated cellulose membrane discs and polysulfone hollow fibers) are analyzed by Langmuir model and Suen model. The isotherm results at 25 °C of the dissociation constant (K_d) and maximum adsorption capacity (q_m) by the Langmuir model for gel beads (Blue Sepharose CL-6B) and hollow fibers show values which compare well with those found with the Cell-Cibacron. Values of the dissociation constants show similarity, gel beads ($K_d = 6.8 \pm 1.8$ μM); hollow fibers ($K_d = 4.0 \pm 1.8$ μM) and Cell-Cibacron ($K_d = 7.8$ μM). As both studies use the batch adsorption procedure then it is reasonable to assume that the Lysozyme–Cibacron molecular interaction is the major contributing factor (or dominant factor) in the result of the dissociation equilibrium constant. On the other hand there are differences in the results of the adsorption capacity of the affinity adsorbents. The magnitudes are expressed as adsorbed lysozyme concentration based on volume of solid adsorbent: gel beads ($q_m = 2100 \pm 700$ μM); hollow fibers ($q_m = 9700 \pm 700$ μM) and Cell-Cibacron ($q_m = 7500$ μM). The maximum adsorption capacity of the Cell-Cibacron is significantly higher than gel beads and lower than hollow fiber. The difference found in this adsorption parameter may be due to the adsorbent ligand density and the physical–chemical characteristics of the solid support used.

4. Conclusions

Dye–ligand affinity adsorbents play an important role in the separation and purification of proteins. An affinity adsorbent or macroligand has been prepared by immobilizing Cibacron blue 3GA to yeast cells used as an insoluble microsupport. Results indicate that the macroligand obtained have a significant affinity and selectivity for lysozyme (K_d: 10^{-6} M). Therefore, under appropriate conditions Cell-Cibacron blue 3GA has a high adsorption capacity for lysozyme and low non-specific interactions with the remaining proteins in solution. However, enzyme elution from Cell-Cibacron–Lys complex was complicated due to the strong interaction. The affinity showed by Cell-Blank microparticle towards lysozyme, attributed to the cell wall chitin, is a novel result which has a great potential for lysozyme purification. The affinity particles have an appropriate size and chemical/physical stability required to be used in ultrafiltration. Future work will involve the design of a large scale process for lysozyme purification from hen egg white using Cell-Cibacron or Cell-Blank adsorbents by affinity ultrafiltration.

Acknowledgements

The authors are grateful to Lic Gómez Barroso and to Dr. Carlos Aguilar for useful comments in the manuscript.

References

[1] A. Denizli, E. Piskin, J. Biochem. Biophys. Methods 49 (2001) 391.
[2] S. Angal, P.D.G. Dean, J. Biochem. 167 (1977) 301.
[3] I.Y. Galaev, Biochemistry (Mosc) 64 (1999) 849.
[4] T.G.I. Ling, B. Mattiasson, Biotechnol. Bioeng. 34 (1989) 1321.
[5] D.C. Herak, E.W. Merrill, Biotechnol. Prog. 6 (1990) 33.
[6] B. Mattiasson, M. Rampstorp, J. Chromatogr. 283 (1984) 323.
[7] N. Garg, I.Y. Galaev, B. Mattiasson, J. Mol. Recognit. 9 (1996) 259.
[8] X. Zeng, E. Ruckenstein, J. Membr. Sci. 117 (1996) 271.
[9] S. Rao, A.L. Zydney, J. Membr. Sci. 280 (2006) 781.
[10] K. Nakamura, T. Suzuki, T. Kamichika, M. Hasegawa, Y. Kato, H. Sasaki, K. Inouye, J. Chromatogr. A 972 (2002) 21.
[11] J.A. Rodríguez, C.F. Aguilar, A. Pérez Padilla, Desalination 130 (2000) 131.
[12] D. Shugar, Biochem. Biophys. Acta 8 (1952) 302.
[13] M. Bradford, Anal. Biochem. 72 (1976) 248.
[14] U.K. Laemmli, Nature 277 (1970) 680.
[15] L. Hsien-Chang, J.R. Fried, AIChE J. 40 (1994) 40.
[16] E. Ruckenstein, X. Zeng, J. Membr. Sci. 142 (1998) 13.
[17] B. Aguilar-Uscanga, J.M. François, Lett. App. Microbiol. 37 (2003) 268.
[18] F.M. Klis, P. Mol, K. Hellingwerf, S. Brul, FEMS Microbiol. Rev. 26 (2002) 239.
[19] Y.H. Yu, B. Xue, I. Sun, Bioproc. Biosyst. Eng. 24 (2001) 25.
[20] E. Ruckestein, X. Zeng, Biotechnol. Bioeng. 56 (1997) 610.
[21] S. Suen, R. Chen, Y. Tsai, J. Liq. Chromatogr. Rel. Technol. 23 (2000) 223.