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Separation and purification of lysozyme by Reactive Green 19 immobilised membrane affinity chromatography

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Abstract

A poly(hydroxyethylmethacrylate)/chitosan (pHEMA-chitosan) composite membrane was prepared by UV-initiated photopolymerisation. A dye ligand (i.e. Reactive Green 19; RG 19) was immobilised onto the membrane. The water content of the dye ligand-immobilised membrane was $50 \pm 2\%$ and the amount of immobilised Reactive Green 19 on the membrane was 0.865 μmol/ml. The rates of adsorption of lysozyme on plain composite and dye ligand-immobilised composite membranes were measured in a stirred cell. The adsorption capacity of the composite membrane was determined by changing pH and the concentration of lysozyme in the adsorption medium. Separation and purification of lysozyme from aqueous solution and egg white were investigated, respectively. The lysozyme adsorption capacity of the RG 19 immobilised membrane was 60.8 mg/ml. The adsorption capacity of the plain composite membrane was 7.2 mg/ml. Separation and purification were monitored by determining the lysozyme activity using *Micrococcus lysodeikticus* as substrate. The lysozyme was purified 25.4-fold in a single step with a recovery 82%, as determined by HPLC. The membranes were stable when subjected to sanitization with sodium hydroxide after repeated adsorption-elution cycles.

Keywords: Affinity chromatography; Composite membrane; Dye-ligand; Lysozyme; Adsorption; Purification

1. Introduction

Egg white contains a complex mixture of proteins, such as ovalbumin, conalbumin, ovomucoid ovomucin, lysozyme and G2 and G3 globulins. One of them, lysozyme, is a commercially important enzyme and is currently used in food technology as a potent antibacterial agent in milk products, in wine production processes, and in pharmacological technology as a drug for treatment of ulcers and infections. The potential use of lysozyme as an anticancer drug and in the treatment of HIV infection has also been discussed (Bayramoğlu & Arıca, 2002; Ghosh, 2003; Tong, Dong, & Sun, 2002).

In affinity membrane chromatography, pseudo specific ligands, e.g., dyes, amino acids and metal chelates have been used instead of biospecific ligands (Bayramoğlu, Kaya, & Arıca, 2002; Fisichella et al., 2003). Recently, dye-ligand chromatography has gained considerable importance in protein purification, for both laboratory- and large-scale separation (Bayramoğlu, 2003; Bayramoğlu et al., 2002; Finette, Mao, & Hearn, 1997; Fisichella et al., 2003; Grasselli, Camperi, del Canizo, & Cascone, 1999). A reactive dye in solution can function as a competitive inhibitor for the substrate, coenzyme or effectors of a variety of proteins, often with an affinity greater than that exhibited by the competitive molecule. This affinity results from the flexibility of the dye, which can assume the polarity and geometry of the surface of a variety of competitive biomolecules (Zhang & Sun, 2001a, 2001b), Cibacron Blue F3GA, Cibacron Blue 3GA, Active Red K2BP, Procion Red HE-3B, Reactive Yellow 86 or Procion Blue MX-R, immobilised on various types of membrane supports, such as cellulose membrane (Suen, Lin, & Chiu, 2000), hollow fibre membrane (Grasselli et al., 1999), poly(2-hydroxyethylmethacrylate) membrane (Arıca, Testereci, & Denizli, 1998) Agarose resins (Grasselli et al., 1999) or polyethylene hollow-fibre membrane-Cibacron Blue (Wolman,

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Grasselli, Smolko, & Cascone, 2000). The use of chitin affinity membrane (Ruckenstein & Zeng, 1997), styren-acrylate-Cibacron Blue (Chen & Lee, 2001), Red HE-3B fibre membrane and PVDF membrane (Ghosh, 2003; Grasselli et al., 1999; Owen & Chase, 1997; Tong et al., 2002) for purification of lysozyme has also been reported.

The selection of the membrane material and the preparation are dominant factors affecting the chromatographic performance. The poly(hydroxyethylmethacrylate) (pHEMA) possesses high mechanical strength, resistance to many chemicals and microbial degradation. The combination of the useful properties of the synthetic pHEMA and natural chitosan could introduce a composite matrix to the chromatographic area. In addition, the preparation of composite material from these polymers is a simple method and the presence of hydroxyl and amino groups on the composite material offer easy attachment sites for a variety of dye-ligands (Bayramoğlu et al., 2002; Bayramoğlu, Yılmaz, & Arıca, 2003; Gebauer, Thommes, & Kula, 1997; Ruckenstein & Zeng, 1997; Suen & Tasai, 2000; Suen et al., 2000).

In the present study, the pHEMA/chitosan composite membrane was prepared by UV-initiated photo-polymerization. A reactive dye-ligand (i.e. Reactive Green 19) was immobilised covalently onto the composite membrane via a nucleophilic substitution reaction under alkaline conditions. The RG 19 dye ligand molecule has six sulfone groups, one primary and four secondary amino groups and the chitosan has several amino groups on the carbohydrate moiety. The resulting ion-exchange surface of the composite membrane was then utilised for its propensity to separate the target protein, lysozyme, in a batch system. The adsorption conditions (i.e. concentration of lysozyme, temperature, medium pH, ionic strength) were varied to evaluate their effects on the performances of the affinity membrane. Finally, the dye ligand-immobilised composite membrane was used for the purification of lysozyme from egg white. The purity of the eluted lysozyme was determined by HPLC and the activity of the purified lysozyme was measured using *Micrococcus lysodeikticus* as a substrate microorganism.

2. Materials and methods

2.1. Materials

Lysozyme (chicken egg white, EC 3.2.1.17), was supplied from Sigma Chemical Co. (St Louis, MO, USA) and used as received. 2-Hydroxyethylmethacrylate (HEMA) was obtained from Fluka AG (Switzerland), distilled under reduced pressure in the presence of hydroquinone and stored at 4 °C until used. α-α′-Azoisobutyronitrile (AIBN), chitosan, Reactive Green 19 and trifluoroacetic acid (TFA) were obtained from Sigma Chem. Co. All other chemicals were of analytical grade and were purchased from Merck AG (Darmstadt, Germany). The water used in the following experiments was purified using a Barnstead (Dubuque, IA, USA) ROpure LP reverse osmosis unit with a high flow cellulose acetate membrane (Barnstead D2731), followed by a Barnstead D3804 NANOpure organic/colloid removal and ion-exchange packed-bed system.

2.2. Preparation of composite pHEMA/chitosan membrane

The synthesis of composite membrane was achieved by mixing a chitosan solution (1.0% chitosan in 1.0% acetic acid, 4.0 ml) with HEMA monomer (2.0 ml) containing 20 mg AIBN. Following flushing with nitrogen, the solution was poured into a round glass mold (diameter: 9.0 cm), sealed and exposed to UV for 1.0 h at ambient temperature. The mold was kept in an atmosphere of nitrogen during polymerisation. After the polymerisation, the resultant product was washed with 1.0% NaOH solution and then distilled water. The composite membrane was cut into circular pieces (diameter: 0.75 cm) with a perforator and left in the wet state at 4 °C.

2.3. Immobilisation of dye-ligand on the composite membrane

RG 19 was covalently immobilised onto the membrane via the nucleophilic reaction between the chloride of its triazine ring and hydroxyl groups of the composite under alkaline conditions. RG 19 (300 mg) was dissolved in distilled water (10 ml), and transferred to NaCl solution (1.0 M, 60 ml) in which composite membrane discs (6 g) were equilibrated for 1 h. After this period, sodium carbonate solution (3.2 M, 30 ml) was added to the medium (about pH 10) and heated at 80 °C for 4 h in a sealed reactor. After the reaction period, the solution was cooled to room temperature and composite membrane disks were washed several times with distilled water, 2.0 M NaCl and 10% methanol. The dye ligand-immobilised composite membranes were stored at 4 °C until used.

2.4. Lysozyme separation studies from aqueous solution

Separation of lysozyme from solution on the RG 19-immobilised membrane was studied at various pHs, either in acetate (7.5 ml, 50 mM, pH 4.0–5.5) or in phosphate buffer (7.5 ml, 50 mM, pH 6.0–8.0). The initial concentration of lysozyme was 1.0 mg/ml in each corresponding buffer solution. The adsorption experiments were conducted at 25 °C, for 2 h, with continuous stirring. After this period, the affinity membranes were

removed from the solution. The amount of adsorbed lysozyme on the RG 19-immobilised affinity membrane was determined by measuring the initial and final concentrations of lysozyme within the adsorption medium. The effects of temperature and ionic strength on lysozyme adsorption were determined in phosphate buffer (7.5 ml, 50 mM, pH 7.0, containing 1.0 mg/ml of lysozyme) at four different temperatures (namely 5, 15, 25 and 35 °C) and at three different NaCl concentrations (namely 0.1, 0.5 and 1.0 M), respectively. To determine the adsorption capacity of the affinity membrane, the initial concentration of lysozyme was changed between 0.05 and 3.0 mg/ml. The adsorption experiments were carried out for 2 h at 25 °C at a stirring rate of 100 rpm. The concentration of lysozyme was measured at 280 nm by using a double beam UV/Vis spectrophotometer (Shimadzu, Tokyo, Japan, Model 1601). The amount of adsorbed lysozyme was obtained by using the following equation:

$$q = [(C_o - C)V_s]/v_m, \quad (1)$$

where q is the amount of lysozyme adsorbed onto affinity membranes (mg/ml); C_o and C are the concentrations of the lysozyme in the initial solution and in the aqueous phase after adsorption, respectively, (mg ml^{-1}); V_s is the volume of the aqueous solution (ml); v_m is the volume of the affinity membranes in the adsorption medium (ml).

2.5. Lysozyme purification from egg white

Chicken egg white was separated from fresh eggs and diluted to 50% (v/v) with phosphate buffer (50 mM, pH 7.0). The diluted egg white was homogenised in an ice-bath and centrifuged at 4 °C, at 12,000 rpm for 15 min. The supernatant fluid was used as a lysozyme source. The purification of lysozyme was carried out as described above except that diluted egg white was used as an adsorption medium instead of pure lysozyme solution. The elution of proteins from the affinity membrane was carried out with phosphate buffer (50 mM, pH 8.0) containing 0.5 M NaCl.

In the purification experiments, the activity of lysozyme was determined spectrophotometrically at 620 nm, the decrease in the turbidity of o/n culture of *M. lysodeikticus* cells suspended in phospate buffer (0.1 M, pH 7.0) was followed for 6 min after addition of lysozyme and/or elution. One unit of lysozyme activity was defined as the amount of enzyme causing a decrease of 0.001 OD value per min at 25 °C and pH 7.0. The purity of lysozyme in the purified samples was analysed using HPLC.

2.6. HPLC conditions

A Dionex HPLC system (Dionex Co., Germering, Germany) was used for the chromatographic studies. The HPLC system consisted of a quaterary pump with an on-line vacuum degasser (Model P580 A), an autosampler with a variable injection capacity from 1 to 250 μl (Model ASI-100), a column oven (Model STH 585) and an UV–Vis diode array detector (Model 340 S). Chromatographic separation of proteins was achieved on a VYDAC 259 VHP 5415 column (150 mm × 4.6 mm i.d.) protected by a guard column (20 mm, 4.6 i.d.).

All protein solutions used in the chromatographic studies were pre-filtered through a syringe membrane filter (0.2 μm, Millipore) to remove particles and large aggregates. HPLC mobile phases A and B were prepared by adding TFA (0.01%, v/v in Milli Q water) and 95% acetonitrile and 5% Milli Q water, respectively. The mobile phases were filtered prior to use. The chromatographic separation was performed using a gradient at 1.0 ml/min flow-rate (0–20 min, phase B from 25% to 60% and 21–25 min, from 60% to 25%) and the sample injection volume of the autosampler was 20 μl. The UV–Vis detector was set at 220 nm and the temperature was maintained at 25 °C. Dionex CHROMELLEON® software was used and operated under Windows 98 for data acquisition and integration.

2.7. Lysozyme elution and stability of RG 19-immobilised membrane

The lysozyme elution was performed in a buffer solution containing 0.5 M NaCl at pH 8.0. The elution ratio was calculated from the amount of lysozyme adsorbed on the RG 19-immobilised composite membrane and the amount of lysozyme desorbed. To determine the reusability of the dye ligand-immobilised composite membrane the adsorption and desorption cycle was repeated eight times by using the same composite membrane. The desorption ratios of lysozyme were calculated by using the following expression:

$$\text{Desorption ratio} = [\text{amount of lysozyme released} \times 100] / [\text{amount of lysozyme adsorbed on the composite membrane}]. \quad (2)$$

3. Results and discussion

3.1. Properties of dye ligand-immobilised composite membranes

The presented pHEMA/chitosan composite membrane is essentially a hydrophilic matrix. The hydroxyl or amino groups of the composite membrane can react with the chloride of the triazine ring of the Reactive Green 19 (dye-ligand) under alkaline conditions, thus giving rise to covalent bonds. The main physical and morphological properties of dye-immobilised pHEMA/

chitosan composite membrane matrix were as follows: the water content of the dye-immobilised membrane was $50 \pm 2\%$; the thickness in the wet state of the composite membrane was 0.06 cm; the density of the composite membrane in the dry state was 1.26 g cm^{-3}; the plain surface area of the 1.0 ml wet composite membrane was 38.5 cm^2. Elemental analyses of the plain and Reactive Green 19-immobilised composite membranes were carried out, and the immobilised dye on the membrane was found to be 0.865 μmol/ml from the nitrogen and sulphur stoichiometry. Dye leakage was not observed from any of the dye-immobilised membranes during adsorption studies, or even over a long period of storage time (more than three months).

3.2. Effect of pH and temperature on lysozyme separation

Fig. 1 shows the effect of pH on the amount of lysozyme adsorbed on the dye ligand-immobilised composite membranes. The maximum lysozyme adsorptions was obtained at pH 7.0 (about 32.9 mg/ml membrane). Significantly, lower lysozyme adsorption were observed on the dye ligand-immobilised composite membrane in the acidic and in the alkaline pH regions. These results indicate that the pH of the medium has an important effect on the adsorption equilibrium of lysozyme, and there is a preferential interaction between lysozyme and dye ligand-immobilised composite membrane at pH 7.0.

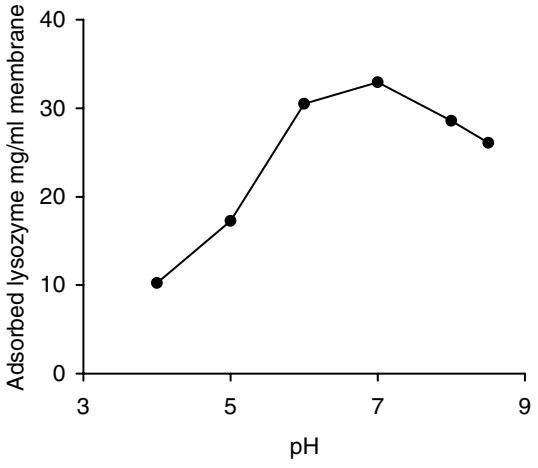

Fig. 1. Effects of pH on lysozyme adsorption on the RG 19 immobilised composite membranes; initial concentration of lysozyme: 1.0 mg/ml; temperature: 25 °C.

The isoelectronic (pI) value of lysozyme is 11.2. The lysozyme molecules would be cationic at pH values below 11.2. The RG-19 dye-ligand molecule has six acidic sulfones, one primary and four secondary amino groups and several hydrophobic groups (Fig. 2). On the other hand, one of the components of the composite membrane was chitosan, that is positively charged between pH 5.8 and 7.0 on the basis of $-NH_3^+$ groups of glucosamine. At pH 4.0, a small amount of lysozyme was adsorbed on the dye-immobilised composite membrane because of the protonation of the acidic sulfonate groups (pK value about 5.0) of the dye molecules and deprotonation of $-NH_3^+$ groups of glucosamine at this low pH value. Because of these effects, the primary interaction between lysozyme molecules and the dye molecules (Reactive Green 19), at around pH 7.0, could result in an ion exchange effect. From this point of view, the binding of lysozyme on the dye ligand-immobilised composite membrane should be a combination of ion-exchange and hydrophobic interactions (Bayramoğlu et al., 2002). These interactions between lysozyme and dye-immobilised membrane could arise from the co-operative effect of different mechanisms, such as hydrophobic and electrostatic interactions, caused by several ionic and aromatic structures, on immobilised dye ligands and the amino acid side-chain hydrophobic groups of the lysozyme molecules (Arıca et al., 1998; Bayramoğlu, 2003; Wolman et al., 2000; Zhang & Sun, 2001a, 2001b).

The equilibrium adsorption of lysozyme onto all the tested adsorbents significantly increased with increasing temperature (Fig. 3). At higher temperatures, the contact area between the protein and the dye ligand on the matrix should increase, resulting in increase in the affinity of proteins for the adsorbents (Finette et al., 1997). From 5 to 35 °C, the adsorption capacity of the RG 19-immobilised composite membranes for lysozyme increased by about 33%. This could be attributed to the chemical interaction between the dye-ligand and the lysozyme molecules as the temperature increased.

3.3. Effect of ionic strength

The initial concentration of lysozyme, temperature and pH were fixed at 1.0 mg/ml, 25 °C and 7.0, respectively, and the salt concentration (NaCl, ionic strength) was varied between 0 and 1.0 M (Fig. 4). The adsorption capacities of RG 19-immobilised membrane

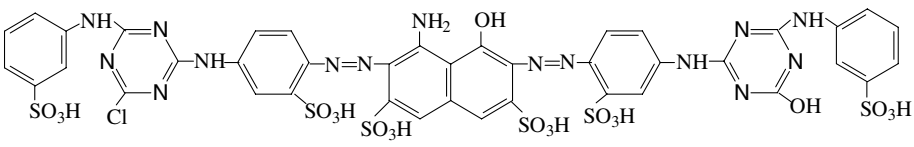

Fig. 2. Chemical structure of Reactive Green 19.

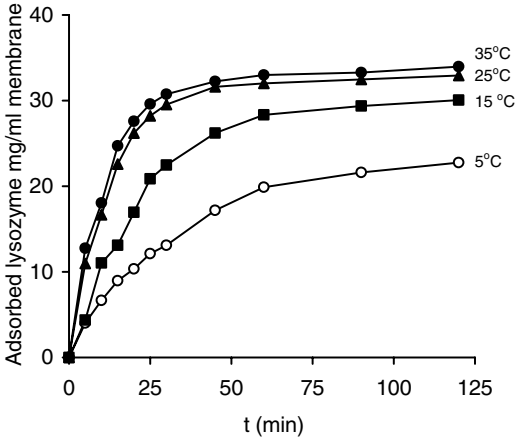

Fig. 3. Effect of temperature on the lysozyme adsorption rate and capacity on the RG 19-immobilised composite membranes; initial concentration of lysozyme: 1.0 mg/ml; pH: 7.0.

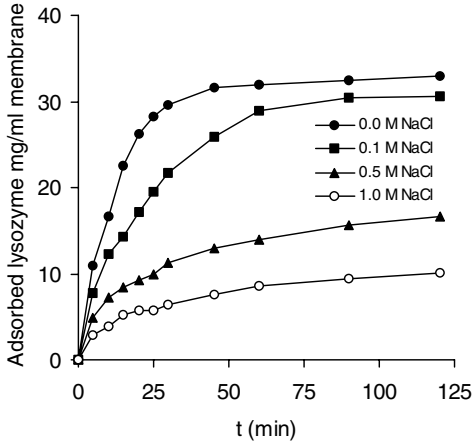

Fig. 4. Effects of ionic strength on lysozyme adsorption on the RG 19-immobilised composite membranes; initial concentration of lysozyme: 1.0 mg/ml; temperature: 25 °C, pH: 7.0.

to lysozyme was reduced 3.3-fold with increasing salt concentration, from 0 to 1.0 M. The decrease in lysozyme adsorption capacity of the dye ligand-immobilised composite membrane with increasing ionic strength could result from decrease in the electrostatic interactions between lysozyme and the dye-molecules. RG 19 dye ligand contains both hydrophobic and charged groups. It is to be noted that the combination of these two forces should be involved during the adsorption process between dye ligands and lysozyme molecules. As the ionic strength increased in the adsorption medium, the electrical double layer around the molecules, given by the Debye–Hückel length, decreased.

3.4. Adsorption isotherms

The lysozyme adsorption isotherm of the Reactive Green 19-immobilised composite membrane is presented in Fig. 5. As seen from the figure, an increase in the

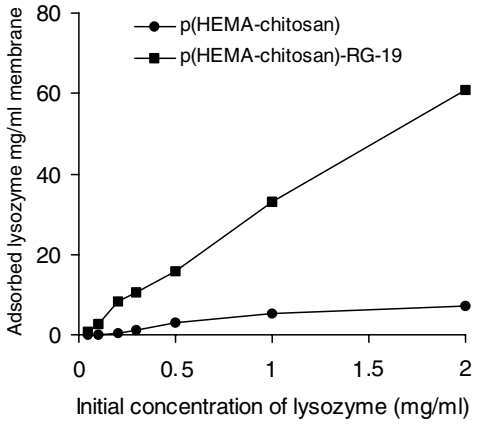

Fig. 5. Effect of lysozyme initial concentration on lysozyme adsorption on the RG 19 immobilised composite membranes. RG 19 content: 0.855 μmol/ml, pH: 7.0; temperature: 25 °C.

lysozyme concentration in the adsorption medium led to an increase for lysozyme on the membrane (about 60.8 mg/ml). It should be noted that a negligible amount of lysozyme was adsorbed non-specifically on the plain pHEMA/chitosan membrane (7.2 mg/ml). The RG 19 dye ligand-immobilisation significantly increased the lysozyme adsorption capacity (about 8.4-fold) of the membrane. It is clear that this increase is due to specific interaction between the immobilised Reactive Green 19 and lysozyme molecules.

After the values of C^* and q^* are obtained from experimental data, the semi-reciprocal plot of C^*/q^* versus C^* was employed to generate the intercept of K_d/q_m and the slope of $1/q_m$. It is necessary to determine which theoretical isotherm best fits the data. Two theoretical isotherm models were used to fit the experimental data, namely the Langmuir and the Freundlich. The Langmuir model is based on assumption of homogeneity, such as equally available adsorption sites, monolayer surface coverage, and no interaction between adsorbed

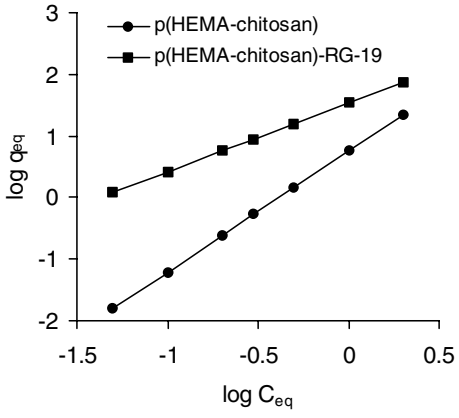

Fig. 6. The Freundlich isotherm plot for RG 19-immobilised composite membranes.

Table 1
Freundlich constants for adsorption of lysozyme on the plain and dye ligand-immobilised composite membranes

Membrane types	q_{ex}	K_F (mg/ml)	n	R^2
Plain	7.2	5.66	0.51	0.967
RG 19	60.8	34.2	0.90	0.988

species. Since the Langmuir model is formulated for homogeneous adsorption (Lan, Bassi, Zhu, & Margaritis, 2001), the Freundlich isotherm models are usually adopted for heterogeneous adsorption. The Freundlich relates the adsorbed concentration to the power function of solute concentration. One limitation of the Freundlich model is that the amount of adsorbed solute increases indefinitely with the concentration of solute in the solution. This empirical equation takes the form:

$$q_{eq} = K_F(C_{eq})^{1/n}, \quad (3)$$

where K_F and n are the Freundlich constants characteristic of the system. K_F and n are indicators of the adsorption capacity and adsorption intensity, respectively. The slope and the intercept of the linear Freundlich equation are equal to $1/n$ and $\ln K_F$, respectively (Fig. 6).

The corresponding semi-reciprocal plots and Scatchard plots gave a non-linear plot for the RG

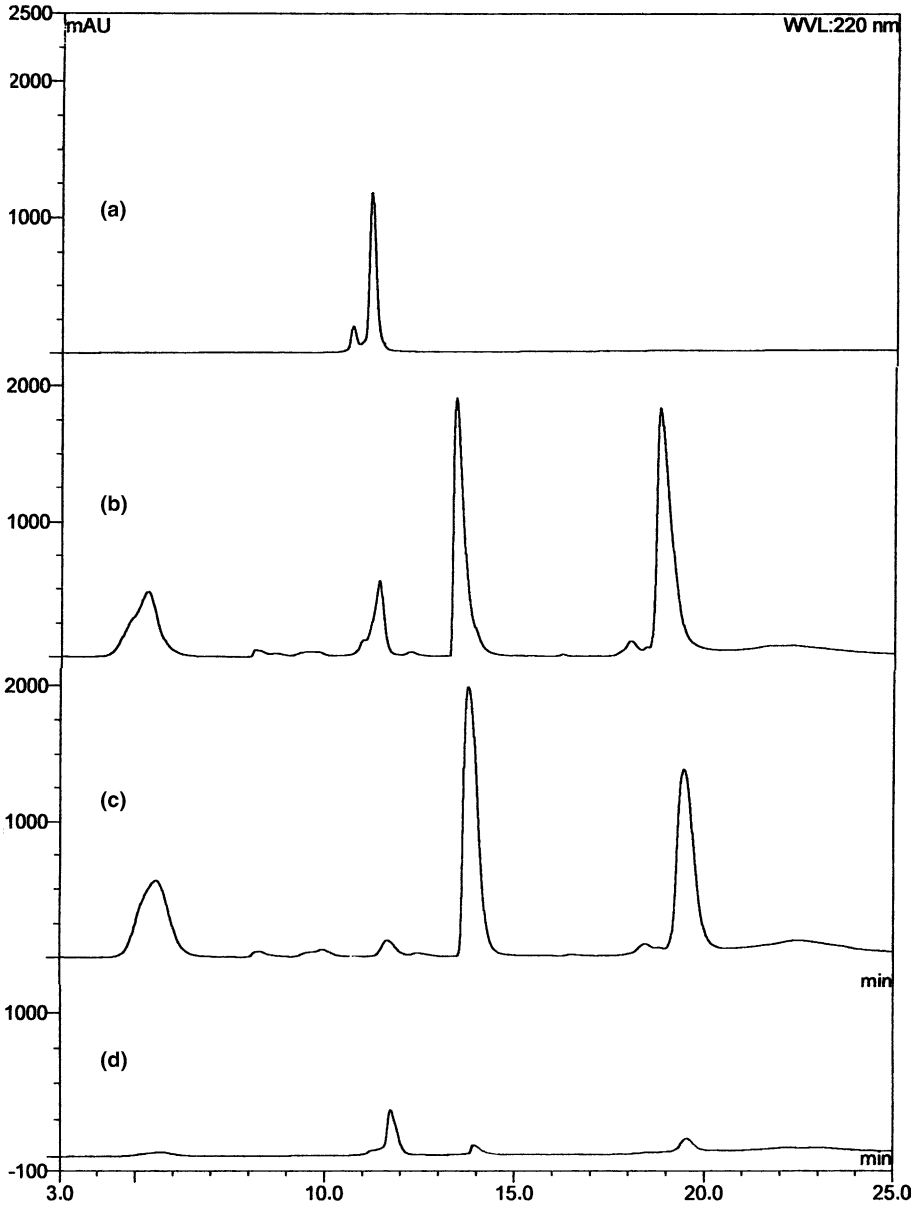

Fig. 7. HPLC chromatogram showing the separation of lysozyme: (a) commercial lysozyme; (b) whole egg white; (c) after lysozyme separation of egg white; (d) purified lysozyme.

19-immobilised pHEMA/chitosan membranes. In other words, a non-linear Scatchard plot indicates adsorption heterogeneity (Finette et al., 1997; Lan et al., 2001), since the adsorption of lysozyme onto the dye ligand-immobilised membrane cannot be described in terms of the Langmuir model.

The magnitude of K_F and n values of the Freundlich model showed easy uptake of lysozyme from the aqueous medium with a high adsorption capacity of the dye ligand-immobilised composite membranes. Values of n close to 1 for the dye ligand-immobilised composite membrane indicate positive co-operativity in binding and a heterogeneous nature of adsorption (Table 1).

Note that there are a wide variety of adsorbents with a wide range of adsorption capacities reported in the literature for lysozyme (Suen & Tasai, 2000; Suen et al., 2000; Tong et al., 2002; Zeng & Ruckenstein, 1999). The maximum lysozyme adsorption that was achieved with the adsorbents developed in this study was 60.8 mg/ml with the RG 19 dye ligand-immobilised composite membrane that was quite comparable with previous reports (Ghosh, 2003; Grasselli et al., 1999; Owen & Chase, 1997; Tong et al., 2002).

3.5. Purification of lysozyme from egg white

In this study, single step lysozyme purification has been reported. Purification of lysozyme from egg white was studied in a batch system. The purity of the lysozyme eluted from both dye ligand-immobilised membranes was determined by HPLC (Fig. 7). The lysozyme was purified 25.4-fold in a single step with a recovery of 82%, as determined by HPLC. The RG 19 dye ligand-immobilised composite membrane provided a good method to purify lysozyme from egg white, showing a high binding capacity and a high selectivity for lysozyme. The specific activity of the lysozyme purified with RG 19 composite membrane 43.5 U/mg. The presented dye ligand interaction system was based on electrostatic and hydrophobic interactions, therefore a small amount other egg white proteins had interacted during the purification of lysozyme.

3.6. Elution of adsorbed lysozyme and regeneration of membranes

The elution of the adsorbed lysozyme from the RG 19-immobilised composite membrane was studied in a batch system. The lysozyme-loaded membrane was placed within the elution medium containing 0.5 M NaCl at pH 8.0, and the amount of lysozyme released in 2 h was determined. Up to 99% of the adsorbed lysozyme was removed when 0.5 M NaCl was used as an elution agent. The elution results showed that NaCl is a suitable desorption agent for lysozyme from both membranes. In order to show the reusability of the composite membrane, the adsorption–desorption cycle of lysozyme was repeated eight times, using the same affinity membrane. The adsorption capacity of the affinity composite membrane did not change during repeated adsorption–desorption operations. After washing the affinity membranes with 0.1 M NaOH and 1.0 M NaCl for 4 and 2 h, respectively, the affinity membranes recovered their original capacities.

4. Conclusion

The pHEMA/chitosan composite membrane was prepared from HEMA monomer and chitosan by UV-initiated photo-polymerization. Reactive Green 19 was then covalently immobilised to the composite membrane with a membrane phase concentration was 0.865 μmol/ml as a dye ligand. Separation/purification studies of lysozyme on the dye-ligand immobilised composite membrane led to the following conclusions: lysozyme adsorption capacity of the dye ligand-immobilised membrane was 60.8 mg/ml. The lysozyme was purified 25.4-fold in a single step with a recovery of 82%, as determined by HPLC. More than 90% of the adsorbed lysozyme was desorbed using 0.5 M NaCl at pH 8.0 as the elution agent. Repeated separation/desorption processes showed that the dye ligand-immobilised composite membrane gave good separation of lysozyme from egg white in aqueous solution. It could be used in large-scale applications for separation of lysozyme from egg white.

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