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Preparation of reversibly immobilized lysozyme onto Procion Green H-E4BD-attached poly(hydroxyethylmethacrylate) film for hydrolysis of bacterial cells

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Abstract

The flat sheet support for enzyme immobilization was prepared by UV-initiated photopolymerization of 2-hydroxyethyl-methacrylate (HEMA) in the presence of an initiator (αα′-azoisobutyronitrile; AIBN). An affinity dye, Procion Green H-E4BD, was attached covalently under alkaline conditions and the pHEMA-Procion Green H-E4BD-attached film was used for the immobilization of lysozyme via adsorption. The amount of attached dye on the pHEMA film was 160 μmol m^2 and the water content of the dye-attached pHEMA film was 69%. The lysozyme adsorption capacity of the dye-attached pHEMA film was determined under conditions of different pH and with different initial concentrations of enzyme in the medium. The maximum lysozyme adsorption capacity of the dye-attached pHEMA film, under the specified experimental conditions was 3.92 g m^{-2}. Non-specific adsorption of the lyzozyme on the pHEMA film was negligible. Optimum reaction pH was 6.0 for the free and 7.0 for adsorbed enzyme. The free enzyme had an optimum temperature of 35°C, whereas it shifted to 40°C for the immobilized enzyme system. The enzyme could be repeatedly adsorbed and desorbed from the dye-attached pHEMA film without any significant loss in adsorption capacity.

Keywords: Lysozyme; Procion Green H-E4BD; pHEMA affinity film; Immobilization; Adsorption

1. Introduction

Polymeric hydrogels are playing an increasingly important role in the immobilization of enzymes, especially for use in the development of enzyme membrane reactors and enzyme electrodes (Arıca, Denizli, Salih, Pişkin & Hasırcı, 1997; Godjevarguva, Konsulov, & Dimov, 1999; Yin, Jonson, & Liu, 2000). The desirable properties of a support designed for enzyme immobilization are high water content, high mechanical strength and presence of chemical groups which permit derivatization.

Poly(2-hydroxyethyl methacrylate) (pHEMA) can easily be prepared in different shapes and forms (i.e. film and microspheres). It possesses a hydrophilic pendant group (–OH) and can form hydrogels. The hydroxyl groups offer a site for attachment of bioactive species after derivatization. It is non-toxic, biocompatible and is very inert towards microbial contamination and resistant to attack by many chemicals (Arıca, Hasırcı, & Alaeddinoğlu, 1995; Arıca, Kaçar, Ergene, & Denizli, 2001).

Lysozymes (E.C. 3.2.1.17) mucopeptide, 1,4-β-N-acetylmuramidases cleave the glycosidic bond between the C–1 of N-acetylmuramic acid and C–4 of N-acetylglucosamine in bacterial peptidogycan. Lysozyme, isolated from chicken egg white and other sources, has broad potential in food and clinical applications. In the dairy industry, food grade additive lysozyme can be used to prevent the growth of lactate-fermenting, gas forming *Clostridium* spp. in milk, thus overcoming the late gas-blowing in cheese. Another application of lysozyme in the food industry is the enzymatic disruption of whole microbial cells. After this treatment, enzymes and pigments could be easily extracted from microbial cells. Enzymatic lysis of the microbial cell wall offers an advantage over mechanical disruption methods, the

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latter generating high stress and heat during the purification step of enzymes and proteins (Appendini & Hotchkiss, 1997; Chen & Chen, 1997; Lee & Ku, 1994).

Immobilized lysozyme is gaining importance in minimizing the microbial load during continuous operation of a bioreactor and is also a promising way to reduce the enzyme cost. In previous works, lysozyme has been immobilized by different methods including covalent attachment to the activated supports (Chen & Chen, 1997; Lee & Ku, 1994; Marolia & D'Souza,1999) and adsorption on the carboxymethylcellulose microparticles (Mocanu, Merle, Carpov, & Muller, 2000). The activity of immobilized lysozyme was found to change upon immobilization, the extent of this being influenced by the immobilization route and type of support. The operational stability of an immobilized enzyme is determined mainly by the rate of enzyme inactivation; immobilization of the enzyme in hydrogels, on polymeric films or on inert, dispersed carriers is known to significantly increase the stability (Arıca, 2000a, 2000b; Tanyolaç, Yörüksoy, & Özdural, 1998; Tümtürk, Çaykara, Kantoğlu & Güven, 1999). It is, thus, important that the choice of proper support material and immobilization method over the free bioactive agent should be well justified.

Among the immobilization techniques, adsorption may have a higher commercial potential than other methods because it is simpler, less expensive, and retain high catalytic activity. The method also offers the reusability of expensive support after inactivation of immobilized enzyme. However, the adsorption is generally not very strong. Some of the adsorbed protein will desorb during washing and operation (Arıca et al., 1997; Baran, Arıca, Denizli, & Hasırcı, 1997; Lu, Lee, & Park, 1991; Roni, Das, Satyana, & Rayama, 2000; Sosnitza, Farooque, Saleemmuddin, Ulber, & Scheper, 1999). Thus, immobilization of enzyme via adsorption requires a strong hydrophobic or electrostatic interaction between the enzyme and support. Procion Green H-E4BD is a reactive dye and has large polysulphonated aromatic groups and the dye can provide a greater affinity for lysozyme biofunctional sites for immobilization via adsorption than that of previously reported reactive dyes such as Cibacron Blue F3GA that was used for the adsorption of lysozyme molecules.

The aim of this work was to investigate the feasibility of immobilization of the antibacterial enzyme (lysozyme) via adsorption onto the polymer network which is suitable for food contact. In order to achieve this, a dye ligand (Procion Green H-E4BD) attached pHEMA flat sheet film was prepared for reversible immobilization of lysozyme. The properties of the immobilized lysozyme system were characterized and compared with those of free lysozyme.

2. Materials and methods

2.1. Materials

Lysozyme (chicken egg white, EC 3.2.1.117) 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 use. α-α′-Azoisobutyronitrile (AIBN) and Procion Green HE-4BD (Reactive Green 19) were obtained from Sigma Chem. Co. All other chemicals were of analytical grade and were purchased from Merck AG (Darmstadt, Germany).

2.2. Preparation of pHEMA film

The poly(2-hydroxyethylmethacrylate) pHEMA film form was prepared by an UV initiated photopolymerization. The polymerization was carried out in a round glass mould (diameter: 9.0 cm) at 25°C under nitrogen atmosphere for 1 h. The film preparation mixture (5 ml) contained 2 ml HEMA, 10 mg AIBN as polymerization initiator and 3 ml phosphate buffer (0.1 M, pH 7.0). The resulting mixture was equilibrated at 25°C for 30 min in a thermostatic water bath. The mixture was then poured into the mould and exposed to long-wave ultraviolet radiation for 20 min. The pHEMA films were washed several times with distilled water and cut into circular pieces (diameter: 1.0 cm) with a perforator.

2.3. Procion Green H-E4BD attachment onto pHEMA film

Procion Green H-E4BD was covalently attached to the pHEMA film via the nucleophilic reaction between the chloride of its triazine ring and the hydroxyl groups of the HEMA molecules under alkaline conditions. Procion Green H-E4BD (300 mg) was dissolved in distilled water (10 ml), and transferred to same medium (80 ml) in which pHEMA film (15 g) were equilibrated. Sodium hydroxide (1.0 M, 10 ml) was then added to the medium (about pH 13) and heated at 80°C for 4 h in a sealed reactor. After the reaction period, the Procion Green H-E4BD-attached films were then removed, and washed exhaustively, first with distilled water and then with methanol, until all the physically-bound dye was removed. The dye attached film were stored at 4°C until use.

2.4. Immobilization of lysozyme via adsorption

Lysozyme adsorption by the dye attached-film was studied at various pH values, in either acetate (20 ml,

0.1 M, pH 4.0–5.5) or in phosphate buffer (20 ml, 0.1 M, pH 6.0–8.0). The initial lysozyme concentration was 2.0 mg ml^{-1} in each corresponding buffer. The adsorption experiments were conducted for 2 h at 25°C with continuous stirring. At the end of this period, the enzyme film was removed from the lysozyme solution and washed with the same buffer three times. It was stored at 4°C in fresh buffer until use.

In order to determine the adsorption capacities of the dye-attached pHEMA film disks, the concentration of lysozyme in the medium was varied from 0.5 to 2.0 mg ml^{-1}. The adsorption experiments were carried out at pH 7.0.

2.5. Desorption of lysozyme from dye-attached film

In order to determine the reusability of the Procion Green H-E4BD-attached film, each lysozyme adsorption and desorption cycle was repeated three times by using the same dye-attached film. The lysozyme desorption experiments were performed in a buffer solution containing 0.5 M KCl at pH 6.0 by magnetic stirring at 25°C for 2 h. The film disks were removed and washed several times with phosphate buffer (0.1 M, pH 7.0) and then reused in enzyme immobilization. The desorption ratio was calculated by using the following expression:

$$\text{Desorption ratio} = [(\text{Amount of lysozyme released})/(\text{Amount of lysozyme adsorbed})] \times 100$$

2.6. Determination of the immobilization efficiency

The amounts of lysozyme in enzyme preparation and in the wash solution were determined spectrophotometrically. A calibration curve was prepared using lysozyme solutions as standards (0.02–2 mg ml^{-1}). The lysozyme solution was measured at 280 nm by using a UV/Vis spectrophotometer (Model 1601, Shimadzu, Tokyo, Japan).

2.7. Activity assays of free and immobilized lysozyme

The activity of free lysozyme was determined spectrophotometrically at 620 nm, the decrease in the turbidity of o/n culture of *Bacillus subtilis* cell suspended in phosphate buffer (0.1 M, pH 7.0) was followed for 6 min after addition of lysozyme. One unit 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.

Five dye-attached pHEMA film disks (diameter = 1.0 cm) were introduced to the assay mixture to initiate the reaction as above. After 6.0 min, the reaction was terminated by removal of the pHEMA film disks from the reaction mixture. The absorbance of the reaction mixture was determined and the immobilized lysozyme activity was calculated.

These activity assays were carried out over the pH range 4.0–8.0 and temperature range 20–45°C to determine the pH and temperature profiles for the free and immobilized enzyme. The results of pH, temperature and substrate concentration of the medium are presented in a normalized form with the highest value of each set being assigned the value of 100% activity.

2.8. Characterization studies

2.8.1. Elemental analysis

The amount of covalently-bound Procion Green H-E4BD on the film was evaluated by using an elemental analysis instrument (Leco, CHNS-932, USA), by considering the nitrogen and sulphur stoichiometry.

2.8.2. FTIR spectra

FTIR spectra of the pHEMA, Procion Green H-E4BD and dye attached films were obtained by using a FTIR spectrophotometer (FTIR 8000 Series, Shimadzu, Japan). The dry film (about 0.1 g) was mixed with KBr (0.1 g) and pressed into a tablet form. The FTIR spectrum was then recorded.

2.8.3. Scanning electron microscopy

The dried films were coated under reduced pressure and their scanning electron micrographs were obtained using a JEOL (JSM 5600) scanning electron microscope.

3. Results and discussion

3.1. Procion Green H-E4BD attached pHEMA film

The microporous pHEMA film was prepared by UV initiated photopolymerization and the biomimetic dye Procion Green H-E4BD was covalently immobilized under alkaline conditions. The main physical and morphological properties of pHEMA film were as follows: the water content of the dye-immobilized film was 69%; the thickness in the wet state of the film was 0.06 cm. Procion Green H-E4BD is an aromatic polysulphonated dye and it contains six acidic sulfonate groups and five basic primary or secondary amino groups (ratio 6:5). It also contains a pendant hydroxyl group (Fig. 1). The strong binding of the Procion Green H-E4BD to proteins occurs largely at binding sites for substrates, coenzymes and other prostetic groups (Gunzer & Hendrick, 1984).

pHEMA and dye attached films were subjected to elemental analysis. The amount of Procion Green H-E4BD attached to the film was calculated from this data

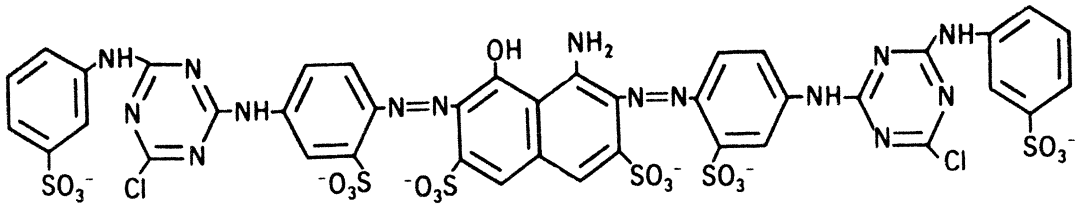

Fig. 1. Chemical structure of Procion Green H-E4BD.

(by considering the stoichiometry), to be 160 μmol Procion Green H-E4BD m^{-2}.

Studies aimed at detecting leakage of Procion Green H-E4BD from the dye-attached pHEMA film revealed no leakage in any of the adsorption and desorption media, and implied that the washing procedure was satisfactory for the removal of the physically adsorbed Procion Green H-E4BD molecules from the pHEMA films.

A representative SEM micrograph is presented in Fig. 2a and b and shows the surface and cross-sectional structure of pHEMA film, respectively. As clearly seen from the figures, the SEM micrographs show that pHEMA film have a porous surface and cross-section. These surface and cross-section properties of the pHEMA films would favour higher immobilization capacity for the enzyme due to increase in the surface area.

To examine the nature of the interaction between the dye (Procion Green H-E4BD) and the pHEMA film, FTIR spectra of the plain Procion Green H-E4BD, pHEMA and dye immobilized pHEMA were obtained. As shown in Fig. 3, The FTIR spectra of the dye-immobilized pHEMA has some absorption bands different from those of pHEMA. These are 3375, 1520 and 650 cm^{-1} and the characteristic N–H stretching, N–H bending (scissoring) and S–O stretching, respectively, were also observed in Procion Green H-E4BD dye (Fig. 3). The dye-immobilized pHEMA spectrum has a sharp shoulder-adsorption band at $\sim$3375 cm^{-1} and this is interpreted as the N–H absorption. The bands at 1075, 1155 and 1280 cm^{-1}, representing symmetric stretching of S=O, asymmetric stretching of S=O and aromatic C–N vibration, respectively, are due to the dye Procion Green H-E4BD bonded to pHEMA.

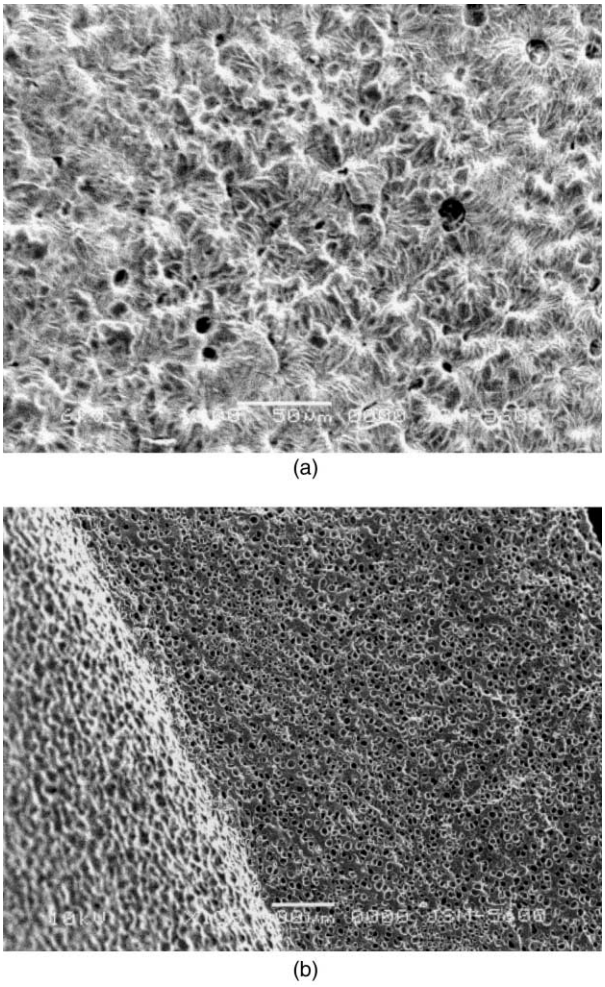

Fig. 2. The SEM micrographs of Procion Green H-E4BD-immobilized pHEMA film: (a) surface, (b) cross-section.

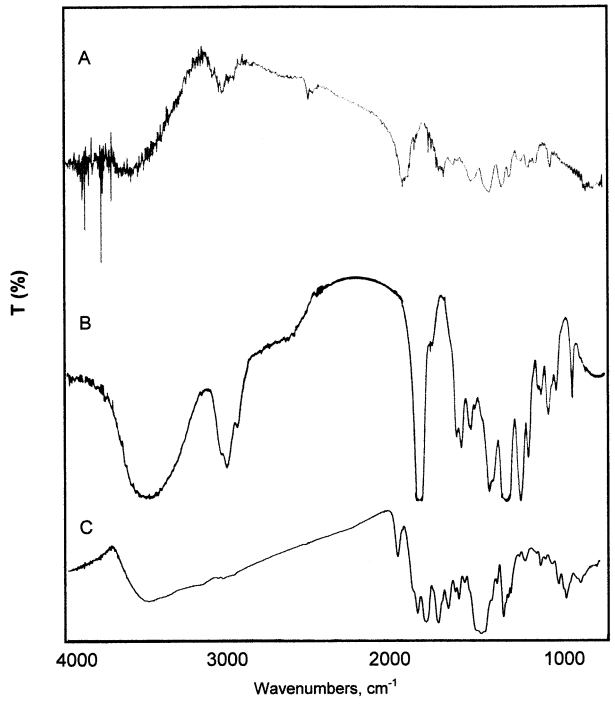

Fig. 3. FTIR spectra: (A) Procion Green H-E4BD-immobilized pHEMA film, (B) pHEMA film, (C) Procion Green H-E4BD.

3.2. Adsorption efficiency of lysozyme

In order to maximize the immobilization of lysozyme onto the dye-attached film, medium pH and initial enzyme concentration were changed within predetermined ranges for individual sets of batch immobilization reactions. The amount of adsorbed lysozyme on the pHEMA film was determined by measuring the initial and final concentrations of protein within the reaction medium. Although insignificant, the amount of enzyme desorbed into the washing solution was also accounted in the calculation of adsorbed enzyme on the film. The optimal pH values for the immobilization of lysozyme onto Procion Green H-E4BD-attached films were scanned in the range 4.0–8.0. The maximum lysozyme adsorption was obtained at pH 7.0 (about 3.92 g m^{-2} film). Significantly lower lysozyme adsorption was observed for the dye-attached pHEMA film in the other tested pH ranges (Fig. 4). The isoelectric point (pl) of lysozyme is 11, so the lysozyme molecules would be cationic at pH values below 11 (Li-Chan, Nakai, Sim, Bragg, & Lo, 1986). The dye Procion Green H-E4BD contains, on the surface, six acidic sulfonate groups and bears six negative charges under neutral and basic conditions. At pH 4.0, no lysozyme was adsorbed on the dye-immobilized film because of the protonation of acidic sulfone groups of the dye at this low pH value. From this point of view, the primary interaction between lysozyme molecules and the dye molecules (Procion Green H-E4BD) at about pH 7.0 could be the result of an ion exchange effect. It should be noted that, secondary interactions between lysozyme and dye attached film may arise from the co-operative effects of different mechanisms, such as hydrophobic interactions, caused by several aromatic structures, on attached dye (Procion Green H-E4BD), and the amino acid side-chain hydrophobic groups of the lysozyme molecules.

The effect of lysozyme concentration on the immobilization efficiency is presented for both plain pHEMA and dye-attached pHEMA film in Fig. 5. An increase in lysozyme concentration in the medium led to an increase in immobilization efficiency but this levelled off at a lysozyme concentration of 1.0 mg ml^{-1}. As shown in Fig. 5, with increasing enzyme concentration in solution, the amount, per unit area, of lysozyme adsorbed by the pHEMA film increases almost linearly at low concentrations, below about 1.0 mg ml^{-1}, then increases less rapidly and approaches saturation. It becomes constant when the enzyme concentration is greater than 2.0 mg ml^{-1}. This could be explained by saturation of interacting groups of the immobilized Procion Green H-E4BD with the adsorbed lysozyme molecules, as a result of which they achieve maximum adsorption capacity. It should be noted that a negligible amount of lysozyme adsorbed non-specifically on the plain pHEMA film (22 μg m^{-2}). Procion Green H-E4BD immobilization significantly increased the lysozyme adsorption capacity (about 180-fold) of the film up to 3.92 g m^{-2}. It is clear that this increase is due to specific interaction between the immobilized Procion Green H-E4BD and lysozyme molecules.

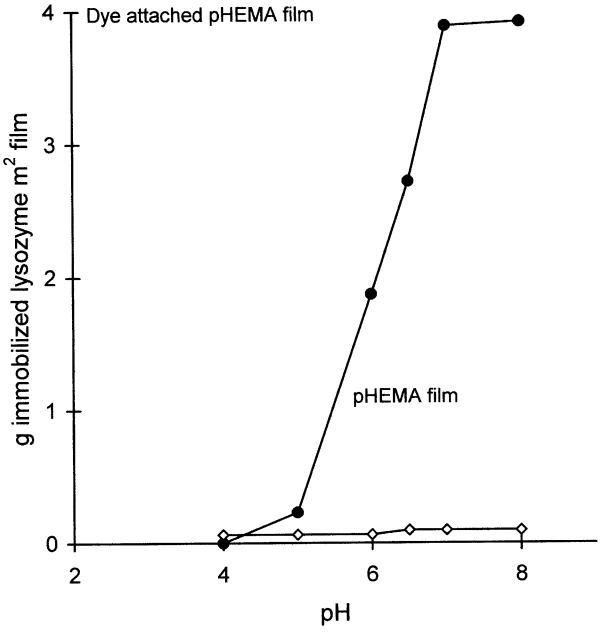

Fig. 4. Effects of pH on lysozyme adsorption on the Procion Green H-E4BD-attached film: the adsorption of lysozyme on the pHEMA film at various pH values was studied under the following conditions; initial concentration of lysozyme: 2 mg ml^{-1}; Procion Green H-E4BD, loading: 160 μmol m^{-2} film; Temperature: 25°C.

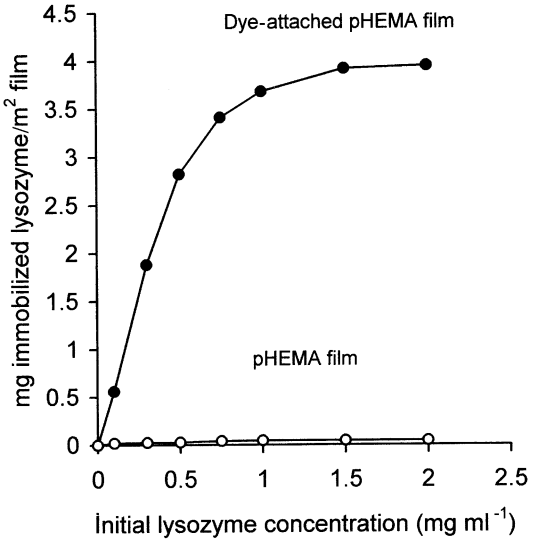

Fig. 5. Effect of lysozyme initial concentration on lysozyme adsorption on the Procion Green H-E4BD-immobilized film: the experimental conditions are; Procion Green H-E4BD loading: 160 μmol m^{-2} film; pH: 7.0; Temperature: 25°C.

3.3. Activity retention of adsorbed lysozyme

The activity displayed by the immobilized enzyme will be related to the amount of enzyme loaded onto the support, and also its adsorption capacity. Fig. 6 shows the effect of enzyme loading on the bacterial cell wall hydrolysis rate and the activity of the adsorbed enzyme on the pHEMA film. The highest retention of enzyme activity (57%) was obtained with the lowest enzyme content (1.85 g m^{-2}). As the enzyme content increased (from 1.85 to 3.92 g m^{-2}), retention of activity decreased, dropping to a minimum of 31%. A high enzyme load on the support generally leads to a low retained activity. This is brought about by over-saturation of the matrix with the enzyme, as a result of which bacterial cell wall binding is restricted toward active sites of the enzyme molecule. Similar results have been reported for lysozyme by other researchers (Chen & Chen, 1997; Crapisi, Lante, Pasini, & Spettolo, 1993). For example, lysozyme was immobilized onto polyamide beads via covalent bonding. The specific activity of the immobilized enzyme decreased with increasing enzyme loading (Datta, Arminger, & Ollis, 1973).

With an increase in the adsorbed lysozyme content on the pHEMA film (1.85–3.92 g m^{-2}), activity also increased (7.3×10^2–16.8×10^2 U m^2 pHEMA film), but this was not at the same rate, because of the loss in retained activity with increased load (Fig. 6).

3.4. Effect of temperature and pH on the catalytic activity

The temperature dependence of the activities of the free and immobilized lysozyme were studied in phosphate buffer (0.1 M, pH 6.0) in the temperature range 15–45°C (Fig. 7). The apparent temperature optimum for free lysozyme was about 35°C, while that for the adsorbed enzyme and the form stabilized by immobilization was about 40°C. This can be explained by the creation of conformational limitations on the enzyme movements as a result of several types of interactions (i.e. hydrophobic and ionic) between the enzyme and dye-attached molecules on the pHEMA film. Similar observations were also reported by other researchers. For example, hen egg white lysozyme was immobilized onto enteric coating polymer and the optimum reaction temperature of the immobilized enzyme was 5°C higher than that of the free enzyme (Cheng & Cheng, 1997).

The change in optimum pH depends on the charge of the enzyme and/or of the support. This change is useful in understanding the structure–function relationship of enzyme and to compare the activity of free and immobilized enzyme as a function of pH. The pH effect on the activity of the free and immobilized lysozyme preparations for bacterial cell wall hydrolysis was studied at various pHs at 35°C. The free enzyme showed a maximum activity at pH 6.0. This value shifted to neutral region upon immobilization (Fig. 8). The optimum pH value for the immobilized lysozyme was about 7.0. It is well known that polyionic matrices cause the partitioning of protons between the bulk phase and the enzyme micro-environment, causing a shift in the optimum pH value. In this work, the shift to neutral region (one pH unit) for the lysozyme adsorbed on the Procion Green H-E4BD-attached pHEMA film can be explained by the presence of negatively charged sulfone groups on the dye molecules at this pH value.

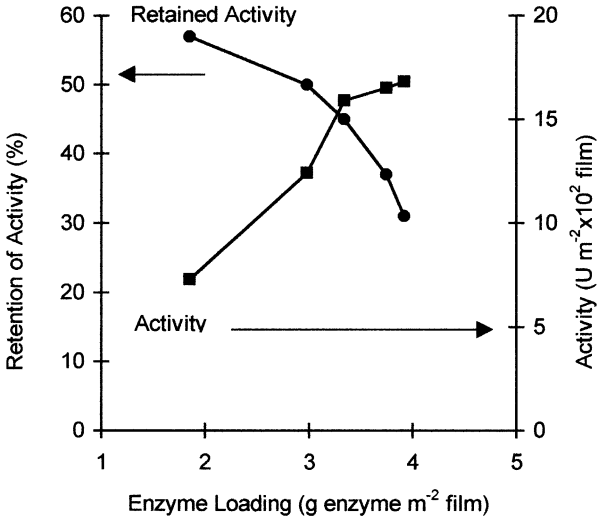

Fig. 6. Effect of enzyme loading on the adsorbed enzyme activity retention and adsorbed enzyme activity.

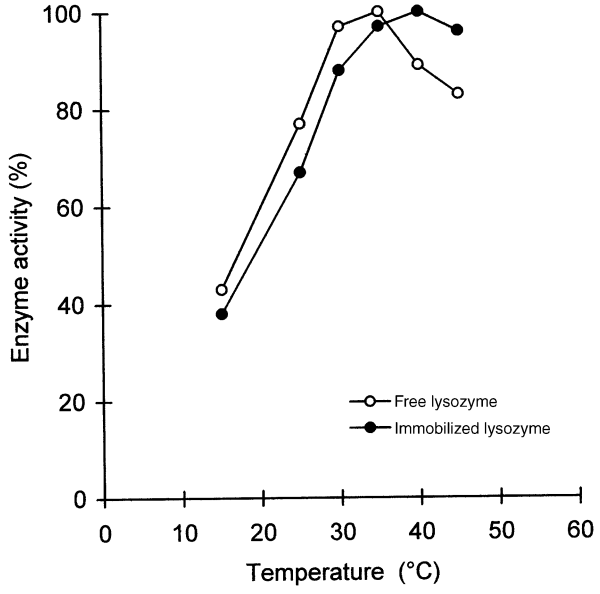

Fig. 7. pH profiles of free and immobilized lysozyme: influence of pH was studied in a batch system under the following conditions: temperature: 25°C; pH range 4.0–5.0 in acetate buffer and 6.0–8.0 in 0.1 M phosphate buffer.

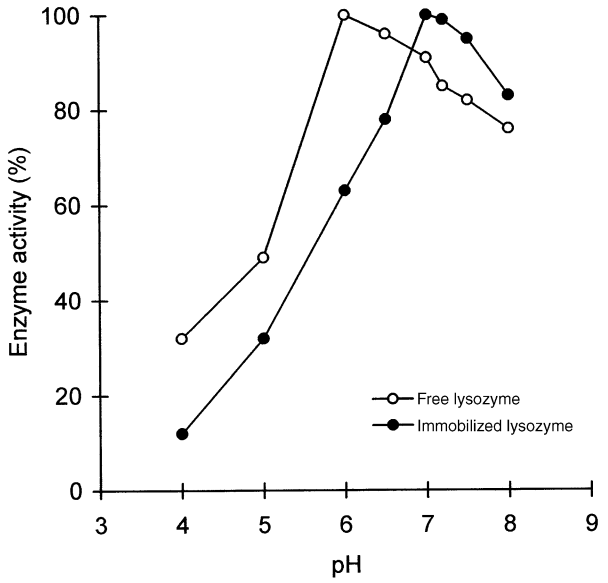

Fig. 8. Effect of temperature on the activity of free and immobilized lysozyme: The effect of temperature (in the 15–45°C range) on the adsorbed lysozyme activity was studied in a batch system by using *Bacillus subtillis* cells suspended in 0.1 M phosphate buffer at pH 6.0.

3.5. Repeated loading of pHEMA films with the enzyme

Desorption of adsorbed lysozyme from dye-attached pHEMA film was carried out in a batch system. The enzyme film preparation was placed within the desorption medium containing 0.5 M KCl at pH 6.0 at room temperature for 2 h as described above and was then repeatedly used in the adsorption of lysozyme. The lysozyme adsorption capacity of the dye attached pHEMA film was not changed during three successive adsorption–desorption cycles. The adsorbed enzyme activity on the pHEMA film did not significantly change during these adsorption–desorption cycles (loss of its original capacity about 5%, end of the third use). These results showed that novel Procion Green H-E4BD-attached pHEMA films can be repeatedly used in enzyme immobilization without detectable losses in their initial adsorption capacity and enzymatic activity.

More than 95% (up to 97%) of the adsorbed lysozyme was removed when KCl was used as a desorption agent. The desorption result showed that KCl is a suitable desorption agent for the Procion Green H-E4BD-attached pHEMA film, and allows repeated use of the dye-attached pHEMA film developed in this study.

4. Conclusion

The pHEMA film was prepared by UV initiated photopolymerization of HEMA monomer and, Procion Green H-E4BD was then covalently attached onto the pHEMA film with a phase concentration of 160 μmol dye per m^{-2} film. The results presented in this communication showed that lysozyme was adsorbed, with a high affinity interaction, on the dye attached pHEMA film. The maximum enzyme loading was 3.92 g m^{-2} film. The pHEMA film has been shown in our earlier studies to be a useful support for the immobilization of enzymes. As previously mentioned, the pHEMA film, modified by the attachment of Procion Green H-E4BD, revealed good adsorption properties and the dye attached pHEMA film can be repeatedly used for lysozyme immobilization. Its non-toxic nature and bacteriolytic property makes it a suitable candidate for hydrolysis of microbial cells in the food industry.

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