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Biospecific Immobilization of Lactoperoxidase on Con A-Sepharose 4B

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The aim of this work was to evaluate the possibilities of immobilization of lactoperoxidase (LPO) on concanavalin A-Sepharose 4B support. Significant biospecific interaction of this heme-containing glycoenzyme with Con A was established by using α-D-mannopyranoside and α-D-glucopyranoside, which are involved in the linking of the carbohydrate moieties of the enzyme with lectin. The preparation obtained indicated improved kinetic parameters (K_m and $V_{\max}$) compared with the soluble form. No significant differences were observed between the optimal pH and temperature of the anchored and free enzymes. The thermal stability of the biospecifically immobilized preparation was substantially higher than that of the unbound enzyme. In addition, seven cycles of enzymatic conversion and washing of the column showed the remarkable operational stability of immobilized LPO.

INTRODUCTION

Meeting the demand for "white" biotechnology (application of nature's toolset to industrial production), enzymes have enormous potential as useful tools for replacing conventional inorganic catalysts in production processes. Their use, impaired, as yet, by not quite satisfactory reliability, is predicted to become widely accepted, once their storage and operational stabilities have been improved. A tailoring tool for this achievement is enzyme immobilization with two main benefits, enhancement of storage/operational stability and usability. Among various immobilization methods, biospecific adsorption has emerged as a gentle strategy for enzyme immobilization [1,2]. In one of the bioaffinity-based immobilization procedures, the affinity of carbohydrate moieties of glycoenzymes to lectins plays a crucial role in the immobilization of enzymes [3]. The obtained preparations exhibited high catalytic activity, improved stability against denaturation and reuse of the support matrix. Moreover, bioaffinity immobilization finds potential applications in the construction of sensitive enzyme-based analytical devices and in separation techniques, as well as other applications [4].

Lactoperoxidase (LPO) is a well studied enzyme, due to its antimicrobial and antiviral action, degradation of various carcinogens and protection of animal cells against peroxidative effects [5], with extensive industrial applications in the dairy industry [6], food production [7], in the preservation of pharmaceuticals [8] and iodine detection [9]. Bovine lactoperoxidase is a heme-containing glycoprotein of 612 amino acids giving a molecular mass of, approximately, 78 kDa [10]. Calcium ions are strongly bound to LPO, stabilizing its molecular conformation, and calcium ion activity appears to be of vital importance for its structural integrity [11]. The LPO molecule has a carbohydrate content of about 10% and possesses five potential N-glycosylation sites [12], which make it a good candidate for a Con A-based affinity immobilization protocol. Con A is a multiple-site sugar-binding protein from the lectin class that recognizes different sugars even after they are randomly immobilized [3,13]. Little is reported in the literature regarding LPO immobilization with slight relation to its catalytic activity [14]. This article describes a study, in which milk lactoperoxidase was affinity-bound to Con A-Sepharose 4B and its behavior compared with the soluble enzyme. The immobilized enzyme has also been evaluated in terms of kinetic parameters, stability and reusability.

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MATERIALS AND METHODS

Reagents

Lactoperoxidase, 2,2-Azinobis [3-Ethyl Benzothiazoline-6-suiphonic acid] diammonium salts (ABTS), phospho-cellulose and Con A-Sepharose 4B were purchased from Sigma Chemical Co., α-D-mannopyranoside and α-D-glucopyranoside were obtained from Merck. All chemicals used in this work were of analytical grade.

Preparation of Skim Milk

Skim milk was prepared by centrifugation at (2000× g) for 20 min at 25°C from pooled milk of Holstein cows. Skim milk was acidified to pH 4.6 at 20°C by addition of 2 N HCl, and casein was removed by centrifugation at (2000× g) for 20 min at room temperature. Acid whey was neutralized to pH 6.8 by the addition of 2 N NaOH, and a volume of 1100 ml acid whey was obtained from 2800 ml whole milk for batch wise chromatography.

Batchwise Chromatography for Preparation of LPO

Phosphocellulose, with a capacity of 0.96 mg/g, was prepared for cation exchange chromatography by washing in a Buchner funnel with 0.1 N NaOH, followed by water, then, suspended in water and decantated to remove fines. It was finally washed in a funnel with 0.1 N HCl, followed by water. After use, it was regenerated in the same manner. To perform chromatography, the resin was equilibrated with 50 mM phosphate buffer (pH 7.0) and, then acid whey was suspended in the resin. The mixture was stirred for 3 h and lactoperoxidase was adsorbed to the cation exchanger. Then, the mixture was allowed to precipitate from the adsorbed exchanger and the supernatant was removed through decantation. The cellulose phosphate was recovered in a Buchner funnel under a moderate vacuum, taking care not to dry the cellulose phosphate pad. The resin pad was washed with 300 mL of 0.2 M NaCl in 50 mM phosphate buffer at pH 7.0, under a moderate vacuum. LPO was eluted with 0.4 M NaCl in a phosphate buffer (50 mM, pH 7.0). To decrease the volume of enzyme solution, an amount of 191 g ammonium sulphate was added to 250 ml of enzyme solution and stood for 3 h at room temperature. The remaining solution was centrifuged (1200× g, 15 min), the collected pellet was dissolved in the phosphate buffer (50 mM, pH 7.0) and then, dialyzed overnight against 100 volume of the same buffer. Fractions were recovered and its purity measured at A_{412}/A_{280}, which was approximately 0.92 [15]. A product of Sigma chemical Co. (St. Louis, MO) was also examined.

Enzyme Assay

Lactoperoxidase activity (EC 1.11.1.7) was determined by using 2,2- azinobis diammonium salts (ABTS), based on the assay given by Putter et al. [16]. Briefly, whey skim milk or a solution of isolated LPO in permeates or buffer (usually 50 mg LPO/L) was first diluted 20-fold with 0.1 mL phosphate buffer (0.12 M, pH 6.4). 750 μl were added to 50 ml of diluted enzyme solution, 100 μl , 10 mM ABTS (in water) and 100 μl, 1 mM H_2O_2 (also in water). The absorbance at 412 nm was measured as a function of time at 20°C, using a Perkin Elmer spectrophotometer. The rate of change of absorbance at 412 nm was constant for at least 2 min.

Determination of Kinetic Parameters

K_m and $V_{\max}$ values of the native and immobilized enzyme (1 mL containing 3.8 U each) were determined by measurement of the enzyme activity with various concentrations of ABTS. To calculate these parameters, one unit activity is defined as the amount of enzyme catalyzing the oxidation of 1 μmol of ABTS $\min^{-1}$ at 25°C ($\varepsilon^{1\%}_{1\text{cm}} = 32400\ \text{M}^{-1}\ \text{cm}^{-1}$) [17]. Then, K_m and $V_{\max}$ values were obtained from a Lineweaver-Burk graph.

Immobilization Procedure

The immobilization process was achieved by the following approach: An enzyme solution of known concentration (30 μg of 2 mg/ml stock solution in 50 mM phosphate buffer pH 7.8) was allowed to contact with 0.2 g of Con A-Sepharose 4B in a tube at 4°C for 1 h, which was found sufficient to reach binding equilibrium. The sample was centrifuged and the pellet was rinsed three times with phosphate buffer (50 mM, pH 7.8) to remove unbound protein. Protein concentrations were determined using the BioRad reagent, according to Lowry [18] and BSA as a standard.

RESULTS AND DISCUSSION

Evaluation of LPO Adsorption on Con A-Sepharose

Biospecific interaction of LPO with Con A-Sepharose 4B was tested by using α-D-mannopyranoside and α-D-glucopyranoside, which has been shown to inhibit the linking of carbohydrate moieties of glycoenzymes to lectins [19]. Figure 1 indicates the maximum catalytic activity of the immobilized preparation in the absence of these derivative sugars. When increasing concentrations of sugars are deposited to the Con A matrix, a marked decrease in the lactoperoxidase activity of the preparations was observed, showing that sugars may inhibit the enzyme from anchoring to the support.

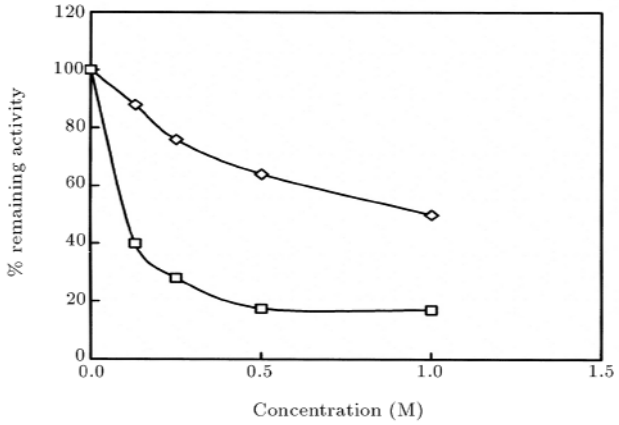

Figure 1. Effect of α-D-mannopyranosid (□) and α-D-glucopyranoside (◊) on inhibition of LPO adsorption on Con A-Sepharose 4B.

α-D-mannopyranoside was more effective than α-D-glucopyranoside in a concentration dependent manner, indicating that the enzyme linkage to the lectin is via its pendant mannosyl moieties. The glycosyl chains may act as long spacers and contribute to the high access of LPO to glycospecific sites of Con A. This trend would provide a unique opportunity for favorable orientation of affinity-bound enzyme molecules and is similar to what has been observed by other researchers [20].

Effect of pH and Temperature on Enzyme Activity

Activity profiles of soluble and affinity bound LPO were investigated at different pH and temperatures. Figure 2 shows that the pH optima of free enzyme remained unaltered at pH 6.0 on immobilization. Although the bound enzyme retained a greater amount of initial activity in all tested pH conditions, it is obvious that the catalytic activity of both forms decreased markedly upon acidic or basic environments. Soluble LPO is less stable under harsh pH conditions, possibly due to the release of calcium ions from the molecule [11]. The higher retention of activity by the affinity bound preparation may be related to an overall improvement in resistance to the unfolding and/or the rigidity of structural conformation, thereby, hindering calcium mobilization from the enzyme molecules. The results are in agreement with those reported by Ozdemir [21].

The optimum temperature of the soluble and immobilized enzyme preparations was studied. The Native LPO and affinity bound preparation were optimally active at 45°C (Figure 3), while the anchored enzyme retained a greater fraction of maximum activity at higher temperatures. This optimum temperature and pH for LPO activity was in good agreement with that observed by other workers [14].

Thermal Stability and Reusability

Figures 4 and 5 show the thermal stability of the enzyme activity at different temperatures. On incubation at 4 and 25°C, the affinity bound preparation retained 60% and 77% of initial peroxidase activity after incubation for 14 days, while the half-life of the soluble LPO was 8 and one day at these temperatures, respectively (Figure 4). At higher temperature, the affinity-bound enzyme retained 20% of its original activity by heating at 75°C for 60 min (Figure 5), while the free enzyme inactivates entirely in less than 5 min at the same temperature. At 65°C, the immobilized enzyme retained more than 60% of its initial activity for 200 min, while the catalytic activity of the free enzyme dropped to 20% after the same time of exposure (Figure 5 inset). These results indicate that the thermal stability of bound LPO increases considerably, as a result of immobilization on Con A-Sepharose 4B. One explanation for the observed stability of the affinity bound

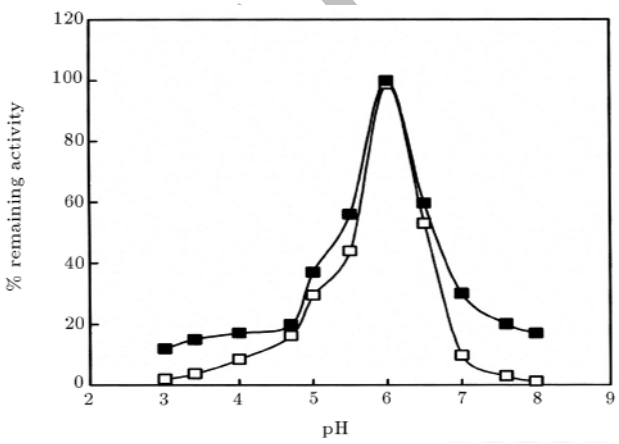

Figure 2. Effect of pH on enzyme activity of free (□) and immobilized (■) lactoperoxidase.

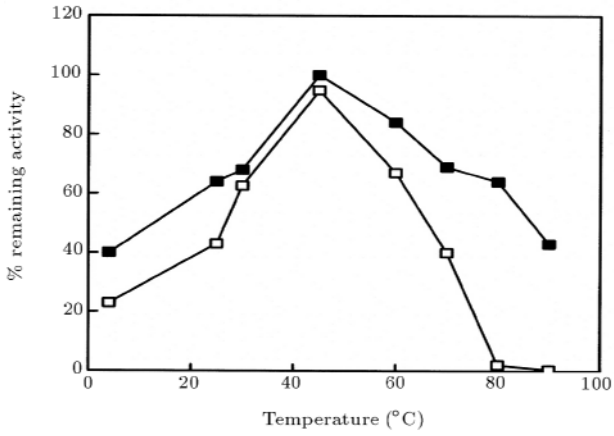

Figure 3. Temperature optima for soluble (□) and immobilized (■) lactoperoxidase.

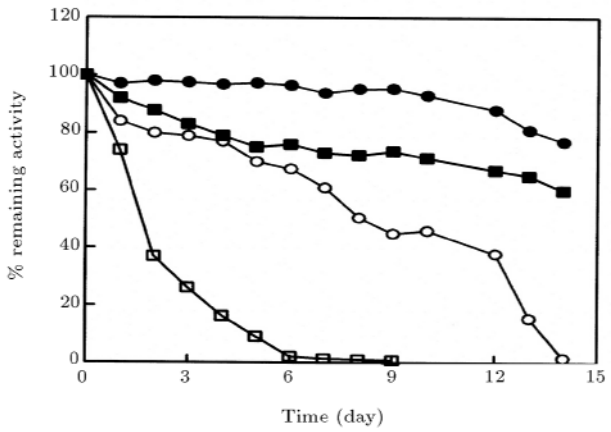

Figure 4. Thermal stability of free (○) and immobilized (●) LPO at 4°C, compared with its stability at 25°C for free (□) and immobilized (■) enzyme.

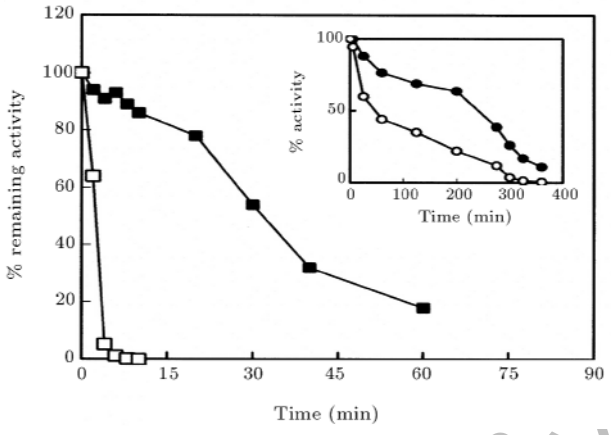

Figure 5. Thermal stability of free (□) and immobilized (■) LPO at 75°C, compared with its stability at 65°C (insert figure) for free (○) and immobilized (●) enzyme.

preparation may be reduced conformational flexibility as a consequence of linkage to Con A through the glycosyl chains, providing facilities for the enzyme to refold after thermal denaturation. This enhancement in stability has been shown for LPO [14,22] and several other glycoenzymes [23] attained greater resistance to various forms of denaturation when immobilized via their glycosyl residues.

Table 1 gives the kinetic parameters determined from Michaelis-Menten kinetics. The K_m and $V_{\max}$ of immobilized LPO are higher than the unbound enzyme. A simultaneous increase of K_m and $V_{\max}$ was also observed for the immobilized malic enzyme [24]. This effect has been attributed to the diffusion limit of the substrate to the active site of the immobilized enzyme [25]. A similar interpretation may be made for LOP. The immobilized enzyme, in fact, shows higher catalytic efficiency (K_{cat}/K_m) when compared to the free enzyme, which may be caused by the higher thermal stability of the immobilized form. The immobilized enzyme could be reused seven times without a remarkable loss of activity (Figure 6).

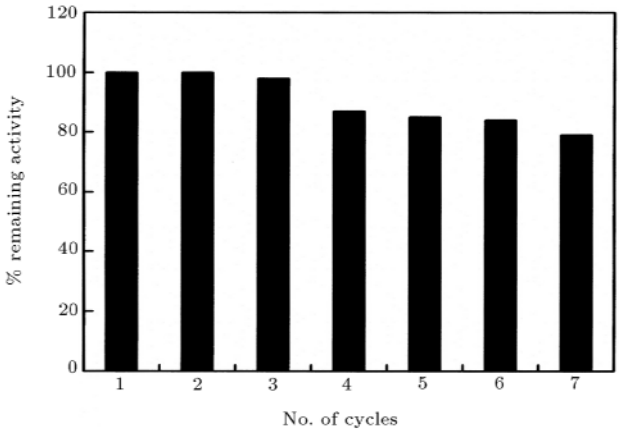

Figure 6. Reusability of immobilized lactoperoxidase on Con A-Sepharose 4B.

Table 1. Determination of kinetic parameters for free and immobilized LPO.

Kinetic Parameters	Free Enzyme	Immobilized Enzyme
K_m	0.57	0.64
K_{cat}	4.2	5.4
K_{cat}/K_m	7.36	8.34

CONCLUSION

The results of this work suggest that the strategy of biospecific interaction between glycosyl chains of LPO and Con A allows a sufficient strong bond with the matrix to be established. The advantages of this biospecific glycolenzyme immobilization are in the designing of efficient and stable biocatalysts, the potential reuse of lectin matrix and the possibility of using the technique of biospecific adsorption for enzyme modification in the design of new biosensors.

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