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Immobilization of Pancreatic Lipase on Chitin and Chitosan

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Abstract: In this study, porcine pancreatic lipase (EC 3.1.1.3) was immobilized on chitin and chitosan by adsorption and subsequent crosslinking with glutaraldehyde, which was added before (conjugation) or after (crosslinking) washing unbound proteins. Conjugation proved to be the better method for both supports. The properties of free and immobilized enzymes were also investigated and compared. The results showed that the pH optimum was shifted from 8.5 to 9.0 for both the immobilized enzymes. Also, the optimum temperature was shifted from 30 to 40°C for chitin-enzyme and to 45°C for chitosan-enzyme conjugates. The immobilization efficiency is low, but the immobilized enzymes have good reusability and stability (storage and operational). Besides these properties, the immobilized lipases were also suitable for catalyzing esterification reactions of fatty acids and fatty alcohols, both with a medium chain length. According to our results, esterification activities of immobilized lipases were two- and four-fold higher for chitosan- and chitin-enzyme, than for the free enzyme, respectively. The immobilization procedure shows a great potential for

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commercial applications of the immobilized lipase, a relatively low cost commercial enzyme.

Keywords: Enzyme immobilization, Enzymatic esterification, Chitin, Chitosan, Pancreatic lipase

INTRODUCTION

Immobilization of enzymes on natural and synthetic supports is advantageous because; it extend the stability of the enzyme by protecting the active material from deactivation; it enables repeated use; it provides significiant reduction in the operating costs; it facilitates easy separation and speeds up recovery of the enzyme. The application of enzymes, in their native forms, in biomedical, biotechnological, biochemical, and food industrial fields is not always suitable and optimal. There are a large number of support materials and methods for the immobilization of enzymes. So, it is important that the choice of suitable support materials and immobilization method over the free enzyme should be well justified.[1–3]

Lipases (triacylglycerol ester hydrolyses, EC 3.1.1.3) are ubiquitous enzymes that catalyze the hydrolysis of fats and oils, and synthesis of fatty acid esters. To fully exploit the technical and economic advantages of lipases, it is recommended to use them in an immobilized state to reduce the cost and the stability of the soluble form. In recent years, the lipases have become of great interest to the pharmaceutical industries and for hydrolysis of oils and fats, and for synthesis of fatty acid esters as cosmetic ingredients or surfactants. Owing to economical considerations, their application to an industrial scale process requires their immobilization and, thus, reusability. Many immobilization techniques and support materials for lipases have been tried and reviewed recently.[4–9] After immobilization, changes were observed in the activity, optimum pH and temperature, affinity to the substrates, and stability of the enzyme. The extent of these changes depended upon the source of enzyme, the type of support material, and the method of immobilization.[10–13]

Chitin, a homopolymer of *N*-acetylgalactosamine and a major component of the shells of crustacea such as crab, shrimp, and crawfish, is a by product of the fishing industry. It has been reported, that every one amino group out of six in the chitin molecule is in the deacylated form.[14] After cellulose, chitin is the second most abundant natural biopolymer found in nature. Chitosan is a natural carbohydrate biopolymer derived from deacetylation of chitin. Over the last several decades, chitinous polymers, especially chitosan, have received increased attention as promising renewable polymeric materials for their extensive applications in the pharmaceutical and biomedical industries, for enzyme immobilization and purification, in chemical plants for wastewater

treatment, and in the food industries for food formulations as binding, gelling, thickening, and stabilizing agents.[15–18]

The search for an inexpensive support has motivated our group to undertake this work dealing with the use of chitin and chitosan as matrix for immobilizing lipase. In addition to its low cost, these biopolymers have several advantages for use as a support, including their lack of toxicity and chemical reactivity, and easy fixation of enzymes.[19–22] The presence of the free amino groups in chitin and chitosan provides binding sites for proteins, which permits their use as a matrix for the immobilization of enzymes.

The aim of the present work was to produce an immobilized form of lipase with advantageous catalytic properties, stability, and esterificaton activity. Adsorption of enzymes is simple and cheap. Crosslinking is also effective and durable, so pancreatic lipase was adsorbed onto chitin and chitosan under optimum conditions and then crosslinked with glutaraldehyde, which is added either in the absence (crosslinking) or in the presence (conjugation) of the unbound proteins. This latter procedure gave the best results. The influence of several parameters on the activities of the enzymes has been also investigated.

EXPERIMENTAL

Materials

Porcine pancreatic lipase, chitin, chitosan, glutaraldehyde and fatty acids (caprylic, lauric, palmitic) were obtained from Sigma Chem. Co. (St. Louis, MO). Alcohols (n-butanol, octanol) and cyclohexane were purchased from E. Merck (Darmstadt, Germany). All other chemicals were analytical grade and were obtained commercially.

Assay of Hydrolytic Activity

The hydrolytic activities of free and immobilized lipases were determined at 37°C by pH-stat titration using an automatic titrator (718 Stat Titrino. Metrohm Ltd., Switzerland). The released free fatty acids from tributyrin were titrated with 0.01 M NaOH. One unit of lipase activity was defined as 1 μmol of free fatty acid released per minute under the assay conditions (pH 7.0, 37°C).[12]

Protein Determination

Protein concentrations were determined by the dye binding method of Bradford, using bovine serum albumin as a standard.[23] The amount of bound protein was determined indirectly from the difference between the amount of protein introduced into the coupling reaction mixture and the amount of protein in the filtrates and also in washings after immobilization.

Immobilization of Lipase

The immobilization procedure for both supports (chitin and chitosan) is similar. Five grams of carrier equilibrated in 50 mL of phosphate buffer (50 mM, pH 7.0) for 15 min at room temperature. 25 mL of solution containing 40 mg/mL of lipase (107.4 Unit/mg) was added to the support suspension and then agitated gently (150 rpm) at 4°C, overnight. After the adsorption of lipase onto the support, crosslinking with glutaraldehyde (0.5%) was carried out by two different procedures. In the first procedure, the enzyme was adsorbed under the optimum adsorption conditions onto supports and then enzyme molecules were crosslinked with glutaraldehyde (croslinking). In the second procedure, after the adsorption of the enzyme, glutaraldehyde (0.5%) was added directly to the suspension without washing the unbounded proteins (conjugation) and the reaction was carried out for 1 hour at room temperature. Then, immobilized enzyme preparates were recovered by filtration and washed several times with 1 M NaCl in order to remove the unbound proteins. The immobilized enzymes were then stored in wet form at 4°C until use. The filtrates and washings were collected and used for protein determination.

Determination of Esterification Activity

Ester synthesis took place in 100 mL closed flasks containing 20 mL of cyclohexane containing 0.12 M of alcohol (n-butanol or n-octanol) and 0.12 M of fatty acids (caprylic, lauric or palmytic). Substrate solutions were preconditioned by incubation for 30 min, with agitation, at 40°C, prior to the addition of the immobilized lipase. 400–800 mg of immobilized lipase or free enzyme, which contain the same amount of protein, was added to the reaction mixture and incubated at 40°C for 6 h with continuous stirring (150 rpm). In the course of the reaction, aliquots (0.5 mL) were taken and centrifuged. Analysis of the alcohols in supernatants were carried out on a Thermo Finnigan TraceGC Ultra chromatograph (equipped with a flame ionization dedector (FID) on a split/splitless injector) by using a fused-silica capillary column Zebron ZB1 (Phenomenex-USA, 30 m length, 0.32 mm ID, 0.25 mm film thickness). Amyl alcohol was used as the internal standard.

Catalytic Properties of Lipase Preparations

The effect of pH on activity of free and immobilized lipase was assayed in the phosphate buffer (50 mM), ranging from pH 7.5 to 10.5, by using the standard activity assay procedure mentioned above.

The effect of temperature on both lipase activities was determined at temperatures from 25 to 55°C under assay conditions.

Free and immobilized enzymes were stored at 4°C and the storage stabilities of the enzymes were determined by measurement of the activities of samples taken at regular time intervals and compared.

The stability of free and immobilized enzymes on repeated use was also examined by measuring the activity towards the hydrolysis of tributyrin (pH 7.0 37°C). After each activity determination, the immobilized enzyme was washed with distilled water and reintroduced into a fresh medium, this procedure being repeated for up to 10 cycles.

Operational Stability Test

The reaction of tributyrin hydrolysis, catalyzed by the immobilized lipase, was carried out for 30 h at 30°C in a continuous stirred tank reactor with measurement of the activity of samples taken at regular time intervals. The relationship between operating time and the decrease in the enzyme activity was determined and the half life ($t_{1/2}$) of the biocatalyst was also estimated.

RESULTS AND DISCUSSION

Lipase Immobilization on Chitin and Chitosan

Enzymes are often immobilized onto solid supports to increase their thermal, pH, and operational stability, and recoverability. Various methods available for enzyme immobilization can be divided into two general classes: chemical methods and physical methods.[24–27] Among these methods, adsorption, being a simple and economical method, was found to be most suitable for large scale lipase immobilization.[28]

In the present work, porcine pancreatic lipase was immobilized by adsorption onto chitin and chitosan and then crosslinked with glutaraldehyde. One of the most important aims of the enzyme immobilization technology is to enhance the conformational stability of the enzyme. The extent of stabilization depends on the enzyme's structure, the immobilization methods, and the type of support. The natural polymers, chitin and chitosan, were used for lipase immobilization, as support materials. Optimum lipase adsorption conditions for both supports (pH, enzyme concentration, and adsorption time) were determined as: pH 7.0, 11.2 mg lipase/g support (107.4 U/mg), 24 h for chitin; and pH 7.0, 11.2 mg lipase/g support (107.4 U/mg), 2 h for chitosan. Under these conditions, lipase was immobilized with an adsorption yield of 50% for chitin and 65% for chitosan.

After the adsorption onto the support, the enzyme was crosslinked with glutaraldehyde, which is a bifunctional agent. This agent is capable of reacting with the amino groups present, both in the support and in the enzyme molecules, thus forming inter- and intra-molecular bonds, so that

the interactions produced by adsorption are strengthened and the stability of the enzyme over time is increased. According to the results, the conjugation procedure was determined to be more suitable and efficient than the crosslinking procedure. The immobilization results for both conjugates are summarized in Table 1.

Properties of the Free and Immobilized Lipases

A comparative study between free and immobilized enzymes is provided in terms of pH and temperature. The activities of free and immobilized lipases were determined at different pH values. The pH-activity relationships were determined from the graph of pH plotted against the percentage of relative activity (Fig. 1). The optimum pH values were found to be 8.5 and 9.0 for free and for both immobilized enzymes, respectively. This range in pH optima upon immobilization has been reported by several researchers.[25] This pH shift is possibly due to the method of immobilization, as well as secondary interactions (e.g., ionic and polar interactions, hydrogen bonding) between the enzyme and the polymeric matrix.[21,29] The pH profiles of the immobilized enzymes were also much broader than that of the free enzyme; this is probably due to the product fatty acid forming layers and causing external diffusion limitations on the enzyme's surface.

The temperature dependence of the lipolytic activity was investigated in the temperature range 25 to 55°C. The relative activity, expressed as percentage of the maximum activity, is presented in (Fig. 2) as a function of temperature. The optimum temperatures were found to be 30, 40, and 45°C for free, chitin- and chitosan-immobilized lipase, respectively. A significant decrease in the activity was observed above these temperatures. The

Table 1. Immobilization of pancreatic lipase on chitin and chitosan

Free enzyme		
Activity (Unit)	6000.5	
Protein (mg)	55.9	
Specific activity (Unit/mg)	107.42	
Carrier	Chitin	Chitosan
Immobilized enzyme		
Bound protein (mg)	12.9	50.7
Bound protein (%)	23	90.6
Activity (Unit)	188	818
Spesific activity (Unit/mg)	14.6	16.1
Activity yield (%)	13.6	15.1

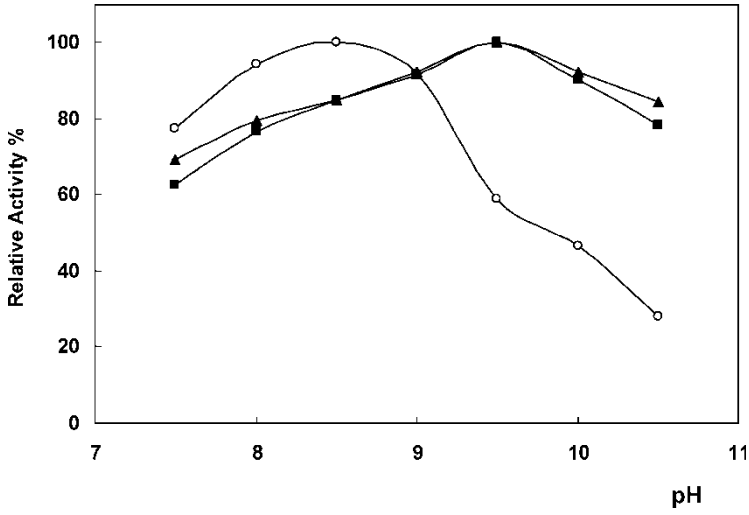

Figure 1. The effect of pH on the enzymatic activity. (○) native lipase, (■) lipase immobilized on chitin, (▲) lipase immobilized on chitosan.

reaction rate increased between 25 to 30°C for free enzyme and then rapidly decreased at higher temperatures. For immobilized enzymes, the temperature profile was very different in the first part of the curve. However, the decrease of reaction rate at temperatures above the optimum was much slower than that of the free lipase. These results suggested that the immobilization enhanced

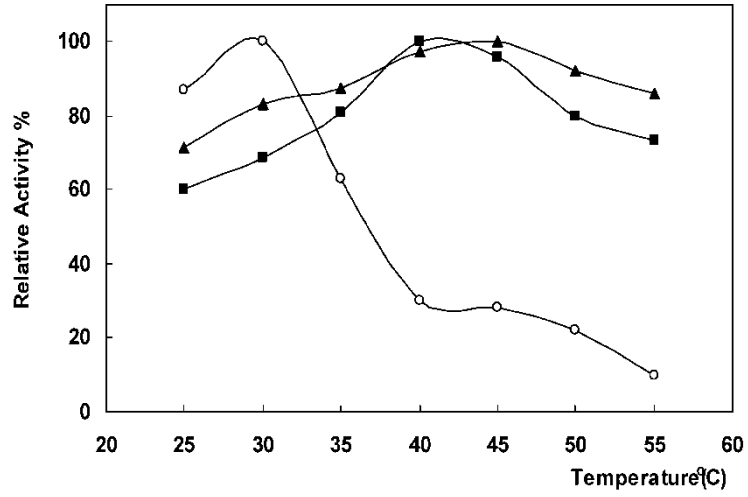

Figure 2. The effect of temperature on the activity. (○) native lipase, (■) lipase immobilized on chitin, (▲) lipase immobilized on chitosan.

the lipase stability. This could be explained by either creation of conformational limitations on the enzyme movements as a result of multipoint attachment and hydrophobic interaction between the enzyme and support or a low restriction in the diffusion of the substrate and products at a higher reaction temperature. Similar changes in the optimum temperature have also been reported.[29,30]

The stability of immobilized lipase on repeated use was examined by measuring the activity for the hydrolysis of tributyrin (Fig. 3). As is seen from (Fig. 3), the immobilized lipase retained 88% and 67% of its initial activity for chitin and chitosan-enzyme, respectively, after 10 times reuse. Such reusability is advantageous for the continuous use of this enzyme in biotechnological applications.[11] The free and immobilized enzymes were stored, in wet form, at 4°C. The chitin-enzyme retained 86.5% and chitosan-enzyme retained 67% of their original activities for about 45 days, whereas the free enzyme retained only 20% of its activity over the same period of time. Various reports confirm that the storage stability of the immobilized enzyme depends on the immobilization method applied and the storage conditions.[3,31]

It is important, for economical use of an enzyme, as a means for the mass production of the desired product, that the enzyme reaction is continuous. One of the problems in continuous enzyme reactions is the operational stability of the enzyme immobilized on the support. When comparing the catalysts intended for preparative or industrial use, characterization of their operational stabilities is of extreme importance. An increased stability could make the immobilized enzyme more advantageous than its free counterparts. The operational stability of immobilized lipases was monitored for 30 h for chitin- and chitosan-enzyme. The chitin-enzyme conjugate lost about nearly 66% of its activity, whereas the chitosan-enzyme conjugate lost about 76% of its

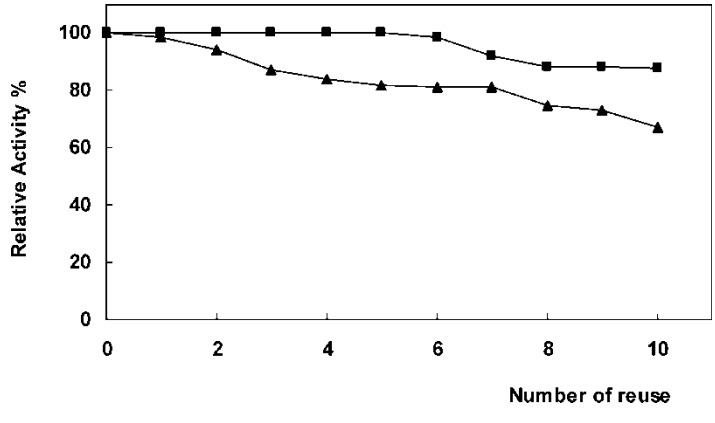

Figure 3. Operation stabilities of immobilized lipase. (■) lipase immobilized on chitin, (▲) lipase immobilized on chitosan.

activity, within 30 h. The operational half life ($t_{1/2}$) of the enzyme was calculated by using the formula:

$$t_{1/2} = 0.693/k_D \quad k_D = 2.303/t \times \log(A_o/A)$$

where t is the operation time, k_D is decay constant, A_o and A are the enzymatic activities at the beginning and at t time. The half-life of chitin bound porcine pancreatic lipase was determined as 14 h. The half-life of immobilized enzymes was determined as 14 h and 11 h for chitin- and chitosan-enzyme, respectively.

Lipases are known to catalyze esterification reactions through an acyl-intermediate formed between the fatty acid substrate and the enzyme. Free enzyme can either bind the fatty acid to produce this intermediate or the ester product. In an excess of alcohol, the acyl intermediate will be consumed and the enzyme may then start to bind the product and catalyze the hydrolysis. On the other hand, in an excess of fatty acid, most of the enzyme is found in the acylated form, preventing it from binding the product. For immobilized lipase preparations, a more complex mechanism is expected to occur, since esterification efficiency is also highly dependent upon the hydration state of the enzyme preparation, which can be greatly modified by the nature of the substrate and the support, particularly for chitin, recognized as a hydrophilic support.[31]

In this work, the effect of carboxylic acid chain length [caprylic acid (C8), lauric acid (C12), and palmitic acid (C16)] and fatty alcohols (n-butanol and n-octanol) on the substrate polarity and activity of free, and on chitin and chitosan immobilized lipases were investigated and compared to each other. The results are shown in Table 2.

Immobilized lipases favoured the esterification of fatty acids of medium chain length (C12). This is clear when comparing the activity results of the three saturated straight chain fatty acids. The mechanism of the lipase

Table 2. The esterification activity of free and immobilized lipase

	Activity(μmol/minute) $\times 10^{-3}$		
Acids	Free	Lipase immobilized on chitin	Lipase immobilized on chitosan
Caprilic acid(C8)		NA[a]	NA[a]
Lauric acid(C12)	1.23	5.23	2.53
Palmitic acid(C16)		NA[a]	NA[a]

Substrates: acid(0.12 mol/L)+ n-octanol(0.12 mol/L); lipase: 1–2 mg/mL for free enzyme, solvent: cyclohexane, reaction temperature: 40°C, reaction time: 6 h.

[a]NA: no any esterification activity was measured under test conditions.

reaction is that the fatty acid first combines with lipase to form an acyl-enzyme complex, which is then reacted with the alcohol to give the ester and free enzyme.[32,33] The fatty acid with a shorter chain length is of higher polarity, which is unfavourable to its combination with the hydrophobic active site of lipase. On the other hand, the fatty acid with a longer chain is of a larger size, which might create a steric hindrance towards the formation of the acyl-enzyme complex. Accordingly, the maximum enzyme activity was observed for the fatty acid with a medium chain length. The activity results showed that the esterification of butanol with the fatty acids has no activity for free and for both immobilized enzymes. The maximum esterification activity of the immobilized lipase preparations were observed for n-octanol with lauric acid. The chitosan-lipase activity was nearly two-fold and chitin-lipase was four-fold higher than that of the free enzyme. The chitin-lipase is most suitable for catalyzing the esterification reactions of medium length fatty acids and fatty alcohols.

The possibilities and benefits of the uses of pancreatic lipase have not yet been exploited in biotechnology. But, on the basis of recent publications, the use of pancreatic lipase would be more advantageous than the microbial and fungal lipases, especially for enzymic esterification. Protein stabilization has been achieved by several methods, including immobilization and crosslinking.[34,35]

In conclusion, immobilization of porcine pancreatic lipase on chitin and chitosan was carried out successively. The supports used in this work are good matrices for the immobilization of lipase. The immobilization procedure is simple and easy to carry out. The immobilized enzymes were shown to have good properties and stabilities. These characteristics of the immobilized enzymes are very important factors when selecting an appropriate enzymatic system for various biotechnological applications.

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