Preparation and Characterization of Pancreatic Lipase Immobilized in Eudragit-Matrix

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ABSTRACT

Pancreatic lipase (EC 3.1.1.3) was immobilized by entrapping in a commercial preparation of acrylic/methacrylic acid ester-based copolymer (Eudragit E 30 D). The activity of the immobilized lipase beads with a diameter of 1.5–2.0 mm was found to be lower than that of the free lipase. The optimum pH was shifted to the alkaline region and the thermal stability increased, whereas the optimum temperature level remained unchanged. The most important reason for the decreased activity was diffusion limitations. The diffusion of the substrate and products became more pronounced, and lipolytic activity increased upon addition of *n*-hexane into the reaction medium. The storage and operational stabilities of the immobilized lipase were investigated, and both characteristics were found to be increased when compared to the free enzyme. Furthermore, mechanical or magnetic stirring during the operation were found to have no influence on the carrier-matrix as determined by nephelometric measurements.

Index Entries: Immobilized lipase; pancreatic lipase; enzyme immobilization; characterization of immobilized lipase.

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INTRODUCTION

There are many areas of application for lipases, such as food and leather processing, as well as medical therapy, and so on (*1–3*). An exciting development is the new application of enzymes to oleochemical processing. Three key areas of oleochemical processing with potential for improvement, by enzymology, are,

1. Fat splitting for fatty acid production (*4,5*);
2. Lipid synthesis by reversal of hydrolysis (*6*); and
3. Lipid modification by ester interchange or interesterification (*7–10*).

The use of an immobilized lipase for interesterification allows retention of the lipase in batch, or continuous-flow reactor operation. The choice of support is important in determining the expressed interesterification activity of the lipase. Eudragit E was used as the carrier matrix for the entrappment of the enzyme. This carrier is very stable, both in water and in *n*-hexane, as well as in other organic solvents. For this reason, lipolytic and interesterification activities of the immobilized lipase can be potentially utilized.

MATERIALS AND METHODS

Pancreatic lipase (EC.3.1.1.3), fatty acids, and triglycerides were obtained from Sigma Chem. Co., St. Louis, USA, Protanal LF 20/60 was purchased from Protan A/S, Dramnen, Norway, Eudragit E 30 D was obtained from Röhm Pharma, Darmstadt, FRG, and all other chemicals were purchased from E. Merck, Darmstadt, FRG.

Enzyme Immobilization

Pancreatic lipase was immobilized in the matrix of an acrylic-based resin, Eudragit E 30 D. This technique has been used previously for cell immobilization (*11*). To 44g of resin suspension (approx 30%), 0.8g of enzyme was slowly added with continuous stirring. After the enzyme was dissolved, the suspension was filtered through a 0.1 mm sieve. Thirteen grams of sodium alginate solution (2%) was added to the suspension; this mixture was homogenized and then transferred to the immobilization apparatus (Fig. 1). The suspension was added dropwise to 1000 mL of gently stirred 2% calcium chloride in bidistilled water. The gel beads were filtered through large filter papers and then left for a night at room temperature.

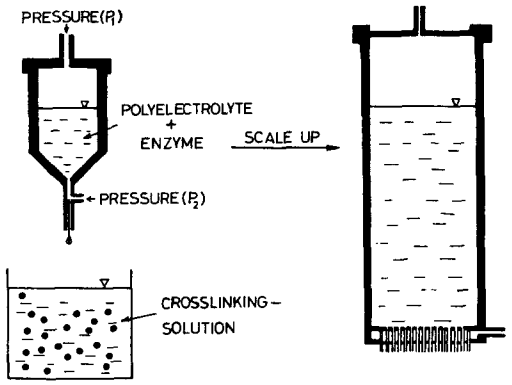

Fig. 1. Technology of bead formation (*15*).

In order to remove calcium ions and alginate from the bead structure, the beads were washed with phosphate buffer (pH 7.5) and bidistilled water, respectively.

Determination of Enzymic Activity

Two enzyme assay methods were used: 1. To determine the interesterification activity (*12*), and 2. to determine the lipolytic activity (*12*). The interestificaiton activity of the immobilized enzyme was measured by following the incorporation of the myristic acid into palm mid-fraction (PMF). This triglyceride is obtained by solvent fraction of palm oil, and myristate composes one molecular percent of its fatty acid chains. Thus, low levels of incorporation of myristic acid into the 1 or 3 position of the triglyceride could be readily measured. The reactions were carried out at 40°C in a 500 mL stirred glass reactor, containing 20g PMF and 10g myristic acid dissolved in 100 mL *n*-hexane. The reaction was initiated by the addition of 2g of wet, immobilized enzyme. Samples were taken at regular intervals to determine the myristic acid content of the triglyceride, as determined by GLC (*13*). One unit of interestification activity was defined as that activity that would interesterify one micromole of triglyceride in one minute at 40°C. The substrate for the lipolytic reaction was prepared by adding 5g of olive oil to 100 mL of 2%(w/w) Acacia in aqueous solution, emulsifying the mixture by ultrasonification, and adding 2.5 mL of 22% (w/w) calcium chloride solution to the emulsion. After adjustment of 10 mL aliquots to a pH of 7.5, pancreatic lipase solution or immobilized lipase preparation was added to start the reaction. The release of fatty acids was automatically titrated by addition of 0.1 *M* sodium hydroxide. Activities were expressed in units, where one unit is the formation of one micromole of free fatty acid per minute at 30°C.

Physical and Chemical Characterization

The protein content of the immobilized enzyme preparation was measured by a modified Lowry method (*14*). The shapes and sizes of the formed gel particles were examined with the help of a magnifying glass, the diameters of the particles were measured, and the range of diameter distribution was established. The abrasion in a stirred vessel was measured by a procedure similar to that described by Vorlop et al. (*15*). A defined amount of gel beads was suspended in a stirred $CaCl_2$ solution (0.25% beads, 2.5% $CaCl_2$ w/v 30°C), and the turbidity of a sample of the supernatant was measured nephelometrically at regular intervals.

RESULTS AND DISCUSSION

The air pressure applied from one side of the immobilization apparatus was adjusted so as to produce gel beads with wet diameters ranging from 1.5 to 2.0 mm in 2% $CaCl_2$. It was observed that, when the beads were removed from the $CaCl_2$ solution, they shrank in diameter by approx 50%. When the beads were transferred to an aqueous medium, there was no significant change in their diameter. The major component in the immobilized lipase is Eudragit. However, there is a need for a gel that would help the Eudragit suspension assume a spherical shape. Alginate, carrageenan, and chitosan can be used for this purpose (*16–18*). Alginate was used in this study. After the beads were hardened, the alginate was withdrawn, either by complexing calcium ions with EDTA, or by forming a weakly soluble salt with phosphate. The removal of calcium ions, and hence alginate, from the immobilized lipase preparation did not result in a change in the mechanical stability of the matrix.

During lyophilization, immobilized enzyme beads were cracked. This suggested the impracticality of storing lipase preparations in dry form.

Lipolytic activities of free and immobilized lipase preparations were measured by pH-Stat method (*19*). Following immobilization, 17% of the original enzymatic activity was found to be retained. Protein determinations in the washings, and the immobilization medium, indicated that approx 70% of the added enzyme remained in the beads. The basic reason for the decrease in lipase activity after immobilization was the diffusion effect (*20*). Thus, when *n*-hexane was added (2% final concentration) into the lipase reaction mixture, this caused a significant increase in the enzymic activity. This increase was owing to the high diffusion rate from the matrix, caused by the organic solvent. There was no effect of *n*-hexane on the free pancreatic lipase activity. This decrease in lipolytic activity had no effect as far as the interesterification activity was concerned, because interesterification reaction takes place in organic solvents.

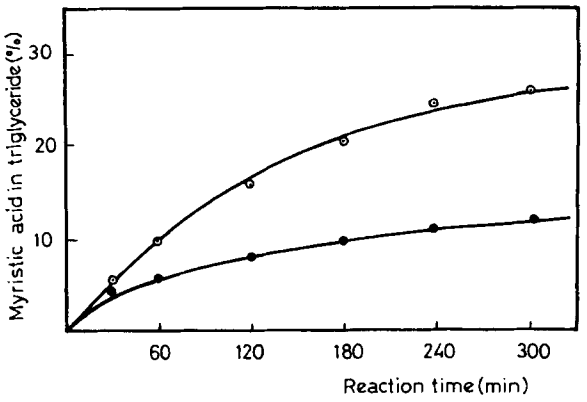

Fig. 2. The progress curves for interesterification reactions catalyzed by free (●) and immobilized (○) lipase preparations.

The interesterification activity of the immobilized enzyme was measured by following the incorporation of myristic acid into PMF by GLC. The interesterification potential of the pancreatic lipase immobilized in the Eudragit E is shown in Fig. 2.

The reasons for using *n*-hexane as the solvent in the measurements of interesterification activity are: The use of *n*-hexane in food processing is universally acceptable, and, the lipase is more stable in *n*-hexane than it is in the other hydrocarbon solvent (*21*).

The enzyme was inactivated by heating immobilized lipase beads in 2% $CaCl_2$ solution (pH 8.0) at 80°C for 30 min, and the gel beads separated by filtration were used in the diffusion test. The gel beads (5g) were mechanically stirred in 100 mL, 0.1% trioleine solution, pH 8.9, 30°C, for 5 h. After mixing, an aliquot was taken, and the amount of trioleine was measured with GLC (*22*). Substrate saturated biocatalyst beads were added to 100 mL, 2% $CaCl_2$ solution, pH 8.9, in a thermostatic reaction vessel at 30°C. Samples taken at different times from the solution were assayed for trioleine by GLC (Fig. 3).

D_e was measured graphically from Fig. 4, drawn as in $(\bar{c}-c_\infty)/(c_o-c_\infty)$ vs t. As seen in Fig. 4, D_e in *n*-hexane is several times greater than that in $CaCl_2$ solution. The increase in the activity of immobilized lipase in the presence of *n*-hexane is owing to the increase in the effective diffusion coefficient.

SUMMARY

Pancreatic lipase (EC 3.1.1.3) was immobilized by entrapping in a acrylic/methacrylic acid ester based copolymer, that is known as Eudragit E

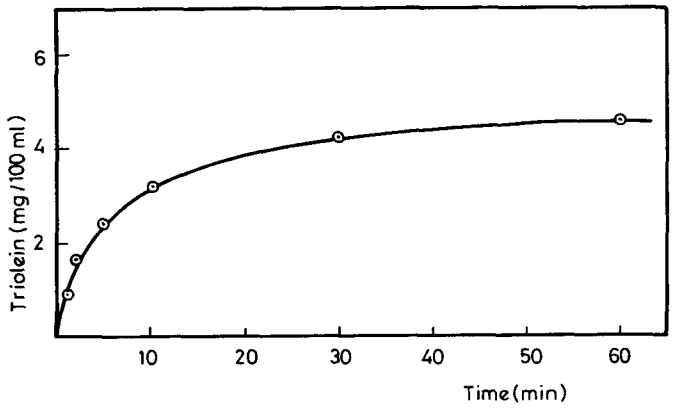

Fig. 3. Effusion curve for triolein from Eudragit E beads.

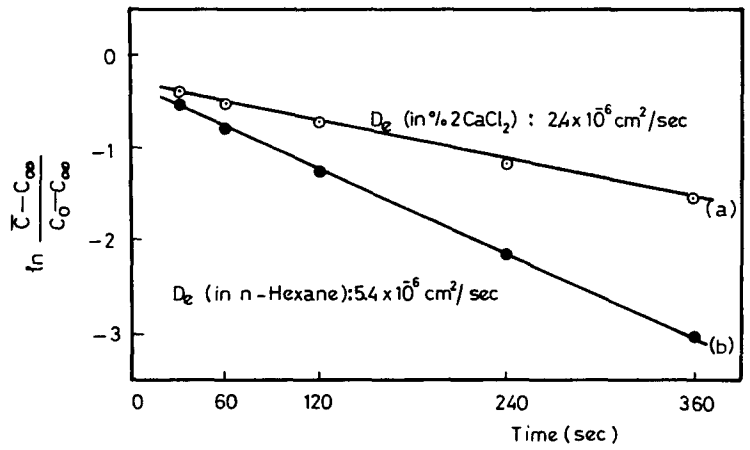

Fig. 4. Determination of D_e for triolein in Eudragit E matrix.

30 D commercially. The protein content of immobilized enzyme preparation was measured by modified Lowry method. Two enzyme assay methods were used, one to determine the interesterification activity, and the other to measure the lipolytic activity. It was found that after immobilization, the enzymatic activity decreased, optimal pH shifted to the alkaline region, and the thermal stability increased, whereas, optimal temperature remained unchanged. The most important reason of the decrease in enzymatic activity was diffusion-limitation. The diffusion of the substrate and products became easier and lipolytic activity increased by the addition of *n*-hexane into the reaction medium. This increase was owing to the high diffusion rate from the matrix caused by the organic solvent. There was no diffusion effect of *n*-hexane on the free pancreatic lipase activity. The decrease in lipolytic activity of immobilized enzyme

had no effect as far as the interesterification reaction took place in organic solvent, and in practically water-free medium. The storage and operational stabilities of immobilized lipase were investigated, and these parameters were found to be greater in comparison to those of the free enzyme. Furthermore, the mechanical or magnetic stirring during the operation were found to have no influence on the carrier-matrix as determined by nephelometric measurements.

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