Indian Journal of Chemical Technology
Vol. 7, March 2000, pp. 64-67

Immobilization of porcine pancreas lipase on zirconia coated alkylamine glass using glutaraldehyde

Sarita V Malik, V Kalia & C S Pundir*

Biochemistry Research Laboratory, Department of Bio-Sciences, Maharshi Dayanand University, Rohtak 124001, India

Received 14 May 1999; accepted 14 December 1999

Commercially available lipase from porcine-pancreas has been immobilized onto zirconia coated alkylamine glass-beads through the process of glutaraldehyde coupling with 100% retention of its initial activity. The enzyme showed changes in its physical properties after immobilization. The utility of immobilized enzyme in removal of oil stain from cotton cloth is demonstrated.

Lipases have attracted more attention compared to other classes of enzyme because of their commercial use in the food industry[1] as well as in the high tech. production of fine chemicals and pharmaceuticals[2] and in diagnostic kit for the estimation of triglyceride[3]. Lipases are known to hydrolyze triglycerides to fatty acids and glycerol, by acting on ester bonds. Recently, lipases have found a great significant role in detergents, as they can remove the fat stain from the clothes more easily under alkaline conditions, compared to that by proteases[4]. But the currently known lipases are not generally stable in detergents and need to be protected from the inhibitory action of proteases and other surfactants. Further, the use of lipases in manufacturing of detergents requires its bulk quantity, which is expensive. The immobilization of lipase on insoluble support not only provides its reuse but also protects it from protease action and surfactant inhibition. In order to get their reuse, lipases from different sources have been immobilized onto various supports such as microporous polypropylene[5], porous polyurethane particles[6], organic polymer beads[7], Sepharose 6-B[8], polystyrene butadiene rubber[9], polyvinyl chloride membrane, anion exchange resin[10], and diatomaceous earth[11]. However lipases have not been immobilized onto alkylamine glass beads, which being inorganic in nature, are resistant to microbial attack and stable over a broad *p*H range or various solvents[12]. The present report describes the immobilization of lipase on zirconia coated alkylamine glass beads, study of changes in its physical properties after immobilization and use for removal of oil stain from cotton cloth in presence of commercial detergents.

Experimental Procedure

Materials—Zirconia coated alkylamine glass beads (pore diameter 55nm) from Corning Glass Works, New York, USA; Lipase from porcine-pancreas (40-70 U/mg protein) from M/S SISCO Research Laboratories Pvt. Ltd., Mumbai; Gum-Arabic, olive oil and detergents were purchased from local market.

Assay of soluble lipase—Lipase was dissolved in 0.1 M tris buffer, *p*H 8.0 (1 mg/mL). The activity of soluble lipase was measured by titration of fatty acid released from fats of olive oil by lipase action against NaOH according to Gotthiff Naher[13]. The titration was carried out manually using burette. 5.0 mL olive oil emulsion and 1.0 mL lipase solution (1 mg/mL) was added to 5.0 mL, 0.1 M tris buffer, *p*H 8.0 and incubated at 45°C for 10 min. Control was made for each sample to correct any drop in *p*H, due to any factor other than lipase or incomplete termination of reaction by acetone and methanol mixture (1:1). In case of control, 5.0 mL 0.1 M tris buffer (*p*H 8.0) was added to 1.0 mL enzyme sample, kept in boiling water bath for 10 min and then 5.0 mL olive oil emulsion was added to it and incubated at 45°C for 10 min as for test. After keeping at room temperature for 20 min., 10 mL acetone-methanol mixture (1:1) was added to both test and control to stop the reaction. Each sample was titrated against 0.025 N NaOH using 1% phenolphthalein as indicator. The volume of NaOH used in the titration was noted. To prepare olive oil emulsion, 180 mL distilled water, 20 mL olive oil, 0.4 g of sodium benzoate and 1.0 g gum-

*For correspondence.

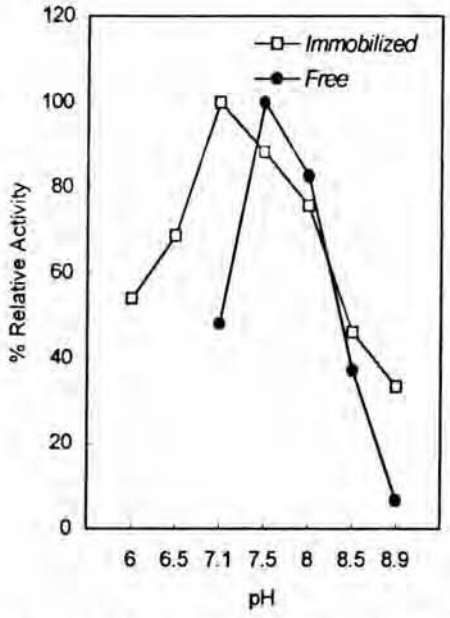

Fig. 1—Effect of *p*H on free and immobilized lipase.

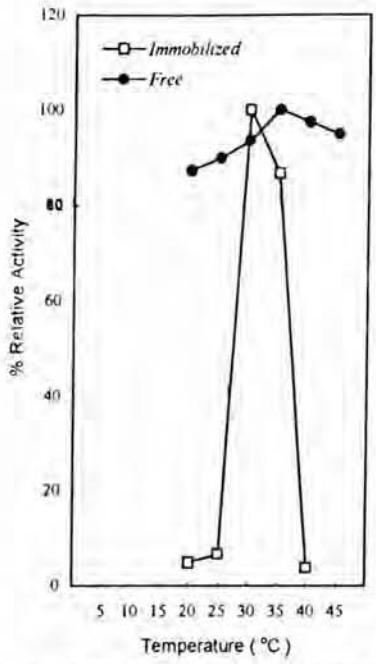

Fig. 2—Effect of temperature on free and immobilized lipase.

arabic were emulsified in mixer. One unit of lipase is defined as the amount of enzyme required to liberate 1μmol of free fatty acid from olive oil per min under the standard assay conditions (*p*H 8.0 and temp. 45°C).

Immobilization of lipase—The lipase was immobilized onto alkylamine glass through the process of glutaraldehyde coupling using the method of Guo *et al.* with modification[14]. To 100 mg glass beads, 1.0 mL of 2.5% glutaraldehyde prepared in 0.1 M tris-buffer, *p*H 8.0 was added and allowed to stand for 2 h at room temperature with occasional shaking. After 2 h, excess of glutaraldehyde was decanted and the glass beads were washed thoroughly with distilled water until the *p*H of the washing discard was 8.0. Finally, the beads were washed with 0.1 M tris buffer, *p*H 8.0. One-mL lipase solution was added to the glutaraldehyde-activated beads and kept at 4°C for 48 h with occasional shaking for coupling. The unbound enzyme was decanted and tested for activity and protein. The glass beads were washed 2-3 times with the same tris buffer, until no activity of enzyme was detected in the washing. The enzyme protein bound to glass-beads was estimated by determining the loss of protein from the solution during immobilization using the method of Lowry *et al.*[15].

Assay of immobilized lipase—It was carried out in a 25 mL conical flask (at *p*H 8.0 and temp. 45°C) in the same manner as described for free lipase except that 1.0 mL enzyme solution was replaced by 100 mg alkylamine glass bead bound lipase. For control, 100 mg glutaraldehyde activated alkylamine glass beads were used in place of free enzyme.

Application of immobilized lipase—The immobilized lipase was used for removal of oil stain from cotton cloth in presence of commercial detergents. For this purpose the cotton cloth pieces (size 5 × 4 cm) were stained with 0.2 mL mustard oil. The stock solutions of three commercially available detergents namely Ariel, Surf and Fena in distilled water (2.0 g/L) were prepared. For each detergent, three test cloth pieces were taken. One piece was not washed at all; other was washed in detergent alone while the third piece was washed with the detergent in presence of immobilized lipase. Washing was done at 30°C for 20 min (presoaking-condition) with continuous shaking in a shaker to make the sufficient contact of the lipid with immobilized lipase, after which it was rinsed with distilled water. The washing performance in each case was noted.

Reusability and storage—To reuse the immobilized lipase, the glass beads were allowed to settle down on the bottom of container and the washing discard was decanted carefully, leaving the glass beads in the container. The beads were washed 2-3 times with distilled water prior to their next use. The immobilized enzyme was stored in distilled water at 4°C when not in use.

Results and Discussion

Commercially available lipase from porcine-

Table 1—Immobilization of porcine pancreas lipase onto zirconia coated alkylamine glass beads (pore diameter 55 nm)

Lipase added to 100 mg glass beads (mg)	Lipase coupled (mg)	% Lipase coupled	Total activity μmol FFA/min	Retention of specific activity
0.8	0.6	75%	9.375	100%

[Specific activity of free lipase was 14.75 μmol/min/mg protein. One enzyme unit is defined as the amount of enzyme required to produce 1μmole FFA/min under the standard assay conditions].
FFA = Free Fatty Acid

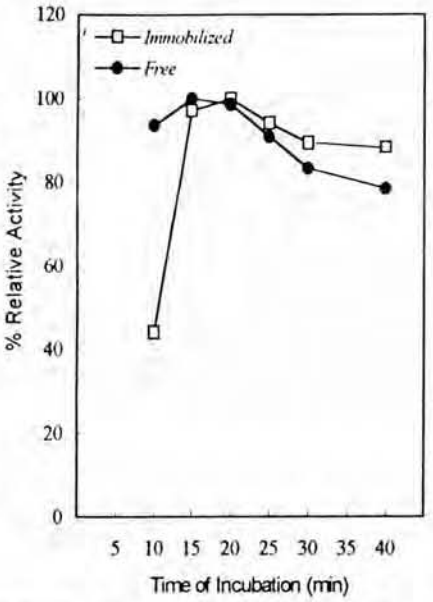

Fig. 3—Effect of time of incubation on free and immobilized lipase.

Fig. 4—Washings of cotton cloth having mustard oil stains in detergent with and without immobilized lipase.

pancreas has been immobilized onto zirconia coated alkylamine glass beads (pore diameter 55 nm) through the process of glutaraldehyde coupling with a conjugation yield of 6.0 mg/g support. The immobilized enzyme retained about 100% of its initial activity (Table 1), which is higher than that reported for cell wall immobilized lipase (80%)[16]. These results showed that alkylamine glass is a suitable support for immobilization of lipase.

Physical properties of immobilized lipase—Some physical properties of alkylamine glass bound lipase were studied and compared with those of free enzyme in Table 2. The maximum activity of alkylamine conjugated lipase was attained at *p*H 7.1, which is slightly lower than that of free enzyme (*p*H 7.5) (Fig. 1). The immobilized enzyme was active in *p*H range 7.0 to 7.5. This may be attributed to the loss of amino group from the surface of enzyme upon conjugation to alkylamine glass, since glutaraldehyde coupling involves the amino group of the enzyme for covalent coupling[17]. The immobilized enzyme showed maximum activity when incubated at 30°C, which is lower than that of free enzyme (35°C) (Fig. 2). The immobilized enzyme was active in the temperature range 30–35°C. These results show comparatively narrow range of the *p*H and temperature for maximum functioning of the enzyme after immobilization. The time of incubation for the maximum activity of enzyme was increased from 15 to 20 min after immobilization (Fig. 3). This indicated the decreased rate of reaction after immobilization, which might be due to the diffusion problem of the substrate from the bulk to the active centre of the immobilized enzyme.

Storage and reusability—The immobilized enzyme showed about 50% loss of its activity during its

Table 2—Comparison of some properties of free and alkylamine glass bound porcine pancreas lipase

Parameter	Free	Alkylamine conjugated
Optimum *p*H	7.5	7.1
Temp. for maximum activity	35°C	30°C
Time of incubation (min)	15	20

regular use 100 times over a period of 2 months when stored in distilled water at 4°C.

Application of immobilized lipase—The results of application of immobilized lipase, as visually interpreted from washed cloth pieces and as shown in Fig. 4, revealed that the detergents mixed with alkylamine glass bead bound lipase gave better washing than that by detergents alone. The removal of the old stain from the cotton cloth by the combination of lipase with three different detergents was found in the following order: Ariel + immobilized lipase > Surf Excel + immobilized lipase > Fena + immobilized lipase. Lipases are well known to hydrolyze the fats present in the oil and thus, remove the oil stain from the cloth freely and rapidly. Hence on the basis of visual assessment, the detergents gave better washing results in the presence of immobilized lipase compared to that with detergents alone. The lipases have been employed along with the alkaline proteases in laundry detergents. Generally, the lipases in free form are not safe as these might be attacked by proteases and inhibited by surfactants[4]. The use of alkylamine glass beads is expected to be less susceptible to the inhibitory effect of surfactants and the proteolytic action of proteases. The use of alkylamine glass bound lipases in the detergents would not only increase their washing efficiencies but also likely to reduce the cost of washing, as it can be reused. However, the modality and the technicality of the use of the glass beads coupled to lipase in the detergents for the washing of clothes at large scale requires to be studied further.

Conclusion

Zirconia coated alkylamine glass beads constitute good supporting material for immobilization of lipase. The alkylamine glass bound enzyme gives maximum activity at *p*H 7.1 and 30°C temperature and can be employed in commercial detergents for better washing of cotton cloth.

References

1 Pencreac'h G, Leullier M & Baratti J C, *Biotech Bioengg*, 56 (1997) 92, 181.

2 Bjorkling F, Godffredsen S E & Kirko, *Trends in Biotech*, 9 (1991) 360.

3 *Sigma Catalogue for Biochemicals, Organic Compounds and Diagnostic Reagents*. (Sigma Chemical Company St. Louis MO, USA.), 1998.

4 Andree N, Muller W R & Schmid R D, *Appl Biochem Biotechnol*, 2 (1980) 218.

5 Montero S, Blanco A, Vitro M D, Landeta L C, Agud I, Solozabal R, Lascaray J M, de Renobales M, Llama M J & Serra J L, *Enz Microb Technol*, 15 (1993) 239.

6 Wang X & Ruckenstein E, *Enz Microb Technol*, 15 (1993) 239.

7 Basri M, Ampon K, Yunus W M, Razak C N & Salleh A B, *J Chem Technol Biotechnol*, 59 (1994) 34.

8 Otero C, Ballesteros A & Guisan J M, *Appl Biochem Biotechnol*, 19 (1988) 163.

9 Iso M, Shirahase T, Hanamura S, Urushiyama S & Omi S, *J Microb Technol*, 6 (1989) 165.

10 Rucka M & Turkiewicz B, *Enz Microb Technol*, 12 (1990) 52.

11 Mustranta A, Forssell P & Poutanen K, *Enz Microb technol*, 15 (1993) 133.

12 Ramesh V & Singh C, *Biochem Biophys Res Commun*, 97 (1980) 779.

13 Naher G, *Methods of Enzymatic analysis*, Bergmeyer, HV ed, vol 2, 814.

14 Guo J A, Mo P S & Li G X, *Appl Biochem Biotechnol*, 23 (1990) 15.

15 Lowry O H, Rosenbrough N J, Farr A L & Randal R J, *J Biol Chem*, 193 (1951) 266.

16 Starauss A & Gotz F, *Mol Microbiol*, 21 (1996) 491.

17 Pundir C S, Kuchhal N K & Satyapal, *Ind J Biochem Biophys*, 30 (1993) 54.