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Immobilization of porcine pancreas lipase onto free and affixed arylamine glass beads and its application in removal of oil stains

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Porcine pancreas lipase has been immobilized through diazotization onto free and affixed arylamine glass beads with 75.59% and 54.26% retention of the initial activity of free enzyme and conjugation yield of 16 mg g^{-1} and 7 mg g^{-1}, respectively. Optimum *p*H of the enzyme was decreased on free glass beads but increased on affixed glass beads. Optimum temperature, energy of activation (Ea), time of incubation and K*m* for triolein were increased but Vmax remained almost unchanged after immobilization on both free and affixed arylamine glass beads. The utility of immobilized enzyme in the removal of oil stains from cotton cloth by various detergents was tested by chemical method. All the detergents gave better wash performance in the presence of immobilized lipase (both onto free as well affixed glass beads) than that by detergent alone. Furthermore, the washing by cheaper (non-enzymic) detergents in the presence of immobilized lipase was almost similar to that by expensive (enzymic) detergents. The free and affixed bead-bound enzyme could be used repeatedly about 100 times without any considerable loss of activity.

Keywords: Porcine pancreas lipase, arylamine glass beads, immobilization, detergent, cloth washing, oil stains

Introduction

Lipase hydrolyses emulsified triglycerides of long chain fatty acid by acting on ester bond[1]. The lipases are used in various industries, such as pharmaceuticals and agrochemical industries, synthesis of structural triglycerides, polymer synthesis, personal care products and cosmetics[2]. Lipase can remove the fat stains more easily under alkaline conditions than the alkaline proteases used for laundry purpose. Hence, lipases have found a new significant role in detergent industries. Lipases used currently in detergents are not generally stable and, hence, required to be protected from the proteases attack and other components of detergents, such as surfactants, which otherwise inhibit lipase activity. The use of immobilized lipase on a suitable support provides its reuse and protects it from protease action and surfactant inhibition. Among the various supports employed for immobilization of lipase[3-15], alkylamine and arylamine glass beads are considered better support. Being inorganic in nature they are resistant to microbial attack, remain stable over a wide *p*H range and in various solvents, such as ethanol and acetone, and possess a long working life span[16]. Immobilization of commercially available porcine pancreas lipase onto free and affixed alkylamine glass beads through glutaraldehyde coupling and its usefulness in removal of oil stains from cotton cloths has been reported from this laboratory[17,18]. Though, a better washing of cotton clothes was observed in the presence of alkylamine glass-bound lipase compared to the detergent alone, yet immobilization of an enzyme onto alkylamine glass beads through glutaraldehyde involves Schiff's base formation, which has a drawback of reversibility of the enzyme reaction[16]. Immobilization of enzyme onto arylamine glass beads through diazotization has no such problem. The present report describes the covalent immobilization of lipase onto both free and affixed arylamine glass beads and their application in the removal of oil stains.

Materials and Methods

Zirconia coated arylamine glass beads (pore diameter 55 nm) were a gift from Prof H H Weetall, Corning Glass Works, New York, USA. Lipase from porcine pancreas (40 U mg^{-1} protein) was from SISCO Research Laboratory Pvt. Ltd, gum-arabic, olive oil and detergents were purchased from local market. All other chemicals used were of AR grade.

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Assay of Free Lipase

The porcine pancreas lipase was dissolved in 0.1 *M* Tris buffer, *p*H 8.0 (5 mg mL^{-1}) and assayed by titration of fatty acid, released from hydrolysis of fat by lipase[16]. The activity of lipase was assayed according to Gotthiff Naher with modifications. The titration was carried out manually by burette. In a 100 mL conical flask, 5.0 mL olive oil emulsion was added to 5.0 mL 0.1 *M* Tris buffer (*p*H 8.0) and incubated at 35°C for 20 min. The reaction mixture was then kept at room temperature for 20 min and 10 mL acetone and methanol mixture (1:1) was added to stop the reaction and titrated it against the 0.025 N NaOH after adding 1% phenolphthalein as an indicator. 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. In the control, 1 mL of lipase solution was kept in boiling water bath for 5 min, so that it became inactive due to denaturation of protein and further processed by the similar method as for the test. One unit of lipase is defined as the amount of enzyme required to liberate 1 μmole of free fatty acid from olive oil per min under the standard assay conditions (at 35°C and *p*H 8.0).

Immobilization of Lipase

On Free Arylamine Glass Beads

It was carried out as described by Lynn[18]. Arylamine glass beads (100 mg) were diazotized in a flask, kept in an ice bath. Solid $NaNO_2$ (100 mg) and 2 mL 2*N* HCl were added to the flask and kept for 30 min in ice bath. Subsequently, excess of HCl was decanted and diazotized glass beads were washed about 7-8 times with 0.1*M* sodium phosphate buffer (*p*H-7.0) until the *p*H of discard was 7.0. Lipase solution (2 mL) was added to the activated beads and then left for 48 h at 4-10°C with stirring at an interval of 2 h.

On Arylamine Glass Beads Affixed Inside a Plastic Beaker

The inner side of a 100 mL plastic beaker was scratched with a sand paper and coated uniformly up to 1 cm height with an fixative (Araldite) by a brush. The powder of arylamine glass (300 mg) was sprinkled uniformly on this fixative layer and allowed to stand at room temperature for 24 h. The immobilization of lipase onto these affixed arylamine glass beads was carried out as described by Lynn[19]. The affixed arylamine glass beads were diazotized, by keeping the beaker on an ice bath and adding 300 mg solid $NaNO_2$ followed by 10 mL 2*N* HCl in it. After keeping it for 30 min in ice bath, the excess of HCl was decanted and diazotized glass beads were washed 7-8 times with 0.1*M* sodium phosphate buffer (*p*H-7.0) until the *p*H of washing discard became 7.0. Lipase solution (5 mL containing 1.2 units) was added to the activated beads and then left for 48 h at 4°C with shaking at an interval of 2 h. The unbound enzyme was decanted and assayed for activity and protein[20]. The glass beads (free and affixed) were washed 3-4 times with the same buffer, until no activity of enzyme was detected in the washings. The protein bound to the glass beads was estimated by determining the loss of protein from the solution during immobilization[20].

Assay of Immobilized Enzyme

It was carried as described above for free lipase in the same flask/beaker containing glass beads with bound lipase. It was termed as 'reaction flask/beaker'. It was carried out in the same flask/beaker in which enzyme was immobilized and the reaction mixture did not contain soluble enzyme and kept under continuous stirring during incubation for both free and affixed glass beads, respectively. Further, a mixture of acetone and methanol was not added to stop the reaction as the reaction mixture was taken off the reaction beaker after the assay. In case of control, the similar conditions were used except that glass beads did not contain immobilized lipase.

Application of Immobilized Lipase

The immobilized lipase was employed for the removal of oil stains from a cotton cloth in presence of solution of commercial detergents (Surf Excel, Ariel Compact, Super Nirma, Multi-action Surf, Henko, Rin Shakti, Nirma and Wheel). For this purpose the cotton cloths pieces (size: 4.5 × 4.5 cm) were stained with 0.2 mL mustard oil. The stock solutions of these detergents in distilled water (2.0 g L^{-1}) were prepared. Three stained cloth pieces were taken for each detergent. One piece was washed with detergent alone, second piece was washed with detergent in presence of immobilized lipase, while the third piece was washed with detergent and free lipase solution at 30°C for 20 min with continuous stirring to ensure that the enzyme bound to the affixed glass beads was in contact with lipid stain on cloth piece. One stained piece was washed in distilled water and immobilized lipase. Each washed cloth piece was rinsed twice manually with distilled water. The

washing performance in each was determined as follows: The oil retained in test cloth after washing was extracted by dipping the washed cloth piece in 10 mL of petroleum ether for 20 min with gentle stirring. The fat extracted was transferred to a 100 ml round bottom distillation flask containing 25 mL alcoholic KOH (0.5 *M*). The flask was attached to a reflux condenser and the mixture was refluxed in a boiling water bath for 30 min. The flask was removed, cooled to room temperature (30°C) and the reaction mixture was titrated against 0.5 *M* HCl using 1% phenolphthalein as an indicator. The blank was set-up similarly but no oil was taken in it. The volume of HCl consumed in each titration was noted.

Results and Discussion

Commercially available lipase from porcine pancreas has been immobilized through diazotization onto free arylamine glass beads affixed inside a plastic beaker by a nonreactive fixative. It gave a conjugation yield of 16 mg g^{-1} support and 7 mg g^{-1} support, respectively, which was higher than that of free alkylamine glass beads (6 mg g^{-1})[1]. The immobilized enzyme on free and affixed glass beads retained 75.6% and 54.3%, respectively of the initial specific activity of the soluble/free enzyme as shown in Table 1.

Table 1—Immobilization of porcine pancreas lipase onto free arylamine glass beads and glass beads affixed inside a plastic beaker with a non reactive fixative

	Lipase bound to free glass beads (100 mg)	Lipase bound to affixed glass beads (300 mg)
Lipase added (mg)	2.4	22.5
Lipase coupled	1.6	19
Coupled lipase (%)	66.6	84.4
Total activity added	2.66	28.75
Total activity bound	1.34	13.25
% Retention	75.59	54.2
Conjugation yield (mg/g)	16	190

Specific activity of free lipase = 1.7 μ*M* FFA $min^{-1}mg^{-1}$
Specific activity of lipase immobilized on free arylamine glass beads = 0.86 μ*M* FFA $min^{-1}mg^{-1}$
Specific activity of lipase immobilized on affixed arylamine glass beads = 0.70 μ*M* FFA $min^{-1}mg^{-1}$
Specific activity of immobilized lipase = 0.86 μ*M* FFA min^{-1} mg^{-1}, the enzyme activity is defined as the amount of enzyme required to produce 1 μ*M* FFA min^{-1} under the standard assay conditions, FFA = Free fatty acid

Table 2—A comparison of kinetic parameters of free porcine pancreas lipase, enzyme bound to free arylamine glass beads and lipase bound to arylamine glass beads affixed inside a plastic beaker

Parameter	Lipase		
	Free[1,2]	Bound to free arylamine glass beads	Bound to affixed arylamine glass beads
Optimum *p*H	7.5	6.5	8.9
Optimum temperature (°C)	35	37	37
E_a (k Cal $mole^{-1}$)	2.05	5.08	5.08
Time of incubation (min)	15	40	30
Saturating concentration for triolein (m*M*)	50	100	100
K_m for triolein ($\times 10^{-3}$ *M*)	4.242	8.33	14.3
V_{max} (μmol min^{-1})	1.515	1.33	1.43
Storage stability (Distilled water at 4ºC)	-----	3 months	3 months

Kinetic Properties of Immobilized Lipase

A comparison of some kinetic properties of lipase coupled to free arylamine glass beads and arylamine glass beads affixed inside the walls of a plastic beaker with those of free enzyme is given in Table 2. The maximum activity of free arylamine glass beads bound lipase was attained at *p*H 6.5, which is slightly lower than that of free enzyme (*p*H 7.5)[1,2], while lipase bound to affixed arylamine glass beads had a optimum *p*H at *p*H 8.9, which is higher than that of free enzyme. The shifting of optimum *p*H of lipase into alkaline range after immobilization onto affixed beads is advantageous for the removal of fat stains. Optimum *p*H of an enzyme is displaced upon immobilization particularly when the support material is charged. The lipase bound to free as well as affixed arylamine glass beads showed maximum activity at 37°C, which is slightly higher than the free enzyme (35°C)[1,2]. The energy of activation (E_a) of the immobilized enzyme, as calculated from Arrhenius plot between 1/T vs log activity, was increased from 2.05 k Cal mol^{-1} to 5.08 (free arylamine glass beads) and 5.34 (affixed arylamine glass beads) k Cal $mole^{-1}$ after immobilization. The time of incubation was also increased from 15 min to 40 and 30 min after immobilization onto free and affixed beads, respectively. This increase might be due to diffusion of the substrate from bulk to the active centre of the immobilized enzyme. The substrate (triolein) concentration required for the maximum activity or saturation of immobilized lipase was two times higher

for enzyme bound to both free and affixed arylamine glass beads than that of free enzyme. A Lineweaver-Burk plot between reciprocal of substrate concentration (1 S^{-1}) and initial activity (1 v^{-1}) of immobilized enzyme was linear. K_m for triolein, as calculated from this plot, increased (8.33 m*M* for enzyme bound to free arylamine glass beads and 14.2 m*M* for lipase bound to affixed arylamine glass beads) after immobilization (4.242 m*M* for free enzyme), indicating a decreased affinity of the enzyme towards the substrate (triolein) after immobilization. V_{max} was unchanged after immobilization on both free and affixed glass beads[17,18]. The changes in kinetic properties of enzyme after immobilization are controlled by four factors: (i) change in enzyme conformation, (ii) its microenvironment, (iii) stearic effects, and (iv) bulk and diffusion effect.

Application of Immobilized Lipase

The oil stained cotton cloth pieces were washed with detergents alone and in the presence of free and immobilized lipase. The volume of HCl used in the titration of unused KOH in each case is given in Table 3, which is directly proportional to the amount of unused KOH, and this in turn reflected the residual oil in cloth piece. The content of residual oil in the cloth piece reflected the washing performance. The lesser residual oil content meant better washing. Two types of detergents were tested, expensive (enzymic) detergents, such as Surf Excel, Ariel Compact, Multi-action Surf and Henko, and non-enzymic detergents, such as Super Nirma, Rin Shakti, Nirma and Wheel. The washing performance of detergents in the presence of immobilized lipase or free lipase was found in the following order: Surf Excel > Ariel Compact >Super Nirma > Rin Shakti > Multi- Action Surf > Henko > Nirma > Wheel > Distilled water. Similar order of washing performance was obtained by detergents alone.

The combination of free/immobilized lipase with anyone of the detergents gave better wash performance than that by detergent alone. But free lipase cannot be preferred over immobilized one, due to its exhaustible property. The amount of HCl consumed in washing by non-enzymatic detergents, such as Rin Shakti and Multi-action Surf, + immobilized lipase was almost similar to that by enzymic detergents alone, such as Surf Excel or Ariel Compact, and revealed the same washing performance of cheaper detergents in the presence of immobilized lipase as that of expensive detergents. The immobilized lipase was reused about 100 times in such washings without any considerable loss of activity. Lipase is well known to hydrolyze the fats present in the oil and thus removes the oil stains from the cloth rapidly. The lipase has been employed along with the alkaline proteases in the detergents. Thus, the use of arylamine glass bead-bound lipase in washing of oil stained cloth by detergents has not only increased their washing efficiencies without consuming them in the process but also made cheaper detergents equivalent to expensive detergents for washing purpose. The immobilized lipase on arylamine glass beads showed no noticeable loss of activity during repeated uses (100 times) for about 2 months, when stored in distilled water at 4°C.

Table 3—Titrimetric determination of residual oil content in the cotton cloth after washing with various detergents alone and in the presence of lipase bound to free arylamine and lipase bound to affixed arylamine glass beads

Detergents	Amount of HCl used in titration (mL)		
	Without lipase	Lipase bound to free arylamine glass beads	Lipase bound to affixed arylamine glass beads
None (Distilled water)	21.8	23.0	23.0
Surf Excel	24.3	25.8	25.6
Ariel Compact	24.1	25.6	25.5
Rin Shakti	23.7	25.5	25.5
Multi Action Surf	23.3	25.1	25.0
Henko	24.0	24.8	24.7
Nirma	22.5	24.3	24.3
Wheel	22.0	24.2	24.2

Conclusions

The washing by cheaper (non-enzymic) detergents in the presence of immobilized enzyme was almost similar to that by expensive (enzymic) detergents. The immobilized enzyme was reused about 100 times without any loss of its activity. The enzyme bound to free arylamine glass beads had a disadvantage of loss of beads during washing, which was overcome by affixing glass beads on inner wall of a plastic beaker before immobilization. The shifting of optimum *p*H of lipase after immobilization on affixed arylamine glass beads in the alkaline range was an added advantage. The removal of fat stains is easier in alkaline medium generated due in the presence of detergents.

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