Characteristics of Poly(AAc_5-*co*-$HPMA_3$-*cl*-$EGDMA_{15}$) Hydrogel-Immobilized Lipase of *Pseudomonas aeruginosa* MTCC-4713

S. S. Kanwar,[1] S. Pathak,[1] H. K. Verma,[1] S. Kumar,[2] R. Gupta,[1] S. S. Chimni,[3] G. S. Chauhan[2]

[1]*Department of Biotechnology, Himachal Pradesh University, Summer Hill, Shimla, Himachal Pradesh 171 005, India*
[2]*Department of Chemistry, Himachal Pradesh University, Summer Hill, Shimla, Himachal Pradesh 171 005, India*
[3]*Department of Chemistry, Guru Nanak Dev University, Amritsar, Punjab 143 005, India*

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ABSTRACT: Four series of noble networks were synthesized with acrylic acid (AAc) copolymerized with varying amount of 2-hydroxy propyl methacrylate or dodecyl methacrylate (AAc/HPMA or AAc/DMA; 5:1 to 5:5, w/w) in the presence of ethylene glycol dimethacrylate (EGDMA; 1, 5, 10, 15, and 20%, w/w) as a crosslinker and ammonium per sulfate (APS) as an initiator. Each of the networks was used to immobilize a purified lipase from *Pseudomonas aeruginosa* MTCC-4713. The lipase was purified by successive salting out with $(NH_4)_2SO_4$, dialysis, and DEAE anion exchange chromatography. Two of the matrices, E_{15a}, i.e. [poly (AAc_5-*co*-DMA_1-*cl*-$EGDMA_{15}$)] and I_{15c}, i.e. [poly (AAc_5-*co*-$HPMA_3$-*cl*-$EGDMA_{15}$)], that showed relatively higher binding efficiency for lipase were selected for further studies. I_{15c}-hydrogel retained 58.3% of its initial activity after 10th cycle of repetitive hydrolysis of *p*-NPP, and I_{15c} was thus catalytically more stable and efficient than the other matrix. The I_{15c}-hydrogel-immobilized enzyme showed maximum activity at 65°C and pH 9.5. The hydrolytic activity of free and I_{15c}-hydrogel-immobilized enzyme increased profoundly in the presence of 5 m*M* chloride salts of Hg^{2+}, NH_4^+, Al^{3+}, K^+, and Fe^{3+}. The immobilized lipase was preferentially active on medium chain length *p*-nitrophenyl acyl ester (C:8, *p*-nitrophenyl caprylate). © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 100: 4636–4644, 2006

Key words: *Pseudomonas aeruginosa*; MTCC-4713; lipase; immobilization; synthetic hydrogels

INTRODUCTION

Lipases (EC 3.1.1.3) are triacylglycerol hydrolases that catalyze the hydrolysis of fats and oils to glycerol and fatty acids at the oil–water interface.[1] Lipases catalyze a wide range of reactions in aqueous as well as organic solvents,[2,3] which include hydrolysis and interesterification. In addition, they also catalyze alcoholysis, acidolysis, esterification, and aminolysis. Lipases are produced by animals, plants, and microorganisms such as bacteria, yeast,[4,5] molds,[6] and a few protozoa.[7] Moreover, lipase possesses high regioselectivity and stereoselectivity for its catalyzed reactions. Thus, there are number of industrial applications of lipases, especially in food and pharmaceutical industries.[8]

A range of fatty acid esters is now being produced commercially using immobilized lipase in nonaqueous solvents,[9,10] and interest in industrial processes employing lipase as a biocatalyst to synthesize more such esters is still growing because of their important and multiple applications. Esters produced from long-chain fatty acids (12–20 carbon atoms) and short chain alcohols (3–8 carbon atoms) have been used increasingly in the foods, detergents, cosmetics, and pharmaceutical industries.[11] Use of synthetic hydrogels as support for enzyme immobilization has attracted the attention of scientists and technologists worldwide.[12–15] Acrylic acid (AAc) and ethyl diamine (EDA) based polymers absorb water and are strongly hydrophilic. The balance of polymer–polymer and polymer–solvent interaction determines the solubility of a polymer in a solvent. A polymer contains group with very different chemical affinities, for instance polar hydrophilic and nonpolar hydrophobic group; each group contributes independently to solubility. Hydrogel can be used as a polymeric support to immobilize enzymes. Varying the concentration of copolymers and crosslinker can customize the hydrophobicity/hydrophilicity of the polymer. Moreover, the important aspect is that the enzyme is not actually attached to anything,[16] and so it retains the same conformation as that of a free enzyme. The hydrogel besides providing water needed for enzyme activity can also absorb water produced during esterification reaction, thus enhancing the conversion to product(s). Also, immo-

Correspondence to: S. S. Kanwar (kanwarss2000@yahoo.com).
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bilized biocatalyst is readily separable from soluble support and products.

In the present study, among various hydrogels, a weakly hydrophilic poly(AAc_5-*co*-$HPMA_3$-*cl*-$EGDMA_{15}$)-hydrogel has been used to immobilize lipase from a thermotolerant *Pseudomonas aeruginosa* MTCC-4713, and various aspects of the bound enzyme have been evaluated.

EXPERIMENTAL

Chemicals

Acrylic acid (AAc), 2-hydroxy propyl methacrylate (HPMA), *N*,*N*-methylene bisacrylamide (MBAm), ethylene glycol dimethacrylate (EGDMA), dodecyl methacrylate (DMA), ammonium per sulfate (APS), benzoyl peroxide (BPO) (MERCK Ltd., Mumbai, India), methanol, acetone (Qualigens Fine Chemicals, India), *p*-nitrophenyl palmitate (*p*-NPP), *p*-nitrophenyl laurate (*p*-NPL), *p*-nitrophenyl caprylate (*p*-NPC), *p*-nitrophenyl acetate (*p*-NPA), *p*-nitrophenyl formate (*p*-NPF), *p*-nitrophenol (Lancaster Synthesis, England), and molecular sieve (3 Å × 1.5 mm) were from E. Merck Pvt. (India) Ltd., Worli, Mumbai, India. All chemicals were used as received.

Microorganism and lipase

The thermophilic *Pseudomonas aeruginosa* MTCC-4713 originally isolated from a sweet shop waste was obtained from the Department of Biotechnology, Himachal Pradesh University, Summer Hill, Shimla (India). The purified lipase was immobilized on various synthetic hydrogels.

Preparation of crude lipase

The *P. aeruginosa* MTCC-4713 culture was repeatedly subcultured at 56°C on a mineral based (MB) broth supplemented with 1% (v/v) cottonseed oil as the sole carbon source. The MB broth contained (g l^{-1}) $NaNO_3$, 3.0; K_2HPO_4, 0.1; $MgSO_4$ $7H_2O$, 0.5; KCl, 0.5; $FeSO_4.7H_2O$, 0.01; yeast extract, 40; and sucrose, 5.0. The cottonseed oil (100 mL) warmed to 70°C was initially emulsified with gum acacia (0.5 g) by continuous stirring (Magnetic Stirrer, Remi Equipments, Bombay, India). pH of the medium was adjusted to 7.5. For lipase production, 7.5% (v/v) of 48-h-old inoculum was used to inoculate production medium (50 mL each in 250-mL capacity Erlenmeyer flasks). The lipase producing broth was rendered free of cells by centrifuging at 10,000 × *g* for 10 min at 4°C (SIGMA 3K30, Germany). The supernatant filtered through Whatman paper No. 1 was subjected to precipitation with 80% (w/v) ammonium sulfate at 4°C. The precipitates sedimented by centrifugation were reconstituted in 40 mL of Tris buffer (0.05*M*, pH 7.5) and extensively dialyzed against same buffer to remove ammonium sulfate. Finally, lipase activity[17] and protein[18] were assayed. This crude lipase fraction was stored at −20°C till further use. One unit (U) of hydrolytic activity of immobilized lipase was expressed as micromoles of *p*-nitrophenol produced per minute by hydrolysis of *p*-nitrophenyl palmitate (Lancaster Synthesis, UK prepared as 20 m*M* stock in 2-propanol) at 65°C by 1 g of immobilized enzyme (including the weight of matrix) under assay conditions.

TABLE I
Networks (Xerogels) with Varying Concentration of AAc/DMA using EGDMA as Crosslinker

Matrix code	AAc/DMA (volume ratio)	EGDMA (%, w/w)
E_a	5:1	1
E_b	5:2	1
E_c	5:3	1
E_d	5:4	1
E_e	5:5	1

Purification of the enzyme

Purification of lipase enzyme was performed by anion exchange (DEAE-Cellulose, Pharmacia Amersham, Sweden) chromatography, as previously described.[19]

Synthesis of hydrogels

The hydrogels employed for the immobilization of lipase were based on the AAc. The hydrogels were obtained when AAc was copolymerized separately with HPMA in 1:1 water/acetone, using APS as initiator, and with DMA in acetone, using benzoyl peroxide as initiator. EGDMA was used as crosslinker to get different series of hydrogels. AAc (2 mL) was taken in 4.0 mL of a solvent system comprising acetone/water (1:1, v/v) along with fixed concentration of initiator APS or benzoyl peroxide (1 m*M*) and known concentration of a crosslinker EGDMA (1%, w/v). The reaction was designed to have AAc/DMA or AAc/HPMA in a ratio of 5:1, 5:2, 5:3, 5:4, and 5:5 (v/v). In other four reactions, only the concentration of the crosslinker was varied (5, 10, 15, and 20%, w/v) with respect to the total weight of the monomers. The reaction mixture was heated in water bath at 50°C when APS was used as initiator and 80°C when benzoyl chloride was used as initiator, for 30 min. Thus, four different series of hydrogels were synthesized (Tables I–IV). Insoluble products were separated from the reaction mixture by filtration to remove the soluble constituents. Sol fraction, if any, trapped in the body of the network was separated from the synthesized networks by polarity

TABLE II
AAc/DMA Based Networks (Xerogels) with Varying Concentration of EGDMA

Matrix code	AAc/DMA (volume ratio)	EGDMA (%, w/w)
E_{5a}	5:1	5
E_{10a}	5:1	10
E_{15a}	5:1	15
E_{20a}	5:1	20

gradient method as the networks were treated with water, methanol, and acetone, separately in Soxhlet apparatus by shifting from solvent of higher to lower polarity. The product was dried in an air-oven at 40°C for 24 h to obtain xerogels.

Immobilization of purified lipase

All immobilization reactions were performed by taking 10 mg of matrix (presoaked in 200 μL of 0.05*M* Tris buffer, pH 7.5 for 20 min and the buffer was separated by decantation) and 200 μL of enzyme in Teflon stoppered glass vials (5 mL capacity). The suspension was incubated at 8°C overnight. Immobilization of lipase on networks was performed in 0.05*M* Tris buffer (pH 7.5) at 55°C in shaking incubator water bath for 10 min. The lipase activity was assayed in the aqueous phase unless specified otherwise.

Reusability of immobilized lipase for hydrolytic activity towards *p*-NPP

To study the reusability of immobilized lipase, the hydrolytic activity of the E_{15a} and I_{15c}-hydrogel-bound lipases was repetitively assayed up to 10 cycles using immobilized enzyme preparation. After each cycle, the matrices were washed thrice with 2 mL of Tris buffer (pH 7.5).

Effect of incubation time on immobilization of lipase

To study the effect of incubation time on optimal immobilization of lipase, 200 μL of enzyme was incubated with 10 mg of presoaked matrix in Teflon stoppered glass vial at 8°C. The unbound lipase was assayed by taking out 5 μL of liquid phase from the preparation at periodic intervals (1 h), up to a maximum of 8 h.

TABLE III
Networks (Xerogels) with Varying Concentration of AAc/HPMA using EGDMA as Crosslinker

Matrix code	AAc/HPMA (volume ratio)	EGDMA (%, w/w)
I_a	5:1	1
I_b	5:2	1
I_c	5:3	1
I_d	5:4	1
I_e	5:5	1

TABLE IV
AAc/HPMA Based Networks (Xerogels) with Varying Concentration of EGDMA as Crosslinker

Matrix code	AAc/HPMA (volume ratio)	EGDMA (%, w/w)
I_{5c}	5:3	5
I_{10c}	5:3	10
I_{15c}	5:3	15
I_{20c}	5:3	20

Effect of temperature on free and I_{15c}-hydrogel-immobilized enzyme

To study the effect of temperature on free and immobilized lipase preparations, the reaction mixtures were incubated separately at different temperatures (45–85°C) in shaking water bath. Assay for the free lipase was performed using 5 μL of enzyme in the reaction mixture.

Effect of pH of reaction buffer on free and I_{15c}-hydrogel-immobilized enzyme

To evaluate the effect of pH of reaction buffer on catalytic potential of free and immobilized lipase, I_{15c}-immobilized lipase or 5 μL of free lipase in Tris buffer (0.05*M* with 0.4% v/v Triton X-100 and 0.1% w/v gum acacia) was incubated in 0.05*M* Tris buffer of varying pH (7.0–12.0) at 55°C (for free lipase) or 65°C (for matrix-immobilized lipase) in shaking water bath. Thereafter, the hydrolytic activity of lipase was assayed by sampling aqueous phase.

Thermostability of free and I_{15c}-hydrogel-immobilized enzyme

Thermostability of the immobilized and free lipase was examined at optimized temperature of 65 and 55°C, respectively. Immobilized and free lipases were incubated separately in aqueous environment (0.05*M* Tris buffer; pH 9.5 for bound enzyme and pH 7.5 for free enzyme) up to 5 h with continuous shaking. The residual lipase activities of the free and immobilized enzyme were determined at different time intervals under standard assay conditions.

Effect of metal ions on the activity of free and I_{15c}-hydrogel-immobilized enzyme

The effect of various metal-ions (K^+, Na^+, Co^{2+}, Cu^{2+}, Zn^{2+}, Hg^{2+}, Mg^{2+}, Fe^{3+}, NH_4^+, and Al^{3+}) on the

TABLE V
Summary of Purification of Lipase

Lipase preparation	Lipase [U ml^{-1}]	Volume [ml]	Total lipase [U]	Protein [mg ml^{-1}]	Specific activity [U mg^{-1}]	Fold purification	Yield [%]
Crude	0.86	500	430.0	20.8	0.04	1.0	100.0
Dialyzed	4.35	44	195.8	9.8	0.44	11.0	50.5
DEAE purified	5.40	15	81.0	5.5	0.98	24.5	18.8

activity of free and immobilized lipase was studied. Matrix-immobilized lipase was preincubated for 10 min at 65°C in the reaction buffer containing each of the metal ions (at a final concentration of 1 m*M*), separately. The final pH of the reaction buffer was adjusted to 7.5. The free and immobilized enzyme preparations were used for assaying hydrolytic activity of lipase under standard assay conditions. The metal ions (K^+, Hg^{2+}, Fe^{3+}, NH_4^+, and Al^{3+}), which led to increase in activity, were further used at varied concentrations (1, 3, 5, 7, and 10 m*M*) in the reaction mixture and the residual lipase activity was assayed.

Effect of various acyl substrate on hydrolytic activity of free and immobilized lipase

To study the substrate specificity of free and immobilized lipases, substrates of varying C-chain length (*p*-NPP, *p*-NPL, *p*-NPC, *p*-NPA, and *p*-NPF) were used. The lipase assay was performed with each of the substrates at 55°C for free enzyme and at 65°C for immobilized enzyme in 0.05 m*M* Tris buffer (pH 7.5 for free enzyme and pH 9.5 for immobilized enzyme).

RESULTS

Production of lipase

The extracellular lipase produced by *P. aeruginosa* MTCC-4713 was harvested at 48 h post-inoculation at 55°C when an optimal amount of enzyme (0.86 U ml^{-1}) was produced in the culture broth in consonance with the growth profile of the bacterium. During the period of incubation, the final pH became alkaline (pH 9.1–9.3) from an initial pH 7.5. The cell free broth (500 mL) after precipitation with ammonium sulfate, followed by extensive dialysis, resulted in a lipase activity of 0.44 U mg^{-1} protein.

Purification of lipase

The cell-free broth had a lipase activity of 430 U with 20.8 mg protein ml^{-1} (specific activity 0.04 U mg^{-1}). The protein was optimally precipitated at 80% (w/v) ammonium sulfate saturation. The precipitates reconstituted in 44 mL of Tris buffer (0.05*M*, pH 7.5) were extensively dialyzed against the same buffer. The dialyzate showed lipase activity of 195.8 U with a specific activity 0.44 U mg^{-1} protein (Table V). The chromatography of the dialyzed lipase on DEAE-Cellulose column resulted in a single peak (Fig. 1). The fractions showing lipase activity were pooled (15 mL, 81 U, 5.40 mg protein ml^{-1}, specific activity 0.98 U mg^{-1} protein).

Protein binding efficiency of hydrogels and their characteristics

Four series of hydrogels were evaluated for their ability to bind the protein (lipase). The purified lipase of *P. aeruginosa* MTCC-4713 was optimally immobilized/bound to a weakly hydrophilic matrix-I_{15c}, which retained 91.8% of the total protein used for immobilization and specific activity (1.16 U mg^{-1} protein) increased by about 18.4% as compared to the specific activity of the free enzyme (0.98 U mg^{-1} protein) used for immobilization reaction (Table VI). However, results of protein binding efficiency were also satisfactory with hydrogel E_{15a}, which bound 89.1% of the total protein (specific activity 1.2 U mg^{-1} protein). However, binding efficiency of the hydrogels for protein decreased with an increase in the concentration of copolymer.

The two matrices, E_{15}a [poly(AAc_5-*co*-DMA_1-*cl*-$EGDMA_{15}$)] and I_{15}c [poly(AAc_5-*co*-$HPMA_3$-*cl*-$EGDMA_{15}$)] showed the maximum activity of 5.8 and 6.0 μM (min g^{-1}) hydrogel, respectively.

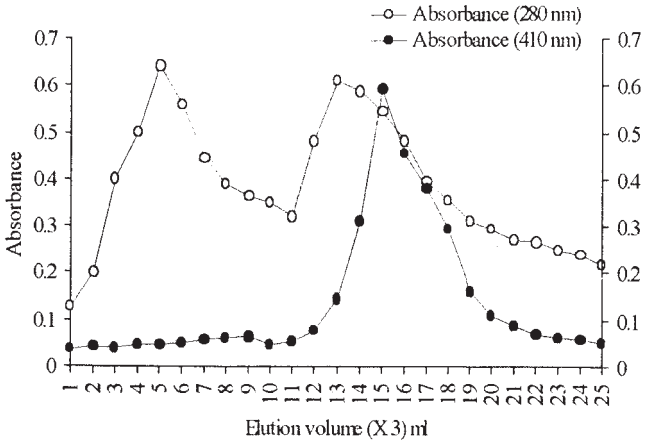

Figure 1 Purification of lipase of *P. aeruginosa* MTCC-4713 by DEAE-cellulose chromatography.

TABLE VI
Efficiency of Various Hydrogels for Binding Lipase

Matrix code	Protein binding efficiency (%)	Lipase activity (U mg^{-1} protein)
E_a	93.6	0.60
E_b	84.5	0.41
E_c	83.6	0.45
E_d	85.5	0.50
E_e	78.2	0.46
E_{5a}	82.7	0.45
E_{10a}	82.7	0.46
E_{15a}	89.1	1.20
E_{20a}	86.4	0.37
I_a	82.7	0.48
I_b	80.9	0.94
I_c	82.7	1.05
I_d	84.6	0.62
I_e	82.7	0.94
I_{5c}	88.2	1.00
I_{10c}	84.6	1.10
I_{15c}	91.8	1.16
I_{20c}	80.9	0.83

Reusability of immobilized lipase for repetitive hydrolysis of *p*-NPP

To select one of the matrices for further experiments, the repetitive use of the two matrices E_{15a} and I_{15c} in the hydrolysis of *p*-NPP was studied. Lipase bound to I_{15c}-hydrogel, i.e., poly(AAc_5-*co*-$HPMA_3$-*cl*-$EGDMA_{15}$), was found to be relatively more stable and efficient during the repeated cycles of hydrolysis as it retained 58.3% (specific activity 1.02 U mg^{-1} protein) of its initial hydrolytic activity after 10th cycle of reuse (Fig. 2). This matrix was selected for performing esterification reactions by studying the effect of various kinetic parameters.

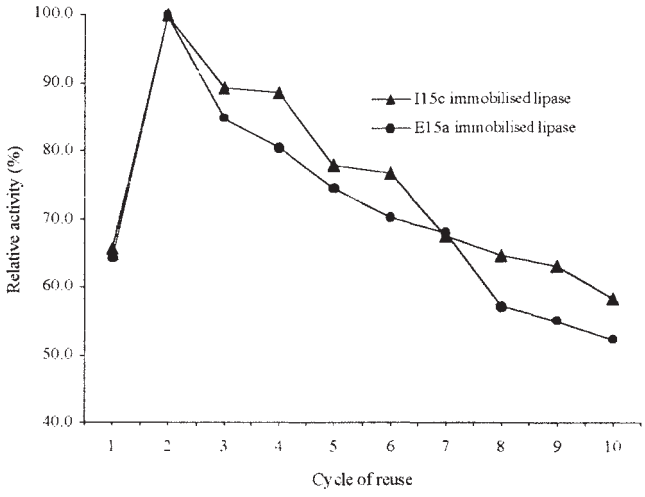

Figure 2 Comparison of I_{15c}- and E_{15a}-matrices-bound lipase for repetitive hydrolysis of *p*-NPP.

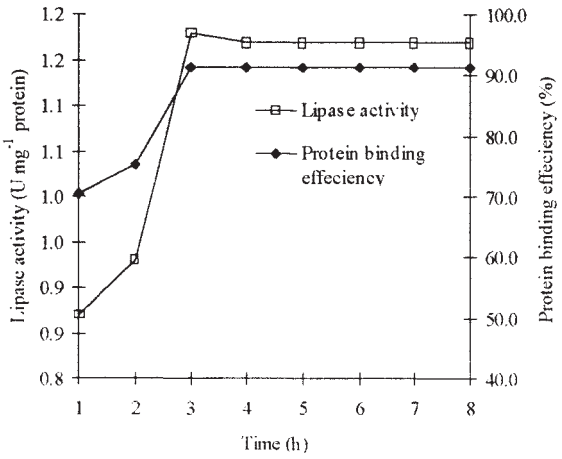

Figure 3 Effect of incubation time on immobilization of lipase.

Effect of incubation time on immobilization of lipase

Immobilization of lipase on to the I_{15c}-hydrogel increased till 3 h of incubation and became static thereafter (Fig. 3). The specific activity of immobilized lipase was found to be 1.18 U mg^{-1} protein at 3 h when 91.4% binding of the protein was recorded. Further incubation did not improve the binding efficiency of the I_{15c}-hydrogel.

Effect of temperature on the activity of immobilized lipase

Maximum activity of I_{15c}-hydrogel-immobilized lipase was observed at 65°C, whereas free enzyme was optimally active at 55°C having specific activities of 1.2 and 0.98 U mg^{-1} protein, respectively (Fig. 4). A drastic decrease (33.0%) in activity was observed at 70°C for I_{15c}-matrix-immobilized enzyme.

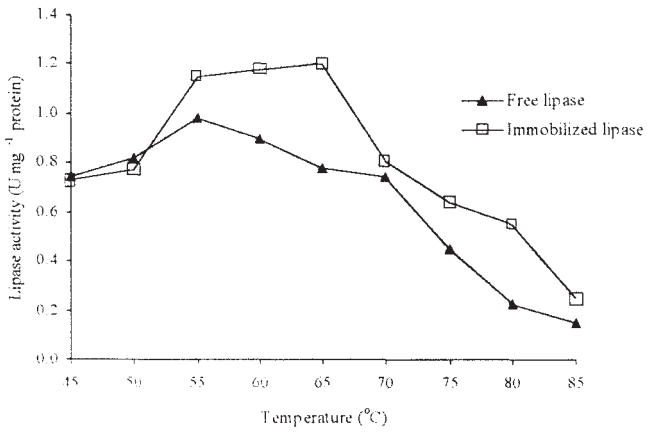

Figure 4 Effect of incubation temperature on hydrolytic activity of free and I_{15c}-bound lipase.

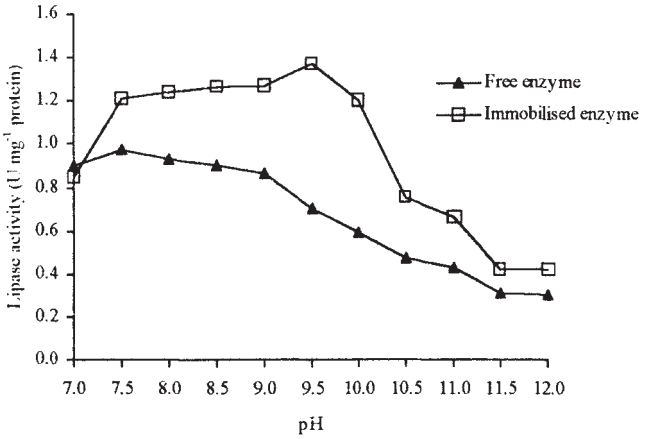

Figure 5 Effect of pH on hydrolytic activity of free and I_{15c}-immobilized lipase.

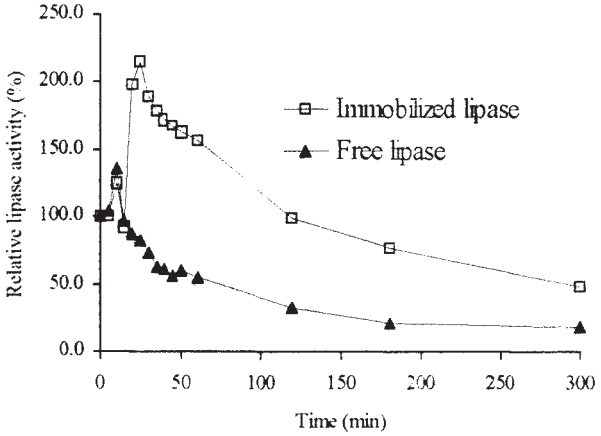

Figure 6 Thermostability of free (at 55°C) and matrix (I_{15c})-immobilized lipase (at 65°C).

Effect of pH on immobilized lipase

The effect of pH of the reaction buffer (0.05*M* Tris buffer) on hydrolytic activity of the bound as well as free lipase was evaluated by adjusting the pH of the buffer in the range of 7.0–12.0. A pH of 7.5 for the free lipase and 9.5 for the I_{15c}-hydrogel-immobilized lipase was found to be optimum for achieving efficient hydrolysis of *p*-NPP (Fig. 5). The immobilized lipase exhibited specific activity of 1.37 U mg^{-1} protein at pH 9.5 and free lipase had a specific activity of 0.97 U mg^{-1} protein at pH 7.5. The pH beyond 9.5 resulted in a decrease of the hydrolytic activity of the I_{15c}-hydrogel bound catalyst.

Thermostability of free and immobilized lipase

When the free and immobilized biocatalysts were subjected to prolonged exposure at their optimum temperature of 55 and 65°C, respectively, an increase in hydrolytic activity (36.0% for free enzyme and 114.7% for matrix-immobilized lipase) was observed after 10 and 25 min incubation, respectively. The free enzyme retained 54.0% and the immobilized enzyme retained 47.8% of its original activity after 1 and 3 h of incubation, respectively (Fig. 6).

Effect of C-chain length of acyl substrate on hydrolytic activity of immobilized lipase

It was observed that free lipase was highly specific towards longer C-chain length esters, whereas the I_{15c}-hydrogel-immobilized catalyst showed the specificity towards medium C-chain length ester, i.e., p-NPC (Fig. 7).

Effect of metal salts on the activity of free and immobilized lipase

Addition of some of the chloride salts ($FeCl_3$, $HgCl_2$, $AlCl_3$, NH_4Cl, and KCl) at 1 m*M* in the reaction buffer led to an increase of 4–13% in lipase activity of bound and free lipase. $CoCl_2$ strongly antagonized the lipase activity of free and bound catalyst [Fig. 8(a)]. The presence of Mo^{2+}, Zn^{2+}, Mg^{2+}, and Cu^{2+} ions also exerted an antagonistic effect on free as well as the immobilized enzyme, leading to a decrease of 20–70% of initial lipase activity. Above five salt ions were then used at relatively higher concentrations (3, 5, 7, and 10 m*M*). The presence of chloride salts of K^+, Hg^{2+}, Fe^{3+}, NH_4^+, and Al^{3+} at a concentration of 5 m*M* resulted in a further increase in the lipase activity that was 14–26% more than that of the control in case of free lipase [Fig. 8(b)] and 40–60% more in case of immobilized lipase [Fig. 8(c)]. The presence of Hg^{2+} ions resulted in maximum increase (22% in free and 83% in immobilized) in lipase activity. NH_4^+, Al^{3+}, K^+, and Fe^{3+} ions also modulated the hydrolytic activity of

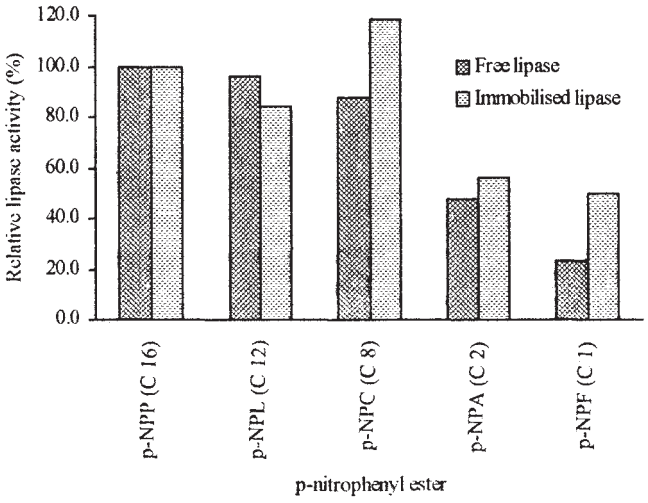

Figure 7 Substrate specificity of free and I_{15c}-hydrogel-bound lipase.

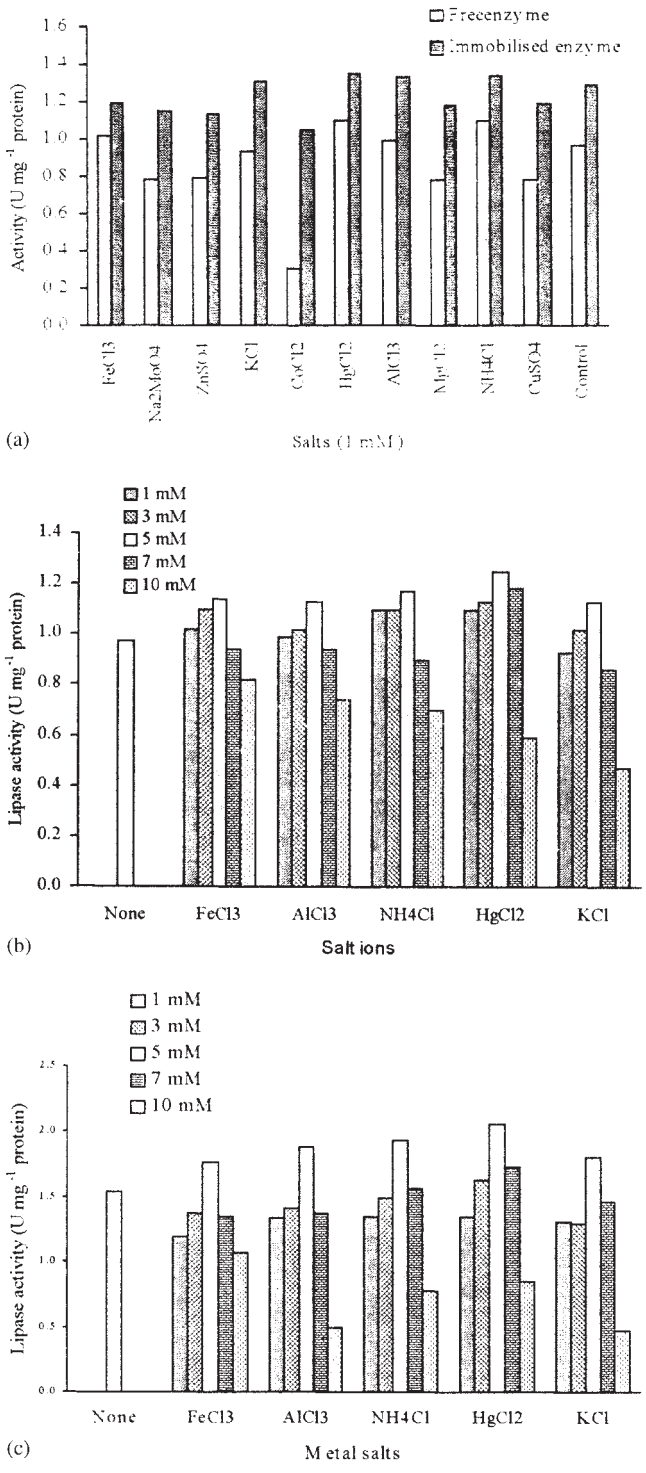

Figure 8 (a) Effect of salt ions (1 m*M*) on hydrolytic activity of free and I_{15c}-bound lipase. (b) Effect of varying concentration of salt ions on hydrolytic activity of free lipase. (c) Effect of varying concentration of salt on hydrolytic activity of I_{15c}-bound lipase.

immobilized lipase in that order. Further increase in the concentration of metal salts led to decrease in activity of both free as well as immobilized enzyme.

DISCUSSION

Lipases (triacylglycerol acyl hydrolases, EC 3.1.1.3.) are hydrolytic enzymes that catalyze the hydrolysis and synthesis of a variety of acylglycerols at the interface of lipid and water.[20,21] Of bacterial extracellular lipases,[22] those from the *Pseudomonas* species have been extensively studied for their industrially applicable properties. Lipases display maximum activity towards water insoluble long chain ($\geq$12) acyl glycerides.[23] A range of fatty acid esters is now being produced commercially using immobilized lipases in nonaqueous solvents[9,10] and the interest in industrial processes employing lipase biocatalysts to synthesize more such esters is still growing because of their important and multiple applications.[24]

In the present study, four series of different network polymers with varying hydrophilic nature (differing in the concentration of the copolymer hydroxy propyl methacrylate or dodecyl methacrylate) were synthesized and employed for immobilization of an alkaline lipase from a moderate thermotolerant *P. aeruginosa* MTCC-4713. One of the polymer networks, I_{15c} i.e., poly(AAc_5-*co*-$HPMA_3$-*cl*-$EGDMA_{15}$), that was obtained using lowest concentration of a weakly hydrophilic copolymer HPMA showed highest binding efficiency towards lipase of *P. aeruginosa* MTCC-4713. In the present study, it was established that *P. aeruginosa* MTCC-4713 lipase when immobilized on I_{15c}-hydrogel exhibited a relatively higher hydrolytic activity as was evident by its repetitive use for hydrolysis of *p*-NPP than the free enzyme. The lipase of *P. aeruginosa* MTCC-4713 performed a highly efficient hydrolysis at alkaline pH. Lipases that are hydrolytic at alkaline pH are conventionally used in detergent and laundry. Most lipases act in a wide range of temperature and pH, although alkaline bacterial lipases are more common.[25–27]

Immobilized lipase offers the economic incentives of enhanced thermal/chemical stability, ease of handling, recovery, and reuse relative to nonimmobilized forms.[28,29] Various features of reaction selectivity of lipase are modulated by exogenous factors, such as the choice of cosubstrate\reactants, water activity, pH, temperature, and immobilization.[30–33] The lipase of *P. aeruginosa* MTCC-4713 after immobilization on I_{15c}-hydrogel became relatively more thermotolerant than the free lipase. The bound lipase retained its hydrolytic activity at 65°C for an extended period (3 h) than the free form. Thus, immobilization enhanced the thermal stability of the *P. aeruginosa* lipase. It appeared that temperature has an important effect on the physical state of substrate dispersion too. Higher temperature and liquefaction can be assumed to make the substrate more acceptable for the enzyme.[34] It was quite likely that the structure of lipase immobilized onto I_{15c}-hydrogel became more fluid at an elevated

temperature. It appeared that specialized support matrices with distinguishing chemical character such as the one used in the present study might have greater influence on the lipase activity and selectivity by virtue of support matrix effects directly on enzyme conformation or microenvironment, differences in substrate diffusion rates, or physiochemical interaction directly with substrate and products, as previously postulated.[35,36] Moreover, immobilization also facilitated dispersal of enzyme on a solid surface to provide far greater interfacial area and accessibility of substrate relative to the use of enzyme powders in low water reaction media.[37] The emergence of commercial applications of lipases for preparing value added speciality products from lipids or by esterification/transesterification will be dependent on understanding and controlling reaction selectivity.[38]

Structural elucidation of lipase has shown that catalytic site of most lipases resembled that of serine proteases.[39] The active site is a catalytic triad of serine, aspartic (or glutamic) acid, and histidine; serine is a highly conserved residue in various lipases. The presence of ionizable salts of metals as well as nonmetals is quite likely to cause structural change(s) at the catalytic site of the immobilized or free lipase. Interestingly, both divalent (such as Ca^{2+}, Ba^{2+}, Mg^{2+}, Mn^{2+}, and Hg^{2+}) as well as monovalent cations (Na^{+} and K^{+}) are strong modulators of microbial lipase activity.[40–45] In the present study, some of the salt ions (Hg^{2+} NH_4^{+}, Fe^{3+}, Al^{3+}, and K^{+}) were found to modulate the hydrolytic activity of the lipase sourced from *P. aeruginosa* MTCC-4713. This observation was highly noticeable, because previously the Hg^{2+} ions nowhere in the literature were reported to strongly modulate the lipase activity. In one of the previous studies, exposure to Ca^{2+} ion to an extracellular lipase of *P. flourescens* 2D was reported to effect an ~360% increase in the lipase activity, but the presence of Hg^{2+} and Co^{2+} strongly inhibited the enzyme activity.[43] Hg^{2+}, Al^{3+}, Mn^{2+}, and Co^{2+} ions exerted a drastic decline in the lipase activity of *Rhizopus oryzae*.[46] The presence of metal ions has been known to influence the hydrolytic activities of microbial lipases of both bacterial as well as fungal origin. Na^{+} ions have been reported to be essential for lipase activity of *P. pseudoalkaligens*.[47]

The present study showed that the immobilization of lipase on I_{15c}-hydrogel promoted the thermostability as well as pH tolerance of the bound catalyst than the free form. Unlike free lipase, which was more active towards a relatively longer C-chain length *p*-nitrophenyl ester (*p*-NPP), the immobilized lipase showed greater specificity towards hydrolysis of *p*-NPC, a medium C-chain length ester. It appeared that immobilization on I_{15c}-hydrogel limited the access of relatively longer esters (substrates) to the catalytic site of the bound enzyme. Thus, it may be concluded that immobilization not only provided stability to the lipase to tolerate a relatively higher temperature, pH, and prolonged thermal stability but also promoted interactive-binding of Hg^{2+} ions at the catalytic site, which enhanced the hydrolytic potential of the I_{15c}-hydrogel bound lipase.

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References

1. Sunna, A.; Hunter, L.; Hutton, C. A.; Bergquist, P. L. Enzyme Microb Technol 2000, 31, 472.
2. Zaks, A.; Klibanov, A. M. Proc Natl Acad Sci USA 1985, 82, 3192.
3. Zaks, A.; Klibanov, A. M. J Biol Chem 1988, 263, 3194.
4. Aires-Barros, M. R.; Taipa, M. A.; Cabral, J. M. S. In Lipases–Their Structure, Biochemistry and Applications; Woolley, P., Peterson, S. B., Eds.; Cambridge University Press: Cambridge, 1994; p 243.
5. Sztajer, H.; Maliszewska, I.; Wierezorek, J. Enzyme Microb Technol 1988, 10, 492.
6. Dalmou, E.; Montesinos, J. L.; Lotti, M.; Casas, C. Enzyme Microb Technol 2000, 26, 657.
7. Vadhera, D. Lipids 1974, 9, 158.
8. Svendson, A. Biochem Biophys Acta 2000, 1543, 233.
9. Macrae, A. R.; Roel, E. L.; Brand, H. M. Seifen Oele Fette Wachse 1984, 116, 201.
10. Shimada, Y.; Hirota, Y.; Baba, T.; Sugihara, A.; Moriyama, S.; Tominaga, Y.; Terai, T. J Am Oil Chem Soc 1999, 76, 713.
11. Bauer, A. R.; Garbe, D.; Surgurg, H. Common Fragrance and Flavour Materials, 2nd ed.; VCH: New York, 1990; p 67.
12. Basri, M.; Harun, A.; Ahmad, M. B.; Razak, C. N. A.; Salleh, A. B. J Appl Polym Sci 2001, 82, 1404.
13. Kaushal, R. K.; Sen, U.; Kanwar, S. S.; Chauhan, G. S. In Proceedings of IXth International Workshop on Bioencapsulation; Illkirch, Strasbourg, France, May 25–27, 2003.
14. Kaushal, R. K.; Chimni, S. S.; Kanwar, S. S. In Proceedings of XIIth International Workshop on Bioencapsulation; Vitoria-Gasteiz, Spain, September 24–26, 2004.
15. Harun, A.; Basri, M.; Ahmad, M. B.; Salleh, A. B. J Appl Polym Sci 2004, 92, 3381.
16. Basri, M.; Samsudin, S.; Ahmad, M. B.; Razak, C. N. A.; Salleh, A. B. Appl Biochem Biotechnol 1999, 81, 205.
17. Winkler, U. K.; Stuckmann, M. J Bacteriol 1979, 138, 663.
18. Lowry, O. H.; Rosenbrough, N. J.; Farr, A. L.; Randall, R. J. J Biol Chem 1951, 193, 265.
19. Kanwar, S. S.; Verma, H. K.; Kaushal, R. K.; Gupta, R.; Kumar, Y.; Chimni, S. S.; Chauhan, G. S. World J Microbiol Biotechnol, in press.
20. Gilbert, E. J. Enzyme Microb Technol 1993, 15, 634.
21. Jaeger, K. E.; Reetz, M. T. Trends Biotechnol 1998, 16, 396.
22. Arpigny, S. N.; Jager, K. E. Biochem J 1999, 343, 177.
23. Eaggert, T.; Pencreach, G.; Douchet, I.; Verger, R.; Jaeger, K. E. Eur J Biochem 2000, 267, 6459.
24. Zaidi, A.; Gainer, J. L.; Carta, G.; Mrani, A.; Kadiri, T.; Belarbi, Y.; Mir, A. J Biotechnol 2002, 93, 209.
25. Kanwar, S. S.; Sultana, H.; Chimni, S. S.; Kaushal, R. K. Acta Microbiol Immunol Hung, in press.
26. Kanwar, S. S.; Srivastva, M.; Ghazi, I. A.; Chimni, S. S.; Kaushal, R. K.; Joshi, G. K. Acta Microbiol Immunol Hung 2004, 51, 57.
27. Gupta, M.; Gupta, P.; Chimni, S. S.; Kanwar, S. S. Indian J Biotechnol, in press.
28. Malcata, F. X.; Reyes, H. R.; Gareia, H. S.; Hill, C. G. T.; Amundson, C. H. J Am Oil Chem Soc 1990, 67, 890.

29. Yahya, A. R. M.; Anderson, W. A.; Moo-Young, P. Enzyme Microb Technol 1998, 38, 438.
30. Bornscheuer, U. T.; Yamane, T. Enzyme Microb Technol 1994, 16, 864.
31. Klien, R. R.; King, G.; Moreau, R. A.; Mcneill, G. P.; Villeneuve, P.; Hass, M. J. J Am Oil Chem Soc 1997, 74, 1401.
32. Rhee, J. S.; Kwon, S. J. J Microbiol Biotechnol 1998, 8, 191.
33. Lee, C. H.; Parkin, K. L. Biotechnol Bioeng 2001, 75, 219.
34. Konthanen, H.; Tenkanen, M.; Fagerstrom, R.; Reinikainen, T. J Biotechnol 2004, 108, 51.
35. Goncalves, A. P. V.; Lopes, J. M.; Lemos, F.; Ribeiro, F. R.; Prazeres, D. M. F.; Cabial, J. M. S.; Aires-Barros, M. R. Enzyme Microb Technol 1997, 20, 93.
36. Pouilloux, Y.; Abro, S.; Vanhove, C.; Barrault, J. J Mol Catal A 1999, 149, 243.
37. Bell, G.; Halling, P. J.; Moore, B. D.; Patridge, J.; Rees, D. G. Trends Biotechnol (TIBTECH) 1995, 13, 468.
38. Jensen, R. G.; Galluzzo, D. R.; Bush, V. J. Biocatalysis 1990, 3, 307.
39. Winkler, F. K.; D'Arcy, A.; Hunziker, W. Nature 1990, 343, 771.
40. Muderhwa, J. M.; Ratomawenia, R.; Pina, M.; Gaille, J.; Grazy, P. Appl Microbiol Biotechnol 1986, 23, 348.
41. Omar, C. I.; Nishio, N.; Nagai, S. Biotech Lett 1988, 10, 799.
42. Kojima, Y.; Yokoe, M.; Mase, T. Biosci Biotechnol Biochem 1994, 58, 1564.
43. Makhzoum, M.; Knapp, J. S.; Dwusu, R. K. Food Microbiol 1995, 12, 277.
44. Sidhu, P.; Sharma, R.; Somi, S. K.; Gupta, J. K. Indian J Microbiol 1998, 38, 9.
45. Hiol, A.; Donzo, M. D.; Druet, D.; Comeau, L. C. Enzyme Microb Technol 1999, 25, 80.
46. Hiol, A.; Donzo, M. D.; Rugani, N.; Druet, D.; Sarda, L.; Comeau, L. C. Enzyme Microb Technol 2000, 26, 421.
47. Lin, S. F.; Chiou, C. M.; Tsai, Y. C. Biotechnol Lett 1995, 17, 959.