Appl Biochem Biotechnol (2008) 151:160–166
DOI 10.1007/s12010-008-8163-x

# Protein-Coated Microcrystals of *Pseudomonas aeruginosa* PseA lipase

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Received: 16 December 2007 / Accepted: 28 January 2008 /
Published online: 9 August 2008


**Abstract** Highly active *Pseudomonas aeruginosa* lipase protein-coated microcrystals (PAL PCMC) have been prepared by immobilization of protein onto $K_2SO_4$ as excipient solid support carrier and *n*-propanol as precipitating solvent. Transmission electron micrographs confirmed the formation of PAL PCMC. These PCMC were found to be a catalytically more active and stable preparation for *p*-nitrophenyl palmitate hydrolysis in *n*-heptane, compared to free lipase. The $V_{max}$, $K_m$, and temperature optimum for PAL PCMC increased from 0.49 to 5.66 nmol $min^{-1}$ $mg^{-1}$, 589 to 679.8 mmol, and 40°C to 45°C, respectively. These were thermally more stable with 4.65, 2.56, and 1.24-fold improvement in half lives at 45°C, 55°C, and 60°C compared to free *P. aeruginosa* PseA lipase. Their catalytic efficiency was enhanced by tenfold over that of free enzyme. PAL PCMC offer a simple and effective technique for obtaining stable and efficient lipase preparation for biocatalysis in nonaqueous medium.

**Keywords** *Pseudomonas aeruginosa* · Protein-coated microcrystals (PCMC) · Lipase · Immobilization · Nonaqueous medium

## Introduction

Lipases constitute one of the most important groups of biocatalysts due to their ability to catalyze three different kinds of reactions viz. hydrolysis, esterification, and trans-esterification with unique chemo-, regio-, and enantiospecific selectivity. Lipase-based processes are employed for oil/fat processing, synthesis of industrially important oleo-chemicals, enantiopure pharmaceuticals, agrochemicals, flavor esters, structured lipids, and biodiesel production [1, 2]. As the lipase catalyzed reactions are carried out in nonaqueous/ biphasic medium, the enzyme should be sufficiently solvent stable. However, lipases

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exhibit reduced catalytic activity in nonaqueous media compared to the aqueous systems [3]. Several techniques have been developed to improve this loss of catalytic activity. The operational viability of these processes necessitates lipase preparations with enhanced catalytic efficiency, stability, and reusability [4, 5].

Immobilization often imparts prerequisite improvements in enzyme properties, hence it has been an extensively employed technique for developing industrially useful enzyme preparations. Wide range of matrices and array of coupling techniques have been developed. Apparently, the suitability of support and method of immobilization vary from enzyme to enzyme and their intended use. It needs to be optimized for each system. A search therefore continues to work out newer and efficient immobilization methods [6, 7]. Protein-coated microcrystals (PCMC) have emerged as a very efficient method of immobilization in recent years. PCMC are formed by the process of simultaneous precipitation and immobilization of protein on microcrystal surface [8]. For making PCMC, aqueous solution of protein is mixed with concentrated solution of an excipient, such as a salt (e.g., $K_2SO_4$)/sugar/amino acid. This protein–excipient mixture, when added to water-miscible organic solvent, co-precipitates both protein and excipient instantly, resulting into micron-sized crystals of excipient with protein molecules adsorbed on its surface [9]. PCMC as a technique has drawn considerable attention due to its simplicity, rapidity, amazing efficiency, and cost effectiveness.

We have previously reported a novel lipase from *Pseudomonas aeruginosa* PseA exhibiting remarkable stability in a wide range of organic solvents [10]. With a view point to make it a viable biocatalyst with enhanced catalytic rate and reusability, PCMC of this lipase were studied. Current work describes the preparation and characterization of *P. aeruginosa* PseA lipase (PAL) protein-coated microcrystals (PCMC). Formation of PAL PCMC has been confirmed by transmission electron microscopy (TEM). Their efficiency towards hydrolysis of *p*-nitrophenyl palmitate (*p*NPP) substrate in completely nonaqueous medium increased by tenfold compared to the free *P. aeruginosa* PseA lipase.

## Materials and Methods

*p*-Nitrophenyl palmitate (*p*NPP) substrate for lipase was purchased from Sigma Chemical Co., USA. *Candida rugosa* lipase was purchased from Sisco Research Laboratories (SRL), India. All the chemicals and solvents used were of analytical grade. All the organic solvents used in the assay were dried on molecular sieves (4 A°) before use.

### Preparation of PAL PCMC

*P. aeruginosa* PseA was cultivated on lipase production media as described previously [10]. The cell-free supernatant after 48 h growth was lyophilized and used as crude lipase. PAL PCMC were prepared by the method of Kreiner et al. [9] using $K_2SO_4$ as excipient carrier for crystal formation. Briefly, lipase powder (40 mg) was dissolved in 1 ml Tris–HCl buffer (0.1 M, pH 8.0) and then mixed with 3 ml of a saturated aqueous solution of $K_2SO_4$. This solution was then added drop wise to a flask containing 60 ml of anhydrous *n*-propanol under constant shaking. Formation of microcrystals was observed as precipitate which was centrifuged at 8,000×*g* at 4°C to collect the PAL PCMC as pellet. These were washed with *n*-propanol, air dried, and stored at 4°C as dry powder for further use.

The PCMC of *C. rugosa* lipase (CRL PCMC) were also prepared in a similar manner.

## Lipase Assay

The assay of lipase in organic media was carried out by the modified method of Pencreac'h and Baratti [11]. The reaction mixture consisting of 1.5 ml *n*-heptane and 50 mM of *p*NPP in a sealed cap glass vial was preincubated at 30°C for 10 min. The reaction was started by the addition of appropriate amount of PAL PCMC lipase/free lipase. The shaking speed was set at 150 rpm. Aliquots of 50 μl of the supernatant were taken from the reaction mixture at fixed intervals and mixed with 1 ml of 0.1 M NaOH in a quartz cuvette. The absorbance as a result of extraction of *p*-nitrophenol (*p*NP) from *n*-heptane phase to aqueous NaOH phase was measured at 400 nm. One unit is defined as the amount of enzyme releasing 1 nmol of *p*-nitrophenol (*p*NP) under standard conditions.

Lipase activity in aqueous medium was determined by following the method of Kilcawley et al. [12].

## Transmission Electron Micrography

The air-dried PAL PCMC were viewed under TEM Philips, CM-10 model and visualized by negative staining. TEM pictures of $K_2SO_4$ crystals formed in the absence of enzyme were also recorded as control.

## Characterization of PAL PCMC

To study the effect of pH on the activity of lipase, the assay was performed at various pH values (5.5–9). The buffer systems used were: 0.1 M sodium acetate buffer (pH 5.5), 0.1 M sodium phosphate buffer (pH 6.0–7.5), and 0.1 M Tris–HCl buffer (pH 8.0–9.0). The relative lipase activity was calculated taking values of pH 8.0 as 100%.

To determine the effect of temperature on free *P. aeruginosa* lipase and PAL PCMC, the enzyme was assayed at various temperatures (25–50°C) at pH 8.0 under standard conditions. Residual lipase activities (%) were determined taking values of 40 and 45°C (for free and PAL PCMC, respectively) as 100%.

Thermal stability of enzyme was investigated by incubating PAL PCMC (in dry form) and free lipase (dissolved in buffer) at three different temperatures: 45°C, 55°C, and 60°C. Residual lipase activity was determined at different time intervals under standard assay conditions. Half-life time ($T_{1/2}$) was estimated as the time required for decreasing lipase activity to half of the initial values. Relative half lives at each temperature were determined by taking the ratio of $T_{1/2}$ of PAL PCMC to that of free lipase at that temperature.

Kinetic constants were determined by carrying out lipase assay with varying concentrations of *p*NPP under standard conditions. $K_m$ and $V_{max}$ were determined by the Lineweaver–Burk plot.

## Hydrolysis of pNPP in Organic Medium

To investigate the effect of different organic solvents on PAL PCMC catalyzed *p*NPP hydrolysis, the hydrolysis was carried out in solvents of different log *P* values (logarithm of partition coefficient between *n*-octanol and water) viz. hexane (log *P* 3.0), heptane (log *P* 3.5), and isooctane (log *P* 4.0). The substrate (50 mM *p*NPP) for the assay was also prepared in respective solvents. The hydrolysis was monitored as described for the assay procedure but replacing heptane with hexane/isooctane.

All the experiments were carried out in duplicate, and the variation between the sets was within ±5%.

## Results and Discussion

Lipases are unique type of enzymes which need interfacial activation for their activity. These are most efficient at the oil–water interface. This necessitates the lipase stability in both aqueous and organic medium. It may be mentioned that fats and oils, the natural substrate of lipase dissolve in solvents only. Thus, ideal immobilization method for lipase should impart activity and stability in both aqueous and nonaqueous system. PCMC offer such an advantage by the way that the biocatalysis in nonaqueous medium can be carried out using the insoluble form of enzyme, whereas crystals can be dissolved in water/buffer to work in aqueous medium.

PAL PCMC were prepared by using $K_2SO_4$ as crystal forming carrier salt (excipient) and *n*-propanol as precipitating solvent. The choice of solvent is very critical for PCMC yields because solvent is responsible for precipitation of excipient and enzyme, and also for dehydration in such a way that enzymes denaturation is minimized, and active conformation is retained [9]. In our case, *n*-propanol was found to be the best solvent compared to other solvents. Solvents other than *n*-propanol, viz. tetrahydrofuran, *n*-butanol, and acetonitrile resulted in lower expressed lipase activity of 86%, 78%, and 53.8%, respectively, with respect to that in the case of *n*-propanol. The choice of solvent may vary from enzyme to enzyme. As reported by Shah and Gupta [13], the precipitation with propanol resulted in substantial loss of *Burkholderia cepacia* lipase activity (up to 60%) upon PCMC formation as evaluated by hydrolysis of *p*-nitrophenyl palmitate, whereas acetone worked better in their case.

The choice of $K_2SO_4$ as excipient salt for PAL PCMC formation was largely prompted by their successful application in PCMC by other researchers including pioneers Kreiner et al. [9].

By using $K_2SO_4$ as carrier salt and *n*-propanol as solvent, we obtained 54% expressed activity in the form of PAL PCMC with respect to initial lipase activity. Shah et al. [13] have reported 40% expressed activity in case of *Burkholderia cepacia* lipase with $K_2SO_4$ and *n*-propanol as solvent.

To confirm the formation of microcrystals and lipase coating onto them, TEM was carried out. TEM pictures clearly showed formation of PAL PCMC (Fig. 1a). The crystals

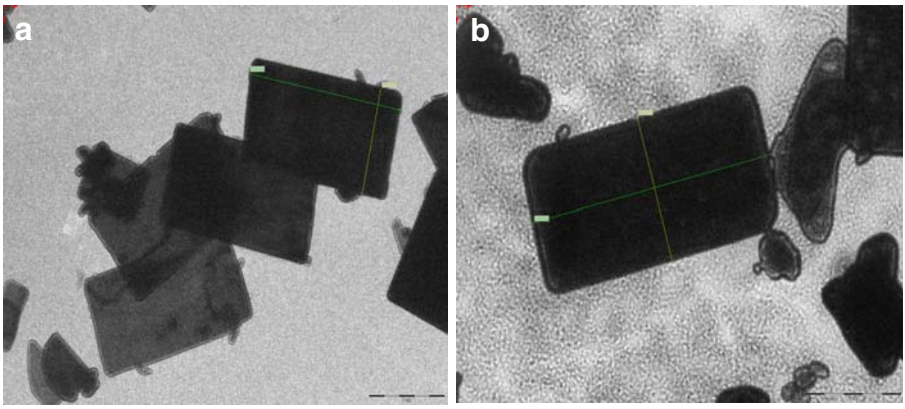


**Fig. 1** Transmission electron micrographs. The PAL PCMC and $K_2SO_4$ microcrystals were prepared as described in the Materials and methods section. These were visualized under TEM after negative staining. **a** PAL PCMC; **b** $K_2SO_4$ microcrystals (control). *Scale bar* 1 μm

were of uniform rectangular shape (length 2.06 μm and breadth 1.19 μm). As a control, TEM of $K_2SO_4$ crystals formed in the absence of protein was also recorded, which showed larger-sized crystal (length 0.53 μm; breadth 1.31 μm) without protein coating (Fig. 1b). The smaller size in case of PAL PCMC is due to the fact that protein molecules retard the growth of normal $K_2SO_4$ crystals by adhering onto their surface. Similar reduction in size due to poisoning effect of protein upon PCMC formation has been reported by Kreiner et al. [9].

As the objective of the study was to develop a catalytically efficient, stable, and reusable lipase, the characteristics of the PAL PCMC were investigated.

Media optimization was performed by assaying the PAL PCMC in different organic solvents. The *p*NPP assay was carried out in *n*-hexane (log *P*=3.5), *n*-heptane (log *P*=4), and *iso*-octane (log *P*=4.5) as solvent medium. PAL PCMC showed a maximum activity and linear trend for *p*NPP hydrolysis in heptane medium. Henceforth, all the characterization was carried out in *n*-heptane medium.

PAL PCMC exhibited an 11.5-fold increase in rate of hydrolysis compared to free enzyme. Kinetic constants $K_m$ and $V_{max}$ for PAL PCMC and free lipase were found to be 679.82 and 589 mmol and 5.66 and 0.49 nmol $min^{-1}$ $mg^{-1}$, respectively (Fig. 2). On immobilization, kinetic parameters $K_m$ and $V_{max}$ undergo variations with respect to the corresponding parameters of the free form, revealing an affinity change for the substrate. These variations are attributed to several factors such as protein conformational changes induced by the support, steric hindrances, and diffusional effects. These factors may operate simultaneously or separately, alternating the microenvironment around the bound enzyme [14].

The significant enhancement in the $V_{max}$ value for PAL PCMC reflects its high hydrolytic efficiency in organic medium. A highly attractive added feature of lipase immobilization by PCMC is increase in catalytic activity of the enzyme. In addition, the PCMC are reusable preparations [8].

The enzymatic efficiency of free and immobilized forms of *P. aeruginosa* lipase (PAL free and PAL PCMC) and commercially available *C. rugosa* lipase (CRL free and CRL PCMC) were compared for hydrolysis of *p*NPP in nonaqueous medium by monitoring the release of *p*-nitrophenol (Fig. 3). Matching enzyme activities were used. CRL PCMC

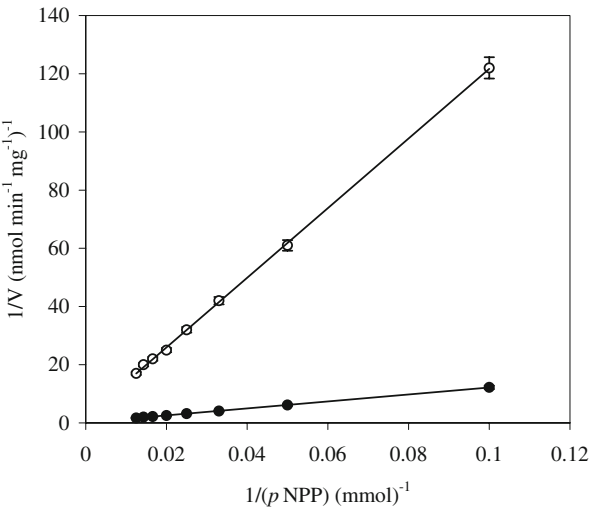


**Fig. 2** Lineweaver–Burk plot for free and immobilized lipase. The kinetic constants $K_m$ and $V_{max}$ for PAL free and PAL PCMC were determined by assaying them at various concentrations of *p*NPP substrate in *n*-heptane medium as described in the Materials and methods section. PAL free (*empty circles*), PAL PCMC (*filled circles*)

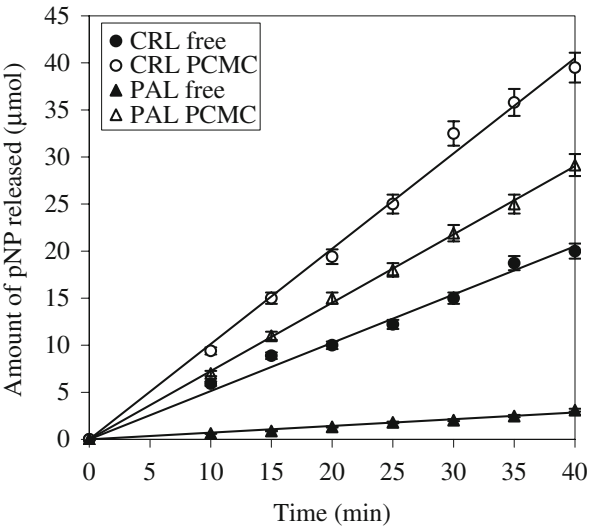


**Fig. 3** Hydrolytic efficiency of free and immobilized lipase. The rate of *p*NPP hydrolysis in heptane medium was determined for free and immobilized *C. rugosa* and *P. aeruginosa* lipases as described in the Materials and methods section. CRL free (*filled circles*), CRL PCMC (*empty circles*), PAL free (*filled triangles*), PAL PCMC (*empty triangles*)

showed 1.8 times more hydrolysis than that of its free form. Figure 3 also showed that PAL PCMC were ten times more efficient than the free form. The significantly enhanced activity of PCMC compared to free enzyme in organic media may be attributed to the probable extra hydration achieved by the water-retaining capacity of salt maintaining enzyme flexibility. Coated microcrystals of *Candida antarctica* and *Mucor miehei* lipase have been reported to have 5- and 15-fold increase in catalytic activity [9]. Kreiner et al. found

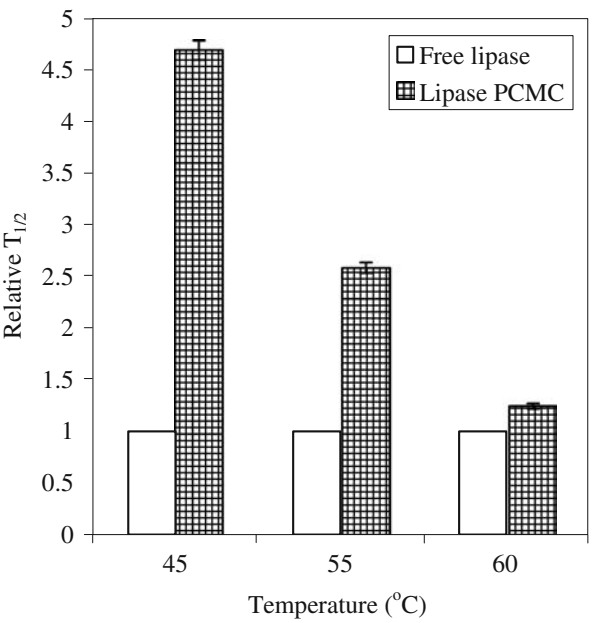


**Fig. 4** Thermal stability of lipase PAL free and PCMC. The thermal stability was determined by incubating lipases at different temperatures (45°C, 55°C, 60°C). Residual lipase activity was determined in the aliquots withdrawn at time intervals, as described in the Materials and methods section. Relative half-lives ($T_{1/2}$): PAL free (*empty bars*), PAL PCMC (*crossed bars*)

*Pseudomonas* sp. lipase PCMC to be 200-fold more active than the free preparation for kinetic resolution of (±)-1-phenylethanol.

Other kinetic properties were less altered upon immobilization. PAL PCMC did not show any change in the pH optima (pH 8.0) and showed only slight increase in temperature optima (40°C to 45°C). Similar results have been observed for *C. rugosa* and *Bacillus* sp. lipases [15, 16].

Thermal stability data in Fig. 4 show enhancement in stability after immobilization as PAL PCMC. The half lives ($T_{1/2}$) at 45°C, 55°C, and 60°C enhanced by 4.65-, 2.56- and 1.24-fold over the free lipase. This acquired stability will extend the applicability of *P. aeruginosa* lipase at higher temperatures.

As the PAL PCMC worked as better catalyst for hydrolysis of *p*NPP in *n*-heptane medium, it was worthwhile to investigate its synthetic activities as well. Preliminary experiments to synthesize industrially important esters by PAL PCMC revealed enhancement in esterification activity by 1.4- and twofold for butyl butyrate and ethyl butyrate synthesis, respectively. The product yields were low. Further experiments on this aspect are in progress.

From the present study, it is concluded that the simple technique of PCMC formation proved to be effective to obtain fairly stable PAL PCMC preparation for efficient catalysis in nonaqueous medium.

**Acknowledgement** The financial support granted by Department of Biotechnology (DBT), Department of Science and Technology (DST) and Ministry of Human Resource Development (MHRD), Govt. of India, is gratefully acknowledged. Authors are thankful to Council for Scientific and Industrial Research (CSIR), Govt. of India, and IIT Delhi for research fellowship.

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