Indian Journal of Biotechnology
Vol. 4, April 2005, pp. 222-226

Lipase from *Pseudomonas aeruginosa* MTCC 2488: Partial purification, characterization and calcium dependent thermostability

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Received 30 September 2003; revised 19 April 2004; accepted 5 May 2004

Psuedomonas aeruginosa MTCC 2488 produced lipase on Rhodamine B agar plates containing olive oil. Extra-cellular lipase activity was analyzed spectrophotometrically using Tween-20 as well as olive oil as substrate. The semipurified enzyme, precipitated by 30% saturated ammonium sulphate, showed 20.79 fold increase in specific activity (U/mg) and reduction in carbohydrate content to 1.7% as compared to the crude enzyme. The enzyme hydrolyzed Tween-20 and -40 better than Tween-60 and -80. Lipase has been found to be thermostable with maximum activity at 55-60^{o}C but marked decrease was observed above this temperature. Ca^{2+} seemed to play an important role in the thermostability as 97% of enzyme activity was retained after 2 hr incubation at 65^{o}C and 1hr incubation at 70^{o}C in presence of 10 m*M* $CaCl_2$. However, thermostability of the enzyme was decreased considerably in presence of 5 m*M* EDTA, confirming the enzyme to be a metalloprotein. Lipase has been found to be stable in presence of 30% acetone, methanol and ethanol. While, the enzyme activity was decreased by 30-50% in presence of n propanol, 2 propanol, n methyl propanol, isooctane and hexane. Further, 30% butanol resulted in ~65% decrease in the enzyme activity. Lipase has been found to be stable in presence of nonionic detergents, whereas anionic detergent, SDS completely inactivated the enzyme.

Keywords: calcium dependent thermostability, lipase, lipase activity, *P aeruginosa*, Tween-20
IPC Code: Int. Cl.[7] A01N63/02; C12N9/16, 9/20; C12R1: 385

Introduction

Lipases (triacylglycerol acylhydrolases; EC 3.1.1.3), which are widely distributed in animals, plants and microorganisms, exhibit great potential for commercial applications for they catalyze production of free fatty acids, interesterification of oils and fats as well synthesis of esters and peptides[1-3]. Lipases are available with a wide range of properties depending upon their source[4,5]. Based on the fact that certain reactions are performed most efficiently at higher temperatures and in organic solvent phase, attempts have been made to find thermostable lipases, which would have advantages over labile enzymes in such applications[6-8]. In this regard, microbial lipases have currently received considerable attention. A wide variety of Gram positive and negative bacterial species are reported to produce lipases but the most widely used enzymes originate from genus *Bacillus* and *Pseudomonas*[3].

In the present study, lipase from *P. aeruginosa* MTCC 2488 has been isolated, partially purified and characterized with respect to its stability at different temperatures and in presence of organic solvents and detergents.

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Materials and Methods

Bacterial Strain

P. aeruginosa MTCC No. 2488, procured from Institute of Microbial Technology, Chandigarh, was maintained in nutrient broth (NB). The strain was grown on nutrient agar containing olive oil (1% w/v) and Rhodamine B 0.001% (w/v) to assay the production of lipase[9].

Partial Purification of Lipase Enzyme

P. aeruginosa was grown in NB for 20 hrs at 30°C. The cell free supernatant, prepared by centrifugation (6000 rpm, 20 min), was passed through a 0.45 μm pore size membrane and then ammonium sulphate was added to achieve 30% saturation. The suspension was further centrifuged (6000 rpm, 20 min, 4°C) and ammonium sulphate was added to the supernatant to reach 80% saturation. The precipitates (0-30% and 30-80%), collected by centrifugation, were separately dissolved in minimal volume of 20 m*M* Tris buffer

(*p*H 7.0) at 4°C and the solution was dialyzed against the same buffer to remove residual ammonium sulphate. Lipase activity in both the fractions was analyzed spectrophotometrically using Tween-20 as well as olive oil as substrate. Protein[10] and carbohydrate[11] contents of the enzyme were measured. The partially purified enzyme was used for further experiments.

Lipase Assay

Lipase activity of *P. aeruginosa* in NB was measured spectrophotometrically using either Tween-20, ester of lauric acid[12] or olive oil[13] as substrate.

Effect of Temperature on Lipase Activity and Stability

The optimum temperature for enzyme activity was determined using Tween-20 as substrate by incubating the reaction mixture at various temperatures. Precipitates thus formed were measured turbidimetrically at 400 nm. Thermostability of the enzyme was determined by incubating aliquots of semipurified lipase solution in 20 m*M* Tris buffer (*p*H 7.0) at various temperatures for 0, 1, 2 and 3hrs. Residual activity was measured using Tween-20 as substrate. To examine the effect of calcium ions or EDTA on the stability of enzyme, the residual activity of the enzyme was assayed after it was incubated at various temperatures in presence of 10 m*M* $CaCl_2$ or 5 m*M* EDTA[14].

Effect of Detergents and Organic Solvents on Lipase Activity

The effect of various detergents on lipase activity was analyzed by incubating the enzyme at 30°C in 20 m*M* Tris buffer containing 1% (w/v) detergent for various time intervals[15]. The control contained no detergent. Activity was measured by using olive oil as substrate.

The effect of organic solvents on the lipase activity was analyzed by incubating the enzyme mixture with organic solvents (30%) for different time intervals at 30°C. The control contained no organic solvent. Residual activity was measured using Tween-20 as substrate. All the measurements were performed six times and average value was calculated.

Results and Discussion

In the present work, *P. aeruginosa* strain MTCC 2488 procured from IMTECH, Chandigarh was used for the production of lipase enzyme. Lipase production by the organism was confirmed on Rhodamine B agar plates containing olive oil. The colonies showed orange coloured fluorescence when exposed to UV light. This is due to the formation of complex between cationic Rhodamine B and the uranyl fatty acid ion.

The enzyme was partially purified using ammonium sulphate precipitation and the maximum lipase activity was recorded in the fraction precipitated by 30% saturation. As compared to the crude fraction, the specific activity of the semi purified enzyme (U/mg protein) increased by 20.79 fold and the carbohydrate content decreased to 1.7% (Table 1).

Hydrolysis of various Tweens by the enzyme was performed as it offers a fast and convenient technique for assaying the free fatty acids, besides providing substrate. The enzymatic hydrolysis was maximum with Tween-20 (100%) followed by Tween-40 (95.69 %). Relative hydrolytic activity reduced by approximately 12% when Tween-60 (87.39%) or Tween-80 (88.99%) was added to the reaction mixture as substrate, showing the preference of enzyme towards lauric (12 C) and palmitic acids (16 C). For further experiments Tween-20 was used as substrate.

Generally, lipases of *Bacillus* and *Pseudomonas* strains are found relatively thermostable[6,16,17]. In the present study, lipase of *P. aeruginosa* MTCC 2488 has also been found to be thermostable. The optimum temperature for the activity of the enzyme was 55-60°C when Tween-20 was used as the substrate. Above the optimum temperature, the activity decreased very quickly (Fig. 1).

The enzyme showed fairly stable activity up to 60°C but a marked decrease was observed above this temperature (Fig. 2). On the other hand, presence of calcium ions (10 m*M*) in the reaction mixture stabilized the enzyme activity. Approximately 97%

Table 1—Partial purification of lipase

Purification steps	Carbohydrate content (μg/ml)	Total protein (mg)	Activity (U/ml)	Total activity	Specific activity (U/mg)	Purification folds
Crude enzyme (400 ml)	222	882	277.53	111012	125.86	1
Ammonium sulphate precipitated (15 ml)	3.75	5.59	975.67	14635.03	2616.44	20.79

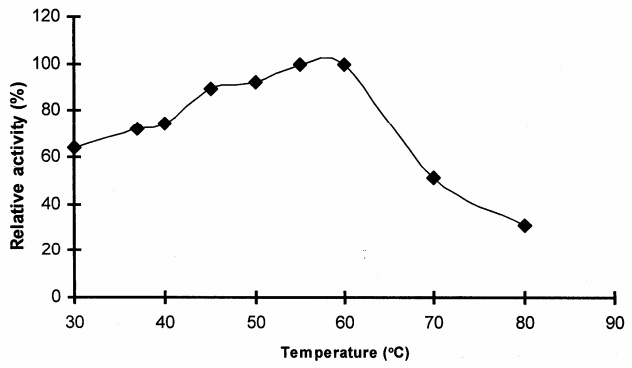

Fig. 1—Effect of temperature on lipase activity. Activity was measured using Tween 20 as substrate and the reaction mixture was incubated at various temperatures for 2hr. Precipitates formed were measured turbidimetrically at 400 nm.

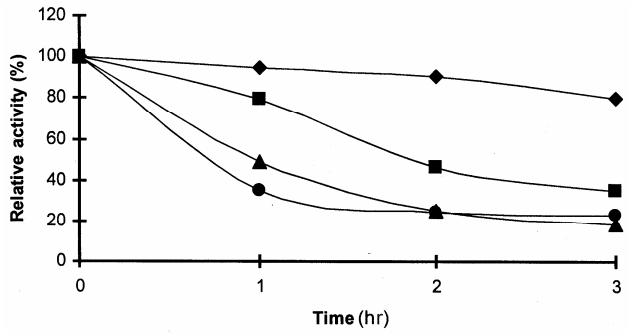

Fig. 2—Effect of temperature on lipase stability. Enzyme was incubated at various temperatures for 0, 1, 2 and 3hr and the activity was measured using Tween 20 as substrate (—♦— 50°C, —■— 60°C, —▲— 70°C, —●— 80°C).

enzyme activity was retained after 2 hr incubation at 65°C and after 1 hr at 70°C (Fig. 3). However, the thermostability of the enzyme decreased considerably in presence of 5 m*M* EDTA with only 32.48, 30.15 and 10.35% residual activity left at 60, 65 and 70°C, respectively after 1hr incubation, while the control figures for the same were 79.26, 48.73 and 34.87% (Fig. 3). Inhibition of activity by EDTA suggested that the lipase might be a metalloprotein. Lipase produced by *P. aeruginosa* KKA-5 has also been found to be Ca^{2+} dependent[18]. Enzyme having lost its activity in presence of EDTA could be reactivated by the addition of $CaCl_2$. Kim *et al*[14] have also shown reported an increase in the thermostability of lipase enzyme from *B. stearothermophillus* by about 8-10 degrees in the presence of calcium ions. In case of *P. aeruginosa* MTCC 2488, calcium ions have also shown stimulatory effect on the cell growth and lipase production as enzyme activity increased by 1.8 times compared to control on addition of calcium ions in the media (data not shown).

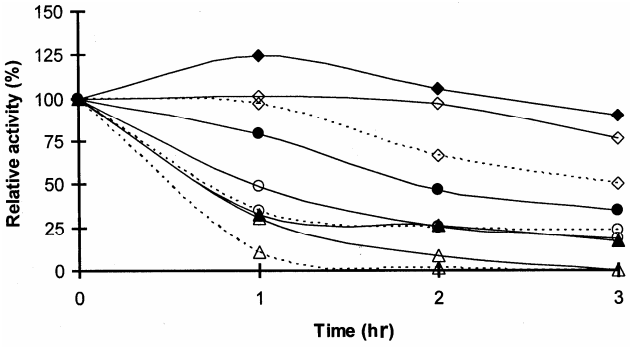

Fig. 3—Effect of $CaCl_2$ and EDTA on thermostability of lipase enzyme. Enzyme was incubated at various temperatures in presence of 10 m*M* $CaCl_2$ or 5m*M* EDTA for different time intervals. Activity was measured using Tween 20 as substrate. ($CaCl_2$: —♦— at 60°C, —◊— at 65°C, ---◊--- at 70°C; EDTA: —▲— at 60°C, —Δ— at 65°C, ---Δ--- at 70°C; Control: —●— at 60°C, —○— at 65°C, ---○--- at 70°C)

Lipases catalyze reactions involving triglyceride hydrolysis, ester synthesis, transesterification and interesterification of fats, etc. Since substrates of such reactions are often insoluble in aqueous solution, organic solvents or organic aqueous two-phase media are required. However, enzymes generally denature in the presence of organic solvents and require methods for stabilizing[19]. On the other hand, the enzymes that remain stable in the presence of organic solvents could be industrially very useful. Lipase produced by *P. aeruginosa* MTCC 2488 showed good stability in organic solvents (Table 2). Incubation of the enzyme in a strong dehydrating solvent, like acetone, methanol or ethanol, did not cause much loss of its activity; 90% activity remaining after 1h incubation. However, n propanol, 2 propanol, n methyl propanol, isooctane and hexane resulted in considerable loss in in activity (30-50%). A sharp decrease in activity was observed in the presence of butanol. Lipases produced by other *Pseudomonas* species showed varied stabilities in organic solvents. Enzyme produced by *P. aeruginosa* KKA-5 was found stable in the presence of acetone, methanol and ethanol[18]. While, lipase from psychotroph *Pseudomonas* sp. (strain B11-1) showed increased activity in methanol, which decreased sharply in ethanol and acetonitrile[20]. Other lipases from *Pseudomonas* and *Bacillus* could also be activated in the presence of several water miscible organic solvents[21]. It has been proposed that organic solvents disaggregate the enzyme and keep it in open conformation. This may lead to enhanced enzyme activity or at least stabilization of the enzymes. However, certain organic solvents completely change

Table 2—Effect of organic solvents on lipase activity

Organic solvents used	Relative activity (%)				
	0 hr	0.5 hr	1 hr	1.5 hr	2 hr
Control	100	100	100	100	100
Acetone	100	93.25	92.96	92.75	92.65
Methanol	99.32	92.32	89.84	90.25	90.35
Ethanol	90.45	90.43	88.83	88.65	88.92
n Propanol	65.36	60.91	50.99	49.27	48.65
Butanol	50.23	40.27	37.57	36.53	36.62
2 Propanol	85.63	79.23	78.18	78.23	79.07
N methyl propanol	84.53	79.45	73.31	73.67	72.25
Isooctane	69.27	67.71	66.89	67.01	67.53
Hexane	70.57	63.97	60.76	60.21	59.89

Table 3—Effect of detergents on lipase activity

Detergents used	Relative activity (%)				
	0 hr	0.5 hr	1 hr	1.5 hr	2 hr
Control	100	100	100	100	100
Tween-20	101.35	104.54	104.36	105.22	105.26
Tween-40	105.48	103.26	100.89	101.02	101.10
Tween-60	99.88	90.72	84.63	82.52	82.32
Tween-80	114.52	110.86	103.72	100.81	100.35
Triton-X-100	94.61	91.82	91.85	91.67	91.65
SDS	14.35	9.05	0.205	0.105	0.00
Brij-35	80.235	60.72	58.62	54.84	54.69

the enzyme structure, resulting in the inactivation of the enzyme[22].

The effect of detergents was studied after incubating the enzyme with 1% detergent at 30°C for varying time intervals. Addition of non-ionic detergents, like Tween-20, -40 and -80, did not alter the lipase activity. Instead, the lipase activity was enhanced initially on their addition (Table 3). On the other hand, Tween-60 and Triton-X-100 resulted in 10-20% decrease in enzymatic activity, whereas Brij-35 was responsible for approximately 45% decrease. However, anionic detergent SDS resulted in complete loss of enzymatic activity. Similar effects have also been seen in case of *P. aeruginosa* KKA-5 strain[18] and *P. pseudoalcaligenes* F-111[23]. Non-ionic detergents seem to weaken the hydrophobic interactions within the protein, causing disaggregation and thus stabilizing its activity, while SDS acts upon the disulphide linkages and so causes the inactivation of the enzyme.

Thus, the lipase produced by *P. aeruginosa* MTCC 2488 can be summarized as having high working temperature, good thermostability and high stability towards organic solvents as well as detergents. By virtue of these features, this enzyme can be widely used in industries, especially in fats and oil industry.

Acknowledgement

This work was supported by a grant (No. 9/797(2)/2001-EMR1) from Council of Scientific and Industrial Research, New Delhi, India to Dr Vandana Kukreja. Laboratory facilities provided by the Director, SLIET, Longowal is also duly acknowledged.

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