Polish Journal of Microbiology
2005, Vol. 54, No 3, 233–240

Purification and Characterization of Two Extracellular Lipases from *Pseudomonas aeruginosa* Ps-x

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Received 2 March 2005, received in revised form 25 May 2005, accepted 27 May 2005

Abstract

Two different extracellular lipases were isolated and purified from *Pseudomonas aeruginosa* Ps-x to apparent homogeneity using ammonium sulfate precipitation followed by ion exchange chromatography on Q- and S-Sepharose column. Both of the purified lipases are monomeric protein with molecular weight of 15.5 and 54.97 KDa respectively. The optimal activities of the enzymes were at 45 and 50°C and pHs 10.0 and 9.0. Calcium ions increase thermostability of both purified lipases I and II. The purified lipase I showed no metal ion dependence for its activity since EDTA up to 10 mM has no effect on the enzyme activity. However purified lipase II showed slight inhibition by EDTA at the same concentration. Moreover, a serine protease inhibitor, PMSF showed an inhibitory effect on both purified enzymes.

Key words: Lipases, *Pseudomonas aeruginosa* Ps-x, 16S rRNA

Introduction

Lipases (triacyl glycerol acylhydrolases, EC 3.1.1.3) constitute a diverse and ubiquitous family of enzymes that in biological system initiate the catabolism of fats and oils by hydrolyzing the fatty acylester bonds of acylglycerols (Carriere *et al*., 1994 and Jose *et al*., 2004). Most of lipases remain active in a variety of organic solvents, where they can catalyze various transformations other than hydrolytic reaction by which they are defined (Margolin and Klibanov, 1987). Lipases are widely distributed in nature and have been found in many species of animals, plants, bacteria, yeast and fungi. Although their wide distribution, the enzymes from microorganisms are most interesting because of their potential application in various industries ranging from the use in laundry detergent to stereospecific biocatalysts (Maliszewska and Przemyslaw, 1992). Most of the microbial lipases are secreted into the culture medium and they differ from one another in their physical and biochemical properties. Since each industrial application requires specific properties of lipases, there is still an interest in additional lipases that could be used in new applications (Jaeger *et al*., 1994; Lambit and Goswami, 2002 and Kyu *et al*., 2005). The synthesis and secretion of extracellular lipases by various microorganisms appear to be controlled in a variety of ways which are only now beginning to be elucidated and the limited data on this subject have been reviewed by Jaeger *et al*., 1994. One of the best studied case, *P. aeruginosa*, lipase only appears in the culture medium at the end of logarithmic growth when a number of other hydrolytic exoenzymes are also released. However, lipases are produced during logarithmic growth in a minimal media when growth is dependent for carbon and energy upon hydrolysis of either a triglyceride or a detergent such as a Tween (Jaeger *et al*., 1994 and Stuer *et al*., 1986). This study described the production, purification and characterization of two extracellular lipases from *P. aeruginosa* Ps-x.

Abbreviations: SDS-PAGE; sodium dodecyl sulfate polyacrylamide gel electrophoresis, Q-Sepharose; Quaternary amino methyl Sepharose, EDTA; ethylene diamine tetraacetic acid.

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Experimental

Materials and Methods

Bacterial isolate identification. The bacterial isolate used in this study was a gift from Dr. Yossry Gohar, Department of Microbiology, Faculty of Science, Alexandria University, Egypt. This isolate was tested for lipase production using nutrient agar medium, pH 7.5 containing per liter: peptone, 10.0 g; NaCl, 5.0 g; $CaCl_2$, 0.1 g; and agar, 20.0 g. The medium was sterile by autoclaving and let to cool to 45°C after that 1% of sterile Tween-20 or Tween-80 was added to the medium and mixed well then poured (20–25 ml) into 100 mm Petri dishes. The identification of the bacterial isolate was based on cell morphology, colony morphology, growth on nutrient broth and nutrient agar as well as several biochemical tests. The identification process was performed at the Fermentation and Biotechnology Center, El-Azhar University, Cairo. To confirm the biochemical tests results for isolate identification, 16S rRNA (rDNA) technique was carried out. DNA was isolated and purified according to Sambrook *et al.*, 1989. Amplification of the 16S rDNA gene from the genome was carried out by polymerase chain reaction (PCR) using primers designed to amplify 16S rRNA gene. The forward primer was 5'-AGAGTTTGATCMTGGCTCAG-3' and the reverse primer was 5'- TACGGYTACCTTGTTACGACTT-3'. The polymerase chain reaction analysis was performed with 100 ng of genomic DNA in a final volume of 50 µl, including a reaction buffer 1x, 30 pmole of each primer, and 2 units of *Taq* polymerase. Thermocycling consisted of an initial denaturation of 5 minutes at 94°C and of 30 cycles of 1 minute at 94°C (denaturation), 1 minute at 55°C (primers annealing), and 1.5 minutes at 72°C (extension). Polymerase chain reaction products were analyzed for purity check on 1% agarose gel by electrophoresis, stained with ethidium bromide (0.5 µg/ml), and visualized using ultraviolet transillumination.

DNA sequencing. DNA was sequenced by the dideoxy chain termination method according to Sanger *et al.*, 1977 using ABI Prism Ready Reaction Dye Terminator Sequencing Kit and analyzed on an ABI 377 automated sequencer. The nucleotide sequence obtained about 326 base pairs were then analyzed using nucleotide Blast search data base and have been deposited in the GenBank sequence data base and have the accession number AF419219.

Lipase assay. Lipase activity was determined colorimetrically according to Kordel *et al.*, 1991, where two solutions were prepared for the assay. Solution 1 contained 90 mg of *p*NPP (*p*-nitrophenyl palmitate), dissolved in 30 ml propane-2-ol. Solution 2 contained 2 g Triton X-100 and 0.5 g gum Arabic dissolved in 450 ml (Tris-HCl 50 mM) buffer at pH 8.0. The assay solution was prepared by adding 1 ml of solution 1 to 9 ml of solution 2 drop wise to get an emulsion that remained stable for 2 hrs. The assay mixture contained 900 µl of the emulsion and 100 µl of the appropriately diluted enzyme solution. The liberated *p*-nitrophenol was measured at 410 nm using Novospek, Pharmacia spectrophotometer. One unit of enzyme was defined as the amount of enzyme that releases 1 µmol of p-nitrophenol from the substrate.

Protein analysis. Total protein concentrations of cell free supernatant and purified samples were assayed by the method of Bradford (Bradford, 1976) using a calibration curve established with bovine serum albumin as a standard. Proteins in these preparations were analyzed by SDS-PAGE on 10% gels according to the method of Laemmli (Laemmli, 1970). Gel analysis and molecular weight determination was done using Alpha Imager 1200 Tm gel documentation system.

Monitoring bacterial growth and extracellular lipase production. The growth and activity of the extracellular lipase was monitored throughout the growth of the *P. aeruginosa* Ps-x strain on modified Williams Basal medium II (Williams *et al.*, 1990) containing per liter: NH_4Cl, 5g; NaCl, 5g; K_2HPO_4, 3.0 g; KH_2PO_4, 4.0 g; $MgCl_2 \times 6H_2O$, 1.0 g and yeast extract 1.0 g. Deionized water was added to approximately 1 L, then the pH was adjusted to 7.5 with 10 N NaOH and autoclaved. Cells were activated by growing them overnight at 37°C on nutrient agar plates. Several recently growing colonies were transferred to a 50 ml of production medium and incubated at 37°C overnight with agitation at 150 rpm. Then, 1 ml was taken to inoculate 100 ml of the production medium and allowed to grow at 37°C with shaking at 150 rpm. Growth was monitored by measuring the absorbance at 420 nm. At the indicated time, 1.0 ml of the growing cultures was taken and centrifuged in a microcentrifuge at 8,000 rpm for 2 minutes and the supernatants were assayed for extracellular lipase using *p*-nitrophenylpalmitate as a substrate as described earlier.

Enzyme production and purification. The extracellular lipase produced by *P. aeruginosa* Ps-x was first purified by salting out precipitation using ammonium sulfate at 80% saturation. *P. aeruginosa* cells were cultured aerobically in 500 ml production medium containing per liter: 5 g NaCl, 0.05 g $CaCl_2 \times 2H_2O$, 10 g yeast extract and 10 ml Tween 20 for 18 hours at 37°C with agitation at 150 rpm. The culture was then centrifuged at 8,000 rpm for 20 minutes at 4°C using the small SS-34 small rotor. Solid ammonium sulfate was then added slowly to the culture filtrate at 70% saturation with gentle stirring on ice bath. After that, the mixture was allowed to stand at 4°C for overnight. The mixture was then centrifuged at 12,000 rpm for 30 minutes using the above rotor. Pellet was dissolved in 5 ml 0.02 M Tris-HCl pH 8.0 and dialyzed overnight against 2 liters of the same buffer. The protein content and the lipase activity were determined as described earlier. The concentrated dialyzed cell free supernatant was then applied to a column (2.5×7 cm) containing Quaternary amino methyl Sepharose fast flow (Q-Sepharose), which previously was equilibrated with 20 mM Tris-HCl, pH 8.5. The column was washed with 3 bed volumes of the same buffer at a flow rate of 60 ml/hour and the bound proteins were eluted with a linear gradient of NaCl (0–1.0 M) in the same buffer. Active fractions that contain lipase enzyme were then polled and concentrated using ammonium sulphate as mentioned before. The concentrated and dialyzed enzyme was further purified using S-Sepharose fast flow column (2.5×5 cm) prequilibrated with 20 mM Tris-HCl, pH 8.5 at a flow rate of 45 ml/hour. The column was washed with three bed volumes of the same buffer and the bound proteins were eluted using linear gradient of NaCl (0–0.5 M) in the same buffer. Lipase containing fractions were polled and concentrated as described before.

pH optima, temperature and thermostability studies. Extracellular lipase optimum pH was determined over a pH values range from 6.0 to 10.0. Sodium phosphate buffer 0.1 M was used for pH 6.0 and 7.0, Tris-HCl 0.1 M for pH 8.0 and 9.0 and carbonate buffer 0.1 M for pH 10.0 and 11.0. The temperature optimum of the purified *P. aeruginosa* lipases was determined over a temperature range of 25–90°C in 50 mM Tris-HCl buffer pH 8.0. Thermostability of the purified enzymes was examined at a temperature range 40–70°C for different time intervals (10–60 minutes) in 50 mM Tris-HCl buffer pH 8.0 in absence and in the presence of 5.0 mM $CaCl_2$. The residual activity was determined by taking 25 ml of the enzyme solution after specified time (10–60 minutes) and the assay was carried out as described before.

Effect of some compounds on the activity of the purified enzymes. To examine the effect of EDTA (ethylenediamine tetraacetic acid), SDS (sodium dodecylsulphate), PMSF (phenylmethane sulfonyl fluoride) and DMSO (dimethyl sulfoxide) on

the activity of the purified lipases, different concentrations of these compounds were prepared and the enzymes solution were pre-incubated with these compounds for 30 minute on ice after which the residual activity was determined as described before using *p*-nitrophenyl palmitate substrate.

Effect of some metal ions on the activity of the purified enzymes. For determining the effect of metal ions on lipase activity, the purified enzymes were pre-incubated with 1–10 mM of the following metal ions $MgCl_2$, $ZnCl_2$, $CaCl_2$ and NaCl for 30 minute on ice and then the residual activity was determined.

Results and Discussion

Identification of bacterial isolate. Based on cell morphology and colony morphology, growth on nutrient broth and nutrient agar as well as several biochemical tests, the bacterial isolate was identified as *Pseudomonas aeruginosa.* To confirm the biochemical tests results for bacterial isolate identification, 16S rRNA methodology was carried out. The sequencing data obtained utilizing this strategy indicated that the isolate under study was 98% *P. aeruginosa* and the nucleotide sequence was deposited in the GenBank database, and given the accession number AF419219.

Monitoring the bacterial growth and extracellular lipases enzymes production. The growth and extracellular lipase production level of *P. aeruginosa* Ps-x was monitored on modified William's Basal medium II supplemented with 2% Tween-20. Figure 1 illustrates the growth behavior with incubation time and lipase production. The level of extracellular lipase enzyme production of *P. aeruginosa* increased gradually at the end of log phase (6 hours after inoculation) and reached its maximum level (74.66 U/ml) after 48 hours of inoculation. It was reported that the extracellular lipase production normally appears in the fermentation medium when the bacterial cell growth reach to the end of the logarithmic. Moreover, the observation of similar growth rates for *Pseudomonas* strains during the logarithmic growth phase is in accordance with the concept that the production of these enzymes is advantageous to the microorganism only when nutrients become limiting *i.e.*, during the late log phase or early stationary phase (Lee and Rhee, 1993).

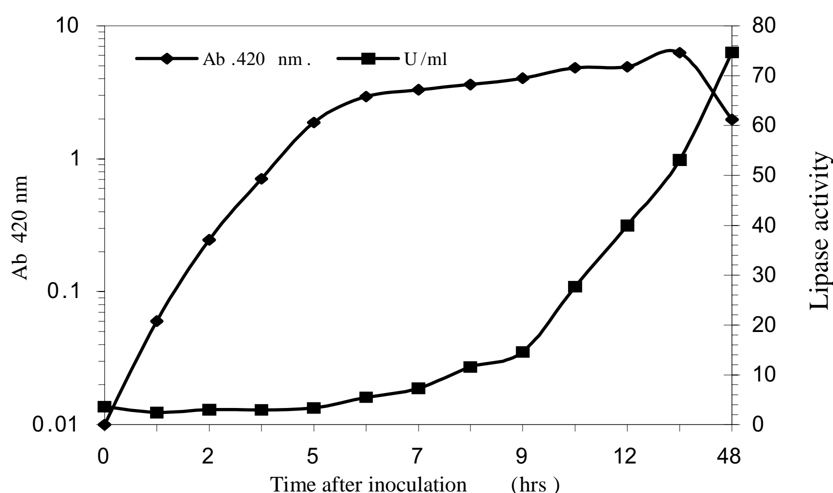

Fig. 1. Monitoring of growth and extracellular lipase production by *P. auroginosa* strain Ps-x on modified basal medium

Purification and characterization of *P. aeruginosa* extracellular lipases. *P. aeruginosa* lipases enzymes were purified using $(NH_4)_2SO_4$ selective precipitation (70%) followed by ion exchange chromatography on Q and S-Sepharose Fast Flow columns. It was found that the unbound proteins were eluted first using 20 mM Tris-HCl, pH 8.5 buffer giving only one protein peak that overlapped with the lipolytic activity. Upon using NaCl gradient (0.0–1.0 M) in the same buffer, one major and one minor protein peaks appeared as shown in Figure 2. Surprisingly, the minor protein peak showed a lipolytic activity as well. This result indicated that, at least *P. aeruginosa* Ps-x strain under study produced two extracellular lipases of different biochemical characters. Thus, upon using Q-Sepharose Fast Flow anion exchanger, lipase enzyme contained in the first peak (LipI) was purified to 6.36 fold with specific activity of 165.5 (Units/mg protein/ml). While lipase enzyme contained in the second minor peak that eluted with NaCl gradient (LipII)

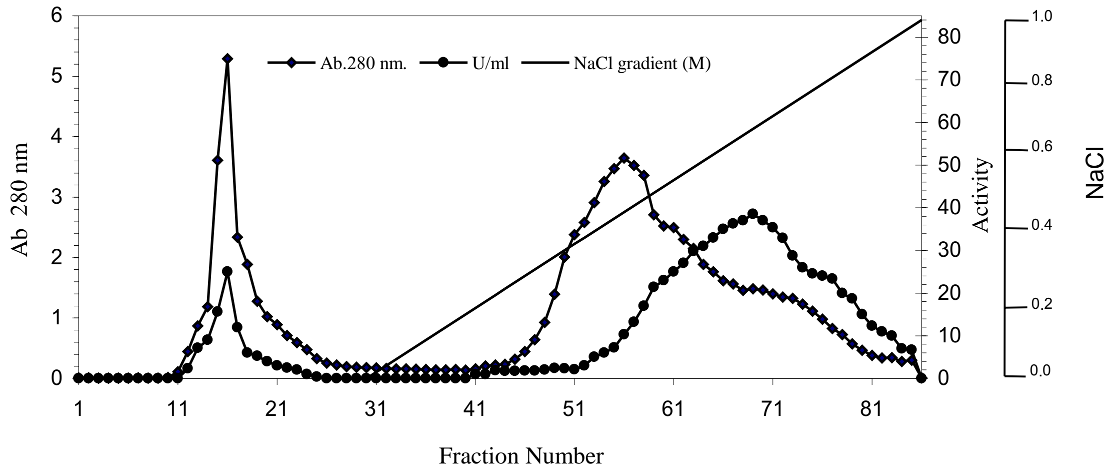

Fig. 2. Fractionation pattern of the extracellular lipase enzyme produced by *P. aeruginosa* upon using Q-Sepharose Fast Flow anion exchange column

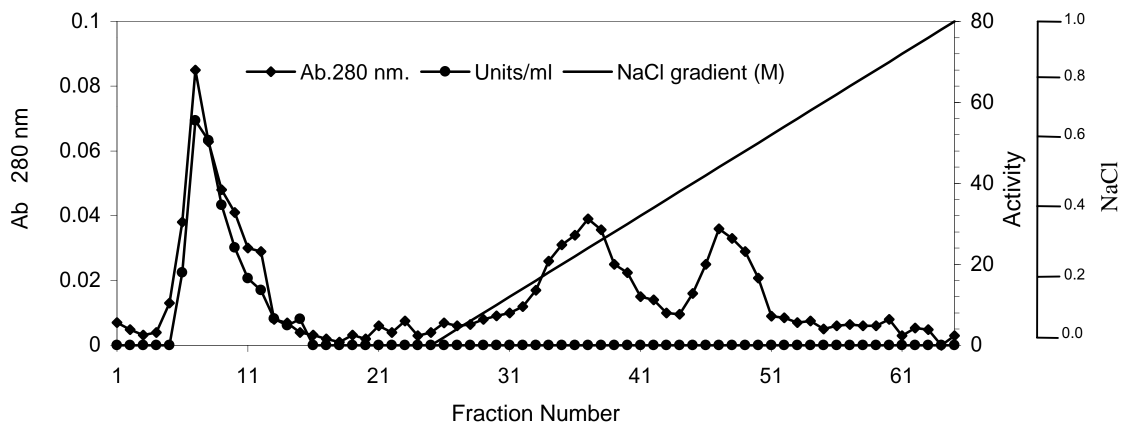

Fig. 3. Fractionation pattern of extracellular lipase II (LipII) from *P. aeruginosa* on S-Sepharose Fast Flow column

was found to be purified to almost 3.77 fold and with specific activity of 98.2 (Units/mg protein/ml) as indicated in Table 1. Interestingly, the eluted lipase I (LipI) was found to be almost pure enzyme as judged by SDS-PAGE as shown in Figure 4 just by selective precipitation with ammonium sulphate followed by anion exchanger chromatography on Q-Sepharose column. Figure 3 show the fractionation pattern of lipase II on S-Sepharose cation exchanger. It was found that; one distinctive protein peak was appeared in the elution buffer 20 mM Tris-HCl pH 7.4 that overlapped with the lipase activity. By applying NaCl gradient

Table I
Purification of extracellular lipases produced by *P. aeruginosa* Ps-x

Purification steps	Volume (ml)	Lipase activity Units/ml[a]	Total Units	Protein content mg/ml	Specific activity U/mg protein	Fold purification	Recovery (%)
$(NH_4)_2SO_4$ concentrated and dialyzed enzyme	18	496.5	7448	19	26	1	100
Q-Sepharose Column							
First Peak (Lipase I)	8	503.2	4025.6	3.04	165.5	6.36	54
Second Peak (Lipase II)	20	88.4	1768.6	0.9	98.2	3.77	23.7
S-Sepharose Column							
Second Peak (Lipase II)	18	38.4	691.2	0.1	384	14.76	9

[a] One unit of enzyme is the amount of enzyme that liberate one micromole of ρ-nitrophenol per minute at 37°C

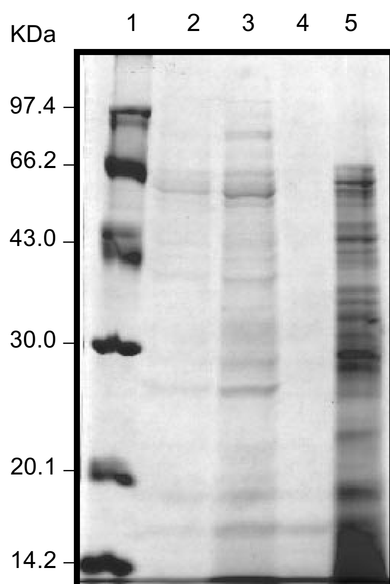

Fig. 4. Sodium dodecyl sulfate polyacrylamide gel (10%) electrophoresis of *P. aeruginosa* cell free supernatant (Lane 2), ammonium sulfate concentrated and dialyzed sample (Lane 3), Q-Sepharose purified lipase I (Lane 4), and Q-Sepharose unbound eluted proteins (Lane 5). Lane 1 represents molecular weight markers protein

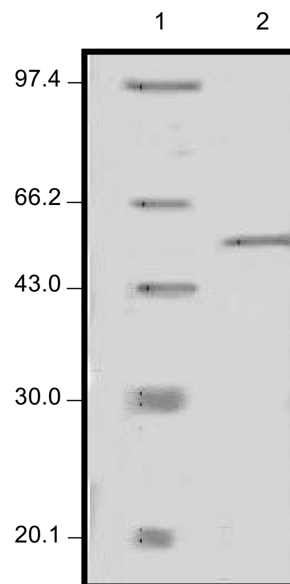

Fig. 5. Sodium dodecyl sulfate gel (10%) electrophoresis of purified lipase II from *P. aeruginosa* (lane 2). Lane 1 represents molecular weight markers protein

(0–1.0 M) in 20 mM Tris-HCl pH 7.4, two major protein peaks appeared that retained no lipolytic activity. Ion-exchange chromatography on S-Sepharose Fast Flow resulted in increase in both the specific activity and fold purification, 384 and 14.74 versus 98.2 and 3.77 after Q-Sepharose Fast Flow column (Table I). SDS-PAGE showed that both of the purified lipases were composed of a single type of subunit with a molecular weight of 15.5 (lipase I) and 54.97 KDa (lipase II) respectively as shown in Figure 4 and 5.

pH and temperature optima of the purified lipase. The pH range of purified *P. aeruginosa* lipase I and II was detectable over a wide range between 6–10 with an optimum pH value at 9.0 and 10 respectively. It has been reported that most of lipases produced by *Pseudomonas* sp. have pH optimum around pH 7.0−9.5 (Lee and Rhee, 1993; Dong *et al*., 1999). It was found that, *P. aeruginosa* purified lipase I showed lipolytic activity over a wide range of temperature from 40 to 70°C with an optimum temperature of 50°C which is in agreement with that of *P. aeruginosa* EF2 and significantly higher than that of *Pseudomonas* PACIR and psychrotrophic strain of *P. fluorescens* (Gibert *et al*., 1991; Yong and Rhee, 1993 and Lee *et al*., 1993). On the other hand, purified lipase II showed an optimum temperature of 45°C.

Temperature and pH stability of the purified enzymes. Temperature stability of the purified *P. aeruginosa* lipase I and lipase II was investigated for a period of one hour at temperature range from 40−70°C in absence and in the presence of calcium ions. It was noticed that, the residual activity for both lipase I and lipase II decreased as the exposure time and temperature increased in absence of calcium chloride. It was found that, after 60 minutes of exposure to 70°C, purified lipase I retained about 47.74% of its original activity. On the other hand purified lipase II retained about 36.7% of its original activity after exposure to 70°C for 60 minutes. Thermostability in the presence of calcium ions indicated that, at a temperature of 40 and 50°C, $CaCl_2$ not only stabilize lipase I against thermal inactivation but also enhanced the lipolytic activity of the purified enzyme (146.19 and 116.4% respectively after exposure time of 60 minutes). On the other hand, $CaCl_2$ at the same concentration and under identical assay condition had no effect on thermostability of purified lipase II at all studied temperatures (from 40–70°C). These results indicated that *P. aeruginosa* lipase I that have a molecular weight of 15.5 KDa, can be stabilized against thermal inactivation by the addition calcium chloride ion while calcium chloride ion has no effect on the thermostability of the purified lipase II (molecular weight 54.97 KDa). The effect of metal ions on thermostability and activity of some enzyme has been investigated before and it has been reported that metal ions such as Ca^{2+}, Mn^{2+} and Co^{2+} can stabilize and activate some enzymes such as xylose isomerases which bind two metal ions, one cation is directly involved in catalysis and the second is mainly structural (Whitlow *et al*., 1991 and Kasumi *et al*., 1982). The role of calcium ions in relation to thermostability was also investigated

and extensively studied in *Thermoactinomyces vulgaris* subtilisin-type serine-protease thermitase and it was found that, thermitase contains three Ca^{2+}-binding sites; one of them is not present in its mesophilic homologues (Teplyakov *et al.*, 1990). A thermophilic homologue of thermitase, the *Bacillus* AK1 protease, contains one more Ca^{2+} than thermitase does, and it is significantly more kinetically stable than thermitase in the presence of Ca^{2+}. It has been reported that, in certain *P. aeruginosa* lipase, the Ca^{2+} ion might be involved in the correct positioning of the histidine residue of the catalytic triad, since three of the Ca^{2+} ion ligand are contained in a loop together with the histidine residue (Mohamed *et al.*, 2003 and Umesh *et al.*, 2003). Thus, it was clear from these results that, both *P. aeruginosa* purified lipase I and II can be affected by the exposure to temperature in presence and in absence of $CaCl_2$ metal to different degree. Difference in thermostability of both enzymes may be explained by the presence of metal binding site that can bind for example calcium ions and hence increase thermostability and enhance the lipolytic activity of purified lipase I but not lipase II that lack this binding site.

One of the most important characteristic features of the enzymes involved in detergent industries is the pH stability of these enzymes. Stability of *P. aeruginosa* lipases against pH was examined at pH's range from 6–10 for 20 days (480 hours) at room temperature and it was found that, both enzymes are stable at pHs from 6–9 for 20 days. Interestingly, both enzymes showed some sort of activation after 36 hours of storage at pHs from 6–8 and this activation phenomenon increased gradually with the incubation time at indicated pHs. On the other hand both purified lipase I and II showed pH instability at pH 10 and were gradually inactivated as the incubation time increases at pH 10 and the residual activities were found to be 28.69 and 50.0 % for lipase I and II respectively after 20 days. One possible explanation for this observation is that different pH values affected the ionization properties of some amino acid residues in the enzyme, which may be resulted in the variation of the activity and stability of the enzymes (Dong *et al.*, 1990).

Effect of some metal ions and some compounds on lipase activity. The effect of some metal ions on the activities of *P. aeruginosa* purified lipases was also investigated. It was found that, NaCl, $CaCl_2$ and $MgCl_2$ enhance the lipolytic activity of purified lipase I and II and the residual activity was higher in the case of $CaCl_2$ than that of NaCl and $MgCl_2$ (Table II). This observation is also exhibited by other *Pseudomonas* lipases (Fox and Stepaniak, 1983 and Yamamoto and Fujiwara, 1988). The observation that lipase activity was significantly enhanced in the presence of these metal ions probably reflects the ability of these salts to react with free fatty acids adhering to the oil droplets to diminish interfacial charge effects and/or to increase droplet surface area. Interestingly, $ZnCl_2$ at a concentration of 10 mM inhibits the lipolytic activity for both lipase I and II and the inhibition was higher in the case of lipase I than lipase II as shown in Table II. This inhibitory effect of Zn^{+2} is also exhibited by the other *Pseudomonas* lipases (Yamamoto and Fujiwara, 1988) and could probably be due to the direct interaction of metal with the catalytic site, although the alteration of the properties of the interface must be considered and it may be attributed to their binding of the thiol group of the enzyme or sulfhydryl groups which may be present in the active center of some microbial lipases (Schrag *et al.*, 1991).

Table II
Effect of some compounds on the activity of *P. aeruginosa* purified lipase I and II

Compound	Lipase residual activity* (%)	
	Lipase I	Lipase II
None	100	100
NaCl	111.04	101.01
$CaCl_2$	122.04	270.32
$MgCl_2$	97	108.89
$ZnCl_2$	106.72	30.64

Compound	Lipase residual activity* (%)	
	Lipase I	Lipase II
EDTA	20.03	100
DMSO	173.5	173.5
SDS	94.54	101.21
PMSF	15	41.84

* Lipase residual activity (%) reflects the percentage of enzyme activity at a given compound concentration when compared to the enzymatic activity at zero concentration and under identical assay conditions.

The effect of EDTA, DMSO, SDS and PMSF was also studied. Data of Table II indicated that the divalent metal-chelating agent, EDTA at a concentration of 10 mM showed slight inhibitory effect on the activity of lipase I and no effect on the activity of lipase II. It was reported that lipase enzyme produced by *P. aeruginosa* EF2 is not affected by metal chelating agent, EDTA which indicate that this kind of lipolytic activity is independent of metal ions (Gibert *et al.*, 1991) in contrast to some other metal ions dependent

Pseudomonas lipases (Fox and Stepaniak, 1983 and Yamamoto and Fujiwara, 1988). Dimethyl sulfoxide (DMSO) enhanced the lipolytic activity of both purified lipase I and II and this observation was agreed with the lipase of *P. aeruginosa* YS-7. It was found that sodium dodecyl sulphate (SDS) at a concentration of 10 mM showed little inhibition of purified lipase II while the same concentration had no effect on the activity of purified lipase I (94.541% and 101.219% respectively). Since most of the microbial true lipases contain a catalytic triad Ser-Asp-His, inhibition study using PMSF was carried out. PMSF at a concentration of 10 mM showed a drastic inhibitory effect on both lipases as shown in Table II and the residual activity was found to be 15% and 41.84% for lipase I and II respectively. This inhibitory effect of PMSF also exhibited for some *Pseudomonas* sp. (Schrag *et al*., 1991; van Oort *et al*., 1989 and Svendsen *et al*., 1995).

Conclusion. In the present work, two lipases from *Pseudomonas aeruginosa* Ps-x were isolated, purified and biochemically characterized. When compared with lipases from other *Pseudomonas* sp. (Stuer *et al*., 1986; Brady *et al*., 1990; Sugiura, 1977 and Mencher and Alford, 1967), these lipases differ in the following properties: molecular weight, wider stable pH and temperature ranges and purified lipase II being of higher molecular weight compared to other *Pseudomonas* sp. lipases. The satisfactory pH and thermal stability will make these two lipases very attractive for future synthetic applications. Genomic library was constructed from purified *P. aeruginosa* DNA and screened for extracellular lipases. A number of potentials positives lipases producing clones were obtained. Future research will focus on the sequencing, subcloning and overexpression of these genes in *E. coli*. Moreover, studies of the substrate specificities, stereospecificities and some aspects of the kinetics of their secretion into the fermentation medium will be undertaken.

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