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# Purification and characterization of a lipase from *Pseudomonas aeruginosa* KKA-5 and its role in castor oil hydrolysis

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**An extracellular lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) from *Pseudomonas aeruginosa* KKA-5 hydrolyzed castor oil by 90%. Purification of this castor oil-hydrolyzing lipase included ammonium sulfate precipitation and successive hydroxylapatite column chromatography. The enzyme was purified 518-fold. It was homogeneous electrophoretically and its molecular weight was estimated to be 30 kDa. The enzyme was stable up to 45°C and retained its activity in the alkaline pH range. Lipase was highly stable in the presence of aqueous organic solvents like methanol and ethanol. It was weakly inhibited in the presence of acetone. The anionic surfactant, sodium dodecyl sulfate, was inhibitory while the cationic surfactants, Triton X-100 and Tween-80 appreciably enhanced activity. Lipase was stabilized significantly by $Ca^{2+}$. Inactivation of the enzyme by EDTA was overcome by sequential $CaCl_2$ treatment. This finding suggests the existence of a calcium-binding site in *Pseudomonas aeruginosa* KKA-5 lipase.**

**Keywords:** calcium; castor oil; hydrolysis; lipase; *Pseudomonas aeruginosa*; purification

## Introduction

Lipases are widely distributed in animals, plants and microorganisms. Microbial lipases have potential industrial value due to their thermostability [5], substrate specificity [2], ability to remain active in organic solvents [10] and other diverse enzymatic properties. Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are capable of catalyzing hydrolysis of long chain triglycerides at the oil–water interface [17], esterification [7] and interesterification reactions [12]. Due to their diverse properties they are being used effectively in medicine, food additive, detergent, clinical reagent, oleochemical and cleaner industries.

Castor oil consists of approximately 90% 12-hydroxy-*cis*-9-octadecenoic acid (ricinoleic acid). The presence of two reactive functional groups, a hydroxyl group and a double bond, leads to the formation of different derivatives of castor oil. Ricinoleic acid produced by enzymatic hydrolysis of castor oil is odorless and light-colored and hence is useful in a number of cosmetic and food industries. A detailed study of castor oil hydrolysis by lipase could clarify the relation between a lipase and its substrate. Reports have been published on castor oil hydrolysis using lipase from *Humicola lanuginosa* No.3 [2], *Pseudomonas* sp f-B-24 [17] and oat seeds (*Avena sativa* L.) [13].

There are many reports on lipase purification and elucidation of the primary and secondary structure of lipase. Examples include the alkaline lipase from *Pseudomonas pseudoalcaligenes* F-111 [14], lipase from *Penicillium cyclopium* [6] which is stable from pH 4.5–6.0 and a thermostable phospholipase D from *Streptomyces* sp [3]. X-ray crystallographic studies have shown that calcium ions are bound to acidic residues in *Pseudomonas glumae* lipase [11]. Recently the amino acid sequence identity of *Pseudomonas* lipase has been analyzed in order to investigate the calcium-binding site [16].

In this work, we report a highly effective lipase from *Pseudomonas aeruginosa* KKA-5, which in crude and purified forms could hydrolyze castor oil. Other work deals with purification and characterization of this lipase. The presence of a calcium-binding site in this lipase is suggested.

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## Materials and methods

### *Bacterial strain and culture condition*

*Pseudomonas aeruginosa* KKA-5 was isolated from industrial wastes of Kitakyushu city, Japan. Cells were grown on Luria-Bertani plates (1% tryptone, 0.5% yeast extract, 0.5% NaCl and 1.5% agar, pH 7.2) for 24 h at 37°C, and then inoculated in the growth media, medium A, (0.1% $KH_2PO_4$, 0.1% NaCl, 4% polypeptone, 0.05% yeast extract, 0.02% $MgSO_4 \cdot 7H_2O$, pH 6.9) for 24 h at 30°C on a hot water bath; 2 ml medium A pre-culture broth was inoculated in 100 ml lipase production medium, medium B, (0.1% $KH_2PO_4$, 0.1% NaCl, 4% polypeptone, 0.05% yeast extract and 2% castor oil, 0.02% $MgSO_4 \cdot 7H_2O$, pH 6.9) in a 500-ml conical flask, at 121 rpm at 30°C.

### *Assay of lipase activity*

Lipase activity was measured using 2,3-dimercaptopropan-1-ol tributyrate as the substrate (Lipase Kit S, Dainippon Pharmaceuticals Co, Osaka, Japan). One unit of lipase activity was defined as the amount of enzyme that liberated 1 $\mu$mol $min^{-1}$ of fatty acid at 30°C. Specific activity is expressed as units $mg^{-1}$ protein.

### *Protein assay*

Protein concentration was determined by Bradford's method [1] using Bio-Rad protein assay, dye reagent concentrate (Bio-Rad Laboratories, Hercules, CA, USA). Bov-

ine serum albumin (Bio-Rad Laboratories) was used as the standard protein.

### *Lipase purification*

Ammonium sulfate was added to the medium B culture supernatant fluid to 45% saturation, at 4°C. The precipitate obtained was collected, centrifuged at 9500 × *g* for 20 min at 4°C and redissolved in 0.01 M potassium phosphate buffer (pH 7.0) containing 0.05 mM DTT (buffer A). The preparation was dialyzed overnight at 4°C, against the same buffer. Four millilitres of dialyzed enzyme solution were applied to a hydroxylapatite column (2 × 15 cm) (Bio-Rad Laboratories), previously washed and equilibrated with buffer A, and eluted at a flow rate of 24.2 ml $h^{-1}$. Fractions showing lipase activity were pooled (16.8 ml), characterized and later used for castor oil hydrolysis.

### *Electrophoresis*

To establish molecular weight of the enzyme, sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described by Laemmli [8], using 12.5% polyacrylamide gel slabs. As reference protein, standard mixture, low molecular weight electrophoresis calibration kit (Pharmacia Biotech, Uppsala, Sweden), was used.

### *Hydrolysis of castor oil*

Castor oil (Nacalai Tesque, Kyoto, Japan) was hydrolyzed using crude and purified lipase preparations. The hydrolysis mixture consisting of 0.25 g castor oil, 1 ml 0.01 M $CaCl_2$, 10 ml buffer A and the lipase preparation (100 U), were placed in 100-ml air-tight erlenmeyer bottles and incubated at 30°C on a reciprocal shaker. The reaction was stopped by adding 20 ml acetone/ethanol (1:1, v/v). Liberated fatty acids were titrated with 0.1 M alcoholic KOH, using phenolphthalein as an indicator. The percentage of castor oil hydrolysis was calculated as:

$$\text{Hydrolysis (\%)} = (\text{acid value}/\text{saponification value}) \times 100.$$

The saponification value of castor oil was 181, the value being measured by the supplier.

## Results and discussion

### *Growth and lipase production*

*Pseudomonas aeruginosa* KKA-5 produced maximum lipase activity on the fourth day of cultivation after which a significant decrease in activity was observed. This decrease in activity could be due to the presence of an accompanying protein. The possible role of magnesium ions in lipase production was also examined. Exclusion of magnesium ions from medium B led to about 50% reduction in lipase activity (Figure 1). A concentration of 0.8 mM $MgSO_4 \cdot 7H_2O$ was most suitable for maximal lipase production. In addition, the presence of magnesium ions did not affect the sigmoidal growth curve. The foregoing results implied that the presence of magnesium ions stimulated lipase production. Addition of calcium ions to the medium containing 0.8 mM $MgSO_4 \cdot 7H_2O$ did not give further increase in lipase production (data not shown). Accordingly, 0.02% 0.8 mM $MgSO_4 \cdot 7H_2O$ was included in all media.

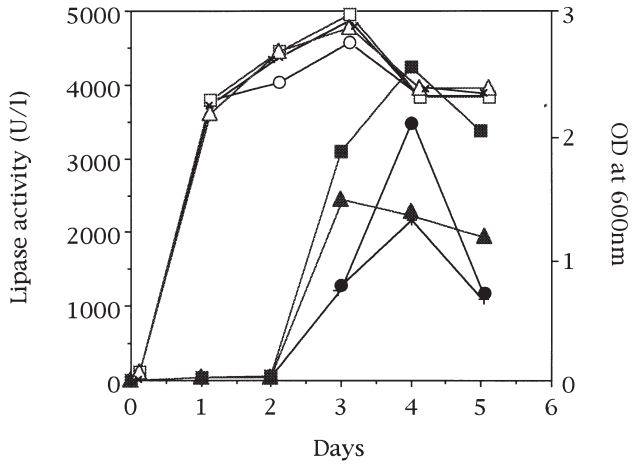


**Figure 1** Effects of various concentrations of $MgSO_4 \cdot 7H_2O$ on growth and lipase production. Symbols for OD at 600 nm and lipase activity are: ×/+: Control; ○/●: 0.4 mM; □/■: 0.8 mM; △/▲: 1.6 mM.

### *Purification of lipase*

A 4-day-old culture was centrifuged at 3400 × *g* for 15 min at 4°C. The supernatant medium is hereby called crude lipase. The precipitate formed by addition of ammonium sulfate to the crude lipase was dissolved in buffer A and dialyzed overnight against buffer A to remove the ammonium ions. The specific activity was increased about 8-fold. This solution (4 ml) was applied to a hydroxylapatite column. The chromatogram of purification is shown in Figure 2 in which 5.6-ml fractions were eluted. The above purification procedure is summarized in Table 1. A 518-fold purification was obtained with a production yield of

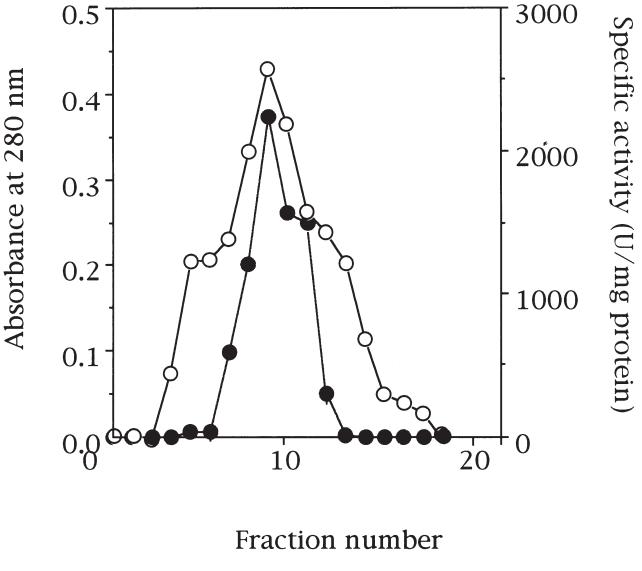


**Figure 2** Elution of lipase from *Pseudomonas aeruginosa* KKA-5 on a hydroxylapatite column. The enzyme from the second step of purification was applied on a (2 cm × 15 cm) hydroxylapatite column. The column was eluted with 0.01 M potassium phosphate buffer (pH 7.0) containing 0.05 mM DTT. Flow rate: 24.2 ml $h^{-1}$; fraction volume per tube: 5.6 ml; ●: lipase activity (U $L^{-1}$); ○: absorbance at 280 nm.

**Table 1** Summary of purification of lipase from *Pseudomonas aeruginosa* KKA-5

| Steps | Total volume (L) | Total protein (mg) | Lipase activity (U) | Specific activity (U $mg^{-1}$ protein) | Yield (% lipase activity) | Purification (fold) |
|---|---|---|---|---|---|---|
| Crude lipase | 0.500 | 694.17 | 2998.80 | 4.32 | 100.0 | – |
| 45% Ammonium sulfate precipitate dissolved in buffer A | 0.013 | 64.00 | 1868.21 | 29.19 | 62.30 | 6.76 |
| Dialyzed ammonium sulfate precipitate dissolved in buffer A | 0.009 | 41.28 | 1295.08 | 31.37 | 43.19 | 7.26 |
| Hydroxylapatite | 0.015 | 0.0265 | 59.53 | 2238.16 | 2.60 | 518.09 |

2.6%. The specific activity of lipase increased to 2238 U $mg^{-1}$ protein. The lipase activity was eluted in fractions 9~11. These fractions were pooled and used as enzyme source for further studies.

## *Characterization of purified lipase*

*SDS-Page:* Polyacrylamide gel electrophoresis of the purified enzyme solution (Figure 3, lane 2), showed a single dark band of protein. The molecular weight of this enzyme was estimated to be 30 kDa.

*Effects of pH and temperature on lipase stability:* The enzyme showed maximum activity at pH 8.5 and remained stable in the pH range from 7 to 10 for 24 h at 30°C. The enzyme was stable in this alkaline range similar to lipase from *Pseudomonas aeruginosa* EF-2 [4]. The purified lipase showed an excellent thermostability at 45°C for 30 min. Above this temperature there was a gradual decrease in activity. A 75% loss in activity was observed on heat treatment of the enzyme at 70°C. This result was similar to that for *Pseudomonas* sp KWI-56 lipase [5]. In contrast to the present results, Mencher *et al* [9] reported total loss in activity of lipase from *Pseudomonas fragi* NRRL B-25, when it was heated at 40°C for 10 min.

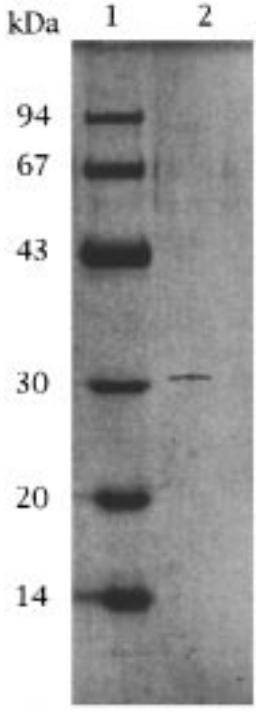


**Figure 3** Sodium dodecyl sulfate polyacrylamide gel electrophoresis pattern of purified lipase. SDS-PAGE was conducted in 12.5% gel, with a current of 10 mA for 4 h. Protein was stained with Coomassie Brilliant Blue R 250. Lane 1: Marker—phosphorylase (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) and alpha-lactalbumin (14.4 kDa); lane 2: purified sample.

*Effects of metal ions on lipase activity:* Buffer A containing chlorides of metal ions at 1 mM were incubated with the purified lipase at 30°C for 20 min. Lipase activity was retained with $Ca^{2+}$ or $Mg^{2+}$, whereas $Mn^{2+}$, $Cd^{2+}$, and $Cu^{2+}$ were slightly inhibitory. Salts of heavy metals ($Fe^{2+}$, $Zn^{2+}$, $Hg^{2+}$ and $Fe^{3+}$) strongly inhibited lipase, suggesting that they were able to alter the enzyme conformation. Lipase from *Pseudomonas fragi* 22.39 B could sustain about 84% of its activity on exposure to $Hg^{2+}$ [10], in contrast to lipase from *Pseudomonas aeruginosa* KKA-5, which lost all activity on exposure to $Hg^{2+}$.

*Effects of organic solvents and surfactants:* The enzyme showed good stablity in protic solvents (Table 2). Incubation of the lipase in a strong dehydrating solvent like acetone did not cause loss of much of its activity. Loss in activity was not observed on incubation of lipase in the presence of 50% methanol or 50% ethanol. A slight loss in activity was observed on incubation of lipase in the hydrophobic solvent, hexane, at 30°C. The non-ionic surfactants, Triton X-100 and Tween-80, effectively enhanced activity, whereas the anionic surfactant, sodium dodecyl sulfate inhibited activity. Similar effects were reported by Shuen-Fuh Lin *et al* in the case of partially purified lipase from *Pseudomonas pseudoalcaligenes* F-111 [14]. Inhibition of the lipase activity by EDTA suggested that this lipase may be a metallo-protein. These results indicate that this lipase has a high solvent-tolerance capacity. A 20–50% decrease in activity was observed on incubation of *Pseudomonas pseudoalcaligenes* F-111 lipase in water-miscible solvents [15].

## *Reactivation of lipase*

Incubation of purified lipase with 0.05 M EDTA for 15 min at 30°C resulted in total loss of activity. Sequential addition of 0.02 M and 0.1 M $CaCl_2$ reactivated the EDTA-treated inactivated enzyme. We therefore conclude that calcium ions are necessary to maintain enzyme stability. However, Svendsen *et al* [16] reported once it was inactivated by

**Table 2** Effects of organic solvents and surfactants on lipase stability. The enzyme was incubated in 0.01 M potassium phosphate buffer (pH 7.0) containing 0.05 mM DTT and the respective reagent, for 20 min at 30°C. After incubation, the residual activity was measured and was expressed as a percentage of non-treated enzyme solution, which was taken as 100%

| Reagents | Residual activity (%) |
|---|---|
| *I. Organic solvent (50%)* | |
| 1. Methanol | 100 |
| 2. Ethanol | 100 |
| 3. Acetone | 92.86 |
| 4. Hexane | 87.13 |
| 5. Control | 100 |
| *II. Surfactant (0.1%)* | |
| 1. Triton X-100 | 183.3 |
| 2. Tween-80 | 180 |
| 3. SDS | 66.7 |
| 4. 0.05 M EDTA | 0 |
| 5. Control | 100 |

EGTA, *Pseudomonas* lipase could not be reactivated by addition of calcium ion. In addition, a calcium-binding site in the lipase of *Pseudomonas glumae* was reported by Noble *et al* [11] who demonstrated that loss of the calcium site could decrease the protein's stability and could also disrupt the structure near the active site.

Thus, calcium ion had no stimulatory effect on cell growth or lipase production, but strongly stabilized the purified lipase. These results suggest the possibility of a calcium-binding site in *Pseudomonas aeruginosa* KKA-5 lipase. We are pursuing evidence for the presence of a calcium-binding site.

### *Hydrolysis of castor oil*

About 80% hydrolysis of castor oil was obtained on incubation of the oil with crude lipase (100 U) and 100 mM $CaCl_2$, over a period of 9 days (Table 3). In the absence of $CaCl_2$, only 60% hydrolysis was obtained. Using the same units of lipase activity, purified lipase was incubated with castor oil. In contrast to the result of crude lipase, the hydrolysis percentage was negligible when the reaction mixture lacked calcium ions. Under the same conditions 90% hydrolysis was obtained in a period of 4 days when 10 mM $CaCl_2$ was included in the reaction mixture with the purified lipase. Crude and purified lipase from *Pseudomonas aeruginosa* KKA-5 could hydrolyze castor oil in the absence of organic solvents. Using the same number of units of activity, purified lipase took less than half the time to hydrolyze castor oil and was hence more efficient and economical.

**Table 3** Hydrolysis of castor oil using lipase in the presence or absence of 10 mM $CaCl_2$. For the reaction mixture and culture conditions see Materials and Methods

| | Crude lipase (% hydrolysis in 9 days) | Purified lipase (% hydrolysis in 4 days) |
|---|---|---|
| Presence of $CaCl_2$ | 80% | 90% |
| Absence of $CaCl_2$ | 60% | 1.3% |

A detailed study on castor oil hydrolysis with this lipase is in progress. Construction of a bioreactor to attain continuous production of lipase will also be described in a forthcoming paper.

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