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Purification and characterization of lipase from *Bacillus subtilis* Pa2

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Abstract

The extracellular lipase produced by *B subtilis* Pa2 was purified by acetone precipitation and ion exchange chromatography. The molecular weight of the pure protein was estimated to be 19.4 & 19.2 kDa by SDS-PAGE. The lipase formed high molecular weight aggregates (>100,000 kDa). The enzyme was most active in the pH range of 7 to 9 with maximum activity at pH 8, whereas it was most stable in the pH range of 5 to 10, retaining more than 70% activity. The present lipase was most active in temperature between 30 and 50 °C. Mg^{2+} was found to stimulate lipase activity.

Key words: Lipase, SDS-PAGE, *Bacillus subtilis*, Chromatography

Introduction

Bacterial lipases are mostly inducible enzymes, requiring some form of oil, fatty acid, fatty acid alcohol or fatty acid ester for induction. However, there are a few reports of constitutive lipase production by bacteria. (Elwan et al. 1983, Gao et al. 2000). Lipases are usually secreted out in the culture medium; but membrane bound lipases and intra-cellular lipases have been reported. (Mourey 1981; Lee 1989; Large et al. 1999). The onset of lipase production is organism-specific, but, in general, it is released during late logarithmic or stationary phase (Matselis and Roussis 1992; Makhzoum et al. 1995). Studies have been made for optimization and fermentative production of lipases. (Gawel and Chen 1977; Nakanishi and Ikeda 1986; Inoue et al. 1987; Holmes 1990; Ishida et al. 1995; Ishikawa et al. 1995; Lawler and Smith 2000).

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Lipase purification, for extracellular lipases, normally the first step is the removal of cells by centrifugation or filtration. In case of intracellular lipases, an additional step of cell lysis is required. The crude lipase preparation can then be concentrated by ultrafiltration or can be subjected to optional solvent or salt precipitation. Usually ion exchange chromatography or hydrophobic interaction chromatography has been effectively used for further purification of the concentrated enzyme. The final step of gel filtration normally yields a homogenous product. In most of the lipase purification procedures, the diethylaminoethyl (DEAE) anion exchanger is used. Lipases show natural affinity for hydrophobic substances, as their substrates are hydrophobic molecules.

The lipases from the two mesophilic strains *B. subtilis* and *B. pumilus* were different from other *Bacillus* lipases. They were the smallest true lipases known (approximate molecular mass 20 kDa) and shared very little sequence homology (approximately 15%) with the other lipases. Nthangeni (2001) classified *Bacillus* lipases in two subfamilies, based on amino acid analysis and biochemical characteristics. The subfamily I.4 included lipases with low molecular mass in the range 19-20 kDa from *Bacillus licheniformis*, *Bacillus subtilis*, and *Bacillus pumilus*. The lipases from *Bacillus themocatenulatus, Bacillus thermoleovorans*, and *Bacillus stearothermophilus* were included in the subfamily I.5 and they had molecular mass around 43 kDa. Arpingy and Jaeger (1999) suggested that the Staphylococcal lipases should be included in separate family of their own.

Materials and Methods

DEAE Cellulose G-50 was obtained Hi-Media and molecular weight calibration kits for electrophoresis from Bangalore Genei. Metal ion salts and buffer ingredients and all other chemicals were AR grade, produced from SRL and S. D. Fine chemicals.

Protein estimation

Protein content has been estimated according to Lowry et al. (1951) using Bovine Serum Albumin (BSA) as a standard and direct method of BSA using U.V. spectrophotometer at 280nm.

Lipase assay

The lipase activity was estimated by spectrophotometer at 410nm method, using p-Nitro- phenyl acetate as substrate. The amount of enzyme that librates 1µg p- Nitrophenol per minute is considered as one unit. Lipase activity was detected by measuring the hydrolysis of p-nitrophenyl esters. Assays were performed in phosphate buffer. Para-nitrophenyl-esters of various chainlength fatty acids have also been used as substrates for lipase assay (Winkler and Stuckmann 1979).

Lipase purification

Crude enzyme preparation

The culture of Pa2 is grown in the optimized medium for inoculum preparation and then inoculated in fermentation medium, incubated for period of 48h. At the end of incubation, the bacterial cells in the fermentation broth are removed by centrifugation at 10,000g for 15 min at 5 °C. The cell-free supernatant is used as a crude enzyme preparation and purified further as described below.

Acetone precipitation

The crud enzyme is precipitated by 60% (v/v) volume of chilled acetone. The precipitate is stored overnight, at -5°C and recovered by centrifugation at 10,000 rpm for 10 min. The precipitates were dried in vacuum dessicator at room temperature so as to remove traces of acetone.

The precipitates were then resuspended in 10 mM phosphate buffer, pH 7.2 and allow to stand overnight at 4^{o}c. after incubation the enzyme was dialyzed against 40% sucrose solution to get concentrated enzyme free from salt and metal ions.

Ion exchange chromatography

The concentrated enzyme solution is loaded on a DEAE Cellulose G-100 column (2 cm dia × 150 cm length) pre-equilibrated with 10 mM sodium phosphate buffer, pH 7.2. The elution is carried out by 0 -0.5 M NaCl in the same buffer at a flow rate of 5.4 ml/30min at room temperature and 5 ml fractions are collected. The protein content of fractions is determined by measuring optical density at 280 nm. The protein-containing fractions are assayed for lipase activity.

Properties of lipase

Molecular weight determination

Molecular weight of the protein is estimated by SDS-PAGE, according to Laemmli (1970) on a vertical slab 7.5% (w/v) polyacrylamide gel, at 200v a constant voltage of 200 V, for 5 h.Using α- Lactalbumin (14.2 kDa), Carbonic anhydrase (29 kDa), Fumarase (48.5 kDa), bovine serum albumin (66 kDa) are used as standard protein molecular weight markers.

Optimum pH

The effect of pH on enzyme activity is studied by incubating the enzyme with p-nitrophenyl acetate substrate, prepared in different buffers in the pH range 4 to 10.The buffers used are, citrate-phosphate (pH 4-7), sodium phosphate (pH 7-8) and glycine-NaOH (pH 9-10) .

Optimum temperature

The temperature optima for the enzyme is determined in the range 20 to 70°C, at pH 7.4, as above. The enzyme stability at different temperatures is studied by incubating the enzyme in 10 mM phosphate buffer pH 7.4 at different temperatures for 2 h, followed by the activity estimation at 37 °C.

Effect of metal ions and EDTA on lipase activity

The effect of metal ions is determined by estimation of the activity in presence of 10mM solution of metal salts. The effect of EDTA is studied by estimating activity in the presence of 10mM EDTA. The enzyme was incubated in presence of metal ions and EDTA for 15 min followed by estimation of activity by p-Nitrophenol liberation.

Results and Discussions

Purification

The crude lipase is purified in three steps. I) Centrifugation- the enzyme is concentrated two fold. II) Acetone precipitation and dialysis step - removed traces of acetone as well as the low molecular weight proteins. III) Ion exchange chromatography is carried out. The purification protocol resulted in a pure protein estimated by direct method. Pure protein giving a band on a stained SDS-PAGE gel which is corresponding to 19.4 and 19.8 KDa(Figure 2).

Properties

Molecular weight

The molecular weight of the enzyme is estimated to be 19.4 and 19.8 KDa by SDS-PAGE (Figure 1). The present lipase bind to ion exchange and hydrophobic interaction chromatography matrices tested. The pure protein smeared badly on native PAGE but showed two clear bands nearby each other corresponding to molecular weight of 19.4 and 19.8 kDa on SDS-PAGE. This indicated that the lipase might have two polypeptide chains and include low molecular weight true lipase of *B.subtilis* Pa2. *B.subtilis* lipase has two chain (LipA and LipB), lipases ware reported by Droege et al. (2005). And also lipase has ά\β Hydrolase fold enzymes as reported by Gertie, *et al.,* (2001). Among Gram-positive bacteria, the *Bacillus* lipases were classified in to two subfamilies by Nthangeni *et al.,* (2001). The first subfamily included lipases with low molecular mass in the range 19-20 kDa from *Bacillus licheniformis*, *Bacillus subtilis*, and *Bacillus pumilus*. The lipases from *Bacillus themocatenulatus, Bacillus thermoleovorans*, and *Bacillus stearothermophilus* were included in the second subfamily and they had molecular mass around 43 kDa. *Pseudomonas fluorescens* lipases are reported to have different molecular masses viz., 20.9 kDa, 33kDa, 48kDa and 45 kDa (Chung et al. 1991; Kojima et al. 1994; Costa et al. 1997).

Effect of pH on the enzyme activity and stability

The enzyme is most active in the pH range of 7 to 9 (Figure 1) with maximum activity at pH 7, whereas it is most stable in the pH range 5 to 10, retaining more than 70% activity. Most of the bacterial lipases are reported to have pH optima on alkaline side. Among Gram positive bacteria, *Bacillus subtilis* lipase is shown to have a very high alkaline pH optimum between 10 and 11.5 (l.c.). The lipases from *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Propionibacterium acnes a*re found to be exceptions to other

bacterial lipases (Ingham et al. 1981; Simons et al. 1996 and 1998) with pH optimum around 6. Talon et al. (1995) have

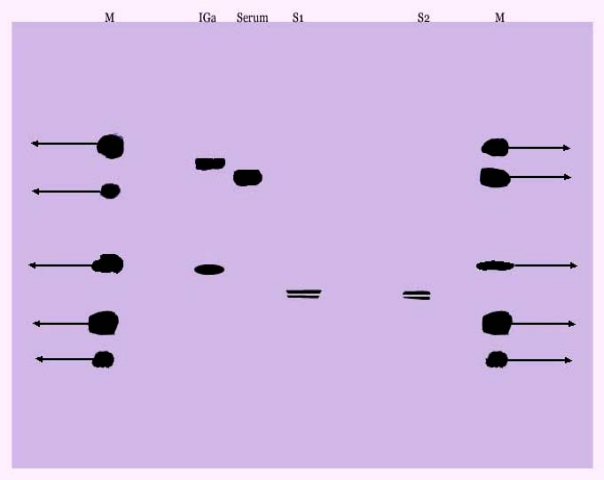

Figure 1: (M) 66kDa,48.5kDa,29 kDa,14.5 kDa ,9kDa and S1 and S2 purified lipase.

reported pH optimum of 9 for *Staphylococcus warneri*. *Staphylococus haemolyticus* and *Staphylococus hyicus* also have alkaline pH optima (Rosenstein and Götz 2000). *Pseudomonas* lipases are reported to have optimum pH in acidic (Iizumi et al. 1990) as well as alkaline environment (Castellar et al. 1997). The lipases from *Pseudomonas* and *Bacillus* lipases are reported to be more stable in alkaline pH range. The present *B. subtilis* lipase is found to be stable over wider pH range than the *Bacillus* lipases studied before with more than 70% activity retention in the pH range 5-10.

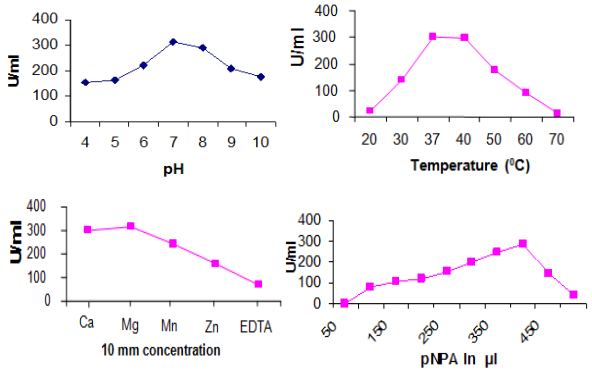

Figure 2: Effect of pH, temperature, substrate (pNPA), and concentration of EDTA and metal ions on lipase production

Effect of temperature on lipase activity and stability

The *B. subtilis* Pa2 lipase is most active at temperature between 30°C and 50 °C. It retained more than 70% activity till 45 °C (Figure 1). The activity drop rapidly above 60 °C Sztajer *et al.*, (1991b) have reported a temperature optimum for oil hydrolysis between 50 and 55 °C, for a lipase from *Pseudomonas fluorescence*. (l.c). The temperature optima of lipases from mesophilic microorganisms are found to be normally in the range of 45°-60 °C. Many thermophilic *Bacillus* strains have been reported to produce lipases that are active at temperatures between 60 and 75 °C. The lipase from thermophilic bacilli is relatively more stable at higher temperatures. Many researchers have observed an interesting phenomenon of low temperature partial inactivation (LTI) for lipases from psychrotrophic *Pseudomonas* sp. (Swaisgood et al. 1984; Kumura et al. 1993; Baral et al. 1997; Konstantinou et al. 1998).

Effect of metal ions

As presented (Figure 1) the enzyme is inhibited by metal ions like Hg^{2+}, Zn^{2+} and Cu^{2+} while Mg^{2+} has been found to stimulate lipase activity. Mg^{2+} and Ca^{2+} do not have any significant effect on the lipase activity. EDTA is found to inhibit lipase activity Metal ions like Hg^{2+}, Zn^{2+} and Cu^{2+} are reported to have inhibitory effect on *Pseudomonas* lipases by several workers (Yamamato et al. 1988; Iizumi et al. 1990; Kumura et al. 1993; Chartrain et al. 1993). Several workers have found that Mg^{2+} and Ca^{2+} are able to stimulate lipase activity (Chartrain et al. 1993; Lee et al. 1993; Jansen 1996; van Oort et al. 1989) in *Bacillus* and *Pseudomonas* sp.

Table 1: Substrate specificity of the lipase

Substrate	Relative lipase activity (U/ml)
Castor oil	65.7
Coconut oil	156.8
Soya oil	109.3
Groundnut oil	189.9
Olive oil	303.7
Tributyrin	24.6

Substrate specificity of enzyme

The lipase caused rapid hydrolysis of the vegetable oils containing C-16 and C-18 fatty acid esters (Table 1). This indicated that the present lipase is a "true" lipase. It acts preferentially on oils with lower chain fatty acid esters. It shows more than three-fold activity with tributyrin as compared to triolein. It is also observe that the lipolytic activity on vegetable oils with C-18 unsaturated fatty acids increased with increase in the degree of unsaturation and the percentage of unsaturated fatty acids. Schmidt-Dannert et al. (1997) have reported that the lipases from *Bacillus thermocatenulatus*, *Staphylococcus hyicus* and *Staphylococcus epidermidis* hydrolyzed tributyrin preferentially. Lipase from Pa2 failed to hydrolyze triacetin. This observation agrees with the reports of Schmidt-Dannert et al. (1997) for *Bacillus thermocatenulatus*, *Staphylococcus hyicus* and *Staphylococcus epidermidis* Chartrain et al. (1993) have reported that *Pseudomonas aeruginosa* MB 5001 lipase was more active towards lipids containing C-18 unsaturated fatty acid and showed similar increase in activity as the percentage of unsaturated fatty acid in the oils increased, as found with the present lipase of *B. Subtilis* Pa2.

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