Purification of Lipase Enzyme Produced by *Bacillus Stearothermophilus* HU1

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***Abstract*—**An extracellular lipase produced by a locally isolated *Bacillus stearothermophilus* HU1 was recovered and purified by ammonium sulfate precipitation by bringing it to 40% saturation, followed by DEAE-cellulose ion exchange chromatography. This purification resulted in a 9.53 fold purification of lipase with 2.49% recovery yield. The molecular mass of the purified lipase enzyme was estimated to have an approximate value of 35-50 kDa by Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE). The enzyme kinetics study showed that the enzyme has a km and Vmax values of 0.2353 mM and 161.2 nmol/min/mL, respectively.

***Keywords*—** Lipase, Bacillus stearothermophilus, purification, Enzyme kinetics.

I. Introduction

LIPASES (triacylglycerol acylhydrolase, EC 3.1.1.3) have been defined as carboxylesterases that catalyze the hydrolysis, esterification and transesterification of acylglycerides with acyl chains with more than ten carbon atoms at an oil-water interface [15], [18]. Due to the commercial importance of extracellular lipases, many microorganisms have been studied for their lipase production ability [3]. *Bacillus* lipases attract attention because they have unique protein sequences and many uncommon biochemical properties. Therefore, purification of lipase allows for better understanding of the kinetic mechanisms action of the enzyme. Lipases have been purified and biochemically characterized from many *Bacillus* species such as *B. subtilis*, *B. pumilus*, and few from thermophilic *Bacillus* have been isolated, purified and their genes cloned and sequenced [7], [17]. Nevertheless, the enzyme source is of major importance in industry and its properties depend on the environmental conditions surrounding the enzyme along with its producer. Most of the enzymatic applications require a certain degree of purity depending on the final application in industries. [3] stated that the purification strategies should be inexpensive, rapid, efficiency and feasible to large-scale operations. The aim of this study was therefore, to purify and study the kinetics of lipase enzyme produced by *Bacillus stearothermophilus*, a local strain isolated from a raw petrol sample.

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II. Material And Methods

A. Lipase Production

In a previous work, *B. stearothermophilus* HU1, was isolated from a raw petrol sample (Hashemite University, Al-Zarqa/Jordan), identified, and studied for its ability to produce lipase enzyme [13]. According to [10] the enzyme was produced as a 2% v/v of the seed culture was transferred to a medium containing g/L: KNO_3 3.54; K_2HPO_4 1.0; $MgSO_4 \cdot 7H_2O$ 0.5; NaCl 0.38; $FeSO_4 \cdot 7H_2O$ 0.01, yeast extract 5.0; Tween 80 0.6% (v/v) and olive oil 1% (v/v), adjusted to pH 8.0 using 1.0 M NaOH and HCl. The culture was incubated at 45 °C for 24 hrs and 150 rpm. After incubation, the culture was centrifuged at 6000 rpm for 30 min, and the supernatant was used for protein and enzyme analysis.

B. Lipase Assay and Protein Determination

Lipase activity was determined using p-nitrophenyl palmitate (pNPP) as substrate [2]. The substrate solution was prepared by freshly mixing solution A (30mg of pNPP (Sigma, USA) in 10 ml of isopropanol) with solution B (0.1 g of gum Arabic and 0.4mL Triton X-100 in 90 ml of 50 mM Tris–HCl buffer, pH 8) while stirring until all was dissolved. The mixture of 9 ml of substrate solution and 1mL of enzyme solution was incubated at 60±0.1 oC for 15 min and absorbance was measured at λ = 410 nm. The coefficient of extinction (ε) of p-nitrophenol (pNP), under the conditions described, was determined from the absorbance at λ = 410 nm of standard solutions of pNP (0.01-0.1 μmol/ml) (ε410 = 1.4653 L/mol/cm). Suitable controls were made for each experiment. One unit of enzyme activity was expressed as one nmol of p-nitrophenol released per minute under the assay conditions. Dissolved protein concentration was determined according to the Lowry method, using bovine serum albumin (BSA) as a standard [11].

C. Purification of Lipase

Ammonium sulfate precipitation: Solid ammonium sulfate was added to the supernatant at 20% saturation and allowed to stand for 4 hrs. The precipitate obtained was separated by centrifugation and solid ammonium sulfate was added to the supernatant at 40% saturation. The precipitate obtained was collected by centrifugation. The supernatant was further treated with ammonium sulfate at 60 and 80% saturation. All the precipitates were resuspended in a minimal amount of deionised water and dialyzed against it by routinely replacing

the water. The process was continued for overnight. All the concentrated fractions were subjected to protein and enzyme activity assay to choose the fraction containing maximum activity [15].

Ion exchange chromatography: The purification process of the enzyme sample was performed using Ion exchange chromatography system equipped with Fraction collector; Biologic LP Chromatography Systems (Biorad, USA). The dialyzed sample was removed from the tubing and filtered through a 0.2 µm filter. The sample was applied to a DEAE-cellulose column previously equilibrated with 1.0 M Tris-HCl buffer (pH 8.0) slowly percolating large volume of buffer through the packed material. The elution was accomplished with 15-column volume gradient 1.0 M Tris-HCl buffer pH 7.0. Flow rate was controlled at 1.0 ml/min by 5 ml of fractions collected and analyzed for protein content and enzyme activity. After assaying the fractions for lipase activity, fractions showing highest activity were pooled together and stored at -20 °C.

D. Enzyme Kinetic Study

The effect of substrate concentration (pNPP) (0.1 – 1.5 mM) on the reaction rate was assayed for 2 min at pH 8.0 by using spectrophotometric method. The Michaelis Menten constant (Km) and the maximum velocity for the reaction (Vmax) were calculated using the GraphPad PRISM software.

E. Protein Mass Determination

In order to study the protein profile of lipase from *B. stearothermophilus* HU1, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12.5% polyacrylamide) was performed according to [9] with few modifications. Boiling time in sample buffer containing 7.5% 2-mercaptoethanol was 10 min and gels were silver stained. For measuring molecular weight of the protein, commercial broad range molecular mass standard proteins were used.

III. Results And Discussion

A. Enzyme Purification

In a previous work, the enzyme was studied in shake cultures and bioreactor system [13]. The culture optimum conditions were determined and the enzyme characterization was performed. The extracellular lipase of *B. stearothermophilus* was purified employing ammonium sulfate precipitation technique followed by DEAE-cellulose ion-exchange chromatography. Table 1 shows that 40% ammonium sulfate saturation of the supernatant precipitated the largest amount of lipase. It led to 9.66% recovery yield with 1.03 purification fold (Table 2). Ammonium sulfate precipitation was followed by ion exchange chromatography as Fig. 1 shows the DEAE-cellulose column chromatogram of DEAE–cellulose chromatography where lipase fraction was eluted with a gradient of 1.0 M Tris-HCl buffer pH 8.0. The purified lipase fraction exhibited a final specific activity of 815.42 U/mg with a 9.53 fold of purification and a recovery yield of 2.49 (Table 2). Low yield of the purified enzyme might be attributed to the loss during ammonium sulphate precipitation. [16] used ammonium sulphate (60% saturation) followed by S-200 chromatography to purify a lipase enzyme from *Talaromyces thermophilus* fungus that led to a 7.87 fold purification with a 62.97% recovery rate. While [1] attempted to purify a lipase enzyme from *B. thermoleovorans* CCR11 using ammonium sulfate but was unsuccessful. [12] used a three step purification protocol to purify the lipase enzyme from B. subtilis involving ammonium sulfate precipitation, ion exchange chromatography, and gel filtration chromatography. Their results revealed 19.7 fold purification with a 30% recovery rate and the characterizations of the purified enzyme revealed a molecular mass of 24 kDa in SDS-PAGE. [6] purified a lipase from *B. stearothermophilus* MC7 with the final purification step being ion-exchange chromatography. The purification led to a 10.2% recovery and 19.25 fold purification. On the other hand, [4] purified an extracellular lipase from Staphylococcus aureus to homogeneity by ammonium sulfate precipitation followed by heat treatment, ion exchange chromatography and ended with gel filtration chromatography. They achieved 23% recovery and 420 fold purification.

TABLE 1
PURIFICATION OF LIPASE BY AMMONIUM SULFATE PRECIPITATION AT DIFFERENT CONCENTRATIONS.

Ammonium sulphate saturation	Fraction activity (U/ml)	Total activity (U)	Fraction dissolved protein concentration (mg/ml)	Total protein (mg)	Specific activity (U/mg)
0%	82.67	12,400	0.966	144.9	85.580
20%	5.642	84.624	0.166	2.490	33.986
40%	79.847	1,1978	0.910	13.65	87.744
60%	6.506	97.591	0.128	1.920	50.829
80%	11.511	172.66	0.112	1.680	102.77

TABLE 2
PURIFICATION OF LIPASE BY AMMONIUM SULFATE PRECIPITATION AND ION EXCHANGE CHROMATOGRAPHY

Purification step	Total activity (U)	Total dissolved protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Cell free extract	12,400	144.9	85.58	100%	1.00
Ammonium sulfate	1,1980	13.65	87.74	9.66%	1.03
DEAE-cellulose	308.23	0.378	815.42	2.49%	9.53

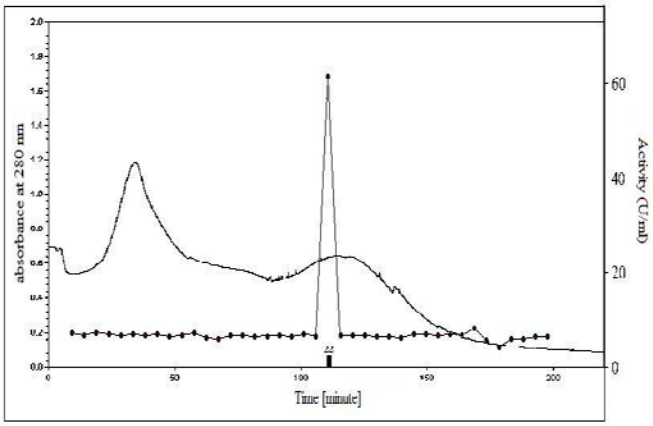

Fig. 1 Ellution profile of lipase from DEAE-cellulose column chromatogram. (—) represents protein concentration by absorbance at 280 nm, (●) represents lipase activity in the collected fractions. The active fraction is marked with a bold rectangle (fraction no. 22).

B. Molecular Weight

The active fraction from DEAE-cellulose showing the highest lipase enzyme activity was run on SDS-PAGE along with the crude extract sample (Fig. 2). In the crude extract, four distinct bands were noticed at 15, 25, 35 and 50 kDa, two of which were seen in the purified fraction lane (3) at 35 and 50 kDa. [5] claimed that most Bacillus lipases have low molecular weight of about 20 kDa, and *B. stearothermophilus* lipases have a molecular mass of approximately 45 kDa. [8] reported a lipase enzyme from *B. stearothermophilus* having a 43 kDa, while [6] reported a lipase having a 62.5 kDa molecular weight.

C. Enzyme Kinetics

The Km and Vmax values were determined as a function of pNPP concentration to be 0.2353 mM and 161.2 nmol/min/mL respectively. [6] reported a lipase from *B. stearothermophilus* that had a Km and Vmax value of 0.33 mM and 188 µM/min/mg, respectively, when using pNPP as substrate. While [4] reported a lipase from *Staphylococcus aureus* that had a Km and Vmax value of 14.53 mM and 1485 µM/min/mg, respectively, when using triolein as substrate.

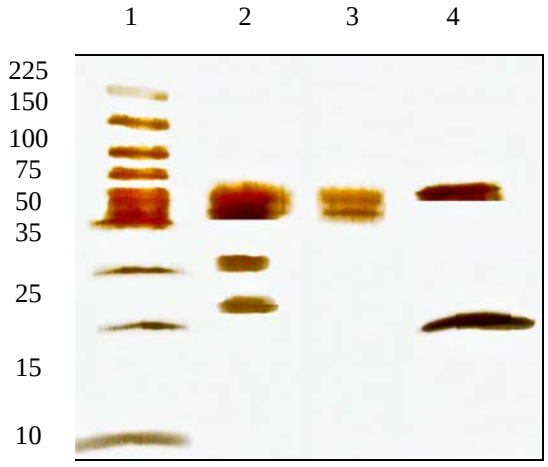

Fig. 2 Molecular Mass of Crude and Purified *B. stearothermophilus* Lipase. Lane 1: Broad range protein molecular marker, lane 2: Crude extract, lane 3: purified fraction and lane 4: Lipase from *Pseudomonas* sp.

In conclusion, the purification of lipase from *B. stearothermophilus* resulted in obtaining a fraction that showed two bands on SDS-PAGE having approximately 35 and 50 kDa molecular weight; the use of ammonium sulfate to purify this enzyme had a negative affect on the yield of the enzyme. It is important to find or employ other purification methods to obtain higher yields.

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