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Purification and Zymography of Lipase from *Aspergillus niger* PTCC5010

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In this study, lipase from *Aspergillus niger* after extraction of medium culture was precipitated with different percentages of acetone and purified by ion exchange chromatography using SP-Sepharose HP and Q-Sepharose HP. The process of purification of the enzyme was studied by electrophoresis and the molecular weight was detected and determined by Zymography using overlying containing phenol red and Rhodamine B. The results showed that the vast majority of lipase from this strain has been precipitated by 70% saturation acetone, and leads to the 1.67 fold the purified enzyme, with special activities of 32.8 U. mg^{-1} and efficiency of 38.5%. Using two-phase chromatography, enzyme specific activity reached 246.47 U. $mg^{-1}$and 12.59-fold purification were achieved. The results of Zymography and electrophoresis indicate a lipase band weighing about 30 kDa.

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1. INTRODUCTION

Lipases (triacylglycerol acyl hydrolases, EC 3.1.1.3) are natural catalysts of the hydrolysis of triacylglycerols into di- and monoacylglycerols, fatty acids, and glycerol at an oil–water interface. Under certain conditions, they are also able to catalyze synthetic reactions. These enzymes are used in various industrial processes, such as food ingredients, additives to detergents and purified drugs, and other refined products [1].

Fungi are broadly recognized as one of the best lipase sources and are used widely in the food industry [2]. *Aspergillus niger* is one of the most important microorganisms used to produce extracellular lipase. It is also of great importance in food and pharmaceutical industries, in which producing a safe and non-pathogenic microorganism enzyme is essential [3].

Various methods have been used to purify lipase from various sources. About 80% of the purification schemes use a precipitation step followed by a combination of several chromatographic methods, such as gel filtration and affinity chromatography. Usually, a single chromatographic step is not sufficient foracquisition of the required level of purity [4].

Zymographyis an electrophoretic technique based on SDS PAGE used for determination of enzyme or protein presence in the solution. This technique can be widely used in detecting and characterizing microbial enzymes such as lipase [5, 6]. Detection of lipase in the native state requires an enzyme zymogram.The zymography can be used for detection of lipase in crude as well as purified forms obtained from all kinds of sources.

The objectives of this study were: to extract and purify extracellular lipase from *A. niger* by precipitation with solvent;and optimize the concentration of the solvent and ion exchange chromatography using SP-Sepharose HP and Q-Sepharose HP, pursue purity of lipase by electrophoresis, determine the molecular weight, and approve the presence of lipase with zymography method.

2. MATERIALS AND METHODS

2. 1. Microbial Strain and Culture Condition In this work, *Aspergillus niger* PTCC 5010, from the

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Persian Type Culture Collection, wasused. It was maintained by monthly transferring to PDA slant tubes, incubated at 30 °C and stored at 4 °C. A concentration of 4×10^7 spores·mL^{-1} (estimated based on the 0.5 McFarland methods) was inoculated into 500 mL Erlenmeyer flasks with 100mL of date waste media [7]. The fermentation medium comprised of: $MgSO_4{\cdot}7H_2O$, KH_2PO_4, yeast extract, peptone, date waste, and olive oil (0.05, 0.2, 0.53, 0.53 and 2.73 %). The pH was adjusted to 7.5 with NaOH. Other conditions were 30 °C, 190 rpm and 5 days incubation time [8]. The mycelium was harvested by filtration and later centrifuged at 12000 rpm for 5 min. The clarified supernatant used as a source of extracellular enzyme [2].

2. 2. Lipase Assay The hydrolytic activities of lipase were measured by titrimetric method asdescribed by Mala et al. [9], using olive oil (Oila Company, Iran) as the substrate. Activities are expressed in international units, where 1 U of the lipase is the amount of enzyme able to catalyze the release of 1 µmol of fatty acid per minute at pH 7 and 37°C [10]. All the assay of activities were repeated three times.

2. 3. Protein Determination Enzyme Protein was determined by Lowry method [11]. Bovine Serum Albomin was used as standard protein.

2. 4. Enzyme Precipitation This operation was studied at temperature below zero. To achieve suitable concentration of solvent for precipitation, acetone with different concentrations (40, 50, 60, 70 and 80%) was added to the enzyme extract, at -20°C. The precipitate for each sample was collected by refrigerated centrifuge at 6000 rpm for 20 min at 4°C. Supernatant was discarded and the remaining solvent was evaporated. This sample was held in the freezer, then the activity and protein of each sample precipitate was determined [12].

2. 5. Determination of Suitable pH for Binding Protein to Ion Exchangers In orderto determine the optimum pH for binding proteins in the culture medium to the column, pH range of 6.4 to 8, for SP-Sepharose High Performance column and 7.4 to 8.6 for Q-Sepharose High Performance column were considered. One mL of the substrate was added to each tube with 10 mL phosphate buffer (50 mM) for the first column and Tris-HCl buffer (50 mM) for the second column for each of the pH levels understudy. To balance the ion exchangers, the buffer was changed 5 times. Then, an equal amount of protein solution was added to each tube, and the mixture was incubated for 24 h at 4 °C. Absorption of the supernatant was measured at 280 nm, and the enzyme activity was also measured [13].

2. 6. Ion Exchange Chromatography on SP-Sepharose HP (Cation Exchanger) A chromatography column of 25 × 2 cm (height × diameter) XK 16 and a chromatography system AKATA prime plus obtained from Amersham Bioscience were employed in the cation exchange chromatography step. The elution flow rate was 2 mL. min^{-1} at 25 °C and fractions of 5 mL were collected.

A 100 mL of SP-Sepharose HP was washed with 5–10 gel volumes of distilled water on a glass filter. To decrease the surface tension of the suspension gel, 250 µl Tween 20 was added and poured into the column. After the bed was compressed, the column was connected to the device, equilibrated with sodium phosphate buffer (pH = 7.2), and the sample was injected into the system. The enzyme bound to the gel for 20 min and then eluted with a linear gradient of NaCl solution.

2. 7. Ion Exchange Chromatography on Q-Sepharose HP (Anion Exchange) For purification of lipase in the second stage, the above-mentioned conditions were used, and the washing buffer and ion exchanger were changed. These partially purified lipolytic fractions were precipitated with solvent, dissolved in Tris HCl buffer (pH = 8.2), and filtrated through an acetate membrane (pore: 0.22 mm). The Q SepharoseHP column was equilibrated with a Tris HCl buffer (pH = 8.2; 50 mmol. L^{-1}). 1 mLof sample was injected into the system. The enzyme bound to the gel for 15 min, and the illusion was achieved using a non-linear NaCl gradient for better separation of proteins, with a final concentration of 1 M of salt and a flow rate of 2 mL. min^{-1}. Fractions of 5 mL were collected.

Samples with the highest enzyme activity levels were collected and mixed. Then they were pelleted by centrifugation at rpm 14000 after an equal volume of cold acetone (-20 °C) was added, and the final samples were stored in a freezer (-20 °C).

2. 8. Following the Steps of Purification

2. 8. 1. Electrophoresis SDS-PAGE (5% stacking and 12% resolving) was performed to evaluate the purity of the enzyme after each stage of purification. The gel was stained using Coomassie blue solution. To estimate the molecular weight, standard molecular weight markers obtained from Sinacolon Company, Iran were used. Lipozyme TL IM lipase from Novozymes Company were used as control. Sample of crude enzyme for greater clarity bands in electrophoresis condensed by the filter pipe cut off 10 kDa in the centrifuge 13000 × g.

2. 8. 2. Zymography To confirm the presence of lipase in various stages of purification and determination of molecular weight, after electrophoresis, gel was put in renaturing buffer containing 2.5% Triton X-100 and 0.02% NaN_3 was spun on rotator with speed of150 rpmuntil SDS was removed from the gel. To avoid the solid medium and the gel from drying out, a little phosphate buffer was added.

The composition of phenol red media was 0.01% phenol red, 1% Olive oil, 10 mM $CaCl_2$, and 2% agar. The pH was adjusted to 7.3-7.4 using 0.1 N NaOH where it was pink, then autoclaved. This layer on gel electrophoresis caused the hydrolysis of olive oil; the pH decreased with the colour yellow, and lipase was identified [14]. The composition of Rhodamine agar was 0.4% NaCl, 1% agar, and 2% olive oil. The medium was adjusted to pH 7.0, autoclaved, and cooled to about 60 ºC. Then, 31.25 mL of olive oil and 10 mL of Rhodamine B solution (1.0 mg. mL^{-1} distilled water and sterilized by filtration) was added with vigorous stirring. It was then poured into plates under aseptic conditions and allowed to solidify [15]. The assay plates were incubated at 37 °C, and the lipase band was identified as an orange halo around the colonies under UV light at 350 nm [16].

3. RESULTS AND DISCUSSION

3. 1. Precipitation of Enzyme with Solvent To determine the best concentration for solvent, the enzyme extract was precipitated by different percentages of acetone. Maximum specific activity 32.64 U. mg^{-1} and the highest purification factor 1.66 were obtained in 70% acetone. The results are shown in Table 1.

3. 2. Determination of Optimal Ph for Ion Exchange Chromatography for Purification of Lipase To increase the efficiency of the column, the maximum target protein specifically bound to the bed was considered; somehow the other proteins were not bound (unlike the cation column). The protein was boundwith thebed by electrostatic interactions as a function of pH and ionic strength of the medium. Since lipase from *A. niger* has pI 4.5, they are negatively charged and bound to the anionic column. Therefore, by providing ion exchange mediums with pH values ranging between 6.8 to 8.4 and 7.4 to 8.6 for SP-Sepharose HP for Q-Sepharose HP, respectively, equal amounts of the protein sample were added to each bed. The amounts of free enzymes in different pH levels in SP-Sepharose HP were almost the same, as this enzyme was not absorbed to a cation bed. Therefore, in order to perform the most efficient purification using this bed, the pH level at which the highest protein absorbed to the column and the lowest free protein observed were selected. According to the reported results in Figure 1 (a), the pH of 7.2 was chosen. The results of lipase that were not connected to the media at different pH levels in the context of Q-Sepharose HP showed that the optimum pH for purification by anion exchange column chromatography is pH 8.2 (Figure 1 (b)). At this pH, the values of the protein and enzyme activity showed a remarkable decline. Low absorption at 280 nm indicated the binding of more proteins to the bed, while low lipase activity in the solution on the bed indicated the specific binding of the enzyme to the bed.

TABLE 1. Precipitation of the enzyme with acetone.

Acetone (%)	Volume (mL)	Activity (U. mg^{-1})	Total activity (U)	Protein (mg. mL^{-1})	Specific activity (U. mg^{-1})	Purification factor	Yield (%)
0 (crude enzyme)	10	11.16	111.6	0.57	19.578	1	100
50	10	1.29	12.9	0.128	7.01	0.35	11.5
60	10	2.49	24.9	0.140	17.79	0.9	22.39
70	10	4.29	42.9	0.131	32.64	1.66	38.52
80	10	5.49	54.9	0.182	30.14	1.54	49.27

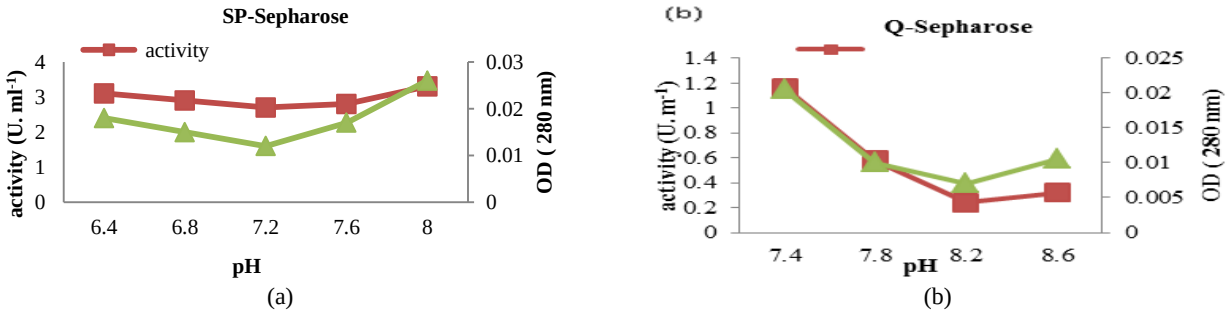


Figure 1. Amount of lipase activity and protein absorbed with (a) SP-Sepharose HP and (b) Q-Sepharose HP in different pH.

TABLE 2. Purification step of lipase from *A. niger*

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U. mg)	Purification factor	Yield (%)
Crude supernatant	111.6	5.7	11.57	1	100
precipitation with acetone	42.99	1.31	32.81	1.67	38.52
SP-Sepharose HP	38.69	0.774	50	2.6	34.6 (90% previous step)
Q-Sepharose HP	25.14	0.0102	246.47	12.59	22.5 (65% previous step)

3. 3. Purification by Column SP-Sepharose HP

SP-Sepharose HP column was the cation exchange, and negatively-charged lipases cannot be absorbed. Therefore, after the absorption of other proteins to the column, lipase was taken out in the initial fraction (11 to 20) by washing (Figure 2). The fractions having lipase activity were pooled and precipitated with acetone to be used in the next stage of chromatography. The enzyme recovery rate of the system was 90%. A recovery rate of 93% for lipase by CMC cation exchanger has been reported by Kashmiri et al. [17]. The specific activity of 50 U. mg^{-1} and a 2.6-fold purification were achieved using this stage.

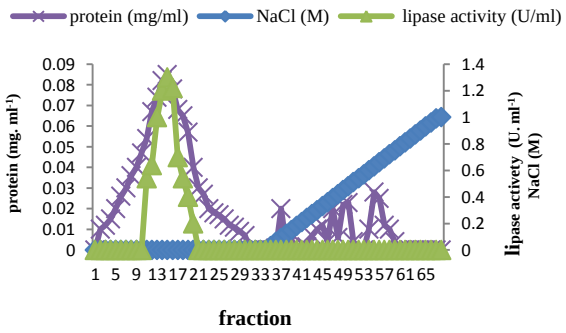


Figure 2. Chromatogram of lipase from *A. niger* on SP-Sepharose HP.

3. 4. Purification by Column Q-Sepharose HP

For purification of lipase from other proteins, the contents of fractions 11 to 20 SP-Sepharose HP, after precipitation with acetone, were dissolved in a washing buffer column and added to the column which was in equilibrium. An isolated sample of 0.25 M with the fraction of 50 to 52, had lipase activity (Figure 3). Special activities of 246.47 U. mg^{-1} and 12.59-fold purification were achieved.

The results obtained at various stages of *A. niger* lipase purification can be seen in Table 2. The specific activity obtained in the last step of purification by this method was 246.47 U. mg^{-1}, which is comparable with data reported in other papers.For example, 85.94 U. mg^{-1} for *Penicillium expansum* PED-03 lipase [18], 189 U. mg^{-1} for *R. oryzae* lipase, and 11.1 U. mg^{-1} for *R. rhizopodiformis* lipasehas been reported by [19].

The ultimate recovery of approximately 30% compared to the average of the various methods that have been reported are moderate [20]. Kim et al., 2000, cloned lipase from *Bacillus stearothermophilus* in *Escherichia coli* using two-stage chromatography with CM-Sepharose and DEAE-Sepharose. Cation and anion were purified at the rate of 11.6-fold and a recovery of 62.2% [21].

In the study with both strong cation and anion exchange, SP and Q-Sepharose were used; enzyme was purified 12.59-fold and recovery was 22.5%. The most effective step in this procedure was Q-Sepharose HP chromatography, which gave a 4.8-fold increase in specific activity.

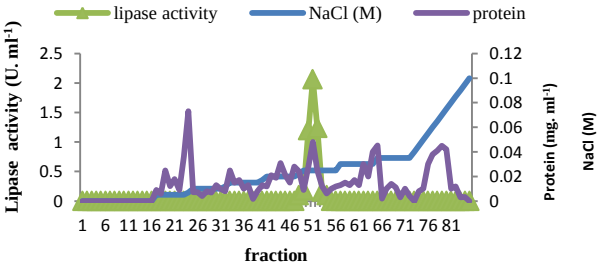


Figure 3. Chromatogram of lipase from *A. niger* on Q-Sepharose HP.

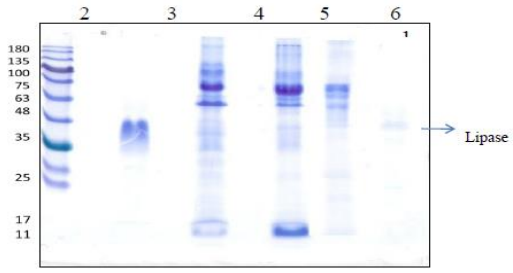
3. 5. Electrophoresis and Zymography

Electrophoresis of *A. niger* Lipase on 12% SDS-PAGE for observation amount of purity enzyme during purification stages can be seen in Figure 4 (a). The enzyme is shown by an arrow in the figure.

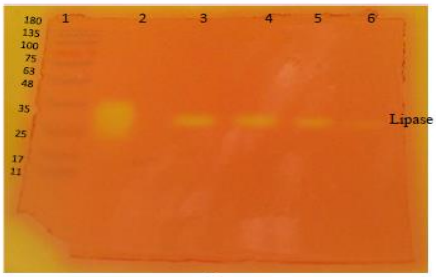
In this study, zymography revealed a yellow band, indicating that reduced pH resulted in the release of fatty acids on the orange gel, and this result shows that the purified enzyme had lipase activity.

The fatty acids released by the action of enzymes in the environment leads to the acidification of the environment and visible colour changes (Figure 4(b)). The observation of the fluorescent orange band due to the reaction of lipase with Rhodamine B on pink gel is also indicative of the activity of purified enzyme lipase. Mono- or diglycerides and fatty acid liberated into the

medium by the enzyme complexed with Rhodamine B to form dimers which can be visualized as fluorescent halos (Figure 4(c)) [22]. Based on the results of electrophoresis and zymography, the molecular weight was found to be about 30 kDa (Figure 4). Other fungal lipases have approximately the same molecular weight, e.g., *A. niger* 35.5 kDa [23], *Penicillium cyclopium* 37 kDa [24], *Rhizopus niveus* 34 and 30 kDa [25]. Zymography with various reagents and substrates such as tricaprylin [26], Tween-20 [27], and Victoria Blue [28] is used by various researchers to determine the molecular weight and properties of lipase.



(a)



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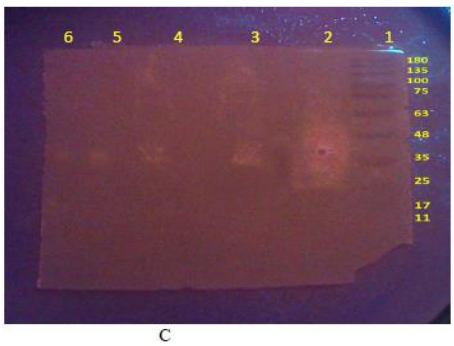

(c)

Figure 4. (a)SDS-page of *A. niger* lipase at various stages of purification. Line 1: molecular weight marker; line 2: lipozyme TL IM (as control); line 3: crude; line 4: 70% acetone; line 5: SP-Sepharose HP and line 6: Q-Sepharose HP; (b) Zymogram in Phenol Red Medium; (c) Zymogram in Rhodamine B medium.

4. CONCLUSION

The lipase was purified after submerged culture and extraction of the crude enzyme solution by precipitation method by acetone solvent and two chromatography steps. Different percentages of acetone were used to determine the optimal solution of acetone to precipitate surveyed, and 70% acetone saturated with purified enzymes to 1.6-fold purification was chosen as the best. For ion exchange chromatography usingcationic and anionic exchange, an initial optimum pH for ion exchange chromatography was used for purification of lipase. Optimal pH values were found to be 7.2 for SP-Sepharose HP and 8.2 for Q-Sepharose HP, respectively. A specific activity of 50 U· mg^{-1} and 2.6-fold purification was achieved using SP-Sepharose HP, and a specific activity of 246.47 U·mg^{-1} and 12.59-fold purification was achieved using Q-Sepharose HP. The study of the purification procedure using electrophoresis method indicated only one band with a molecular weight of about 30 kDa that presented lipase conformed by zymography, and the colour yellow on the phenol red layer and orange florescence on the Rhodamine B layer were observed. Therefore, lipase from *A. niger* PTCC 5010 produced in a date waste medium and purified using precipitation by solvent and two chromatography steps and showed to a 12.59-fold purification and desirable specific activity.

5. ACKNOWLEDGMENT

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Zymography

چکیده

در این تحقیق لیپاز حاصل از کپک آسپرژیلوس نایجر، پس از استخراج از محیط کشت توسط رسوب دهی با درصدهای متفاوت استون و کروماتوگرافی تعویض یونی با استفاده از SP-Sepharose HP و Q-Sepharose HP خالص سازی شد. جهت بررسی روند خالص سازی از الکتروفورز، و برایردیابی آنزیم و تعیین وزن مولکولی آن از زایموگرافی با استفاده از پوشاننده حاوی فنول رد و ردامین B، استفاده شد. نتایج نشان می دهند بخش اعظم آنزیم لیپاز این سویه، به وسیله استون70٪اشباع رسوب می کند که این امر باعث تخلیص آنزیم به میزان 1/67 برابر، با فعالیت ویژه $32/8U\cdot mg^{-1}$ و بازده 38/5٪ می شود. با استفاده از دو مرحله کروماتوگرافی آنزیم با فعالیت ویژه $247/47\ U\cdot mg^{-1}$ و 12/59 برابر تخلیص به دست آمد. نتایج الکتروفورز و زایموگرافی نشان دهنده باند لیپاز با وزن حدود 30 کیلو دالتون می باشد.

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