



Purification and Characterization of the Lipase from Marine *Vibrio fischeri*

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Abstract

Lipolytic enzymes from marine microbes have been the focus of intense and growing research. The bioluminescence bacterium *Vibrio fischeri* was produced lipase enzyme when the medium contained specific substrate. The lipase was purified from the concentrated culture supernatant. The most active fractions were obtained using the technique of precipitation with ammonium sulphate. The precipitated fraction was purified by desalting and ion exchange chromatography. The purified active fraction exhibiting final specific activity of 121U/mg and characterized; the optimum pH was likely between 7 to 8, the optimum temperature was 30°C and about 80 % of activity at 5°C. The enzyme was very stable at the pH 8, at the temperature 30°C. The enzyme was monomeric protein having molecular mass of 57 KDa estimated by native PAGE assay.

Keywords: *Vibrio fischeri*, Lipolytic enzymes, Extracellular, Lipase

1. Introduction

Marine microorganisms which are salt tolerant, provide an interesting alternative for therapeutic purposes. Marine microorganisms have a diverse range of enzymatic activity and are capable of catalyzing various biochemical reactions with novel enzymes. Especially, halophilic microorganisms possess many hydrolytic enzymes and are capable of functioning under conditions that lead to precipitation or denaturation of most proteins. Further it is believed that sea water, which is saline in nature and chemically closer to the human blood plasma, could provide microbial products, in particular the enzymes, that could be safer having no or less toxicity or side effects when used for therapeutic applications to humans (Sabu, 2003).

The *Photobacterium (Vibrio) fischeri* group consists of rod-shaped cells with a light yellow, cell-associated pigment and a tuft of sheathed flagella (Hendrie et al., 1970). The species is restricted to the marine environment and has a specific requirement for sodium ion for growth (Reichelt and Baumann, 1973). It occurs both free living in sea water (Ruby and Nealson, 1976) and as the specific luminous symbiont of the monocentrid fish and squid.

Lipases, triacylglycerol hydrolases, catalyse both the hydrolysis and synthesis of esters formed from glycerol and long chain fatty acids. These enzymes exhibit broad substrate specificity and degrade tweens, phospholipids often with positional, stereo and chain length selectivity (Jaeger et al., 1994). Lipases have been recognized as very useful biocatalysts because of their wide ranging versatility in industrial applications such as food technology, detergent,

chemical industry and biomedical sciences (Jaeger and Reetz, 1998; Jaeger et al., 1999; Ghanem et al., 2000; Gupta et al., 2004). Information of lipolytic enzymes produced by marine *Vibrio* spp is particularly limited. Therefore, the objective of this study is to focus on the lipase production of *V. fischeri* isolated from squid and on the purification and partial characterization of its lipase.

2. Experiments

2.1 Bacteria and growth conditions

The squids (*Sepia* sp.) were collected and cooled to about 8°C before opening the mantle cavity along the ventrum. The following two methods were used to obtain samples of symbiotic luminescent bacteria. Light organ fluid containing bacteria was obtained from pore that leads into channels with in the organ tissue. Whole light organs were removed by dissection and homogenized in 700µl of sterile seawater (Ruby and Nagai, 1992). Material obtained in either of these ways was serially diluted in seawater complete (SWC) broth and the samples were spreaded on SWC (0.38M NaCl, 0.02M MgCl₂.6H₂O, 0.025M MgSO₄.7H₂O, 8mM KCl, 0.5% peptone, 0.3% yeast extract, 2% agar and 0.3% glycerol) agar plates and identified the luminous bacteria based on the work of Reichelt and Baumann (1973) and VHA (*Vibrio* Hareyi Agar) differential media (Harris et al., 1996).

2.2 Screening of Lipase Enzyme

2.2.1 ZoBell 2216E modified media

All strains were precultivated on the solid maintenance SWC medium. For detection of lipolytic activity the following basal medium (Zobell 2216E, slightly modified) was used. It contains 10g of peptone, 1g of yeast extract, 0.05g of CaCl₂, 15 g of bacto agar made to 1 L with aged seawater, pH was adjusted to 7.6. The media supplements with either of 1% tweens (20, 80) or 0.25% Triacylglycerols (tributyryn, triolein) were then poured into petri dishes, after solidification of these media inoculated and incubated at 30°C for 10 days. The total diameter, minus the diameter of the colony was considered to be proportional to the lipolytic activity rate. After 1-10 day incubation the halos, clear (on tributyrin, triolein) or turbid (on all other substrates) were measured.

2.2.2 Spirit Blue Agar

Broth culture was streaked on the spirit blue agar plates with substrate (Tweens or Triacylglycerol). Then the plates were incubated at different temperature 6°C, 17°C, 30°C for up to 15 days. The plates were observed after 6 hrs and every 12 hrs for the clearing of the blue or deep blue color around each streak. Lipolytic activities at different hours were compared by measuring the width (millimeter) of areas of cleaning or area of deep blue color around the colonies.

2.2.3 Rhodamine B Agar

A plate assay to detect bacterial lipase in a medium containing olive oil or triglycerol and the florescent dye rhodamine B. Growth medium was adjusted to pH 7.0, autoclaved and cooled about 60°C. Then 1% of olive oil, 10 ml of rhodamine B solution (0.001% w/v) was added with vigorous stirring and emulsified by mixing for 1 min with an homogenizer. 20 ml of medium was poured into each petri dish. The overnight culture was spotted in the centre of the plate and incubated for 48 hrs. The plates were incubated for 16 hrs at 30°C. Lipase production was monitored by irradiating plates with UV light at 350nm. After 16 hrs of incubation bacterial colonies began to show an orange fluorescence, with continuing incubation time orange fluorescent halos were formed around the colonies.

2.3 Enzyme Production and Preparation of Cell Free Filtrate

150µl of culture was inoculated into 200ml of sea water medium with substrate and incubated at 30°C for 3 days. The portion was centrifuged at 20,000 xg at 4°C for 30 min. The supernatant was filtered using 0.45 µm cellulose acetate filter units. The cell free filtrate was used as the crude enzyme in the purification experiments.

2.4 Purification of extracellular lipase

2.4.1 Ammonium Sulfate Fractionation and Dialysis Against Buffer (Desalting)

Solid ammonium sulphate was added to the fraction 1 at 20% saturation and allowed to stand for 30 min. The precipitate obtained was separated by centrifugation (Fraction II) and the resulting supernatant was further treated with solid ammonium sulphate at 40% saturation. The precipitate obtained was collected by centrifugation (Fraction III). The supernatant was similarly treated with ammonium sulphate at 60 and 80% saturation and the precipitates obtained were termed as fractions IV, V respectively. All the precipitates (II-V) were resuspended in a minimal amount of buffer (0.1M Tris-HCl, pH 7.0) and dialyzed against the same buffer by using successive large volume of buffer. The process was continued till the last trace of ammonium sulphate was removed. The desalted fractions were used for further process. All the concentrated fractions (II-V) were subjected to protein and enzyme activity assay to choose the fraction containing maximum activity.

2.4.2 Ion-exchange chromatography (DEAE-Cellulose Column)

The dialyzed sample was removed from the tubing and filtered through a 0.45µm filter. Then applied to a column previously equilibrated with 0.01M Tris HCl buffer (pH 7.2) slowly percolating large volume of buffer through packed material. A sample of desalted enzyme preparation was loaded onto the column. The elution of the pure lipolytic enzyme accomplished with 15 column volume gradient of NaCl obtained by 270 ml of 0.6M NaCl in 0.01 M Tris HCl buffer (pH 7.2). Flow rate was controlled at 0.5 ml/ min by 5 ml of fractions collected and analyzed for protein and enzyme activity. After assaying the fractions for lipase activity, fractions collected from DEAE-cellulose chromatography were impregnated on to a small disc (5mm) of filter paper and placed on top of chromogenic substrate plates. The plates were incubated at 30°C for 15-30 min. Those with highest activity were pooled together and used for further enzyme characterization.

2.5 Enzyme Activity Assay

Enzyme assay used for the determination of lipase activity upon emulsified substrates and Tween solutions. The final concentration of oil was 10%, gum arabic 8%, Triacylglycerols (TAGs) 10-50mM, Tweens 0.8-20% (v/v). The mixture of the substrate and buffer (final volume 8ml) was adjusted to pH 8.0 by 1N NaOH and the pH was maintained for 3 min by titration with 50mM NaOH solution (blank). Then 1 ml of the lipase solution in the buffer was added and the lipolytic reaction was observed for 40 min by titration as mentioned above. Enzyme activity is expressed as U/ml and one unit (U) of activity is defined as µmols of free fatty acids liberated /min/ml by the enzyme solution under assay conditions.

2.6 Protein Mass Determination (Native PAGE)

In order to study the undenatured protein profile of lipase enzyme from *V. fischeri* in cell free broth, performed electrophoresis by the method described by Lammeli et al., (1970) with some modifications (Vorderwulbecke et al., 1992). Gel was casted by using discontinuous buffer system having 10% resolving gel and 5% stacking gel. For measurement of molecular mass of protein, commercial broad range molecular mass standard proteins were used. Protein bands were located by coomassie blue staining and molecular mass was determined using TotalLab v2.01 software.

2.7 Characterization of Lipase Enzyme

2.7.1 pH stability

Enzyme solutions at a concentration of 70mg/ml were adjusted to various pH ranging from pH 2 to 11 with either 0.1N NaOH or 0.1 N HCl and aliquots were incubated at 30°C for 4 hrs. Then aliquot was removed and assayed for activity.

2.7.2 pH optimum

Enzyme assays were conducted at various pH in an emulsified reaction mixture containing 0.4 ml of oil or Tween or TAG, 4ml 50mM Tris, 0.1 ml enzyme. The pH was maintained by pH stat with 0.02 NaOH. After incubation of the reaction mixture for 40 min at 30°C, it was titrated to pH 9. The quantity of free fatty acids released was calculated from the total quantity of base used. Control reaction mixture contained heat inactivated enzyme.

2.7.3 Temperature stability

The solution of enzyme at concentration of 45mg/ml was adjusted to pH 7.0 with 0.02 NaOH and aliquots were incubated at temperatures ranging from 5°C to 65°C for 4 hrs. Then the aliquots were assayed for activity.

2.7.4 Temperature optimum

Enzyme activity was determined at various temperatures ranging from 5°C to 65°C. The reaction mixtures (except enzyme) were held at the respective temperature for 5 min before the addition of the enzyme.

2.7.5 Substrate and Enzyme Kinetics

The lipase enzyme was incubated with various concentrations of substrates and the final substrate concentration ranged from 1 to 10 mM of tributyrin, triolein. Different concentrations of enzymes also studied in the concentration of 5, 10, 15 and 20 microlitres.

2.7.6 Enzyme Stability on metal ions and other chemicals

Enzymes were preincubated for 1 h at 30°C (pH 7.0) and in 0.1M Tris-HCl buffer with various ions and other chemicals (one at a time). Assay was performed with the mixture, which did not contain CaCl₂ (except in test sample). The ions used were included NaCl (10mM), BaCl₂ (0.001M), MgCl₂ (0.001M), KCl (2mM), FeSO₄ (0.001M), CaCl₂ (0.001M), SrCl₂ (0.001M), NaF (2mM), MnCl₂ (2mM), CuO₂ (2mM). Other chemicals tested were ethylene diamine tetra acetic acid (0.5% EDTA), the ammoniac detergent, sodium dodecyl sulphate (0.5% SDS).

2.7.7 Enzyme Stability During Storage

Enzyme solutions from 2 days culture were stored at -20, 1, 8, and 20°C. Formaldehyde was added to a final concentration of 0.04% to prevent bacterial growth. 30 ml of a 2 days culture of lipase solution was sterilized by membrane filtration (0.22µm, Millipore corp.,). The lipase activity in the stored samples was determined periodically by the pH stat technique and expressed as percent initial activity.

3. Results and Discussion

3.1 Bacteria and growth condition

V. fischeri encountered 100% of luminous bacteria. Total viable luminous count range was varied from 4 to 18 CFU/ml. The habitats of this squid species must receive a significant input of cells of symbiotic *V. fischeri* (Lee and Ruby, 1994). The luminous *V. fischeri* isolate was motile Gram negative rods. They produced yellow colonies on SWC agar plates. They were halophiles, unable to grow in the absence of NaCl. The colony morphology of the luminous *V. fischeri* strains tested on VHA was small (2 to 5mm) dark blue green colonies. Harris et al., (1996) reported that the VHA media displays great potential as primary isolation medium and offers significant advantages over thiosulfate-citrate- bile salts- sucrose agar.

3.2 Screening of Lipase Enzyme

In ZoBell modified media, the *V. fischeri* was showed 40 mm of halos after 10 days incubation indicates that the strain *V. fischeri* showed significant lipase activity. In fact it actively splits tributyrin and tween 80 than triolein as good substrates. Bruni et al., (1982) reported that the most strains of *Pseudomonas* sp. NCMB 1082 was split all tweens, tributyrin, but not triolein, 9 strains showed good activity on water soluble tweens, 4 on tween 85. In spirit blue agar, the width of hydrolysis areas were measured to observe the lipase activity of luminous *V. fischeri* bacteria at different temperatures. At 30°C, the lipase activity of strain was appeared after 7 hrs with a large discoloration area or dark blue halos and the widest area observed after 1 day. At 17°C, the activity began after 12 hrs, the size of halos increase after 3 days but the area of hydrolysis was smaller than that at 30°C. Apparently low activity was observed at 6°C. More or less similar mm of halos as that in ZoBell modified agar was observed in spirit blue agar with the substrate (tweens, triacylglycerol) at 30°C. The higher activity at 30°C may be attributed to maximum growth of the organism and subsequently increased lipase secretion. Similar results were observed by Makhzoum et al., (1995) during their study of lipase production by *P. fluorescens*. In Rhodamine B agar, the strains of *V. fischeri* showed 12mm of orange halos around the bacterial colonies at the 10 day incubation. Kouker and Jaeger (1987) detected a plate assay for bacterial lipase in a medium containing triacylglycerol and the fluorescent dye rhodamine B. Substrate hydrolyses causes the formation of orange fluorescence halos around bacterial colonies visible upon UV irradiation.

3.3 Purification of Lipase Enzyme

Highest yield was obtained in the medium after 3 days at 30°C. To minimize the loss of enzyme activity, the purification procedures were performed at 4°C. Results of several trials indicated that 60% saturation of the supernatant fluid with ammonium sulfate precipitated the largest amount of lipase. After dialysis, the specific activity of lipase containing dialysate was increased than that in culture supernatant fluid. In DEAE – cellulose chromatography, lipolytic fraction could be eluted with 0.6M NaCl (pH 7.0). Hundred lipolytic fractions on the top of chromogenic substrate plates showed yellow zone around the disc, those highest activity was observed in between the fractions of 40-46, similar result was observed in titrimetric assay. DEAE-cellulose column chromatogram is shown in Figure 5. The purified lipase was exhibiting final specific activity of 121U/mg and also showed single band in native PAGE. Nawani and Kaur (2000) have been used phenyl sepharose chromatography as a single step process that yielded a high active and pure lipase. Low yield of purified enzymes can be attributed to loss during ammonium sulphate precipitation as well as tight binding of lipase to hydrophobic column as a significant amount of lipase activity never eluted from the column. Like most of *Bacillus* lipases, the lipase from *Bacillus* J33 does not form aggregates (Lesuisse et al., 1993; Schmidt-Dannert et al., 1994). On the CM-cellulose column, the highest lipase activity was eluted very early in fractions 6 to 20 and about 93% of its activity was recovered. Active fractions were applied to the DEAE-cellulose and the lipase activity was highest between 0.23 and 0.35 M NaCl in fractions 21 to 32 and the recovery was 45%. The purified lipase showed only one protein band on SDS-PAGE (Abdou, 2003).

3.4 Molecular Mass

The lipase from *V. fischeri* was showed molecular mass of 57 KDa was calculated by Rf value. The crude lipase (culture supernatant) was showed multiprotein bands, indicates the impurities of other proteins (Fig 6). Lipases from *P. fluorescens* had a molecular mass of 55, 52, 32 KDa (Bazoglu, 1984; Kumura et al., 1993; Stepaniak and Fox, 1985). The lipase molecular mass was estimated to be 52 KDa (Abdou, 2003). Matsumae and Shibatani (1994) found that the molecular mass for *S. marcescens* lipase was 62 KDa, while that of *S. grimsii* was 57 KDa (Abdou, 1997). The lipase from a bacterium (*Pseudomonas aeruginosa* YS-7) showed the molecular size of 40 KDa hydrolysed a variety of fatty acid esters (Shabtai and Mishne, 1992).

3.5 Characterization of Lipase

3.5.1 pH stability

The purified lipase showed maximum stability at pH 8. Under the pH 5 the lipase lost about 40% of its activity after holding at 4 hrs. The stability range was 7 to 9. Similar results were observed by Matsumae and Shibatani (1994). *Serratia marcescens* Sr41 8000 lipase was stable between pH 6 to 9, also that of *Serratia grimisii* was stable over the pH range of 7 to 9 (Abdou, 1997). Other lipase showed stability with in the pH range of 5.5 to 9 (Fox and Stepaniak, 1983; Kumura et al., 1993).

3.5.2 Optimum pH

The optimum pH was likely between 7 and 8. The pH activity curve (Fig 1) showed that the enzyme reached about 100% of its maximum activity at pH 8. Abdou (1997) reported that the purified lipase from *Serratia grimisii* exhibited an optimum pH over the range 8 to 9, while that from *S. marcescens* Sr41 8000 showed maximum activity at about pH 8 (Matsumae and Shibatani, 1994). Previous investigation (Fox and Stepaniak, 1983) showed that the optimum pH for lipases from different psychrotrophs was 8.

3.5.3 Thermal Stability

The lipase of *V. fischeri* showed 80% stability at 35°C, but the least residual activities was at 5, 10 and 50°C. No activity was observed in 60, 65°C and above. Petersen and Daniel (2006) determined the effects of temperature and pH on the activity of purified lipase was 6-9.5 and 15-60°C respectively. The maximal activity was recorded at 40°C and at pH 8.

3.5.4 Optimum Temperature

The effect of temperature on the lipase activity (Fig 2) showed that optimum temperature for the enzyme activity occurred at 30°C and about 80% of its maximum activity occurred at 5°C. This high activity at low temperature was observed by some workers. Fox and Stepaniak (1983) found that purified lipase of *P. fluorescens* strain AFT 36 exhibited about 15% of its maximum activity at 4°C, Stepaniak et al., (1987) reported that *P. fluorescens* P1 lipase showed about 30% at 4°C.

3.5.5 Enzyme and Substrate Kinetics

The lipase activity was reached at maximum in between the 1 to 2 mM tributyrin concentration. The 50% of the activity was inhibited by 7 mM of tributyrin. This results was supported by Abdou (2003), he reported that the enzyme was inhibited by a concentration more than 6.25 mM. A linear relationship was also evident when enzyme activity was measured as a function of protein concentration. The same direct proportionality was found when crude extract were used as the source of enzyme (Oterholm et al., 1970).

3.5.6 Effect of Metal Ions, Chelator and Other Chemicals on Lipase

Figure 3 shows the results on the effect of various inhibitors on the activity of the lipase. Except NaCl, CaCl₂, which were showed no effect on enzyme activity. All other ions tested in the present study showed inhibition with relative degree of variation. Evidently even the low concentration of NAF and CuO₂ highly retarded the lipase activity. The ion chelator, EDTA and anionic detergent SDS, retarded lipase activity. The inhibitory action of former was stronger than that of latter. Action of these two compounds could be attributed to their effect in creating the imbalance of ions in the reaction mixture by absorption or release, respectively. Benjamin and Pandey (2000) reported that the chelator, EDTA and anionic detergent SDS effect 75% of lipase activity and the effect of EDTA is higher than the latter. In this study 80% of activity inhibited by SDS and no lipase activity was observed in the presence of EDTA.

3.5.7 Enzyme Stability During Storage

Figure 4 shows the lipase activity in the time of storage at different temperatures. Frozen storage did not affect the enzyme activity to any greater extent. The lipase enzyme stability suggests that the differences are due to a real decrease in enzyme production, rather than to an accelerated destruction of enzyme at higher temperatures. An explanation might be that the enzyme systems involved in lipase formation are repressed at higher temperatures, thus resulting in a lower productivity (Andersson, 1980).

4. Conclusions

V. fischeri is the bioluminous bacteria secreting extracellular lipase has been purified and characterized. The lipase hydrolytic activity upon tributyrin (C4) was found to be higher than upon triolein (C16). This higher effect of lipase was due to the different chain length of substrates. The characteristics such as pH stability in the alkaline range and high hydrolytic reaction on tweens and triacylglycerols will be useful for further research. Further study will focus on cloning and molecular characterization of the *V. fischeri* lipase coding gene.

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Vorderwulbecke, T., Kieslich, K. & Erdman, H. (1992). Comparison of lipases by different assays. *Enzyme Microb. Technol*, 14: 631-639.

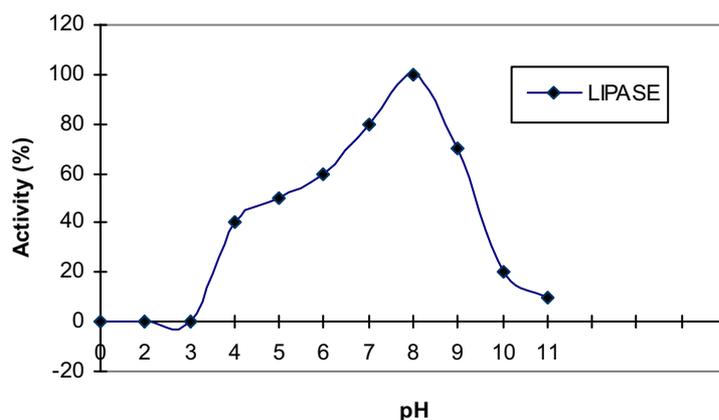


Figure 1. Effect of pH on the Lipase Enzyme from *Vibrio fischeri*

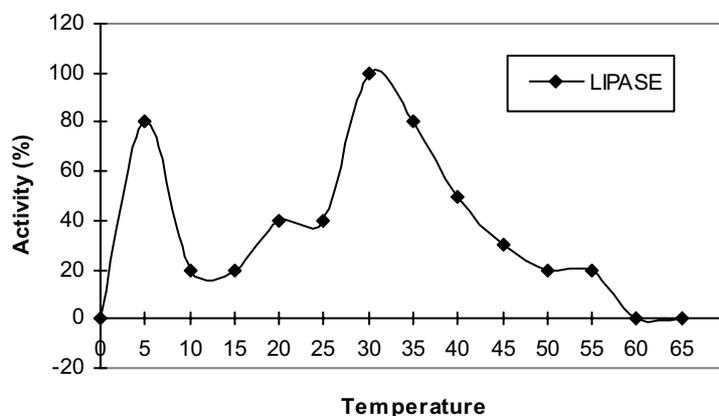


Figure 2. Effect of Temperature on the Lipase Enzyme from *Vibrio fischeri*

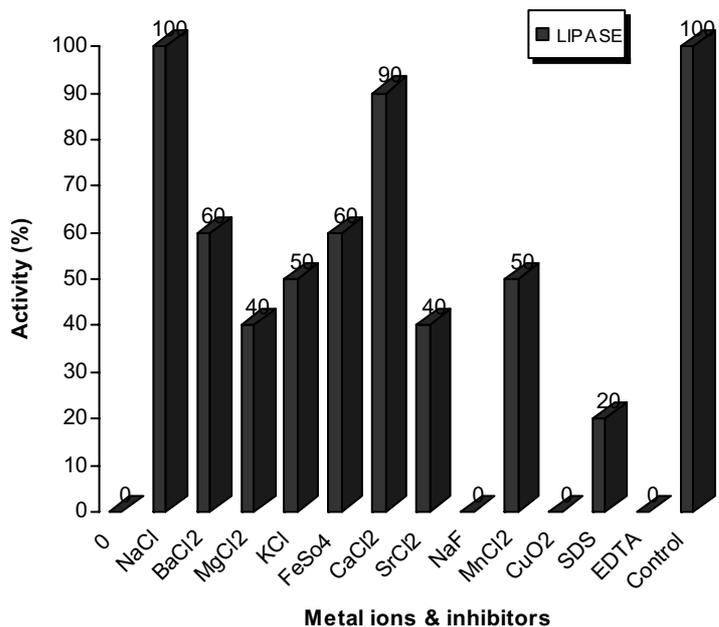


Figure 3. Effect of inhibitors on lipase enzyme

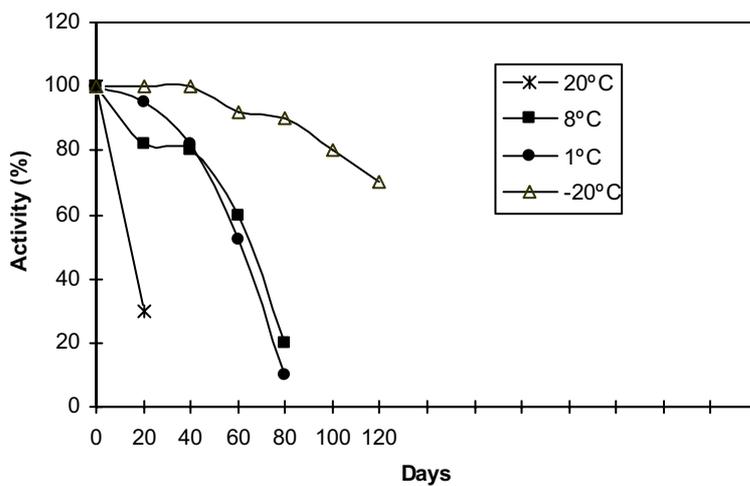


Figure 4. Storage Stability of *Vibrio fischeri* Lipase at Different Temperature

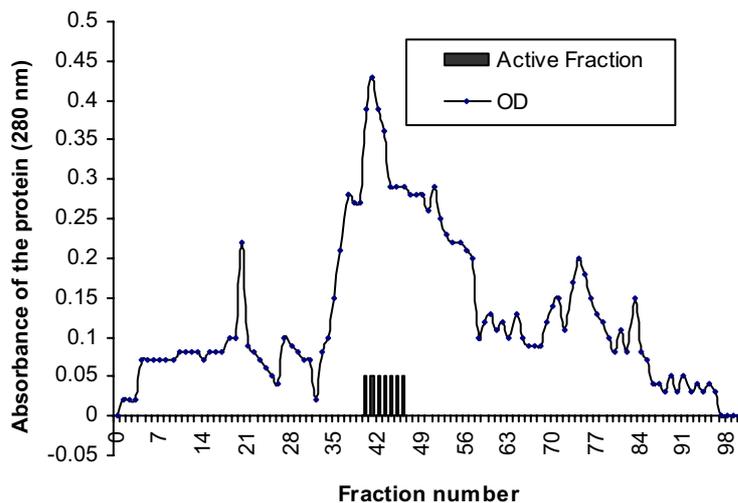


Figure 5. Elution Profile of Lipase from the DEAE-Cellulose Column

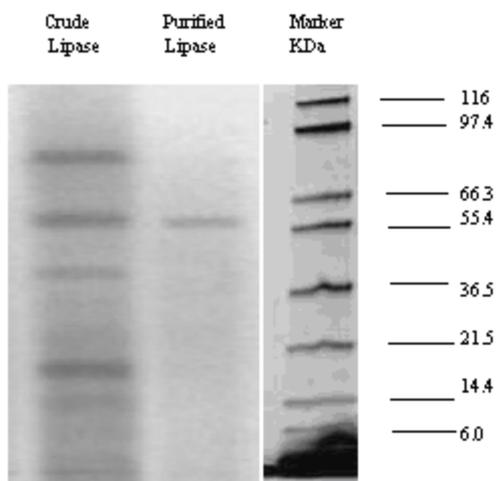


Figure 6. Molecular Mass of Crude and Purified *V. fischeri* Lipase