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ISOLATION, PURIFICATION AND CHARACTERIZATION OF LIPASE ENZYME FROM *RHIZOPUS JAPONICUS*

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Abstract

Lipase from *R.japonicus* was purified to apparent homogeneity and characterized. The purification protocol for lipase involved a combination of Phenyl Sepharose chromatography, $(NH_4)_2SO_4$ precipitation and gel filtration chromatography. The molecular weight is determined by HPLC and SDS – PAGE was found to be 29.5 ± 0.5 kDa. Kinetic studies of lipase were done with PNPA as the substrate. The optimum pH with PNPA as the substrate was found to be pH 8.0. The enzyme was stable at this pH for over a period of 24h. The temperature optimum of the enzyme was found to be 45 °C. the enzyme was stable for a period of 24h at 27 °C. Linear regression analysis of V vs (S) indicated the apparent Km for PNPA to be 0.042mM V_{max} for PNPA to be 1.105μmoles/min/ml. Polyclonal antibodies developed against pure *R.japonicus* lipase cross reacted with lipase isolated from several other fungi such as Aspergillus and Penicillium indicating the presence of common antigenic determinants.

Keywords: Lipases, *R.japonicus,* SDS-PAGE, Chromatography Techniques.

Introduction

Lipases are versatile enzymes that catalyze the hydrolysis of ester linkages primarily in neutral lipids such as triglycerides. They hydrolyze the acyl chains to free fatty acids and glycerol. The unique characteristic of lipases is the phenomenon of interfacial catalysis. Lipases are regarded as distinct class of esterases that act specifically on oil water interface. The reaction moves to equilibrium process. The enzyme must penetrate the interface in order to act. Once this occurs catalysis occurs and products are formed.

Lipases catalyse a series of different reactions. Most of the lipases cleave ester bond of triacyl glycerol with concomitant consumption of water molecules (hydrolysis). Under microaqueous conditions lipases are also able to catalyze the reverse reactions i.e. formation of ester bonds between alcohol and –COOH moiety (ester synthesis). These two basic process can be combined in a sequential fashion to give rise to a set of reactions known as transesterification. Depending upon the particular starting point in terms of substrate acidolysis occurs when an aryl moiety is displaced between acyl glycerol and carboxylic acid. Alcoholysis when acyl moiety is displaced between acyl glycerol and alcohol and transesterification.

Occurrence of lipases

Lipases are of widespread occurrence and occur throughout the Earth's flora and fauna comprising, bacteria, fungi and yeast.. A large number of microbial strains have been used for the enzyme production. *Candida* sp., *Pseudomonas* sp. and *Rhizopus* sp. are the important sources. In plants, during post germination the metabolism of oil reserves provide energy and carbon skeleton for embryonic growth and is controlled by the action of lipase (Huang, 1987).

Structure of Lipase

In 1990, the first crystal structure of two unrelated triglyceride lipase were reported. One of an enzyme purified from fungus *Rhizomucor miehel* (R ML) of 1.9 Å resolution by (Brady *et al*., 1990) and the other of human digestive enzymes, pancreatic lipase (hpL) at 2.3 Å resolution (Winkler *et al*., 1990). It has a central, predominantly parallel βsheet structure and a catalytic centre made up of a triad of Asp, His and Ser reminiscent of that found in serine proteinases (Derewanda *et al*., 1992)

Specificity of lipase

The substrate specificity of a lipase is defined by its positional specificity or stereo-specificity or its preference for long or short chain saturated or unsaturated fatty acids (Hoff and Jensen, 1974).

There are 3 major specificities of lipase

1. **Substrate specificity:** The enzyme hydrolyses various acyl glycerols (monoglycerols, diacyl glycerol, triacyl glycerol) or types of fatty acids at different positions.
2. **Positional specificity:** Enzyme catalyses the release of free fatty acids at preferential positions primary, secondary esters or random hydrolysis on the acylglycerol molecule.
3. **Stereo Specificity:** The enzyme hydrolyses the two primary esters at different rates. The stereo chemistry of glycerol derivatives is expressed by the stereo specific numbering which recognizes the two primary carbinol group of the parent glycerol that are not identical in their reactions with non symmetric structures.

Physiological Significance of lipases

Today lipases are the enzymes of choice for organic chemists, pharmacists, biophysicists, biochemists, biotechnologists, microbiologists, food technologists, biochemical- and process engineers due to its versatility. Information on lipolytic enzymes in higher plants is important in understanding their physiological roles as well as their action in agricultural products during storage. In post-germination of oil seeds, the mobilization of oil reserves is essential in providing energy and carbon skeleton for embryonic growth. The turnover of membrane lipids in various tissues is dependent upon lipolytic enzymes. Lipolytic enzymes catalyze the initial steps during lipid mobilization and thus may be rate controlling in germination and post germination growth.

Applications of lipase

Enzyme – mediated reactions are alternative to tedious and expensive chemical methods. However, with the realization of the biocatalytic potential of lipases in both aqueous and non-aqueous media in the last one and a half decades, industries have shifted towards utilizing this enzyme for a variety of reactions of immense importance.

1. **Lipases in Dairy industry:** Lipases are used extensively in the dairy industry for the hydrolysis of milk fat. Current applications include the flavor enhancement of cheese, the acceleration of cheese ripening, the manufacture of cheese like products, enzyme modified cheese (EMC) and the lipolysis of butterfat and cream.
2. **Lipases in pharmaceutical and agrochemical industries:** The resolution of 2 – halopropionic acids, the starting materials for the synthesis of phenoxy propionate herbicides, is catalyzed by porcine pancreatic lipase.
3. **Lipases in oleo chemical industry:** Lipases have tremendous potential in small-scale enzymatic fat splitting process for the production of high value polyunsaturated fatty acids and the manufacture of soaps.
4. **Lipases in detergents:** At present, lipases have not played a significant role in household detergents mainly because of the lack of enzymes that are stable and active under alkaline conditions.

Isolation and Purification

Lipase from different sources have been isolated by the classical methods of protein isolation. The common methods employed for the purification of lipase include, ammonium sulfate fractionation, gel filtration chromatography and affinity chromatography. Affinity chromatography is the only technique, which enables purification of almost any biomolecule on the basis of its biological function or individual chemical structure. The different affinity adsorbents, which are commonly used, are melibiose-Sepharose, melibiose – agarose and ConA-Sepharose.

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Materials and Methods

Chemicals

R.japonicus was isolated from mangrove soil samples. Petroleum ether, disodium phosphate, mono sodium phosphate, Tris, $CaCl_2$, Phenyl-Sepharose, Sephadex G-150 were obtained from Pharmacia Chemicals, Upsala, Sweden. *p*–nitrophenyl acetate, Bovine serum albumin (BSA), Coomassie brilliant blue R – 250, N, N, N′, N′ – tetra methyl 1, 2 diamino ethane (TEMED), α-naphthyl acetate, tetrazotized *o*-dianisidine, BCIP(5-bromo-4chloro 3-indolyl phosphate), NBT (nitroblue tetrazolium) were from Sigma. Sodium dodecyl sulfate (SDS), SDS-PAGE low molecular weight markers kit was from Bangalore Genei, Bangalore.

Methods

Preparation of R.japonicus Lipase

One hundred grams *R.japonicus* mycelium was taken in 300 mL of petroleum ether (60 - 80 °C) and stirred for 12-16 h. Then filtered through Whatman filter paper No. 3 and air dried. The fat content was estimated to be ~ 2 % after defatting.

Preparation of Crude Extract

100 mL of 50 mM sodium phosphate buffer pH 7.4 is added to 10 g of defatted *R.japonicus* mycelium kept for cold overnight at 4 °C centrifuge at 14000 rpm for 45 min at 4 °C.

Preparation of Phenyl-Sepharose

Phenyl-Sepharose purchased from Sigma chemical co. was supplied as a suspension in 0.5 NaCl containing 0.02 % thimerosal. The Phenyl-Sepharose medium was washed extensively with distilled water and then equilibrated in 10 mM Tris-HCl buffer, pH 7.0 containing 1 mM $CaCl_2$. The medium was packed in a glass column of dimensions, 11.5 × 3.5 cm at a flow rate of 20 mL/h. the column was washed with 5 bed volumes of the equilibrating buffer and the pH of the eluent was checked before loading the sample on the column.

After each purification cycle, bound substances were washed out from the column to restore the original function of the column. Hydrophobic interaction chromatography absorbents can normally be regenerated by washing with distilled water after each run. To prevent slow build up of contaminants on the column over time, after every three cycles, a more rigorous cleaning protocol was applied (Pharmacia, 1993). According to this sanitization protocol, the Phenyl Sepharose column was washed with 4 bed volumes of 0.5 M NaOH to remove any precipitated proteins, followed by 2-3 bed volumes of distilled water. The column was further washed with 6 bed volumes of 30 % isopropanol to remove strongly bound hydrophobic proteins, lipoproteins or lipids. The column was then washed with 3-4 bed volumes of distilled water. The column matrix after sanitization was equilibrated in the starting buffer and was reused. The column was stored in starting buffer containing 0.05 % sodium azide when not in use.

Preparation of Sephadex G-150

Sephadex G-150 of particle size of 40-120 µm, which give bed volume of 20-30 mL/gms of dry gel was used. The exclusion limit of Sephadex G-150 is 5 kDa - 300 kDa for globular proteins.

Fifteen grams of Sephadex G-150 was allowed to swell in 500 mL of 0.5 M sodium phosphate buffer, pH 7.4 and fines removed by decanting. The slurry was packed into a glass (100 × 2 cm) at a flow rate of 15 mL/h. Gel filtration chromatography on Sephadex G-150 was used for the first column chromatography step in the purification of fungal lipase. The column was stored in buffer containing 0.05 % sodium azide when not in use.

Enzyme Assay

R.japonicus lipase activity was determined by using *p*-nitrophenyl acetate as a substrate. Lipase acts on *p*-nitrophenyl acetate and release p-nitrophenyl (PNP) detected at 410nm (Σ = 15000 M^{-1}/cm^{-1}). The activity of the enzyme was assayed using 0.01 mL of 5 mM *p*-nitrophenyl acetate in acetonitrile, 0.1 M sodium phosphate buffer pH 7.4 containing enzyme in a total volume of 1 mL. The assay mixture was incubated for exactly 10 min at 25 °C and the absorbance was read at 410 nm. One unit of lipase activity is defined as the amount of enzyme that produces one micromole of *p*-nitrophenol per min at 25 °C and pH 7.5.

Protein Estimation

The protein concentration of the lipase was estimated according to the method of Bradford (1976) using bovine serum albumin as standard. Bradford reagent. Coomassie brilliant blue R-250 (100mg) dissolved in 50 mL of 95 % ethanol, 100 mL of phosphoric acid 85 % (w/v) added and diluted to 100 mL. Filtered twice and stored at 4 °C.

Results and Discussion

Purification of lipase was performed using *R.japonicus* as starting material. A crude extract of *R.japonicus* mycelium lipase was prepared by extraction for 16h, at 4 °C. The specific activity of the crude extract was 0.05 units/mg. The crude extract was concentrated using an Amicon Ultra-15 centrifugal filter device with a 10kDa cut off. This resulted in the removal of small molecular weight proteins. The concentrate was then chromatographed on a Phenyl Sepharose CL-4B column equilibrated in 0.01M Tris-HCl buffer, pH 8.0 containing 1 mM $CaCl_2$. Elution of the sample was carried out using the same buffer at a flow rate of 10 mL/h. The elution profile is shown in Figure 3. In this step, an inactive protein fraction eluted in the wash followed by a second protein peak. All the lipase activity eluted as a single symmetrical peak on the descending shoulder of the second protein peak. The specific activity was 0.150U/mg. A purification of 2.6 fold was obtained (Table 1).

To the pooled sample, solid $(NH_4)_2SO_4$ (51.6 g/100 mL) was added to obtain 80 % saturation at 4 °C. The precipitate formed was removed by centrifugation at 15000 rpm for 30 min at 4 °C. the precipitate was dissolved in minimal amount of 10 mM Tris-HCl containing 1 mM $CaCl_2$ pH 8.0and used in the next step.

R.japonicus lipase was further purified by gel filtration chromatography. The sample was chromatographed on a Sephadex G-150 column pre-equilibrated in 10 mM Tris-HCl buffer containing 1 mM $CaCl_2$, pH 8.0 at a flow rate of 10 mL/h. The void volume was 60 mL. RBL lipase eluted as a single peak well resolved from a high molecular weight fraction. The pooled fractions had a specific activity of 0.212 U/mg. A purification of 5.30 fold was obtained. Yield 8 % was obtained. The purification is summarized in Table.1.

Table 1. Summary of purification of lipase.

Step	Total Protein (mg)	Total activity	Specific activity (U/mg)	Fold purification	Yield (%)
Crude	212.9	12.17	0.040	---	100
Phenyl-Sepharose chromatography	7.4	1.12	0.150	2.70	13
SephadexG-150 chromatography	2.14	0.787	0.212	5.30	8

Criteria for Homogeneity

The homogeneity of the purified protein was assessed by native protein gel, specific activity staining and analytical size exclusion chromatography. In the native PAGE, the purified enzyme was electrophoresed in 12.5 % T polyacrylamide gel in Tris–Gly buffer pH 8.3 and located by protein staining and enzyme activity. The purified enzyme revealed a single band by specific enzyme staining with naphthyl acetate and Coomassie Blue indicating the presence of a single isoform.

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The purify of lipase was also determined by analytical size exclusion HPLC using a BIOSEP – SEC – S 3000 (300 × 7.8 mm) column. The single symmetrical peak observed in the profiles indicates the homogenous nature of the enzyme (Figure 1).

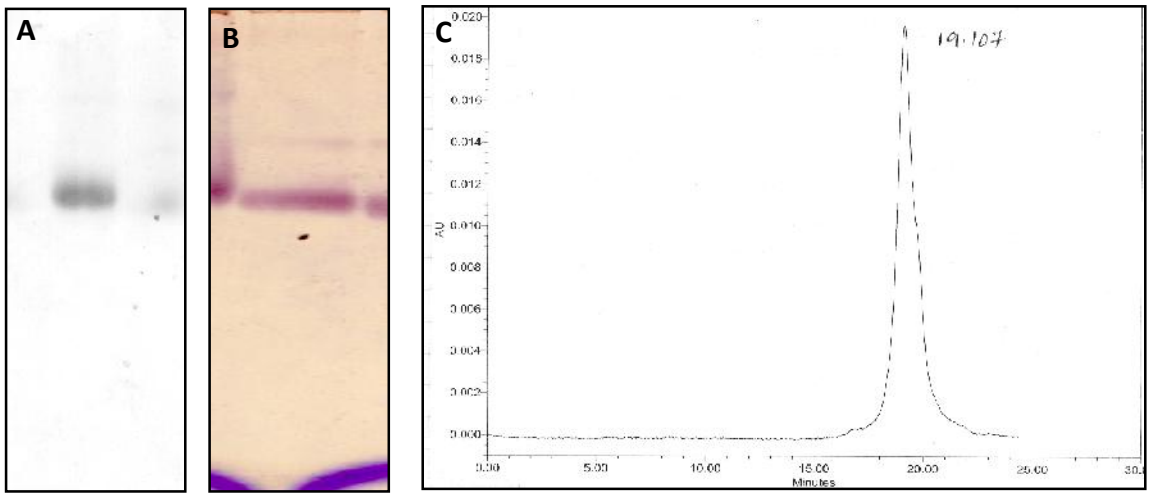

Figure 1. Homogeneity of lipase. A) Protein gel; B) Activity staining and C). Analytical Gel filtration chromatography.

Molecular Weight Determination

The apparent Mr weight of lipase was determined by size exclusion on BIOSEP – SEC – S 3000 column and by SDS – PAGE. The molecular weight of the purified enzyme estimated by HPLC size exclusion was 29.5 kDa from a plot of log Mr versus retention time (Figure 2).

SDS – PAGE of purified lipase on 12.5 % T gel was carried out in a discontinuous buffer system. The molecular markers used were Phosphorylase b (97.4 kDa), BSA (66.3 kDa), Ovalbumin (43.0 kDa), Carbonic anhydrase (29.0 kDa), Soybean trypsin inhibitor (20.0 kDa), Lysozyme (14.3 kDa. SDS-PAGE followed by protein staining using silver staining showed a single protein band of 30 kDa calculated from a plot of log Mr vs retention factor (Figure 7 A and B). The molecular weight of the purified enzyme estimated by analytical gel filtration and SDS-PAGE indicate that RBL is a single polypeptide of 29.50±0.5 kDa

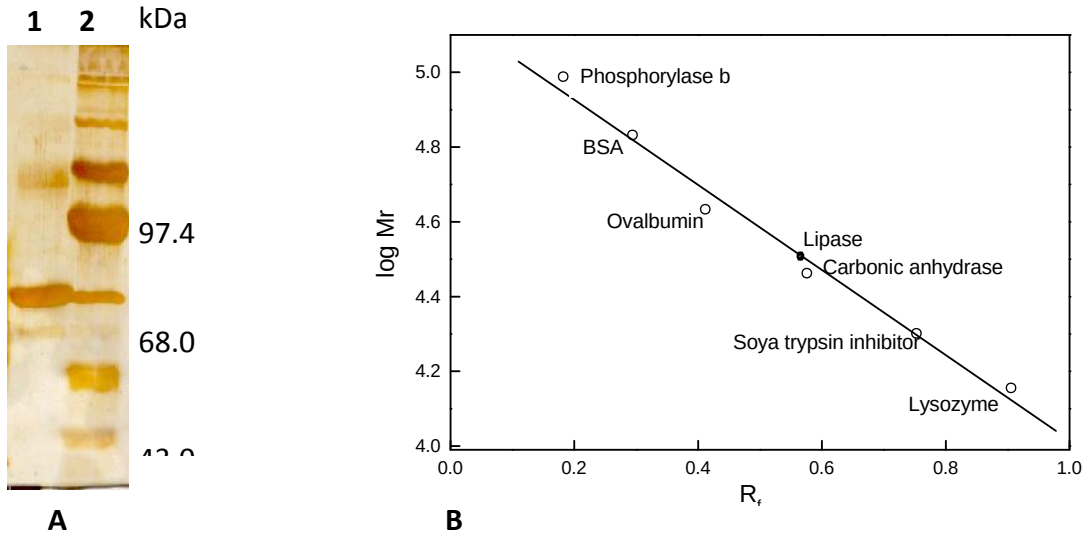

Figure 2. Molecular weight determination of lipase.
A) SDS-PAGE (12.5 % T, 2.7 % C) and B) Plot of relative mobility vs log Mr of standard proteins.

Characterization of lipase

Every enzyme has specific conditions for optimum activity. These include temperature, pH, varying concentrations of substrate and presence of inhibitors. Characterization studies were undertaken with respect to the above factors.

Effect of pH

pH is a measure of the concentration of the hydrogen ions in a solution.. Enzymes require specific pH requirements for optimum activity. The optimum pH of lipase was determined by measuring the activity at various pH using different buffers. The buffers used were McIlvaine buffers (0.1 M citric acid and 0.2 M Na_2HPO_4) (pH 2.5 – 7.5), 0.1 M Tris buffer (pH 7.5 – 9). The maximum activity of lipase with PNPA as the substrate was found to be pH 8.0 (Figure 8). A second optima was observed at pH 4.0, which had 75% of the activity at pH 8.0. The pH optimum of 8.0 indicates presence of a protonated His residues at the active site.

Effect of Temperature

Temperature has profound effect on the activity of the enzyme reaction. Every enzyme has a temperature at which its activity as maximum. Some enzyme are totally inactive at high temperatures owning to denaturation.

The effect of temperature on the activity of the enzyme was performed by assaying the purified enzyme at different temperatures ranging from 4-55 °C, using 5 mM PNPA as the substrate in 0.1 M Tris-HCl buffer pH 8.0 as shown in the Figure 9. The optimum temperature for the activity of lipase was found to be 45 °C. However at this temperature *lipase* is inactivated rapidly therefore the assays were performed at 25 °C, which corresponds to 80% of the activity at 45 °C

Temperature Stability

The temperature stability of the enzyme was examined. Fungal lipase was pre-incubated at different temperatures ranging from 27–45 °C in a water bath preset to the appropriate temperatures. Aliquots were removed at different time intervals and assayed for the residual activity using PNPA at pH 8.0. The enzyme retained its activity over a period of 24 h at 27 °C. However at 37 °C the enzyme retained 63% of its activity after 4 h. The activity decreased to 55 % when pre-incubated at 45 °C for 4 h (Figure 10).

Effect of Time

The optimum time of the lipase reaction was determined at various times ranging from 5 – 25 min using 5 mM PNPA as the substrate in 0.1 M Tris-HCl pH 8.0. As shown in Figure 11the increase in activity was liners with increase in time. These results suggest that the time of assay can range from 5-25 mins to get optimal activity. The assay procedure used in this investigation is limited to 10 mins.

K_m and V_{max}

One of the important factors affecting the rate of the reaction is the concentration of the substrate. The effect of varying PNPA concentration on the initial velocity of the RBL catalysed reaction was studied. The results showed that the reaction followed Michaelis–Menten kinetics. Linear regression analysis

of 1/V vs 1/[S] indicated the apparent K_m for PNPA to be 0.042mM mM. Linear regression analysis of 1/[V] Vs 1/[S] indicated the apparent V_{max} was 1.105μmoles/min/mL .

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