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Biochemical Studies of Lipase from Germinating Oil Seeds (*Glycine max*)

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Abstract: Problem statement: Lipase is one of the important enzymes in food, pharmaceutical, detergent and biofuels industries. Search for the lipase with distinct features, possibly from germinating seeds, is of interest for industrial applications. **Approach:** The lipase produced by soybean oil seeds was partially purified and characterized in terms of the optimal pH and temperature for activity as well as substrate specificity. **Results:** The lipase was extracted and partially purified from germinating soybean seeds using chilled acetone and ammonium sulfate precipitation. Partially purified and dialyzed enzyme profile was observed on native-Polyacrylamide Gel Electrophoresis (PAGE). The lipase was optimally active at pH 8 and temperature of 24°C. In the presence of Ca^{2+} and Mg^{2+} enhance the activity at low concentration, while the Hg^{2+} and Ethylene Diaminotetracetic Acid (EDTA) showed inhibitory effect. The enzyme was found to be metalloenzyme. Enzyme kinetics with olive oil emulsion substrate showed k_m and v_{max} of 7.67 mg and 0.0125 µm mL min^{-1}, respectively. **Conclusion:** The mettaloenzyme enzyme was able to attack specifically on oil in seeds to generate free fatty acids as the major end product. This understanding may help in devising efficient methods to overcome the problem of soybean seed oil in stability.

Key words: Soybean seed, germinating seeds, partial characterization, mettaloenzyme enzyme, industrial enzymes, lipolytic enzymes, food industry, partial purification, soybean germinating

INTRODUCTION

Lipases (EC 3.1.1.3) are among the most important classes of industrial enzymes. In recent years the growing demand of lipolytic enzymes has been increased due to its potential use in the various manufacturing processes of industrial goods such as detergent industry, food industry, cosmetics, flavour enhancers and in pharmaceutical industry (Gandhi, 1997; Corzo and Revah, 1999; Cancino *et al*., 2008). Lipase may be used to produce fatty acids (Linder *et al*., 2002), aroma and flavor compounds (Athawale *et al*., 2003), lubricant and solvent esters (Hills, 2003), polyesters (Kumar and Gross, 2000), amides, thiol esters (Gandhi, 1997) and biomodified fats (Neklyudov *et al*., 2002).

Lipases are widespread in nature and have been found in animals, higher plants and microorganisms. In plants lipase activity has been identified in various tissues but relatively high concentration is found in seeds. Seeds are generally rich in triacylglycerols, which serve as compact source of energy for the newly emerging plant. During germination of the seed, the reserved triacylglycerols are disappeared, since the fatty acids can't be oxidized to provide energy until they are released from the triacylglycerol. Lipases are

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probably rate controlling during germination and the activity of the lipase is high during germination (Brockerhoff and Jensen, 1974; Ejedegba *et al*., 2007). Recently, seed lipases have been the focus of much attention as biocatalysts. In some cases, these enzymes present advantages over animal and microbial lipases due to some quite interesting features such as specificity, low cost, availability and ease of purification, representing a great alternative for potential commercial exploitation as industrial enzymes (Barros *et al*., 2010). In the present study we report the isolation and partial characterization of a lipase from germinating soybean seeds.

MATERIALS AND METHODS

Seed collection and germination: Blackseeds (*Guizotia abyssinica*), seeds of soybean (*Glycine max*), groundnut (*Arachis hypogaea*), pea (*Pisum sativum*) and caster (*Recimus communis*) were purchased from local market. All seeds were soaked in water for six h and were allowed to germinate for 24 h at room temperature (28±2°C) on moist germination studys.

Extraction and partial purification of lipase: After 24 h of seed germination, the seed coats were removed manually and 20 g seed cotyledons were homogenized in chilled acetone at 4°C. The suspension was centrifuge at 3000 rpm and residue obtained was dissolved in 100 mL distilled water followed by centrifugation at 7500 rpm. The supernatant was used as source of crude enzyme and was precipitated by ammonium sulphate (80% saturation) according to Michael *et al.* (2001). The precipitate was obtained by centrifugation at 10,000 rpm for 20 min. Precipitate was dissolved in 20 mL Tris-Cl buffer (10 mM, pH 8.5) and dialyzed overnight against the same buffer. The dialyzed enzyme was used as partially purified enzyme and used for enzyme characterization.

Lipase assay: The titrimetric method of Maliks *et al* (2000) was used for determination of lipase activity. Olive oil emulsion was prepared in 180 mL distilled water containing 20 mL olive oil, 0.4g of sodium benzoate and 1g gum-arabic. Assay mixture contained 5 mL olive oil emulsion, 5 mL 0.1M Tris buffer (pH 8) and 1 mL crude enzyme and incubated at 35°C for 10 min. The reaction was stopped by 10 mL of acetone and methanol mixture (1:1). Each sample was titrated against 0.025 N NaOH using 1% phenolphthalein as indicator. The volume of NaOH used in the titration was noted and used for enzyme activity calculations. One unit of lipase is defined as the amount of enzyme required to liberate 1µmol of free fatty acid from olive oil per min under the standard assay conditions.

Protein estimation: Protein concentration of soluble enzyme preparation was quantified by method of Lowry *et al.* (1951) using Bovine Serum Albumin (BSA) as a standard.

Lipase characterization:

Polyacrylamide gel electrophoresis: Enzyme purification or purity was checked on non denaturing native PAGE using 10% gel concentration with slight modification on method described by Holt and Hartman (1994). Gel was stained using the Coomassie Brilliant Blue R-250 staining solution. Total enzyme protein used for PAGE was of 10 µg for crude as well as partially purified lipase.

Effect of pH and temperature: Optimum pH for lipase activity was determined covering the range (3-9) using 0.1M buffers of different pH. The buffers were: pH 3-6 (acetate); pH 7 (phosphate); pH 8-9 (Tris-Cl). For optimum temperature, the enzyme assay was performed as discussed above except that incubation was done at temperatures from 20-70°C.

Enzyme kinetics: Lipase was assayed in reaction buffer (pH 8) at 24°C with different concentrations (10-120 mg mL^{-1}) of olive oil emulsion as a substrate. The values of v_{max} (maximum velocity) and k_m (Michael is constant) were calculated from Lineweaver-Burk (LB) plot.

Statistical analysis: All experiments were conducted in triplicates and results were represented with standard deviation calculated by Microsoft excel program.

RESULTS

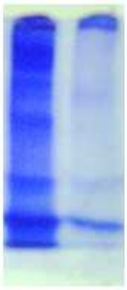
Lipase screening: Among the all five germinating seeds, only soybean germinating seeds (10.37±0.28 $\times 10^{-3}$U/mL) showed maximum lipase activity followed by caster seeds (Table 1). Moderate lipase production was observed in blackseeds. Whereas, less and significantly same lipase activities were found in groundnut and pea seeds.

Table 1: Lipase activity profile of various Indian germinating oil seeds

Plant source	Botanical names	Lipase activity $\times 10^{-3}$ (U/ml/min)
Black seeds	Guizotia abyssinica	6.25±0.52
Soybean	Glycine max	10.37±0.28
Ground nut	Arachis hypogaea	4.37±0.48
Pea	Pisum sativum	4.12±0.34
Caster	Recimus communis	7.25±0.53

±: Indicate the values of activities and standard deviation of triplicate analysis

Enzyme characterization: Lipase was partially purified by acetone and ammonium sulphate precipitation followed by dialysis. Partial purification showed cut off of unnecessary proteins and was evidenced on native PAGE (Fig. 1).



(a) (b)

Fig. 1: Electrophoresis pattern of crude and partially purified lipase isolated from soybean germinating seeds. (a) Crude enzyme; (b) Partially purified dialyzed enzyme

Effect of pH on lipase activity: The enzyme activity increased with an initial increase in pH and optimum activity was noted at pH 8 suggesting alkaline nature of the enzyme. Further increase in pH beyond optimum caused a rapid decrease in the enzyme activity (Fig. 2).

Effect of temperature on lipase activity: The temperature activity profile of soybean germinating seed lipase is shown in Fig. 3.

Table 2: Effect of metal ions on lipase activities from germinating soybean seeds

Metal ion	Concentration (mM)	Residual lipase activity (%)
Control	-	100.00
$CaCl_2$	0.001	107.36
	0.005	86.31
$MgCl_2$	0.001	104.21
	0.005	80.00
EDTA	0.001	87.15
	0.005	62.10
$HgCl_2$	0.001	82.10
	0.005	68.42

EDTA- Ethylenediaminotetraacetic acid

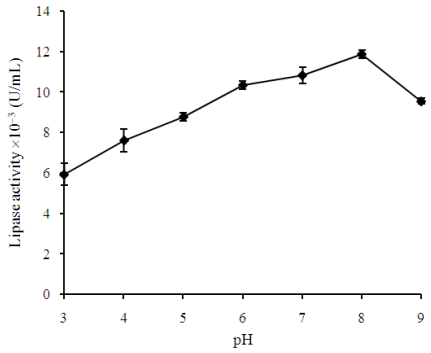


Fig. 2: Effect of pH on lipase activity isolated from soybean germinating seeds. Lipase assay was performed at 35°C and at various pH values. Error bars indicate standard deviation between replicates (n = 3)

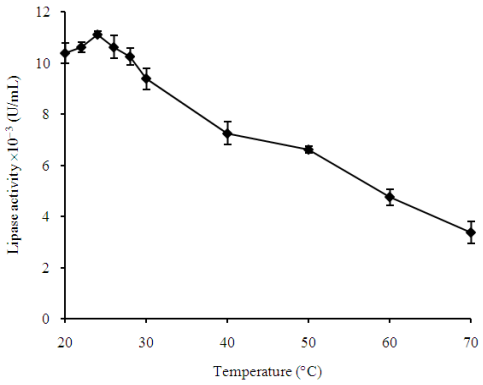


Fig. 3: Effect of temperature on activity of the lipase isolated from soybean germinating seeds. Lipase assay was performed at pH 8 and at various temperatures. Error bars indicate standard deviation between replicates (n=3)

Effect of substrate concentration: The k_m and v_{max} for the lipase were determined using olive oil emulsion as a substrate. The Km value for the free enzyme, estimated from Lineweaver-Burk plot (Fig. 4) was 7.67 mg with olive oil emulsion as substrate. The v_{max} value obtained from the L.B. plot was 0.0125 µm mL min^{-1}.

Effect of metal ions and inhibitors: Metal ions as Ca^{+2}, Mg^{2+} at lower concentration (0.001 mM) showed enhanced effect on lipase activity where as at higher concentration the lipase activities were found to be inhibited. EDTA and Hg^{+2} inhibited the enzyme activity (Table 2).

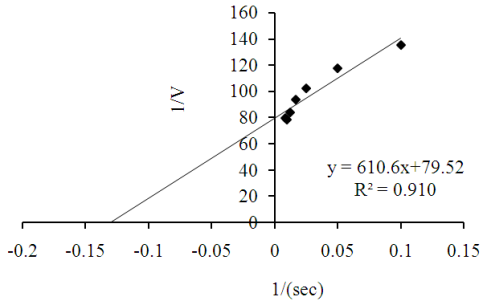


Fig. 4: Lineweaver-Burk plot for lipase from soybean germinating seeds. Lipase assay was conducted at various substrate concentrations at pH 8 and temperature 24°C. The data were plotted according to Lineweaver-Burk. Each value is average of three independent experiments

DISCUSSION

Plants are the major source of oil and due to storage these oil seeds also produced lipases. Present study showed 5 germinating oil seds for lipase characterization. Likewise but three different varieties of *Cucumeropsis* were studied for lipase characterization by Eze and Chilaka, (2010). Lipases were produced from a germinating seeds by various researchers (Abigor *et al*., 2002; Miled *et al*., 2000) and purified with the help of acetone precipitation and different chromatographic steps as reported earlier (Opute, 1975; Bahri, 2000; Sammour, 2005; Su *et al*., 2010). (Bahri, 2000) showed SDS electrophoresis pattern of germinating linseeds.

Using soybean lipase, Huang, (1982) observed an optimum pH of 9.0 with glycine-HCI buffer and 6.5 with imidazole-HCI Buffer. A pH optimum from 7.5 has previously been reported for lipases from some seeds of *Jatropha curcas* L. (Abigor *et al*., 1985). However, lipase from *Cucumeropsis manii* showed pH optima at 4.5 and 7.5 suggesting the presence of both acidic and alkaline lipases (Eze and Chilaka, 2010). Enujiugha *et al*. (2004) also isolated a lipase from coconut seeds that was found to be active at pH 7.5-8.5 and 35°C.

The lipase had an optimum temperature of 24°C that is close to (30°C) the previously reported optimum temperatures for lipases from *Cucumeropsis manii* (Eze and Chilaka, 2010). A optimum temperature of 45^{o}C has previously been reported for lipases from some seeds of *Jatropha curcas* L. (Abigor *et al*., 2002). Abigor *et al*. (1985) purified oil palm lipase which had optimum temperature of 30°C, above which there was a steady decline. Enujiugha *et al*., 2004 observed a gradual decline in the activity of the lipase from conophor nut with successive increases in temperature, from 30-80°C.

The k_m of 0.23 mM with olive oil substrate for a lipase from germinating oil seeds (*Brasicca napus* L) was found by Sana *et al.* (2004).

Likewise, EDTA has inhibitory effect on lipase activity of linseed was observed by Sammour, (2005). Strong inhibition of the lipase activity in our case shows that the enzyme requires metal ions that are chelated out with EDTA. Mg^{2+} has been reported to increase the lipase activity from linseed (Sammour, 2005). Our results were in agreement with the Enujiugha *et al*. (2004) which showed Ca^{+2} as activator while Hg^{+2} and EDTA as inhibitors of African oil beans lipase.

CONCLUSION

Lipase was isolated and partially purified from germinating soybean seeds. An alkaline lipase (optimum pH 8.0) with a fair thermoactivity was isolated from the cotyledons of germinating soybean seeds (*Glycine max*). The germinating soybean seed lipase could prove useful in industrial biocatalytic hydrolysis. It could also be inferred from the present preliminary characterization that the germinating soybean seed lipase could prove useful in processes that require lower cooling costs and minimal corrosion problems. Ca^{2+} and Mg^{2+} enhanced the enzyme activity at lower concentrations, while EDTA and Hg^{+2} caused various degrees of inhibition. The results show that the germinating soybean seed lipase could be exploited in industrial processes. This understanding may broaden the use of lipases in industry and medicine and may help in devising efficient methods to overcome the problem of soybean seed oil instability.

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