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Identification, Purification and Characterization of Lipase from Germinating Oil Seeds (*Brassica napus* L.)

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Abstract: Ionic effect on the degradation of oil seeds (*Brassica napus* L) storage lipid was investigated during germination. Lipid degradating enzyme lipase was identified in germinating *Brassica napus* L and the enzyme was purified to homogeneity by extraction, Sephadex G-50, DEAE and CM-cellulose column chromatography. The enzyme was purified to 67.59 fold with the final specific activity of 39.10 mmol min^{-1} mg^{-1} at 37°C using triolein as a substrate. The purified enzyme showed a single band when it was subjected to SDS / PAGE electrophoresis. The SDS/PAGE electrophoresis indicated a molecular mass of 34 kDa for this lipase which corresponding to the high-resolution gel filtration. The optimum pH and temperature for hydrolysis of Oleic acid were 7.0 and 37°C, respectively. In the presence of Ca^{2+} and $Bi^{3+,}$ the lipase activity was dramatically enhanced by 165 and 124%, respectively. Fe^{3+}, Fe^{2+}, Zn^{2+}, Hg^{2+} and Cu^{2+} could inhibit this lipase but Al^{3+}, Pb^{2+} showed no influence on hydrolysis activity. K*m* of this enzyme was 0.23 mM.

Key words: Germinating, *Brassica napus* L, lipase, hydrolyzing lipid, Km value

INTRODUCTION

In plant seeds the storage nutrient substances used up for seedling growth during germination[1]. Lipases (EC 3.1.1.3) are versatile enzyme that catalyzes the hydrolysis of ester linkages, primarily in neutral lipids such as triglycerides. They hydrolyze the acyl chains either at primarily or secondary positions[2,3]. Mammals, bacteria and plants in large amounts produce lipase. Plant lipases hydrolyze triacylglycerols as much lower rates (usually <0.5 μmol min^{-1} mg^{-1}) as compared with animal or microbial lipases. Oil seed (*Brassica napus* L) lipases have been shown to be localized in oil bodies[4,5] or glyoxysomes[6,7]. They are known to play an essential role in the mobilization of seed storage lipids to support germination and post-germinative embryonal growth. Since the last decade, lipases often have been perceived by research scientists as one of the most important class of industrial enzymes[8]. For instant, lipases have been used extensively in the dairy industry, in the oleo chemical industry and to produce structured triacylglycerols[9,34]. Very recently, the application of lipase as catalysts in organic synthesis has been paid much more attention due to its several advantages for synthetic chemists. In addition to triglycerides, lipases are also known to degrade water-soluble and insoluble esters. So far lipases have been purified from several different sources by using several different methodologies[10-12]. Lipases are generally considered to be absent in most dry seeds and are probably synthesized de novo after the germination. In plants the exact physiological roles of lipase are not very clear. Till now there is no detailed study about the enzymatic degradation of lipids in oilseeds during germination. Here we report the effect of ionic concentration on degradation of oil seeds (*Brassica napus* L.) storage lipids during germination and extraction, purification and characterization of lipid degradating enzyme from germinating *Brassica napus* L.

MATERIALS AND METHODS

Plant material: For experimental purpose oil seeds (*Brassica napus* L) were collected from Bangladesh Agriculture Research Institute, Irshardi, Pabna and Rajshahi Local Shaheb Bazar Market. Good and mature seeds shorted out and was allowed to germinate in petri-dishes on filter paper moistened with distilled water (control); NaCl solutions and Na-Phosphate (Na_2HPO_4-NaH_2PO_4) buffers at different concentrations (0, 50, 100, 150 and 200 mM) in the dark room at 25°C for 120 h. The germinating seeds at different time interval (24, 48, 72, 96 and 120 h), cotyledons were separated from seedlings, rinsed with distilled water and store at 4°C for further experimental purposes.

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Lipid degradation study: Degradation of lipids in oilseeds (*Brassica napus* L), treated with different salts and buffers concentrations during germination were determined by using standard procedures[13-15]. Seeds (500 mg)/ Cotyledons (280 mg) were homogenized in liquid nitrogen and lipases were inactivated by adding 1.2 ml of isopropanol and heating at 70°C for 10 min. After centrifugation, the powder was dried under nitrogen and the lipids were extracted in a chloroform /methanol mixture as described by Bligh and Dyer[15]. Filtered over glass wool and was concentrated the oil materials using a Rotovapor (Flawil, Switzerland).

Measurements of hydrolysis activity of lipase

Enzymatic hydrolysis activity was assayed by two methods

Method A: Lipase activity was assayed essentially as described by Sugihara *et al.*[16]. Olive oil was used as substrate. The lipase activity was measured by estimating the release of free fatty acids. One unit of lipase activity is defined as the amount that liberates one mole of fatty acid under the specific condition. Specific activity of lipase was expressed as the enzyme unit per mg of protein.

Method B: The lipase activities preparations were quantified by the hydrolysis of triolein[17]. One unit (U) of lipase activity was defined as 1mmol of oleic acid released per minute at 37°C. All the experiments were carried out in triplicate.

Purification of lipase

The typical procedure was as follows: 2 g of crude cotyledons (germinated at 40 h) were placed into pre-cooled 25 ml of de-ionized distilled water and then ground gently with a mortar and pestle. The suspension was centrifuged at 8000 g for 20 min at 4°C. The supernatant was collected into a beaker while the precipitate was remixed with 25 ml of de-ionized distilled water. This extraction procedure was repeated four times with de-ionized distilled water. The clear supernatant was adjusted to 80% saturation by adding solid ammonium sulfate. The precipitate was dissolved in minimum volume of pre-cooled de-ionized water and dialyzed against 5 mM phosphate buffer (pH 7.0) for 24 h and lyophilized for column chromatography.

Gel filtration: The lyophilized material was dissolved in 10 ml of 50 mM phosphate buffer, pH 7.2, and loaded into a Sephadex G-50 column (2.5x120 cm) pre-equilibrated with 50 mM phosphate buffer, pH 7.2, for 3 column volumes. The column was eluted with the same buffer at a flow rate of 1.0 ml min^{-1}. The active fractions were lyophilized.

DEAE cellulose chromatography: The Enzymatically active protein fractions after gel filtration were collected and dialyzed against 10 mM Tris-HCl, pH 8.2 for overnight and then concentrated to its 1/4th volume and loaded into DEAE-cellulose column (40x2.5 cm). The column was washed with same buffer and eluted by stepwise increase of NaCl concentration at a flow rate of 1.0 ml min^{-1}.

CM-cellulose column chromatography: The active lyophilized fractions was dissolved in 2 ml of distilled deionized water and applied to a CM-cellulose column (40x2.5 cm), which was equilibrated with 5 mM sodium phosphate, pH 7.0. The column was washed with same phosphate buffer and elution was done with sodium chloride gradient (0-0.5 M) containing higher concentration of buffer (upto 2M) at a 0.5 ml min^{-1} flow rate. The fraction showing maximum activity was lyophilized immediately.

Characterization of the lipase from *Brassica napus* L.

Determination of optimum pH: The activity of the lipase enzyme was measured at different pH values (3.0-10.0) using phosphate buffer at 37°C by the method Sugihara *et al.*[16].

Determination of optimum temperature: The activity of the lipase enzyme was measured by the method of Sugihara *et al.*[16] at different temperature using phosphate buffer, pH 7.0.

Substrate and metallic ion specificity: Activity of the lipase from germinating Brassica seeds was performed using different triglycerides as substrate and metallic salts as an a ctivator and inhibitor by the method Sugihara *et al.* [16] and the result were presented in Table 4 and 5.

Molecular weight determination: The molecular weight of the Brassica lipase was determined by gel filtration on a sephadex G-75 column (90x0.9 cm). Trypsin inhibitor (12.028 kD); Carbonic anhydrase (29 kD); α-amylase (58 kD); Albumin (bovine, 66 kDa) and β-galactosidase (116 kD) were used as marker proteins[18].

Electrophoresis: SDS/PAGE was performed according to the method of Laemmli on a Bio-rad Mini electrophoresis system[35]. The standard proteins (purchased from Sigma) were lysozyme (14 kDa), β-lactoglobuline (18.4 kD), trypsinogen (24 kDa), Pepsin (36 kD), albumin (chicken, 45 kDa) and albumin (bovine, 67 kDa). PAGE was performed with 7% gels and the electrophoresis was run at 2000 V and 50 A.

Determination of Km value: The initial velocity is equal to the amount of product formed per unit time. The initial velocity (Vi) was determined by measuring quantitatively the amount of one of the product at various times[19].

Protein assay: Protein concentration of each fraction was determined by UV-visible spectrophotometer at 280 nm. The amount of protein was estimated by the published method of Lowry *et al.*[20] using BSA as standard.

Chemicals: BSA, SDS / PAGE-chemicals and Sephadex G-75 were purchased from Sigma Chemicals Ltd. USA. Standard proteins, DEAE and CM- cellulose were purchased from Pharmacia Chemicals Ltd. Sweden. All other chemicals use for this research purpose was commercially available and were high purity.

RESULTS AND DISCUSSION

Time course study of lipase from germinating *Brassica* seeds: The lipase from *Brassica napus* L. variety of oilseeds in distilled water treatment showed their maximum activity after 40 h of germination (in crude and in partial purified) and then declined rapidly (Fig. 1). So for the extraction of lipase we used the extract of *Brassica napus* germinated at 40 h.

Degradation study of the lipid storage substances during germination: Oil component in the Brassica seeds degraded sharply during germination due to the high lipase activity, which was strongly inhibited by salts, but there was a little effect on buffer treatments (Table 1, 2 and Fig. 2, 3). This finding is in good agreement with previous studies on sunflower[21], groundnut[22] and Jojoba[23] seeds germinating in saline conditions. From the results it was concluded that, the decrease in seedling growth caused by salts and Na-buffer treatment may be correlated with inhibition of TAG hydrolysis, a process that generates sugars for the growth of the germinating embryo. Furthermore, inhibition of lipase in cotyledons from salts and buffer treated seedlings could be explained by a direct effect of Na^+, PO_4^- and Cl^- ions on the enzyme, reducing its activity or by an indirect effect of salts by modifying gene expression of the enzyme and then affecting its de novo synthesis.

Table 1: Effect of various NaCl concentrations on TAG degradation of Brassica cotyledons (Results are expressed in mg of TAG/280 mg cotyledon)

Days	Time in h	0 mM NaCl	50 mM NaCl	100 mM NaCl	150 mM NaCl	200 mM NaCl
0	0	13.65±1.2	13.65±1.2	13.65±1.2	13.65±1.2	13.65±1.2
1	24	10.20±2.1	11.86±1.9	12.67±0.9	12.88±1.1	13.02±0.9
2	48	02.21±1.1	08.51±0.8	10.30±0.9	10.87±1.2	10.88±1.1
3	72	01.89±0.6	03.99±0.9	06.40±0.7	08.25±1.1	10.23±1.0
4	96	01.05±0.8	02.55±0.6	04.12±1.2	05.99±0.9	08.85±0.9
5	120	00.88±0.9	01.99±0.3	02.98±0.4	03.98±0.7	04.98±0.9

Table 2: Effect of various Phosphate buffer concentrations on TAG degradation of Brassica cotyledons. (Results are expressed in mg of TAG/280 mg cotyledon)

Days	Time in h	0 (mM) Na-Phosphate buffer	50 (mM) Na-Phosphate -buffer	100 (mM) Na-Phosphate -buffer	150 (mM) Na-Phosphate -buffer	200 (mM) Na-Phosphate -buffer
0	0	13.65 ±2.2	13.65±4.2	13.65±2.2	13.65±3.2	13.65±2.2
1	24	10.20±3.1	10.36±2.9	10.67±4.9	11.58±4.1	11.02±1.9
2	48	02.21±1.1	03.51±1.8	3.80±2.9	5.87±7.2	9.98±3.1
3	72	01.89±0.6	02.09±2.9	04.49±3.7	03.25±3.1	7.98±1.0
4	96	01.05±0.8	01.50±3.6	03.00±3.2	02.90±5.3	05.80±1.9
5	120	00.98±0.9	01.09±2.3	01.08±4.4	01.98±2.1	02.68±3.0

Table 3 (A): Purification of Brassica lipase by the method[17] (Triolein used as substrate)

Purification steps	Total protein (mg)	Total activity (Units)	Specific activity (units mg^{-1})	Yield (%)	Purification (-fold)
Crude extract	1090.56	630.45	0.58	100	1.00
Salting out and dialysis	344.00	394.90	1.15	62.64	1.98
Sephadex G-50 gel	61.23	351.70	5.74	55.79	9.90
DEAE-cellulose	12.31	245.90	19.98	39.00	34.50
CM-cellulose	3.08	120.75	39.20	19.15	167.59

Table 3 (B): Purification of Brassica lipase by the method[16] (Olive oil used as substrate)

Purification steps	Total protein (mg)	Total activity (Units)	Specific activity (units mg^{-1})	Yield (%)	Purification (-fold)
Crude extract	1597.00	15942	9.98	100.00	1.00
Salting out and dialysis	407.33	11324	27.80	71.03	1.98
Sephadex G-50 gel	91.95	6749	73.39	42.33	9.90
DEAE-cellulose	18.29	5742	313.94	36.01	34.50
CM-cellulose	10.70	3922	366.54	24.60	67.59

Table 4: Substrate specificity of Brassica seeds lipase (Action of lipase on different oils, buy from local Market)

Substrate	Specific activity, mU mg^{-1} min^{-1}
Castor oil	165
Coconut oil	60
Corn oil	178
Cottonseed oil	134
Linseed oil	153
Olive oil	170
Peanut oil	186
Rapeseed oil	164
Soybean oil	142

Table 5: Effect of metal ions on enzyme activity 20 µl of Brassica lipase solution (50 µg ml^{-1}) was added to 0.20 ml of 5 mM sodium phosphate buffer, pH 7.0 with various metal ions (10^{-3} M) and incubated at 37°C for 2 h. An aliquot of 10 µl of the solution was to measure the remaining activity according to method Ihara *et al.*[24]

Metal ion	Remaining Activity (%)	Metal ion	Remaining Activity (%)
None	100.00	Ba^{2+}	101.90
Fe^{3+}	64.80	Mg^{2+}	103.50
Fe^{2+}	51.20	Mn^{2+}	103.00
Cu^{2+}	61.20	Sn^{2+}	107.20
Ca^{2+}	165.30	Al^{3+}	89.90
Zn^{2+}	62.40	Hg^{2+}	53.10
Pb^{2+}	93.10	Bi^{3+}	115.20

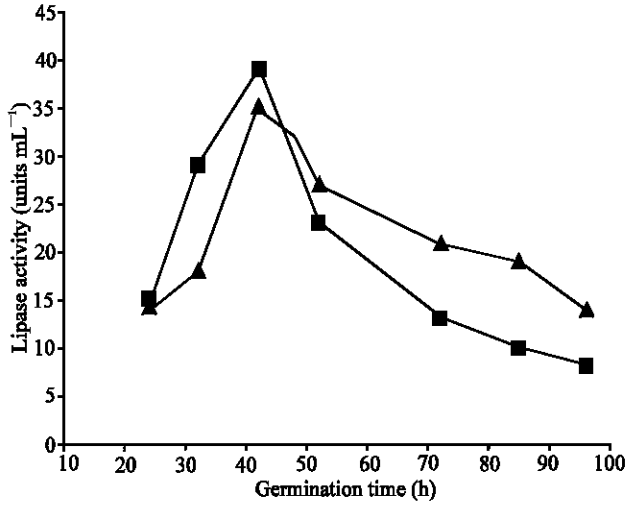

Fig. 1: Time course study of crude lipase extract (-▲-) and Partial purified (-■-) lipase from *Brassica seeds*. (Lipase activity Unit ml^{-1})

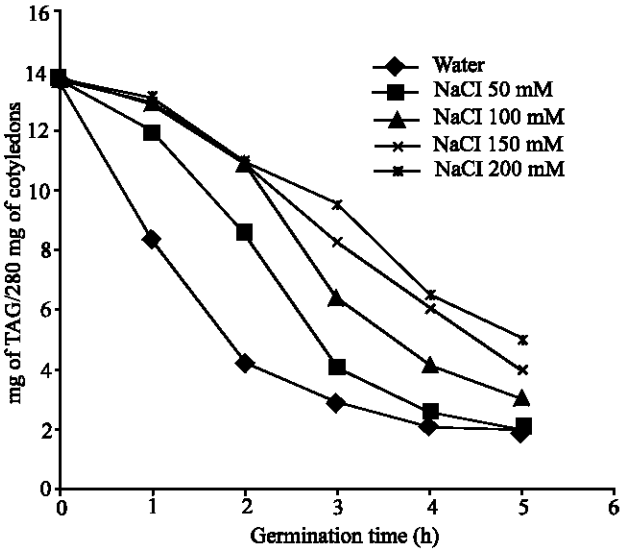

Fig. 2: Effect of NaCl solution on TAG mobilization during germination of *Brassica seeds*

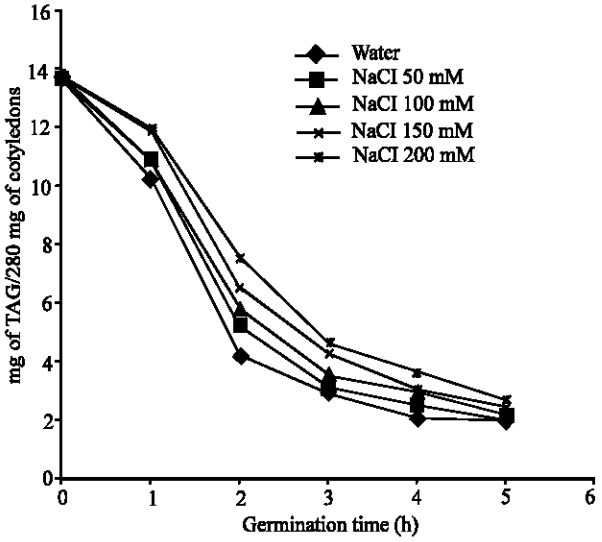

Fig. 3: Effect of Na-phosphate buffer on TAG mobilization During germination of *Brassica* seeds

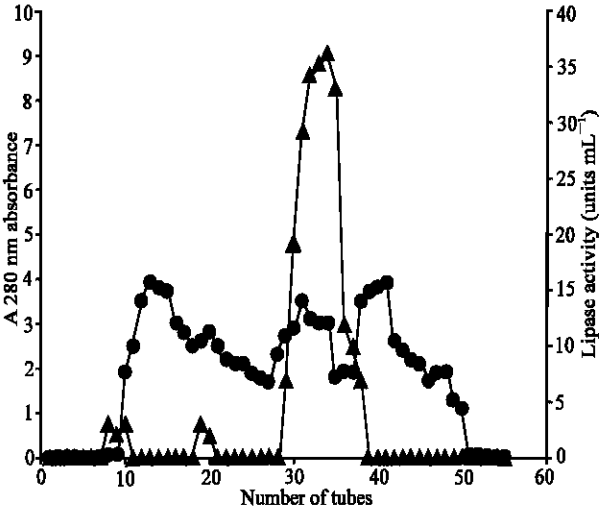

Fig. 4: Gel filtration pattern of ammonium sulphate saturated crude extract Of Brassica lipase on Sephadex G-50 column. Lipase activity (-▲-) and (-●-) Protein concentration. [Lipase activity Units ml $^{-1}$]

Purification of lipase: The purification results of lipase from germinating Brassica seeds are summarized in Table 3A and 3B. The specific activities of extracted enzyme increases at each steps and the purification fold was achieved from crude extract nearly 67.59. The specific activity of the final preparation was 39.2 U mg^{-1} with 19.2% overall yield of enzyme where triolein used as a substrate (3A). In (Fig. 3B) the purification results of Brassica lipase are summarized, where olive oil was used as a substrate. Due to high-resolution of Sephadex G-50, gel filtration was very useful for excluding the low-molecular-mass fractions (under 15 kDa) (Fig. 4). It was found that fractions (no. 32-37) contained the lipase activity while others showed no activity. Active fractions (no. 32-37) was pooled, dialysis against 10 mM Tris-HCl buffer, pH 8.2, concentrated and further purified by DEAE-cellulose column chromatography.

The components of fraction no. (32-37) from gel filtration were separated into three peaks using sodium chloride gradient (0.1; 1.0 and 2.0 mM) on DEAE cellulose column (Fig. 5). Fraction numbers (11-16) eluting with

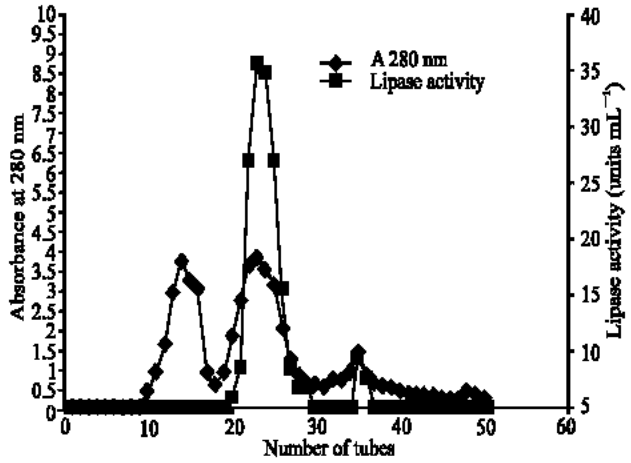

Fig. 5: DEAE-cellulose column chromatography of lipase active fractions from Gel filtration. [Lipase activity Units ml^{-1}]

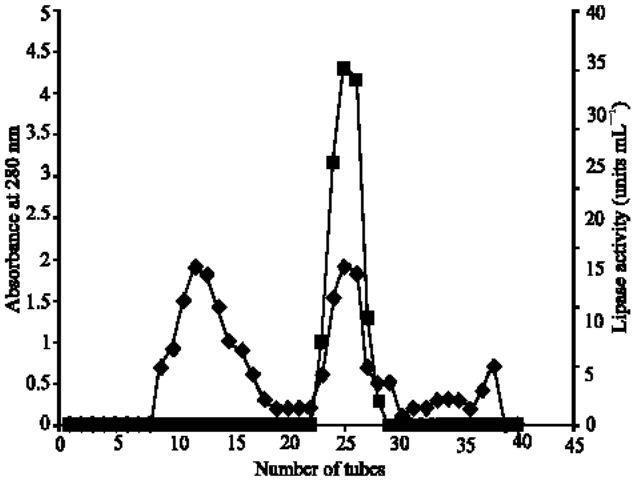

Fig. 6: CM-cellulose chromatography of the lipase active fraction from DEAE-cellulose column [Lipase activity Units ml^{-1}]

buffer containing lipase activity and other peaks eluted with salt gradient possesses no lipase activity. Enzymatically active fractions were collected separately dialyzed against 5 mM phosphate buffer (pH 7.0) and then concentrated to apply CM-cellulose column chromatography.

Active fractions no (11-16) were separated into main two peaks by CM-cellulose column when eluted with phosphate buffer (pH 7.0) using sodium chloride gradient (0.1-0.5 M). Here enzymes are adsorbed on CM-cellulose and eluted by high concentration of buffer with sodium gradient. Fraction no. (24-26) from CM-cellulose column showed high lipase activity (Fig. 6). These fractions were collected, dialysis and concentrated.

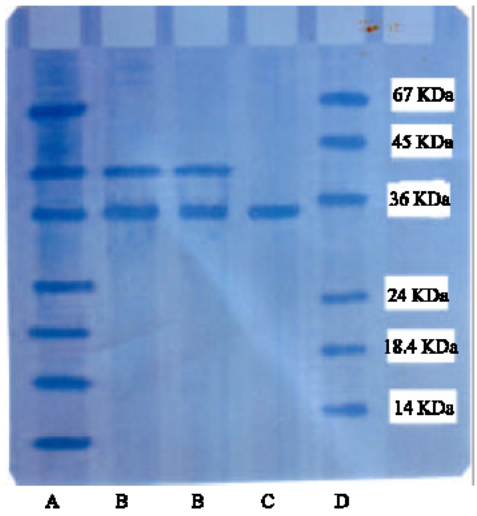

Fig. 7: Photographic representation of the sodium dodecyl sulfate polyacrylamide slab gel electrophoresis. [A=Crude lipase extract from germinating Brassica seeds; B= Lipase after DEAE cellulose column; C = Lipase after CM cellulose column; D = Marker Protein]

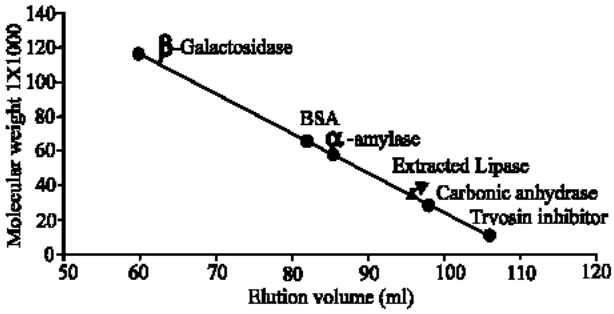

Fig. 8: Molecular weight determination by gel filtration. [Sephadex-G 75]

By using CM-cellulose with phosphate buffer and sodium chloride gradient (0.1-0.5M) active enzyme was eluted as a sharp peak, which was proved to be homogeneous by SDS/PAGE (Fig. 7). The SDS/PAGE of revealed that the protein band corresponded to a molecular mass of 34 kDa which corresponding to the high-resolution gel filtration (Fig. 8).

Determination of optimum temperature and pH: The effect of temperature on the Brassica lipase activity were examined in the range of 20-50°C. The activities of the purified enzyme increased sharply at 37°C and then began to decrease gradually with the rise in temperature (Fig. 9). Very little activities were found below 20°C and at or above 50°C. The optimum temperature for two lipase from *Pen. cyclopium* were 35 and 40°C[25] and

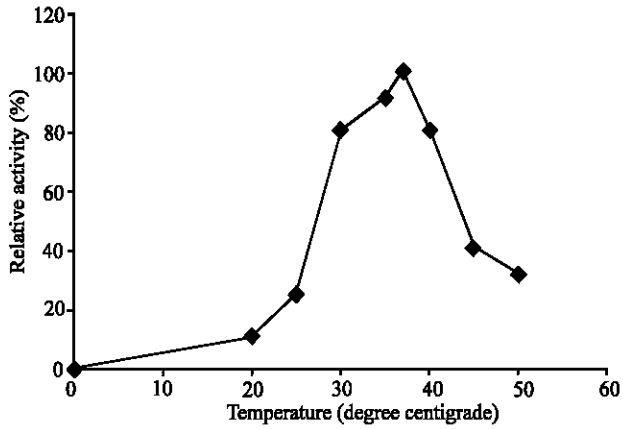

Fig. 9: Effect on temperature on lipase activity 10 μl of lipase containing 50 μg ml^{-1} protein were placed into 0.5 ml of 5 mM phosphate buffer, pH 7.0 and the activity measured according to method Sugihara *et al.*[16].

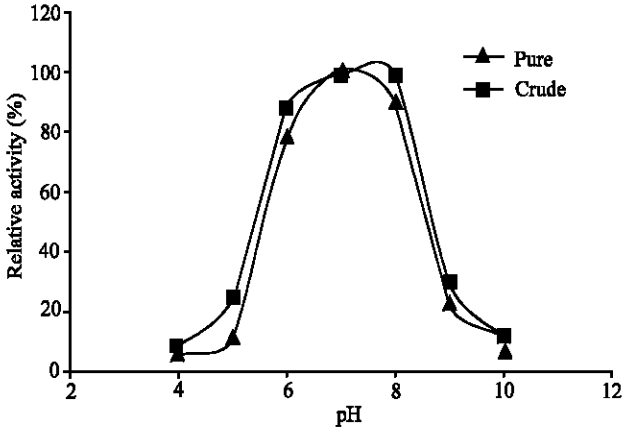

Fig. 10: Effect of pH on lipase activity 50 μl of lipase containing 50 μg ml^{-1} protein were placed into 0.4 ml of 5 mM phosphate buffer, pH 7.0 and the activity measured according to method Sugihara *et al.*[16].

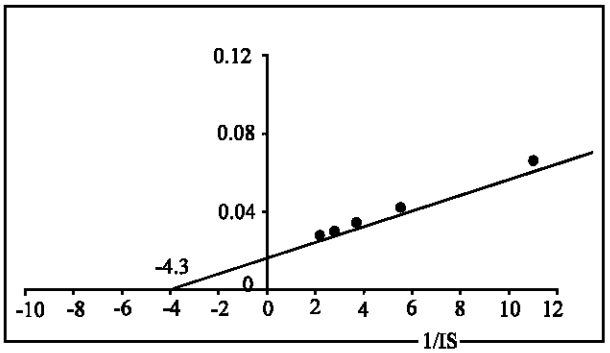

Fig. 11: Lineweaver-Burk double reciprocal plots for the determination of Km value of the *Brassica napus* seed lipase

those of three lipases from *Rh.Delemer* were range from 30 to 35°C[26]. The optimum pH calculated for the lipase of Brassica seeds was 7.0 (Fig. 10). From the result it might be concluded that the lipase isolated from Brassica napus seeds belong to the category of neutral lipase similar to lipase in peanut as reported by Sanders and Pattee[27] and Awny[28].

Activators and inhibitors: Ca^{2+} is required for activity [Sr^{2+} and Mg^{2+} are less effective activators][29]. Metals ion Ba^{2+} and Mg^{2+} slightly stimulate the lipase at 1 mM, 2 mM and 3 mM concentration whereas Mn^{2+}, Al^{3+} produces no effect on the activity. Activity of Brassica seed lipase inhibited by heavy metals such as Cu^{2+}, Zn^{2+}, Hg^{2+} and Fe^{2+} (Table 5). These results supported for pancreatic lipase[30], lipase from *Aspergillus niger*[31] and rat adipose tissue[32]. Based on the Lineweaver-Burk plots, apparent Km for Brassica lipase for olive oil and triolein were 0.23 mM (Fig. 11) and 5.8 mM, respectively. An earlier reports that the Km for 44 kD rice barn lipase for triolein was 7.4 mM[33].

In conclusions, the catalytic rates of the Brassica lipase are comparable to the reported plant lipase, reasons for its lower catalytic efficiency under physiological condition of pH 7.0 and temperature 37°C as compared with most other known animal or microbial lipase.

Brassica lipase being a small protein (34 kD) and hence amicable for manipulations, provides a good model enzyme to study the interaction with substrate. Unique properties like neutral pH, stability and broad substrate specificity of lipase are of particular interest for their implications in various industrial applications.

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