Purification and Biochemical Characterization of Lipase from the Dorsal Part of *Cirrhinus reba*

M.A. Islam[1], F. Parveen[1], K. Hossain[1], S. Khatun[1], Md. R. Karim[1] G.S. Kim[2], N. Absar[1] and Md. S. Haque[1,*]

[1]*Laboratory of Protein and Enzyme Research, Department of Biochemistry and Molecular Biology, Rajshahi University, Rajshahi-6205, Bangladesh*
[2]*Laboratory of Biochemistry, School of Veterinary Medicine Korea National Animal Bio Resources Bank (ABRB), Korea*

Corresponding author. Email: haque_drshahidul@yahoo.co.in

Abstract

Lipase obtained from dorsal part of *Cirrhinus reba* (designate *C. reba*) was purified to the homogeneity by 85% $(NH_4)_2SO_4$ fractionation followed by simultaneous desalting and concentrating by ultrafiltration, and then chromatography as Sephadex G-50 gel filtration and DEAE- cellulose. The molecular weight of the enzyme was 87 KDa as determined by gel filtration on Sephadex G-150 and by SDS- polyacrylamide slab gel electrophoresis. The enzyme is a monomer in nature. The purified lipase was active within the pH range of 4.5-5.5, with an optimum pH of 5.5, and within the temperature range of 30-60^oC, with optimum temperature for the hydrolysis of olive oil at 35^oC. The hydrolytic activity of the enzyme was enhanced by Ca^{+2} but strongly inhibited by heavy metals Zn^{+2} and Hg^{+2} as well as EDTA while slightly inhibited in the presence of Cu^{+2} salts.

Keywords: triglycerides, bata fish, gel electrophoresis, thermo stability, enzyme activity, heavy metals

Introduction

Lipases are involved to catalyze hydrolysis of long chain triglycerides into free fatty acids and glycerol at the interface of emulsified lipid substrates. The fatty acids are oxidized endogenously to get energy available for doing mechanical work while the glycerol moiety produces energy in some specific tissues through oxidation procedure. Lipases play the role in the postmortem quality deterioration of seafood (other foodstuffs) during handling, chilled frozen storage, and widely used for biotechnological applications in such dairy industry, oil processing etc. Compared with other hydrolytic enzymes (e.g., proteases), lipases from fish sources are relatively less well studied and in this regard, lipases from aquatic animals are even less well known than mammalian, plant and microbial sources (L´opez-Amaya et al., 2001). The presence of a lipase activity has been described for some aquatic organisms such as lobster (Brockerhoff et al., 1970), crab (Vonk, 1960) and few lipases that have been studied from fish and other aquatic animals include lipases from the leopard shark (Patton et al., 1977), rainbow trout (Tocher and Sargent, 1984), Atlantic cod (Lie and Lambersten, 1985), dog fish (Raso and Hultin, 1988) and sardine (Mukundan et al., 1985).

Several lines of evidences suggested the specificities and importances of lipase in producing various essential and nutritional products. Lipase can hydrolyze lipids and produce undesirable rancid flavor in milk products, meat, fish and other food products containing fat. For instance lipases have been used extensively in the dairy industry as house

hold detergent, in the oleo chemical industry and to produce structural triglycerides (Verger et al., 1982). Lipases are also used in the synthesis of polymers, agrochemical leather textile, baking pharmaceutical and paper industry. Recently, several applications of lipases have been identified ranging from their use in laundry detergents, the modification of the nutritional, sensory and physical properties of the triglycerides in foodstuffs, and the production of optically pure enantiomers (Hemachander et al., 2000; Undurraga et al., 2001).

The prospect of lipase catalysis in organic solvent with its associated advantages has also received widespread attention (Faber and Franssen, 1993; Hazarica et al., 2002). Therefore, it is speculated that this enzyme might be involved not only in catalyzing the metabolic pathway but also in other aspects of chemical and biochemical importances. The *Cirrhinus reba* fish is very popular due to be high nutritious and delicious in Bangladesh. The dorsal part of the fish contains unsaturated fatty acid as well as lipase activity. This fish plays the vital role in supply of adequate protein to the people in Bangladesh. However, no reports are available on the isolation, purification and biochemical characterization of Lipase from the dorsal part of the bata fish (*C. reba)*. Moreover, the regulatory mechanism of lipase involved in lipid metabolism in this fish is not yet clarified. Therefore, the present study has been undertaken to purify and to characterize lipase from the *C. reba* which is a part of ongoing research in our laboratory to discover new sources of this enzyme as potential food with emphasis on its biotechnological applications in future.

Materials and Methods

Biological Materials

Adult *C. reba* (1-1.5 kg) were purchased from local fish market (Shaheb bazaar, Rajshahi, Bangladesh) and the dorsal part was removed by knife and stored in plastic bags with crushed ice and transported to the laboratory which were then stored frozen at –20°C until used for experimental purpose.

Preparation of Sample for Enzyme Extraction

The frozen dorsal part of fish was thawed at 4°C and cleaned by flushing with distilled water followed by rinsing with ice-cold 0.85% NaCl solution to remove blood. The tissue was chopped into small pieces and rapidly frozen in liquid nitrogen and fine powder was prepared by blender. The powder was defatted with successive changes of cold acetone, chloroform: *n*-butanol (9:1, v/v), chloroform: *n*-butanol (8:2, v/v), acetone and diethyl ether, all at −20°C, with intermittent stirring and then filtration. The ratio of tissue to solvent was 1:10 (w/v), and after each solvent treatment, the homogenate was filtered via vacuum suction with a Bijchner funnel. The defatted material was air dried at room temperature in a fume hood and then stored at −20°C.

Preparation of Enzyme Extract

The dried defatted powder was homogenized in 25 mM Tris–HCl buffer, pH 7.8, containing 5 mM benzamidine–HCl, 1 mM EDTA and 10% (w/v) glycerol (TBEG buffer). The defatted powder to TBEG buffer ratio was 1:10, (w/v); the homogenate was gently stirred at 4°C for 1 h and centrifuged at 8000 × *g* for 20 min at 4°C. The supernatant was filtered through several layers of cheese cloth to remove the floating fatty material and then fractionated with solid $(NH_4)_2SO_4$ up to 85% saturation. The mixture was gently stirred for 3 h at 4°C and re-centrifuged at 8000 × *g* for 20 min at 4°C. The resulting precipitate was re-dissolved in TBEG buffer (pH 7.8) and was dialyzed overnight against three times changes of 4 L TBEG buffer (pH 7.8) in a cellulose membrane dialysis tubing (12 KDa MW. CO, Sigma Chemical Co., St. Louis, MO, USA). The dialyzed fraction was centrifuged again at 8000 × *g* for 20 min at 4°C and the resulting dialysate was simultaneously desalted and concentrated using a Millipore Amicon® ultra centrifugal filter device (30 kDa MWCO, Amicon Co. Ltd., Bedford, MA, USA). The concentrated and desalted, ultrafiltrate extract (UF fraction) was stored frozen at −20°C.

Isolation and Purification of Lipases

The crude enzyme extract was loaded into the gel filtration column previously equilibrated with Tris-HCl buffer, pH 8.4 for 24 hours and the proteins were recovered from the column by step wise elution with the same buffer at 4°C. The fraction showing the lipase activity was pooled and dialyzed against 10 mM Tris-HCl buffer, pH 8.4 for overnight with four times changes of buffer at 4°C. After

centrifugation the supernatant was applied into a Sephadex G-50, which was previously equilibrium with 5 mM PBS, pH 7.6 at 4°C and the proteins were eluted from the column with the same buffer. The fraction containing the lipase activity was pooled and dialyzed against 10 mM Tris-HCl buffer, pH 8.4 for overnight with four times changes of buffer at 4°C and finally concentrated by sucrose. After centrifugation, the supernatant was applied into the DEAE-cellulose column previously equilibrated with Tris-HCl buffer, pH 8.4 for 24 hours and the proteins were eluted from the column by stepwise elution with the same buffer containing different concentration of NaCl at 4°C. The active fraction containing lipase activity was pooled and tested for homogeneity according to Alberta et al. (2007).

Electrophoresis

Polyacrylamide slab gel electrophoresis was conducted at room temperature, pH 8.4 on 7.5% gel and amido black was used as staining reagent. The molecular weight (MW) of the purified native enzyme was determined by gel filtration on Sephadex G-150 column (0.75 × 100 cm) as described by Laemmli (1970). The marker proteins used were β-galactosidase (116 KDa), BSA (67 KDa), α-amylase from *Bacillus subtilis* (58 KDa), egg albumin from white (45 KDa), pepsin (36 KDa), trypsin inhibitor (20 KDa) and lysozyme (14 KDa). The molecular weight was also determined by SDS-PAGE according to the method of Laemmli (1970) and Sugihara et al. (1990) who used the marker proteins, myosine (205 KDa), β galactosidase (116KDs), BSA (66 KDS), carbonic anhydrase (29 KD), β lactoalbumin (18 KD) and aprotinin (6.5 KD).

Enzyme Assay

Lipase activity was assayed as reported by Sugihara et al. (1990) using olive oil as substrate. The lipase activity was measured by estimating the release of free fatty acids and one unit of lipase activity is defined as the amount that liberates one micromole of fatty acid under the specified conditions. Specific activity of lipase is expressed as the enzyme unit per mg of protein.

Estimation of Protein

Protein concentration was estimated by the method of Lowry et al. (1951) using BSA as standard as well as from of the absorbance at 280 nm.

Effect of pH on the Activity and Stability of Lipases

The activity of lipase was examined within the pH range of 2.0-7.5 using the following buffer solutions of 0.2M: HCl-KCl, pH 2.0; glycine-HCl, pH 3.0; AcONa-HCI, pH 4.0-5.0; AcONa-CH_3COOH, pH 5.5-8.0, with olive oil as substrate as described by Alberta et al. (2007). The results were expressed as percentage of the activity obtained at pH 5.5. Furthermore, the data obtained for the measurements above pH 5.5 were excluded from the results presented here due to substrate instability. The effect of pH on lipase stability was determined by incubating the lipase fraction in various buffer solutions ranging from 1.5 to 8.0 for 30 min at 35°C in a Haake circulating water bath. The compositions of the buffer solutions used for the pH stability studies were as follows: 0.2M acetate buffer, pH 4.0; 0.2M AcONa-HCl buffer, pH 3.10; 0.2M citrate-phosphate buffer, pH 6.0; 0.2M phosphate buffer, pH 8.0. After the incubation period, 100 µl aliquots of the buffered enzyme solutions were added to 900 µL of the olive oil substrate, and lipase activity was assayed spectrophotometrically at 410 nm as described previously.

Effect of Temperature on the Activity and Stability of Lipases

The temperature dependence of lipase activity was measured by equilibrating olive oil at 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60°C for 30 min. In each assay, 100 µL of the enzyme extract was added to 900 µL of pre-equilibrated substrate. The thermo stability of the lipase fraction was studied by incubating the enzyme extract at various temperatures (10, 15, 20, 25, 30, 35, 40 and 45°C) for 10, 30 and 60 min. At the end of the incubation period, the enzyme extract was rapidly cooled and the remaining lipase activity was assayed using olive oil.

Effect of Various Chemicals on Enzyme Activity and Stability

Aliquots of the enzyme were incubated with equal volumes of the various chemicals and metal ions at various concentrations at 35°C in a Haake circulating water bath (Haake D1-G, HAAKE Mess-Technik GmbH Co., Germany) for 30 min. Appropriate blanks and control were performed for each chemical tested. The relative activity of lipase after incubation period was assayed with olive oil as substrate as previously described and the results were expressed as percentage of the activity obtained without the chemical agent.

Statistical Analysis

Statistical analysis was carried out using analysis of variance followed by Duncan's Multiple Range Test. Mean differences with $P<0.05$ were considered statistically significant

Results and Discussion

Isolation and Purification of Lipase

The crude enzyme solution prepared from 85% ammonium sulphate saturation of *C. reba* was dialyzed against 10 mM Tris-HCI buffer, pH 8.4 for 24 hrs and was applied to gel filtration (G-50) at 4°C, which was previously equilibrated with the same buffer. The column bound proteins were eluted as a two major and one minor peaks indicating the presence of more than one component (Figure 1). It was found that only the fraction F-1 had contained the lipase activity on the basis of the experiment of Iijima et al. (1998). Fractions from F-1 peak were rechromatographed against on sephadex G-50 column under identical condition and the elution pattern showed one sharp peak (F-1) and another minor peaks (F-2, F-3) (Figure 1). Moreover, polyacrylamide slab electrophoresis (Figure 1) of active fraction of enzyme indicated that it did not contain pure enzyme. To separate the components from the sample, it was further subjected to DEAE-cellulose chromatography.

DEAE-Cellulose Chromatography

As shown in Figure 2, the components of the enzyme solution were eluted as one major peak F-1a and one minor peak F-1b. It was found that only the fraction, F-1a had contained the lipase activity. The purity of F-1a was checked by polyacrylamide slab gel electrophoresis. The photographic representation of the electrophoretic patterns of crude protein, F-1 and F-1a are shown in Figure 3. The enzyme fraction characterized in this study (referred to as "fish lipase") was the F-1a fractions obtained after DEAE cellulose chromatography. The data presented in Table 1 indicate that the steps used for the recovery of fish lipase resulted in 34.5-fold purification, which was higher than that reported (Gjellesvik et al., 1992; Iijima et al., 1998). Total protein content was 3.3 mg mL^{-1} and the enzyme activity was 20.5 U mL^{-1} showing the higher purity and higher efficiency of enzyme.

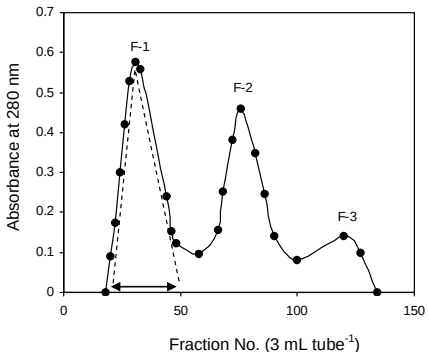

Figure 1 Gel filtration of F-1 fraction on Sephadex G-50. F-1 fraction was applied to the column (3x120) pre-washed with 10mM Tris-HCl buffer, pH 8.4 at 4°C and eluted by the same buffer. Flow rate: 25 mL h^{-1}. Dashed line indicates enzyme activity.

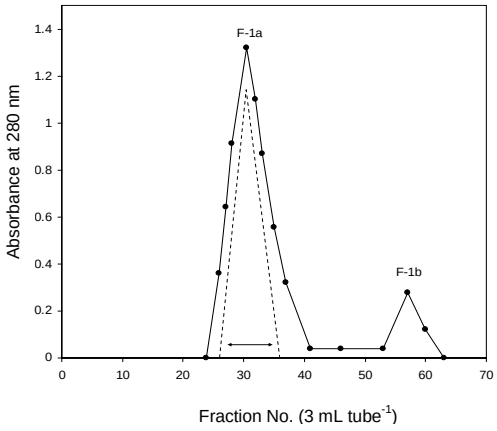

Figure 2 Ion exchange chromatography of lipase enzyme on DEAE-cellulose. The enzyme solution (50 mg) was applied to the column (2.1×25 cm) pre-equilibrated with 10 mM Tris-HCI buffer, pH 8.4 at 4°C and eluted by a linear gradient of NaCl (0 to 0.3 M) in the same buffer. Flow rate: 25 mL h^{-1}. Dashed line indicates lipase activity.

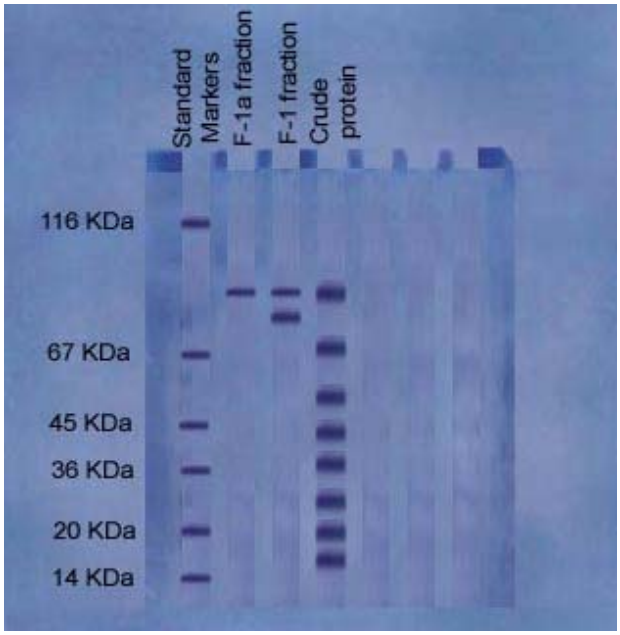

Figure 3 Photographic representation of the SDS-PAGE (7.5% gel) of different fractions of fish lipase obtained during purification steps and standard proteins (Staining reagent 1% Amido black).

Lane 1: standard solution
Lane 2: F-1a fraction from gel filtration on Sephadex G-50
Lane 3: F-1 fraction obtained from gel filtration on Sephadex G-50
Lane 4: Crude protein extracts from dorsal part of the bata fish

Determination of Molecular Weight (MW)

The molecular weight of the enzyme was determined by gel filtration on Sephadex G-150 using β-galactosidase from E.coli (116 KDa), BSA (67 KDa), α-amylase from Bacillus subtilis (58 KDa), egg albumin (45 KDa), pepsin (36 KDa), trypsin inhibitor from corn kernels (20 KDa) and lysozyme (14 KDa) as standard proteins. The molecular weight was calculated from the standard curve of reference proteins and was constructed by plotting log of molecular weight against elution volume on gel filtration (Figure 4A) and estimated to be 87 KDa for the purified lipase. Iwai et al. (1975) purified two lipases from *Penicillium cyclopium* having MW of 27 KDa and 36 KDa, while Aloulou et al. (2007) reported the MW of the lipase from *Yarrowia lipolitica* a was 38.48 KDa.

The MW of the enzyme was also determined by SDS- PAGE using the same marker proteins and found to be almost same as that found by gel filtration 87 KDa (Figures 3 and 4B). Abbas et al. (2002) reported that the molecular weight of lipase from *Mucor sp* strain isolated from palm fruit was 42 KDa. Further, the MW of the enzyme was found to be unchanged in the presence or absence of β-mercaptoethanol indicating that the lipases contained only one sub unit. The results and the evidences suggest that the molecular weight of the lipase is variable in species.

Table 1 Summary of purification procedure of the lipase from the dorsal part of *C. reba.* Percentage of yield is calculated by dividing total enzyme activity by starting total activity multiplied by 100. Purification fold is obtained by dividing specific activity with total protein.

Purification step	Volume (mL)	Protein (mg mL^{-1})	Total protein (mg)	Enzyme activity (U mL^{-1})	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)	Purification (fold)
Supernatant (Crude extract)	195	25.1	4894.5	4.5	877.5	0.18	100	1
85% $(NH_4)_2SO_4$ precipitation	212	14.5	3074	5.4	1144.8	0.37	130.42	2
Gel filtration on Sephadex G-50 (F-1)	30	5.5	165	15.4	462	2.8	52.65	15.5
DEAE cellulose \(F-1a)	18	3.3	59.4	20.5	369	6.21	42.05	34.5

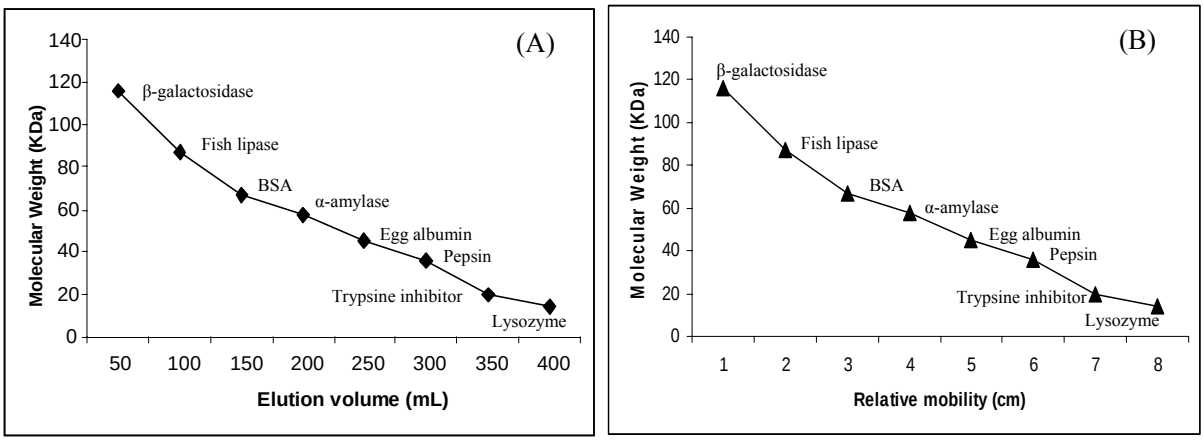

Figure 4 (A) Standard curve for the determination of molecular weight by gel filtration. Size of column: 1.8 × 90 cm, buffer: 10 mM Tris-HCI, pH 8.2, Flow rate: 15 mL h^{-1} and (B) molecular weight by SDS-polycrylamide slab gel electrophoresis. M.W. of fish lipase (87KDa) was determined by using β-galactosidase (1,16 KDa), bovine serum albumin (67 KDa), α -amylase (58 KDa), egg albumin (45 KDa), pepsin (36 KDa), trypsin inhibitor (20 KDa) and lysozyme (14 KDa) as standard proteins.

Effect of pH on the Activity of Lipases

The purified lipase hydrolyzed olive oil as substrates appreciably over a relatively broad alkaline pH range from pH 2.0 to 7.5. The enzyme gave maximum activity at pH 5.5. Beyond this pH values, the activity of the enzymes decreased gradually and more than 50% activities were destroyed below pH 3.5 as well as above 6.5 (Figure 5A). These trends were similar to the findings reported for other fish lipases (Gjellesvik et al., 1992; Raso and Hultin, 1988) and are also consistent with the pH optima reported for lipases from other sources (Lima et al., 2004; Nthangeni et al., 2001). In addition, the enzyme activity was found to be stable between pH 4.6-5.5 after incubation at 35°C for 30 min (Figure 5B). This observation is also similar to those found for other fish lipases (Mukundan et al., 1985) as well as lipases from microorganisms such as *Aspergillus carneus* (Saxena et al., 2003) but different from scorpion lipase that was reported to be most stable at pH 11.0 (Borlongan, 1990).

Effect of Temperature on the Activity of Lipases

The effect of temperature on the activities of fish lipase was examined in the range of 10 to 60°C. Lipolytic activities of fish lipase were found to be profoundly affected by temperature. As shown in Figure 6A, the activity of lipase was increased gradually with rise in temperature and the maximum activity was observed around 30-35°C. The result was similar to the lipase from cod demonstrated by Gjellesvik et al. (1992). With further rise in temperature, the activities were decreased abruptly and the enzymes lost their activities almost completely at 60°C. Lipases purified from other sources were found to be highly active in the temperature range from 30 to 40°C (Destain et al., 1997; Aloulou et al., 2007).

The thermal stability of purified lipase was studied at 15°C increments from 10 to 40°C. The data, shown in Figure 6B, indicates that fish lipase had appreciable stability (76-100%) after prolonged incubation about 60 min within 10 to 35°C. The enzyme exhibited maximum stability at 10°C but also found to be fairly stable up to 35°C with concomitant loss of activation after 60 min incubation. At higher temperatures (≥ 45°C), the degree of inactivation was markedly increased. For instance, only 25%, 22%, and 0% residual activities were observed after incubation at 40°C for 10, 30, and 60 min, respectively, while the enzyme was completely inactivated after only 10 min incubation at 45°C. The results reveal that fish lipase is quite heat-labile and in this respect, very similar to lipases from other cold-water marine fish (Choo et al., 1998; Patton et al., 1977).

Effect of Metal Ions on the Activity of Lipases

The role of lipase inhibitors may provide a better perceptive of their mechanism of action and successful identification of potent and specific

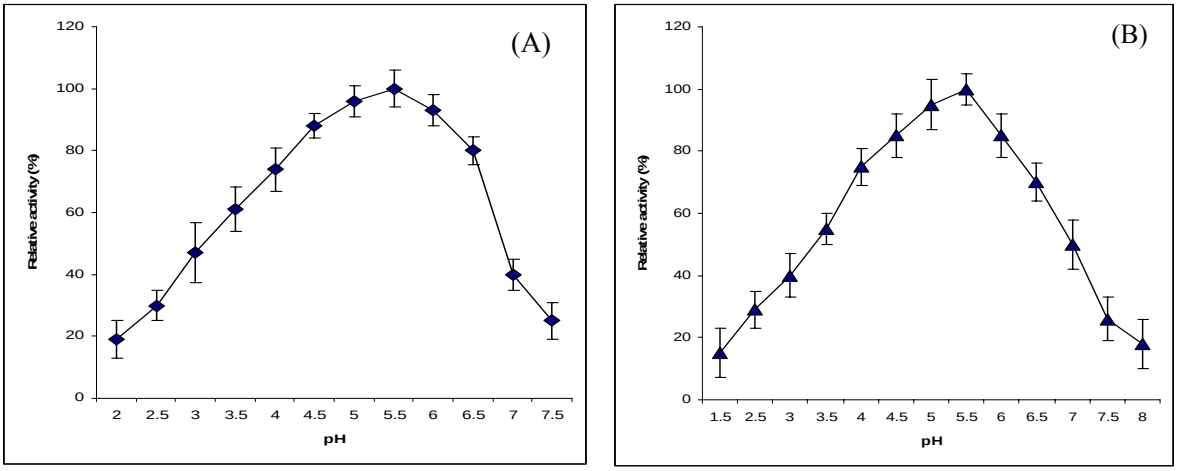

Figure 5 (A) Effect of pH on the activities of fish lipase. The buffers used within pH 2.0-7.5 are HCl-KCl, pH 2.0; glycine-HCl, pH 3.0; AcONa-HCI, pH 4.0-5.0; AcONa-CH_3COOH, pH 5.5-8.0. (B) Effect of pH on the stability of lipase activity. The enzyme extract was incubated at 35°C for 30 min in various buffers: HCl-KCl, pH 2.0; glycine–HCl, pH 3.0; acetate, pH 4.0 and 5.0; and phosphate, pH 6.0-8.0. All the buffers were 0.2 M. Relative activity of fish lipase was expressed as percentage of the initial activity. Values are means ± SD (n=3).

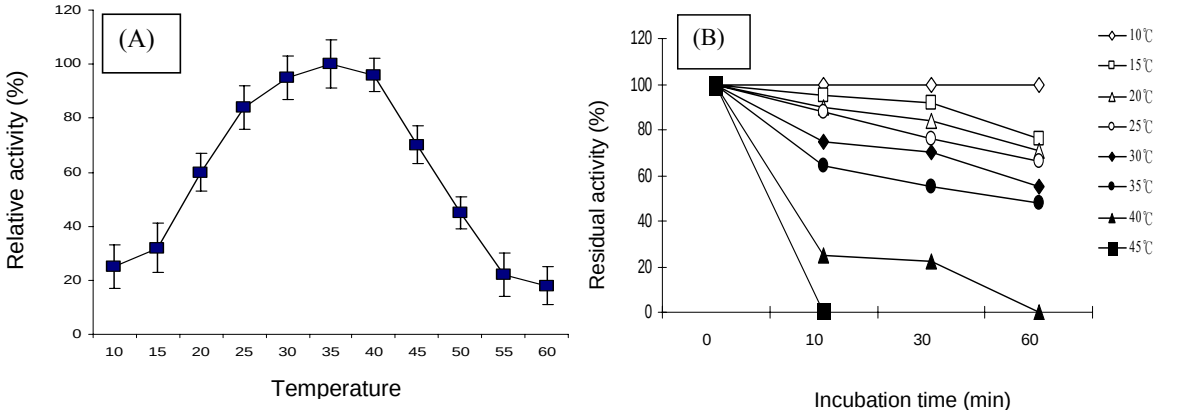

Figure 6 (A) Effect of temperature on the activities of fish lipase. The enzyme was incubated at the indicated temperature for 30 min using olive oil as substrate. (B) Effect of temperature on the stability of fish lipase. The enzyme was incubated at various temperatures: 10, 15, 20, 25, 30, 35, 40 and 45°C for 10, 30 and 60 min and the residual activity was assayed with olive oil as substrate. Relative activity of fish lipase was expressed as percentage of the initial activity. Values are means ± SD (n=3).

inhibitors has resulted in their application in certain treatments (Kotsovolou et al., 2007). In this study, the enzyme was incubated with various compounds and their relative activities were measured after 30 min of incubation at 25°C. From the Table 2, it is evident that the activities of fish lipases were inhibited by heavy metals such as Cd^{2+}, Zn^{2+} and Hg^{2+}. The presence of Zn^{2+} and Hg^{2+} potently inhibited lipolytic activities of the lipases from fish, while activities were slightly inhibited in the presence of Cu^{2+} salts. This is likely due to the proximity of the -SH group to the catalytic and interfacial binding site but partially remote from the catalytic site which may have induced the marked loss of activity (Raso and Hultin, 1988; Verger et al., 1971). The catalytic effect of lipases might be considered to be of Ser, His, and Glu or Asp residues (Guidoni et al., 1981; Jin et al., 2002), thus the bulky Hg^{2+} group might cause steric interference to the approach of the substrate to the active site. This was similar to the previous report (Choo et al., 1998). Salts of Fe were found to

inhibit lipase from *Aspergillus niger* and lipases from fungus *Humicola lanuginose* S-38 (Liu et al., 1973). Hg has been also shown to inhibit the lipase of rat adipose tissue (Fredrikson et al., 1981). Treatment with Zn^{2+} at both 3mM and 5mM significantly inhibited the activity of lipase with only 40% and 20% relative activity after 30 min incubation which was very similar to the findings of Choo et al. (1998).

The activity s of lipase s was found to be increased slightly in presence of lower concentration of Ca^{2+} but at significantly higher concentration of Ca^{2+}, the activities were decreased slightly which is consistent with the results reported elsewhere (Shastry and Raghavendra Rao, 1971). The primary role of Ca^{2+} seems to be to remove the released fatty acid as its calcium salt but the catalytic effect of calcium on a lipase derived from *Humicola lanuginosa* was explained by the removal of free fatty acids from the interface (Liu et al., 1973). In a calcium free system, the lipase can not adsorb at the water-fat interface, and consequently no lipolytic activity occurs. Possibly, the calcium ions compensate for the electrostatic repulsion created between the enzyme and the substrate. EDTA at 1 mM concentration deactivated the fish lipase by 60%, similar to the results of Lima et al. (2004) which might be due to the removal of metal ions located on or near the active site.

Table 2 Effect of metallic salts on the activity of fish lipase (*Cirrhinus reba*).

Salts added[1/]	Concentration[2/] (mM)	Residual activity[3/] (%)
Control	0	100
EDTA	1	60
	3	55
	5	25
$CuCl_2$	1	85
	3	90
	5	65
$CaCl_2$	1	116.6
	3	111.5
	5	76
$CdCl_2$	1	86
	3	65
	5	35
$HgCl_2$	1	42
	3	31
	5	25
$ZnCl_2$	1	48
	3	39
	5	22

[1/] Different types of metallic salts added to obtain the activity of fish lipase enzyme.
[2/] Concentration of 1mM, 3mM and 5mM of each salt used for the determination of enzyme activity.
[3/] Residual activity defined as the percentage of fish lipase enzyme activity comparing with the control at 35°C and pH 5.5.

Conclusions

The identification and characterization of lipase from fish (*C. reba*) is an important aspect of knowledge for the clarification of the mechanism of the enzyme. It might be speculated that the enzyme involved in the hydrolysis of olive oil as substrate is a monomeric in nature. The pure enzyme, tentatively named fish lipase, is a glycosylated protein with molecular weight of 87 KDa. The optimum pH and temperature of lipase is 5.5 and 35°C. Fish lipase activity was stimulated by Ca^{2+} and Cu^{2+} and inhibited by Hg^{2+} and Zn^{2+}. Further studies are needed to clarify its role in esters synthesis and specificity in hydrolysis of unsaturated fish oil.

Acknowledgments

We are grateful to Dr. H. Rahman, Dr. M.A. Bari, Dr. P. Hasan and Mr K.A. Salam for excellent laboratory assistance. This work was financially supported by the Ministry of Science and Technology, Bangladesh.

References

Abbas, H., A. Hiol, V. Deyris and L. Comeau. 2002. Isolation and characterization of an extracellular lipase from *Mucor sp* strain isolated from palm fruit. Enzyme Microb. Technol. 31: 968-975.

Alberta, N.A. Aryee, K. Benjamin Simpson and R. Villalonga. 2007. Lipase fraction from the viscera of grey mullet (*Mugil cephalus*) iolation, partial purification and some biochemical characteristics. Enzyme and Microb. Tech. 40: 394-402.

Aloulou, A., J.A. Rodriguez, D. Puccinelli, N. Mouz, J. Leclaire, Y. Leblond and F. Carriere. 2007. Purification and Biochemical characterization of the LIP2 lipase from Yarrowia lipolytica. Biochim. Biophys. Acta. 1771: 228-237.

Borlongan, I.G. 1990. Studies on the digestive lipases of milkfish. Chanos chanos. Aquaculture. 89: 315-25.

Brockerhoff, H., R.J. Hoyle and P.C. Hwang. 1970. Digestive enzymes of the American Lobster (Homarus americanus). J. Fish. Res. Board Can. 27: 1357-1370.

Choo, D.W., T. Kurihara, T. Suzuki, K. Soda and N. Esaki. 1998. A cold-adapted lipase of an Alaskan psychrotroph, *Pseudomonas* sp. Strain B11-1: gene cloning and enzyme purification and characterization. Appl. Envir. Microbiol. 64: 486-91.

Destain, J., D. Roblain and P. Thonart. 1997. Improvement of lipase production from Yarrowia lipolytica. Biotechnol. Lett. 19: 105-7.

Faber, K. and M.C.R. Franssen. 1993. Prospects for the increased application of biocatalysts in organic transformations. Trends Biotechno. l11: 461-70.

Fredrikson, G., P. Stralfors, N.O. Nilsson and P. Belfrage. 1981. Horon-sensitive lipase from adipose tissue of rat. Method Ezymol. 71: 636-646.

Gjellesvik, D.R., D. Lombardo and B.T. Walther. 1992. Pancreatic bile salt dependent lipase from cod (*Gadus morhua*): purification and properties. Biochim. Biophys. Acta. 1124: 123-134.

Guidoni, A.A., F. Bendouka, J.D. De Caro and M. Rovery. 1981. Characterization of the serine reacting with diethyl-nitrophenyl phosphate in porcine pancreatic lipase. Biochim. Biophys. Acta. 660: 148-50.

Hemachander, C. and R. Puvanakrishnan. 2000. Lipase from *Ralstonia pickettii* as an additive in laundry detergent formulations. Process Biochem. 35: 809-14.

Hazarika, S., P. Goswami, N.N. Dutta and A.K. Hazarika. 2002. Ethyl oleate synthesis by porcine pancreatic lipase in organic solvents. Chem. Eng. J. 85: 61-8.

Iijima, N., S. Tanaka and Y. Ota. 1998. Purification and characterization of bile saltactivated lipase from the hepatopancreas of red sea bream, *Pagrus major*. Fish Physiol. Biochem. 18: 59-69.

Iwai, M., S. Okunura and Y. Tsujisaka. 1975. The comparism of theproperties of two lipases from Penicillium cyclopium. Agr. Bio. Chem. 39: 1063-1070.

Jin, W., U.C. Broedl, H. Monajemi, J.M. Glick and D.J. Rader. 2002. Lipase H. a new member of the triglyceride lipase family synthesized by the intestine. Genomics. 80: 268-73.

Kotsovolou, S., A. Chiou, R. Verger and G. Kokotos. *2007.* Bis-2-oxo amide triacylglycerol analogues: a novel class of potent human gastric lipase inhibitors. J. Org. Chem. 66: 962-7.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nutr. 227: 680-685.

Lie, Ø. and G. Lambersten. 1985. Digestive lipolytic enzymes in cod (*Gadus morhua*): Fatty acid specificity. Comp. Biochem. Physiol. 80B: 447-50.

Lima, V.M.G., N. Krieger, D.A. Mitchell and J.D. Fontana. 2004. Activity and stability of a crude lipase from Penicillium aurantiogriseum in aqueous media and organic solvents. Biochem. Eng. J. 18: 65-71.

Liu, W., T. Beppu and K. Arima. 1973. Effect of various inhibitors of lipase action on thermophilic fungus *Humicola lanuginose* S-38. Agric. Biol. Chem. 37: 2487-2492.

L´opez-Amaya, C., A.G. Marangoni, N.F. Haard and B.K. Simpson. 2001. Lipases: Seafood Enzymes. Utilization and Influence on Postmortem Fish Quality. NY.

Lowry, O.H., N.J. Rosenbrough and R.J. Randall. 1951. Protein measurement with the Folin-phenol reagent. J. Biol. Chem. 183: 265-275.

Mukundan, M.K, K. Gopakumar and M.R. Nair. 1985. Purification of a lipase from the hepatopancreas of oil sardine (Sardinella longiceps Linnaceus) and its characteristics and properties. J. Sci. Food Agric. 36: 191-203.

Nthangeni, M.B., H.G. Patterton, A.V. Tonder, W.P. Vergeer and D. Litthauer. 2001. Over expression and properties of a purified recombinant Bacillus licheniformis lipase: a comparative report on Bacillus lipases. Enzyme Microb. Technol. 28: 705-12.

Patton, J.S., T.G. Warner and A.A. Benson. 1977. Partial characterization of the bile saltdependent triacylglycerols lipase from the leopard shark pancreas. Biochim. Biophys. Acta. 486: 322-30.

Raso, B.A and H.O. Hultin. 1988. A comparison of dogfish and porcine pancreatic lipases. Comp. Biochem. Physiol. 89B: 671-677.

Saxena, R.K., W.S. Davidson, A. Sheoran and B. Giri. 2003. Purification and characterization of an alkaline thermostable lipase from Aspergillus carneus. Process Biochem. 39: 3239-47.

Sugihara, A., Y. Shimada and Y. Tominaga. 1990. Separation and characterization of two molecular forms of Geotrichum candidum. J. Biochem. 107: 426-430.

Shastry, B.S. and M.R. Rao. 1971. Studies on Rice Bran Lipase. Indian J. of Biochem. and Biophys. 8: 327-332.

Tocher, D.R. and J.R. Sargent. 1984. Studies on triacylglycerol, wax ester and sterol ester hydrolases in intestinal caeca of rainbow trout (Salmo gairdnerii) fed diets rich in triacylglycerols and wax esters. Comp. Biochem. Physiol. 77B: 561-571.

Undurraga, D., A. Markovits and S. Erazo. 2001. Cocoa butter equivalent through enzymic interestierification of palm oil midfraction. Process Biochem. 36: 933-939.

Verger, R., L. Srada and P. Desnuelle. 1971. On the sulfhydryl groups of porcine pancreatic lipase and their possible role in the activity of the enzyme. Biochim. Biophys. Acta. 242: 580-92.

Verger, R., F. Ferrato, C.M. Mansbach and G. Pieroni. 1982. Novel intestinal phospholipase A2: purification and some molecular characteristics. Biochemistry. 21: 6883-6889.

Vonk, H.J. 1960. Digestion and metabolism, pp. 291-316. *In* T.H. Waterman, ed., The physiology of Crustacea, Vol. I. Academic Press, Inc., New York, NY.

Manuscript received 29 January 2009, accepted 3 June 2009