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Isolation, Purification and Characterization of Lipase from Grey Mullet (*Liza parsia* Hamilton, 1822)

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Abstract: Two lipases designated as Lip-1 and Lip-2 were purified from dorsal part of grey mullet (*Liza parsia*) to homogeneity by 85% $(NH_4)_2SO_4$ fractionation followed by simultaneous desalting and concentration by ultrafiltration and Sephadex G-50 and DEAE-cellulose chromotography and CM-cellulose chromotography. The molecular weight of two lipases was determined by SDS-PAGE and gel filtration about 46.5 and 41.2 KDa, respectively. Both the enzymes were dimer in nature remained unchanged the presence and absence of reducing agent under SDA-PAGE. The Lip-1 and Lip-2 lipases were active within the pH range of 7-8.5, with an optimum pH of 8 and 8.5.0 and were stable from 2.0-10.5. The enzyme was active within the temperature range of 30-60°C and maximum activities were observed around 33 and 35°C, respectively and beyond which it lost activity progressively. The hydrolytic activity was enhanced by Ca^{+} and EDTA (concentration 0.001-0.003 M) but strongly inhibited by heavy metal s Cd^{++}, Zn^{++} and Hg^{++}. The presence of Zn^{++} and Hg^{++} potently inhibited lipolytic activities of the lipases from grey mullet, while activities were slightly inhibited in the presence of Cu^{++} salts.

Keywords: *Liza parsia*, grey mullet, lipases, characterization, purification

INTRODUCTION

Lipases are hydrolytic enzymes (EC 3.1.13) act at fat-water interfaces to catalyze the hydrolysis of tryglycerides to fatty acid and glycerol (Sarda and Desnuelles, 1958, Desnuelle *et al*., 1960; Norin *et al*., 1993). Lipases are very important because of the role they play in the postmortem quality deterioration of seafood and other foodstuffs during handling, chilled frozen storage and widely used for biotechnological applications in dairy industry, oil processing, production of surfactants and preparation of enantiomercally pure pharmaceuticals. Compared with other hydrolytic enzymes (e.g., proteases and carbohydrases), lipases are relatively less well studied and in this regard, lipases from aquatic animals are even less well known versus their counterparts from mammalian, plant and microbial sources (L'opez-Amaya *et al*., 2001). The aquatic world contains a wide variety of living species and, hence represents great potential for discovering new enzymes (Cherif *et al*., 2007). the presence of a lipase activity has been described for some aquatic organisms such as lobster (Brockerhoff and Stewart, 1967), crab (Vonk, 1960) and *Homarus americanus* (Brockerhoff *et al*., 1970) 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; Gjellesvik *et al*., 1992), dog fish (Raso and Hultin, 1988), sardine (Mukundan *et al*.,

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1985), anchovy, striped bass and salmon (Leger *et al*., 1977), as well as red sea bream (Iijima *et al*., 1998). Other investigators have been reported lipases and lipolytic activities in the digestive glands, liver and adipose tissues of different fish species (Izquierdo and Socorro, 2000; Liang *et al*., 2002).

From few years ago, considerable attention has been devoted to lipases of botanical origin and microorganisms (Aquino, 1994; Desnuelle, 1961). Several lipases of animal origin have been purified and their chemical and enzymatic properties were elucidated in details (Verger, 1984; Mc Crae, 1983). 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 for house hold detergent in the oleochemical industry and to produce structural triglycerides (Verger *et al*., 1982). Lipase also used in the synthesis of polymers, agrochemical leather textile, baking pharmaceutical and paper industry.

Most of the purification procedures for lipases reported involve series of non-specific techniques, such as ammonium sulphate precipitation, gel filtration and ion-exchange chromatography. In recent years, affinity chromatographic techniques have become frequently used, decreasing the number of steps necessary for lipase purification.

Most recently, reversed-micellar and aqueous two phase systems, ultra filtration membranes and immune purification have also been applied to purify some lipases, mainly of microbial origin. In the present study purification of grey mullet lipases was carried out mainly by ion-exchange chromatography followed by gel filtration.

MATERIALS AND METHODS

Biological Materials

Adult grey mullet (1-1.5 kg) were purchased from local fish landing centre and the dorsal part was removed with knife and stored in crushed ice in plastic bags and transported directly to the laboratory where were stored frozen at -20°C until used for experimental purpose.

Chemicals and Reagents

Sephadex G-50 and deae-cellulose and cm-cellulose were purchased from pharmacia Biotech Inc. Sweden. Diisopropylfluorophosphate (DIP) was from ICN and phenylmethyl sulphonylfluoride (PMSF) was from Wako pure chemical Industry Ltd, Japan, Sodium cholate (NaC), sodium taurocholate (NaTC), sodium deoxycholate (NaDC), dioxane, arabic gum, glycerol, benzamidine hydrochloride, calcium chloride ($CaCl_2$), Bovine Serum Albumin (BSA), sodium hydroxide (NaOH), ammonium sulphate ($(NH_4)_2SO_4$), ethylenediamine tetraacetic acid (EDTA), tris(hydroxymethyl) aminomethane (Tris), mercaptoethanol, Sodium Dodecyl Sulfate (SDS) and mercury chloride ($HgCl_2$) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Acetone, chloroform, butanol, methanol, diethyl ether, iso-propanol, hydrochloric acid, glacial acetic acid, benzene, toluene, hexane, n-heptane, iso-octane, dimethyl sulfoxide, dimethyl formamide (HPLC/analytical grade) were also purchased from Fisher Scientific (Fair Lawn, NJ, USA).

Sample Preparation for Enzyme Extraction

The frozen dorsal part of fish were thawed at 4°C and cleaned by flushing with distilled water followed by thorough rinsing with ice-cold 0.85% NaCl solution to remove adhered blood. The dorsal part of fish were chopped into small pieces and rapidly frozen in liquid nitrogen and then comminuted into fine powder in a Waring blender. The powder was defatted using a modified version of the procedure by Verger *et al*. (1982) and Andrews (1965), 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 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 B¨uchner funnel. The defatted material was air dried at room temperature (23°C) in a fume hood then stored at 20°C until needed.

Enzyme Extract Preparation

The dried defatted powder was homogenized in 25 mM Tris-HCl buffer, pH 7.8, containing 5 mM benzamidine-HCl, 1mM 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 then centrifuged at 8000×g for 20 min at 4°C. The precipitate was discarded and the supernatant was filtered through several layers of cheesecloth (to remove the floating fatty material) and then fractionated with solid (NH4) 2SO4 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 collected and redissolved in TBEG buffer (pH 7.8) to form the ammonium sulfate fraction. The fraction was dialyzed overnight against three changes of 4 lTBEG 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 extract, ultrafiltrate (UF fraction) was stored frozen at 20°C until needed.

Isolation and Purification of Lipases

The enzyme extract was loaded into the DEAE-cellulose column previously equilibrated with Tris-HCl buffer, pH 8.4 for 24 h and the proteins were recovered from the column by step wise elution with the same buffer containing different concentration of NaCl 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 changes of buffer at 4°C. After centrifugation the supernatant was applied into a CM-cellulose column previously equilibrium with 5 mM PBS, pH 6.5 and the proteins were eluted from the column with the same buffer. The fraction showing the lipase activity were pooled and dialyzed again against 10 mM Tris-HCl buffer, pH 8.4 for overnight with four changes of buffer at 4°C. After centrifugation the supernatant was applied into gel filtration on 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. Lipase containing active fraction was pooled and tested for homogeneity.

Electrophoresis

Polyacrylamide disc gel electrophoresis was conducted at room temperature, pH 8.4 on 7.5% gel and amido black was used as staining reagent.

Determination of Molecular Weight (MW): MW of the purified native enzyme was determined by gel filtration on Sephadex-50 column (0.9×90 cm) as described by Laemmli (1970). The marker proteins used were lysozyme (14.6 KD), Egg albumin (4.5 KD), BSA (66 KDS), α galactosidase (116 KDs), β-amylase and tripsinogen (24 KD). The MW also determined by SDS-PAGE according to the method of Laemmli (1970) and Sugihara *et al*. (1990) using the marker proteins, Myosine (205 KD) α galactosidase (116 KDs), BSA (66 KDS), Carbonia anhydrase (29 KD), β lactoalbumin (18 KD) and Aprotinin (6.5 KD)

Enzyme Assay

Lipase activity was assayed as reported (Sugihara *et al*., 1990) using olive oil 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 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 by Lowry *et al*. (1951) using BSA as standard as well as by measurement of absorbance at 280 nm.

Effect of pH on the Activity and Stability of Lipases

The activity of two lipase denoted as Lip-1 and Lip-2 was investigated within the pH range of 2.0-8.0 using the following buffer solutions: 0.2 M phosphate buffer, pH 6.0 and 8.0; 0.2 M Tris-HCl buffer pH 8.5 and 9.0; 0.2 M carbonate-bicarbonate buffer pH 9.5 and 10.5 with olive oil as substrate as described above. 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 pH on lipase stability was determined by incubating the lipase fraction in various buffer solutions ranging from 2.0 to 8.0 for 30 min at 25°C in a Haake circulating water bath. The compositions of the buffer solutions used for the pH stability studies were as follows: 0.2 M acetate buffer, pH 4.0; 0.2 M AcONa-HCl buffer pH 3.10; 0.2 M citrate-phosphate buffer, pH 6.0; 0.2M phosphate buffer, pH 8.0; and 0.2M bicarbonate-carbonate buffer, pH 10.5. 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 Lip-1 and Lip-2 activity were measured by equilibrating olive oil at 10, 15, 20, 25, 30, 32, 33, 34, 35, 40, 45, 50, 55, 60 and 70°C for 30 min. In each assay, 100 μL of the enzyme extract was added to 900 μL of pre-equilibrated substrate. The thermostability of the lipase fraction was studied by incubating the enzyme extract at various temperatures for 10, 30, or 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 25°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 Lip-1 and Lip-2 after incubation period was assayed with olive oil as substrate as previously described in duplicates and the results were expressed as percentage of the activity obtained without the chemical agent.

RESULTS AND DISCUSSION

Isolation and Purification of Lipases

Few scientists have been studied fish lipases and reported different purification methods and yields (Gjellesvik *et al*., 1992; Iijima *et al*., 1998). The crude enzyme solution after dialysis against 10 mM Tris-HCl buffer, pH 8.4 for 24 h, at 4°C was applied to firstly ion exchange chromotography a DEAE for purification.

In Fig. 1, the proteins of crude enzyme solution were separated in two major peaks: F-1 and F-2 and one minor peak F-3. Of these fractions F-1 was eluted by buffer only while F-2 and F-3 were eluted by the buffer containing 0.1 M NaCl and 0.2 M NaCl, respectively. Further, it was found that only F-1 fraction contained lipase activity. So, the area as indicated by solid bar of this fraction was pooled together and used for further purification by CM-cellulose chromatography.

The enzyme active fraction F-1 was dialyzed against distilled water for 12 h and against the eluting buffer (5 mM sodium phosphate buffer, pH 6.5) for 12 h at 4°C. After centrifugation the

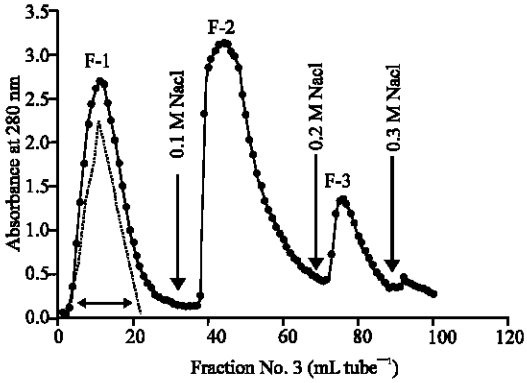

Fig. 1: Ion exchange chromatography of crude enzyme solution on DEAE-cellulose. The crude solution (20 mg) was applied to the column (2.1×25 cm) pre-equilibrated with 10 mM Tris-HCl buffer, pH 8.4 at 4°C and eluted by stepwise increases of NaCl concentration in same buffer. Flow rate: 25 mL h^{-1}

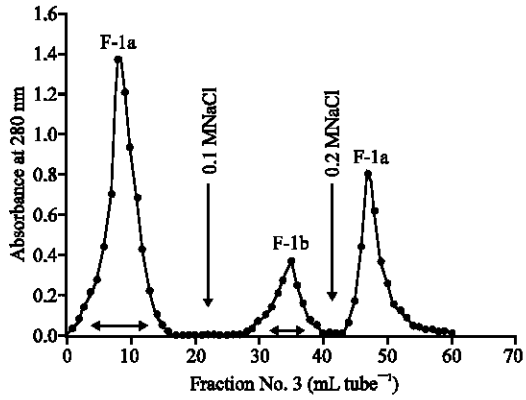

Fig. 2: CM-cellulose chromatography of F-1a fraction obtained from ion exchange chromatography on DEAE-cellulose. Fraction F-1a (12 mg) was applied to the column (1.5×15 cm) pre-washed with 5 mM sodium phosphate buffer, pH 6.5 at 4° C and eluted by stepwise increases of NaCl in the same buffer. Flow rate was 20 mL h^{-1}

clear supernatant was applied to the CM-cellulose column. As shown in Fig. 2, the components in F-1 was separated into two sharp peaks F-1a and F-1c and one small peak, F-1b. Of these fractions F-1a was eluted by the buffer only while F-1b and F-1c were eluted by the buffer containing 0.1M and 0.2 M NaCl, respectively. It was found that the fractions F-1a and F-1b contained lipase activities while F-1c' possessed no such activity

The area as indicated by solid bar of these fractions (F-1a, F-1b) were pooled separately and their purity was checked by SDS-PAGE.The phtographic representation of the elctrophoretic patterns of F-1a and F-1b are presented in Fig. 3. From the result it might be concluded that the fraction F-1a was not pure as it gave more that one band on the gel which the fraction F-1b must be contained pure protein as it gave single band on the gel. The fraction F-1a was then further purified by gel filtration, on Sephadex G-50.

The fraction F-1a was dialyzed against distilled water for 12 h and then against 10 mM Tris-HCl buffer, pH 8.4 for 12 h. After concentration, this fraction was applied to a Sephadex G-50 column at 4°C, which was previously equilibrated with the same buffer. In Fig. 4, the components present in

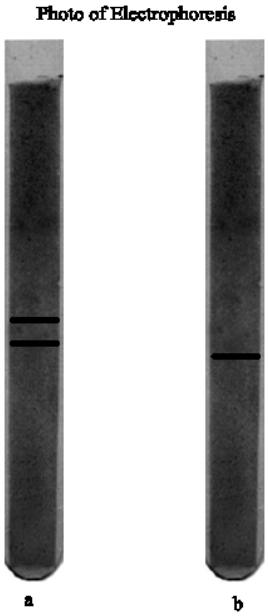

Fig. 3: Polyacrylamide disc gel electrophoretic pattern of the protein at room temp. pH 8.3 on 7.5% Polyacrylamide gel. Staining reagent : 1% amido black. Protein concentration : 50 µg. a : F-1a fraction b: F-1b fraction

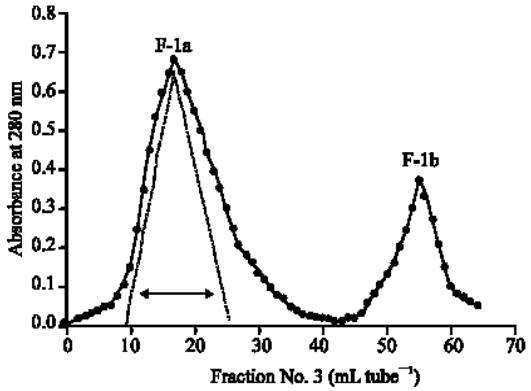

Fig. 4: Gel filtration of F-1a fraction obtained from CM-cellulose chromatography on Sephadex G-50. F-1a fraction (6 mg) was applied to the column (3×120 cm) pre-washed with 10 mM Tris-HCl buffer, pH 8.4 at 4°C and eluted by the same buffer. Flow rate: 25 mL h^{-1}

this fraction was eluted as one major peak, F-1a and one minor peak F-1b. It was found that only the fraction F-1a contained the lipase activity while F-1b contained no lipase activity. The active enzyme fraction, as indicated by solid bars was pooled and its homogeneity was checked by Polyacrylamide gel elctrophoresis.

In Fig. 5, the fraction F-1a gave clear single band on polyacrylamide gel indicating that this kaj korchifraction contained pure lipase enzyme. Lipase activities in the course of purification steps are shown in the following table (Table 1).

Although the activities of the enzymes were decreased at each subsequent purification steps but the purification fold of the enzymes were increased (Helisto *et al*., 2001; Yu *et al*., 2007) at each step

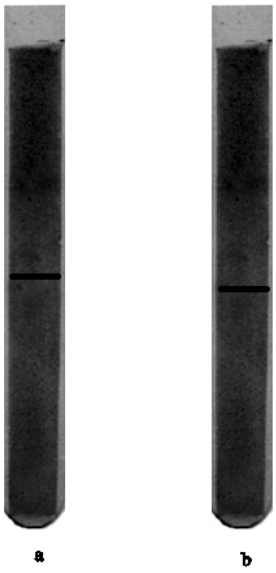

Fig. 5: Disc electrophoretic pattern of F-1a′ protein on 7.5% polyacraylamide gel. Protein concentration: 60 μg

Table 1: Lipase activities in the course steps of purification of phaisa fish lipase

Steps of purification		Total protein (mg)	Total activity (mU)	Specific activity (mU mg^{-1})	Yield (%)	Purification fold
Crude extract		4895.78	41124.55	8.40	100.00	1.00
85% Ammonium sulphate precipitation (Crude enzyme)		1040.59	15296.67	14.70	36.95	1.70
DEAE-cellulose fractions	F-1	58.75	4456.18	75.80	10.40	9.00
CM-cellulose fractions	F-1a	12.10	1413.88	116.85	3.40	11.95
	F-1b	6.85	1099.76	160.55	2.95	18.55
Gel filtration on Sephadex G-50	F-1a'	2.85	913.42	32.50	2.00	37.75

and the purifical fold of F-1a and F-b became about 38 and 19 folds, respectively The decrease in yield might be due to denaturation of the enzymes during lengthy purification procedure.

Determination of Molecular Weight (MW) of Lipases

The MW of the enzymes were determined by gel filtration on Sephadex G-150 using β-galactosidase, bovine serum albumin, α-amylase, ovalbumin, trypsin inhibitor and lysozyme as standard proteins. The MW of purified lipases were estimated to be 46.5 KDa for F-1a′ and 41.2 KDa for F-1b. Iwai *et al*. (1975) purified two lipases from *Penicillium cyclopium* having MW of 27 KDa and 36 KDa, while Yu *et al*. (2007) and Aloulou *et al*. (2007) reported the MW of the lipase Lip2 from *Yarrowia lipolitica* a were 38 and 38, 48 KDa, respectively.

The MW of these enzymes were also determined by SDS-PAGE using the same marker proteins and the MW of F-1a' and F-1b were found to be 46.5 and 41.2 KDa, respectively. Abbas *et al*. (2002) reported the molecular weight of lipase from *Mucor sp* strain isolated from palm fruit was 42 KDa Choo *et al*. (1998) and Helisto *et al*. (2001) showed two protein bands on SDS-PAGE with MW 35,000 and 46,000 Da. Further, the MW of the enzymes were found to be unchanged in the presence or absence of β-mercaptoethanol indicating that both the lipases contained only one subunit. The lipases, F-1a and F-1b were denoted as Lip-1 and Lip-2, respectively.

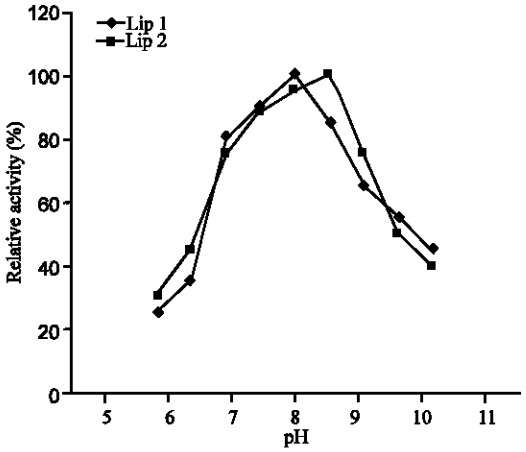

Fig. 6a: Effect of pH on the activity of Lip-1 and Lip-2L. The buffers used were phosphate, pH 6.0-8.0; Tris-HCl, pH 8.4-9.0; carbonate-bicarbonate, pH 9.4 and 10.0; all the buffers were 0.2 M. Data used to plot figure are average values of duplicate results for two experiments

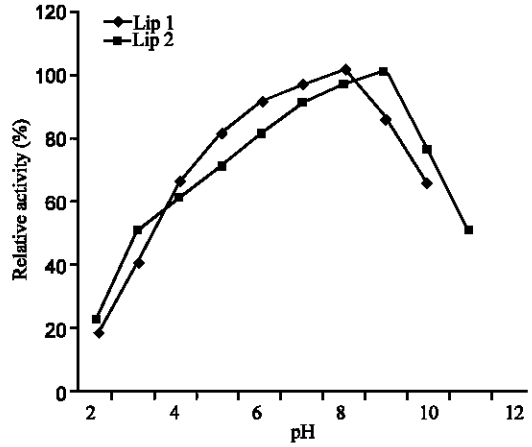

Fig. 6b: Effect of pH on the stability of Lip-1 and Lip-2L. The enzyme extract was incubation at 25 °C for 30 min in various buffers; HCl-KCl, pH 2.0; glycine-HCl, pH 3.0; acetate, pH 4.0 and 5.0; phosphate, pH 6.0-8.0; and carbonate-bicarbonate pH 10.0; all the buffers were 0.2 M. Data used to plot figure are average values of duplicate results for two experiments

Effect of pH on the Activity of Lipases

Lip-1 and Lip-2 hydrolyzed olive oil appreciably over a relatively broad alkaline pH range (from pH 7.0 to 10.0). Beyond pH 9.0, the activity of the enzymes decreased gradually to pH 10.0 (Fig. 6a). These trend were similar to findings reported for other fish lipases (Gjellesvik *et al*., 1992; Raso and Hultin, 1988; Mukundan *et al*., 1985; Iijima *et al*., 1998) and is also consistent with the pH optima reported for lipases from other sources (Lima *et al*., 2004; Nthangeni *et al*., 2001). The Lip-1 and Lip-2 both enzymes were stable between pH 7.0 and 10.0 during incubation at 25°C for 30 min. There was 55% residual activity above pH 7.0. However, there was a marked loss of activity in the acidic region below pH 4.0 with 55% decrease in activity, compared to the activity at pH 8.0 (Fig. 6b).

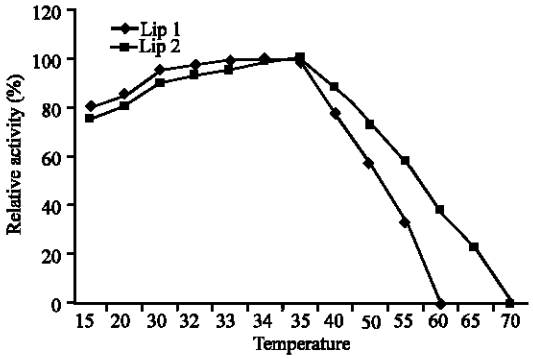

Fig. 7: Effect of temperature on the activity of Lip-1 and Lip-2. The substrate (Olive oil) was incubation at the indicated temperature for 30 min. Data used to plot figure are average values of duplicate results for t experiments

This observation is also similar to those found for other fish lipases (Mukundan *et al*., 1985) as well as lipases from microorganisms such as *Pseudomonas* sp. strain B11-1, *Bacillus licheniformis* and *Aspergillus carneus* (Lima *et al*., 2004; Saxena *et al*., 2003; Zouari *et al*., 2005), but different from scorpion lipase that was reported to be most stable at pH 11.0 (Borlongan, 1990) It is worth mentioning here that Lip-1 and Lip-2 was both also exhibited greater stability in phosphate buffers than in Tris-HCl buffer. For instance, Lip-1 and Lip-2 retained about 60, 50 and 44 and 6%, 48 and 40% of initial activity when incubated in phosphate, citrate-phosphate and Tris-HCl buffer, respectively, at pH 7.0 (data not shown). This observation was similar to the findings of Lima *et al*. (2004) and Nthangeni *et al*. (2001).

Effect of Temperature on the Activity of Lipases

The effects of temperature on the activities of Lip-1 and Lip-2 were examined in the range of 10 to 70°C. Lipolytic activities of grey mullet lipases were found to be profoundly affected by temperature. In Fig. 7 the activities of Lip-1 and Lip-2 were increased gradually with rise in temperature and the maximum activities were observed around 33 and 35°C, respectively. These observed temperature optimum in this study was similar to the lipase from sardine and cod were 37 and 25°C with tributyrine and p-nitrophenyl myristate as substrate, respectively (Gjellesvik *et al*., 1992; Raso and Hultin, 1988), For instance, the reported optimum temperature of lipase from milkfish, 45-50°C with olive oil as substrate (Tan *et al*., 2004). and lipases from the psychrotroph *Pseudomonas* sp. strain B11-1 and *Penicillium camembertii* Thom PG-3 with temperature optima of 45 and 48°C with *p*-nitrophenyl butyrate and olive oil as substrates (Lima *et al*., 2004; Destain *et al*., 1997). With further rise in temperature the activities were decreased abruptly and the enzymes lost almost 100% of their activities at 60 and 70°C for Lip-1 and Lip-2, respectively. Grey mullet fish lipases are found to be highly active in the temperature range from 30 to 35° 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) reported that the optima pH and temperature of extracellular lipase from *Y. lipolytica* was 7.0 and 37 8°C, while this lipases from *Yarrowia lipolitica* was 40°C (Aloulou *et al*., 2007). Data used to plot figure are average values of duplicate results for experiments.

Effect of Metal Ions on the Activity of Lipases

Understanding the role of lipase inhibitors may provide a better perceptive of their mechanism of action (Marguet *et al*., 1994) and successful identification of potent and specific inhibitors have resulted in their application in certain treatments (Bray, 2000; Kotsovolou *et al*., 2007). In this study,

Table 2: Effect of metallic salts on the activities of phaisa fish lipases

		Relative activity	
Salts added	Concentration (Molar)	Lip-1 (%)	Lip-2 (%)
None	-	100.0	100.00
EDTA	0.001	65.0	75.00
	0.003	50.0	55.00
	0.005	25.0	35.00
$CuCl_2$	0.001	85.0	80.00
	0.003	80.0	75.00
	0.005	75.0	65.00
$CaCl_2$	0.001	114.4	105.20
	0.003	100.5	90.95
	0.005	84.0	75.00
$CdCl_2$	0.001	71.0	63.00
	0.003	56.0	54.00
	0.005	38.0	28.00
$HgCl_2$	0.001	45.0	50.00
	0.003	25.0	35.00
	0.005	20.0	25.00
$ZnCl_2$	0.001	50.0	45.00
	0.003	30.0	25.00
	0.005	20.0	18.00

the enzyme was incubated with various compounds and relative activities were measured after 30 min of incubation at 25°C From the Table 2, it is evident that activities of grey mullet fish lipases inhibited by heavy metals such as Cd^{++}, Zn^{++} and Hg^{++}. The presence of Zn^{2+} and Hg^{+} potently inhibited lipolytic activities of the lipases from grey mullet 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, this may have induced the marked loss of activity (Raso and Hultin, 1988; Verger *et al*., 1971). The catalytic triad of lipases has been recognized to consist of Ser, His and Glu or Asp (Guidoni *et al*., 1981, Fredrikson *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. Salts of Fe were found to inhibit lipase from *Aspergillus niger* (180) and lipases from fungi of the genus *Geotrichum*. Mercury is known to bind to thiol groups of proteins forming stable complexes. Mercury has been shown to inhibit the lipse of rat adipose tissue (Fredrikson *et al*.1981). It has been shown conclusively that some heavy metals inhibit lipase activity, However, treatment with Zn^{2+} at both 0.003 and 0.005 M significantly inhibited the activity of Lip-1 and Lip-2 with only 30 and 20% and 25 and 18% relative activity after 30 min incubation and this was similar to the findings of Choo *et al*. (1998).

Calcium ions carry out distinct role in the lipase action. The activities of both the lipases were found to be increased slightly in presence of lower concentration of Ca^{2+} but at higher concentration of Ca^{2+}, the activities were decreased slightly which is consistent with the results reported elsewhere (Shastry and Raghavendra Rao, 1971). Like pancreatic lipase (Liu *et al*., 1973) grey mullet lipases were stimulated in the presence of calcium ions at certain concentrations. The primary role of Ca^{++} seems to be to remove the released fatty acid as its calcium salt. The activating effect of calcium on a lipase derived from *Humicola lanuginosa* was explained by the removal of free fatty acids from the interface. 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 (Shahani, 1975).

EDTA, 0.001 activated the Lip-1 and Lip-2 by 65 and 75%, respectively, similar to the results of Lima *et al*. (2004). The decrease in activity may be due to the removal of metal ions located on or near the active site.

CONCLUSIONS

The purification of a lipase from grey mullet fish (*Liza parsia*), is described in this paper for the first time. The pure enzyme, tentatively named Lip-1 and Lip-2, is a glycosylated protein with molecular weight of 46.5KDa and 41.2KDa, respectively. The optimum pH and temperature of Lip-1 and Lip-2, 8 and 8.3, 33 and 35°C, respectively. Lip-1 and Lip-2 activity were stimulated by Ca^{++} and Cu^{++} and inhibited by Hg^{++} and Zn^{++}. Further studies to determine its performances in esters synthesis and specificity in hydrolysis of unsaturated fish oil.

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