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A Study on the Intestinal Lipase of Indian Major Carp *Labeo rohita*

J. NAYAK, P.G.V. NAIR*, S. MATHEW and K. AMMU

Department of Biochemistry and Nutrition
Central Institute of Fisheries Technology
Willingdon Island, Matsyapuri, Cochin-682029
India

Abstract

Lipase from rohu(*Labeo rohita*, cyprinidae) intestine was extracted, fractionated with ammonium sulphate and purified on Sephadex G-100 column. It was found that the specific activity increased 3.6 times after ammonium sulphate precipitation and Sephadex G-100 Chromatography of crude extract. Lipase activity at different levels of ammonium sulphate fractionations was tested. Precipitates obtained at 30 and 60% saturation of ammonium sulphate showed lower activity. Fractions precipitated with 80% saturated ammonium sulphate had the maximum activity and no activity was detected in the filtrate from this fraction. Lipase was found to have optimum activity at temperature 45°C and at pH 7.0. The amino acid composition of the enzyme showed that lipase had high amount of polar amino acids than nonpolar amino acid content.

Introduction

Lipolytic enzymes play an important role in digestion of lipids in biological systems (Walton and Cowey 1982). Apart from physiological activity, lipase catalysed reactions are important in various industries, such as dairy (Lin et al. 1996), oleochemical (Mc Neill and Yamane 1991), food (Kim et al. 1998) etc., as an alternative to expensive and tedious chemical reactions. In an enzyme linked reaction, maintenance of optimal reaction conditions is essential to obtain maximum yields of the product.

Past studies on lipase in different species of aquatic origins like whale (Isihara 1960-1962), skate (Brockerhoff and Hoyle 1965), amazon fish

**Corresponding author*

(Reimer 1982) and turbot (Koven et al. 1997) have documented the properties of this enzyme. These data show that the characteristics of lipase vary depending on the source from which it is obtained. Therefore detailed investigations are required to understand the properties of lipase from various sources. Lipase from fish pancreas (Patton et al. 1975; Leger et al. 1977; Mukundan et al. 1985; Sukarno et al. 1996) and pyloric caeca of cod (Gjellesvik et al. 1989; 1992) and tuna (Maurin and Gal 1996) have been purified and partially characterised. Experiments on intestinal lipase are so far restricted to crude preparations (Kitamikado and Tachino 1960; Kayama et al. 1979; Borlongan 1990; Mukhopadhyay and Rout 1996) and literature on its purification is scarce. The present paper focuses on the partial purification of rohu(*Labeo rohita*, Cyprinidae) intestinal lipase and determination of the optimum temperature and pH conditions for the enzyme activity.

Materials and Methods

Experimental animal

Fresh water fish, rohu, *Labeo rohita* weighing between 500 to 600 g each, were collected from a nearby aquaculture farm and immediately after harvesting were washed, iced and transported to the laboratory.

Chemicals

Polyvinyl alcohol (PVA), tryptophan standard, amino acid standards, tributyrin, bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St Louis, MO, USA). The column chromatographic materials, Sephadex G-25 and Sephadex G-100, were from Pharmacia Fine Chemicals, Uppasala, Sweden. All other chemicals used were of Analytical Grade.

Lipase assay and protein estimation

The enzyme activity was assayed by estimating the amount of fatty acids released following the method of Mukundan et al. (1985). Protein content of the enzyme was determined by the method of Lowry et al. (1951) using BSA as standard.

Purification

CRUDE EXTRACT PREPARATION AND AMMONIUM SULPHATE FRACTIONATION

Throughout the purification process temperature was maintained between 0-4°C unless otherwise specified. Specimens were dissected and the intestines were removed, washed to remove the gut contents and washed intestines were pooled together for extract preparation. Crude extract was prepared as described by Mankura et al. (1984). The crude extract was frac-

tionated with 30, 60 and 80% saturated ammonium sulphate and subsequently desalted following the procedure of Mukundan (1982).

SEPHADEX G-100 CHROMATOGRAPHY

Volume of desalted extract was reduced by addition of dry Sephadex G-25 and applied to a column of Sephadex G-100 (2.5 x 45 Cm) previously equilibrated with 0.01 M Mcllvaine Phosphate buffer (pH 7.0) (Mukundan 1982). The sample was eluted with 0.01 M Mcllvaine Phosphate buffer and 5 ml fractions were collected at a flow rate of 20 $ml \cdot hr^{-1}$. The elution was monitored by observing the absorbance of fractions at 280 nm. Enzyme activity of each fraction was measured and fractions showing higher activity were pooled together and used for further characterization.

Effect of temperature and pH on lipase activity

Effect of temperature on enzyme activity was assayed with 1.0 ml portions of purified lipase at temperatures of 3°, 15°, 30°, 37°, 45° and 50°C at a pH of 7.0 (Mukundan et al. 1985). The effect of pH on lipase activity was determined by conducting the assay as described by Mukundan et al. 1985, at pH of 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 using 1.0 ml of purified extract at 37°C.

Estimation of amino acids

Amino acids except Tryptophan were estimated according to the procedure of Ishida et al. (1981). To 1.0 ml of partially purified enzyme extract 6.0 ml of 6N Hydrochloric acid was mixed and digested overnight at 110°C. The hydrolysate after flash evaporation was made up to 2.0 ml with Buffer C (Sodium citrate tribasic, Perchloric acid, n-Caprylic acid pH 2.2) and 20 ml was analysed on a Shimadzu HPLC LC-10 AS amino acid analysis system (Shimadzu Corporation, Japan) using ISC-07/S/Na column (19 x 5 cm) packed with Styrene Divinyl benzene copolymerized with sulfinic group.

Tryptophan content of the enzyme was estimated by the method of Sastry and Tummuru (1985). To 1.0 ml of partially purified sample 3.0 ml of 5% NaOH was added and hydrolysed at 110°C for 24 hours. The hydrolysate was neutralized, volume was made up to 25 ml with distilled water, filtered and the filtrate was used for estimation.

Results and Discussion

Enhancement of specific activity

The enhancement of the enzyme activity at different stages of purification is summarized in table 1. Specific activity of the extract increased 3.6 fold by ammonium sulphate fractionation and Sephadex G-100 chromatography. Kayama et al. (1979) purified carp hepatopancreas lipase 12.5 fold by

$(NH_4)_2SO_4$ fractionation and Sephadex G-200 column chromatography. However poor recovery of activity due to the addition of ammonium sulphate that may change the native structure of the enzyme during the treatment has been reported (Holland and Coolbear 1996, Chich et al. 1997 and Castillo et al. 1999). Gjellesvik et al. (1992) reported that practically all activity was lost during desalting or affinity chromatography regardless of desalting method in the case of lipase from cod pyloric caeca. Gjellesvik et al. (1992) purified a bile salt dependent lipase of defatted powder of cod pyloric caeca by combined affinity chromatography on Cholate-Sepharose and gel filtration on Sephacryl S-200 HR. The authors noticed that the specific activity of the enzyme increased by 1.5 fold after $(NH_4)_2SO_4$ fractionation which was nearly to the extent of enhancement of enzyme activity obtained in the present study (1.3 fold).

Ammonium sulfate fractionation

Enrichment of enzyme activity at different concentrations of ammonium sulphate is shown in figure 1. The precipitation of the enzyme was not noticeable at 30% saturation of ammonium sulphate. The maximum precipitation of the enzyme was obtained at 80% saturation. The filtrate from this step did not show any lipase activity. The result of the study is similar to

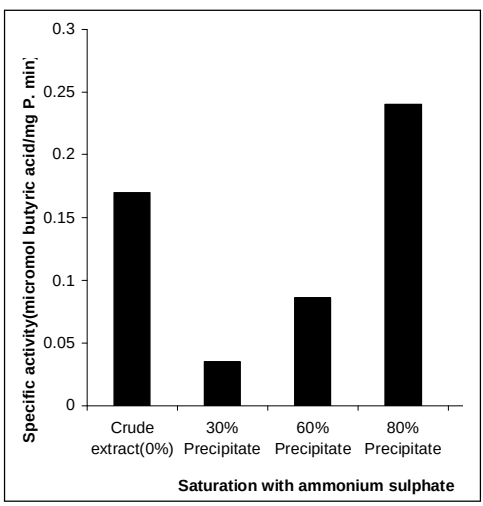

Fig. 1. Salting out rohu intestinal lipase at different saturation of ammonium sulphate.

Table 1. Purification of lipase from intestine of rohu

Steps	Total Protein (mg)	*Total activity x 10^3	**Specific Activity x 10^2	Yeild (%)	Purification fold
Crude Extract	2850	506.33	10.66	-	1.00
Desalted extract	917.85	220.28	14.4	43.5	1.35
Sephadex G-100 Chromatography	120.0	76.8	38.4	15.16	3.61

*Expressed as nmol butyric acid • min^{-1}
**Expressed as nmol butyric acid • min^{-1} • mg $protein^{-1}$

the observations of Mukundan et al. (1985) who salted out oil sardine hepatopancreatic lipase at 80% saturation. Kayama et al. (1979) obtained carp hepatopancreatic lipase precipitated at 30% saturation. Gjellesvik et al. (1989) precipitated cod pyloric caeca lipase with 47% saturation of ammonium sulphate. The salting out of enzyme depends on the hydrophobic nature of the protein (Scopes 1984). Amount of ammonium sulphate needed for precipitation of protein is dependent upon the hydrophobicity of the enzyme which in turn is decided by its nonpolar amino acid content (Scopes 1984)

Amino acid composition of the enzyme

The amino acid composition of partially purified lipase is shown in Table 2. The enzyme contained high proportion of glutamic acid and aspartic acid and the level of methionine was comparatively low. The result showed that the enzyme was composed of more acidic amino acids than basic ones. It was also noticed that quantum of hydrophilic amino acid was more than that of hydrophobic amino acids. Albro et al. (1985) also noted an excess of acidic amino acids over basic ones in rat pancreatic lipase. Mukundan et al. (1985) reported that purified oil sardine hepatopancreas lipase contained higher proportion of hydrophilic amino acid but cystine and methionine were absent. Gjellesvik et al. (1992) noticed high serine and glutamate levels compared to other amino acids in cod lipase. This shows that there are considerable differences in the amino acid composition of lipase from different sources.

Effect of temperature and pH on lipase activity

Effects of temperature and pH on rohu intestinal lipase activity are presented in figures 2 and 3 respectively. At 3°C there was no activity but it gradually increased and maximum activity was noticed at 45°C (pH 7.0).

Table 2. Amino acid composition of partially purified lipase

Amino acids	μg amino acid residue • mg protein^{-1}
Aspartic acid	102.00
Threonine	47.33
Serine	53.73
Glutamic acid	143.62
Proline	40.26
Glycine	64.46
Alanine	65.66
Valine	50.68
Methionine	06.81
Isoleucine	41.66
Leucine	71.26
Tyrosine	37.80
Phenyl alanine	37.06
Histidine	33.40
Lysine	24.96
Arginine	39.00
Tryptophan	9.18

The pH optimum of lipase was noticed at pH 7.0 (37°C). Brockerhoff et al. (1970) reported that partially purified digestive lipase of adult lobster showed optimum activity at pH 7.0. Vonk and Western (1984) observed that the lipolysis in teleost fish occurred between pH 6.2-8.5. Digestive lipase (pyloric ceaca and anterior ileum) of cod showed optimum activity at pH 7.0 and at temperature 37°C (Lie and Lambertsen 1985). The optimum temperature of milkfish intestinal lipase was found to be at 45°C (Borlongan 1990). In contrast to the present study, reports on slightly alkaline pH and lower temperature optima in different fishes are available. Kitamikado and Tachino (1960) noticed a relatively low temperature optima (20°C-25°C) and an alkaline pH optima (7.5-8.0) in rainbow trout intestinal lipase. Patton and Quinn (1973) established pH 8.0 as the optimum for surf clam digestive lipase. Rasco and Hultin (1988) found that dogfish lipase had pH and temperature optima at 8.5 and at 35°C, respectively. Variations in temperature optima of different fishes depend on the temperature of the habitat of the fish (Borlongan 1990) and thermal stability of the lipases (Sukarno et al. 1996). Indian major carps thrive well in the temperature range 18-37°C, temperature below 16.7°C and above 39.8°C prove fatal to them (Jhingran

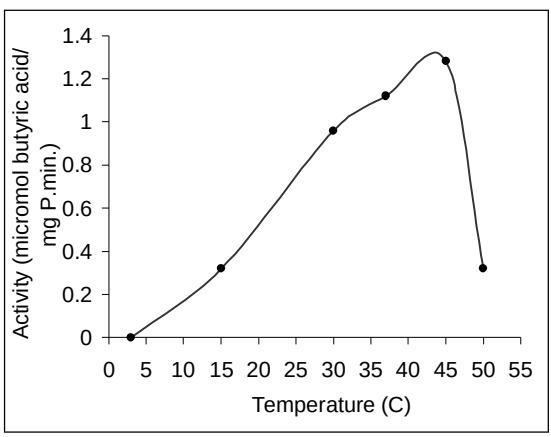

Fig 2. Effect of temperature on lipase activity

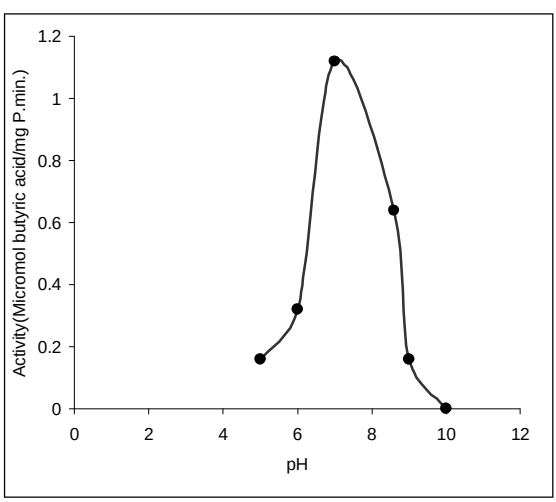

Fig 3. Effect of pH on lipase activity

1991). However, the observed optimum temperature for the digestive lipase activity is not compatible to normal surrounding water temperature condition.

Conclusion

The present study demonstrated that lipase present in intestine of rohu could be precipitated at a comparatively high concentration of ammonium sulphate (80%). Presence of more amount of polar amino acid might be responsible for such behavior of the enzyme. The pH optimum for the enzyme was found to be 7.0 which was nearer to the pH of the gut fluid (>6.0) of stomachless filter-feeding carps (Bitterlich 1985). From previous studies it has been observed that the characteristics of lipase vary from species to species of fishes and the results obtained during this investigation support this view. Therefore, purification and characterization of the enzyme from different species are necessary for proper understanding of the behavior of the enzyme.

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