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Partial purification and characterisation of α-amylases from the digestive tract of the Indian major carp *Labeo rohita* (Hamilton, 1822)

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ABSTRACT

Partial purification of α-amylases from the digestive tract of the Indian major carp *Labeo rohita* (Hamilton, 1822) through acetone fractionation and ion exchange chromatography (DEAE-SephadexA-50) resulted in 8-fold purification with 86% recovery. Characterisation of amylase activity revealed two pH optima at 4.5 and 6.5. Activity was stable over wide pH ranges of 3.5 to 4.5 and 7 to 12. Optimum incubation temperature was 35°C. The enzyme lost 91% activity at 60°C within 15 min and was inhibited by Amylase inhibitor Type-1 (wheat); 1, 10 Phenanthroline; Ethylene diamine tetra-acetate (EDTA) and Phenyl methyl sulphonyl fluoride (PMSF). Heavy metal ions Hg^{++} and Cu^{++} strongly inhibited the enzyme activity, while Zn^{++} and Bi^{++} inhibited to a lesser extent. Native polyacrylamide gel electrophoresis of the purified α-amylase fractions revealed four bands, with corresponding molecular weights of 43.59; 52.36; 55.42 and 54.01 kDa. α-Amylase activity from *L. rohita* exhibited linear hydrolysis of starch upto 7% concentration in 60 min.

Keywords: α-Amylase, Characterisation, Digestive tract, *Labeo rohita*, Purification

Introduction

The production of freshwater fish in India was 5.29 million t during 2017-18 and about 33% of this production comprised the Indian major carp (IMC) *Labeo rohita* (Hamilton, 1822) which contributed 1.75 million t next to *Catla catla* (GOI, 2018). The rohu *Labeo rohita* (Family Cyprinidae) is the most popular species among the IMCs. In aquaculture operations, feed accounts for 50% of the cost of production and the protein sources for the feed are more expensive than the carbohydrate sources. The protein sparing action of carbohydrate is well known in fishes (Wilson, 1994; Stone, 2003; Krogdohl *et al.*, 2005). Starch, the predominant carbohydrate in fish feed is made available to the fish by the action of α-amylases and therefore understanding the nature of amylases in fish species will pave ways for selection of appropriate carbohydrate source. The information pertaining to the purification of amylases in *L. rohita* and their characterisation is limited (Moreau *et al.,* 2001; Roychan and Chaudhari, 2001; Kushwaha, *et al.* 2012). This paper reports on the partial purification and characterisation of α-amylases in *L. rohita.*

Materials and methods

Enzyme extracts

Specimens of *L. rohita* (average length 45 cm; 995 g) were obtained from the culture ponds of the Regional Research Centre of ICAR-Central Institute of Freshwater Aquaculture (ICAR-CIFA) at Bangalore, Karnataka, India. The digestive tract (DT) and liver (L) of the specimens were dissected out under ice cold conditions and washed repeatedly with ice-cold distilled water. The tissues were homogenised individually with distilled water (4 ml g^{-1}; 15 g tissue in 60 ml) and centrifuged at 16,000 rpm for 20 min at 4°C. The supernatants (crude enzyme extract) were frozen and stored at -20°C in 20 ml aliquots for use in purification studies.

Enzyme estimation

α-Amylase activity was estimated using 1% starch solution in Tris-HCl buffer (0.1 M, pH 7.0) as the substrate. The assay mixture contained 0.05 ml crude enzyme extract plus 1.0 ml substrate and was incubated at 25°C for 1 h. The resulting reducing sugars were determined by the method of Nelson (1944) and Somogyi (1952) using glucose as the standard. Enzyme activity was expressed as µg glucose liberated per mg protein per hour. Protein in the crude enzyme extract and other enzyme fractions was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard. All assays were carried out in triplicate.

Acetone fractionation (AF)

The crude enzyme extract obtained was subjected to solvent fractionation by the addition of chilled acetone

(-20°C) at 100% saturation v/v, followed by centrifugation after 2 h at 16,000 rpm for 20 min at 4°C. The resulting supernatant was again treated with chilled acetone (300% saturation v/v) and stored at 4°C overnight, followed by centrifugation as above. The precipitate obtained in each step was suspended in chilled distilled water and subjected to amylase and protein assay as described previously. The fold purification and enzyme recovery were calculated in each case.

Ion exchange chromatography (IExC)

The amylase fraction obtained from the acetone fractionation step (equivalent to 16 mg protein) was layered on a DEAE Sephadex A-50 column (column length = 25 cm, r = 0.75 cm) and equilibrated with Tris-HCl (20 mm, pH 7.0). Flow rate was maintained at 2 ml min^{-1} and 5.0 ml fractions were collected by establishing a linear gradient of 100 ml of Tris-HCl (20 mM, pH 7.0) and 100 ml Tris-HCl (20 mM, pH 7.0) containing sodium chloride (1 mM). Active major enzymes fractions IExC 1 (4-9), were pooled (designated as partially purified amylase) and fold purification and enzyme recovery were calculated. The pooled fractions were dispensed into aliquots of 0.5 ml in 1% glycerol and stored at -20°C for use in characterisation studies. An aliquot of 25 µl from this pool was used in each characterisation study and assay was conducted as above in triplicate.

Characterisation of α-amylase (*EC 3.2.1.1*)

The partially purified amylase was incubated with substrate for 15-180 min and enzyme activity was estimated for optimising the time of incubation. Reaction mixture containing the partially purified amylase and substrate was incubated at temperatures ranging from 10-40°C for 60 min and enzyme activity was assayed for determining the optimum temperature of incubation. The partially purified amylase was exposed to temperatures ranging from 10-50°C for 10 min and residual activity estimated for determination of heat stability of the enzyme. The partially purified amylase was exposed to different temperatures of 25ºC, 30ºC, 37ºC, 40ºC, 50ºC and 60ºC for 15-120 min; cooled in an ice bath and thermostability was assayed for residual activity.

In order to determine the pH stability, amylase activity was assayed at pH levels from 2 to 12 using the following buffers: 0.1 M KCl-HCl for pH 2; 0.2 M glycine-HCl for pH 3; 0.1 M phosphate buffer for pH 4-7; 0.1 M Tris-HCl for pH 7.0-9.0 and 0.1 M glycine-NaOH for pH 10-12. The partially purified amylase was exposed to the buffers for 30 min and residual activity was estimated.

The partially purified amylase was incubated with solutions of the following metal ions: $HgCl_2$; $CaCl_2$; $CdCl_2$; $ZnSO_4$; $CoSO_4$; $FeSO_4$; $CuSO_4$; Li_2SO_4; $PbNO_3$; $MgSo_4$; $Bi(NO_3)_2$ and $Ag(NO_3)$ at concentrations of 0.1 mM and 1 mM for 30 min and then assayed for enzyme activity. Relative activity was calculated in relation to activity in enzymes not exposed to metal ions. The effect of inhibitory compounds on amylase activity was studied by incubating partially purified amylase in p-chloro mercuric benzoate (PCMB); Amylase inhibitor (Type-1) (wheat); 1, 10 Phenanthroline; Ethylene diamine tetra acetate (EDTA); Thiomersol; N-Ethylmaleimide and Phenylmethylsulphonyl fluoride (PMSF) at various concentrations, then assaying enzyme activity. Relative activity was calculated against activity in partially purified amylase not exposed to the inhibitors.

Using starch as the substrate at concentrations of 1-10%, the rate of hydrolysis was measured for 60 min in terms of increase in hydrolytic product.

Polyacrylamide gel electrophoresis (PAGE)

Partially purified fractions PPA [IExC - (4 - 9)] and crude enzyme extract of DT and L were subjected to native slab polyacrylamide gel electrophoresis (PAGE) at 7% gel concentration (Garfin, 1990). Fresh gel containing the enzyme was soaked in 2% starch solution in Tris-HCl buffer (pH 7.0) for 30 min, followed by staining with iodine solution (I/KI: 5 mM I_2 in 2% KI solution). Active amylase fractions appeared as colourless bands.

Molecular weight determination by non-denatured protein electrophoresis

Relative mobility (Rf) of partially purified enzymes and standard proteins such as α-lactalbumin (14.2 kDa), carbonic anhydrase (29 kDa); albumin (chicken egg white; 45 kDa), albumin (bovine serum) monomer (66 kDa) and dimer (132 kDa) were determined at gel concentrations of 5, 6, 8 and 10% by native PAGE (Davis, 1964). The ordinates of Rf values {100[Log (Rf x 100)]} were plotted against the % gel concentrations to obtain the retardation coefficient (Kr) as the slope of the plot for each standard protein. Logarithms of negative slopes were plotted against logarithms of standard molecular weights to obtain linear plots using the equation Y = 0.4628x - 0.0455. Molecular weights of the amylase isomers were determined using this plot, after determining the retardation coefficient as above.

Results

Purification

A two-fold purification with 87% recovery of amylase activity was obtained in the 300% acetone fraction. The fold purification increased to eight with 86% recovery (Table 1) by ion exchange chromatography (Fig. 1).

Table. 1. Purification of α-amylase from *L. rohita.*

Sample fraction	Protein (mg)	Total glucose liberated (µg)	Specific activity (µg mg^{-1})	Recovery %	Fold purification
CEE	197	1068853	5426	100	1
AF	64	931728	14641	87	3
AF*	16	232932	14641	87	3
PPA(4 - 9)	5	202650	42763	87	8

*Correspond to one run of the column (16 mg). AF- Acetone fraction; CEE- Crude enzyme extract; PPA- Partially purified amylase fraction by ion exchange chromatography.

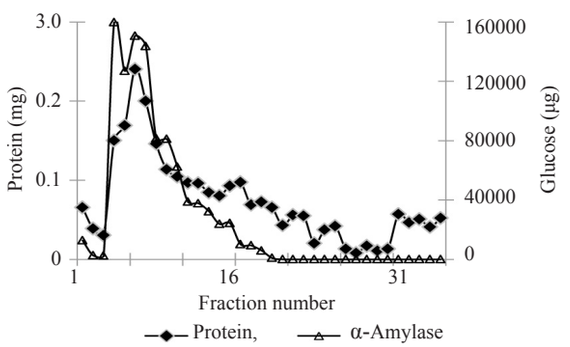

Fig. 1. Purification of α-amylases by ion exchange chromatography

Characterisation of α-amylase

The incubation time for optimum hydrolysis of the substrate was 60 min (Fig. 2) and the optimum incubation temperature was found to be 35°C. The partially purified amylase was heat stable up to 40°C and the loss of activity was observed to be minimal at 50ºC (Fig. 3). Thermostability for the amylases was recorded at 30ºC upto 120 min and beyond this temperature, the activity declined. The enzyme retained 75% activity at 40ºC upto 120 min, which decreased to 25% at 50ºC after 60 min. Loss in activity up to 91% at 60ºC was recorded within 15 min.

The partially purified amylases had two pH optima: sharp optima at 4.5 and 6.5 pH (Fig. 4) and were stable

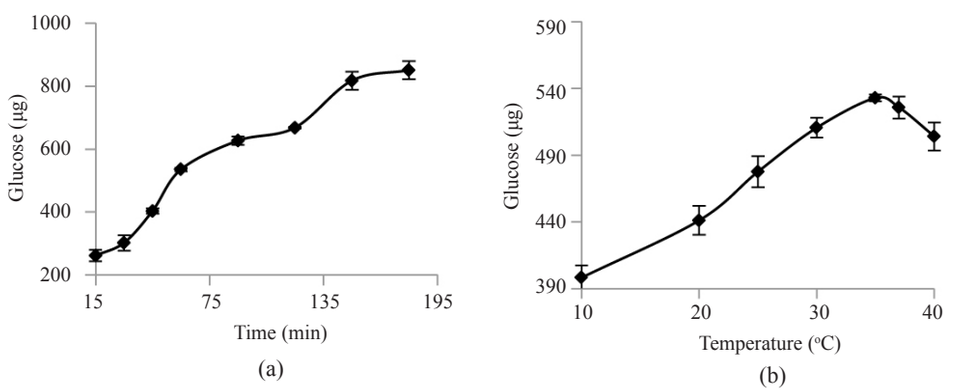

Fig. 2. Effect of (a) incubation time and (b) temperature on α-amylase activity

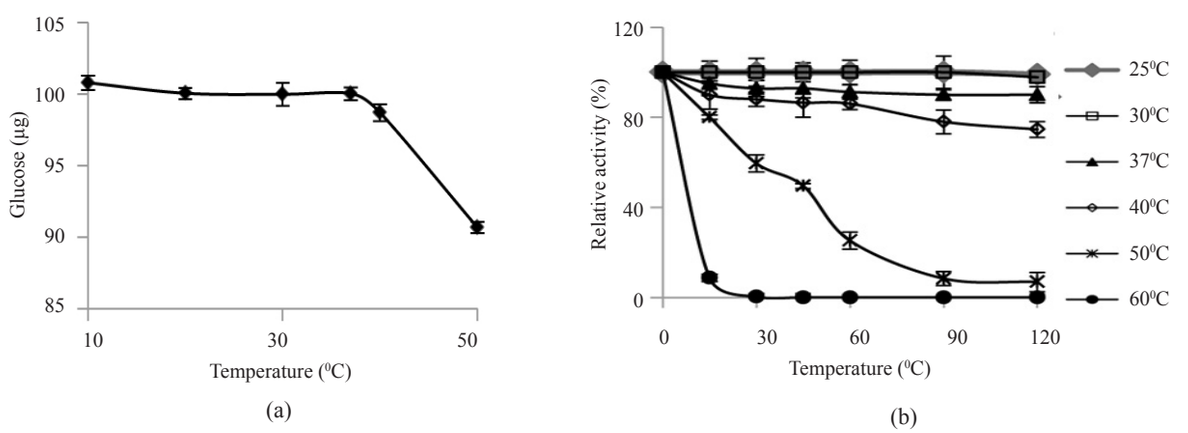

Fig. 3. Effect of (a) heat treatment and (b) thermostability on α-amylase activity

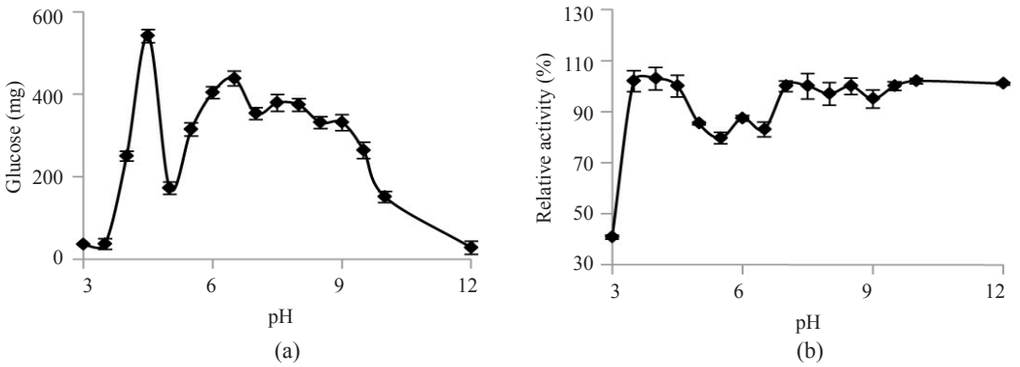

Fig. 4. Effect of (a) incubation pH and (b) pH stability on α-amylase activity

over a wide range of pH from 3.5 to 4.5 and 7.0 to 12. A minimal activity loss of 15% was recorded between pH 5.0 and 6.5 Starch hydrolysis was linear upto 7% starch concentration and attained a plateau thereafter (Fig. 5).

Heavy metal ions Hg^{++} and Cu^{++} strongly inhibited α-amylase activity to the extent of 52 and 93% respectively at 1 mM concentration, while Zn^{++} and Bi^{++} inhibited to an extent of 30% (Table 2). Amylase inhibitor Type-1 (wheat) inhibited amylase activity by 51% (Table 3).

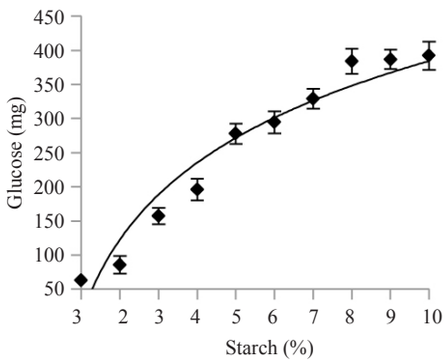

Fig. 5. Effect of starch concentration on α-amylase activity

Table 2. Relative activity (%) of metal ions on α-amylase activity

Concentration	Metal ion								
	Bi^{++}	Hg^{++}	Cu^{++}	Pb^{+}	Zn^{++}	Co^{++}	Ag^{+}	Mg^{++}	Li^{++}
1mM	68	48	7	88	70	89	99	97	93
0.1mM	100	100	82	100	94	99	100	100	97

All digestive tract fractions (purified and crude enzyme extract) had four amylase activity bands (Fig. 6) in native-PAGE, while crude enzyme extract from the liver had only two bands.

Retardation coefficients (Kr) of the standard proteins were 3.34; 4.45; 5.11; 7.22 and 11.21 respectively. Kr of the four amylase isozymes (AM1-4) were 5.71; 6.31; 6.51 and 6.42 and extrapolation of these values in the equation Y = 0.4628x - 0.0455, recorded molecular weights equal to 43.59; 52.36; 55.42 and 54.01 kDa, respectively.

Discussion

Carbohydrates, the most economical and the least expensive energy source in fish feed, not only function as a binder during feed manufacturing but also help to reduce the feed cost. However in nature, fish has limited access to carbohydrates (Wilson 1994). Besides dietary inclusion level, the efficiency of carbohydrate utilisation by fish has been associated to factors such as botanical origin, complexity of molecules and technological treatments applied (Wilson, 1994; Stone, 2003; Krogdahl *et al*., 2005). This study thus unravels the properties of amylases in *L. rohita.*

The amylases of the fish was partially purified by 8 folds with a recovery of 87% in this study. However, in *C. catla,* a predominantly cultured IMC species, the fold purification was very high (27) with an overall low recovery of 6.6% through Sephadex G-100 column

Table 3. Relative activity (%) of enzyme modulators on α-amylase activity

Concentration	Modulator						
	Amylase inhibitor	PCMB	EDTA	PMSF	Cys-HCl	1,10 Phenanthraline	Thiomersal
1mg ml^{-1}	49	---	---	---	---	---	---
10mM	---	---	92	---	---	---	---
5mM	---	---	---	92	---	---	---
1mM	---	102	---	---	101	86	96
0.1mM	---	102	100	--	100	93	98

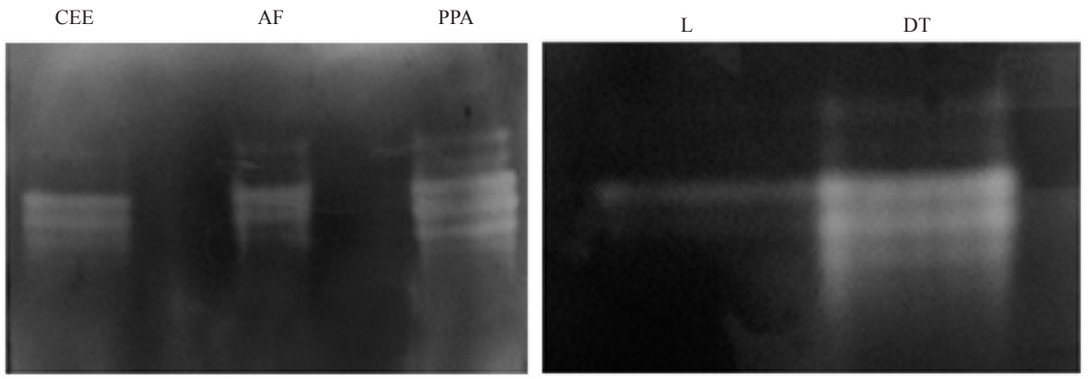

Fig. 6. Native PAGE gel showing amylase activities in substrate-stained (a) crude enzyme extract (CEE), acetone fraction (AF) and partially purified amylase (PPA); (b) Digestive tract (DT) and Liver (L).

(Roychan and Chaudhari, 2001). Kushwaha *et al.* (2012) purified amylase from a medium carp *Labeo fimbriatus* with 5 fold purification and 29% recovery by Sephadex G-75 column. Amylases from the intestinal cavity were also purified by 100 fold in two tilapia species, *Oreochromis niloticus* and *Sarotherodon melanotheron* (Moreau *et al.,* 2001). The eight fold purification and high recovery obtained in the present study inferred the predominant nature of the enzymes as reported in other omnivorous and herbivorous fishes (Philips, 1969; Hofer *et al*., 1982; Hidalgo *et al*., 1999; Kushwaha *et al.,* 2012). The remarkable difference in the fold purification and yield reported by different workers could be attributed to physiological and nutritional factors as well as source of biological samples taken for the extraction of crude enzyme extracts (Hidalgo *et al*., 1999; Roychan and Chaudhari, 2001; Kushwaha *et al.,* 2012).

The enzyme amylase exists as isozymes in fishes such as *Tilapia nilotica* (Yamada *et al*., 1991) and sparids (Fernandes *et al*., 2001). Castro *et al.* (2012) reported a difference in number of isoforms of amylase in different growth stages of *Penaeus (=Litopenaeus) vannamei.* In the present study, four isozymes of amylases ranging in molecular weight from 43 to 55.42 kda were demonstrated. Similar observations were made in *L. fimbriatus* with four bands corresponding to the molecular weights of 72; 68; 66 and 65kDa. Zymogram represents a useful tool for the analysis of difference in digestive enzymes among different species, allowing the identification of active enzyme forms (Castro *et al.,* 2012).

Two additional amylases observed in the intestine in this study were corroborated by the observations made in *L. fimbriatus* (Kushwaha *et al.,* 2012). The additional amylases observed could be of microbes inhabiting the intestine or synthesised in the intestine of the species (Kushwaha *et al*., 2012). The amylases produced by the pancreas and intestinal microflora had also been demonstrated by Sugita *et al*. (1997). The presence of isozymes is closely related to the ability of the fish to digest different kinds of foods, showing activity in a wide range of pH and temperature in addition to sensitivity to inhibitors (Natarajan *et al*., 1992) which may represent an ecological advantage (Fernandes *et al*., 2001).

The optimal temperature of incubation measured in this study for amylase was 35°C similar to the results obtained in *Clarias gariepinus* (Uys and Hecht, 1987). Temperature optimum ranging from 25 to 50°C and above had been reported in studies with different fish species (Morishita *et al.,* 1964; Moran and Rey, 1996; Hidalgo *et al*., 1999; Fernandez *et al*., 2001). The amylases of *L. rohita* lost 90% activity at 60**ºC** within 15 min and were stable at 40**ºC** exhibiting 75% activity upto 120 min. The thermostability pattern observed for amylase in *L. rohita* was similar to the result obtained in *L. fimbriatus* (Kushwaha *et al*., 2012). In contrast, the amylases of *Pagrus pagrus* and *Diplodus annularis* were observed to be highly resistant to temperature retaining 100% activity even after 90 min at 60ºC (Fernandez *et al.*, 2001). The above results confirmed *L. rohita* to be a eurythermic fish living in the environment having habitat temperature above 14°C (FAO, 2013).

The amylase isozymes in the study exhibited two pH optima at 4.5 and 6.5 and further observed to be stable over a wide range of pH from 3.5 to 4.5 and 6.5 to 12. This was supported by the observations made in *L. fimbriatus* (Kushwaha *et al.,* 2012). Optimum pH of 6.5 was also reported for amylases in other fishes (Takahashi, 1960; Ikeda and Kawai, 1966; Clark *et al.,* 1984; Glass *et al.,* 1987; Roychan and Chaudhari, 2001).

The highest activity in the neutral pH (7.0-7.5) region of intestinal and pyloric caecal amylase of *Sparus aurata* and *Scophthalmus maximus*; in the acidic pH (4.5 - 5) range in *Sebastus mentalla* (Munilla-moran and Saborido Rey, 1996) and at alkaline pH 8.5 for salmon (Ushiyama *et al*., 1965) and 7.5 for flat fish (Yasunsaga, 1972) shows that pH optimum of amylase in fish seems to be species dependent. Broad spectrum of pH for amylase activity as observed in our study was also observed by Parra *et al*. (2007) in Pacific blue fin tuna *Thunnus orientalis* with highest activity in the range 7-9, with loss of less than 40% activity in the pH range of 3-5.

Certain enzymes require non-protein chemical groups for catalytic activity. These groups should be organic complexes or metalorganic molecules (coenzymes) or simply an additional chemical component (cofactor) such as inorganic ions (Nelson and Cox, 2005). A number of metallic ions, such as Ca^{2+}, Ba^{2+}, Ag^{3+}, Mn^{2+}, Hg^{2+} and Cu^{++} are important as their presence or absence may regulate enzyme activity (Castro *et al.,* 2012). Uchida (1973) reported that α-amylase requires Ca^{2+} for their stability in chum salmon. Kushwaha *et al*. (2012) observed the activation of amylases of *L. fimbriatus* with 1 mM calcium chloride. In this study, enhancement of amylase activity by calcium chloride was not observed in the concentration tested attributing the activation due to metal ions to be species specific. Unlike amylases of *L. fimbriatus* which were inhibited by EDTA, the amylases of *L. rohita* were not inhibited, thus reiterating the negative role of metal ions. However, heavy metal ions Hg^{++} and Cu^{++} strongly inhibited amylases in this study at both the concentrations tested (0.1 and 1 mM), while Zn^{++} and Bi^{++} inhibited amylase activity to a lesser extent (30% and 11% respectively) at 0.1 mM concentration. Inhibition by copper sulphate has been reported in turbot and red fish (Moran and Rey, 1996). These studies are important as these ions are commonly included in the vitamin mineral mixtures supplemented in formulated feeds, or may be present as effluents or contaminants in the aquatic environment which could negatively influence food digestion, consequently reducing the dietary carbohydrate absorption, thereby affecting energy balance in fish (Castro *et al.,* 2012).

The variation in sensitivity to modulators by amylases from different species can be attributed to the difference in the molecular structure of amylases (isoforms). Amylases of *L. rohita* were found to be sensitive to amylase inhibitor from wheat with inhibition upto 50% which was supported by the observations made in many other fish species (Fernandez *et al*., 2001; Castro *et al*., 2012).

α-Amylase, a dominant enzyme in *L. rohita*, was purified 8-fold with 86% recovery with an optimum incubation temperature of 35°C. Acidic and neutral forms of amylases and their stability in a wide range of pH demonstrated the ability of the fish to utilise carbohydrate from different sources. The four isomers of amylases could be a gift of nature for functional diversity, to regulate the digestive physiology of the animal during variations in environmental conditions and food availability. Future investigations on deciphering the properties of each of the amylases individually might reveal their significance in this species and thus pave way for further understanding of their energy needs.

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