

A comparative study on partially purified amylase isozymes from three carp species - *Ctenopharyngodon idella*, *Cirrhinus mrigala* and *Hypselobarbus pulchellus*

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Abstract

Effective utilisation of starch from feeds by herbivorous fishes depends on the competency of amylases in their system. In this study, a comparative evaluation of partially purified α -amylases from economically important carp species such as grass carp (*Ctenopharyngodon idella*), mrigal (*Cirrhinus mrigala*) and *Hypselobarbus pulchellus* was undertaken along with their characterisation, to understand the nature of the enzyme and its isozymes with their molecular weight. The analysis of partially purified enzyme (PPE) revealed the presence of three (~64, 54 and 52.5 kDa), two (~65.5 and 55 kDa) and six (~79, 78, 66, 58, 49 and 43 kDa) isozymes for grass carp, mrigal and pulchellus respectively with optimum pH ranging between 4.5-7.5 and optimum temperature between 25-55°C. Amylases of three fish species were characterised to be metalloenzymes with serine and thiol groups at their active site.



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Introduction

Carbohydrates are the most economical component of fish feed, significantly reducing feed costs, which account for nearly 60% of aquaculture production expenses and ultimately determine the success of aquaculture operations (Hasan *et al.*, 2001). Amylases, the enzymes responsible for making carbohydrates, the primary energy source available to fish, are produced in the form of isozymes and their occurrence in fish has been well established (Yamada *et al.*, 1991; Yamada *et al.*, 1996; Kushwaha *et al.*, 2012; Budriang and Champasri, 2017; Champasri and Champasri, 2017; Umalatha *et al.*, 2020). They catalyse the same reaction *i.e.*, the hydrolysis of starch, the major carbohydrate in the fish feed (Hidalgo *et al.*, 1999; Chakrabarti and Rathore, 2010; Liu *et al.*, 2016; Gutierrez *et al.*, 2017; Maalej *et al.*, 2021) with a glucopolysaccharide with α -1,4 glucoside linkage (Saini *et al.*, 2017). Thus the biosynthesis of a digestive enzyme with same catalytic action in many

forms appear to be contrary to regulation of protein expression on need basis *viz.* Threonine biosynthesis (Galili 1995). Though well known for their protein-sparing action (Wilson 1994) only up to 20% of dietary carbohydrates can be utilised by fishes (Craig *et al.*, 2017). Furthermore, the herbivorous carps, the exotic grass carp (*Ctenopharyngodon idella*), mrigal (*Cirrhinus mrigala*), one of the Indian major carps (IMCs) and *Hypselobarbus pulchellus*, a medium carp are domesticated for their economic importance (Pillai and Katiha, 2004; Yusuf *et al.* 2017; Jayasankar 2018). They are weed feeders and used as weed controllers in many countries. Of the three, *H. pulchellus* is listed as a critically endangered species in IUCN threatened species list (<https://indiabiodiversity.org/species/show/232566>) which has been successfully induced bred (Sridhar *et al.*, 2014). Grass carps are surface feeders consuming submerged terrestrial vegetations and aquatic macrophytes (Ni and Wang, 1999); mrigala is a bottom feeder feeding on detritus and

phytoplankton (Jhingran and Khan, 1979), while *H. pulchellus* is a macrophyte feeder (David and Rahman, 1982). This study presents the comparative account of partially purified α -amylases from the digestive tract of the three species of fish, *C. idella*, *C. mrigala* and *H. pulchellus*, detailing their isozymes, molecular weights, starch splitting efficiency and their characterisation.

Materials and methods

Enzyme extracts

Fishes *C. idella* and *C. mrigala* were purchased afresh from the local market in Bangaluru, while live specimens of *H. pulchellus* (average 45 cm; 995 g) from culture ponds of the Regional Research Centre of the ICAR-Central Institute of Freshwater Aquaculture (ICAR-CIFA), Hesaraghatta, Bangaluru, were collected. The fishes were anaesthetised with clove oil (1 ml of clove oil in 10 ml of ethanol and added to 40 l of freshwater) and the digestive tracts (DT) of the specimens were dissected under ice-cold conditions, washed repeatedly with cold distilled water and homogenised individually in cold distilled water (4 ml g⁻¹). The homogenised mixture was centrifuged at 16,000 rpm for 20 min at 4°C. The supernatant was treated as a crude enzyme (CE) and stored in aliquots till further analysis at -20°C. Ethical guidelines of the Institute Animal Ethical Committee (IAEC), ICAR-CIFA, Bhubaneswar were followed throughout the study.

Partial purification of amylases from DT of carps

Partial purification was carried out on the pooled digestive CE by acetone fractionation at 100% saturation under chilled conditions. Acetone was precooled and added drop by drop and incubated for 2 h in refrigerated conditions. The mixture was centrifuged at 10,000 rpm at 4°C for 10 min and the precipitate dissolved in a minimum quantity of distilled water. The supernatant was further subjected to 250% saturation with chilled acetone and allowed to incubate at 4°C overnight. The mixture was centrifuged and processed as before.

All purification steps were carried out at 4°C. The enzyme fraction from 250% acetone fractionation was subjected to gel filtration chromatography using Sephadex G75 for *C. idella* and *C. mrigala* (column length = 38 cm, r = 0.5 cm) and Sephadex G-100 for *H. pulchellus*. The columns were equilibrated with approximately two-bed volumes of starting buffer (0.1M Tris HCl buffer-pH 7). The sample was then loaded onto the column and eluted with the same buffer at a flow rate of 0.1 ml min⁻¹. Fractions of 1 ml each were collected, active fractions were pooled, aliquoted in 1% glycerol and stored at -20°C until further use. The purified fractions (PF) from each case were assayed for their amylase activity. The specific activities of respective enzymes are estimated in the pooled fractions.

Bovine serum albumin was used as the standard in the estimation of proteins (Lowry *et al.*, 1951). The enzyme fractions were allowed to react with 1% (w/v) starch in 0.02 M phosphate buffer (pH 6.9) as substrate and maltose formed was taken up as a measure of the enzyme activity (Rick and Stegbauer, 1974). The specific activity of amylases was expressed as μ moles of maltose mg protein⁻¹ h⁻¹. Partially purified enzymes fractions from acetone fractionation (AF) and gel filtration (GF) steps along with CEE were subjected to native vertical polyacrylamide gel electrophoresis (PAGE) at 7% gel

concentration (Garfin 1990). The gels were stained and molecular weight determined (Kushwaha *et al.*, 2012).

Characterisation of amylases

PFs were subjected to a different time of incubation (15-180 min) with starch as substrate and amylase activity estimated as described earlier. PFs were incubated for an hour individually with starch as substrate at different temperatures (10, 15, 20, 25, 30, 35, 37 and 40°C) and the amylase activity estimated to determine the optimum temperature

The heat stability of the PFs was studied by exposing the enzymes to different temperatures for 10 min and their residual amylase activity was estimated using starch as substrate as described earlier. Buffers with different pH were prepared (0.1M KCl-HCl for pH 2; 0.2 M Glycine-HCl for pH 3.0,3.5 and 4.5; 0.2M Phosphate buffer for pH 5.0, 5.5, 6.5, 7.0 and 7.5; 0.1M Tris-HCl for pH 8.5, 9.0 and 9.5; 0.1 mM Glycine-NaOH for pH 10.0) and optimum incubation pH and pH stability of the PFs were determined. For determining the optimum incubation pH, PFs were incubated with starch as substrate prepared with buffers of different pH for an hour at 37°C and their amylase activity was determined. The pH stability of the amylases was estimated by incubating the PFs in different buffers without any substrate for 30 min. Their residual amylase activity was estimated as mentioned before.

PFs were also incubated with various metal ions (ZnSO₄, HgCl₂, AgNO₃, KCl, NaCl, MgSO₄, CoSO₄, MnCl₂, CaCl₂, FeSO₄) at concentrations of 10⁻³ and 10⁻⁴ M and the residual amylase activity was estimated as described above, to study the effect of metal ions on the enzyme activity. Effect of reducing agents, inhibitors and chelating compounds such as para chloro-mercuric benzoic acid (PCMB), cysteine hydrochloride (Cys.HCl), ethylene diamine tetra acetic acid (EDTA), ethyl maleimide, 1,10 phenanthroline, sodium meta bisulphite, thiomersal, phenylmethylsulphonyl fluoride (PMSF), mercaptoethanol, amylase inhibitor on amylase activity was studied by incubating the enzyme fractions with the activators and inhibitors to give a final concentration of 10⁻³ and 10⁻⁴ M and the residual amylase activity estimated.

Statistical analysis

The data obtained from 5 independent experiments were analysed using graph pad prism 9 and MS excel, 2019. Each value denotes the mean of five independent experiments done in triplicates. Two-way ANOVA Bonferroni's multiple comparison test) was used for data analysis, wherever applicable and p<0.05 was considered statistically significant.

Results and discussion

Partial purification

Acetone fractions (250%) from the DT of the three fishes were partially purified *via* gel filtration using Sephadex G 75 or G 100. Proteins were eluted and active fractions: 1-17 from *C. idella*, 4-21 from *C. mrigala* and 3-22 from *H. pulchellus* were pooled individually. The highest activity was observed in fractions 16,17; 15,16 and 17,18 for *C. idella*, *C. mrigala* and *H. pulchellus* respectively (Fig.1a-c). The

highest amylase activity was exhibited by *C. mrigala* and the lowest by *C. idella*. Acetone fractionation (250%) resulted in higher yield and fold purification in *H. pulchellus* in comparison to other carps. Recovery of amylase from *C. mrigala* and *C. idella* was almost equal through gel filtration. Purification fold increased to 9 times through gel filtration for *C. mrigala* and doubled for the other two carps in comparison to their acetone fractionation (Table 1).

Enzyme purification begins with the removal of non-enzymatic components, non-targeted enzymes and other contaminants that may interfere with the performance of the targeted enzyme. Therefore, purification aims to acquire substantial recovery and fold purification of the specific enzyme while conserving its functionality (<https://conductscience.com/enzyme-purification>). In this study, the partial purification of the amylases from the three species of carps has been achieved. High amylase activity exhibited by *C. mrigala*, was similar to that of *L. rohita* reported earlier (Umalatha *et al.*, 2020). Partially purified amylases from species under study exhibited fold purification in the following order: *C. idella* >

H. pulchellus > *C. mrigala*. Their recovery percentage from the CE indicated the labile nature of the amylases (Kushwaha *et al.*, 2012). Moreover, their recovery of nearly 50% and purification fold recorded above 5 was comparatively higher than those reported earlier from other carps *C. catla* and *L. fimbriatus* (Roychan and Chaudhari, 2001; Kushwaha *et al.*, 2012). However, seven-fold purification along with 75% recovery has been reported from the purification of amylases from pyloric caeca of salmon (Hiroshi Ushiyama *et al.*, 1965). Studies on amylases from the intestine of tilapia (*Tilapia nilotica*) reported very high recovery along with a high purification fold of >50 (Yamada *et al.*, 1991, 1996; Moreau *et al.*, 2001). Ion exchange chromatographic purification of amylases from DT of *L. rohita* resulted in 87% recovery and 8-fold purification of the enzyme (Umalatha *et al.*, 2020). The difference among the reported purification results could be attributed to many aspects including the nutritional influences, conditions and methodology adopted for purification including the feeding and rearing conditions apart from species variation concerning inherent physiological factors.

Table 1. Partial purification of α -amylases

Sample	Volume of sample (ml)	Protein (mg)	Specific activity (μ g of maltose mg protein ⁻¹)	Yield (%)	Fold purification
<i>C. idella</i>					
CE	50	731.4	1476.5	100	1.0
Ace 250%	9.5	96.2	3323.9	29.6	2.25
PF	50	39.1	18490.2	45	4.4
<i>C. mrigala</i>					
CE	25	85.7	6080.9	100	1
Ace 250%	4.5	17.1	8533.1	28.1	1.40
PF	26.5	11.8	95492.9	50.8	11.2
<i>H. pulchellus</i>					
CE	50	367.7	1994.2	100	1
Ace 250%	13	57.3	5542.383.1	43.3	2.78
PF	29	17.9	38325.4	45	6.9

*Ace- acetone fractionation

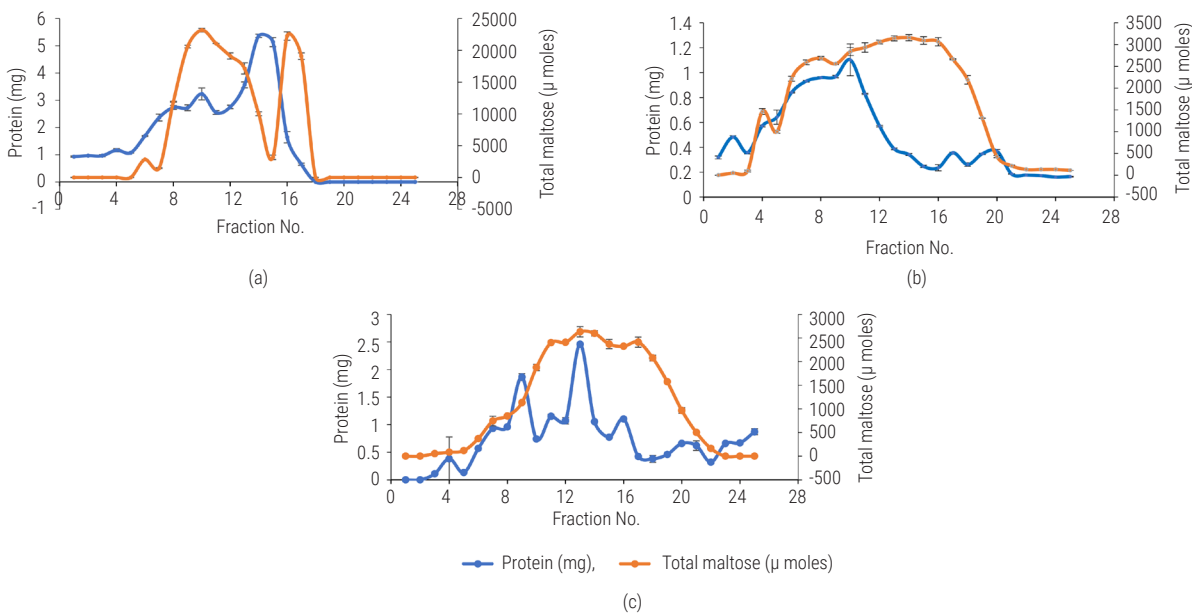


Fig. 1. Purification of α -amylases from (a) *C. idella*, (b) *C. mrigala* and (c) *H. pulchellus*

Substrate zymography

A substrate zymogram using 1% starch revealed the presence of ≤ 2 isoforms of amylases in the DT of fishes under study. Molecular weight ranged between 43-79 kDa for all PFs. *H. pulchellus* exhibited six isozymes of molecular weight ~79, 78, 66, 58, 49 and 43 kDa as compared to three isozymes of molecular weight ~64, 54 and 52.5 kDa and two isozymes of molecular weight ~65.5 and 55 kDa from grass carp and mrigal respectively (Fig. 2a).

These multiple forms, called isozymes or isoenzymes, may occur in the same species, in the same tissue, or even in the same cell. The isoforms of the enzyme commonly differ in their kinetic or regulatory properties/in the cofactor they use, or in their subcellular distribution either soluble or membrane-bound. Isozymes are variants of an enzyme that catalyse the same reactions not only vary in their molecular weight, electrophoretic mobility and charge due to different amino acid compositions but also in their activity potential (Lubert *et al.*, 2015). The occurrence of these forms may be in the same species/tissue/cell and may utilise different cofactors for their activity. The uniqueness of these isoforms is that they may possess similar amino acid sequences but are not identical and mostly share a common evolutionary origin (Nelson *et al.*, 2008). Amino acids synthesis is well regulated by feedback/product inhibition in all living systems. However, isozymes are produced only on requirements and their site of action. Isozymes of amylases have been reported earlier from many freshwater species including Tilapia, *L. fimbriatus*, *L. rohita* (Yamada *et al.*, 1991; Kushwaha *et al.*, 2012; Champasri and Champasri 2017; Umalatha *et al.*, 2020; Champasri *et al.*, 2021). It could be observed that molecular weight of the partially purified amylases of the three fishes in this study correlated well within the range reported from other freshwater species. In addition, the six isozymes of amylases of *H. pulchellus* were found to be unique in their number as compared to grass carp and mrigal in the current study and also to other freshwater species reported (Kushwaha *et al.*, 2012; Champasri and Champasri, 2017; Umalatha *et al.*, 2020; Champasri *et al.*, 2021). To further understand if these six amylases are only from fishes, liver amylases were subjected to substrate zymography and only four amylases were visualised on the page with molecular weight of ~79, 78, 59 and 49 kDa (Fig. 2b). These results indicate that extra amylases present in the DT of *H. pulchellus* must of bacterial origin and open the scope for the use of pre/probiotics to be used in the

feed for the promotion of the bacteria colonised in the gut. This may be an added advantage to the species for reaping the benefits of starch available from different sources and forms. Physically starch exhibits itself differently in different feeds, in rice bran, it is small and granular (Singh and Sogi, 2018) whereas in maize it appears chunkier (Premavalli and Devaki, 2012).

Characterisation of amylases

Time of incubation

The PFs in the study exhibited a different type of activity with different times of incubation (Fig. 3). Amylase activity increased with time of incubation till 90 min of incubation for *C. idella* and till 45 min of incubation for *H. pulchellus* after which the activity reached a plateau in both fish enzymes. Amylase activity from the PF of *C. mrigala* was linear with time and in comparison, higher than other PFs under study.

The time of incubation is evaluated to understand the rate of activity of the enzyme (Nelson *et al.*, 2008). The results from the time of incubation for *H. pulchellus* were similar to that reported by *L. fimbriatus* with a plateau after 45 min of incubation (Kushwaha *et al.*, 2012), however, *L. rohita* amylases reported 60 min as an

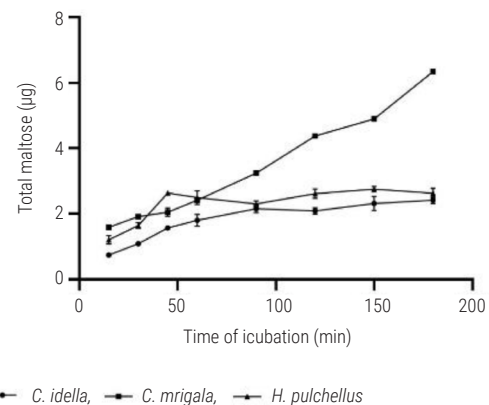


Fig. 3. Effect of time of incubation on α -amylases from *C. idella*, *C. mrigala* and *H. pulchellus*

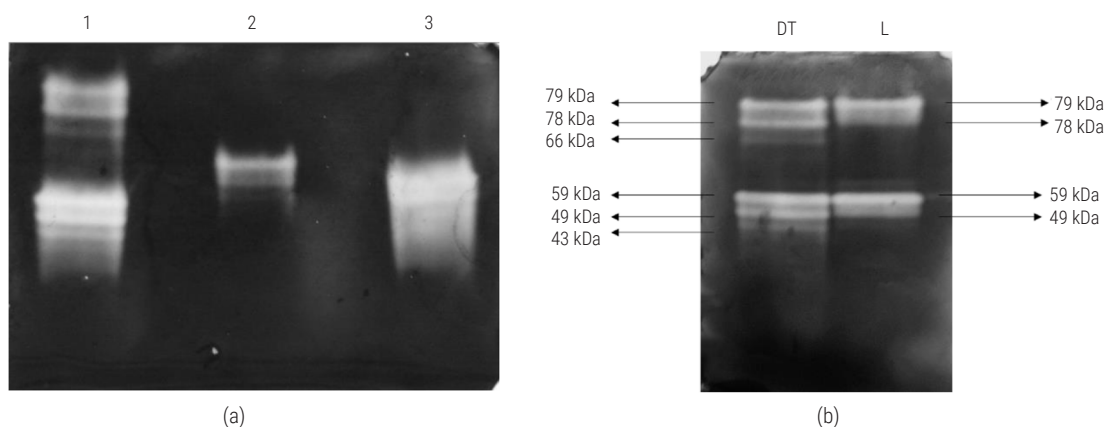


Fig. 2. Substrate zymography of amylases from (a) DT of carps; (b) liver amylases from *H. pulchellus*

optimum time of incubation (Umalatha *et al.* 2020). From the results of our work, it could be observed that the optimum time of incubation for exotic and major carp was higher in comparison to that of medium carp indicating the efficacy with which the given species of fish, maximise the availability and utility of starch.

Optimum temperature and heat stability

The optimum temperature for hydrolysis of starch was found to be 37 and 50°C for *C. idella*, 35°C for *C. mrigala*, 25 and 55°C for *H. pulchellus*. The amylases from *C. idella* and *C. mrigala* were active even at the low temperature of 10–15°C. The major carp exhibited a dip in activity at 37°C, however, the activity showed a linear increase from 40 to 60°C. The exotic carp exhibited optimum temperature at 37°C below and above which the activity was low. A sharp decline in activity was observed at 35°C, nevertheless, these amylases also showed a wide activity range. Amylases from *H. pulchellus* were not active below 25°C and exhibited the highest activity at 25°C. A sharp fall in activity was witnessed till 35°C, above which activity increased gradually to a maximum at 55°C (Fig. 4a) indicating that some of the forms of isozymes are viable at higher temperatures.

C. idella and *H. pulchellus* amylases were heat stable up to 37°C above which the loss of activity was gradual and at 60°C complete loss of activity was recorded. Amylases in *C. mrigala* were highly stable at 30°C above which a gradual reduction in activity was seen and at 60°C, 50% activity was recorded unlike its other counterparts (Fig. 4b).

The optimum temperature for amylases reported from freshwater fishes was in the range of 25°C–65°C (Yamada *et al.*, 1991, 1996; Sabapathy and Teo, 1992; Thongprajukaew *et al.*, 2010; Chaijaroen and Thongruang, 2016; Champasri and Champasri, 2017; Champasri *et al.*, 2021). *L. fimbriatus* amylases recorded 25°C to be the optimum temperature and 30°C to be the maximum temperature at which the enzyme was found to be stable for 10 min and lost 90% activity at 60°C (Kushwaha *et al.*, 2012). *L. rohita* digestive amylases exhibited a temperature optimum at 35°C with heat stability at 40°C (Umalatha *et al.*, 2020). This merged with the results recorded in *L. rohita* and *Anabas testudineus*, the fishes with different feeding habits (Debnath and Saikia, 2020). The optimum temperature and heat stability of amylases from fishes in the present study are comparable to those reported earlier by the researchers. *H. pulchellus* is a region-specific species and is found only in tropical climatic conditions which are reflected in

its temperature optimum and heat stability. The results indicate that the survival of this species would be restricted under cold conditions. However, amylases of Gangetic carps, *C. idella* and *C. mrigala* were active even at low temperatures providing evidence of their ability to survive in the cold habitat. Lethal temperatures for many fishes are above 40°C and hence an optimum temperature of $\geq 40^\circ\text{C}$ is not considered advantageous for fishes (Sabapathy and Teo, 1992). Considering this fact, the optimal temperature exhibited by the fish amylases in this work seems beneficial and merges with their tropical culture conditions.

Optimum pH and pH stability

The amylase activity of the fishes under study recorded two optimum pHs between 4.5 and 7.0 (Fig. 5a). pH stability was recorded at an alkaline pH of 8.5 to exhibit maximum amylase activity (Fig. 5b). Over 95% activity was retained at pH 9 for grass carp and at pH 10 for the other two species.

The optimal conditions of the assay for amylase activities vary with each fish species. The optimum pH is the characteristic pH at which the enzyme exhibits maximum activity (Lubert *et al.* 2015). The presence of more than one optimum pH correlates to the presence of isoforms of amylases in fishes. Similar results were presented from studies on amylases from freshwater fishes: *C. catla*, *L. fimbriatus*, *B. splendens*, Nile tilapia, *L. rohita* (Klahan *et al.*, 2009; Thongprajukaew *et al.*, 2010; Kushwaha *et al.*, 2012; Chaijaroen and Thongruang, 2016; Umalatha *et al.*, 2020). A sharp rise in activity at acidic pH may be attributed to the presence of extracellular amylase-producing bacteria in these carps (possibility for pre and probiotic agents in the feed). Such production of amylases by intestinal bacteria has been reported in freshwater fishes (Kar *et al.*, 2008; Mondal *et al.*, 2008; Kushwaha *et al.*, 2012; Umalatha *et al.*, 2020). The neutral optimum pH of amylases from other freshwater fishes was comparable to that from the current findings. Rabbitfish, tilapia, catla, fimbriatus, Siamese fighting fish, rohu and testudineus were all found to possess neutral or near neutral pH optima (Yamada *et al.*, 1991; Sabapathy and Teo, 1992; Yamada *et al.*, 1996; Roychan and Chaudhari, 2001; Thongprajukaew *et al.*, 2010; Kushwaha *et al.*, 2012; Umalatha *et al.*, 2020). However, reports on cichlids and cyprinids have presented stability over a wide range of pH in both acidic and basic pH (Yamada *et al.*, 1991; Yamada *et al.*, 1996; Kushwaha *et al.*, 2012; Umalatha *et al.*, 2020). Variation in the optimal pHs and stability of amylases under the study would enable

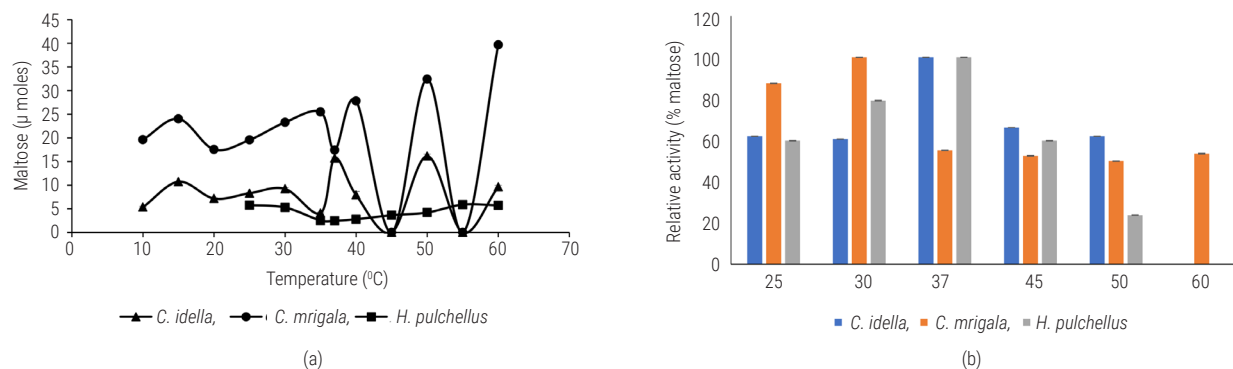


Fig. 4. Effect of (a) temperature and (b) heat treatment on α -amylases from carps; *C. idella*, *C. mrigala* and *H. pulchellus*

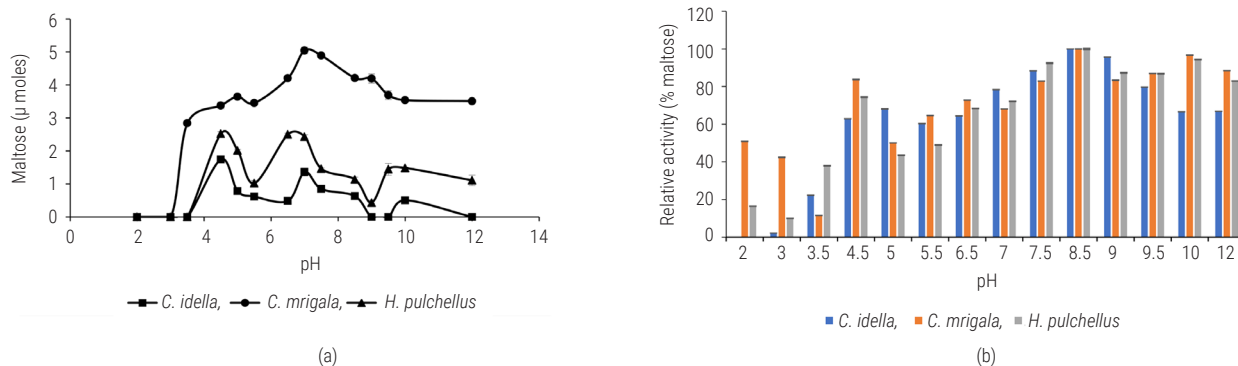


Fig. 5. Effect of (a) pH and (b) pH stability on α -amylases from carps: *C. idella*, *C. mrigala* and *H. pulchellus*

their ability to derive energy from starch or feed at all environmental variations in pHs either acidic or groundwater conditions which generally possess neutral or near neutral pH.

Effect of metal ions

Metal ions that are normally encountered in the aquatic environment influence the activity of amylases of the three species to a varied extent in their two optimum pHs (pH 4.5 and 7). At 0.1 and 1 mM concentrations, amylases of *H. pulchellus* were activated by Ca^{2+} and K at both pHs, unlike Na and Mn^{2+} which had no impact. Heavy metals such as Hg^{2+} and Ag inhibited at 1 mM concentration whereas Zn, Co^{2+} and Fe^{2+} ions had no impact at both concentrations on the amylases of this species at both pHs. At 0.1 and 1 mM concentrations, Na, K, Ca^{2+} , Mg and Mn^{2+} ions in addition to Co^{2+} and Fe^{2+} ions had no influence on *C. mrigala* amylases at both pHs. However, Zn, Hg and Ag ions inhibited the amylases at both concentrations and pH. Amylases of *C. idella* exhibited only inhibitions than activation by most of the metal ions tested in comparison to the former two species, unlike Ca^{2+} ions that enhanced the activity only at pH 4.5 (Table 2).

The characteristic feature of α -amylase is that it is a calcium metalloenzyme (Kumari *et al.*, 2019). The observations from the results indicate Ca^{2+} ions activate amylase at pH 4.5 and 7 in both low and high concentrations for the carps indicating the type of amylase to be α -amylase. Calcium is required for the stability of the secondary and tertiary structure of the enzyme and Cl ions increase the activity (Wigglesworth and Griffith, 1994). Ca^{2+} ions used in this work were in form of CaCl_2 , hence the increase in activity is justified. Activation of amylase activity by CaCl_2 has been reported from amylases of other freshwater species like tilapia, *L. fimbriatus*, *C. catla* (Yamada *et al.*, 1991; Yamada *et al.*, 1996; Roychan and Chaudhari, 2001; Kushwaha *et al.*, 2012). However, amylases from *L. rohita* were not activated by calcium (Umalatha *et al.*, 2020). Two purified amylases from tilapia were activated by Mg, K along with other metal ions similar to the results of *H. pulchellus* from this study (Yamada *et al.*, 1991; Yamada *et al.*, 1996). Though *C. mrigala* belongs to IMC it exhibits more tolerance to metal ions than that reported for *L. rohita* (Umalatha *et al.*, 2020) and *C. catla* (Roychan and Chaudhari, 2001). An earlier study on mrigal to assess the impact of sodium chloride on the digestive enzymes revealed that sodium did not impact the amylases which were in agreement with our results for the same species (Keshavanath *et al.*,

2003). Besides, the observations reveal that *H. pulchellus* amylases belonging to medium carps is more tolerant to heavy metal ions than to other carps in this study. This characteristic feature may be advantageous for the production management of this fish even in hard water conditions in comparison to the other two.

Effect of modulators and inhibitors

The effect of modulators on the partially purified amylases was assayed at their optimum pHs 4.5 and 7 (Table 3). PMSF (5 mM), PCMB (0.1 and 1 mM) and 1,10 phenanthroline (1 mM) inhibited the amylases of the three fishes at both pHs. Amylase inhibitor (1 mg 10 ml⁻¹) inhibited the activity of amylases in the three fishes at pH 4.5. However, activation of amylases of *H. pulchellus* was recorded at pH 7. At 0.1 mM concentration EDTA inhibited *C. mrigala* and *C. idella* amylases at pH 7 and pH 4.5 respectively and did not affect the activity of *H. pulchellus* amylases at both pHs. Activation by EDTA at higher concentrations (1 and 10 mM) was also observed for *C. mrigala* (pH 7) and *C. idella* (pH 4.5) amylases. Mercaptoethanol at 0.1 and 1 mM concentrations inhibited the amylases of three species at pH 4.5. However, activation was recorded for *C. idella* and *H. pulchellus* at pH 7. Thiomersal at 0.1 mM and 1 mM concentrations inhibited amylases of *H. pulchellus* at pH 4.5 whereas at 1 mM concentration inhibited *C. idella* and *C. mrigala* amylases at both pHs. Sodium metabisulphite positively impacted *H. pulchellus* amylases only at 1 mM concentration. At 0.1 and 1 mM concentrations Cys. HCl positively impacted the amylases of *C. idella* at pH 4.5.

The effect of activators and inhibitors of an enzyme is assessed to understand the presence of a particular amino acid in the active site of the enzyme under study (Sethi *et al.*, 2016). It is quizzing to observe that at pH 4.5 the amylases from the fishes under study show more inhibitory reactions than at neutral pH. Inhibition of amylases by PMSF and mercaptoethanol confirms the presence of serine in their active site. These results of complete inhibition at neutral pH for PMSF correlate with amylases from *L. fimbriatus* and tilapia (Yamada *et al.*, 1991; Kushwaha *et al.*, 2012). Inhibition by thiomersal and PCMB suggests the presence of thiol group/ cysteine in the active site of the amylases of all fishes which correlated with similar results from the amylases of fimbriatus and tilapia (Yamada *et al.*, 1991, Kushwaha *et al.*, 2012). The presence of different groups in the active site may be due to two or more isoforms of amylases present in each species. Freshwater fishes

Table 2. Effect of metal ions on the enzyme activity

Metal ions	Concentration (mM)	Relative activity (% maltose)					
		<i>C. idella</i> (mean±SD)		<i>C. mrigala</i> (mean±SD)		<i>H. pulchellus</i> (mean±SD)	
		pH 4.5	pH 7	pH 4.5	pH 7	pH 4.5	pH 7
ZnSO ₄	1	17.89±5.78	74.53±2.5	46.01±3.92	39.62±0.69	93.19±4.08	86.22±6.38
	0.1	8.04±4.42	75.57±5.27	34.48±2.25	48.09±0.22	115.18±5.08	89.93±6.42
HgCl ₂	1	18.73±3.95	10.77±1.33	42.02±4.85	53.28±3.72	2.13±1.60	5.78±2.55
	0.1	35.53±6.36	54.24±5.00	42.57±7.67	77.33±4.45	122.13±6.10	85.48±2.66
AgNO ₃	1	37.22±8.05	10.77±0.5	47.89±5.81	23.20±2.02	18.16±0.74	10.89±0.53
	0.1	59.30±11.4	82.09±3.38	46.56±3.42	47.99±0.24	117.31±11.9	98.67±4.36
KCl	1	76.35±10.2	70.39±1.74	157.98±15.3	97.99±2.32	115.75±4.61	129.93±3.48
	0.1	81.87±14.5	75.05±3.25	104.66±7.43	100.32±2.22	125.53±8.1	104.59±2.99
NaCl	1	86.8±14.96	56.63±0.47	109.98±8.14	85.81±1.92	85.11±7.37	94.67±2.71
	0.1	72.75±10.9	68.74±3.45	109.76±8.06	78.5±0.81	91.77±5.2	99.70±1.36
MgSO ₄	1	51.98±8.56	61.18±4.89	109.42±9.08	87.08±2.47	77.45±7.18	74.67±1.406
	0.1	65.31±11.0	78.99±1.24	102.44±10.4	103.92±0.5	84.11±11.31	92.89±2.372
CoSO ₄	1	32.17±5.73	90.99±1.8	81.82±6.01	78.71±12.7	102.13±7.86	98.67±1.81
	0.1	39.26±8.11	99.07±0.50	107.32±7.83	99.68±4.21	120.43±8.09	88.82±1.93
MnCl ₂	1	46.94±7.59	107.56±7.4	118.74±8.52	111.44±3.32	100±8.06	120.44±2.40
	0.1	46.82±7.06	116.87±6.9	83.04±6.05	115.36±4.14	114.89±4.55	113.93±0.61
CaCl ₂	1	131.9±24.3	97.62±2.3	93.90±6.74	113.45±3.4	96.60±6.77	124.67±1.13
	0.1	93.27±19.8	115.42±2.9	85.58±3.66	127.01±4.79	107.09±2.14	111.78±4.01
FeSO ₄	1	36.74±10.8	103.21±6.0	79.82±5.85	68.54±1.27	94.33±9.58	103.93±0.45
	0.1	93.27±19.8	109.31±8.4	93.90±7.36	91.21±1.15	92.19±16.78	110.81±4.10

Table 3. Effect of activators and inhibitors

Activators/ inhibitors	Concentration (mM)	Relative activity (% maltose)					
		<i>C. idella</i> (mean±SD)		<i>C. mrigala</i> (mean±SD)		<i>H. pulchellus</i> (mean±SD)	
		pH 4.5	pH 7	pH 4.5	pH 7	pH 4.5	pH 7
PCMB	1	19.1±1.18	0±0	37.70±1.28	0±0	77.49±7.16	2.71±3.43
	0.1	44.68±0.83	15.84±2.23	0.78±0.67	34.03±4.25	79.31±3.32	39.18±2.08
Cys.HCl	1	154.72±3.14	87.59±1.36	84.41±4.29	87.10±2.77	112.04±9.57	85.52±37.44
	0.1	123.20±5.63	95.76±3.17	104.65±6.25	91.12±12.19	118.73±2.62	110.86±1.51
EDTA	10	133.74±10.7	80.92±0.05	113.28±2.70	152.00±2.47	90.02±2.56	85.52±20.25
	1	102.17±2.40	91.82±1.94	104.34±6.96	139.53±2.37	94.64±4.25	101.98±21.88
	0.1	46.610±2.46	96.77±1.82	84.36±5.67	33.40±0.05	96.95±0.16	113.84±15.92
Ethyl maleimide	1	116.42±7.25	111.20±3.35	120.76±5.38	153.48±0.56	94.76±10.55	131.60±22.86
	0.1	115.78±5.08	113.82±2.35	118.46±2.81	134.25±3.52	134.42±1.44	139.86±9.32
1,10 Phenanthroline	1	20.48±2.03	0±0	57.21±3.33	53.06±4.61	69.70±2.22	68.96±49.53
	0.1	133.30±6.70	124.02±37.8	80.07±1.94	139.95±0.66	108.27±3.99	101.77±54.68
Sodium meta bisulphite	1	94.50±1.63	107.06±11.8	117.52±2.82	83.72±5.89	126.52±3.36	126.75±12.36
	0.1	101.18±2.03	67.10±55.38	106.01±3.0	110.99±6.69	114.11±1.3	119.95±9.43
Thiomersal	1	69.67±3.93	88.09±4.09	51.46±1.40	33.82±2.13	50.12±42.05	115.56±11.47
	0.1	100.30±6.5	96.27±4.52	92.52±4.11	117.54±2.87	53.64±1.39	126.38±18.00
PMSF	5	39.33±1.58	0±0	3.53±2.84	24.52±3.50	100.12±5.69	27.42±1.06
Mercaptoethanol	1	9.95±0.66	134.81±4.02	34.80±2.53	87.73±7.10	59.73±1.57	156.42±4.53
	0.1	23.35±0.48	142.28±3.3	40.85±4.53	96.40±4.61	54.74±1.59	162.06±19.41
Amylase inhibitor	0.1%	37.85±1.61	91.52±0.74	0±0	103.38±5.9	55.83±1.91	144.67±17.04

(carps and tilapia) exhibiting retarded inhibition / enhanced activity to amylase inhibitor has been reported by Natarajan *et al.* (1992). The report suggested the presence of contaminants in the enzyme fraction such as active proteases or maltose from fish diet to cause such a reaction (Natarajan *et al.*, 1992). These factors may attribute to the varied effects recorded by the amylases of the three carps at neutral pH in this study. A potent divalent metal chelating agent, 1,10 phenanthroline, chelates with metal ions that are responsible for the activation of amylases suggesting the amylases under the study to be metalloenzymes (Regonesi *et al.*, 2013). However, the mixed response was elucidated by the amylases of three fishes when treated with EDTA, another metal-chelating agent. Activation of amylases at higher concentrations can be correlated to the fact that EDTA at higher concentrations chelates with divalent metal ions such as zinc and magnesium (Blanco and Blanco, 2017). This chelation prevents the availability of such metal ions to some of the isoforms of amylases thereby activating them. The low or no sensitivity to EDTA in *L. rohita* (Umalatha *et al.*, 2020) was comparable to our results. On the contrary, amylases of tilapia and fimbriatus were inhibited by EDTA (Yamada *et al.*, 1991, Kushwaha *et al.*, 2012). The difference in the sensitivity to inhibitors may be due to the presence of major structural modifications of amylases in different fish species or due to the isoforms of amylases in each of the fish (Fernandez *et al.*, 2001). The presence of isoforms of amylases proves to be an ecological advantage as they augment a wide range of activity in different pH, temperature and their sensitivity towards inhibitors that may be prevalent in the different environmental conditions in which the fishes are cultured.

Physical forms of starch present in carbohydrate source varies and thus its maximum utilization by herbivores require an efficient enzyme system. The results of this study shed light on the efficacy of amylases present in the carp to digest the starch from all the available conventional sources. The presence of multiple isoforms of amylases possessing broad pH and temperature ranges along with resistance to heavy metal ions provides a path forward approach in the production management of these species in the aquaculture system. Their efficiency in the hydrolysis of starch from different sources revealed the significance of their presence in more than one form. Future studies on the isolation of individual amylases, quantification, characterisation and their hydrolytic ability of different carbohydrate sources may describe their presence in the species cultured and also be of commercial importance to the aquaculture system.

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