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Characterization of amylase and protease activity in the digestive tract of two teleosts (*Labeo rohita* and *Anabas testudineus*) with different feeding habits

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ABSTRACT   Two teleosts (Rohu, *Labeo rohita* and Koi, *Anabas testudineus*), both with contrasting feeding habits (herbivorous versus carnivorous) were studied for amylase and protease activity concerning different regions of their digestive tracts. Significant differences in enzymatic activity across different regions of the digestive tracts were observed. Rohu, with three equal regions of the stomachless gut, showed the highest amylolytic activity at the posterior digestive tract but the highest proteolytic activity is limited to mid region. Contrary to such observation, Koi with three distinct regions of the digestive tract (stomach, pyloric caeca and intestine), the pyloric caeca exhibited the highest specific activity for both amylase and total protease. The optimum pH and temperature conditions were determined concerning the activity for both amylase and protease.   **Acta Biol Szeged 64(2):173-179 (2020)**

Introduction

Like other vertebrates, the activity of digestive enzymes in fish is influenced by the abiotic factors, such as temperature (Kuz'mina 1996), particular timing of day (Kuz'mina and Strelnikova 2008) and different seasons (Kofuji et al. 2005), and biotic factors, including the preference of food (Ugolev and Kuz'mina 1993), the age of the organism (Kuz'mina 1996), state of infestation with parasites (Izvekova and Solovyev 2012), etc. Hence, careful investigation of the pattern of the digestive activity of different enzyme would provide not only the complete information of the digestive physiology of fish, but also the age, health and feeding ecology of the organism. The α-amylase and protease were one of the major digestive enzymes found in digestive tract (DT) of fish, which are widely studied for the above purpose.

In India and rest of South-East Asia, Rohu (*Labeo rohita*) has been the most preferred cultivable fish over many other freshwater species and extensively cultivated in India, Nepal, Bangladesh, Myanmar, Sri Lanka and Pakistan (Talwar and Jhingran 1991). This Indian Major Carp (Rohu), has always been receiving special attention as a potential animal crop over centuries for alleviating malnutrition in human populations because of its capabil-

ity to retain high amounts of vitamins and other micro-nutrients at a reasonable cost (Mohanty et al. 2016). On the other hand, the cultivation of climbing perch (*Anabas testudineus*), locally known as Koi, is gaining importance as one of the most potential candidates for aquaculture because of its high market demand and greater consumer preference throughout all the seasons (Uddin et al. 2016). It is a good source of protein, fat, vitamins, amino acids and fatty acids (Bogard et al. 2015; Paul et al. 2017). Being an air-breathing fish, Koi is also a model of interest for several scientific studies for its low maintenance effort, as well as easy availability and hardy nature (e.g., Munshi et al. 2018). Wide spreading aquaculture of Koi has been a great demand among the entire Indian sub-continent including Bangladesh, Myanmar, and Indonesia.

Voluminous research works have been carried out on the growth and feeding biology of Indian Major Carps (see review Majumder and Saikia 2020). But, except few (e.g., Mandal and Ghosh 2010; Singh et al. 2018), these studies have not addressed the details of the digestive physiology, particularly the activity of the crucial digestive enzymes along the digestive tract. These studies considered the whole intestine as an overall source of activity of a particular enzyme. As a result the specific region wise performance of an enzyme remains shadowed. Similarly, studies on Koi with regard to the effects

of toxicants on the digestive system were performed (Samanta et al. 2014; Kole et al. 2017). Banerjee and Ray (2018) reported the seasonal variation in the activity of digestive enzymes of Koi. However, these studies are not elaborate since these studies treated whole gut as site of activity of a particular enzyme. Of late, more precise explanation of digestive enzyme activity across the gut length of fish has been started (e.g., Weinrauch et al. 2019). More precise observation of the process of the activity of digestive enzymes will provide supportive information to improvise diet formulations for optimum supplementary feeding. In the present study, an attempt has been made to characterize region wise enzyme activities keeping in mind the optimum pH and temperature. This study establishes the optimum pH and temperature for amylase and protease activity in the tissue extracts of digestive tract from Rohu and Koi. The feeding habits of both the fishes were compared keeping in view of the activity of enzymes across different regions of digestive tract.

Material and methods

Experimental animals

A total of 20 fish were collected from local fish pond (23°37'N, 87°49'E) during February, 2020 using gill net (08:00-09:00) and brought alive to the laboratory. In laboratory fishes were kept unfed in glass aquaria (45 x 30 x 30 cm, 30 L) for 24 hours before analysis. Throughout the experiment, the water temperature was 28.67 ± 1.87 °C; dissolved oxygen 7.61 ± 0.26 mg/L; pH 7.48 ± 0.21; conductivity 623 ± 15.2 μS; photoperiod 12 :12 (light hour : dark hour).

Preparation of extracts

On the second day of collection, a small amount of food was introduced to stimulate the digestive function in the two fish species. After one hour, fish were euthanized with the help of ice-cold water and dissected on ice board. The DT was dissected out from esophagus to anus, washed in chilled distilled water and blotted with paper towel. For Rohu, the DT was divided lengthwise into three equal segments viz. anterior, middle and posterior starting from the esophagus to anus. Like Rohu, the short DT of Koi was also divided into three parts based on the morphological difference and identified as the stomach, pyloric caeca and intestine. Tissues were initially stored in -40 °C until use. Later, 10% tissue homogenate were prepared at 4 °C in a buffer containing 100 mM Tris-HCl, pH 7.4, centrifuged at 10 000 g at 4 °C for 10 min, and the supernatants (or the DT extracts) were collected carefully.

Table 1. Activity of amylase and protease in different sections of the digestive tract of *L. rohita* (n = 10). Values are expressed as mean ± SE. Means with different alphabets within a group show statistically significant difference at $p < 0.05$.

Digestive tract (DT)	Specific activity (U/mg protein)	
	Amylase	Total protease
Anterior DT	0.400 ± 0.005[a]	0.316 ± 0.005[c]
Mid DT	0.246 ± 0.004[b]	0.523 ± 0.016[a]
Posterior DT	0.182 ± 0.022[c]	0.483 ± 0.012[b]

Estimation of digestive enzymes

Amylase

The activity of amylase was measured following the method of Bernfeld (1955) using potato-starch as the substrate. The specific activity was expressed as the measure of unit activity per μg protein, where one-unit activity was the amount of enzyme required to liberate 1 μg of maltose per hour form the assay mixture.

Protease

Casein was used as substrate to measure the activity of protease according to Walter (1984). One unit of enzyme activity in each sample was expressed as the amount of enzyme required to liberate 1 μg of tyrosine in one hour per μg protein. Method suggested by Lowry et al. (1951) was followed to estimate the amount of total protein in the DT extracts.

Optimal range of pH and temperature

Firstly, the individual optimum pH for each type of enzyme from different region of DT was determined spectrophotometrically using a range of buffer solutions (pH 1.0 to 10.0) as the assay medium. The buffers used were as follows: 0.2 M KCl-HCl buffer (pH 1.0 and 2.0), 0.2 M glycine-HCl buffer (pH 3.0), 0.1 M citrate buffer (pH 4.0, 5.0, 6.0), 0.2 M Tris-HCl buffer (pH 7.0, 8.0, 9.0), 0.2 M glycine-NaOH (pH 10.0), respectively. Finally, the optimal temperatures for the same digestive enzymes were determined by measuring their activity at several temperatures starting from 20 °C to 45 °C with 5 °C interval where, the pH condition was kept constant to the previously determined optimum level. The spectrophotometric enzyme activity assays were performed in triplicate.

Data analysis

Data from the replicates for digestive enzymes were combined for statistical analysis. One-way ANOVA was performed and multiple comparisons between mean values were made using Tukey's post hoc test. The alpha level

was maintained less than 0.05 for all statistical analysis. Results are reported as mean values with SD. Minitab 18 was used for all statistical analysis.

Results

### *Measurement and distribution of digestive enzymes*

The activity of amylase and protease from different parts of DT in Rohu is summarized in Table 1. Amylase activity in the anterior DT was much higher than the rest. The mid DT showed significantly less amylolytic activity than the anterior part (p < 0.05) but, had higher activity when compared to the posterior region. On the other hand, in the mid DT, the protease activity was much higher than that in other parts. Levels of protease activity were in descending order in the DT were as follows: mid DT, posterior DT, and anterior DT. The distribution of the activity of amylase and protease in different digestive locations throughout the DT in Koi are summarized in Table 2. The pyloric caeca showed the maximum amylase activity followed by the intestine and stomach. Also, for proteolytic activity, pyloric caeca showed the dominant portion of the DT. There are no significant (p < 0.05) difference between the total digestive protease activity in stomach and intestine.

Characteristics of amylase and protease

The results showed that the optimum pH value for amylase activity was 8.0 in both the anterior and mid DT in Rohu (Fig. 1a). On the other hand, the last segment of the DT showed an optimum pH of 7.0 for the amylolytic activity. The optimal temperature for amylase activity was 35 °C throughout the DT (Fig. 1b).

Proteolytic enzyme showed optimum activity at pH 8.0 in both anterior and middle region of the DT in Rohu (Fig. 1c). However, the posterior region of the DT showed the highest proteolytic activity at pH 7. The optimum temperature for proteases, found in the DT of Rohu, to achieve the maximum activity was 35 °C (Fig. 1d)

In Koi, the optimum pH value, at which the amylase
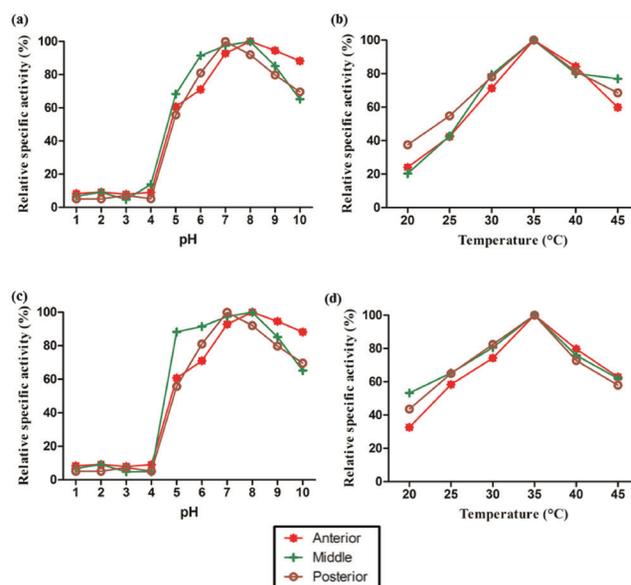
Figure 1. Effect of pH and temperature on the activity of amylase and protease of *L. rohita*. Fig. 1a and 1b represent effect of pH and temperature on relative specific activity of digestive amylase respectively. Fig. 1c and 1d represent effect of pH and temperature on relative activity of total protease, respectively. Enzyme activity was expressed as relative specific activity (RSA). RSA% = (Z_i/Z_{max}) x 100 [Z_i = enzyme activity at specific pH or temperature value; Z_{max} = maximum enzyme activity]. In all cases, n = 10.

activity peaked the highest value was 3 in both stomach and pyloric caeca (Fig. 2a). In intestine, the highest activity of amylase was found at pH 8.0. Protease activity was at its peak in stomach at pH 2.0. In both pyloric caeca and intestine, pH 7.0 was recorded as optimum value for highest activity (Fig. 2c). The optimum temperature for both amylase and protease activity were 35 °C (Fig. 1b, 1d) throughout all regions of DT.

Discussion and conclusions

Biochemical studies of the digestive enzymes reflect the dietary specializations of the respective organism (Day et al. 2011). The present study aimed to study the ability to digest dietary carbohydrate and protein by two freshwater teleosts with different feeding habits and topologically dissimilar DTs. It also aimed to establish the optimum pH and temperature for the studied digestive enzymes along different parts of their DT. In the current study, the highest amylase activity was reported in the anterior DT for Rohu, and the activity was apparently twice than the mid and posterior DT. Here pancreatic amylase, which is found in association to hepatopancreas act as source of higher activity of amylase. It is known as the primary glucosidase available in fish (Candiotto et al. 2018). Fishes generally lack the salivary amylase prevalent

Table 2. Activity of amylase and protease in different sections of the digestive tract of *A. testudineus* (n = 10). Values are expressed as mean ± SD. Means with different alphabets within a group show statistically significant difference at p < 0.05.

Digestive tract (DT)	Specific activity (U/mg protein)	
	Amylase	Total protease
Stomach	0.099 ± 0.014[b]	0.285 ± 0.004[b]
Pyloric caeca	0.175 ± 0.004[a]	0.308 ± 0.001[a]
Intestine	0.113 ± 0.002[c]	0.281 ± 0.001[b]
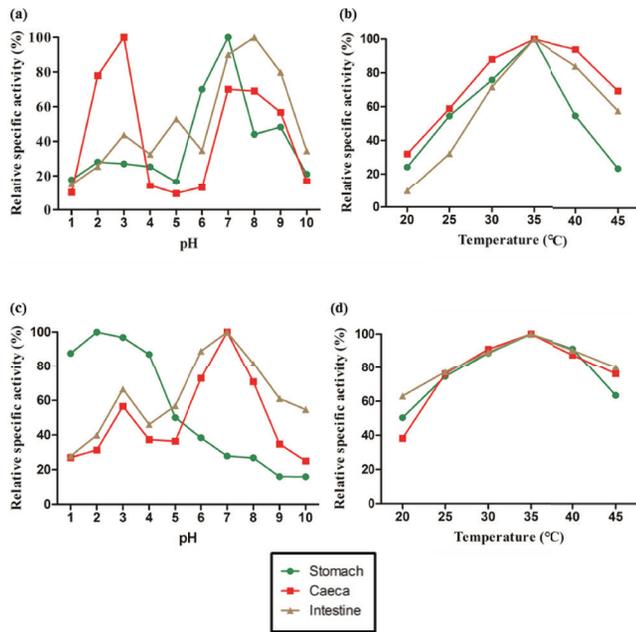
Figure 2. Effect of pH and temperature on the activity of amylase and protease of *A. testudineus*. Fig. 2a and 2b represent effect of pH and temperature on relative specific activity of digestive amylase, respectively. Fig. 2c and 2d represent effect of pH and temperature on relative activity of total protease, respectively. Enzyme activity was expressed as relative specific activity (RSA). RSA% = (Z_i/Z_{max}) x 100 [Z_i = enzyme activity at specific pH value; Z_{max} = maximum enzyme activity]. In all cases, n = 10.

in mammals, but intestinal α-amylase is produced in the exocrine pancreas (Krogdahl et al. 2005). Day et al. (2011) found a similar pattern of amylase activity in *Arrhamphus sclerolepi krefftii*, which was again a stomachless herbivore fish. Similar result was observed by Parra et al. (2007) for α-amylase in Pacific bluefin tuna *Thunnus orientalis* under culture conditions. Hidalgo et al. (1999) observed higher activity of amylase in omnivorous species *Cyprinus carpio* and found that amylase activity determined in the hepatopancreas of carp was high compared to digestive tract. It is known that amylase activity depends on the natural diet of fish species, and herbivorous and omnivorous fish have more amylase activity than carnivorous fish (Liu et al. 2016). Being an herbivore, Rohu has to consume a lot of plant materials rich in starch. Moreover, Ray et al. (2010) reported that Rohu has a sac like region (intestinal bulb) in the anterior portion of the DT, which is responsible for the temporary storage of the ingested food material. The requirement of high amylolytic activity in the anterior DT can be linked with the storage of plant based food at this region. Moderate amylolytic activity was enough for the breakdown of starch in the mid and anterior portion because the fish has a long DT. The longer the length of the DT the greater the chance of the action of digestive

enzymes in herbivore fish and it may compensate the low specific activity of digestive enzyme. On the other hand, the results revealed that the pyloric caeca of Koi is the most active site for both α-amylase and proteases. Similar result was reported in the study of Caruso et al. (2009), where the amylase and total protease activity in pyloric caeca of starved blackspot seabream (*Pagellus bogaraveo*) were potentially higher than the rest parts of the DT. The overall activity of amylase was higher in Rohu than Koi. This fact is in agreement with the popular principal that the amylase activity is always higher in the herbivorous fish species when compared to the carnivorous ones (Krogdahl et al. 2005).

The optimum pH for amylase activity in the Rohu was 8.0 for both the anterior and mid DT but at the posterior region of the DT, it was 7.0. However, in Koi the maximum activity of digestive amylase was obtained at pH 3.0 in stomach in addition with some significant activity of digestive amylase was also found at the range of pH 7.0 to pH 9.0. Subsequently the pyloric caeca and the intestine of Koi showed the optimum amylolytic activity at pH 8.0, having a relatively weak tendency towards acidic amylase activity. Study of several authors (Parra et al. 2007; Xiong et al. 2011; Champasri and Champasri 2017) in different fish reveals that the general trend of digestive amylase activity picks at natural or alkaline pH in the intestine, and in stomach the amylase activity was slightly acidic (Munilla-Moran and Saborido-Rey 1996; Fernández et al. 2001; Xiong et al. 2011). Besides, diverse feeding habits of Koi may be one of the reasons of the activity observed within a wider range of pH. The amylase activity has been reported within a wide range shows quite different feeding habits (Kawai and Ikeda 1971; Kuz'mina et al. 1996). Highest activity of amylase recorded at 35 °C in all parts of the DT in both fish species. It is known that temperature ranging from 30 °C to around 55 °C was responsible for amylase activity in wild fish (Ugolev and Kuz'mina 1993; Parra et al. 2007; Xiong et al. 2011; Champasri and Champasri 2017; Candiotto 2018). But, in most cases, temperature of gut lumen in fish is closely linked to that of the environment as well as the water temperature and may have manifold effects on fish digestion (Munilla-Moran and Saborido-Rey 1996).

In the present study total proteolytic activity of both Rohu and Koi were measured. Study on proteolytic activity means the study of pepsin, trypsin, chymotrypsin, aminopeptidase, carboxypeptidase that, which act as a battery of enzymes (Torrissen 1987; Unajak et al. 2012). In Rohu, highest activity of protease was found in mid region of the DT followed by the posterior region. The lowest activity was found in the anterior DT. Day et al. (2011) made a similar observation in another stomachless fish *Strongylura krefftii*, where proteases were more

active at mid and distal intestine than the proximal parts. However, in Koi the proteolytic activity of the stomach and the intestine showed no significant difference but for the stomach the optimum activity achieved at acidic pH, whereas in the intestine the highest activity was taken place at the neutral pH. Similar result was obtained in case of *Glyptosternum maculatum*, where protease activity was highest in stomach followed by anterior intestine (Xiong et al. 2011). In contrast to stomachless fish, proteolytic activity is highest in low pH condition when a stomach is present in fish (Kuz'mina 1990; Hidalgo et al. 1999**).**

On the other hand, the pyloric caeca in Koi exhibited maximum proteolytic activity at neutral pH (pH 7.0) and in compared to stomach (highest activity at pH < 2.0), the proteolytic activity in intestine and pyloric ceaca were far higher at neutral pH. Thus the proteolytic digestion in Koi takes place near neutral environment outside the stomach. In general, the maximum activity of protease at highly acidic pH in the stomach may be due to the stomach was bearing the gastric cells. Earlier, Lobel (1981) noted that the fish with a thin-walled stomach, with ability to considerably widen in the presence of large food items, the gastric pH was lower than in fish with a thick-walled stomach. Apparently, Koi maintains an ability to digest protein food in all the components across the gut with variable pH. Such adjustment is often noticed in fishes with diversified food habits (Moyano et al. 2001). The optimum pH for total protease activity was 8.0 in both the anterior and mid region of the DT of Rohu, but the posterior portion showed optimum proteolytic activity at pH 7.0. It gives clear indication that neutral to alkaline range of pH has been the environment of gut in Rohu for protease digestion. The absence of stomach may be the probable reason why Rohu lacks acidic protease activity. These findings were similar with other findings where the optimum activity was close to pH 8.0 (Hidalgo 1999; Pena et al. 2015; Aissaoui et al. 2017). All three regions showed some degree of acid protease activity at pH 5.0 to pH 6.0. In the case of the current study, the maximum activity of protease was found to be at 35 °C and it was somewhat in agreement with the study of Ugolev and Kuz'mina (1993), Munilla-Moran and Saborido-Rey (1996) and Aissaoui et al. (2017), where they showed the activity of proteases falls within a range of 35 °C to 40 °C in various other fishes.

Although the observations here are compared for herbivorous versus omnivorous fish species and variable ranges of optimal activity of enzymes are presented under different pH as well as temperatures levels, it is to be kept in mind that there are different other factors, like time of ingestion of food, environmental temperature, emptiness of the stomach, food types available in the stomach for omnivorous, age of fish etc to effect the release of diges-

tive enzymes at different frequencies throughout the gut (Solovyev et al. 2017). Nevertheless, it is also possible that the existence of more than one peak of optimal pH found in the above cases of Rohu and Koi indicates the possible existence of isoenzymes. Optimum pH and temperature for the enzyme activity varies along different regions of the DT (Fernández et al. 2001; Xiong et al. 2011; Solovyev et al. 2015) and within different fish (Fernández et al. 2001; Alarcón et al. 2001) as several isoforms may possibly present for a particular enzyme.

There is a strong relationship between the environment and the internal physiological state of fish, as it is an ectothermic aquatic organism. Concerning this relation, the internal physiological environment of the fish also modulated or affected by the environmental conditions. Various seasons are with various temperatures and as a rule of thumb, pH decreases with an increase in temperature. So, the self-adjustment of the ectothermic aquatic animal obviously affects its gastrointestinal digestive purpose accordingly (Solovyev and Izvekova 2016). Our study mainly conveyed the unique pattern of the complete activity of amylase and protease from different positions along with the DT of *L. rohita* and *A. testudineus* across a series of pH and temperature values. We established the specific distribution of major digestive enzymes working in various digestive sections in the studied fish species. The study also demonstrated typical patterns of varying activity depending on location in the digestive tract, pH and temperature. It is the primary study of the digestive physiology of *L. rohita* and *A. testudineus*, and further research should be carried out to learn greater details regarding its digestion and nutrition. In this context, this study surely going to guide the researchers who are working with the diet preparation or feed formulation of the studied fish species.

Acknowledgments

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