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Studies on Digestive Enzymes in Different Size Groups of *Channa aurantimaculata* Musikasinthorn, 2000

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ABSTRACT

Amylase, cellulase, trypsin, chymotrypsin, lipase and total protease are primary digestive enzymes in the fish study. *Channa aurantimaculata* (Musikasinthorn, 2000) is an omnivore fish species, specifically found in Brahmaputra River of Assam, India. Due to its attractive physical features, this fish is favored as food and is used as an aquarium fish as well. This study aimed to observe digestive enzyme (amylase, cellulase, trypsin, chymotrypsin, lipase, and total protease) activities from the gastrointestinal tract (GI tract) in different size groups (adult, juvenile, and fry) of *C. aurantimaculata* using quantitative enzyme assay. After collection, some of the adult fishes were cultured and fed with small live fishes, whereas others were freshly dissected to conduct experiments. In the fry age group, α amylase activity was the highest (1±0.31 unit·mg protein^{-1}) and in the juvenile group, protease activity was found the highest (4.6±3.1 unit·mg protein^{-1}). In adults, total protease, trypsin, chymotrypsin, and lipase activities were significantly higher ($p<0.05$) than juvenile and fry age groups. A comparative study was conducted on the total protease activity between wild and cultured fish species in adult, juvenile, and fry age groups. A significant difference ($p<0.05$) was only detected in the adult age group of wild and cultured species. The present study concluded that the protease enzyme is the primary digestive enzyme (0.9±0.39 - 17.01±8 unit·mg protein^{-1}) found in *C. aurantimaculata*, which supports the carnivorous type of food habit with relative gut length less than one (0.45-0.59) in different age groups.

INTRODUCTION

The orange spotted snakehead, *Channa aurantimaculata* Musikasinthorn is a species that belongs to Channidae family endemic to the Brahmaputra River of Assam and Arunachal Pradesh, India. The International Union for Conservation of Nature has listed this murrel species in the data of the deficient category **(Musikasinthorn, 2000; Vishwanath & Geetakumari, 2009)**. This species is distinguished with 51-54 lateral line scales, 45-47 dorsal fin rays, 28–30 anal fin rays, 8–12 cheek scales, 3–4 bars on the pectoral fin, 14 -16 pectoral fin rays and striking, vibrant pattern of purple and orange

adorning the length of its body, qualifying it as a potential aquarium fish **(Vishwanath & Geetakumari, 2009; Baruah *et al.*, 2014)**. *Channa aurantimaculata* adapts well to the controlled environmental conditions such as cemented cistern, earthern pits etc. This suggests its potentiality for *ex situ* culture, and provides an additional approach of income, helping in long-term conservation **(Baruah *et al.*, 2014)**.

Channa aurantimaculata has a wide mouth, highly developed gill rakers and very powerful jaws inlayed with sharp canine teeth desirable for carnivorous nature of food habit **(Gogoi & Biswas, 2015)**. Studies revealed that this fish changed its food habit from omnivorous to carnivorous as it grows and prefers feeding on live small fish, insects and earthworms **(Gogoi & Biswas, 2015)**. Numerous studies have revealed that gastrointestinal tract of fish species accommodates diverse microbial communities **(Ramirez *et al.*, 2003; Fidopiastis *et al.*, 2006; Izvekova *et al.*, 2007; Li *et al.*, 2009; Sun *et al.*, 2009; Merrifield *et al.*, 2010; Nayak, 2010)**. Those microbial communitities play important role in the digestion by producing digestive enzymes, especially for substrate such as cellulose, which few animals can digest **(Smith *et al.*, 1989)**. Recently, proteolytic, amylolytic and cellulolytic bacteria were detected in the digestive tracts of fishes **(Ghosh *et al.*, 2002; Saha *et al.*, 2006; Roy *et al.*, 2009)**. Therefore, considering the disease resistance potential, advance level of food and feeding habit, the present study focused on gut-associated amylase, cellulase, lipase, trypsin, chymotrypsin, and total protease enzymes of an endemic species of Assam, *C. aurantimaculata*.

MATERIALS AND METHODS

Collection of samples

Live specimens of *C. aurantimaculata* were collected from different landing centers of Upper Assam and few were reared in a cemented tank and were fed on small live fish, earthworms, mosquito, small insects, egg whites etc. Furthermore, for enzyme assay, both freshly collected and cultured fish specimens of different size groups (fry, juvenile and adult) of *C. aurantimaculata* were stored in a sedentary glass aquarium before dissection and left to starve for 48 hrs. Fry age group of *C. aurantimaculata* selected for this study was about 4.5-6 cm length.

Digestive enzyme assay

The selected specimens were collected and cleaned properly using sterile water, and the micro flora was removed from the outer surface skin of the fish. After being sacrificed by freezing, the whole body of the selected fish was dissected, and gut samples were diluted with iced 0.1% (w/v) peptone water (pH 7.2) and were homogenized **(Trust & Sparrow, 1972)**. For the 4-13 cm length group, the whole digestive glands were removed under prechilled dissecting microscope.

Amylase enzyme assay

Amylase activity of homogenized gut content of fish was assayed by the dinitrosalicylic acid (DNS) following the procedure of **Bernfeld (1955)**. To assay α amylase activity, substrate preparation was done by mixing 1.0 g of soluble starch with 0.02 M phosphate buffer. On the other hand, for β amylase activity, substrate preparation was done by mixing 1.0 g of soluble starch in 0.016 M acetate buffer. For both α and β amylase activities, 1.0 mL of substrate solution was mixed with 1.0 mL enzyme preparation and incubated for 3 min at 20^{o}C. To stop the reaction, 2 mL of di-nitrosalicylic acid (DNS) reagent was added. Afterwards, the enzyme mixture was kept in boiling water for 5 min and then cooled down. Then, 20 mL of H_2O was added, and the absorbance of the solution with brown color reduction product was determined with a colorimeter at 540 nm. Standard curve was constructed with aqueous solution of maltose (0.1 to 1.0 mg.mL^{-1}).

Cellulase enzyme assay

Cellulase activity was assayed by di-nitrosalicyclic acid method by measuring the production of reducing sugar **(Denison & Kohen, 1977)**. For conducting the experiment, 0.05 mL enzyme preparation was mixed with 0.45 mL of 1% carboxymethyl cellulose (CMC) in sodium citrate buffer (pH-5) and then incubated for 15 min at 55^{o}C. After incubation, 0.5 mL DNS reagent was added and kept in boiling water for 5 min. After cooling down to room temperature, the solution mixture was adjusted to 5 mL by adding distilled water, and absorbance was taken at 540 nm. Calibration curve was prepared by using aqueous solution of D-glucose (0.05 mg.mL^{-1} to 1.0 mg.mL^{-1}).

Total protease enzyme assay

Total protease activity of homogenized gut content was assayed by using azocasein as a substrate **(Garcia-Carreno, 1992)**. Proteolytic activity was assayed by mixing 10 mL enzyme preparation with 0.5 mL TRIS HCl (0.5 M, pH 7.5), and then 2 % azocasein in 50 mM TRIS (pH-7.5) was added. After 10 min, to stop the reaction, 0.5 mL of 20% trichloroacetic acid (TCA) was added and incubated for another 10 min at 4^{o}C. For preparation of the control, TCA was mixed before the substrate. The reaction mixture was then centrifuged at 16,500×g for 5 min, and absorbance was noted at 440 nm.

Trypsin and Chymotrypsin enzyme assay

Trypsin activity was assayed using benzoyl-Arg-p-nitroanilide (BAPNA) as a substrate **(Garcia-Carreno & Haard, 1993)**. BAPNA (1.0 mM) was prepared by dissolving it in 1.0 mL DMSO , and then converted to 100 mL by adding 50 mM TRIS buffer (pH-7.5) having 20 mM $CaCl_2$. The whole experiment was conducted at a temperature of 37^{o}C. First, 10 mL enzyme preparation was mixed with 1.25 mL substrate solution and incubated for 10 min, and then 0.25 mL acetic acid (30%) was added. Absorbance was recorded at 410 nm against water blank and used as a control.

Chymotrypsin activity was analyzed **(Garcia-Carreno & Haard, 1994)** by using substrate succinyl-$(Ala)_2$-Pro-Phe -p-nitroanilide (SAPNA). The substrate solution (100

mL of 0.02 mM) and 590 mL of 0.1 M TRIS (Ph-7.8), with 0.01 M $CaCl_{2,}$ were mixed with 10 mL of enzyme solution, and the absorbance was noted at 410 nm for 3 minutes.

BAPNA/SAPNA activity was obtained using the following formula:

(Absorbance at 410 nm.min^{-1} × l000 × mL of reaction)/(8800 × mg protein)]

Where, 8800 is the extinction coefficient of p-nitroaniline.

Lipase enzyme assay

Lipase enzyme activity of gut content was evaluated with p-nitrophenyl palmitate (pNPP) as the substrate **(Winkler & Stuckmann, 1979)**. P-nitrophenyl palmitate (30 mg) was mixed with 10 ml of iso-propanol and added to 90 mL Sorensen phosphate buffer (0.05 M, pH-8), containing 100 mg Gum Arabic and 207 mg sodium deoxycholate. During the experiment, an amount of 2.4 mL substrate solution was heated (37^{o}C), then added to 0.1 mL of enzyme solution and incubated for 15 min at 37^{o}C. Absorbance was taken at 410 nm.

Statistical analysis

All data were analyzed by performing one way ANOVA and paired sample t-test using SPSS version 20 (License provided by statistical Department, Dibrugarh University, Dibrugarh).

RESULTS

Analyses of different enzyme activities from the GI tract (gastrointestinal tract) of *C. aurantimaculata* (Table 2) show the variable occurrence of amylase, lipase, cellulase, trypsin, chymotrypsin and total protease activities in different age groups (Table 1); i.e. in fry, juvenile, and adult of freshly collected species. Fry age group showed the highest levels of cellulase (0.88±0.36), alpha amylase activity (1±0.31) and β amylase activity (0.90±0.25). The highest chymotrypsin activity was observed in the adult group (4.4±2) and was moderate in juvenile age group (1.3±0.82), whereas it significantly decreased in the fry group ($p<0.05$). Similarly, the trypsin activity recorded its highest in both the adult (0.086±0.042) and the juvenile age (0.045±0.025) groups, but decreased significantly in the fry group ($p<0.05$). Lipase activity was almost the same in adult (0.61±0.28) and juvenile groups (0.53±0.29), whereas it differed significantly in the fry group ($p<0.05$). The highest β-amylase activity was observed in the fry group (0.90±0.25), however significant decrease was detected with respect to both adult and juvenile age groups ($p<0.05$).

Table 1. Mean total length (TL), standard Length (SL), weight (W) and gut length (GL) of different age group of *Channa aurantimaculata*

Age group	TL(cm)	SL(cm)	W(g)	GL(cm)
Adult (C)	28.8±7.1	23.8±5.8	183.3±126.1	15.1±6.8
Juvenile(C)	17.3±1.5	14.4±1.2	37.6±9.3	8.4±2.1
Fry(C)	5.5±0.39	4.7±0.4	5.4±0.6	3.2±0.36
Adult (W)	32.7±6.9	26.3±5.2	266.1±102.3	18.6±6.9
Juvenile(W)	17.2±1.8	14.1±1.6	33.8±11.1	7.95±1.8
Fry(W)	5.4±0.47	4.7±0.52	7.62±1	3.1±0.47

Note: Values are mean ± SD of three determinations.

Table 2. Specific activity of different enzymes from the gastrointestinal tract (GI tract) of wild species of *Channa aurantimaculata* Musikasinthorn, 2000

Enzyme	Adult	Juvenile	Fry
Trypsin	0.086 ± 0.042^{a}	0.045 ± 0.025^{a}	0.002 ± 0.001^{b}
Chymotrypsin	4.4 ± 2^{c}	1.3 ± 0.82^{d}	0.11 ± 0.04^{e}
Lipase	0.61 ± 0.28^{f}	0.53 ± 0.29^{f}	0.028 ± 0.012^{g}
α-amylase	0.36 ± 0.33^{h}	0.27 ± 0.23^{h}	1 ± 0.31^{i}
β-amylase	0.08 ± 0.05^{j}	0.52 ± 0.22^{k}	0.90 ± 0.25^{l}
Cellulase	0.23 ± 0.20^{m}	0.57 ± 0.34^{n}	0.88 ± 0.36^{o}

Note: All enzyme activities are expressed in unit·mg protein $^{-1}$; Mean values in the same row with different superscripts are significantly different ($p<0.05$)

A comparison of total protease activity from the GI tract between wild and cultured species of this particular fish was done (Table 3). Protease activity differed significantly among adult, juvenile and fry age groups ($p<0.05$) in wild and cultured species. The highest protease activity was recorded in adult age group, but it decreased significantly in fry group. Both in wild and cultured species of *C. aurantimaculata*, the lowest values of relative gut length (RGL) were found in adult and juvenile, indicating carnivorous type of feeding habit. The comparative study of RLG between the wild and cultured species of *C. aurantimaculata* showed that, the value ranged between 0.45-0 .59 without any

significant difference (P >0.05). In wild species of *C. aurantimaculata*, the relative gut length is positively correlated with total protease activity (r=0.012, P<0.05); whereas, no significant correlation was detected between RGL and total protease activity in the case of cultured species (r=0.303, P>0.05). From correlation curve estimation (Figs. 1 & 2), it can be concluded that the occurrence of total protease activity in wild species of *Channa aurantimaculata* moderately (r^2 =0.1127) depends on the RGL.

Table 3. Relative gut length (RGL) and specific activity of total protease from the GI tract of wild (W) and cultured (C) species of *Channa aurantimaculata* Musikasinthorn, 2000

Age group	RGL (C)	RGL(W)	Total Protease(W)	Total Protease(C)
Adult	0.51 ± 0.09^{a}	0.57 ± 0.03^{c}	17.01 ± 8^{f}	9.4 ± 0.5^{i}
Juvenile	0.47 ± 0.08^{a}	0.45 ± 0.06^{d}	4.6 ± 3.1^{g}	2.8 ± 2^{j}
Fry	0.59 ± 0.046^{b}	0.54 ± 0.1^{e}	0.9 ± 0.39^{h}	0.11 ± 0.04^{k}

Note: Relative gut length (RGL) is calculated as the ratio of gut length (cm) to the total length (cm) of *Channa aurantimaculata*; total protease activities are expressed in unit·mg $protein^{-1}$; mean values in the same column with different superscripts are significantly different (p<0.05).

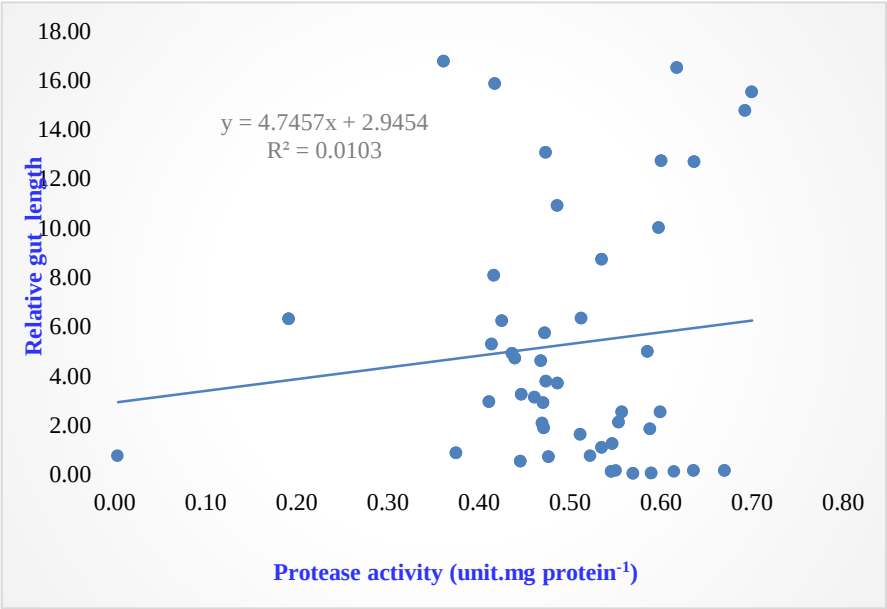

Fig. 1. Correlation between gut total protease activity (unit.mg protein^{-1}) and relative gut length of cultured species of *Channa aurantimaculata*

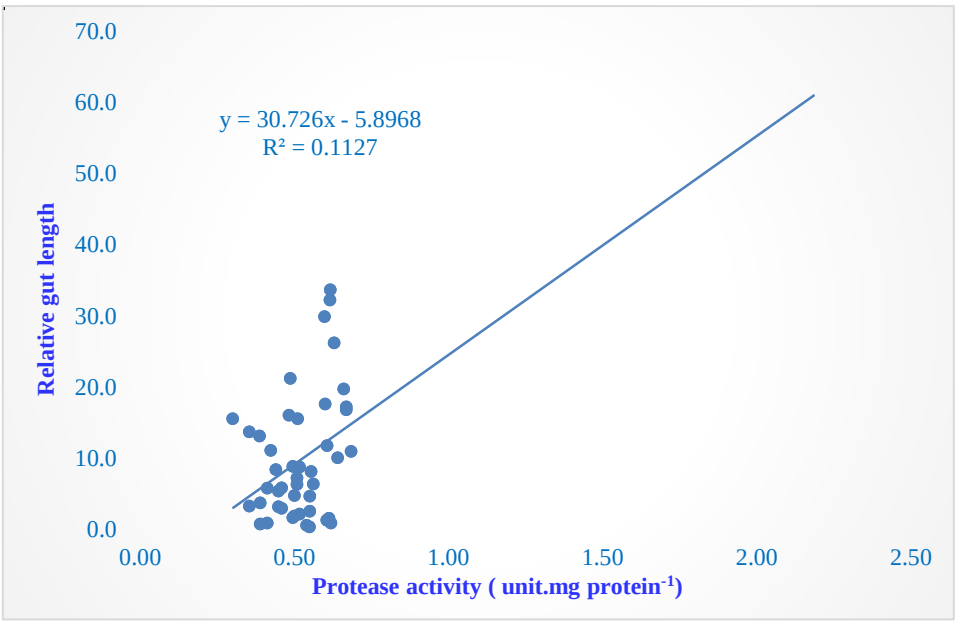

Fig. 2. Correlation between gut total protease activity (unit.mg protein^{-1}) and relative gut length of wild species of *Channa aurantimaculata*

Channa aurantimaculata shows that the omnivore's food habit in the fry and juvenile stages changes to carnivore food habit as it grows, i.e. in adult. Despite carnivore food habit, adult age group of the afore- mentioned fish showed the occurrence of α-amylase, β- amylase and cellulase activities from adult to fry age groups. In adult stage, protease , trypsin, chymotrypsin and lipase activities were quite prominent compared to the juvenile and fry age groups. Hence, a significant correlation was denoted between these three age groups (fry, juvenile and adult) and the digestive enzyme activity from the GI tract of *C. aurantimaculata.*

DISCUSSION

The present study addressed the distribution of amylase, cellulase, trypsin, chymotrypsin and lipase activities throughout the digestive tract of *Channa aurantimaculata*. Previous study indicated that *C. aurantimaculata* is primarily carnivorous although they showed herbivorous mood of food habit during their early stage because the young ones feed on plankton, and after some time in maturity stage, they prefer to eat mosquito larvae. After attaining the fingerling stage, they prefer to eat earthworms, silkworm pupae and insects **(Gogoi & Biswas, 2015)**. Feeding keenness of *C. aurantimaculata* is dependent on temperature; as soon as water temperature drops below 20ºC, this species stops eating and becomes sluggish **(Gogoi & Biswas, 2015)**.

Channa aurantimaculata shows similarity with carnivorous species such as *Sparus aurata* **(Deguara *et al.*, 2003)**, *Pseudoplatystoma corruscans* **(Lundstedt *et al.*, 2004)** and *Scleropages formosus* **(Natalia *et al.*, 2004)** and additionally with omnivorous species, including *Colossoma macropomum* **(De Almeida *et al.*, 2006; Correa *et al.*, 2007)**. Furthermore, the species under study is similar to the omnivorous *Diplodus puntazzo* **(Tramati *et al.*, 2005)**, which has significant occurrence of total protease activity, trypsin and chymotrypsin activities as well as cellulase and amylase activities released from the digestive tract in different age groups. The occurrence of trypsin and chymotrypsin was detected in carnivorous species **(Chiu *et al.*, 2002; Garcia-Carreno *et al.*, 2002)**. In addition, omnivorous species **(Martinez & Serra, 1989)** recorded the same enzymes' activities. Results of this current study implies that the gut enzyme profile of *C. aurantimaculata* is well adopted to omnivores type of food habit, because they are herbivores in fry stage and carnivores in juvenile and adult stages. Previous researches have revealed that protease classification isn't possible depending on feeding habit. Several studies reported that rainbow trout and common carp have high levels of protease activity, but carnivorous fishes, including the European eel and the gilthead seabream have lower protease activity **(Hidalgo *et al.*, 1999; Furne *et al.*, 2005)**. Remarkably, the amylase activity was defined to be more dependent on nutritional habits instead of proteolytic activity. Moreover, it is suggested that the occurrence of amylase activity is quite prominent in herbivorous and omnivorous rather than in carnivorous fish **(Ji *et al.*, 2012)**. Unlike the case with *C. aurantimaculata* carnivorous species, *Notopterus chitala*

(Ghosh, 1985); *Notopterus notopterus* **(Chakrabarti *et al.*, 1995)**; *Oncorhynchus mykiss* **(Hidalgo *et al.*, 1999)** and *Anarhichas minor* **(Papoutsoglou & Lyndon, 2006)** showed no amylase activity in the digestive tract, whereas in *Dentex dentex* **(Perez-Jimenez *et al.*, 2009)**, the presence of amylase activity was clearly detected in its digestive tract. Identically, this was marked with *Lates calcarifer* **(Sabapathy & Teo, 1993)**. However, such type of carnivorous fish mostly eats protein and very little amount of carbohydrates. The occurrence of cellulase and amylase activities in such fishes varies and is quite controversial that needs more study to confirm their role **(Chakrabarti *et al.*, 1995; Hidalgo *et al.*, 1999; Deguara *et al.*, 2000; Lundstedt *et al.*, 2004; Natalia *et al.*, 2004**).

Wild carnivorous fish has the ability to swallow high level of lipids, and thus lipase activity is observed in their digestive tract **(Chakrabarti *et al.*, 1995)**. Lipase activity varies depending on fish feeding habit. Herbivorous fish, such as *Oreochromis niloticus* has limited lipase activity throughout its digestive tract **(Tengjaroenkul *et al.*, 2000),** whereas omnivorous fish, including *Colossoma macropomum* **(De Almeida *et al.*, 2006)** shows high level of lipase activity in the stomach compred to other parts of GSI tract **(Perez-Jimenez *et al.*, 2009)**. Although lipase enzyme activity wasn't detected in the stomach of carnivores fish including *Dentex dentex*, it was detected throughout the digestive tract in some carnivorous species; namely, *Pseudoplatystoma corruscans* **(Lundstedt *et al.*, 2004)** and *Scleropages formosus* **(Natalia *et al.*, 2004)**. In those species, the highest enzyme activity was observed in the intestine portion.

CONCLUSION

It can be concluded that the reason behind the food habit of *C aurantimaculata*, omnivore in fry age group and carnivore in the adult, is the occurrence of cellulolytic and proteolytic enzymes' activities. The lowest activity of enzymes, such as lipase, trypsin and chymotrypsin, and the highest amylase and cellulase activities in fry age group prefer its herbivores' mode of food habit. The juvenile and adult age groups showed mainly carnivores' food habit due to the fact that their enzyme activities showed the same preferences. The highest total protease activity in adult age group of freshly collected fish, especially wild fish compared to cultured fish indicates environmental enhancing enzyme activity in *C. aurantimaculata*. It can be assumed that some gut micro flora are responsible to enhance the enzyme activity in wild species available in natural habitat. More studies are recommended to confirm such facts. Relative gut length did not show much variability in different age groups of this species of fish, and comparative study between wild and cultured groups suggests that environment may influence the morphometric parameters of this fish because RGL in all age groups of wild fish is higher than that in cultured fish. Study with more morphometric parameters is required in relation to digestive enzyme activity to reach a conclusion. In addition, studies on enzyme activity of carnivore and omnivore fish species can be related to the impact of gut micro-

flora on nutrition and the enumeration of gut proteolytic bacteria, and hence analyze its importance to be used as probiotics.

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