Postprandial Patterns of Digestive Enzyme Activity in Male Siamese Fighting Fish (*Betta splendens*)

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ABSTRACT

Postprandial changes in digestive enzyme patterns were investigated in male Siamese fighting fish (*Betta splendens*). The fish (1.58±0.03 g) were fasted for 24 h to empty the gut and then fed once before sampling postprandially. Specific activities of pepsin, trypsin, chymotrypsin, lipase, and amylase were determined, as well as ratios of trypsin/chymotrypsin and amylase/trypsin (n = 4). Over the 24-h postprandial observation, feces were not found for the first four hours of sampling, but 52.5% of the fish defecated within 24 h. Of all feces excreted, approximately 25% were counted at 5–6 h after feeding. Postprandial time had significant effects on all five of the enzymes ($p<0.05$), and higher specific activity than the control (0 h) was observed over the entire 24-h period of observation. Protein-, lipid-, and carbohydrate-digesting enzymes, as well as the trypsin/chymotrypsin ratio, exhibited similar patterns in response to time after feeding. The amylase/trypsin ratio at every postprandial time interval was significantly lower than the control at 0 h. The present study found that postprandial enzyme activities of male Siamese fighting fish changed gradually but were sustained for a relatively long period. These findings could inform the feeding regimen management of this species, particularly regarding suitable feeding frequency.

Keywords: Amylase, Betta, Feces, Lipase, Ornamental fish, Trypsin

INTRODUCTION

The Siamese fighting fish (*Betta splendens*, Osphronemidae) is a commercially important ornamental fish. Wild populations of this species are found only in Thailand and a small region of Laos, while their ornamental form is traded worldwide (Panijpan *et al*., 2020). The United States is the top importer of Siamese fighting fish from Thailand while other important importers are China, France, the European Union, and Singapore (Sermwatanakul, 2019). Only male fish, with their long ventral and caudal fins, and their vivid, uniform body coloration are traded (Srikulnath *et al*., 2021; Panthum *et al*., 2022).

Siamese fighting fish are carnivorous. The typical diet given to ornamental fish consists of live organisms such as mosquito larvae and other aquatic insects (Thongprajukaew *et al*., 2019; Mejia-Mejia *et al*., 2021). Pellet feed formulations have been developed by some researchers (Mandal *et al*., 2010; 2012; Thongprajukaew *et al*., 2011), but to achieve the best appearance and behavior of these fish, an egg-based diet is preferred to commercially available pellet feeds (Saekhow *et al*., 2022). Experimentally, these proposed pellets were given to fish at various frequencies: two

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Received 22 November 2022 / Accepted 20 February 2023

(Thongprajukaew *et al*., 2019; Khatoon *et al*., 2021), three (Sipaúba-Tavares *et al*., 2016; Yuslan *et al*., 2021) or four (de Oliveira *et al*., 2022) times a day. However, a comparison by Norazmi-Lokman *et al*. (2020) reported insignificant results on survival and growth performance between fish reared under these three regimens and fish fed once a day.

Feeding strategies are directly linked with postprandial patterns of digestive enzyme activities. Under some feeding regimens, changes in these patterns have been associated with gut emptying and return of appetite influenced by feeding frequency (Satjarak *et al*., 2022). Postprandial digestive enzyme patterns may also vary by species, age, or diet type (Jia *et al*., 2021; Paulino *et al*., 2022). Therefore, it is essential to investigate these responses to better optimize feeding strategies. The aim of this study was to investigate the postprandial patterns of the main digestive enzymes, namely pepsin, trypsin, chymotrypsin, lipase, and amylase, in male Siamese fighting fish. The findings of this study may help to accurately predict the optimal feeding strategy that stimulates feed efficiency in Siamese fighting fish, and facilitate appropriate schedules for sample harvesting in biological research. Understanding the flexibility of digestive enzymes involved in nutrient catabolism could also provide primary data for making decisions on fish transportation management.

MATERIALS AND METHODS

Animal protocols

The transportation, acclimatization, rearing, specimen collection, and euthanasia of the fish used in this study conformed to the "Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes" of the National Research Council of Thailand (Application No. U1-06514-2560). The use of animals in this project was approved by the Institutional Animal Care and Use Committee, Prince of Songkla University (Project Code 2564-01-101).

Fish acclimatization and postprandial trial

Four-month-old, short-finned male *Betta splendens* were purchased from a private farm in Nakhon Si Thammarat Province, Thailand. They were transported to the Faculty of Science, Prince of Songkla University, where they were individually acclimatized for two weeks in 350 mL of water in polypropylene cups (9.0 cm diameter × 11.5 cm height). They were fed to satiation at 8:30 a.m. and 4:00 p.m. with a floating pellet for Siamese fighting fish containing 40% crude protein (Betta-Bio-Gold; Kyorin Food Ind. Ltd., Himeji, Japan). Every other day, 80% of the water in the cups was replaced with dechlorinated water. During the acclimatization period, the water quality parameters were pH 6.89±0.06, temperature 29.37±0.23 °C, dissolved oxygen 3.60±0.17 $mg \cdot L^{-1}$, total alkalinity 14.00±1.25 $mg \cdot L^{-1}$, total hardness 21.35±2.71 $mg \cdot L^{-1}$, and total ammonia 0.88±0.10 $mg \cdot L^{-1}$.

At the end of the acclimatization period, the fish were screened to obtain forty individuals of similar size (1.58±0.03 g weight and 4.26±0.02 cm length). These fish were reared in the conditions described for acclimatization. They were fasted for 24 h to empty the gut and then fed once prior to sampling. Four fish were collected at 0, 1, 2, 4, 6, 8, 12, 16, 18, and 24 h after feeding. They were euthanized with clove oil at a concentration of 75 $mg \cdot L^{-1}$, then the gastrointestinal tracts were carefully removed on ice and stored at -20 °C until use for enzyme extraction. During the 24 h of harvesting, feces were counted and recorded at two- or three-hour intervals.

Determination of digestive enzyme activities

Gastrointestinal tract samples were weighed and then homogenized in cold 0.2 M Na_2HPO_4-NaH_2PO_4 buffer (pH 8) at a ratio of 1:15 w/v. Centrifugation was performed at 15,000×g for 30 min at 4 °C to obtain supernatant. This portion was kept at -20 °C until use. The Lowry method (Lowry *et al*., 1951) was applied for quantification of soluble proteins in crude enzyme extracts.

The optimal conditions of pH and temperature for assaying digestive enzyme activities were chosen from previous investigations of enzyme activities in Siamese fighting fish by Thongprajukaew *et al*. (2010a; 2010b). The conditions were: pepsin, pH 2 and 40 °C; trypsin, pH 8 and 50 °C; chymotrypsin, pH 8 and 50 °C; lipase, pH 8 and 40 °C; amylase, pH 8 and 50 °C. Activity of pepsin (EC 3.4.23.1) was determined using hemoglobin as the substrate (Worthington, 1993). One unit (U) of this enzyme is defined by 1.0 absorbance at 280 nm. Activities of trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) were determined using *N*-benzoyl-*L*-Arg-*p*-nitroanilide and *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide as the substrates, respectively (Rungruangsak-Torrissen *et al*., 2006). The liberated product was spectrophotometrically measured at 410 nm against the linear range of *p*-nitroanilide standard. Alpha-amylase activity (EC 3.2.1.1) was assayed using soluble starch as the substrate (Areekijseree *et al*., 2004). Colorimetric measurement was performed at 540 nm and the obtained values were compared with maltose standard curve. Activity of lipase (EC 3.1.1.3) was assayed using *p*-nitrophenyl palmitate as the substrate (Winkler and Stuckmann, 1979). The liberation of *p*-nitrophenol was spectrophotometrically detected at 410 nm against its standard curve. The enzymatic conversion of 1 µmol of substrate per minute is defined as 1 U of trypsin, chymotrypsin, amylase, or lipase. To calculate the ratios of trypsin/chymotrypsin (T/C ratio) and amylase/trypsin (A/T ratio), the relevant values were taken from the same fish sample.

Statistical analysis

Ten postprandial times with four fish each were formulated under a Completely Randomized Design. Data are expressed as mean±SEM (n = 4). The Statistical Package for Social Sciences, Version 22 (SPSS Inc., Chicago, USA) was applied for all statistical evaluations. One-way analysis of variance was used to evaluate the differences in digestive enzyme activities between postprandial times by using Duncan's multiple range test as the *post hoc* test. The relationships between pairs of enzymes were evaluated by Pearson's correlation. The statistical significance level was set at $p<0.05$.

RESULTS AND DISCUSSION

Postprandial time had significant effect on the specific activity of all observed enzymes ($p<0.05$). Pepsin specific activity increased progressively with postprandial time but its highest activity was maintained from 12 to 24 h after feeding (Figure 1a). Although pepsin digests up to 20% of ingested amide bonds (Nelson and Cox, 2016), it is a major enzyme contributing to protein digestion in the stomach. Maintaining pepsin activity over a long period of time indicates gradual catabolism of protein in this organ.

The specific activities of the protein-digesting intestinal serine proteases, namely trypsin (Figure 1b) and chymotrypsin (Figure 1c), followed similar patterns to pepsin. Trypsin activates itself and its own and other zymogens for subsequent protein catalysis, so that it contributes 40–50% of the protein digestion in the gastrointestinal tract (Eshel *et al*., 1993). This enzyme cleaves the C-terminal end of peptide chains at arginine and lysine residues. The significant reduction in trypsin specific activity at 24 h after feeding indicates that most protein was partially digested during the previous 23 h. Prolongation of postprandial time would produce the same specific activity observed in the control treatment (0 h). The same trend was observed for the T/C ratio (Figure 1d). This sensitive protease marker is directly linked with the transport rate and level of amino acids to target tissues, and determines growth capacity and protein utilization efficiency (Rungruangsak-Torrissen *et al*., 2006), so that the fish with high growth rate demonstrate higher T/C ratio relative to slow-growing fish. Under starvation conditions, T/C ratio decreases with time, indicating the possible prohibition of growth (Chan *et al*., 2008). However, short postprandial time (within 24 h) from the current study had no effect on fish growth (data not shown). It is possible that the modulation of T/C ratio might be driven by some physiological responses that occur during short periods of starvation, such as protein synthesis/turnover and plasma free amino acids (Rungruangsak-Torrissen *et al*., 2006). In the current study, the activity of chymotrypsin at 24 h was relatively high. Since the cleavage sites of chymotrypsin preferentially

occur where the N-terminal of a polypeptide chain contains large hydrophobic amino acids (tyrosine, tryptophan, or phenylalanine), the high level of specific activity compared to trypsin might be due to differences in the products digested by each enzyme.

The postprandial responses of lipase (Figure 2a) and amylase (Figure 2b) showed broadly similar trends to the three protein-digesting enzymes. These findings indicate that the catabolism of proteins, lipids, and carbohydrates occurred concurrently. This conclusion is supported by the highly positive correlations ($r = 0.607–0.907$, $p<0.01$, $n = 40$) between the activities of every pair of observed enzymes (Table 1). The A/T ratio indicates the utilization of available carbohydrate per unit of protein (Thongprajukaew *et al*., 2011), so that non-starved fish (0 h) appear to utilize carbohydrate per unit of protein at a higher rate than the starved fish (1–24 h). This marker has been applied for investigating the flexibility between carbohydrate and protein, two major constituents in feed, in a number of fish species such as Asian seabass *Lates calcarifer* (Wattanakul *et al*., 2017); Siamese fighting fish (Saekhow *et al*., 2019); and Nile tilapia *Oreochromis niloticus* (Thongprajukaew and Rodjaroen, 2020). The maintenance of this ratio from 1 h after feeding until the end of sampling (Figure 2c) indicates that the utilization of both nutrients was normal in the experimental time period.

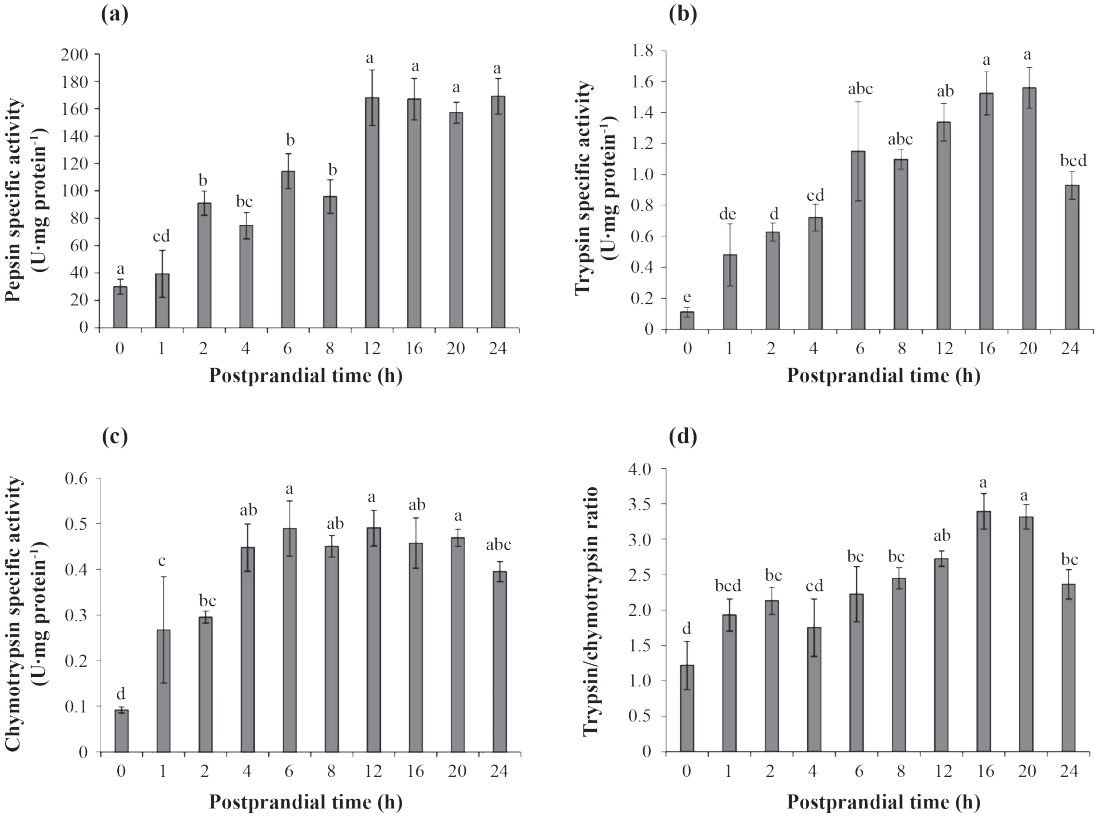

Figure 1. Postprandial patterns of protein-digesting enzyme activities in male Siamese fighting fish: pepsin (a), trypsin (b), chymotrypsin (c), and the trypsin/chymotrypsin ratio (d). Different lowercase letters above bars represent significant ($p<0.05$) difference between treatments and error bars represent SEM.

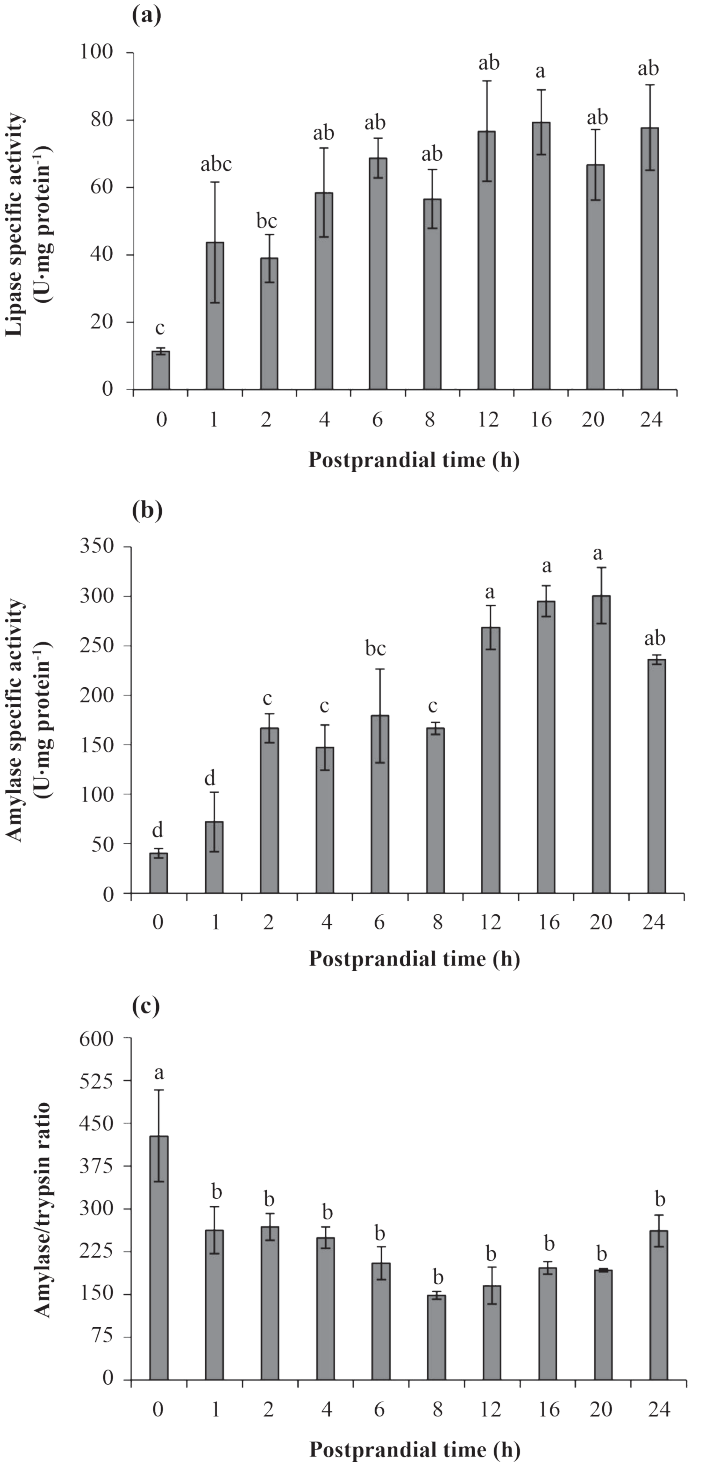

Figure 2. Postprandial patterns of lipase (a) and amylase (b) specific activity, and the amylase/trypsin ratio (c) in male Siamese fighting fish. Different lowercase letters above bars represent significant ($p<0.05$) difference between treatments and error bars represent SEM.

Table 1. Pearson correlation coefficients (r) of specific activity between each pair of digestive enzymes in male Siamese fighting fish.

Traits	Pepsin	Trypsin	Chymotrypsin	Lipase
Trypsin	0.759**			
Chymotrypsin	0.676**	0.816**		
Lipase	0.780**	0.607**	0.836**	
Amylase	0.866**	0.907**	0.742**	0.659**

Note: The values were calculated from a dataset of forty individuals (10 postprandial times×4 fish); ** = $p<0.01$

In general, the gastrointestinal tract of carnivorous fish is always described as short. However, the feces count in the present study of Siamese fighting fish shows that feces were not found for the first four hours after feeding and that only 52.5% of the fish defecated within the 24-h postprandial period (Figure 3). Approximately one quarter of the total feces excreted were recorded in the period from 5 to 6 h after feeding. This indicates that the digestion rate of individual fish covered a wide range, and in general, the digestion rate appears to be rather slow. It is possible that the prolongation of digestion, as well as the activities of digestive enzymes, might conserve energy, since the motions and swimming behavior of this species are rather slow.

Based on observations of liver microstructure (Chansue *et al*., 2003), Siamese fighting fish have the ability to starve. In a mixed-sex group of Siamese fighting fish, no differences in growth or feed utilization were reported between fish fed one, two, three, and four times daily (Norazmi-Lokman *et al*., 2020). In a study of spawning fish, the recommended strategy was feeding two times daily rather than the alternative choices of one meal in three days, one meal in two days, one meal daily, or three meals daily (James and Sampath, 2004). It is possible that male fish might maintain growth and feed utilization for longer time intervals between meals. Therefore, varying the frequency of feeding with longer time intervals between meals might save feed cost and significantly improve feed efficiency.

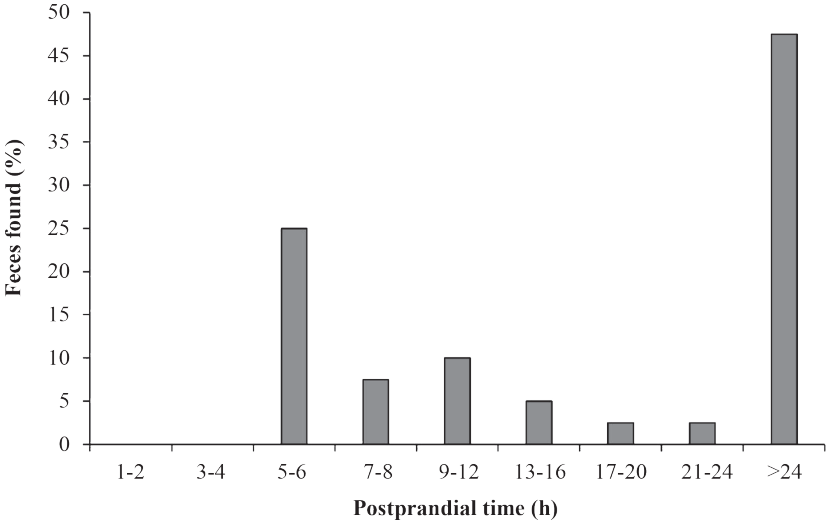

Figure 3. Feces found for male Siamese fighting fish at different postprandial times. Data are expressed as means and calculated from forty observed fish.

Postprandial changes in fish are always investigated during the first 24 h after feeding. The Siamese fighting fish in this work exhibited broad peaks of activity for all enzymes assayed: 12–24 h for pepsin, 6–20 h for trypsin, 4–24 h for chymotrypsin, 1–24 h for lipase and 12–24 h for amylase. Such gradual changes allow the long-distance transportation of these fish in the fasted state via commercial airlines or overland shipping by post office. This presumption agrees with the relatively low weight loss (2.15–5.23%) of the male fish after starvation following 48-h overland transportation (Thongprajukaew *et al*., 2023). These results contrast with the narrow peaks of enzyme activity observed in a number of other carnivorous species. Observations during 2–24 h after feeding of the anterior intestine of the European sea bass *Dicentrarchus labrax*, indicated that trypsin activity peaked at 6 h, and lipase and amylase activities peaked at 2 h (Castro *et al*., 2016). Observations from 0 to 15 h of a hybrid grouper, *Epinephelus moara*×*Epinephelus lanceolatus*, showed that chymotrypsin and lipase activities peaked at 6 h, and amylase from 0–15 h (Jia *et al*., 2021). When considering the results of these investigations, it always has to be taken into account that postprandial enzyme activity patterns may be directly affected by biological factors such as feeding habits or developmental stage, by dietary factors such as feed type, nutrient composition, or feeding frequency, and by the digesting organ involved, whether the stomach, intestine, or whole digestive tract (Castro *et al*., 2016; Parma *et al*., 2019; Jia *et al*., 2021; Camila *et al*., 2022; Paulino *et al*., 2022).

CONCLUSION

In this study of male Siamese fighting fish, postprandial time had significant effects on the specific activities of pepsin, trypsin, chymotrypsin, lipase, and amylase. All the observed enzymes showed similar patterns of activity, which were exhibited concurrently over the 24-h duration of the study. Compared to other carnivorous fishes, the activity of digestive enzymes in this species changed gradually, but were maintained at a high level over a relatively long period. This finding supports the long-distance transportation of these fish in the fasted state due to gradual changes of the main digestive enzymes. Also, when Siamese fighting fish are used as animal models, specimens should be fasted >24 h to avoid background effects from food intake. In addition, the optimization of feeding strategies to include longer intervals between feeding than recommended in previous investigations (James and Sampath, 2004; Norazmi-Lokman *et al*., 2020) should be of interest.

ACKNOWLEDGEMENTS

We would like to thank Asst. Prof. Dr. Sutthiratana Khaopakro, and the Cooperative Education Project from Faculty of Science and Technology, Princess of Naradhiwas University, for supporting the assistant researchers. The authors acknowledge the International Relations Office, Faculty of Science, Prince of Songkla University, for advice in manuscript preparation.

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