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# Research Article
# Postprandial *In vitro* Protease-Specific Activity of Nile Tilapia (*Oreochromis niloticus* L.) Digestive Organs

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## Abstract

**Background and Objective:** Protease-specific activity varies postprandially. This study aimed to identify the optimum time after feeding Nile tilapia (*Oreochromis niloticus* L.) to collect digestive organ samples (stomach, proximal intestine, distal intestine and liver) to obtain maximum protease-specific activity values. **Materials and Methods:** One hundred Nile tilapia (average weight 55.19±1.78 g/fish) were acclimatized and fed two times per day for 14 days. At the beginning of the experiment, 6 fish were randomly taken from the tank before feeding (0 h) and then 6 fish were also randomly taken after feeding at 1, 3, 6, 12, 18, 24, 36 and 48 h for digestive organ collection. Homogenized organ pH was measured and extracted for a protease-specific activity assay using azocasein as a substrate. **Results:** The pH values of all organs differed ($p<0.05$) after feeding and the pH changes were related to chyme movement in these organs. The protease-specific activities of the stomach and proximal intestine were highest at 24 h after feeding ($p<0.05$) but the protease-specific activities of the distal intestine and liver were not significantly different ($p>0.05$) between time points. **Conclusion:** Postprandial digestive organ sample collection was recommended at 24 h to maximize protease-specific activity for further protease characterization.

Key words: Digestive organs, feeding time, fish feed, *Oreochromis niloticus* L., pH, postprandial and protease-specific activity

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Competing Interest: The author has declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

Nile tilapia (*O. niloticus* L.) has a strong economic impact in many countries[1-3]. The global production trends of Nile tilapia in aquaculture have been increasing and exceeded 5.45 million tons in 2014[3]. In aquaculture production, feed accounts for 50-70% of production costs[4,5], while dietary protein sources are the most expensive feed ingredients[6-10].

Protein digestion and utilization are important for fish growth. Many studies on proteolytic enzymes in fish contribute to a better understanding of them and help improve protein utilization for fish production[11-31]. Proteolytic enzymes, known as proteases or proteinases, catalyze the hydrolysis of proteins to peptides and amino acids commonly found in organs such as the stomach, intestine and liver[32].

Many factors affect proteolytic activities include species, dietary composition, developmental stages, bacterial enzyme activities, temperature and intestinal pH[13,33-39]. In an earlier study, digestive organs of Nile tilapia (*O. niloticus* L.) with body weights of 5.70, 35.80 and 92.10 g were collected at 16 h after feeding and revealed different protease activities among the three size groups[15]. Uscanga *et al.*[40] reported varied protease activity at different postprandial times (0, 30, 60 and 90 min) of *O. niloticus* L. (50.30 g). These results suggest that sampling time is an important factor affecting the activity of enzymes. The present study aimed to investigate the effect of time after feeding on the protease-specific activity of Nile tilapia (*O. niloticus* L.) from digestive organs including the stomach, proximal intestine, distal intestine and liver.

## MATERIALS AND METHODS

**Fish and preparation of samples:** One hundred Nile tilapia (*O. niloticus* L.) with similar size (body weight 55.19±1.78 g/fish) obtained from a local fish farm in Khon Kaen province, Thailand, were used in this study. They were acclimatized for 2 weeks prior to the experiment in a round plastic tank with a diameter of 2.00 m containing 2.50 $m^3$ of water. Fish were fed at 06.00 and 18.00 to near satiation using pelletized commercial feed containing 32% crude protein. The experiment used a completely randomized design (CRD). On day 15, fish were randomly taken in 6 fish groups at time points before feeding (0 h) and at 1, 3, 6, 12, 18, 24, 36 and 48 h after feeding. Body weight and standard length of the fish were recorded. Then, fish were euthanized by submersion in ice cold water and the stomach, proximal intestine, distal intestine and liver were dissected out on an ice-chilled tray. The samples were washed with distilled water and weighed and pooled samples of 2 fish and 3 replicates were kept in liquid nitrogen for further analysis. The weights of the stomach, intestine, liver and fish body were calculated as follows:

$$\text{Relative stomach weight (g g}^{-1}\text{ body weight)} = \frac{\text{Stomach weight}}{\text{Body weight}}$$

$$\text{Relative intestine weight (g g}^{-1}\text{ body weight)} = \frac{\text{Intestine weight}}{\text{Body weight}}$$

$$\text{Vesicle somatic index (VSI\%)} = \frac{\text{Vesicle weight}}{\text{Body weight}} \times 100$$

$$\text{Hepatosomatic index (HSI\%)} = \frac{\text{Liver weight}}{\text{Body weight}} \times 100$$

**Digestive organ pH measurement:** Digestive organs were homogenized by a tissue grinder (Wheaton, Potter ELV) on ice and then mixed with distilled water 1: 5 (g: mL). The pH of the homogenates was measured using a calibrated pH meter (Denver instrument, Ultrabasic). The mixtures were kept on ice at 4°C prior to a determination of protease-specific activity.

**Determination of protease-specific activity:** The homogenized sample was mixed with 1 M Tris-HCl buffer pH 7.00 (1:0.05 v/v) and then centrifuged (Centurion Scientific Ltd., K240R) at 15,000×g and 4°C for 60 min. The supernatant was collected and kept in liquid nitrogen for further determination of the enzyme-specific activity of protease (modified from Areekijseree *et al.*[26]). The protein concentrations of crude enzymes from the stomach, proximal intestine, distal intestine and liver were determined by following Lowry's method (1951) using bovine serum albumin as the standard.

Protease-specific activity was analyzed by measuring the increase of oligopeptides, a short chain polypeptide[26]. The activity was determined by using 250 µL of 0.5% azocasein as a substrate and adding 10 µL of crude enzyme. Substrate was dissolved in KCl-HCl buffer at an acidic pH of 2.00 and $NaHCO_3$-$Na_2CO_3$ buffer at an alkaline pH of 9.00. The mixture was incubated at 30°C for 30 min and the reaction was stopped with 1.20 mL of 10% Trichloroacetic acid (TCA). Blank was prepared by mixing with crude enzyme and stopping activity with TCA followed by adding substrate. The mixture was centrifuged at 10,000×g for 15 min at 4°C and the collected supernatant (1.20 mL) was mixed with 1.40 mL of

1.0 M NaOH. Total protease specific activity was determined as U $mg^{-1}$ protein/min. One unit (U) of protease activity was specified as an increase in absorbance at 440 nm per minute at the specific reaction condition, as measured by a spectrophotometer (Biochrom, Libra S80).

**Statistical analysis:** Statistical analysis of data was performed using one-way analysis of variance (one-way ANOVA). Duncan's multiple range test at $p<0.05$ was used when a significant difference existed. A correlation between postprandial time and biometric parameters of fish was calculated.

## RESULTS

**Postprandial physical characteristics of fish:** Body weight, total length and hepatosomatic index (HSI) of Nile tilapia (*O. niloticus* L.) at the time of sample collection are shown in Table 1. There were no significant differences among treatments ($p>0.05$). The average body weight at all periods was 55.19±1.78 g/fish and the total length was 14.59±0.14 cm/fish. The mean HSI was 2.66±0.12. Stomach weight, intestine weight, relative stomach weight, relative intestine weight and visceral somatic index (VSI) were significantly different between time period groups ($p<0.05$). In general, both stomach and intestine weights were significantly decreased ($p<0.01$) after feeding. The highest stomach weight was in the 1st h after feeding (3.16±1.16 g/fish) and the lowest weight was at 48 h (0.69±0.17 g/fish). The average intestine weight was higher than 2.00 g until 6 h and lower onward. The average relative stomach weight was 0.03±0.00 g $g^{-1}$ body weight before feeding and at 6 h after feeding and decreased as time increased. The relative intestine weights were lowest at 36 h and highest before feeding at 0.02 and 0.05 g $g^{-1}$ body weight, respectively. The vesicle somatic index (VSI) was calculated from the sum of stomach, intestine, liver and other entrails per total body weight of fish. VSI was highest at 6 h after feeding (11.24±0.45) and then decreased as time increased ($p<0.05$).

**Postprandial pH changes:** The pH values of the stomach, proximal Intestine, distal intestine and liver of Nile tilapia (*O. niloticus* L.) are shown in Fig. 1. The stomach pH was between 4.18 and 6.61 (Fig. 1a). The lowest stomach pH was 4.18 ± 0.12 before feeding and significantly differed from the others ($p<0.05$). The proximal intestine pH ranged from 6.89-7.53 (Fig. 1b) and significantly differed ($p<0.05$) among collection time points. The distal intestine pH ranged from

Table 1: Alteration in body weight, stomach weight, intestine weight, vesicle somatic index (VSI) and hepatosomatic index (HSI) of Nile tilapia during the period after feeding

| | Time after feeding (h) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Initial | 1 | 3 | 6 | 12 | 18 | 24 | 36 | 48 | p-value |
| Fish | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | |
| Body weight (g/fish) | 49.02±2.68$^{a}$ | 65.50±8.27$^{a}$ | 59.72±6.37$^{a}$ | 59.28±5.22$^{a}$ | 57.20±2.96$^{a}$ | 46.37±2.24$^{a}$ | 57.10±8.31$^{a}$ | 52.80±3.39$^{a}$ | 49.77±1.95$^{a}$ | 0.220 |
| Total length (cm) | 13.67±0.29$^{a}$ | 15.35±0.66$^{a}$ | 14.52±0.35$^{a}$ | 14.97±0.47$^{a}$ | 15.08±0.34$^{a}$ | 14.35±0.20$^{a}$ | 14.73±0.60$^{a}$ | 14.27±0.19$^{a}$ | 14.40±0.24$^{a}$ | 0.165 |
| Stomach weight (g/fish) | 1.62±0.12$^{abc}$ | 3.16±1.16$^{a}$ | 2.35±0.54$^{abc}$ | 2.37±0.52$^{abc}$ | 1.13±0.35$^{c}$ | 0.85±0.22$^{c}$ | 3.08±0.87$^{ab}$ | 1.32±0.27$^{bc}$ | 0.69±0.17$^{c}$ | 0.018 |
| Intestine weight (g/fish) | 2.22±0.04$^{a}$ | 2.22±0.09$^{a}$ | 2.31±0.23$^{a}$ | 2.21±0.24$^{a}$ | 1.40±0.11$^{b}$ | 1.25±0.04$^{b}$ | 1.21±0.12$^{b}$ | 1.39±0.16$^{b}$ | 1.08±0.13$^{b}$ | 0.001 |
| Relative stomach weight (g $g^{-1}$ body weight) | 0.03±0.00$^{bcd}$ | 0.04±0.01$^{ab}$ | 0.04±0.00$^{abc}$ | 0.04±0.01$^{ab}$ | 0.02±0.00$^{de}$ | 0.02±0.00$^{de}$ | 0.05±0.01$^{a}$ | 0.03±0.00$^{cde}$ | 0.01±0.00$^{e}$ | 0.001 |
| Relative intestine weight (g $g^{-1}$ body weight) | 0.05±0.00$^{a}$ | 0.04±0.00$^{b}$ | 0.04±0.00$^{b}$ | 0.02±0.00$^{c}$ | 0.02±0.00$^{c}$ | 0.03±0.00$^{c}$ | 0.03±0.01$^{c}$ | 0.02±0.00$^{c}$ | 0.02±0.00$^{c}$ | 0.001 |
| VSI (%) | 10.49±0.46$^{ab}$ | 9.23±2.57$^{bc}$ | 11.24±0.45$^{a}$ | 9.36±0.64$^{bc}$ | 7.28±0.53$^{d}$ | 6.68±0.54$^{d}$ | 9.10±0.54$^{bc}$ | 7.74±0.23$^{cd}$ | 7.02±0.60$^{d}$ | 0.001 |
| HSI (%) | 2.53±0.33$^{a}$ | 1.89±0.18$^{a}$ | 3.15±0.27$^{a}$ | 2.76±0.54$^{a}$ | 3.11±0.21$^{a}$ | 2.21±0.36$^{a}$ | 2.11±0.35$^{a}$ | 3.09±0.19$^{a}$ | 3.07±0.30$^{a}$ | 0.071 |

Data represented as Mean±SEM, $^{a,b,c}$Different superscript letters indicate a significant difference between values on a given row ($p<0.05$)

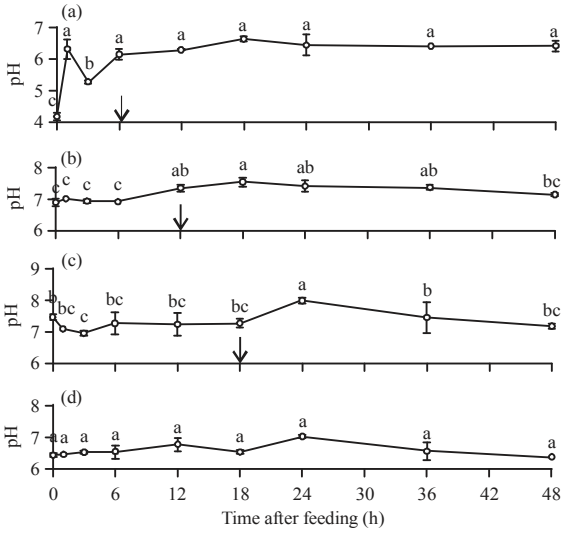


Fig. 1(a-d): Postprandial changes in the pH of the (a) Stomach, (b) Proximal intestine, (c) Distal intestine and (d) Liver of Nile tilapia

Each point represents the mean of 3 replicates±SEM. Different letters indicate a significant difference between values on a point of time after feeding (p<0.05). ↓: Indicates the chyme exhausted from the organ

6.95-7.97 (Fig. 1c). The highest pH in the distal intestine was at 24 h after feeding (7.97±0.05) and was significantly different from other collection time points (p<0.05). The pH of the liver ranged from 6.35-7.01 (Fig. 1d), which was not significantly different (p>0.05) among collection time points in the experiment. The lowest pH (6.35±0.15) of the liver was found at 48 h after feeding. At 24 h after feeding, the liver had the highest measured pH, which was 7.01±0.22.

**Postprandial changes of *In vitro* protease-specific activity:** Postprandial *In vitro* protease-specific activity of the stomach, proximal intestine, distal intestine and liver extracts are shown in Fig. 2. The alkaline protease activities of the stomach are shown as solid lines in Fig. 2a. The highest alkaline protease activity of the stomach was found at 24 h postprandial (0.18±0.01 U $mg^{-1}$ protein/min) and was significantly different (p<0.05) from other collection times. The acidic protease activity of the stomach was the highest at before feeding (0 h), shown in Fig. 2a (dash line). For protease-specific activities in the proximal intestine, a peak of alkaline protease activity was found before feeding (0.23±0.02 U $mg^{-1}$ protein/min) and it increased again after 24 h (0.18±0.05 U $mg^{-1}$ protein/min) (Fig. 2b). The protease-specific activities of the distal intestine were approximately 0.02-0.39 U $mg^{-1}$ protein/min (Fig. 2c) and were not significantly different (p>0.05). The lowest protease

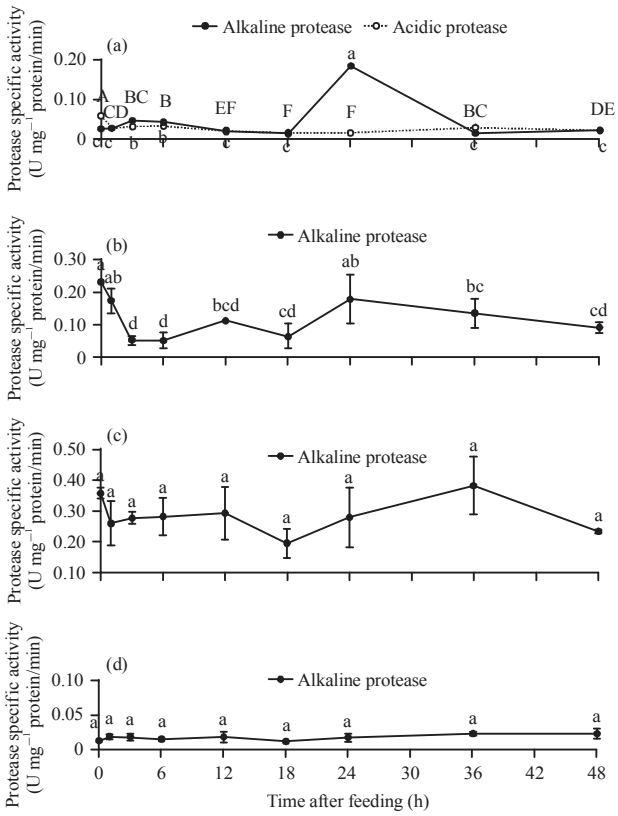


Fig. 2 (a-d): Postprandial changes in protease-specific activity of the (a) Stomach, (b) Proximal intestine, (c) Distal intestine and (d) liver of Nile tilapia

The data points represent the mean of 3 replicates±SEM. Different letters indicate a significant difference between values at a point of time after feeding (p<0.05)

specific activity was 18 h postprandial (0.20±0.05 U $mg^{-1}$ protein/min), while the highest protease specific activity was at 36 h (0.39±0.09 U $mg^{-1}$ protein/min). The alkaline protease activities from liver extracts were 0.01-0.02 U $mg^{-1}$ protein/min in the present study, which were the lowest compared to other organs (Fig. 2d) and there were no significant differences (p>0.05) in protease-specific activities measured in liver extracts from all time points.

## DISCUSSION

In the present study, with a short experimental period (48 h), there were no significant differences in *O. niloticus* L. weight, which was similar to an earlier report in which fish fed and starved for 3 days were not significantly different[41]. However, the results in our study contrasted those of an earlier study with a 7-day starvation period in which *Danio rerio*

weight, after 7-day starvation, was slightly higher than the fed fish[42]. Similarly, the weight of *Oncorhynchus mykiss* decreased when starved for 4 weeks[43].

The HSI of *O. niloticus* L. did not change when starved for 48 h (Table 1). This was in agreement with another study which showed the liver weight was stable until 9 days of starvation in *Pagrus major* [44]. In general, HSI provides an indication of the status of energy reserves and health of the animal because it shrinks under adverse environmental conditions[45]. The correlation coefficient between collecting time and physical characteristics (weight of stomach, intestine and VSI) showed a tendency to decrease, r = -0.293, -0.701 and -0.502, respectively. These results indicated that the weight of digestive organs decreased postprandially. Changes in body weight and HSI from all collection times were not observed following 48 h starvation, which was due to the short experimental period, unlike stomach and intestine weight, where changes after feeding were most likely observed due to chyme movement.

The stomach pH was significantly different ($p<0.05$). The stomach pH was lowest before feeding. Although postprandial stomach pH (1 through 6 h) increased from 6 h until 48 h, pH was relatively stable from 6.13-6.61 ($p>0.05$) on an empty stomach. The stomach pH increased when there was no chyme. This was in agreement with other studies on *O. niloticus* L.[46] and *Alcolapia graham* [47] which showed that pH was higher in fish with empty stomachs and was lower in fish with full stomachs. A lower pH condition in the stomach was caused by the secretion of hydrochloric acid (HCl)[48,49]. The results of this study and earlier reports supported the stomach pH changes in which the pH of a full stomach was acidic[47,50,51]. The pH of the intestine displayed a trend of increasing alkalinity as time increased. The proximal intestine had a low pH (6.89-6.92) at 0 through 6 h after feeding, which was probably caused by the movement of chyme from the stomach into the proximal intestine. The pH was increased to 7.34±0.11 at 12 h and was highest at 18 h (7.53±0.13). The proximal intestine was empty at 12 h postprandial. This was similar to earlier reports in that the proximal intestinal pH was neutral and remained unchanged after feeding. In other species (*T. rendalli*, *O. mossambicus*, *C. gariepinus* and *A. graham*) their proximal intestine pH was 7-8[38,48]. The distal intestine pH had a statistically significant difference ($p<0.05$) between the average intestinal pH of the collection time. At 18 h after feeding, the chyme was not visible in the distal intestine. The distal intestine pH increased after the distal intestine was empty at 24 h after feeding, then pH was maintained from 7.17-7.43. Similarly, *A. graham* had an average hindgut pH of 8.12[47]. In the distal intestine, pH values of *S. aurata* were increased from 6.90-7.80 at 6 h[47]. In the distal intestine, the pH of *S. aurata* increased from 6.90-7.80 at 6 h after feeding[34]. In the distal intestine of *T. rendalli*, *O. mossambicus* and *C. gariepinus*, the pH values were in the alkaline range at all experiment time points[38]. In all intestinal sections of *O. mykiss*, alkaline pH values were recorded over time following feeding (0 through 72 h) and by 48 h, pH values returned to similar conditions before ingestion of a meal[49]. The highest pH values of the proximal and distal intestines in *S. aurata* were alkaline (pH>7.00) at 12 h[34]. The pH variations found in the different parts of the digestive organs could be explained by the flow of chyme along the digestive tract[34,49]. Secretion of bile (bicarbonate) also contributed to the alkaline pH of the proximal and distal intestines[36,52]. In addition, our results showed that the movement of chyme affected the change in pH over time in the digestive organs but not in the liver. In terms of chyme movement, chyme was observed in the stomach for 6 h, then moved to the proximal intestine and remained in both the proximal intestine and distal intestine for 6 h. Eventually, no feed was observed in the digestive tract at 18 h after feeding.

In terms of protease activity, the highest acidic protease activity in the stomach was found before feeding, when pH was the lowest. Similar findings were reported by Simpson[53] and Zhao *et al.*[54] who observed that the acidic proteases from fish stomachs, pepsin displayed high activity between pH 2.0 and 4.0. Similarly, the highest pH for acidic protease activity was 2.5 and 1.6-3.3 in *O. nilotica* and hybrid juvenile tilapia (*O. niloticus*×*O. aureus*) stomachs, respectively[55,56]. In other species, the highest protease specific activity in the stomach of *Symphysodon aequifasciatus* [25], *S. aurata* and *Dentex dentex* [57] were found at pH 2 and at pH 1.8 in *Colossoma macropomum* [58]. In general, the enzyme activities changed when the pH of the stomach changed[18]. The stomach protease-specific activities of *O. mossambicus*, *C. gariepinus* and *T. rendalli* were the highest at 12, 12 and 31 h, respectively, after feeding[38]. Another experiment on *S. aurata* reported acid protease activity between pH 3 and 4.5, which was high at 0 h and 3 h, respectively, after a meal[18]. On the other hand, the alkaline protease activity in the stomach of *S. aurata* was highest at 1 h after feeding but the acid protease activities did not differ from other times after feeding[35]. The activity of alkaline protease from the stomach was highest at pH 8-9 in *O. niloticus* L. (5.7, 35.8 and 92.1 g)[15]. In the stomach and intestine of *S. dumerilii*, the values of protease-specific activities at 6 h after feeding were similar to those measured in an empty stomach (after 2 days of starvation, which was 48 h after feeding)[13]. In all *in vitro* protease-specific activities of the stomach, it was found to be lower than that of the intestine and this was in

agreement with other studies conducted on *O. niloticus* L.[15,59], *T. rendalli*[38], *Pagellus bogaraveo*, *Seriola dumerilii*[13], *C. gariepinus*[38] and *Scleropages formosus*[28].

The proximal intestine protease-specific activities were decreased in low pH before feeding to 6 h after feeding. Then, specific activities of the protease in all intestines of *O. niloticus* L. were highest when chyme moved from the stomach into the proximal intestines at 6 h after stomach emptiness. This was in agreement with the estimated total gut evacuation of *O. niloticus* L. reported in an earlier study at 7.15 h[40]. The highest activity of the alkaline protease in the proximal intestine of *O. mykiss* was 6 h after stomach emptiness[49]. The activity of alkaline protease in the duodenum of *S. aurata* was higher from 0-2 h after feeding but not significantly different from other times[18]. The *in vitro* protease-specific activities of the intestine were higher when the pH increased. This was in agreement with earlier reports in which protease-specific intestinal activities were high when the pH was neutral or alkaline[15,22,25,53,60]. Based on the results of this study, the protease-specific activities of the distal intestine were higher than those of the proximal intestine. In contrast, the protease-specific activity of the proximal intestine of *O. mykiss*[49] and *O. niloticus* L.[40] was higher than that of the distal intestine over 48 h after feeding.

The alkaline protease activities from liver extract in the present study were the lowest compared to the other organs, which was similar to earlier reports[15,22,28]. In addition, liver enzymes have inactive forms known as zymogens for preventing self-digestion[32].

The time after feeding was a key factor in studying the enzyme activities because feeds are involved in the secretion of enzymes[35,38,59]. Protease-specific activity in the digestive tract of *Rutilus rutilus caspicus* was decreased after starvation for 1-3 weeks[61]. Stomach, proximal intestine and distal intestine of *T. rendalli*, *O. mossambicus* and *C. gariepinus* revealed the highest enzyme activity at 12 h after feeding[38]. Differences in gut evacuation may be due to species, dietary composition and chyme movement in the digestive tract, which are also factors affecting the activity of enzymes[13,33,35,38].

Variations in pH and protease specific activity in the digestive system appeared to be affected by the movement of chyme at different times after feeding[18,33,38]. Other factors, including species, developmental stages, temperature and dietary composition, could also affect the pH and protease-specific activity changes[13,33-35,38,39]. In this study, a similar size of Nile tilapia fed the same formula diet was used; thus, species and dietary composition effects were eliminated.

The digestive system was empty at 18 h and the specific activities of the protease in all organs showed an increment from 18 h to their highest at 24 h, suggesting it was the best period after feeding for digestive tract sample collection to obtain the possible highest enzyme activity for further needed enzyme assays.

## CONCLUSION

Starvation for 48 h did not cause a significant effect on body weight, length, or HSI. The movement of chyme at different times after feeding affected changes in the pH values of the digestive system. The highest protease-specific activities of digestive organs were found at 24 h after feeding in this study and thus it is recommended as the optimum time for digestive organ sample collection in further investigations on protease characterization in Nile tilapia (*Oreochromis niloticus* L.).

## SIGNIFICANCE STATEMENT

This study discovers the time after feeding which effect on pH and protease-specific activity changes that can be beneficial for precise *In vitro* protein digestion of Nile tilapia. This study will help researchers uncover the critical area of the *In vitro* protease-specific activity in digestive organs in fish that are essential for an effective feed formulation.

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