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Characterization of Digestive Enzymes from Adult Freshwater Pearl Mussel *Chamberlainia hainesiana* (Lea, 1856)

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Abstract

Freshwater mussel *Chamberlainia hainesiana* is a vulnerable species in Thailand. The species has a lustrous nacreous shell that can be utilized for various purposes, and has a potential industrial use for producing freshwater pearl. Characterization of digestive enzymes would provide critical information prerequisite for studying the development of digestive enzymes during on–growing as well as for *in vitro* digestibility study important for the development of suitable artificial feed formulations for the commercial culture. The aim of this preliminary study was to determine optimum pH and temperature for three digestive enzymes: amylase, protease and lipase, from digestive gland including stomach of adult *C. hainesiana* under various pH's (2–11) and temperatures (25–80^{o}C). The results revealed at least three isoforms of amylase and protease, and at least 4–5 isoforms of lipase. No alkaline protease activity was detected. For future enzyme expression study, the suitable optimal conditions are 40^{o}C pH 6 for amylase activity, 50^{o}C pH 5 for protease activity, and 50^{o}C pH 8 for lipase activity. At natural habitat of around 30^{o}C, these enzymes are most active at pH 6, 6, and 8 (or 10), respectively, which are suitable conditions for future study on *in vitro* digestibility.

Keywords : Freshwater mussel, Amylase, Protease, Lipase

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Introduction

Freshwater pearl mussel *Chamberlainia hainesiana* (Lea, 1856) is a vulnerable species in Thailand. It is multi-utility aquatic animal with economic values (Kovitvadhi et al., 2002). This mussel has a lustrous nacreous shell that can be utilized for various purposes. The species also has a potential industrial use for producing artificial freshwater pearl (Yeemin, 1997). Furthermore, it is food for the native people as well as for domestic animals (Kovitvadhi et al., 2002). Mussels are filter feeders and their filtering activities contribute to maintaining a clean environment and reducing pollution (Dan et al., 2001). The population of freshwater mussels has decreased as a result of low water quality. To increase the population of freshwater mussels, culture techniques have been used for mass production and conservation (Isom and Hudson, 1982, 1984; Hudson and Isom, 1984; Keller and Zam, 1990; Kovidvathi et al., 2002; Kovitvadhi et al., 2003). Recently, Kovitvadhi et al. (2006) have successfully cultured *Hyriopsis (Limnoscapha) myersiana* to adult phase, and it should also be possible for *C. hainesiana.*

At present, the hatchery rearing of juveniles and adults bivalves relies on the mass production of microalgae. Coutteau and Sorgeloos (1992) identified the mass production of live algae as a major bottleneck in bivalve hatcheries and nurseries. Algae production comprises up to 30% of hatcheries' operation costs. Furthermore, algae cultures often vary in nutritional value and are subject to seasonal growth patterns and contamination. The use of artificial feeds with optimized nutritional quality is the need in aquaculture. Utilization of nutrients in aquatic animals depends on digestive enzymes (Rungruangsak-Torrissen et al., 2006; Areekijseree et al., 2006; Rungruangsak-Torrissen, 2007; Rungruangsak-Torrissen and Fosseidengen, 2007; Supannapong et al., 2008). Recent studies on feeding of aquatic animals have reported the correlation among digestive protease activity (trypsin specific activity and activity ratio of trypsin to chymotrypsin), protein digestibility, feed conversion efficiency (FCE), fish weight and specific growth rate (SGR), as well as the quality of muscle and oocyte in Atlantic salmon (Sunde et al., 2001; Sunde et al., 2004; Rungruangsak -Torrissen et al., 2002; Rungruangsak-Torrissen, 2007), Atlantic mackerel (Rungruangsak-Torrissen and Fosseidengen, 2007), and among trypsin specific activity, FCE and SGR in Atlantic cod (Lemieux et al., 1999). Study on amylase activity has helped in the development of more rapid and accurate *in vitro* carbohydrate digestibility assay (Areekijseree et al., 2006; Supannapong et al., 2008) and used as indicator for carnivorous feeding habit (Hofer and Schiemer, 1981). *In vitro* digestibility studies of dietary protein (Rungruangsak-Torrissen et al., 2002; Areekijseree et al., 2006; Rungruangsak-Torrissen, 2007; Supannapong et al., 2008) and carbohydrate (Areekijseree et al., 2006; Supannapong et al., 2008) using bivalves crude enzyme extract and based on the activities of protease and amylase, respectively, could provide informations on nutritional quality of feeds and feed raw materials.

This work is the first part of the project on biochemical evaluation of dietary quality in relation to growth performance quality of *C. hainesiana.* The aim of the work was to determine optimum pH and temperature for the main digestive enzymes (amylase, protease and lipase) collected from digestive gland including stomach of the adult *C. hainesiana,* under various conditions. Data obtained from this study are prerequisite for future studies of digestive enzyme expression during the life cycle, and for future development of artificial feed formulation for mussel culture.

Materials and Methods

Animal and rearing

Adult *C. hainesiana* of 14 months old were transferred from Kanchanaburi Inland Fisheries Research and Development Center to the Department of Aquaculture, Faculty of Fisheries, Kasetsart University, Bangkok, Thailand, where they were sampled for enzyme analyses. They were cultured mussels from glochidia stage until adult, according to Kovitvadhi et al. (unpublished). The mussels from late juvenile (150 days) to adult were fed freely with natural plankton in the pond.

Preparation of enzymes extracts

Eight mussels were cleaned with tap water to remove adhering detritus. The shells were opened by cutting off anterior and posterior adductor muscles, and the digestive glands and stomachs were dissected. The organs were weighed and the digestive gland including stomach from four mussels were pooled and used as one replication. Enzyme extraction was modified from Areekijseree et al. (2004) by adding 1:0.5 (w/v) of 200 mM phosphate buffer, pH 7 during homogenization on ice. After centrifuged at 13,000 g for 15 min at 4°C, the supernatant was collected and kept in aliquots at –80°C for later determination of amylase, protease and lipase specific activities. The protein concentration of the enzyme extracts was determined using the method described by Lowry et al. (1951).

Determination of amylase specific activity

Amylase activity was determined by 3,5-dinitrosalicylic acid (DNS) method according to Areekijseree et al. (2004) based on Benfeld (1951). The reaction mixture contained 125 µl 1% soluble starch (in 100 mM specific buffer containing 6 mM NaCl) and 125 µl enzyme extract of suitable dilution. For pH profile study, the reaction mixture was incubated in buffers pH 2–11 for 15 min at 25°C. Then the reaction was stopped by adding 250 µl 1% DNS (dissolved in 2 M NaOH and 0.6% sodium potassium tartrate), heated in boiling water bath for 5 min. After cooling down, 2.5 ml distilled water was added before measuring the absorbance at 540 nm. The buffers used were glycine–HCl buffer for pH 2, citrate phosphate buffer for the pH range of 3–5, phosphate buffer for the pH range of 6–8, and $NaHCO_3$–Na_2CO_3 buffer for the pH range of 9–11 (Areekijseree et al., 2004). For temperature profile study, the reaction mixtures were performed at various temperatures (25–80°C), at different pH. Amylase specific activity was expressed as U mg protein^{-1}. The unit (U) of amylase activity was defined as µmol maltose produced per hour (under the specified reaction conditions).

Determination of protease specific activity

Protease activity was determined by measuring an increase in cleavage of short-chain polypeptide using azocasein as substrate, modified from Areekijseree et al. (2004) based on Garcia-Carreno (1992). The reaction mixture contained 250 µl 1% azocasein (dissolved in 0.1 M NaOH and 100 mM specific buffer) and 10 µl enzyme extract of suitable dilution. The protease activity was too low to be detected at 25°C. Therefore the pH profile study was performed at 30°C in the pH range of 2–11 using the buffers as described above. After 15 min incubation, the reaction was stopped by adding 1.2 ml 10% TCA and centrifuged at 8,000 g for 15 min. The supernatant was collected and 1.4 ml 1 M NaOH was added before measuring the absorbance at 440 nm. For temperature profile study, the reactions were carried out at 25–80°C, at different pH. Total protease

specific activity was expressed as U mg protein^{-1}. The unit (U) of protease activity was defined as an increase in absorbance at 440 nm per hour (under the specified reaction conditions).

Determination of lipase specific activity

Lipase activity was determined using the method modified from Gupta et al. (2002) based on Winkler and Stuckmann (1979). The reaction mixture contained 200 µl 0.01 M *p*-nitrophenyl palmitate (dissolved in isopropanol), 800 µl 0.2 M buffer and 10 µl crude enzyme extract of suitable dilution. For pH profile study, the reaction mixtures were performed at 25°C in various buffers pH 2–11, as described above, for 15 min. The reaction was then stopped by adding 250 µl 0.1 M Na_2CO_3. After centrifuging at 13,000 g for 15 min, the absorbance of the supernatant was measured at 410 nm. For temperature profile study, the reaction mixtures were performed at various temperatures (25–80°C), at different pH's. Lipase specific activity was expressed as U mg protein^{-1}. The unit (U) of lipase activity was defined as the increase in absorbance at 410 nm per hour (under the specified reaction conditions).

Statistical analysis

Mean and standard error of the mean were calculated for each enzyme specific activity.

Results

Amylase activity had pH optimum at pH 6 (Fig. 1A), and at least three isoforms of amylase were observed at pH 5, 6 and 8 (Fig. 1A). Specific activity of amylase was very low at pH ≤ 4 and at pH ≥ 9 (Fig. 1A), showing less than 400 U/mg protein. Study of temperature profiles at pH 5, 6 and 8 indicated different amylase activities with optimal temperatures at 25°C, 40°C and 40°C, respectively (Fig. 1B). Two isoforms had very low activities at ≥ 50°C, while the other isoform had very low activity at ≥ 60°C (Fig. 1B). Amylase showed highest specific activity at 40°C pH 6.

For protease, it had optimum pH 6 (Fig. 2A), and at least three isoforms were observed at pH 3, 5 and 6 (Fig. 2). Protease specific activity was too low to be detected at 25°C. However, the pH profile study at 30°C revealed the specific activity at pH 6 > pH 5 > pH 3 (Fig. 2A). No protease activity was detected at pH ≥ 7 (Fig. 2A). Study of temperature profiles at pH 3, 5 and 6 indicated different proteases with optimal temperatures at 40–60°C, 50°C and 30°C, respectively (Fig. 2B). The optimal condition at 50°C pH 5 revealed the highest protease specific activity following with the condition at 30°C pH 6.

The pH profile of lipase showed a relatively high specific activity in a broad pH range of 2–11. (Fig. 3A). At least 4–5 isoforms of lipase were observed at pH's 2, 6, 8, 10, and probably 11 (Fig. 3A). By selecting not too extreme pH, the pH 6, 8, and 10 were chosen for temperature profile study, showing lipase specific activities with optimal temperatures at 40°C, 50°C 80°C, and 30°C, respectively (Fig. 3B). Lipase showed highest specific activity at 50°C pH 8.

Discussion

In this study, we observed different isoforms of the digestive enzymes with different optimal conditions of pH and temperature for their activities. At least three isoforms were detected for amylase and protease, and at least 4–5 isoforms for lipase. The temperature range of 30–50°C and pH range of 5–8 for amylase activity in the digestive gland including stomach of *C. hainesiana* are similar to those observed in marine mussel *Mytilus chilensis* (Fernandez-Reiriz et al., 2001) and freshwater

pearl mussel *Hyriopsis bialatus* (Areekijseree et al., 2002; Supannapong et al., 2008). A relatively high amylase activity at 30°C, although not optimum, indicated an importance of *C. hainesiana* amylase for carbohydrate digestion in natural habitat where the temperature range is 28–30°C. However, the general optimum condition suitable for studying amylase expression in *C. hainesiana* is 40°C and at pH 6.

There are many types of protease enzymes with

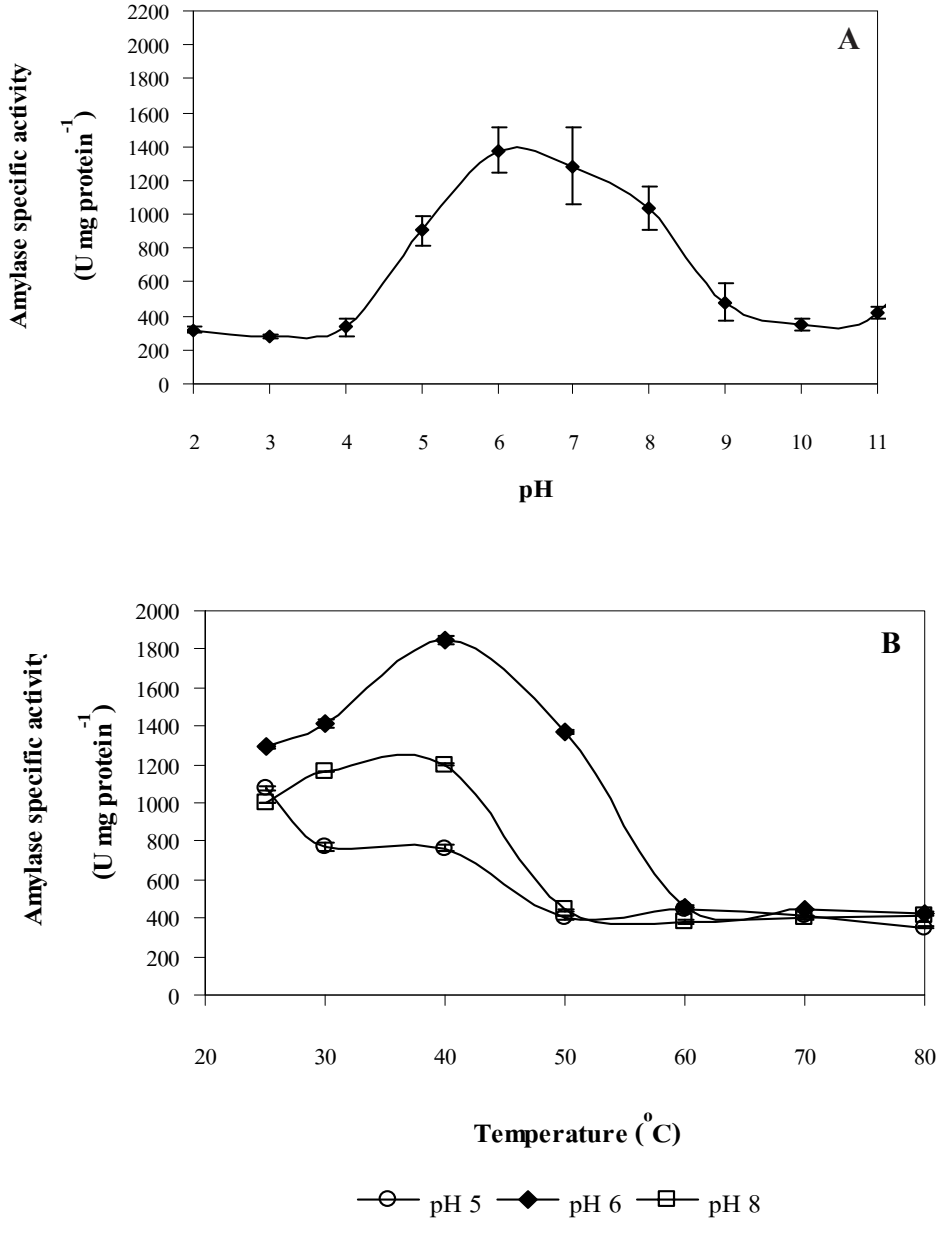

Figure 1. Amylase specific activity (U mg protein^{-1}) showing pH profile at 25°C (A), and temperature profiles at pH's 5, 6 and 8, indicating general optimal condition at 40°C pH 6 (B).

specific activity at different pH (Areekijseree et al., 2004; Supannapong et al., 2008; present work) and temperature (Rungruangsak Torrissen and Male, 2000; present work). Surprisingly, no alkaline protease activity was detected in the digestive gland including stomach of adult *C. hainesiana.* Among the three isoforms of protease detected, two main

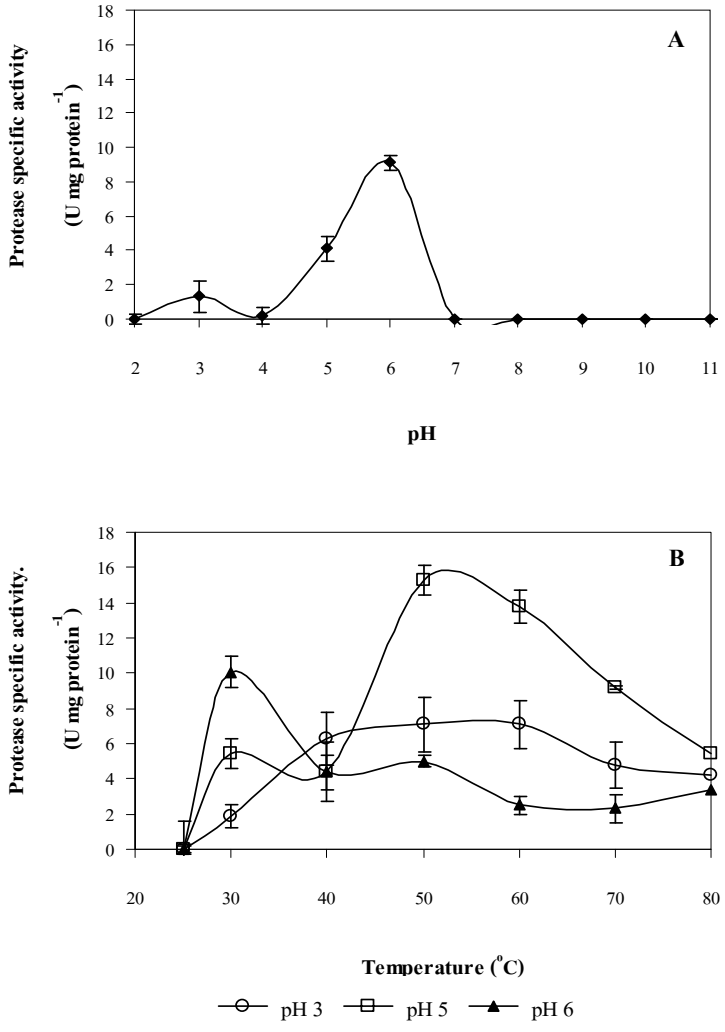

Figure 2. Protease specific activity (U mg protein^{-1}) showing pH profile at 30°C (A), and temperature profiles at pH's 3, 5 and 6, indicating general optimal condition at 50°C pH 5 (B)

protease activities were observed with their optimum conditions at 50°C, pH 5 and 30°C, pH 6. These two main proteases probably represent one from stomach and the other from digestive gland but when extracting was difficult to separate these two organs. pH of the samples extracted from digestive gland and stomach it was neutral. This indicated that, at natural habitat temperature (28–30°C), neutral protease is important for protein digestion in adult *C. hainesiana.* However, the general optimum condition suitable for studying protease expression in *C. hainesiana* is 50°C, pH 5.

Lipase seemed to consist of a large group

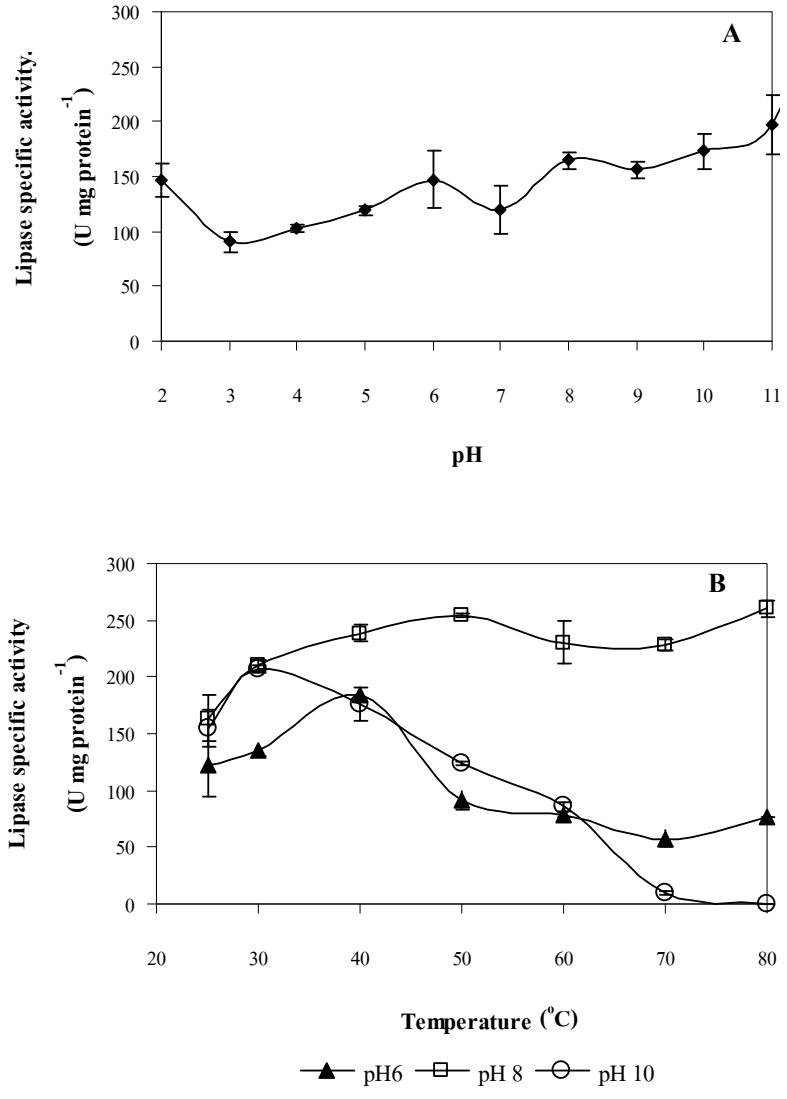

Figure 3. Lipase specific activity (U mg protein^{-1}) showing pH profile at 25^{o}C (A), and temperature profiles at pH's 6, 8 and 10, indicating general optimal condition at 50^{o}C pH 8 (B)

of enzyme types with the activities covering a broad temperature and pH range. Among the different isoforms of lipase observed, and at natural habitat temperature of around 30^{o}C, two alkaline lipases actively at pH 8 and pH 10 are important for lipid digestion of this mussel species. However, the optimum condition suitable for studying lipase expression in *C. hainesiana* is 50^{o}C pH 8.

Temperature and pH, playing important

roles on enzyme activities, can cause changes in enzyme structure as well as its catalytic performance. The temperature and pH for optimizing digestive enzyme activity are important factors for future study of any impact on expression of each digestive enzyme of interest during the animal life cycle, whereas the condition of pH with high temperature optimum is suitable for such comparison study (Rungruangsak-Torrissen et al., 2006, Rungruangsak-Torrissen, 2007). The enzyme characteristics study could also be useful as a prerequisite knowledge for developing artificial feed formulations through nutritional evaluation of diets by *in vitro* digestibility study (Rungruangsak-Torrissen et al., 2002; Areekijseree et al., 2006; Rungruangsak-Torrissen, 2007; Supannapong et al., 2008), whereas the condition of pH with optimal temperature close to natural habitat is suitable. In addition, it could be useful for formulating artificial diets that are suitable for the digestion under different rearing conditions (Rungruangsak-Torrissen et al., 2002; Rungruangsak-Torrissen, 2007). The ability of digestion is species specific (Rungruangsak-Torrissen et al., 2002). Therefore it is important that temperature and pH for optimizing the activities of the main digestive enzymes are studied in each species of interest. By this way, the optimal temperature and pH could be selected for amylase, protease and lipase expressions, for *in vitro* digestion of carbohydrate, protein and lipid, respectively, in different food raw materials using fish crude enzyme extracts. Among different main nutrient digestibility, protein digestion apparently is the key factor determining food quality even in herbivores like mussels (Areekijseree et al., 2006; Supannapong et al., 2008). Protease activity in acid condition (peptic activity) is not related to feed utilization quality and fish growth, unlike protease activity in alkaline condition (tryptic activity) that is related to feed conversion efficiency and fish growth (see Rungruangsak-Torrissen et al., 2006). *In vitro* protein digestibility of diets using fish crude enzyme extracts could indicate the qualities of the diets in fish growth trials (Rungruangsak-Torrissen et al., 2002; Rungruangsak-Torrissen, 2007). Unfortunately, in *C. hainesiana,* no protease activity was observed in alkaline pH (Fig. 2A). Therefore, we could not detect tryptic activity, which is active in alkaline pH. However, for *C. hainesiana,* future studies on *in vitro* protein digestibility of food raw materials in neutral condition of pH 6 should provide reasonably good information for quality of the food raw materials for diet formulations that could be utilized optimally by the mussel. For adult *C. hainesiana,* the *in vitro* digestibility condition for future assessing the qualities of food raw materials for diet formulation should be at 30°C pH 6 for protein and carbohydrate digestion. If lipid digestion would be studied, the *in vitro* digestion condition would be performed separately at 30°C pH 8. These temperature and pH conditions could be used for the *in vitro* digestibility study of food raw materials as well as the formulated diets.

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