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Biochemical characterization of protein-digesting enzymes from viscera of bigfin reef squid (*Sepioteuthis lessoniana*) and implications for *in vitro* protein digestibility

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Abstract

Importance of the work: Proteinase characteristics and digestibility screening can be used for nutritional investigations and the formulation of a suitable diet incorporating bigfin reef squid.

Objective: To investigate the proteolytic activity and *in vitro* protein digestion of enzymes from the viscera of bigfin reef squid.

Materials & Methods: The pH (2–11) and temperature (25–80°C) characteristics, stability, and inhibition of proteinases from viscera extracts of bigfin reef squid were investigated and the *in vitro* digestibility of five alternative protein ingredients were screened.

Results: The highest activity of proteolytic enzymes was at pH 6 and 45°C. Three observed proteinases were stable in the pH range 5–11 and at temperatures of up to 40°C, for exposure times of 30–120 min. An inhibition study indicated that various proteinases were present in bigfin reef squid viscera. Based on *in vitro* digestibility, squid meat and brine shrimp were appropriate sources of alternative protein ($p < 0.05$). Digestibility values did not differ when squid meat was replaced with grade levels of soybean meal or fish meal ($p > 0.05$).

Main findings: The optimal conditions for the major proteinases were pH 6 and 45°C. Based on their characteristics, the major proteinases were most likely chymotrypsin or chymotrypsin-like. The *in vitro* digestibility study suggested that squid meat could be replaced as a protein source with grade levels of soybean meal or fish meal.

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Introduction

Globally, cephalopod production is decreasing because of overfishing (Ospina-Alvarez et al., 2022). Some cephalopods are considered valuable and attractive for aquaculture, including the bigfin reef squid (*Sepioteuthis lessoniana*), the European cuttlefish (*Sepia officinalis*), the Mexican four-eyed octopus (*Octopus maya*) and the common octopus (*Octopus vulgaris*) (Vidal et al., 2014). The bigfin reef squid is a neritic species widely distributed throughout the Indo-West Pacific regions (Lu and Tait, 1983; Walsh et al., 2002). It represents an important marine resource in many regions of the world and is a potential model species for neuroscience, molecular biochemistry and nutritional biochemistry research (Gilbert et al., 1990; Lee et al., 1994). The desirable characteristics of this squid for aquaculture include its large hatching size, tolerance to handling and confinement, short life span and rapid growth rate (Forsythe et al., 2001; Walsh et al., 2002). However, the mariculture of this species is limited by the difficulty of supplying an economic feed (Saekhow et al., 2018).

It is well known that squid are carnivorous, contain high protein and require low lipid requirements (Rosas et al., 2013). The digestive gland of cephalopods has a very high level of enzyme activity that contributes to key roles in digestive processes. In particular, the gland appears to be the main source of the proteolytic enzymes found in the posterior digestive tract (Boucaud-Camou and Roper, 1995), which are essential for digestive processes in both alkaline (trypsin and chymotrypsin) and acidic (pepsin and cathepsin D) conditions (Ibarra-García et al., 2018). The major enzymes from the hepatopancreas of jumbo squid (*Dosidicus gigas*) are cysteine proteinases at pH values of 3–4 and 5–6 (Cardenas-Lopez and Haard, 2005). The proteinases from the hepatopancreas of wild Mexican four-eyed octopus exhibited maximum activity at pH 4, pH 9–10 and pH 10 for total proteinases, trypsin and chymotrypsin, respectively, while the optimal conditions for these enzymes obtained from gastric juice were pH 6, pH 8, and pH 7, respectively (Martinez et al., 2011). For bigfin reef squid, the ontogenic changes of protein-digesting enzymes (pepsin, trypsin and chymotrypsin) were investigated at pH levels of 2, 9 and 9, respectively, without optimizing the favored conditions (including pH and temperature) (Saekhow et al., 2018). Studying these digestive enzymes can help to understand the hydrolysis of main nutrients in the aquaculture of squid species (Ibarra-García et al., 2018).

In vitro digestibility can be used to predict the quality of experimental feeds, as this method is rapid, more economical, simpler than conventional growth studies and has already been utilized for screening feeds in *in vivo* growth trials (Sansuwan et al., 2017; Hahor et al., 2022; Sutthinon et al., 2023). In addition, changes in digestibility are closely related with protein quality (such as solubility, secondary protein structure and disulfide bridges) and the physicochemical properties of feed that directly affect enzymatic digestion (Hahor et al., 2022). Therefore, the current study focused on the characterization of protein-digesting enzymes and the *in vitro* protein digestibility (IVPD) of feed ingredients. The information obtained from enzyme characterization could be used for further investigations into physiological and nutritional responses in bigfin reef squid, while the data from the *in vitro* digestibility study could be used in formulating suitable artificial diets for this species.

Materials and Methods

Chemicals

Casein from bovine milk, bovine hemoglobin, ethylenediaminetetraacetic acid (EDTA), pepstatin A, soybean trypsin inhibitor (SBTI), *N*-*p*-tosyl-*L*-lysine chloromethyl ketone (TLCK), *N*-tosyl-*L*-phenylalanine chloromethyl ketone (TPCK), 1-(*L*-trans-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64), *N*-ethylmaleimide, phenylmethylsulfonyl fluoride (PMSF), *L*-tyrosine, *DL*-alanine, sodium carboxymethyl cellulose and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, USA). Trichloroacetic acid, tris (hydroxymethyl) aminomethane, Folin-Ciocalteu's phenol reagent and acetic acid were obtained from Merck KGaA (Darmstadt, Germany).

Squid specimens and crude enzyme extraction

The animal protocols were approved by the Institutional Animal Care and Use Committee, Prince of Songkla University, Thailand (Project Code 2564-01-075). Freshly caught bigfin reef squid were obtained from Satun province, Thailand. The specimens were transported on ice within 3 hr from capture to the Department of Food Science and Technology, Thaksin University, Phatthalung, Thailand. The viscera of the bigfin reef squid sample were separated, pooled and washed with cold water. The carcasses were soaked in 1% acetic acid at -20°C for 1 min and then cut into pieces with a thickness of 1–1.5 cm. The pieces of carcass were homogenized in an ice box for 3 min with distilled water at

a ratio of 1:5 (weight per volume). To remove tissue debris, the suspension was centrifuged for 30 min at 15,000×*g* and 4°C, using a Sorvall Model RC-B Plus centrifuge (Newtown, CT, USA). The supernatant was collected and referred to as crude enzyme extract (Thongprajukaew et al., 2011).

Characterization of proteolytic enzymes

pH and temperature profiles

Proteolytic activity was measured using a substrate-TCA-Lowry assay (Klomklao et al., 2004). Hemoglobin and casein were used as substrates within pH ranges of 2–5 and 5–11, respectively. The optimal pH was determined at 50°C over the pH range 2–11 (0.2 M McIlvaine's buffer for pH 2–7.5 and 0.1 M glycine-NaOH for pH 8–11). Temperature profiles (25°C, 30°C, 40°C, 45°C, 50°C, 55°C, 60°C, 65°C, 70°C, 80°C) were studied at pH 3, 6 and 8.

pH and thermal stability

The effect of pH on enzyme stability was evaluated by measuring residual activity after incubation at various pH levels for 30, 60 and 120 min at room temperature. The different buffers used were 0.2 M McIlvaine's buffer for pH 2–7.5 and 0.1 M glycine-NaOH for pH 8–11. To investigate the thermal stability of enzymes, the proteolytic activity of the crude enzyme extracts was first analyzed at various temperatures (0–80°C) for 30, 60 or 120 min. Then, the crude enzyme extracts were cooled in iced water. Remaining activity was measured using the substrate-TCA-Lowry method (Klomklao et al., 2004) and reported as the percentage of relative activity compared to the original activity.

Effects of inhibitors

The effects of inhibitors on proteinase activity were determined by incubating viscera extract with an equal volume of proteinase inhibitor solution to obtain the designated final concentration. The inhibitors used were 0.1 mM E-64, 1 mM *N*-ethylmaleimide, 1.0 g/L SBTI, 5 mM TLCK, 5 mM TPCK, 0.01 mM pepstatin A, 5 mM benzamidine, 1 mM PMSF and 2 mM EDTA. After incubating the reaction mixture at room temperature (26–28°C) for 30 min, remaining activity was measured using the substrate-TCA-Lowry method (Klomklao et al., 2004) at pH levels 3, 6 and 8 in 0.2 M McIlvaine's buffer. Then, the percentage inhibition was calculated using the formula $(A_c - A_s / A_c) \times 100$, where A_c and A_s are the control and the sample treated with inhibitor, respectively, with deionized water used as the control, instead of the inhibitor.

In vitro digestibility screening of protein sources

Preparation of feedstuffs and enzymes

The tested ingredients consisted of squid meat (*Loligo* sp., 74.5% crude protein on dry basis), shrimp meat (*Litopenaeus vannamei*, 59.4% crude protein on dry basis), brine shrimp (*Artemia salina*, 57.2% crude protein on dry basis), fish meal (60% crude protein) and de-hulled soybean meal (45% crude protein). Commercially available fish and soybean meals were purchased from Phatthalung Livestock Co., Ltd., Phatthalung, Thailand. All the samples were ground, sieved, freeze-dried for 24 hr, packed in polyethylene bags and kept in desiccators for later analysis of *in vitro* digestibility. For comparison with single-ingredient feeds, mixed-ingredient feeds were also prepared. Dried squid meat (25%, 50%, 75%) was proportionally mixed with grade levels of either fish meal or soybean meal (75%, 50%, 25%, respectively). These mixtures were designated as SQ1, SQ2 and SQ3, respectively.

Crude enzyme extracts were dialyzed (based on a molecular weight cut-off of 10 kDa) overnight against 50-fold volumes of 0.2 M McIlvaine's buffer (pH 6) at 4°C. The dialyzed enzymes were stabilized and concentrated overnight using sodium carboxymethyl cellulose at 4°C (Velderrain-Rodríguez et al., 2019). Before performing the *in vitro* digestibility study, the optimal proteinase concentration was investigated using casein as a substrate. The concentrated enzymes were diluted to obtain 80, 60, 40, 20 or 8 U/mg protein, and then kept at -20°C until used (Hahor et al., 2022).

In vitro protein digestibility

The *in vitro* protein digestibility (IVPD) reaction was performed as described by Thongprajukaew et al. (2011), with slight modifications. The reaction mixtures contained 5 mg of feed ingredients, 10 mM of 50 mM phosphate buffer (pH 6.0), 50 µL of 0.5% chloramphenicol and 125 µL of dialyzed enzyme. The reaction was incubated at the optimal temperature (45°C) for 24 hr. After terminating the enzymatic reaction at 100°C for 10 min, IVPD was determined by measuring the increase in the liberated reactive amino groups of cleaved peptides. The reaction mixture contained 100 µL of digested solution, 1 mL of 50 mM phosphate buffer (pH 8) and 0.5 mL of 0.1% trinitrobenzenesulphonic acid. After incubation in the dark at 60°C for 1 hr, 0.5 mL of 1 M HCl was added and absorbance was measured at 420 nm. Spectrophotometric absorbance was compared with the *DL*-alanine standard curve.

Statistical analysis

A completely randomized design was used throughout. Data were subjected to analysis of variance and means comparison was carried out using Duncan's multiple range test. Data analyses were performed using SPSS version 14 software (SPSS Inc.; Chicago, USA). All data were expressed as mean ± SD values from triplicate observations and significance was tested at the $p < 0.05$ level.

Results and Discussion

pH and temperature profiles

The proteinases from the viscera extracts of bigfin reef squid had three activity peaks. The major activity occurred at an optimal pH of 6, while minor activities occurred at pH 8 and 3 (Fig. 1A).

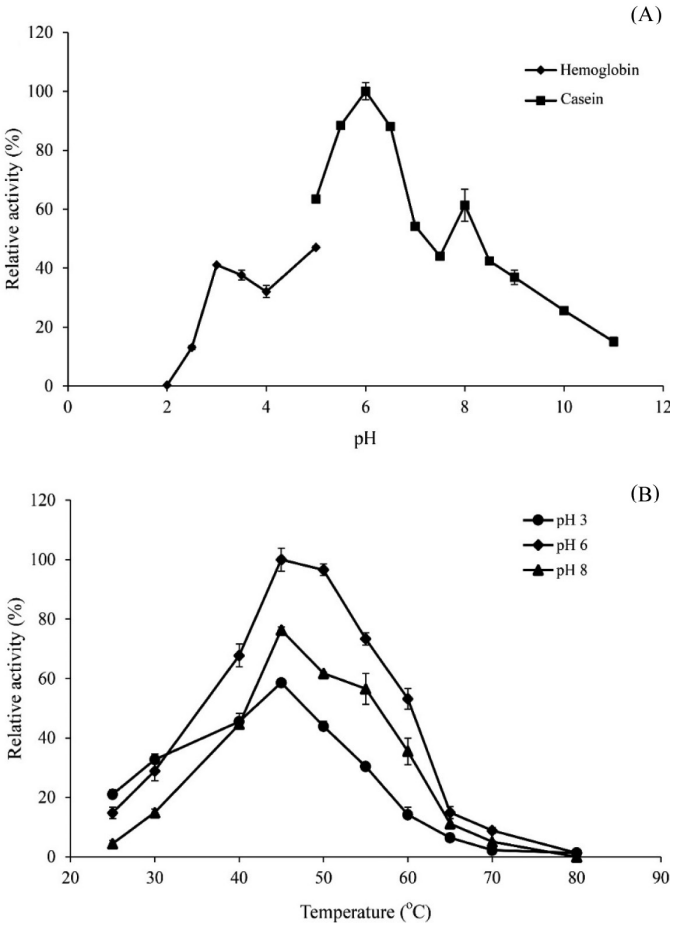

Fig. 1 Proteinase activity of crude enzymes extracted from bigfin reef squid viscera: pH profile (A); temperature profile (B), with hemoglobin and casein used as substrates within the pH ranges 2–5 and 5–11, respectively, and error bars represent ± SD from triplicate determinations.

The major activity involved acidic proteinase, while the other peaks mostly involved alkaline and strongly acidic proteinases. The presence of acid and alkaline proteinases in internal organs of various cephalopod species has been reported. For example, the digestive gland of the California two-spot octopus (*Octopus bimaculoides*) had the highest activity of total proteases at pH 3 (Ibarra-García et al., 2018), with a significant presence of acidic proteinase in this organ. The same pattern was reported for the digestive gland of the Mexican four-eyed octopus, suggesting the presence of aspartic protease, probably cathepsin type D (Martinez et al., 2011). Similar results have been reported for other species of cephalopods, such as in the digestive gland of the jumbo squid, where the highest peaks of protease activity were reported at pH 3 and pH 5, suggesting these two isoforms might be cathepsins (Cardenas-Lopez and Haard, 2005). Two other peaks of enzyme activity were observed, with one isoform between pH 3 and 4 and another between pH 5 and 6, suggesting the presence of cathepsins B, H and L (Cardenas-Lopez and Haard, 2005).

The temperature profile of proteinase activity was determined at pH 3, 6 and 8. At all three pH levels, activity increased from 25°C to 45°C and then decreased at higher temperatures (Fig. 1B). The decrease in activity above 45°C might have been due to the thermal denaturation of enzymes. The optimal temperature (45°C) from the current study was similar to optimal trypsin characteristics at 50°C to 60°C in the California two-spot octopus (Ibarra-García et al., 2018) and to the chymotrypsin characteristic peak at 55°C in the European cuttlefish (Balti et al., 2012). The results of these various studies showed that species-related enzymes had adapted to work in a wide range of temperatures. The high temperatures tested in the current study were outside the natural range that the tested species inhabits and could affect enzyme conformation, which is governed by habitat, environment and genetics (Klomklao et al., 2006). Based on the pH and temperature characteristics observed in the current study, the major proteinases extracted from bigfin reef squid viscera were characterized as heat-activated acidic proteinases.

pH and thermal stability

The proteinase from viscera extract of bigfin reef squid was stable in the pH range 5–11 for exposure times of 30–120 min. At all pH levels, residual activity was greater than 85% (Fig. 2A). Proteinase activity was lost to some extent for longer incubation times, with the loss of activity being more noticeable at pH levels below 5. These findings were consistent with the

behavior of trypsin from the hepatopancreas of cuttlefish that was reported to be extremely stable in the pH range 6–10 and highly stable up to 50°C, after 1 hr of incubation (Balti et al., 2009). In general, the stability of the enzyme at a particular pH is related to the net charge of the enzyme at that pH (Klomklao et al., 2006). At extreme pH levels, strong intramolecular electrostatic repulsion, caused by the high net charge, results in swelling and unfolding of protein molecules (Damodaran, 1996).

The proteinase enzyme was stable when incubated at temperatures up to 40°C for 30–120 min (Fig. 2B). Nevertheless, sharp decreases in activity were observed at temperatures above 40°C. No activity remained at 70°C, suggesting complete loss of activity caused by thermal denaturation of the enzyme. In general, the stability of proteinase decreased as the heating time increased. At every temperature above 40°C, the loss of activity was highest for a heating time of 120 min.

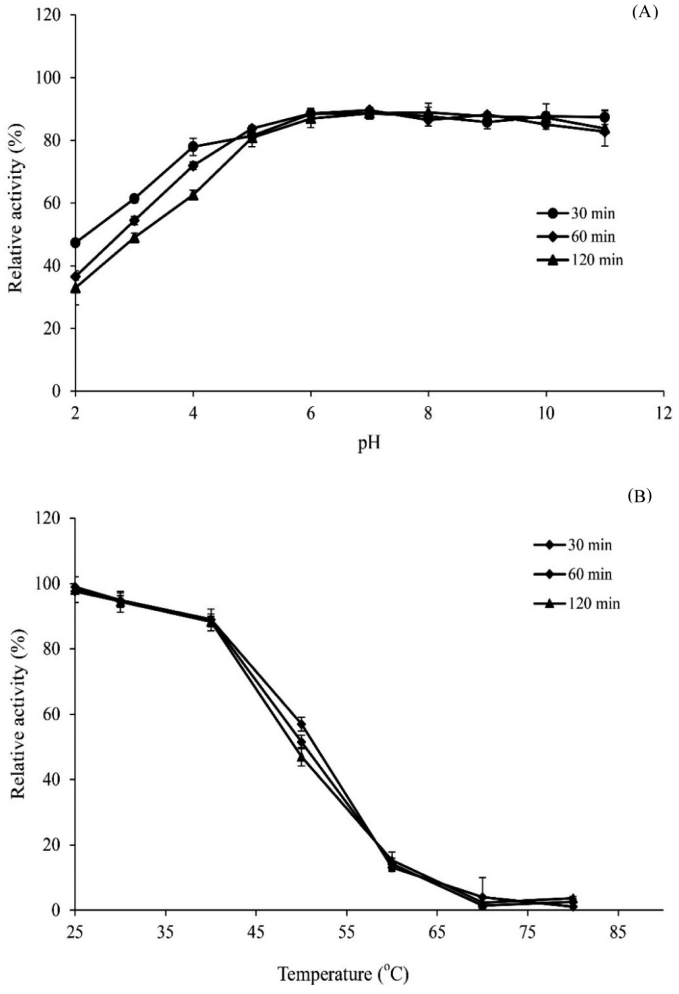

Fig. 2 Proteinases from crude enzymes extracted from bigfin reef squid viscera: (A) pH profile; (B) thermal profile, with error bars representing ± SD from triplicate determinations

It was concluded that proteinases from bigfin reef squid viscera underwent denaturation during high temperature heating, especially for longer times. The observed behavior was in accordance with the reported behavior of cuttlefish chymotrypsin above 50°C (Balti et al., 2012). Enzymes are inactivated at high temperature due to the partial unfolding of the protein molecules, with the thermal stability of proteins being increased by strengthened hydrophobic interactions and disulfide bonds in the structure of the protein (Kim et al., 1994) and by the number of disulfide linkages and strong hydrophobic interactions in the internal protein structure (Klomklao et al., 2009).

Effects of inhibitors

The effects of various inhibitors on the proteinase activity of bigfin reef squid viscera extracts were determined at pH 3, 6 and 8 (Table 1). At pH 3, the proteolytic activity was strongly inhibited by pepstatin A (78% inhibition), identified as aspartic proteinase. At pH 8, TLCK, a specific inhibitor for trypsin, was the major inhibitor of proteolytic activity, contributing 87.6% inhibition. PMSF (a serine proteinase inhibitor) and TPCK (a chymotrypsin inhibitor) also inhibited proteolytic activity at pH 8. The proteolytic inhibitory activity of TPCK was strongest at pH 6 (63.7%). Although the proteolytic activity was most strongly inhibited by serine inhibitor groups at pH 6, the proteolytic activity was also inhibited by pepstatin A (35.2%). The obtained results indicated that the major proteinases extracted from the viscera of bigfin reef squid were serine proteinases, particularly chymotrypsin or chymotrypsin-like enzymes.

Table 1 Effects of various inhibitors on activity of proteinases from bigfin reef squid viscera

Inhibitor	Concentration	% Inhibition		
		pH 3	pH 6	pH 8
Control		0	0	0
E-64	0.1 mM	9.4±2.9	10.2±3.3	10.2±1.9
N-ethylmaleimide	1 mM	8.1±1.0	12.5±0.6	3.1±3.9
SBTI	1.0 g/L	0	17.0±4.0	8.4±3.4
TLCK	5 mM	6.0±2.8	29.2±2.2	87.6±2.5
TPCK	5 mM	5.6±2.5	63.7±1.6	44.1±3.4
Benzamidine	5 mM	0	1.9±0.2	0
PMSF	1 mM	8.1±2.8	53.8±0.2	64.6±2.3
Pepstatin A	0.01 mM	78.0±1.9	35.2±2.3	7.4±0.8
EDTA	2 mM	1.3±2.5	12.7±0.8	21.6±0.8

E-64 = 1-(*L*-trans-epoxysuccinyl-leucylamino)-4-guanidinobutane; SBTI = soybean trypsin inhibitor; TLCK = *N*-*p*-tosyl-*L*-lysine chloromethyl ketone; TPCK = *N*-tosyl-*L*-phenylalanine chloromethyl ketone; PMSF = phenylmethylsulfonyl fluoride; EDTA = ethylenediaminetetraacetic acid. Data presented as mean ± SD from triplicate determinations

Serine proteinases from the intestine of the discus fish (*Symphysodon aequifasciata*) were inhibited by SBTI, PMSF and TLCK, while TPCK and EDTA produced partial inhibition (Chong et al., 2002). In salivary glands and gastric juices, most of the proteolytic activity during extracellular digestion is carried out by serine proteinases, as indicated by the approximately 90% inhibition caused by serine proteinase inhibitors (PMSF, SBTI, TPCK, TLCK) in other studies works (Martinez et al., 2011; Hamdan et al., 2013; Mancuso et al., 2014). The aspartic proteinase from the hepatopancreas of the Mexican four-eyed octopus was inhibited by 72% in the presence of pepstatin A. This characteristic suggested the presence of an aspartic proteinase such as pepsin or cathepsin D (Martinez et al., 2011). Normally, pepstatin A, an aspartic proteinase inhibitor, can bind with pepsin that prevents the binding of enzyme to substrate, resulting in complete inhibition of acidic proteinase (Wu et al., 2009; Zhao et al., 2011). In addition, trypsin activity from the hepatopancreas of cuttlefish was reported to be strongly inhibited by SBTI and PMSF (Balti et al., 2009). Since PMSF performs by sulfonylating the hydroxyl groups at active site serine residues of enzymes, while TLCK and TPCK strongly inhibit at histidine residues (James, 1978; França et al., 2016), these three inhibitors can covalently interact with active enzyme sites and block enzyme-substrate binding (Pavlisko et al., 1997; França et al., 2016). These observations indicated the variety of proteolytic enzyme patterns present in cephalopods and other aquatic animals. Based on this brief review of the relevant literature, enzyme purification and further molecular investigation are needed to clarify the findings from the current study.

Assessment of protein bioavailability through in vitro digestibility technique

Based on the characteristic study of protein-digesting enzymes, the *in vitro* digestibility was conducted at the suitable conditions of pH 6.0 and 45°C. The optimal proteinase concentration in crude enzyme extracts from the viscera of bigfin reef squid is shown in Fig. 3A. IVPD increased significantly within the range 8–60 U/mg protein. There were no significant differences among the observed values in the range from 60 U/mg protein to 80 U/mg protein. This finding suggested that the optimal proteinase concentration for IVPD was 60 U/mg protein.

The IVPD values of different protein sources using crude enzyme extracts from the viscera of bigfin reef squid are shown in Fig. 3B. Squid meat and brine shrimp had the highest IVPD levels ($p < 0.05$), followed by shrimp meat, soybean meal and fish meal, with the latter three having similar IVPD values ($p > 0.05$). In a study of the common octopus (Morillo-Velarde et al., 2012), a diet containing the principal ingredient of European flying squid (*Todarodes sagittatus*) meat promoted good growth and digestibility. In the current study, although brine shrimp had a relatively high level of IVPD, the limited production of this ingredient would complicate its inclusion in an artificial diet.

Although the IVPD values of the mixed feeds were significantly lower than the values of squid meat alone, the IVPD values did not differ across grade levels of squid meat (25–75%) combined with soybean meal or fish meal ($p > 0.05$, Fig. 3C). These results indicated the feasibility of including soybean meal or fish meal in artificial diets for bigfin reef squid. Based on regression analysis across a number of fish species, both soybean meal and fish meal reportedly contained high amounts of protein (35.8–56.2% and 48.1–83.7, respectively), providing ranges of 49.7–99.4% and 56–99%, respectively, for the apparent protein digestibility coefficient

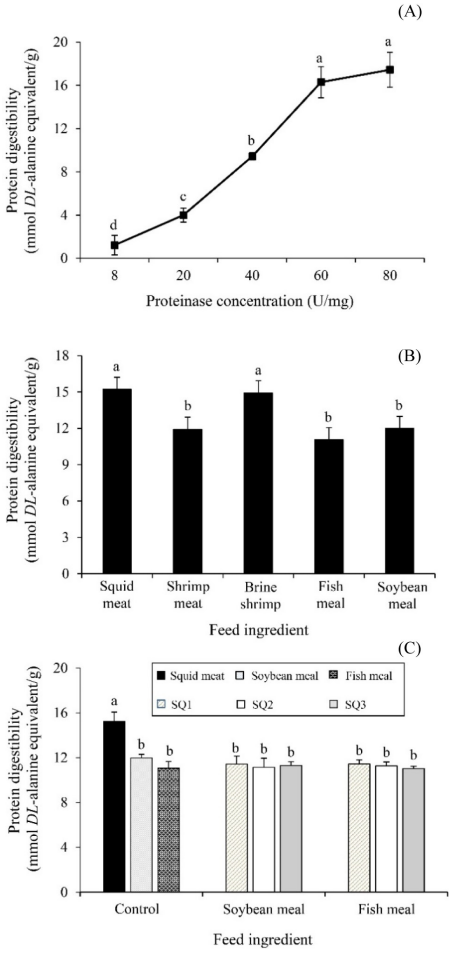

Fig. 3 Determination using dialyzed crude enzyme extract from bigfin reef squid viscera of *in vitro* protein digestibility of: (A) casein; (B) feed ingredients; (C) feed ingredient ratios, where dried squid meat (25, 50, 75%) was proportionally mixed with grade levels of either fish meal or soybean meal (75, 50, 25%, respectively) and designated as SQ1, SQ2 and SQ3, respectively, error bars in (A) represent ± SD and elsewhere represent + SD, all from triplicate determinations and significant ($p < 0.05$) differences between groups are indicated by different lowercase letters above bars.

(Sales, 2008). Although an artificial diet for bigfin reef squid has never been reported, some diets given to cephalopods have included commercially available feed ingredients, such as soy protein meal (Castro et al., 1993), fish powder and protein hydrolyses (Domingues et al., 2008; Ferreira et al., 2010), shrimp diet (Domingues et al., 2007) and fish meal, soybean concentrate, krill meal, poultry meal, wheat gluten and corn gluten (Ma et al., 2022). Recently, newly hatched bigfin reef squid were reported to accept a moist diet, with IVPD testing suggesting that Indian mackerel (*Rastrelliger kanagurta*) fillet and Indian anchovy (*Stolephorus indicus*) fillet were suitable alternative protein sources, followed by yellow-stripe scad (*Selaroides leptolepis*) fillet and Pacific white shrimp (*L. vannamei*) fillet (Chanlek et al., 2023). A moist diet based on screened protein ingredients should be of interest for future feeding trials.

In conclusion, from the current results, the crude enzyme extract from the viscera of bigfin reef squid was active in an acidic environment at around pH 6, with the enzyme proteinase activity maximal at 45°C. The results of the inhibition study indicated it was most likely that the major proteinase in the extract was chymotrypsin or chymotrypsin-like. However, enzyme purification and further molecular investigation should be undertaken to clarify the findings in the current study. The results of *in vitro* digestibility testing using the crude enzyme extract suggested that squid meat and brine shrimp were the most suitable protein sources. When squid meat was replaced with grade levels of commercially available soybean meal and fish meal, the results indicated that both ingredients could be included in artificial diets for bigfin reef squid. However, various circumstances that affect feed ingredient quality might have directly influence the *in vitro* digestibility results. Preparation of experimental diet using these types of feedstuffs and performing *in vivo* feeding trial in bigfin reef squid should be of interest.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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