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Isolating of the endogenous proteases presenting in the shrimp head of *Litopenaeus vannamei* and their enzymology characterization

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Abstract

The aim of this study was to isolate the endogenous proteases in the shrimp head of Litopenaeus vannamei and investigate their enzymology characterization. The results show that there are10 types of endogenous proteases existing in the shrimp head, which are successively identified as chymotrypsin, cathepsin L, collagenase, calpain, pepsin, metalloprotease, trypsin, serine protease, cathepsin B and MBSP, respectively. In addition, pH and temperature have a significant impact on the activities of proteases P1, P4-P8, and their optimal pHs and temperature are in the range of 6.0-9.0 and 30-40 °C, respectively; proteases P5-P7 own better stability than that of other proteases; the optimal temperature for P1, P4-P8 stability is 30 °C, and that of other proteases is 40 °C. In conclusion, proteases P1 to P10 are isolated and characterised, which is an important support to develop high-quality condiments and efficiently recover proteins from shrimp head waste.

Keywords: *Litopenaeus vannamei*, endogenous protease, enzymology characterization, gelatin-PAGE

1. Introduction

Accounting for approximately 35–45% of the whole shrimp weight, shrimp head waste is the main by-product of processing headless and peeled prawns (MEYERS. [7]). It is estimated that the shrimp-head output in China is up to 200,000 tons per year, and 70% of which is *Litopenaeus vannamei* (LIU & al.[8]).Shrimp head contains abundant proteins (50–65%, dry weight basis),chitin (11%,dry weight basis), enzymes and other nutrients (SUBASINGHE[9]; FANIMO & al.[10]; SYNOWIECKI & al,[11];HEU& al.[12]). Therefore, the efficient utilization of shrimp industry waste has attracted a great concern in recent years.

The recovery of protein fractions, which are main components of shrimp h ead waste, has been receiving an increasing interest. The proteins recovered in the form of hydrolysates can be used as flavouring and incorporated into shrim p-based foods, aquaculture feed or as a nitrogen source in growth media for m icroorganisms (CAVALHEIRO &al.[13]). To produce different flavours, the activi ties of endogenous proteases have been adjusted by optimizing the fermentation conditions such as temperature, pH, and the ratio of water to material (CAO & al.[14]). In addition, the hydrolysates in shrimp head waste are good sources of biologically active peptides, which have a considerable potential in pharmac ology (GILDBERG & al[15]). Recovery of protein fractions in shrimp head wa ste has extensively been studied by using enzymatic hydrolysis methods (SIMP SON &al.[16]; CANO-LOPEZ& al.[17]). Certain proteolytic enzymes such as al calase (MIZANI& al.[18];GUERARD&al.[19])and trypsin (SYNOWIECKI & al. [11]) have been used to extract the proteins from shrimp head waste.

Such processes currently used to extract protein fractions from shrimp head waste are extremely expensive due to the high cost of commercial enzymes. To develop an efficient and economical method for extracting proteins from shrimp head waste, it is necessary to analyse the endogenous proteases in shrimp heads. Under certain conditions, these endogenous proteases could easily lead to autolysis of shrimp heads, and this phenomenon also occurred in many species of fish (MUKUNDAN &al.[20]).Autolysis has successfully been utilized to produce fish-protein hydrolysates (LIU &al.[21]; YOSHIOKA &al.[22]; SAMARANAYAKA &al.[23]); EAKPETCH &al.[24]) reported the autolysis of Pacific white shrimp meat.(CAO & al.[25]) developed a new method of autolysis by gradual temperature for recovering

proteins from shrimp head waste, and also evaluated the nutritional value of the autolysis hydrolysate as a spice in food supplements. However, there is little information on the types, contents and properties of endogenous proteases in the shrimp head of *L. vannamei* due to no effective method for isolating and identifying them. Consequently, several key problems such as optimal fermentation conditions, duration and protease dosage remain mysterious. Therefore, the development of an effective method for isolating and characterising endogenous proteases is critical for cost-effectively utilizing shrimp head waste. In this paper, we report a novel method to effectively isolate the endogenous proteases in the shrimp head of *L.vannamei*, and their enzymology characterization was investigated.

2. Materials and Methods

2.1 Materials

2.1.1 Shrimp

L.vannamei was bought in a local shrimp market. They were washed thrice with sterile water. The shrimp heads were sampled and stored in the sealed plastic bags at –20 °C until they were used for extracting enzymes.

2.1.2 Chemicals

Sephadex G-100 was purchased from GE Healthcare Life Sciences Co., Ltd. (Beijing, China). Trypsin inhibitor from *Glycine max* (SBTI) was purchased from CNW Technologies GmbH (Munich, Germany).Phenylmethylsulfonyl fluoride (PMSF), ethylene diamine tetraacetic acid (EDTA) and tosyl-phenylalanine chloromethyl-ketone (TPCK) were purchased from Sigma-Aldrich Co., LLC (St. Louis, USA). Substrate hemoglobin for pepsin, Fluorogenic substrates N-succinyl -Leu-Tyr-AMC for calpain, Z-Phe-Arg-AMC for cathepsin L, Z-Arg-Arg-AMC for cathepsin B were bought from Sigma Chemical Co.(St. Louis, USA). Fluorogenic substrates Boc-Phe-Ser-Arg-AMC for MBSP,succinyl-Gly-Pro-Leu-Gly-Pro-AMC for collagenase were bought from Peptide (Japan). All the other chemicals used were commercial products with the analytical reagent grade. Deionized water was used for preparing all solutions.

2.2 Preparation of a crude enzyme extract

The shrimp heads (250 g) were homogenized for 120 s with 500 ml buffer A (10 mM Tris–HCl, 10 mM $CaCl_2$, pH 7.5). The mixture was then centrifuged at 10,000 ×

g for 20 min at 4 °C. The pellet was discarded, and the supernatant was filtrated through a Waterman No. 1 filter to yield a crude enzyme extract, which was stored at –80 °C.

2.3 Gel chromatography

The crude protease extract was purified by ammonium sulphate fractionation, and the precipitate in the saturation range of 30–60% was collected by centrifugation for 15 min at 10,000 × *g*. The precipitate was suspended in buffer A, and dialysed for 24 h at 4 °C against the same buffer, which was repeatedly changed. The dialysed precipitate was then heat-treated in a water bath at 50 °C for 30 min with continuous stirring and followed by immediate cooling in ice-water. The resulting precipitate was discarded after centrifugation at 10,000 × *g* for 10 min at 4 °C. The heat-treated enzyme was then subjected to gel filtration on a Sephadex G-100 column (2.6 × 100 cm) pre-equilibrated with buffer B (25 mM Tris–HCl buffer, pH 8.0 containing 0.05% Triton X-100). Enzyme fractions (5 ml) were eluted at a flow rate of 28 ml h^{-1} with the same buffer. The protease activity of each fraction was measured. These fractions with the protease activities were collected, and stored at –20 °C for further analysis. All of the purification steps were conducted at temperatures below 4 °C.

2.4 Gelatin protein electrophoresis

Firstly, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) containing 1% gelatin was used to separate protein components, and was performed as previously described (WU & al.[26]). Secondly, the protease activity recovered in the buffer containing divalent metal ions, led to the hydrolysis of the gelatin in the protease location. Finally, the gels were stained with 0.25% Coomassie Brilliant Blue R250 in a 45% ethanol–10% acetic acid mixture for 3 h and destained using a 5% ethanol–7.5% acetic acid mixture for 2 h. White stripes were visible on the gel under the blue staining. The intensities of white stripes were proportional to their corresponding protease activities.

2.5 Analysis of proteolytic activities

The protease activity was measured according to the method described by KEMBHAVI&al.[27]) using casein as a substrate. Briefly, a 0.5-ml aliquot of the enzyme, suitably diluted, was mixed with 0.5 ml of 100 mM Tris–HCl buffer containing 1% casein and incubated for 15 min at 60 °C. The reaction was stopped by the addition of 0.5 ml trichloroacetic acid (20%). The mixture was then allowed to stand at room temperature for 15 min before being centrifuged at 10,000 × *g* for 15

min to remove the precipitate. The absorbance was measured at 280 nm. A standard curve was generated using the solutions of 0–50 mg L^{-1} tyrosine. One unit of the protease activity was defined as the amount of an enzyme required to liberate 1 μg of tyrosine ml^{-1} in 1 min under the experimental conditions. To determine pH and temperature profiles of each endogenous protease, the activity of the enzymes was analyzed over a range of different temperatures (from 20 to 80 °C with an interval of 10 °C) and pH (from 5.0 to 11.0 with an interval of 1.0)

2.6 Effect of temperature on enzyme stability

Experiments were conducted to study the thermal stability of protease enzymes. The deactivation of each endogenous protease was carried out at temperature of 20, 30, 40, 50, 60, 70 and 80 °C under optimal pH. Aliquots of samples were taken at different intervals of time, and then cooled on ice. Residual enzyme activity of samples after deactivation was measured in standard assay conditions. The deactivation of protease enzymes is assumed to follow first-order kinetics, and the half life time of each endogenous protease is calculated as previously described (BHUNIA & al.[28]).

2.7 Determination of inhibition ratios

Four specific protease inhibitors of 5 mM PMSF, 100 mg L^{-1} SBTI, 10 mM TPCK and 10 mM EDTA were selected to further confirm the types of endogenous proteases in the crude enzyme extract. The crude enzyme extract was mixed with a selective inhibitor of the protease using casein as a substrate and incubated for 1 h at 26 °C. The reaction was stopped by the addition of 0.5 ml trichloroacetic acid (15%). The reaction buffer (100 mM Tris–HCl) at optimal pH, adjusted by the addition of HCl or NaOH, and the method for determining the protease activity was the same as described above. The inhibition ratio can be calculated using the following formula:

$$\text{Inhibition Ratio}(\%) = \frac{\text{Specific activity without inhibitors} - \text{Spcific activity with inhibitors}}{\text{Specific activity without inhibitors}} \times 100$$

2.8 Determination of specific proteolytic activities

Pepsin activity was assayed by measuring the extent of hydrolysis of denaturated hemoglobin solution after incubation for 10 min at 37 °C (MERIDOR & al.[29]). The undigested hemoglobin was precipitated with trichloroacetic acid. After filtrating the resulted precipitate, the absorbance of the soluble peptides, present in the filtrate, was measured at 280 nm.

The activities of myofibril-bound serine proteases (MBSP), cathepsin B,

cathepsin L, collagenase and calpain were determined as previously described (MERIDOR &al.[29]; YANG & al.[5]). Briefly, appropriate assay buffer (100 μl) (Buffer A for cathepsins: 150 mM bis-Tris, 30 mM EDTA, 6 mM DTT, pH 6.0; Buffer B for collagenases and calpains: 150 mM bis-Tris, 7.5 mM $CaCl_2$, pH 6.0; Buffer C for MBSP: 150 mM bis-Tris, pH 8.0) was mixed with enzyme extract (100 μl) and the mixture was incubated at 30 °C for 10 min. The reaction was started with the addition of 100 μl 0.09 mM substrate (substrate was dissolved in distiled water). After 15 min of incubation at 30 °C, the reaction was stopped by adding 3.0 ml stopping solution (1% SDS 50 mM bis-Tris buffer, pH 7.0). The solutions were immediately put on ice. Fluorescence of AMC was measured after 10 min at 360 nm (10 nm slits) excitation wavelength and 460 nm(10nm slits) emission wavelength (Fluorescence Spectrometer 3000, Perkin Elmer, UK). The analyses were run in triplicate. The blank was prepared using 100μl corresponding buffer instead of enzyme extract. Activities were expressed as the increase in fluorescence and given in arbitrary units (U) based on the mean of three measurements.

2.9 Statistical analysis

The analyses were performed in triplicate. Mean values and standard deviation were calculated for the different individual samples using Excel (Microsoft Office, USA), and the results were presented as means±SDs.SPSS 11.0 for windows software was used for statistical analysis (SPSS Inc., Chicago, Illinois, USA), and P-values $\leq$ 0.05 were considered to be statistically significant.

3. Results and Discussion

3.1 Isolation and purification of endogenous protease components

The crude enzyme extract of shrimp head could not directly be analysed by SDS-PAGE or gelatin-PAGE due to the existence of abundant impurities, especially pigments. Therefore, this extract had firstly been separated by gel filtration on a Sephadex G-100 column, and then each fraction was further characterized. The enzymatic activities of 20 fractions collected by gel filtration from the extract were determined under acidic, neutral and alkaline conditions. The measurements showed that 15 fractions possess different protease activities (Figure 1). It can be seen that fractions 3 to 5, 13 to 15, and 17 own a higher protease activity under alkaline conditions than those on neutral or acidic conditions, but no significant difference

between neutral or acidic conditions; fractions 6 and 16 have no significant difference in the protease activities in different pH values; fraction 7 and 8 have lower protease activities under acidic conditions than those under neutral and alkaline conditions, but no significant difference between neutral and alkaline conditions; fractions 9 and 10 exhibit a significant pH-dependent change with the highest protease activity under neutral conditions, followed by alkaline and then acidic conditions; fraction 11 owns a higher protease activity under acidic conditions than those on neutral or alkaline conditions, but no significant difference between neutral or alkaline conditions; fraction 12 owns a higher protease activity under neutral conditions than those on alkaline or acidic conditions, but no significant difference between alkaline or acidic conditions.

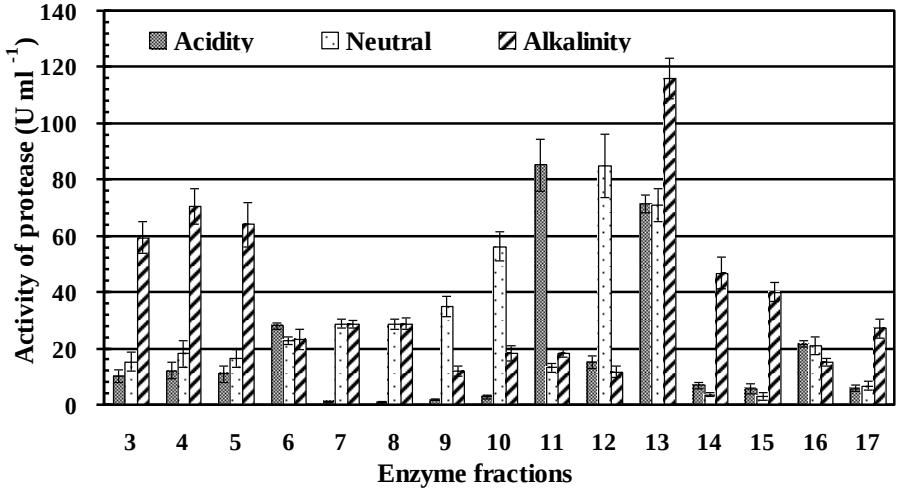

Figure 1. Endogenous protease activities in fractions 3 to17 extracted from the head of *L. vannamei* under acidic, neutral and alkaline conditions.

Notes: One unit of the endogenous protease activity was defined as the amount of an enzyme required to liberate 1μg tyrosine ml^{-1} in 1 min at 50°C. All data are the mean values of three individual determinations, and are presented as the mean ± SD.

3.2 Zymogram identification of endogenous proteases

Gelatin electrophoresis conduces to detect the activities of endogenous proteases. The gelatin electrophoresis patterns of fractions 3 to 17 separated from the crude protease extract by gel filtration are illustrated in Figure 2, and a single white stripe exhibits in every gel lane. This result indicates fractions 3 to 17 all have contributions to the observed hydrolysis activity of gelatine. Because the white stripes occurring at the same location may be regarded as the same endogenous protease, we thus conclude that 10 types of endogenous proteases exist in fractions 3 to 17; fractions 3 to 5, 7-8, 9-10 and 14-15 represent the same protease, respectively; and each of the

others contains a single distinct protease. The endogenous proteases of fractions 3-5, 6, 7-8, 9-10, 11, 12, 13, 14-15, 16 and 17 are named P1 to P10, respectively.

Although many investigators have made important contributions to the studies of endogenous proteases in the shrimp head (ZHANG&al.[30]; NOVILLO&al.[31]; ANWAR & al.[6]; P ÇALIK&al.[1]), its endogenous proteases have roughly been subdivided into three categories, i.e., alkaline, neutral and acidic enzymes, and only a few have been isolated, purified and characterized. To promote the activity of a favourable protease and inhibit the activity of an undesired protease, it is necessary to clearly identify and characterize endogenous proteases in shrimp head waste.

Based on gel filtration and gelatin-PAGE, a novel strategy with four steps may be proposed to isolate and identify endogenous proteases in shrimp heads. Step 1 is to homogenize shrimp heads for yielding a crude enzyme extract by filtration. Step 2 is to purify the crude enzyme extract by ammonium sulphate fractionation, and collect the precipitate in the saturation range of 30–60%. Step 3 is to separate endogenous protease components by gel chromatography at temperatures below 4 °C. Step 4 is to identify and characterize the endogenous proteases by gelatin protein electrophoresis, which is one key procedure for the above strategy (Figure 2). With this new method, we has successfully achieved to isolate, purify and identify 10 types of endogenous proteases in the crude enzyme extract from the shrimp head of *L.vannamei*, which is significant for elucidating the structure of proteases, cloning and expressing their related genes, and producing endogenous protease on a large scale.

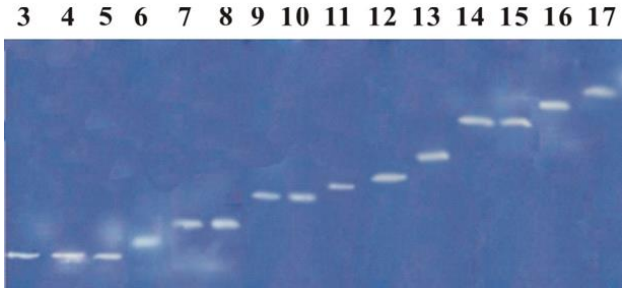

Figure 2. Gelatin electrophoresis of fractions 3 to 17 separated from a crude enzyme extract.

Notes: Numbers 3 to 17 represent the enzyme fractions collected by gel filtration.

3.3 Effect of pH and temperature on enzymatic activity and stability

As shown in Figure 3A and 3B, P1 exhibits a significant pH-dependent change with the highest protease activity at pH 8.0; P2 is shown to have optimal pH at 5.0 and has no significant difference in the protease activities in the pH range of 5.0-10.0; P3 and P4 are shown to have optimal pH at 7.0, and remain less than 5% of the

highest activity at pH 5.0. P5 has no significant difference in the pH range of 7.0-11.0, and the enzymatic activity suddenly increases up to 97.87 U/ml at pH 6.0; P6 has the highest activity at pH 7.0, which is five times than those in the other pHs; P7 has the optimal pH at 10.0, and more than 70% of the highest activity remains at acidic and neutral conditions; the activities of P8 and P10 at pH 8.0-9.0 far above those in the other pHs; P9 has no significant difference in the pH range of 5.0-8.0, and loses activity at pH 11.0. Above all, pH has a significant impact on the activities of P1, P4, P5, P6, P7 and P8, and a very small impact on the activities of P2, P3, P9 and P10.

The effects of temperature on the enzymatic activity are shown in Figure 3C and 3D. It is shown that P1, P5, P7 and P8 have the highest activities at 30 °C; P4 and P6 have the optimal temperature of 40 °C; P2, P3, P9 and P10 have no significant difference in the temperature range of 30-50 °C. However, the effects of temperature on the activity of 10 types of endogenous proteases have a same tendency. The activities of all endogenous proteases dramatically increase the highest, and then gradually decrease to the lowest at 70 °C; from 70-80 °C, the activities of all endogenous proteases have no significant change.

The effects of temperature on half-life time of each endogenous protease have been studied and the results are shown in Fig. 3E and 3F. It is found that half-life times of P1-P10 are 14.18 h, 4.76 h, 6.81 h, 7.41 h, 21.51 h, 19.55 h, 23.39 h, 12.91 h, 4.38 h and 7.52 h at optimum conditions of pH and temperature, respectively, which indicate that P5, P6 and P7 own better stability than that of other proteases. It also is shown that the optimal temperature for stability of P1, P4-P8 is 30 °C, and that of other proteases is 40 °C. In addition, the effects of half-life times of 10 types of endogenous proteases have a similar tendency. The half-life times of all endogenous proteases increase the highest, and then gradually decrease to the lowest at 60-70 °C; from 60-80 °C, the half-life times of all endogenous proteases are very short and have no significant change, which indicates endogenous proteases in shrimp head would likely be suitable for recovering proteins from shrimp head waste under temperature of 30-40 °C.

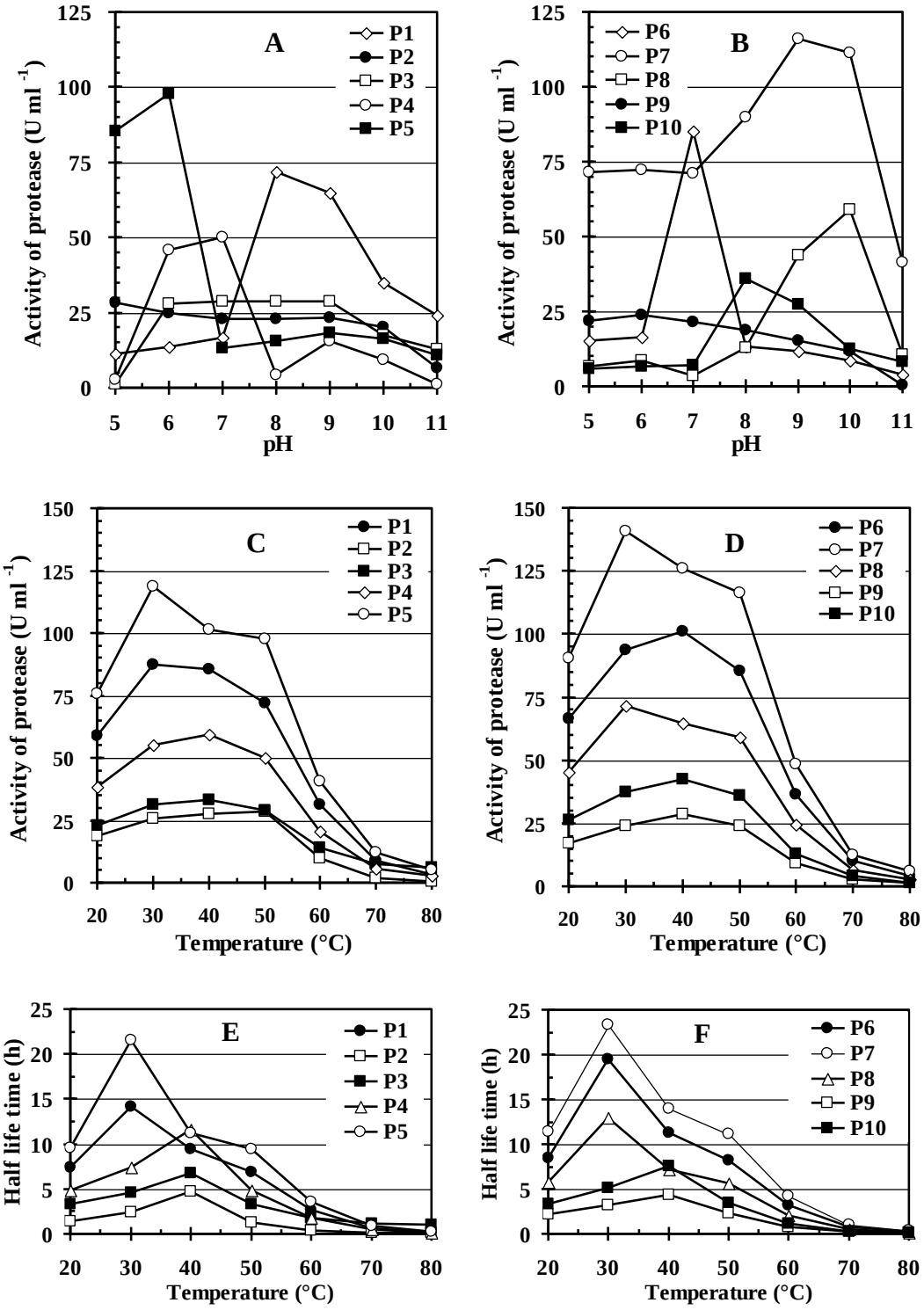

Figure 3.Effect of pH and temperature on enzymatic activity and stability. (A, B) Effect of pH on activity; (C, D) Effect of temperature on activity; (E, F) Effect of temperature on stability under optimal pH

Notes: P1-P10 represent fractions 3-5, 6, 7-8, 9-10, 11,12,13,14-15, 16 and 17, respectively.

3.4 Characteristics of endogenous proteases in shrimp heads

The effects of enzyme inhibitors on the endogenous protease activities are

illustrated in Figure 4. It is demonstrated that PMSF, a serine protease specific inhibitor, has significant impact on the endogenous protease activity of P1, P7, P8 and P10, which indicates these proteases own the serine residue and belong to the serine protease. The protease active of P1 is also significantly inhibited by TPCK with the inhibitory rate of 46.89%, which shows it belongs to chymotrypsin. The protease active of P7 is significantly inhibited by PMSF and SBTI simultaneously, while SBTI has higher inhibitory rate. This indicates that protease P7 belongs to the trypsin. Proteases P3 and P6 are significantly affected by an enzyme inhibitor, EDTA with the inhibitory rates of 34.27% and 38.24%, respectively, implying that the presence of Ca2+ is important for enzyme stabilization, and proteases P3 and P6 are metalloproteases. The protease activities of P2, P4, P5 and P9 are not significant inhibited by enzyme inhibitors of PMSF, EDTA, SBTI and TPCK.

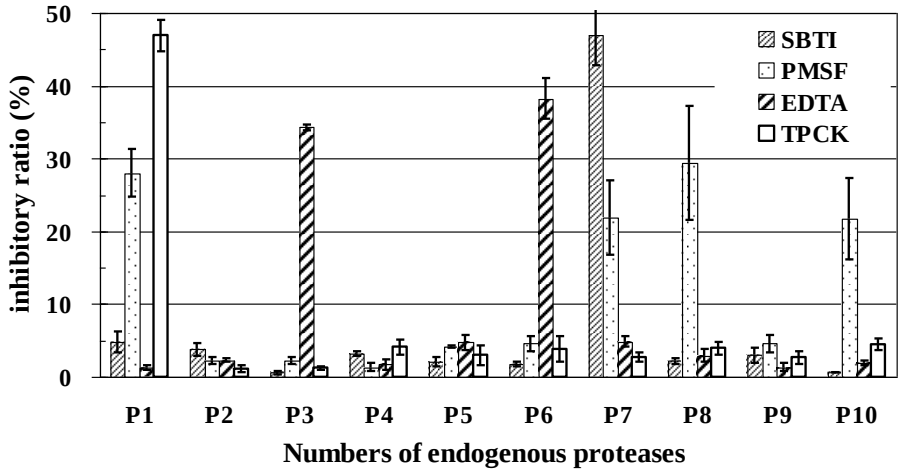

Figure 4. Effects of inhibitors SBTI, PMSF, TPCK and EDTA on the activities of endogenous proteases in the head of *L. vannamei*

Trypsin, pepsin, myofibril-bound serine proteases (MBSP), cathepsin B, cathepsin L, collagenase and calpain are thought to be the main contributors to autolyze fish and shrimp muscle (YANG&al.[5]). Therefore, these specific proteolytic activities of endogenous proteases P1 to P10 are studied, and the results are shown in Table 1. Protease P2 could hydrolyze Z-Phe-Arg-AMC, fluorogenic substrate of cathepsin L, showing it belongs to cathepsin L. Protease P9 could hydrolyze Z-Arg-Arg-AMC, fluorogenic substrate of cathepsin B, showing it belongs to cathepsin B. Cathepsin B (EC 3.4.22.1) and Cathepsin L (EC 3.4.22.15) are acid cysteine lysosomal proteases and can be activated by thiol compounds. It has been reported that cathepsins play a role in protein catabolism of live fish and autolyze fish muscle during post-mortem storage (HULTMANN & al.[2]). When haemoglobin was

adopted as a substrate of protease P5, obvious difference was observed before and after addition, indicating that P5 exhibits a strong activity over haemoglobin hydrolysis. Because haemoglobin is the specific substrates of pepsin, we consider that P5 belongs to pepsin. Protease P3 belongs to collagenase due to its effectively hydrolyzation of succinyl-Gly-Pro-Leu-Gly-Pro-AMC. Collagenases (EC 3.4.24.7) are metalloproteinases found in skeletal muscle. The collagen triple helix is attacked by these specific proteases (collagenases) before they are hydrolysed by other non-specific proteases (KRISTJÁNSSON&al.[3]). Protease P4 could hydrolyze N-succinyl -Leu-Tyr-AMC, fluorogenic substrate of calpain, showing it belongs to calpain. Calpains (EC 3.4.22.17) are neutral Ca^{2+} activated cysteine endopeptidases in sarcoplasm of muscles. Calpains cleave proteins at specific sites and result in limited proteolysis. The large peptides produced after hydrolysis by calpains have increased the susceptibility to other proteases (GAARDER& al.[4]; HULTMANN & al.[2]). Protease P10 could hydrolyze Boc-Phe-Ser-Arg-AMC and belongs to MBSP. It was shown that MBSP had a great impact on skeletal muscle from silver carp when fish was made into surimi (CAO & al.[25]).

Table 1. Specific proteolytic activities of endogenous proteases P1 to P10 isolated from the shrimp head of *L.vannamei*

Substrates	Endogenous proteases									
	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
hemoglobin	+	-	-	-	+	-	-	-	-	-
N-succinyl-Leu-Tyr-AMC	-	-	-	+	-	-	-	-	-	-
Z-Phe-Arg-AMC	-	+	-	-	-	-	-	-	-	-
Z-Arg-Arg-AMC	-	-	-	-	-	-	-	-	+	-
Boc-Phe-Ser-Arg-AMC	-	-	-	-	-	-	-	-	-	+
succinyl-Gly-Pro-Leu-Gly-Pro-AMC	-	-	+	-	-	-	-	-	-	-

Notes: "+", positive reaction; and "-", negative reaction. P1-P10 represent fractions 3-5, 6, 7-8, 9-10, 11,12,13,14-15, 16 and 17, respectively.

Above all, proteases P1, P7, P8 and P10 belong to the serine protease, and P1, P7 and P10 are further identified as chymotrypsin, trypsin and MBSP, respectively; proteases P3 and P6 belongs metalloproteases, and P3 is further identified as collagenase; protease P2 and P9 are identified as cathepsin L and cathepsin B, respectively; proteases P4 and P5 are identified as calpain and pepsin, respectively.

4. Conclusion

In conclusion, an effective method for isolating and identifying the endogenous proteases has successfully been developed. Adopting this novel method, we have already isolated and identified 10 types of endogenous proteases from the head of L. vannamei for first time, and the enzymology characterization of these proteases are also investigated. Our new results may provide important information for developing a cost-effective and environmentally friendly method to recover proteins from shrimp head waste by using endogenous proteases.

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