Characterization of protease activity from hepatopancreas of blue crab

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Abstract. Proteolytic enzymes play an important role in determining the quality of blue crab during postmortem storage. Activity of endogenous proteases is involved in the texture softening and autolysis of blue crab, which limits the customer acceptance and marketing price. This research aimed to characterize the protease activity of crude enzyme extract from the hepatopancreas of blue crab. The optimum activity of crude protease extract was found at pH 7.0 and 50°C. The crude protease enzyme was highly stable over a wide pH range of 4.0-11.0 and showed high stability at temperatures below 40°C. In addition, the protease activity continuously decreased with an increasing concentration of NaCl (0-15% w/v). Therefore, an understanding of the endogenous proteases in the blue crab could be used to develop appropriate storage methods during its distribution process.

1. Introduction
Local distribution of fish and shellfish, especially marine animals, generally involves being stored in ice. Therefore, a major problem occurring during local distribution of fish and shellfish is a rapid deterioration due to autolytic degradation and microbial spoilage. Autolytic degradation is related with the activity of endogenous proteolytic enzymes [1-3]. Proteases are a group of enzymes responsible for protein hydrolysis. The endogenous proteases in fish and shellfish can be classified into four groups: cysteine proteases, aspartic proteases, serine proteases and metalloproteases, which are associated with a loss of textural quality during postmortem storage [3]. The proteolytic enzymes from the hepatopancreas played a probable role in the muscle softening of prawn during postmortem handling and storage [4], and proteolytic activity was suggested as a factor influencing textural quality of salmon meat during iced storage [5]. As a result, understanding the role of proteases during postmortem storage can be valuable for developing techniques to safely preserve the quality of marine food products. Blue crab is a popular local marine food in Pathiu, a district in Chumphon province, and it is generally distributed using iced storage. Postmortem quality loss tends to decrease the taste and texture of the blue crab, which leads to reduction in economic value. However, there is little information associated with the activity of proteases in blue crab. This study aimed to characterize the proteolytic activity from the hepatopancreas of blue crab in order to develop better preservation methods during local distribution.

2. Materials and methods
2.1. Preparation of crude enzyme
Blue crabs were obtained from port of Pathiu district, Chumphon province, Thailand. Their weight ranged between 140 and 160 g. The hepatopancreas was isolated and homogenized in 50 mM Tris-HCl (pH 8.0) at a ratio of 1:3 w/v. After centrifugation for 30 min at 10,000×g at 4°C, the collected supernatant was defined as “crude enzyme extract” and used for protease activity analysis.

2.2. Characterization of proteolytic activity in crude extract from hepatopancreas

2.2.1. pH profile and stability. The optimum pH of the crude protease was investigated by the modified method from [6,7] using 1% (w/v) casein as a substrate. The following buffer system was used for different pH conditions: 100 mM Citrate buffer (pH 4.0-6.0), 100 mM Tris–HCl (pH 7.0-9.0), 100 mM Glycine–NaOH (10.0-11.0) [8]. The crude extract (1 ml) was mixed with the 1% casein (1 ml) and incubated for 15 min at 37°C. The enzymatic reaction was ceased by adding 20% (w/v) trichloroacetic acid (2 ml) and the mixture was kept on ice for 1 h. After centrifugation for 10 min at 10,000×g at 4°C, the collected supernatant was determined the absorbance at 280 nm. One unit of protease activity was defined as releasing 1 µmol of tyrosine/min. The relative activity was computed and reported compared to condition showing the highest value (100% activity).

The pH stability of crude enzyme activity was analyzed by keeping the crude enzyme at different pH values (4.0-11.0) using several buffers as previously described at 4°C for 1 h. Aliquots were taken at 15 min intervals to assay the residual activity under standard conditions.

2.2.2. Thermal profile and stability. The activity of crude protease was determined over the various temperatures (0, 4, 30, 37, 40, 50, 60, and 70°C). The protease activity was carried out as previously described at the optimum pH.

The thermal stability of crude protease was analyzed by keeping the crude enzyme at various temperatures (0, 4, 30, 40, 50, 60 and 70°C) for 1 h. Aliquots were taken at 15 min intervals to assay the residual activity under standard conditions.

2.2.3. Effect of NaCl. The influence of NaCl on the protease activity was measured by adding the NaCl in the reaction mixture to gain the final concentration of 0-20% (w/v). The protease activity was analyzed under standard conditions.

3. Results and discussion

3.1. pH profiles and stability
The influence of pH on the protease activity of the crude enzyme from hepatopancreas was analyzed over a pH range of 4.0-11.0 as represented in figure 1(a). The crude protease exhibited the highest activity at pH 7.0. The crude protease was also active between pH 8.0-10.0, showing more than 80% activity compared to the highest activity observed at pH 7.0. The proteolytic activity consistently decreased when pH was below pH 7.0. Optimum pH value of crude enzyme from the hepatopancreas of the blue crab was in accordance with that of the crude extract from Macrobrachium rosenbergii hepatopancreas [4], which had an optimal pH at 7.0. The proteolytic enzyme from the midgut gland of Farfantepenaeanus paulensis [9] and viscera of Portunus segnis [10] displayed the maximum activity at pH 8.0. Many proteases from fish, such as Oreochromis niloticus [7,11], Stolephorus indicus [1] and Raja clavata [12], showed a high activity within pH ranges of 7.0-9.5. The decline in the proteolytic activity at a pH outside of the optimal pH might be involved in the change of charge distribution and conformation of both the enzyme and substrate [8,13].

The pH stability of the crude protease was represented in figure 1(b). Crude protease from the hepatopancreas was extremely stable over a wide pH range between 4.0-11.0, retaining over 90% of its initial activity after 60 min incubation at 4°C. The pH stability of proteases from fish viscera, such as R. clavata [12,14], Zosterisessor ophiocephalus [14], and Scorpaena scrofa [14,15], was extremely...
stable over a wide broad pH range between pH 5.0-12.0.

Figure 1. (a) pH profiles and (b) stability of crude protease from blue crab hepatopancreas.

3.2. Thermal profiles and stability

The protease activity of the crude enzyme from the hepatopancreas measured at different temperatures at the optimum pH was shown in figure 2(a). The crude protease demonstrated the highest proteolytic activity at 50°C. The crude enzyme showed activity above 80% at 37, 40 and 60°C. The relative activity at 70°C was approximately 62% of that at 50°C. The protease activity sharply declined more than 80% at low temperatures (0 and 4°C). Similar results on the optimum temperature of protease activity were observed in *R. clavata* [12, 14] and *Z. ophiocephalus* [14]; the enzyme extracts had the highest activity at 50°C. The optimum temperature of the crude protease from the hepatopancreas of blue crab was lower than the value reported from *M. rosenbergii* [4], *P. segnis* [10], *S. scrofa* [14, 15], and *Salaria basilisca* [16], which had optimum temperatures between 55-60°C.

Figure 2. (a) Thermal profiles and (b) stability of crude protease from blue crab hepatopancreas.

Thermal stability profile of the crude protease was represented in figure 2(b). The crude protease extract was extremely stable below 40°C, retaining over 80% of its original activity after 60 min incubation. The crude protease extract was quite stable at 50°C with its activity retained about 70% of its original activity after 60 min incubation. A continuous decrease of protease activity was found at 60°C since incubation for 15 min until no protease activity remained after incubation for 60 min. The proteolytic activity was almost absolutely inactivated at 70°C after incubation for 15 min. The thermal stability of the crude protease extract from the blue crab hepatopancreas showed a similar result with the protease from viscera of *P. segnis* [10], *Z. ophiocephalus* [14] and *Sardinella aurita* [17].
Moreover, the crude protease from hepatopancreas showed a higher stability than the visceral protease of *R. clavata* [12,14] and *S. scrofa* [14].

### 3.3. Effect of NaCl concentration

The influence of NaCl on the activity of the crude protease from the hepatopancreas was represented in figure 3. The activity of the crude protease continuously decreased with an increment of NaCl concentration up to 15%. Thereafter, the enzyme activity was constant with a further increment of NaCl concentration at 20%. In accordance with several reports on fish, such as *Pangasianodon gigas* [8], *Katsuwonus pelamis* [13] and *S. aurita* [17], the reduction of protease activity was observed with an increasing NaCl concentration. The decline in activity might be due to an increase in ionic strength, which is described by the salting out effect [18,19].

![Figure 3. Influence of NaCl concentrations on the activity of crude protease from hepatopancreas.](image)

According to [20], the preservation methods used in fish and shellfish can be divided into three groups; physical (e.g. chilling, freezing, ozonation), chemical (e.g. curing with salt or preservatives), and bio-preservation methods. Nevertheless, the use of ice is a popular method for storage and local distribution because it is simple and cheap. Our results revealed that the protease from the blue crab hepatopancreas was also active at pH of ice (pH 6.5-8.5) and highly stable at chilling temperature (0 and 4°C). Moreover, the concentration of salt affected the activity of the blue crab protease. Therefore, an understanding of protease characteristics is valuable for controlling blue crab quality during storage and distribution. To inhibit protease activity during blue crab preservation, the storage condition should be regulated at pH lower than 6.0, chilled between 0 and 4°C, and stored in salt. However, one limitation of the study is that the spoilage and shelf life of blue crab are associated with several other factors, so further research is needed to help improve storage and distribution methods.

### 4. Conclusions

The crude protease extract from the blue crab hepatopancreas was highly active at pH 7.0 and a temperature of 50°C. The crude protease was extremely stable at a pH range of 4.0-11.0 and at temperatures below 40°C. These results are a beneficial data for developing preservation methods during storage and local distribution of the blue crab.

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References

[1] Siringan P, Raksakulthai N and Yongsawatdigul J 2006 Food Chem. 98 678-84
[2] Sriket C, Benjakul S, Visessanguan W and Kishimura H 2011 Food Chem. 124 29-35
[3] Sriket C 2014 Int. Food Res. J. 21 433-45
[4] Sriket C, Benjakul S and Visessanguan W 2010 J. Sci. Food Agric. 91 52-9
[5] Hultmann L and Rustad T 2004 Food Chem. 87 31-41
[6] Mehrotra S, Pandey P K, Gaur R and Darmwal N S 1999 Bioresour. Technol. 67 201-3
[7] Chaijaroen T and Thongruang C 2016 Int. Food Res. J. 23 1432-8
[8] Vannabun A, Ketnawa S, Phongthai S, Benjakul S and Rawdkuen S 2014 Food Biosci. 6 9-16
[9] Buarque D S, Castro P F, Santos F M S, Lemos D, Junior L B C and Bezerra R S 2009 Aquac. Res. 40 861-70
[10] Hamdi M, Hammami A, Hajji S, Jridi M, Nasri M and Nasri R 2017 Int. J. Biol. Macromol. 101 455-63
[11] Bezerra S R, Lins E J F, Alencar R B, Paiva P M G, Chaves M E C, Coelho L C B B and Carvalho Jr L B 2005 Process Biochem. 40 1829-34
[12] Lassoued S, Hajji S, Mhamdi M, Jridi A, Bayoudh A and Barkia M 2015 Int. J. Biol. Macromol. 80 668-75
[13] Klomklao S, Kishimura H, Nonami Y and Benjakul S 2009 Food Chem. 115 155-62
[14] Nasri R, Younes I, Lassoued I, Ghorbel S, Ghorbel-Bellaaj O and Nasri M 2011 J. Amino Acids 2011 913616 http://dx.doi.org/10.4061/2011/913616
[15] Younes I, Nasri R, Bkhairia I, Jellouli K and Nasri M 2015 Food Res. Int. 94 453-62
[16] Ktari N, Khaled H B, Younes I, Bkhairia I, Mhamdi S, Hamza I and Nasri M 2014 J. Food Sci. Technol. 51 3094-103
[17] Khaled H B, Ghorbel-Bellaaj O, Hmidet N, Jellouli K, El-Hadj Ali N, Ghorbel S and Nasri M 2011 Food Chem. 128 847-53
[18] Klomklao S, Benjakul S and Visessanguan W 2004 J. Food Biochem. 28 355-72
[19] Klomklao S, Benjakul S, Kishimura H and Chaijan M 2011 Food Chem. 129 739-46
[20] Mahmud A, Abrah B, Samuel M, Mohammedidris H, Abraham W and Mahmud E 2018 MOJ Food Process Technol. 6 303-10