The Thai Journal of Veterinary Medicine

Volume 46
Issue 3 *September, 2016*

Article 7

9-1-2016

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Pattarawadee Srimeetian

Suriyan Tunkijjanukij

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Srimeetian, Pattarawadee and Tunkijjanukij, Suriyan (2016) "Differential protein expression in haemolymph of first-stage red sternum mud crabs (Scylla serrata)," *The Thai Journal of Veterinary Medicine*: Vol. 46: Iss. 3, Article 7.
Available at: https://digital.car.chula.ac.th/tjvm/vol46/iss3/7

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Original Article

Differential protein expression in haemolymph of first-stage red sternum mud crabs (*Scylla serrata*)

Pattarawadee Srimeetian[1,2*] and Suriyan Tunkijjanukij[3]

Abstract

Mud crab (*Scylla serrata*) is one of the important commercial aquatic animals in Thailand. A cause of death in crab farm is red sternum syndrome. The aim of this research was to characterize differential protein expression in the haemolymph of normal mud crabs and first-stage red sternum mud crabs using gel filtration chromatography on a Sephacryl S-200 chromatography column for protein purification. Results showed that the haemolymph of the red sternum mud crabs presented two fractions (F1 and F2) that were not found in that of the normal mud crabs. Subsequently, these peak fractions were separated by two-dimensional electrophoresis (2DE) technique. Each peak fraction was found to contain 1 spot. These protein spots were successfully identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Results indicated that F1 and F2 were alpha-2-macroglobulin (A2M), which was expressed in the haemolymph of the red sternum mud crabs but not in that of the normal mud crabs. Furthermore, the A2M plays important roles in innate defense of crustaceans. Our results may help understand the mechanism of molecular responses of mud crab to red sternum syndrome.

Keywords: mud crab, red sternum syndrome, gel filtration, two-dimensional electrophoresis, LC-MS/MS

[1]*Center for Agricultural Biotechnology, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand*
[2]*Center of Excellence on Agricultural Biotechnology: (AG-BIO/PERDO-CHE), Bangkok 10900, Thailand*
[3]*Department of Aquaculture, Faculty of Fisheries, Kasetsart University, Bangkok 10900, Thailand*
***Correspondence**: p.srimeetian@hotmail.com*

Introduction

Mud crab (*Scylla serrata*) is one of the important commercial aquatic animals of Thailand with its good taste and nutritious value. They are cultured in several provinces in Thailand such as Trat, Chantaburi, Samutsongkhram and Ranong. In crab culture, a lot of crabs die because of several factors such as inability to molt (Srimeetian, 2010) and red sternum syndrome. Red sternum syndrome is present around 10% of the normal population. Intanakom (2010) reported normal crabs infected with *Vibrio parahaemolyticus*. The haemolymph color of the infected crabs changed from transparent to transparent orange, which demonstrated that *V. parahaemolyticus* was the cause of red sternum syndrome in the mud crabs. A recent study has also demonstrated that *V. parahaemolyticus* is the cause of the haemolymph color change from transparent to transparent orange in mud crabs, which is one of the symptoms of red sternum syndrome (Plainpun, 2014). Characteristics and behaviors of normal and red sternum syndrome mud crabs are different. Normal mud crabs have hard shell, white sternum, fast movement and good appetite, while red sternum syndrome mud crabs have soft shell, red sternum, less movement and loss of appetite (Salaenoi et al., 2006). In addition, the haemolymph of normal mud crabs is colorless, but red sternum mud crabs have different haemolymph color which appears in 3 stages. At the first stage, the haemolymph color changes from colorless to orange. During the second stage, the haemolymph changes its color to orange-white and at the third stage to milky-white, finally the crabs die (Areekijseree et al., 2010). The haemolymph of red sternum syndrome is also similar to milky haemolymph disease in shrimp (*Exopalaemon carinicauda*) caused by *Hematodinium* (Xu et al., 2010). The haemolymph of crab (*Paralithodes camtschaticus*) changed to cream color by *Hematodinium* (Ryazanova, 2008). Moreover, Xu et al. (2007) reported that *Hematodinium* was the causative agent of milky haemolymph disease in the Chinese swimming crabs (*Portunus trituberculatus*). Therefore, red sternum syndrome in mud crabs is a serious problem that decreases productivity and causes farmers loss of income.

Protein is the end product of gene expression which can be post-transcriptionally modified. These proteins function in enzyme catalysis, signal, metabolic control and immune. Several types of protein are produced in different conditions (Thammasirirak, 2002). The red sternum syndrome which appears in mud crab may result in different protein expressions. There are still no reports of the molecular response mechanisms of the syndrome. In regard to other crustaceans, shrimp (*Penaeus monodon*) were studied on differential protein expressions under unchallenged and *V. harviyi* challenged conditions, in order to understand the responses of shrimp against pathogenic bacteria infection (Somboonwiwat et al., 2010). This research studied the protein expression of haemolymph from normal mud crabs and first-stage red sternum mud crabs. For effective control of red sternum syndrome, treatment should be initiated since the first stage of the syndrome. Crabs having the second and third stages will soon become paralyzed and suffer from serious conditions, resulting in death within 24 hours (Intanakom, 2010). In our study, haemolymph samples were purified by gel filtration column and were compared in separation by the 2DE technique. Then, proteins were determined by LC-MS/MS. The outcome may help us to understand the mechanism of red sternum syndrome. Moreover, the obtained results can be used as basic data for further studies in terms of protection, resistance and control of this syndrome.

Materials and Methods

Sample preparation: Five normal (*S. serrata*) and five first-stage red sternum mud crabs were collected from a soft-shell crab farm in Samutsongkhram province, Thailand. Their body weights, carapace widths, carapace lengths and thicknesses were 332-406 g, 7.46-9.04 cm, 5.16-6.32 cm and 2.86-3.47 cm, respectively. Haemolymph samples were collected from the swimming legs of the mud crabs by a syringe. Then, they were centrifuged at 12,000 x rpm for 30 minutes at 4°C and supernatants were collected for protein concentration analysis by the BioRad protein assay (Bio-Rad protein Laboratories, California, USA), which is based on the dye-binding procedure of Bradford (1976).

Gel filtration chromatography: The supernatant was loaded onto a Sephacryl S-200 gel-filtration column (Amersham Biosciences, Uppsala, Sweden) equilibrated with 20 mM Tris-HCl buffer (pH 7.1) at a flow rate of 0.2 ml/min. About 80 fractions were collected, their absorbance levels were measured at 280 nm, and gel filtration standards used protein molecular weight such as blue dextran (2000 kDa), catalase (232 kDa), immonogrobin G (150 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa) and ribonuclease (13.7 kDa).

Two-dimensional gel electrophoresis (2DE): Each protein peak with the 200 µg concentrate from gel filtration chromatography was mixed with rehydration solution and then loaded onto IPG strip gel (Nonlinear pH 3-10; 7 cm long) (GE Healthcare, Uppsala, Sweden). First-dimension isoelectric-focusing (IEF) was then run on an IPGphor II system (Amersham Biosciences, Uppsala, Sweden) at 250 V for 30 min, 500 V for 30 min, and 7500 V for 1 h 40 min. During two-dimensional gel electrophoresis using SDS-PAGE, the IPG strip was incubated for 15 min in 0.5 mg/ml DTT into 5 ml SDS equilibration buffer and was then incubated for 15 min in 25 mM iodoacetamide (IAA) into equilibration buffer. Then, the IPG strip was transferred onto the top of a 12% SDS-PAGE gel. The gel was run in a Mini-Protean Tetra (Bio-Rad Laboratories, California, USA) at 70 V for 20 min and 100 V for 2 h. The gel was stained with Coomassie brilliant blue G-250.

In-gel tryptic digestion protocol: Protein spots were selectively cut from the 2DE gels and transferred to microcentrifuge tubes. Then, the following steps were performed: (1) add 20 mM AmB (ammonium bicarbonate) and remove all liquid, (2) add 20 mM

AmB/ACN (acetonitrile) (1:1) and remove all liquid, (3) add 20 mM ACN and remove all liquid, repeat steps (2)-(3). For reduction and alkylation, the steps were as follows: add 10 mM DTT in 20 mM AmB, incubate at 56°C for 45 min, remove all liquid, add 55 mM IAA in 20 mM AmB, incubate in dark at room temp for 30 min, remove all liquid, add 20mM AmB/ACN (1:1), remove all liquid, add 20 mM ACN, remove all liquid, dry down gel pieces for 5 min, and add 20 µl of freshly prepared enzyme solution to cover the gel. Then, incubation was done at 4°C for 30 min, excess enzyme solution was removed, and 25 mM AmB was added to keep the gel wet overnight. The samples were incubated at 37°C overnight (>16 hr). Supernatants were collected for LC-MS/MS analysis. For peptide extraction, the following steps were performed: add 20 mM AmB, collect all liquid in a microcentrifuge tube, add 1% FA in 50% ACN, collect all liquid in a microcentrifuge tube, add 1% FA in 85% ACN, collect all liquid in a microcentrifuge tube, dry down the solution in speed vac at 45°C, resuspend with 0.1% FA in 2% ACN, centrifuge at 10,000 rpm for 10 min, aliquot 20 µl of sample to limited volume inserts 50 ul glass in vial.

LC-MS/MS analysis: The samples were analyzed with a nano-liquid chromatography system (EASY-nLC II, Bruker) coupled to an ion trap mass spectrometer (Amazon Speed ETD, Bruker, USA) equipped with an ESI nano-sprayer. Each sample volume of 1 µl was loaded by the autosampler onto EASY-Column, 10 cm, ID 75 µm, 3 µm, C18-A2 (Thermo Scientific, USA) using a flow rate of 300 nL/min and linear gradient from solution A (0.1% formic acid) to 40% of solution B (0.1% formic acid in acetonitrile) in 40 min. Bruker Daltonics software package, HyStar v.3.2 was used to control the ion trap device. LC-MS/MS spectra were analyzed using Compass Data Analysis v.4.0. Compound lists were exported as Mascot generic files (mgf) for further search in Mascot program.

Data analysis: Protein identification was performed by searching against the protein database from NCBInr and SwissProt (All entries) using MASCOT MS/MS Ion searches (http://www.matrixscience.com) with the initial searching parameters; enzyme: trypsin, allowed up to one missed cleavage; carbamidomethylation as fixed modification, and oxidation (HW) and oxidation (M) as variable modification; peptide mass tolerance of 0.5 Da and fragment mass tolerance of 0.5 Da; a peptide charge state of +1, +2, +3; instrument type: ESI-TRAP; and report top: auto. All the proteins identified should have protein scores greater than 67 for NCBInr database and 45 for SwissProt database (significant; $p < 0.05$).

Results

Characterization of normal and red sternum mud crabs in orange haemolymph stage: The normal mud crabs and the first-stage red sternum mud crabs collected from the soft-shell crab farm were observed. The sternums of the normal mud crabs were white in color, while the sternums of the red sternum mud crabs were orange. The haemolymph of the normal mud crabs was colorless or pale blue when exposed to oxygen while the haemolymph of the first-stage red sternum mud crabs was orange (Figs. 1A-D). Furthermore, the movement and feeding behavior of the red sternum mud crabs were less than those of the normal mud crabs.

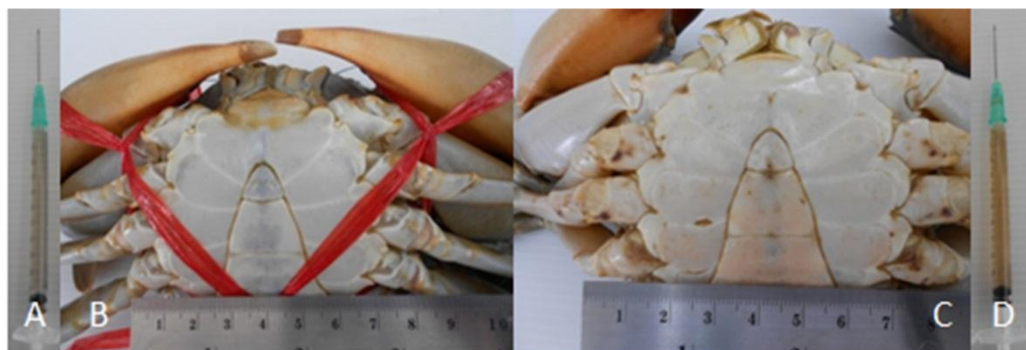

Figure 1 The ventral side of mud crab, the haemolymph (A) and sternum (B) of normal mud crab, the sternum (C) and haemolymph (D) in of first-stage red sternum mud crab

Purification of protein from mud crabs haemolymph: The proteins from the haemolymph of the normal and red sternum mud crabs were purified on the Sephacryl S-200 gel-filtration column. Fractions were obtained after elution with 20 mM Tris-HCl buffer (pH 7.1) and their absorbance at 280 nm was recorded. Seven protein peaks (F1, F2, F3, F4, F5, F6 and F7) were found in the haemolymph of the red sternum mud crabs (Fig. 2A). Their fraction numbers were 4-5, 6-8, 18-23, 32-34, 39-41, 45-48 and 55-58, respectively. Each protein peak was compared to molecular weight of the gel filtration standards. The molecular masses of peak fractions were about 205, 170, 72, 37, 21, 14 and 8 kDa, respectively. Five protein peaks (F3, F4, F5, F6 and F7) were found in the haemolymph of the normal mud crabs (Fig. 2B). Their fraction numbers were 18-24, 31-33, 39-41, 45-48 and 55-58, respectively. Each peak fraction was compared to molecular weight of the gel filtration standards. The molecular masses of peak fractions were about 72, 37, 21, 14 and 8 kDa, respectively. The data demonstrate that the peak fractions of the normal and red sternum mud crabs differ. The peak fractions F1 and F2 were found only in the red sternum mud crabs while the peak fractions F3, F4, F5, F6 and F7 were found in both the normal and red sternum mud crabs.

Protein expression and identification: The proteins were separated by the 2DE technique. The pH range for the first-dimension isoelectric-focusing based separation of haemolymph proteins was determined using the 7-cm IPG strip of pH 3-10. The spots of purified protein (F1, F2, F3, F4, F5, F6 and F7) were cut out from 2DE. Each peak fraction was found to have 1 spot, except peak fraction F3, which was found to have 3 spots (Fig. 3). The spots were digested in-gel with trypsin and analyzed with LC-MS/MS. The peptide masses obtained from LC-MS/MS were used to search the entire NCBInr and SwissProt database using the MASCOT search engine (Matrix Science). Results indicated that F1 was A2M (*S. paramamosain*); F2 was A2M (*S. paramamosain*); F3 was hemocyanin (*Eriphia verrucosa*), hemocyanin subunit 1 (*Macrobrachium nipponense*) and hemocyanin subunit (*Callinectes sapidus*); F4 was cytochrome P450 (*Rattus norvegicus*); F5 was serine protease (*Staphylococcus aureus*); F6 was ribonuclease (*Acinetobacter baylyi*); and F7 was putative cytochrome c oxidase (*Pinus strobus*) (Table 1).

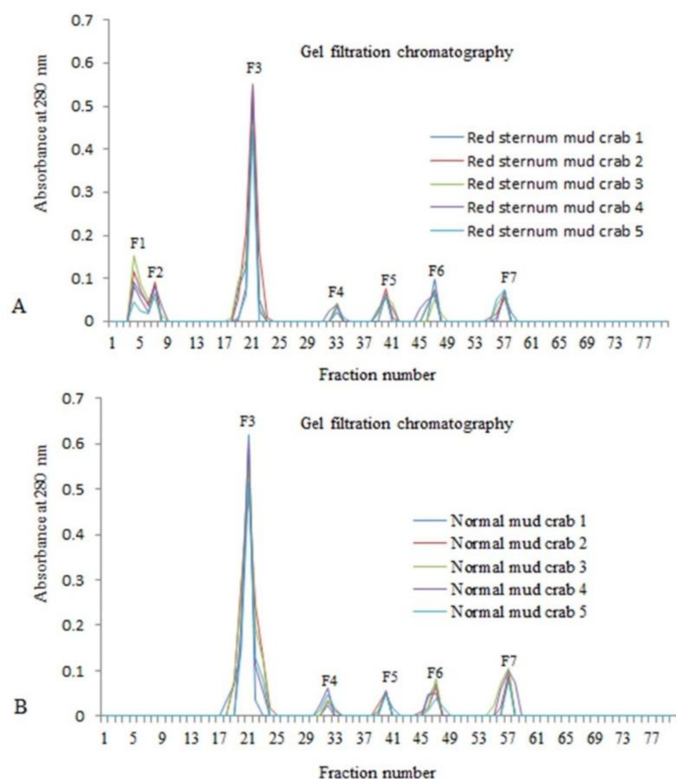

Figure 2 Gel filtration chromatography on Sephacryl S-200 to a column (70x0.8 cm) was equilibrated with 20 mM Tris-HCl buffer, ultraviolet absorption was monitored at 280 nm. Elution profile of the haemolymph of first-stage red sternum mud crabs (A) and elution profile of the haemolymph of normal mud crabs (B).

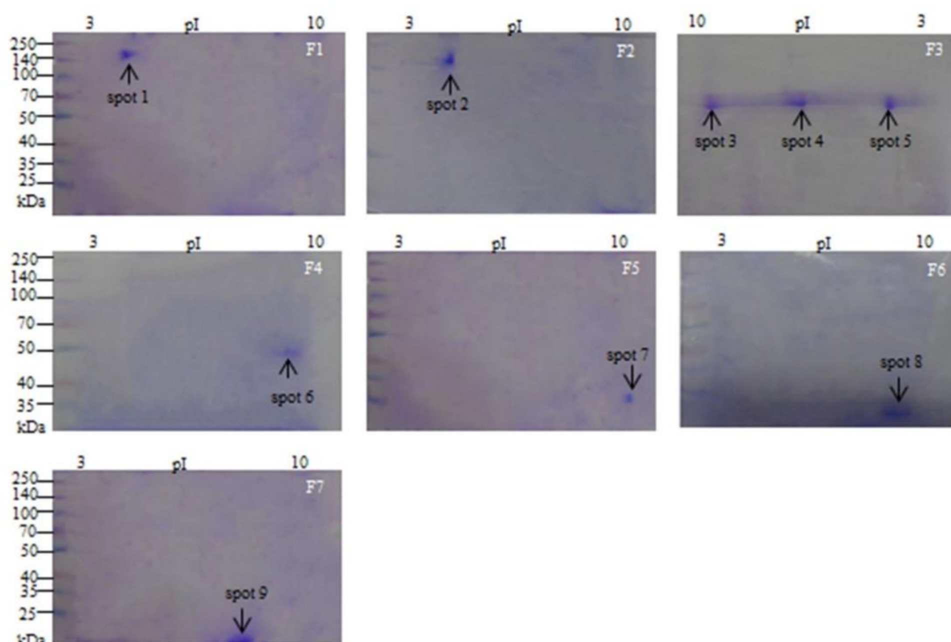

Figure 3 2DE of peak fractions F1, F2, F3, F4, F5, F6 and F7 from gel filtration chromatography

Table 1 List of matching proteins obtained by LC-MS/MS analysis from gel spot of peak fractions F1, F2, F3, F4, F5, F6 and F7

Spot no.	Protein/Species	Accession name	MW(Da)/pI	Mascot score
1 (F1)	Alpha-2-macroglobulin [*Scylla paramamosain*]	gi|520188162	167018/5.6	960
2 (F2)	Alpha-2-macroglobulin [*Scylla paramamosain*]	gi|520188162	167018/5.6	1131
3 (F3)	Hemocyanin [*Eriphia verrucosa*]	gi|930174081	77179/5.32	155
4 (F3)	Hemocyanin subunit 1 [*Macrobrachium nipponense*]	gi|431832960	78012/5.36	79
5 (F3)	Hemocyanin subunit [*Callinectes sapidus*]	gi|7582388	77055/5.26	261
6 (F4)	Cytochrome P450 [*Rattus norvegicus*]	CP270_RAT	56690/8.20	37
7 (F5)	Serine protease [*Staphylococcus aureus*]	SPLA_STAAC	25860/8.98	33
8 (F6)	Ribonuclease [*Acinetobacter baylyi*]	RNH2_ACIA	20806/6.44	30
9 (F7)	Putative Cytochrome c oxidase [*Pinus strobus*]	PS17_PINST	1707/9.62	31

Spots no. 1-5 were derived from NCBInr database and spots no. 6-9 were derived from SwissProt database.

Discussion

Red sternum syndrome affects crab farming in Thailand. Crabs infected with the syndrome have red sternum, easily broken shell, loss of appetite and less movement before they finally die. These crabs smell bad and have a bitter taste (Areekijseree et al., 2010). In the present study, the haemolymph of normal and red sternum mud crabs was purified by the Sephacryl S-200 gel-filtration technique. The results showed that the haemolymph of red sternum mud crabs contained seven peak fractions (F1, F2, F3, F4, F5, F6 and F7) and the haemolymph of normal mud crabs contained five peak fractions (F3, F4, F5, F6 and F7). Only two peak fractions from the red sternum mud crabs were different from those of the normal mud crabs. These peak fractions were separated by the 2DE technique. Each peak fraction was found to contain 1 spot. These protein spots were successfully identified by the LC-MS/MS analysis. The results indicated that F1 and F2 were A2M.

The cause of red sternum syndrome in mud crab is *V. parahaemolyticus* (Intanakom, 2010). *V. parahaemolyticus* is gram-negative bacteria that produce protease enzyme. Protease is a product of pathogens which causes cell damage, illness and death among the hosts (Sudheesh and Xu, 2001). Crustaceans are protected from pathogens by their immune system. Although they do not have acquired immunity, they have an innate immune system (Sritunyalucksana and Söderhäll, 2000). A2M can play a very important role in the innate immune system of crustaceans (Vaseeharan et al., 2007). Moreover, A2M plays a key role in protease enzyme inhibition of pathogens. A2M consists of 3 important regions, which are the bait region, the thiol ester domain, and the receptor-binding domain (Armstrong, 2010). Characteristics of the bait region are similar to a cage and can also be called a cleavage site. The bait region is like a trap that catches protease enzymes of the pathogen; it is also called a unique trap. This mechanism can be found only in the A2M protein. Furthermore, the bait region functions cut and digest protease of pathogen. The thiol ester domain, the second domain, binds protease enzymes covalently and also helps to trap them. The receptor-binding domain, the last domain, is located near the C-terminal region and buried inside the A2M (Armstrong and Quigley, 1999). It acts as a receptor to connect to receptors of other proteins, especially transglutaminase enzymes (Chaikeeratisak et al., 2012), which are key enzymes involved in the haemolymph clotting system (Martin et al., 1991). Furthermore, A2M is involved in the prophenoloxidase (proPO) system, whose function is melanin formation. Melanin is an element that inhibits and prevents the growth of pathogens and other microorganisms (Söderhäll and Cerenius 1998).

The results demonstrated that A2M was expressed in the haemolymph of red sternum mud crabs but was not presented in that of normal mud crabs. A2M may be expressed to destroy protease enzyme of *V. parahaemolyticus*. Plainpun (2014) reported that *V. parahaemolyticus* was found in mud crabs (*Scylla* sp.) with red sternum syndrome. Furthermore, Kaewsangiem (2005) indicated that red sternum mud crabs (*S. serrata*) were infected by rod or curve-rod bacteria but no bacteria were found in normal crabs. Moreover, the haemocytes of crab (*S. serrata*) showed that A2M was increased significantly 24 h and 48 h after induction with lipopolysaccharide (LPS; gram-negative bacteria) (Vaseeharan et al., 2007). In shrimp studies, *P. monodon* was challenged by the bacteria *V. harveyi* to induce vibriosis disease. A2M showed considerably varied expression levels against *V. harveyi* infection (Somboonwiwat et al., 2010). In addition, A2M expression of the haemolymph from white spot syndrome virus (WSSV) affected crustaceans was studied by Liu et al. (2011). Their results showed that A2M was present in the haemolymph of the crabs (*S. serrata*) challenged with WSSV but not present in the haemolymph of the WSSV unchallenged crabs. A2M is an important element in the immune system of crustaceans. Thus, it is produced and present in red sternum syndrome crabs but not found in normal crabs.

Conclusion

The haemolymph of normal mud crabs and mud crabs infected with the first stage of red sternum syndrome was analyzed by gel filtration, 2DE and LC-MS/MS analysis. Our results demonstrated that A2M was expressed in the haemolymph of red sternum mud crabs but was not presented in that of normal mud crabs. A2M is involved in immunity. The results provide important information on the mechanism responses of mud crabs to red sternum syndrome.

Acknowledgements

This work was supported by the Center of Excellence on Agricultural Biotechnology, Science and Technology Postgraduate Education and Research Development Office, Office of Higher Education Commission, Ministry of Education (AG-BIO/PERDO-CHE), National Research Council of Thailand, the Office of the Higher Education Commission, Thailand (grant fund under the Program Strategic Scholarships for Frontier Research Network for the Join Ph.D. Program Thai Doctoral degree for this research).

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บทคัดย่อ

การแสดงออกของโปรตีนที่แตกต่างในเลือดของปูทะเล (*Scylla serrata*) ที่มีอาการอกท้องแดงระยะแรก

ภัทราวดี ศรีมีเทียน[1,2]* และ สุริยัน ธัญกิจจานุกิจ[3]

ปูทะเล (*Scylla serrata*) เป็นสัตว์น้ำเศรษฐกิจที่สำคัญชนิดหนึ่งของประเทศไทย การตายของปูทะเลในฟาร์มสาเหตุหนึ่งมาจากการที่ปูมีอาการอกท้องแดง วัตถุประสงค์ของงานวิจัยนี้เพื่อศึกษาการแสดงออกของโปรตีนที่แตกต่างในเลือดของปูทะเลปกติและปูทะเลที่มีอาการอกท้องแดงระยะแรก โดยใช้เทคนิค gel filtration chromatography บนเจล Sephacryl S-200 เพื่อแยกโปรตีนให้บริสุทธิ์ การศึกษาพบว่าเลือดของปูที่มีอาการอกท้องแดงมีการแสดงออกของ 2 พีค โปรตีน (F1 และ F2) ที่ไม่พบในเลือดของปูปกติ หลังจากนั้นใช้เทคนิค two-dimensional electrophoresis (2DE) พบว่าแต่ละพีคมี 1 จุดโปรตีน จากนั้นนำจุดโปรตีนมาศึกษาชนิดของโปรตีนด้วย liquid chromatography-tandem mass spectrometry (LC-MS/MS) การศึกษาพบว่า F1 และ F2 คือ alpha-2-macroglobulin (A2M) ซึ่ง A2M มีการแสดงออกในเลือดของปูทะเลที่มีอาการอกท้องแดงแต่ไม่แสดงออกในเลือดของปูปกติ โดย A2M มีบทบาทหน้าที่สำคัญเป็นอย่างยิ่งในระบบภูมิคุ้มกันของสัตว์กลุ่มครัสเตเชียน ผลการศึกษานี้ช่วยให้เข้าใจเกี่ยวกับการตอบสนองระดับโมเลกุลของปูทะเลต่ออาการอกท้องแดง

คำสำคัญ: ปูทะเล อาการอกท้องแดง เจลฟิวเตรชัน two-dimensional electrophoresis LC-MS/MS

[1]*ศูนย์เทคโนโลยีชีวภาพเกษตร มหาวิทยาลัยเกษตรศาสตร์ วิทยาเขตกำแพงแสน จ.นครปฐม 73140*

[2]*ศูนย์ความเป็นเลิศด้านเทคโนโลยีชีวภาพเกษตร สำนักพัฒนาบัณฑิตศึกษาและวิจัยด้านวิทยาศาสตร์และเทคโนโลยี สำนักงานคณะกรรมการการอุดมศึกษา กรุงเทพฯ 10900*

[3]*ภาควิชาเพาะเลี้ยงสัตว์น้ำ คณะประมง มหาวิทยาลัยเกษตรศาสตร์ เขตจตุจักร กรุงเทพฯ 10900*

***ผู้รับผิดชอบบทความ** *E-mail: p.srimeetian@hotmail.com*