EFFECT OF POLY I:C AND LPS INJECTION ON IMMUNE RESPONSES OF REDCLAW (*Cherax quadricarinatus*)

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ABSTRACT

*Redclaw (*Cherax quadricarinatus*) is a potential species for aquaculture. It is native to Australia and has beneficial attributes which enhance its suitability for aquaculture. This present study was attempted to investigate the protein expression in redclaw crayfish injected with poly I:C, LPS and PBS (control). Redclaw size of 13-20 gram were put into 60 litre glass aquaria, each aquarium consists of 4 animals. Tank 1 contains animals injected with Poly IC (2 mg/ml), tank 2 contains animals injected with LPS (10 mg/ml) and tank 3 contains animals injected with PBS (as control). Haemolymph and hepatopancreas of the animals were sampled and used for assay. Injection of Poly I:C and LPS resulted in the presence of inducible protein which express just below the 66.2 kDa marker on the gel electrophoresis, then suspected as Mx protein in crustaceans. Besides Mx protein, gel electrophoresis also expressed protein which has molecular weight between 31 and 45 kDa, which could be lipopolysaccharide and β-1,3-glucan binding protein (LGBP). Results from electrophoresis of redclaw hepatopancreas showed the expression of protein which was up-regulated by Poly IC but not by LPS. The protein was constitutively expressed in PBS-injected controls but an noted increase in expression was recorded in poly I:C stimulated animals.*

Key words : Poly I:C, LGBP (lipopolysaccharide and β-1,3-glucan binding protein), Mx protein

ABSTRAK

*Sri Dwi Hastuti & A.C. Barnes. Pengaruh Injeksi Poly I:C dan LPS terhadap Respon Imunitas Udang Cherak (*Cherax quadricarinatus*). Udang cerak merupakan jenis yang potensial untuk akuakultur. Udang ini merupakan hewan asli Australia dan memilliki karakter yang sesuai untuk dibudidayakan. Pada penelitian ini dilakukan pengamatan ekspresi protein dari jenis hewan ini yang disuntik oleh I:C, LPS, dan PBS (kontrol). Udang berukuran 13-20 gram ditempatkan dalam akuarium kaca 60 L, masing-masing 4 ekor per akuarium. Akuarium 1 diisi udang yang disuntik Poly I:C (2 mg/ml), akuarium 2 diisi udang yang diinjeksi LPS (10 mg/ml), dan akuarium 3 diisi udang yang disuntik PBS (kontrol). Haeomlimpha dan hepatopankreas udang-udang tersebut disampling dan digunakan untuk pengamatan. Injeksi Poly I:C dan LPS menyebabkan munculnya protein yang terekspresikan tepat di bawah* marker *66.2 kDA pada elektroforesis gel, yang diduga sebagai protein Mx pada krustasea. Disamping protein Mx, elektroforesis gel juga memunculkan ekspresi protein yang mempunyai berat molekul 31 – 45 kDA, yang kemungkinan berupa lipopolysakarida dan protein terikat β-1,3 glukan (β-1,3 glucan binding protein; LGBP). Hasil dari analisa elektroforesis hepatopankreas memperlihatkan ekspresi protein yang diatur oleh Poly I:C, tapi tidak melibatkan LPS. Protein ini juga ditemukan pada udang yang diinjeksi PBS sebagai kontrol tapi peningkatan ekspresi yang nyata terlihat akibat stimulasi Poly I:C.*

Kata kunci : *Poly I:C, LGBP (lipopolysaccharide and β-1,3-glucan binding protein), Protein Mx*

INTRODUCTION

Redclaw crayfish is potentially an important species for aquaculture, besides being native to Australia, it has many attributes which of benefit to aquaculture: For example, it can tolerate relatively crowded conditions, exhibits fast growth rate, requires relatively simple spawning technique, tolerates a wide range of water quality including low dissolved oxygen, elevated ammonia and nitrite levels (Meade & Watts, 1995; Muzinic et al., 2004). This species of crayfish is now being cultured not only in Australia but also in several countries including Ecuador, Israel and China (Barki, Gur, & Karplus, 2001; Chen & Edgerton, 2001; Jacinto et al., 2004). Increasing demand for crayfish requires intensification of redclaw farming in the future. Intensification may increase the possibility of animals becoming stressed and might lead to disease problem as experienced by other species cultured in intensive conditions (Lorenzon et al., 1997);

Several countries have experienced devastation of shrimp aquaculture as a result of white spot syndrome caused by WSSV (Zhang, Huang, Xu, & Hew, 2002). Recently, it has been reported that WSSV, the most pathogenic virus for shrimp has been found to infect freshwater crayfish (Edgerton et al., 2002). Other viruses have also been identified in redclaw (Edgerton, 1996; Edgerton & Owens, 1999; Edgerton et al., 1994; Edgerton et al., 2000), thus viral infection are of concern if crayfish aquaculture is to intensify and develop into a major international industry. Therefore a better understanding of immune response of redclaw will be very helpful for developing disease control strategies.

As a crustacean, redclaw have no adaptive immune response and hence relies on innate immunity for defence against invading microorganisms. Innate immunity has the ability to limit infectious challenge in the early hours after the infection occurs. The innate immune system of crustaceans is able to recognize the conserved molecular patterns characteristic of pathogens, known as pathogen-associated molecular patterns or PAMPs (Sritunyalucksana, 2001). These patterns include the lipopolysaccharides (LPS) of Gram negative bacteria, the glycolipids of mycobacteria, the lipoteichoic acids of Gram positive bacteria, the mannans of yeast, the ß-1,3-glucan of fungi and double-stranded RNAs of viruses (Hoffman, Kafatos, Janeway, & Ekekowitz, 1999). Host organisms have a set of receptors which called pattern recognition proteins or receptors (PRPs or PRRs). These receptors form the initial basis of the response to the PAMPs (Janeway, 1989). The recognition of microbial substances from pathogens by pattern recognition protein will lead to the activation of several biological molecules responsible for the defence system including antimicrobial peptides (Sritunyalucksana, 2001).

It has been reported that injecting LPS into fish induced the production of complement components (Lunde & Robertsen, 1997) and stimulated the phagocytic activity of macrophages against glutaraldehyde-fixed sheep red blood cells. Recent study conducted by Salinas and colleagues shows that injecting LPS into the Atlantic salmon induced only a very low Mx response (Salinas et al., 2004). In crustaceans, exposing haemocytes to LPS *in vitro* resulted in granulocyte cell degranulation and release or activation of the prophenoloxidase (ProPO) cascade which performs as a protective bactericidal mechanism (Lorenzon et al., 1997). LPS can induce lipopolysaccharide and ß-1,3-glucan-binding protein (LGBP) of crayfish which can recognize and bind pathogens such as bacteria and fungi and is involved in activation of the coagulation process (I. Soderhall & Soderhall, 2002). Lee reported that LGBP of freshwater crayfish has a molecular weight of between 36 and 40 kDA on 10 % SDS polyacrylamide gel

electrophoresis (J. Y. Lee, Wang et al., 2000).

Injecting of synthetic double-stranded RNA (Poly I:C) in vertebrates has been proven to induce antiviral response through inducing interferon (IFN) system. The IFN system is the first line of defence against viruses in vertebrates. The type I interferon system consists of the α and β interferons, which induce synthesis of a variety of proteins, including protein kinase R PKR, adenosine deaminase and the Mx proteins (Plant & Thune, 2004). Studies conducted with fish injected with poly I:C revealed the appearance of Mx protein which also has a roles as an antiviral agent (V. Jensen & Robertsen, 2000; J. Y. Lee, Hirono et al., 2000; Plant & Thune, 2004; Yap et al., 2003). Gel electrophoresis revealed that Mx proteins have molecular weight of between 72.5 and 76 kDa (I. Jensen, Albuqueque, Sommer, & Robertsen, 2002; Nygaard et al., 2000; Plant & Thune, 2004).

To date, there have been no studies conducted to investigate the effect of Poly I;C on crustacean immunity. This present study was attempted to investigate the protein expression in redclaw crayfish injected with poly I:C, LPS and PBS (control).

MATERIAL AND METHODS

Animals and Injection

Redclaw size of 13-20 gram from "Iron Bark" redclaw farm were put into 60 litre glass aquaria, each aquarium consists of 4 animals. Tank 1 contains animals injected with Poly IC (2 mg/ml), tank 2 contains animals injected with LPS (10 mg/ml) and tank 3 contains animals injected with PBS (as control). Injection was done intramuscularly at the ventral side (abdominal muscular) of the animals. After 4 days, haemolymph and hepatopancreas of the animals were sampled and used for further assay.

Sampling of Haemolymph and Hepatopancreas.

Haemolymph was taken from the base of the 5 th walking legs of the animals using a 1 ml syringe and 25 gauge needle, which previously filled with 100 µl modified Alsever's solution (19.3mM Sodium citrate ; 239.8mM Sodium chloride; 182.5mM Glucose and 6.2mM EDTA pH 7.3) to prevent clotting of the haemolymph. Hepatopancreas was taken by dissecting the animal' cephalothorax, cut a part of hepatopancreas then put it into 200 µl of 50 mM phosphate buffer, pH 7.4??? added with 1 µl of PMSF (10 mg/ml in isopropanol) to inhibit proteolytic activity. Samples were frozen until used.

Preparation of Gel Electrophoresis

Haemolymph was centrifuged at 700 x g for 15 minutes at 4 ºC, supernatant was discarded and pellet was resuspended in 50 µl of 50mM phosphate buffer. Protein in the cell suspensions was assayed with BCA protein assay kit (Pierce Biotechnology, USA). Based on the result from protein assay, cell suspension then diluted to reach concentration protein of 1 mg/ml. Cells were extracted in SDS sample buffer, boiled for 5 minutes at 95 ºC. Samples containing 10µg protein were loaded and run on 10% SDS polyacrylamide gels (SDS-PAGE) for 45 minutes at 200V (Laemmli, 1970).

Hepatopancreas was homogenized using a sterile disposable pestle then centrifuged at 10,000 x g for 15 minutes at 4 ºC. Supernatant (aqueous phase) was taken then centrifuged a further 2 times to remove excess lipid. Protein was assayed as above and adjusted to a conncentration of 1 mg/ml. Samples were then added to 2 volumes of SDS sample buffer, boiled for 5 minutes at 95 ºC and run in SDS-PAGE 10% electrophoresis for 45 minutes at 200V, according to the method of Laemmli, 1970.

SDS-PAGE standards (Bio-Rad, Hercules, CA) were used. The resolving gel and the stacking gel were made with Tris–

HCl buffers 1.5 M pH 7.8 and 0.5 M pH 8.8, respectively. The gel was stained with Coomassie Brilliant Blue R-250 (Bio-Rad, Hercules, CA).

RESULTS

Two crayfish from the LPS-injected group died 2 days after injection and a further animal died the day after. Only one crayfish was left when sampling was done and it was also dying.

Haemolymph from dying animals did not give a pellet when centrifuged, suggesting that haemocytes had lysed or the number was very low. Unfortunately, THC was not measured in this study. For that reason haemocytes from LPS tank were not included in the assay. All the animals in Poly I:C and PBS tanks survived until they were sampled.

Result from electrophoresis of haemocytes are shown in Fig.1. The figure clearly shows that that there were no differences of protein expression between haemocytes from Poly I:C and PBS challenged crayfish. Nevertheless, electrophoresis detected that there was protein with molecular weight of 31-45 kDa expressed, which may be LGBP (arrow).

Hepatopancreas

Gel electrophoresis of hepatopancreas soluble extracts is shown in Fig.2.. Fig 2. shows there were many of protein expressed. Lanes 1 and 7 show protein from molecular weight marker, while lanes 2 and 5 contain extracts from Poly I:C injected animals, lane 4 was from LPS (dying animal) and lanes 3 and 6 contained extracts from PBS injected animals (control). Of particular interest was a band running just below the 66.2 kDa marker which, whilst expressed in the PBS control crayfish (Fig 2., lanes 3 and 6), was markedly up-regulated in the hepatopancreas fractions from poly IC injected crayfish (Fig 2., lanes 2 and 5). The protein has been indicated in the figure with an arrow.

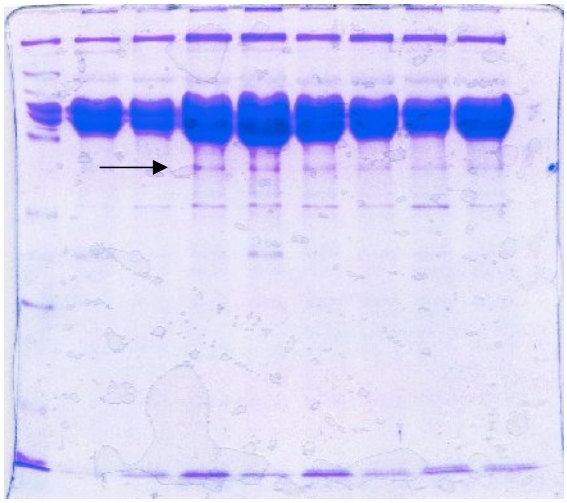

Fig. 1. Electrophoresis of hemocytes protein. Lane 1= marker; lanes 2,3, 6,7 = animal treated with Poly I:C ; lanes 4, 5, 8,9= animal treated with PBS (control)

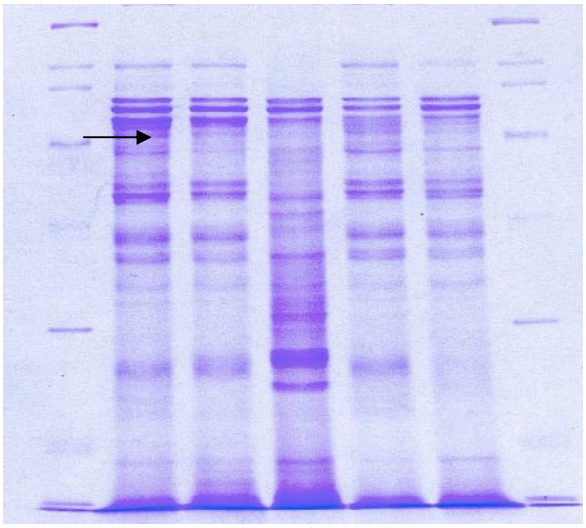

Fig. 2. Gel electrophoresis of protein expressed from redclaw hepatopancreas. Lanes 1 and 7= markers; lanes 2,3= poly I:C treatment; lane 4= LPS treatment; lanes 5, 6= PBS

DISCUSSION

Electrophoresis of redclaw hemocytes revealed the appearance of protein which can be assumed as LGBP based on its molecular weight. It has been reported that LGBP has molecular weight of 36 and 40 kDa (J. Y. Lee, Wang et al., 2000). The expression of LGBP in poly I:C treatment and control might be because the animals were already infected with bacteria in the farm. As reported, LGBP gene expression is upregulated in bacterial and fungal infection. The binding of LPS to LGBP activates the proPO and coagulation cascades and the genes for antibacterial effector proteins (Hoffman, Reichart, & Hetru, 1996; S. Y. Lee & Soderhall, 2002). Histology survey of the redclaw from the same farm revealed severe infection of bacteremia in most identified redclaw (result of histology showed herein a previous chapter of this thesis). Roux and colleagues found that LGBP could be identified in hemocytes of healthy animals and animals infected by WSSV (Roux, Pain, Klimpel, & Dhar, 2002), suggesting that LGBP is an inducible acute phase protein that may play a critical role not only in bacterial and fungal infection but also in viral pathogenesis. It is reasonable then that injecting synthetic double-stranded RNA could induce the expression of LGBP.

Crustaceans haemocyte has been known to role in the innate immunity both cellular and humoral (I. Soderhall & Soderhall, 2002). On the other hand, little is known of immune system in other crustacean organs. Therefore in this study we also investigated the expression of immune gene of hepatopancreas. The hepatopancreas is a key organ involved in the immune response of crustaceans. Hence, it is assumed to be a primary site for the production of immune recognition molecules (Gross, Bartlett, Browdy, Chapman, & Warr, 2001; Johnson, 1987). Gross and colleagues postulated that an organ of haemolymph filtration and digestion such hepatopancreas should play an important role in immune defence (Gross et al., 2001). They mentioned that several immune-function genes have been identified by high-throughput sequencing of expressed sequence tags from a hepatopancreas. Furthermore the arterioles hemal spaces of the hepatopancreas are occupied by fixed phagocytes that are involved in eliminating pathogens and other particulate matter from the hemolymph (Johnson, 1987). Several

studies have reported that hepatopancreas has antimicrobial activity against bacterial, fungi and viruses (J. R. Chisholm & Smith, 1992; Gross et al., 2001; Haug, Kjuul, Stensvag, Sandsdalen, & Styrvold, 2002; Johnson, 1987; Luo et al., 2003; Pan et al., 2000).

Results from electrophoresis of redclaw hepatopancreas showed the expression of protein which was up-regulated by Poly IC but not by LPS. The protein was constitutively expressed in PBS-injected controls but an noted increase in expression was recorded in polyIC stimulated animals.

Poly I:C , a synthetic dsRNA has been shown to effect the innate immune system of vertebrates against virus infection through the induction of interferons (IFN) which in turn upregulate the transcription of many genes that protect cells from damage and death (Loker, Adema, Zhang, & Kepler, 2004; Salinas et al., 2004). Type I IFN change cells into an antiviral state by induction of proteins such as 2', 5'-oligoadenylate synthetase (OAS), dsRNA dependent protein kinase (PKR) and Mx proteins which can inhibit viral replication (Salinas et al., 2004). Type I IFNs are produced by most cell types (Robertsen et al., 2003). Toll like receptor (TLR) which are located in the surface of cells are able to recognize and bind to virus structures which then induce IFN through NFκβ toll pathway to activate non self specific defence mechanism for example by inducing Mx protein, a member of GTP-ases superfamily, which has antiviral activity (S. Lee & Vidal, 2002). However, there are no data to suggest that invertebrate have comparable receptors capable of recognizing viruses and obvious homologs of interferon (Loker et al., 2004). Molecular weight of the polyI:C induced protein in the present study is similar to that of other Mx proteins. Furthermore, the constitutive expression with up-regulation in response to polyI:C is also indicative of Mx-like activity. Sequencing of the protein is required to determine the nature of this inducible protein. If sequencing result proves that the protein is a member of Mx protein, it will become the first Mx protein found in crustaceans, and more importantly may be indicative of a broader ranging innate antiviral activity in invertebrates.. Loker et al. (2004) suggested that mechanism of antiviral action in invertebrate works through RNAi, which is a sequence-specific response triggered by double-stranded RNA that subsequently targets any cytoplasmic RNA species sharing homology with the triggering sequence. It is increasingly viewed as a basic trans-eukaryotic genome defense against molecular parasites like viruses and transposons. (Loker et al., 2004; Sritunyalucksana, 2001).

Despite unclearness of Mx protein and its inducing mechanisms in crustaceans, it is believed that crustacean possess antimicrobial protein which can response against bacterial, fungal and viruses infection. Those antimicrobial properties not only located in the haemolymph, but also detected in other organ or tissue including hepatopancreas (J. R. Chisholm & Smith, 1992; Haug et al., 2002; Luo et al., 2003; Zhang et al., 2004). Accordingly, further research is required in order to reach a better understanding of antiviral mechanism of crustaceans in correlation with Mx protein. At this time, sequencing of protein found in this present work is still underway. In the future study, it will be worth to inject LPS and poly I:C with different doses, then sampling at the different time to find lethal dose of injection and to understand the kinetic of expected protein expression . We also suggest to measure expression of the protein in other organ such as the lymphoid organ of redclaw.

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