Characterization of Gene Expression of Biodefence-Related Genes of Kuruma Shrimp, *Marsupenaeus japonicus* Using Real-Time PCR Technology

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ABSTRACT

An expressed sequence tag analysis was conducted for discovery of biodefence-related genes of kuruma shrimp haemocytes. We sequenced 635 clones and 370 clones from healthy and white spot syndrome virus (WSSV)-infected shrimp cDNA libraries, respectively. Of 635 clones (healthy shrimp), 284 clones (44.7%) had significant homology to known genes in the DNA/protein databases. 174 (47.0%) of 370 clones (WSSV-infected shrimp) were significantly matched with sequences in the DNA/protein databases. There were 27 ESTs for biodefence- and immune-related genes including the prophenoloxidase system, antibacterial peptides and proteins, membrane-associated proteins, and soluble proteins. Oral administration of peptidoglycan (PG), an immunostimulant derived from *Biofidobacterium thermophilum*, resulted in the transcription of several biodefence-related genes in the kuruma shrimp, as shown by quantitative real-time RT-PCR. These genes included the genes for prophenoloxidase, 2 types of prophenoloxidase-activating factor, Masquerade protein, TGase, clottable protein, lysozyme, α-2-macroglobulin and penaeidin. Results from the real-time RT-PCR suggested that all but not the clottable protein dramatically increased following PG stimulation. Our results also suggest that proteins involved in innate defence mechanisms synchronously act against pathogens. Nearly all of the biodefence genes analysed were expressed solely in haemocytes, suggesting that the haemocytes have a major defence responsibility.

INTRODUCTION

Penaeid shrimps are economically important cultured crustaceans, of which the major shrimp producers are in south-east Asia, central America and south America. The total annual production from aquaculture is over one million metric tons, with a value of over 6.8 billion US dollars (FAO Fishery Statistics, 1999). However, a major problem for the industry is the outbreak of epizootic diseases, especially viral and bacterial diseases. The most significant viral diseases are caused by white spot syndrome virus (WSSV), which is found in all penaeids, yellow head virus (YHV) in the giant tiger shrimp (*Penaeus monodon*) and monodon baculovirus (MBV) in many penaeids. The most significant bacterial disease is vibriosis. Invertebrates lack a true adaptive immune system, that is, one that contains a memory system and antibodies. Instead, they have innate immunity that has two defence

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mechanisms: a cellular system and a humoral system. The cellular system is comprised of phagocytosis, nodule formation and encapsulation. In shrimp, agranular haemocytes respond with phagocytotic activity to small particles, nodule formation rids the host of large pathogens or parasites, and granular haemocytes have an ability to encapsulate microbes. The humoral reaction is composed of a coagulation system comprised of coagulation factors that catalyse clottable protein substrates, prophenoloxidase cascade producing bio-melanin, antimicrobial proteins (lysozyme, penaeidin, so on), oxigen radicals, and lectins (Destoumieux *et al*., 2000; Kanost, 1999; Kawabata and Iwanaga, 1999; Johanson, 1999; Minagawa *et al*., 2003; Relf *et al*., 1999; Schnapp *et al*., 1996; Söderhäll and Cerenius, 1998, Sritunyalucksana and Söderhäll, 2000, Sritunyalucksana *et al*., 1999, 2001). However, little is known about the humoral system in shrimp, compared with vertebrates, plants, or even in insects, which are in the same phylum as shrimp. Thus, basic knowledge of biodefence mechanisms in crustaceans is required for research and development purposes to ensure high efficiency of production. Here, we briefly review our recent results of the molecular cloning, characterization of function and quantification of gene expression of defence-related genes of kuruma shrimp (*Marsupenaeus japonicus*).

EXPRESSED SEQUENCE TAG ANALYSIS

Expressed sequence tags (ESTs) are short sequences, a few hundred base pairs in length, which are derived by partial, single-pass sequencing of the inserts of randomly selected cDNA clones (Adams *et al*., 1991). EST analysis is a powerful tool for collection and characterization of expressed genes from organs, tissues, and cells of interest. Recently, ESTs of many organisms have been reported in the EST database (dbEST,www.ncbi.nlm.nih.gov/dbEST). In the last half decade, much effort has gone into EST analysis studies, where such studies have been conducted on a variety of fish and shellfish. As of as November 13, 2002, GenBank (www.ncbi.nlm.nih.gov) had over 5,000 shrimp EST sequences and it has dbEST, deposited for Penaeidae; Kuruma shrimp, black tiger shrimp, white shrimp, and other penaeid shrimp. Recently, four papers including our own work on shrimp ESTs have been published (Gross *et al*., 2001; Lehnart *et al*., 1999; Rojtinnakorn *et al*., 2002; Supungul *et al*., 2002).

Table 1. Overview of the results of EST analysis of kuruma shrimp hemocytes.

	Healthy shrimp	WSSV infected shrimp
Total ESTs	635 (100.0)	370 (100.0)
Matched to known genes	284 (44.7)	174 (47.0)
Penaeid	182 (28.7)	35 (9.5)
Other crustaceans	10 (1.6)	13 (3.5)
Other arthropods	35 (5.5)	36 (10.0)
Other animals	51 (8.0)	80 (21.6)
Uncharacterized ESTs	6 (0.9)	9 (2.4)
Different known genes	65	54
Unknown genes	351 (55.3)	196 (53.0)

Two different cDNA libraries were constructed from the haemocytes of healthy and WSSV-infected kuruma shrimp. We sequenced a large number of clones selected randomly from the haemocytes of healthy and WSSV-infected kuruma shrimp (635 and 370 clones, respectively), and compared them with sequences in the GenBank databases (Table 1). Approximately half of the clones (480 of 1,005 clones, 47.8%) revealed significant homologies with DNA and protein sequences of known genes, whereas the remaining clones did not show any significant homology. We classified homologous cDNA sequences into ten categories; ribosomal proteins, nuclear genes, structural and cytoskeletal proteins, energy and metabolic proteins, transcription and translation proteins, regulation and signal transduction proteins, proteases and their inhibitors, secretory proteins, tumor-related proteins, and unidentified coding proteins (Fig. 1). The ratio of the number of genes in these categories varied somewhat between healthy shrimp and viral infected shrimp. Thirty-four ESTs were for immune- and defence-related proteins, including proteins involved in the prophenoloxidase activating system, antimicrobial peptides, an antimicrobial protein, membrane-associated proteins, secreted proteins, and apoptosis-related molecules (Table 2).

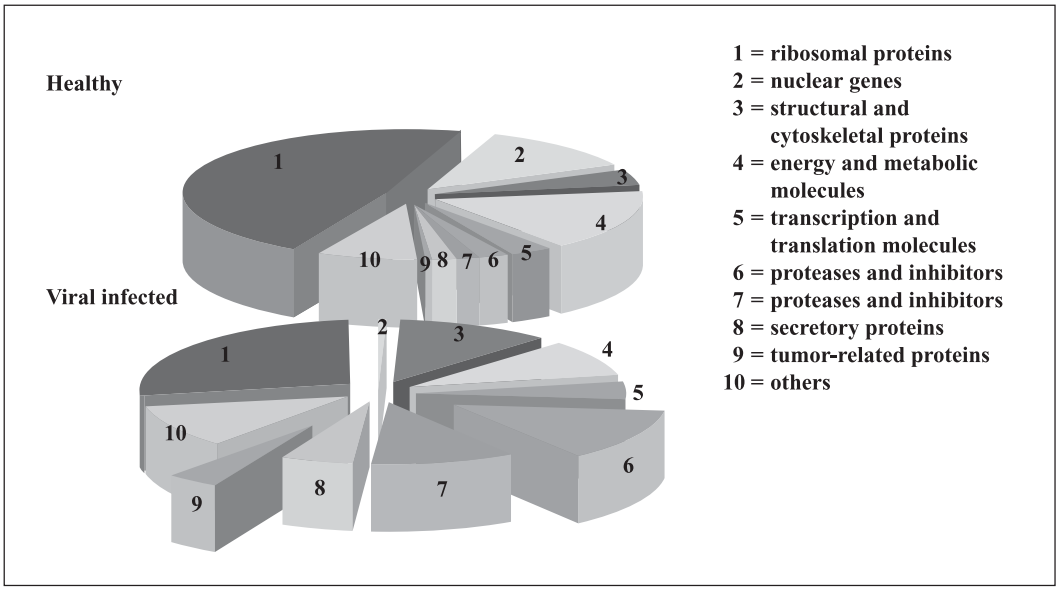

Figure 1. Comparison of the ratio of ten categorized genes between healthy and viral-infected shrimp.

Table 2. Identified putative defense-realted moleculs from kuruma shrimp ESTs.

Accession no.	Gene	Closest species
Prophenol oxidase system related molecules		
AU175227	Prophenoloxidase	*Penaeus monodon*
AU176144	Coagulation factor G - b chain precursor	*Tachypleus tridentatus*
AU175462	Factor D	*Tachypleus tridentatus*
AU176028	Masquerade protease	*Pacifastacus leniusculus*
AU175379	a2-macroglobulin	*Limulus sp.*
AU176036	Antileukoprotease (1)	*Gallus gallus*
AU176305	Antileukoprotease (2)	*Gallus gallus*
AU175528	Chelonianin	*Caretta caretta*
AU176369	Elastase inhibitor	*Anemonia sulcata*
AU176018	Kazal inhibitor (1)	*Pacifastacus leniusculus*
AU176105	Kazal inhibitor (2)	*Pacifastacus leniusculus*
AU175971	Kunitz-type inhibitor	*Ipera ammodytes*
AU175804	Clottable protein	*Penaeus monodon*
AU175508	Hemocyte TGase	*Tachypleus tridentatus*
Anti-microbial substances		
AU175976	Bactinecin 11	*Ovis aries*
AU175469	Lysozyme	*Mus musculus*
AU175636	Penaeidin-2	*Penaeus vannamei*
Putative defense substances		
AU176315	Basophilic leukocyte Interleukin-3 regulated protein	*Homo sapiens*
AU176256	integra transmembrane protein	*Homo sapiens*
AU176124	Drac - Ras-like GTP-binding protein	*D. melanogaster*
AU176073	ESM-1 protein	*Homo sapiens*
AU176327	Natural killer cell - enhancing factor (NK-EF)	*Trypanasoma brucei*
AU176285	Notch homolog	*D. melanogaster*
AU175688	Beta-integrin	*P. leniusculus*
AU175893	Cell adhesion molecule	*Hirudo medicinalis*
AU176002	Collagen alpha 3 (Iva)	*Mus musculus*
AU176210	Collagen IV alpha 4	*Homo sapiens*
AU176045	Collagen,fragment	*Haemonchus contortus*
Apoptosis and tumor related molecules		
AU175974	Ubiquinol-cytochrome c reductase complex	*Bos taurus*
AU176167	Proteasome 26S subunit	*Homo sapiens*
AU176101	Programmed cell death 6- interacting protein (Alix)	*Homo sapiens*
AU176195	KE-3 (ribosomal protein S18)	*Homo sapiens*
AU176183	Rat insulinoma gene	*Homo sapiens*
AU176216	Wilm's tumor-related protein	*Bombyx mandarina*

BIODEFENCE-RELATED MOLECULES

Prophenoloxidase

The prophenoloxidase (proPO) system is a complex cascade reaction leading to the production of melanin, which is induced with high sensitivity upon exposure to picogram levels some of products of pathogenic agents, such as lipopolysaccharide (LPS), peptidoglycan (PG) and β-1,3-glucan (Söderhäll and Cerenius, 1998; Sritunyalucksana and Söderhäll, 2000; Sritunyalucksana *et al*., 1999; 2001). We have cloned proPO cDNA from kuruma shrimp and obtained the full sequence of 3,047 bp (Aoki *et al*., 2004). This gene encodes 688 amino acid residues and the putative activated site was found at Arg44-Ile45 which, is the same as in other proPOs. The position of important amino residues such as 6 histidine residues and domain structures such as a copper (Cu)-binding site, complement-like motif and N-glycosylation sites were found in kuruma shrimp proPO. Phylogenic analysis confirmed that crustacean proPOs differed from those of insects (Aoki *et al*., 2004). *Marsupenaeus japonicus* proPO is closest to those published for *Penaeus monodon* and *Pacifastacus leninuculus* (freshwater crayfish). Tissue expression, as determined by RT-PCR, showed that proPO is expressed only in haemocytes (Aoki *et al*., 2004).

Antimicrobial peptide penaeidin

Cationic antimicrobial peptides play important roles as effectors of the first line of defence in innate immunity of every animal, due to their broad-spectrum of antimicrobial activity and rapid production (Destoumieux *et al*., 1997; Munoz *et al*., 2002). The full sequence of a penaeidin-like peptide from an EST clone of *Marsupenaeus japonicus* was recently determined (Rojtinnakorn *et al*., 2002). This antimicrobial peptide, now named penaeidin-pj, is composed of 4 domains; a Ser-rich domain, a Ser-Arg-rich domain, a Pro-rich domain and a Cys-rich domain. Many isoforms of penaeidin-pj were identified. It was found that they could be classified into 2 groups. One group, consisting of two isoforms (1a and 1b), lacks the Ser-rich and Ser-Arg-rich domains, while the other group, consisting of eight isoforms (2a to 2h) have these domains. The alignment of penaeidin-pj was compared with other penaeidins. The six cys-disulfide bridges are highly conserved and are located in the same position. One of the penaeidin-pj isoforms was tested for antimicrobial activity using agarose gel diffusion. It was found to be effective against *Micrococcus lysodeikiticus* activity (Rojtinnakorn, 2002).

Lysozyme

Lysozyme (muramidase, EC.3.2.1.17) is a widely distributed enzyme, located in many tissues of invertebrates and vertebrates. It catalyzes the hydrolysis of bacterial cell walls and acts in non-specific innate immunity against the invasion of bacterial pathogens (Jollés and Jollés, 1984). Lysozymes are classified into 5 types: chicken-type lysozyme (c-type), which includes insect lysozyme; goose-type lysozyme (g-type); T4 phage lysozyme (phage-type); plant lysozyme and bacterial lysozyme (Prager and Jollés, 1996; Hultmark, 1996; Beintema and Terwisscha van Scheltinga, 1996; Holtje, 1996; Fastrez, 1996; Qasba and Kumar, 1997).

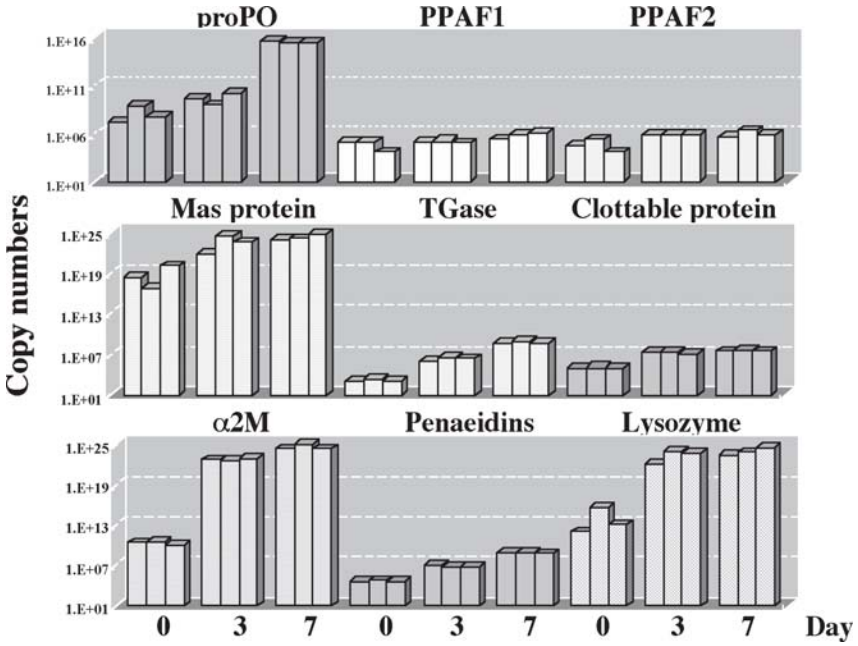

Figure 2. The gene expression patterns of triplicate samples of proPO, PPAF1, PPAF2, Mas protein, TGase, clottable protein, a2-macroglobulin, penaeidins, and lysozyme in *M. japonicus* haemocytes at days 0, 3 and 7 of peptidoglycan administration.

We cloned a lysozyme cDNA from kuruma shrimp (Hikima *et al*., 2003). The lysozyme cDNA consists of 1,055 bps including the initiation codon (ATG) stop codon (TAG) and poly (A) tail and codes for 158 amino acids (Hikima *et al*., 2003). A comparison of amino acid sequences of c-type lysozymes from kuruma shrimp and ten other animals (Pacific white shrimp, cecropia moth, domestic silkworm, tobacco budworm, malaria mosquito, fruit fly, human, chicken, rainbow trout and Japanese flounder) showed that two catalytic residues (glutamic acid and aspartic acid) and the eight cysteine residues are completely conserved in the kuruma shrimp lysozyme. The sequences flanking the catalytic residues are also highly conserved. The sequences of both the kuruma shrimp and Pacific white shrimp had two insertions that were not present in the other sequences. The kuruma shrimp lysozyme gene was mainly expressed in haemocytes, although weak expression was observed in other tissues (Hikima *et al*., 2003).

We expressed the kuruma shrimp c-type lysozyme cDNA in insect cells using a baculovirus expression system (Hikima *et al*., 2003). Lysozyme assays were performed by a lysoplate assay and a turbidimetric assay. The recombinant kuruma shrimp lysozyme clearly showed lytic activity (Hikima *et al*., 2003). The effects of temperature and pH on lysozyme activity were measured using the lysoplate assay. Optimum pH and temperature of kuruma shrimp lysozyme were pH 7.5 and 50°C, respectively. Kuruma shrimp lysozyme was observed to have lytic activity against all the examined Vibrio strains (*V. anguillarum, V. alginolyticus,* and *V. parahaemolitycus*), and another marine fish pathogen (*Pasteurella piscicida*), and *Escherichia coli* (but not *Edwardsiella tarda* and *Lactococcus garvieae*). It also had more lytic activity that hen egg white lysozyme. Thus, the kuruma shrimp lysozyme potentially has a broad range of activities in the innate immune system.

PEPTIDOGLYCAN ENHANCEMENT OF GENE EXPRESSION OF BIODEFENCE GENES IN *PENAEUS JAPONICUS* HAEMOCYTES

Immunostimulants have successfully been used to enhance resistance of fish and crustacea against bacterial or viral infections. Immunostimulants are agents that enhance non-specific immune responses (phagocytosis, macrophage activity and non-specific humoral mediated factors). Prophylaxis has been effectively applied as an alternative for antibiotics in cultivated fish and shellfish. In recent years, immunostimulants such as PG, LPS and β-1,3 glucan were administered orally, by injection or by immersion in a bath, to enhance the resistance of shrimp to bacterial and viral infections (Itami *et al*., 1996; Raa, 1996; Scholz *et al*., 1999; Takahashi *et al*., 2000). However, the molecular mechanisms by which immunostimulants affect the expression of each biodefence gene are unclear. Peptidoglycans are found in the rigid cell wall of Gram-positive and Gram-negative bacteria, mycobacteria and yeast glucan. In order to study the effects of enhancement at the molecular level, we used peptidoglycan, a muramyl dipeptide substance, as an immunostimulant for kuruma shrimp. Expression patterns of the proPO system peptides, proPO and its three activating factors, PPAF1, PPAF2 and Mas protein were analysed by conventional reverse transcription PCR (RT-PCR) and real-time quantitative PCR. The effects of PG on the expression of genes encoding proteins involved in the clotting system (transglutaminase and clottable protein) and antimicrobial proteins (α_2-macroglobulin, lysozyme and a penaeidin homologue called penaeidin-pj) were compared with proPO-system proteins. PG was prepared by lysozyme digestion of the *Bifidobacterium thermophilum* cell wall. Kuruma shrimp with an average size of 19.4 g body weight were acclimated in the laboratory for 1 week at ambient temperature and fed 4 times daily with PG at a rate of 0.2 mg/kg body weight for 7 days. Haemolymph of the three shrimps were collected before feeding of stimulant, and 3 and 7 days after PG feeding. Tissue samples were processed and cDNA was synthesized. Then, we conducted reverse transcription PCR (RT-PCR) and real-time quantitative PCR. Real-time fluorescence PCR was performed using SYBR green PCR core reagents (Perkin-Elmer, USA) as described previously (Rattanachai *et al*., 2004). PCR reactions were performed in a GeneAmp 5700 sequence detector using the sequence detection system version 1.3 (Applied Biosystems, USA), and PCR conditions consisting of 50°C for 2 min, 95°C for 10 min followed by 45 cycles of 95°C for 0.15 min and 60°C for 1 min.

The pro-PO activation system related proteins (PPAF1, PPAF2), the coagulation related proteins (TGase and clottable protein), and antimicrobial protein and peptide ($\alpha_2$macroglobulin, and penaeidions) genes were found to be expressed specifically in haemocytes (Aoki *et al*., 2004). Masquerade protein and α_2- macroglobulin genes were extensively expressed in haemocytes, and also the lymphoid organ, hepatopancreas, gill, heart, midgut and muscle (Aoki *et al*., 2004). The lysozyme gene is mainly expressed in haemocytes although weak expression was observed in other tissues (Hikima *et al*., 2003). This result suggests that haemocytes play an important role in the biodefence mechanisms of *M. japonicus*. The RT-PCR analysis, although not quantitative, suggested that all genes were expressed much more strongly in the PG-administrated shrimp than in the control shrimp at days 3 and 7 (Aoki *et al*., 2004).

The expression patterns of various immune-related genes, as determined by real-time PCR, were similarly elevated in the PG administrated shrimp (Fig. 2). PG treatment was found to significantly enhance the expression of lysozyme, penaeidin-pj and α_2-macroglobulin after 3 days, whereas a significant enhancement of proPO and coagulation systems was not observed until after 7 days. Presumably, the antimicrobial peptides and the specific binding proteins might have responded immediately following the detection of the presence of pathogenic agents, following which the proPO cascade is activated. These results suggest that PG can effectively promote expression of all genes examined which are involved in shrimp innate defence mechanisms, including proPO cascades, coagulation and antimicrobial substances in *P. japonicus*. Interestingly, induced copy numbers of mRNA of characterized genes were different in each gene. This suggests that while PG induces the expression of shrimp biodefence genes, there may exist different regulatory mechanisms for each gene. Haemocytes, which are systematically circulated, might have an intrinsic function for targeting pathogenic agents, releasing biodefence genes or activating the biodefence mechanisms in this species. We have characterized the shrimp biodefence network system by using a cDNA microarray that is based on our EST clones.

In conclusion, we found several biodefence-related molecules using EST analysis and showed that quantitative real-time PCR is a powerful method for the characterization of gene expression pattern of biodefence-related genes of kuruma shrimp. The results of characterization of gene expression suggest that PG feeding activates several different biodefence system in shrimp.

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