Developmental and Comparative Immunology (2008) **32**, 1497–1509

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Shrimp single WAP domain (SWD)-containing protein exhibits proteinase inhibitory and antimicrobial activities

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Received 13 May 2008; received in revised form 5 June 2008; accepted 7 June 2008
Available online 3 July 2008

KEYWORDS
Shrimp;
Penaeus monodon;
Whey acidic protein domain;
Crustin;
Proteinase inhibitor;
Antimicrobial peptide

Summary
Single WAP domain (SWD)-containing proteins are small proteins with a C-terminal region containing a single whey acidic protein (WAP) domain. In the present study, the cDNAs representing three isoforms of SWD proteins (SWD*Pm*1, SWD*Pm*2 and SWD*Pm*3) were identified from hemocytes of the black tiger shrimp, *Penaeus monodon*. The deduced peptides revealed that they contain a putative signal peptide of 24 amino acids and encode for a mature peptide of 69, 68 and 56 amino acids, respectively, which contain typical characters similar to those of the shrimp SWD proteins (type III crustin) with a Pro-Arg region and a WAP domain towards the C-terminus. Tissue distribution analysis by RT-PCR showed that all three SWD*Pm* transcripts were primarily found in hemocytes. Transcript expression of SWD*Pm*1 was down-regulated upon injection with *Staphylococcus aureus* whilst there was no change of SWD*Pm*2 and SWD*Pm*3 expression. In contrast, white spot syndrome virus (WSSV) injection resulted in a biphasic response with up-regulation of SWD*Pm*1 and SWD*Pm*2 transcripts at 6 h followed by significant down-regulation by 24 h after infection. Genomic organization of the SWD*Pm*2 gene revealed the presence of three exons interrupted by two introns. To characterize the biological functions of the SWD protein, the mature SWD*Pm*2 protein encoding cDNA was cloned and expressed in *Escherichia coli*. Purified recombinant (r)SWD*Pm*2 exhibits antibacterial activity against several Gram-positive, but not Gram-negative, bacteria and is a competitive inhibitor of subtilisin A with an inhibition constant (K_i) of 1.98 nM. Thus, rSWD*Pm*2 may contribute to the inhibitory regulation of subtilisin A from bacterial infection and *P. monodon* SWD

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0145-305X/$ - see front matter
doi:10.1016/j.dci.2008.06.005

protein likely function as immune effectors in defense against invasion of shrimp pathogens.

Introduction

Innate immunity in multicellular organisms is the first line of defense against invading microbes such as bacteria, fungi and viruses. Like other invertebrates, shrimp do not have an acquired immunity; instead they have an efficient innate immune system, which includes melanization by the prophenoloxidase-activating system, a clotting process, phagocytosis, encapsulation of foreign material, cell agglutination and a diverse array of general and specific antimicrobial peptides (AMPs) [1].

AMPs and proteinase inhibitors are important components of the host innate immune system and play crucial roles in the host defense against microbial invasion. AMPs, including penaeidins [2], antilipopolysaccharide factors [3] and crustins [4,5] are found in shrimp hemocytes. Serine proteinase inhibitors, which are widely distributed in animals, plants and microorganisms, play a critical role in the regulation of many biological processes. Unsurprisingly then, many pathogens are known to produce extracellular proteinases which are reported to serve an active role in the development of various diseases. Several reports have suggested that a major function of proteinase inhibitors is to combat the proteinase of pathogens [6], and it is no surprise that at least some proteinase inhibitors have been found in crustaceans, such as members of the Kazal [7–9], serpin [10], pacifastin [11] and alpha 2-macroglobulin [12–16] families. These proteinase inhibitors and AMPs both serve defensive roles and exert the effect on pathogens.

Proteins containing a whey acidic protein (WAP) domain, initially characterized as a milk protein, have been found in several species of vertebrates and invertebrates [17,18]. The WAP domain comprises of approximately 50 amino acids including eight cysteine residues which form a four-disulfide core (4-DSC) [17]. The WAP domain is not however exclusive to WAP proteins but is found in numerous other proteins, where it may be present as multiple domains. WAP domain proteins are, however, typically small secretory proteins, which exhibit a variety of functions including proteinase inhibitory and antimicrobial activities [19,20].

Currently, a family of single WAP domain (SWD)-containing proteins in crustaceans has been described as crustins [18]. Type I and II crustins have been characterized in several species of crustaceans and have been shown to be abundant and to exhibit antimicrobial activity mainly against Gram-positive bacteria. Type III crustin classification is based on the protein domain structure which contains a signal peptide and a Pro-Arg region at the N-terminus and a WAP domain towards the C-terminus. Currently, the presence of Type III crustin has only been reported in shrimp and no biological function has yet been reported. The SWD-containing proteins from *Litopenaeus vannamei* (SWD*Lv*) and *Penaeus monodon* (SWD*Pm*) have been identified and current studies have mainly focused on cDNA sequences, gene expression levels and promoter analysis [21–23]. Here, we report the cDNAs encoding three isoforms of the SWD-containing protein from the black tiger shrimp, *P. monodon*, and for the first time, the biological functions were ascribed to this shrimp, or at least the *P. monodon*, SWD protein (type III crustin).

Material and methods

Animals and sample preparation

Three-month-old subadult black tiger shrimp, *P. monodon*, of about 20 g weight, were obtained from a local farm in Thailand. The challenge experiment was performed by injection into the last abdominal segment of each shrimp *either* shrimp salt solution (SSS: 450 mM NaCl, 10 mM KCl, 10 mM HEPES, pH 7.3) as a control, or a suspension of formalin-inactivated *Staphylococcus aureus* (10^8 CFU) in the same volume of SSS. Alternatively, shrimp were challenged *with* a viable white spot syndrome virus (WSSV) virion suspension (7.6×10^5 viral copies of WSSV) in lobster hemolymph medium (LHM), prepared as previously described [24], or with the same volume of LHM alone (control). Hemocytes were collected from the shrimp ventral sinus at 0, 6, 24 and 48 h after *S. aureus*, WSSV and control (SSS or LHM only) injections into an anticoagulant solution of 10% (w/v) trisodium citrate dihydrate pH 4.6. Collected hemolymph was immediately centrifuged at 800*g* for 10 min at 4 °C to separate hemocytes from the plasma and the hemocyte pellet was then immediately resuspended in TRI REAGENT® (Molecular Research Center, USA) for further processing.

RNA isolation and first-strand cDNA synthesis

Total RNA was isolated from dissected tissues, including hemocytes prepared as above, using TRI REAGENT® and treated with DNase I (Promega, USA) following the manufacturer's protocol. Total RNA concentration and integrity was assessed by UV spectrophotometry and agarose gel electrophoresis. First-strand cDNAs were synthesized from 2 μg of DNA-free total RNA sample and 0.5 μg of oligo $(dT)_{18}$ primers using the ImProm-II™ Reverse Transcriptase System kit (Promega, USA) according to the manufacturer's protocol.

Tissue distribution analysis

RT-PCR was carried out to investigate the transcript expression profile of SWD*Pm*1, SWD*Pm*2 and SWD*Pm*3 transcripts in different tissues of *P. monodon* including hemocytes, hepatopancreas, lymphoid organ, gill, intestine and heart. A pair of each SWD*Pm*-specific primers (SWD*Pm*1-F and SWD*Pm*1-R, SWD*Pm*2-F and SWD*Pm*2-R, and SWD*Pm*3-F and SWD*Pm*3-R) was designed (Table 1). The elongation

Table 1 Primer sequences used for amplification of SWD genes.

Primer	Sequence (5′–3′)
SWD*Pm*1-F	5′ CGATATCTTCTCCATCTGCGTC 3′
SWD*Pm*1-R	5′ GAGCCAACCGCGATGACGTCAG 3′
SWD*Pm*2-F	5′ CGGCATCATCACCACGTGCGAG 3′
SWD*Pm*2-R	5′ TCAGTAACCTTTCCAGGGAGAC 3′
SWD*Pm*3-F	5′ TTAAGGGCCATCAGCTGTAACG 3′
SWD*Pm*3-R	5′ ACGATTACCACAGTCCAAGGTC 3′
SWAP-F	5′ CACAGGATCTGATTCCCAAAATGG 3′
SWAP-R	5′ CTCTTACCACCTTCCTTTTCCGTA 3′
NcoI-SWAP-F	5′CATGCCATGGGCCATCATCATCATCATCATATG GTTCCAACGAGACACAGTAAGCC 3′
NotI-SWAP-R	5′ ATAAGAATGCGGCCGCTTACCACCTTCCTTTTC CGTAGGGAGGA 3′
EF1α-F	5′ GGTGCTGGACAAGCTGAAGGC 3′
EF1α-R	5′CGTTCCGGTGATCATGTTCTTGATG 3′

factor 1-α gene (EF-1α) was used as an internal control. PCR amplifications were performed as described in [25]. The amplification product was analyzed on a 1.8% agarose gel, and visualized by UV transillumination following ethidium bromide staining.

Semi-quantitative RT-PCR analysis

The same transcript-specific primer sets (Table 1) were used for the amplification of SWD*Pm*1, SWD*Pm*2 and SWD*Pm*3 transcripts and their expression levels were normalized relative to the EF-1α gene transcript expression level from the same cDNA preparations using Genetools Analysis Software (Syngene). The number of PCR reaction cycles was optimized to 25 cycles for SWD*Pm*1 and SWD*Pm*2 and 35 cycles for SWD*Pm*3 (data not shown). The cycling protocol was 1 cycle of 94 °C for 1 min; 25 or 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s followed by 1 cycle of 72 °C for 5 min.

Genomic organization

Genomic DNA was extracted from the plepod of individual shrimp using a standard phenol–chloroform extraction method. To obtain the sequence of SWD*Pm*2 genomic DNA, gene-specific primers (SWAP-F and SWAP-R, Table 1) were designed from start and stop codons of the SWD*Pm*2 cDNA sequence (EU623980). PCR reactions were performed with the BD Advantage™ Genomic PCR Kit (Clontech, USA) using touch-down PCR with the conditions as follows: 3 min initial denaturation at 94 °C for 1 cycle, then 21 cycles of denaturation at 95 °C for 30 s, primer annealing at gradient temperature from 65 to 55 °C (each cycle 0.5 °C lower than the former) for 30 s, and extension at 72 °C for 3 min. An additional 25 cycles were performed each at 95 °C for 30 s, at 55 °C for 30 s and extension at 72 °C for 3 min. At the end of the last cycle, the PCR mixture was incubated at 72 °C for 10 min. The PCR products were then fractionated on a 1.2% (w/v) agarose gel and the DNA fragments were recovered and cloned into pGEM-T easy vector (Promega, USA). The clones were then screened and sequenced in both directions using M13 forward and reverse primers, with an automated sequencer by a commercial service (Macrogen Inc., Korea).

Sequence data analysis

The complete nucleotide sequence was analyzed with GENETYX (Software Development Inc.). The DNA and amino acid sequence homology were searched using BLASTX/BLASTP [http://www.ncbi.nlm.nih.gov/BLAST/]. The signal peptide cleavage site was determined by SignalP V3.0 [http://www.cbs.dtu.dk/services/SignalP/]. Based on the putative amino acid sequences, multiple sequence alignments were performed using Clustal W. Aligned sequences were bootstrapped 1000 times using Seqboot. Sequence divergence was calculated based on the two Kimura parameter method using Prodist. Boostrapped neighbor-joining trees were constructed using Neighbor and Consense. All phylogenetic reconstruction programs were routine in PHYLIP [26] and phylogenetic tree was appropriately illustrated using TREEVIEW (http://taxonomy.zoology.gla.ac.uk/rod.html).

Construction of expression vector

Sequence encoding for the predicted mature SWD*Pm*2 encoding (no signal sequence) was amplified from the clone using *Pfu* DNA polymerase (Promega, USA). For the convenience of cloning, an *Nco*I site and six His encoded nucleotides were added to the 5′-end of the NcoI-SWAP-F primer and a *Not*I site was added to 5′-end of the Not I-SWAP-R primer after the stop codon (Table 1). *Pfu*-based PCR amplification from cDNA was performed under the following conditions: 94 °C for 3 min (1 cycle); 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min (29 cycles) and 72 °C for 10 min (1 cycle). The PCR products were fractionated on a 1% (w/v) agarose gel and the DNA fragments were recovered, digested with *Nco*I and *Not*I restriction enzymes, and cloned into *Nco*I and *Not*I linearized pET28b. The recombinant plasmid was transformed into *Escherichia coli*

JM109 for expansion with nucleotide sequencing to ensure inframe insertion. The resulting plasmid was then transformed into *E. coli* Rosetta (DE3) pLysS cells for protein expression.

Recombinant protein expression

Cells were grown at 37 °C in LB medium containing 50 μg/ml kanamycin under agitation until they reached an OD_{600} of 0.6. Expression was then induced by the addition of IPTG to a final concentration of 1 mM and monitored by SDS-PAGE resolution (see below) every hour for 4 h. Subsequently, production of the rSWDPm2 protein was attained by 4 h of induction. Cells were harvested by centrifugation at 6000 rpm for 15 min at 4 °C. The pellets were washed twice with 20 mM Tris–HCl buffer, pH 8.0 and then resuspended in the same buffer and disrupted by sonication. The lysate was centrifuged at 13,000 rpm, 4 °C for 20 min and soluble and insoluble fractions were analyzed by 15% (w/v) SDS-PAGE. The protein fraction obtained by centrifugation was stored at −80 °C.

Protein purification

The recombinant rSWD*Pm*2 protein was purified using Ni-NTA affinity chromatography. After analysis of the protein fractions, rSWD*Pm*2 protein was found in the pellet fraction as inclusion bodies. Therefore, the pellet containing insoluble protein (inclusion bodies) was subjected to purification by a prepared Ni-NTA affinity chromatography column according to the manufacturer's suggestions for insoluble proteins (Qiagen, USA). Briefly, inclusion bodies were washed twice in 20 mM Tris–HCl (pH 8.0) followed by centrifugation and dissolved in a denaturing buffer (8 M urea). The solution was loaded onto a nickel-nitrilotriacetic acid (Ni-NTA) agarose column (Qiagen, USA), washed with a denaturing buffer containing 20 mM imidazole, and eluted by a denaturing buffer containing 250 mM imidazole. Purified proteins were analyzed in 15% (w/v) SDS-PAGE.

Protein refolding and molecular mass determination

For recombinant protein refolding, 1 ml of elution fractions were collected and pooled fractions were dialyzed overnight at 4 °C against 20 mM sodium phosphate buffer (pH 5.8). The dialyzed protein sample was then centrifuged at 13,000 rpm for 20 min at 4 °C to remove unfolded or aggregated proteins and analyzed by SDS-PAGE. The concentration of rSWD*Pm*2 protein was determined by Bradford's method, and its molecular weight estimated by coresolution with standard markers through a 15% (w/v) SDS-PAGE gel. Moreover, the purified rSWD*Pm*2 protein was subjected to mass spectrometric analysis, MALDI-TOF spectra, at the Bioservice unit, National Science and Technology Development Agency (NSTDA), Thailand, to confirm its purity and likely sequence identity, which also confirmed its mass.

Antimicrobial assay

The antimicrobial activity of the purified rSWDPm2 protein was tested against five Gram-positive (*S. aureus*, *S. haemolyticus*, *Aerococcus viridans*, *Bacillus megaterium* and *Micrococcus luteus*) and five Gram-negative (*Salmonella typhimurium*, *E. coli* 363, *Enterobacter cloacae*, *Vibrio harveyi* and *Klebsiella pneumoniae*) bacterial species by liquid growth inhibition assays. The antimicrobial activity was determined against each bacterial strain as the minimal inhibitory concentration (MIC), the lowest concentration that caused 100% inhibition of bacterial growth. Serial twofold dilutions of rSWDPm2 protein ranging from 0 to 50 μM were made in 20 mM sodium phosphate buffer (pH 5.8). Ten microliters of each concentration was added to each corresponding well of a 96-well microtiter plate, and each well was inoculated with 90 μl of a suspension of mid-log phase bacteria (10^5 CFU/ml) in Poor Broth (1% (w/v) tryptone, 0.5% (w/v) NaCl, pH 7.5). The negative control was 90 ul of poor broth with 10 ul of 20 mM sodium phosphate buffer, pH 5.8. Cultures were grown for 24 h at 30 °C. The growth of bacteria was evaluated by measuring the culture absorbance at 595 nm using a microplate reader.

Serine proteinase inhibition assays

The inhibition effects of rSWD*Pm*2 on the hydrolysis of synthetic chromogenic substrates by the serine proteinases trypsin (bovine pancreas, Sigma), chymotrypsin (type II bovine pancreas, Sigma), subtilisin A (*Bacillus licheniformis*, Sigma) and elastase (porcine pancreas, Pacific Science) were assayed in 50 mM Tris–HCl, pH 8.0 buffer at 30 °C. *N*-benzoyl-Phe-Val-Arg-*p*-nitroanilide (146.8 and 293.6 μM) for trypsin and subtilisin A, respectively, *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (147.3 μM) for chymotrypsin and *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide (443.1 μM) for elastase were used as a substrate. The proteinase (final concentrations of 5, 3, 10 and 3.9 nM for trypsin, chymotrypsin, substilisin A and elastase, respectively) and different amounts of rSWD*Pm*2 (final concentrations of 0, 2, 4, 8.1, 16.3, 32.5, 65 and 130 nM) were pre-incubated for 15 min at 30 °C and then terminated by adding 50 μl of 50% (v/v) acetic acid. The formation of *p*-nitroaniline was monitored continuously by a spectrophotometric method at 405 nm. The remaining activity was calculated and plotted against the molar ratios of inhibitor to proteinases.

Kinetics of serine proteinase inhibition

The inhibition constant K_i of rSWD*Pm*2 for the hydrolysis of *N*-benzoyl-Phe-Val-Arg-*p*-nitroanilide by subtilisin A was determined according to the method of Somprasong et al. [27]. Four sets of reactions in which each set consisted of five concentrations of substrate in the presence of fixed amounts of serine proteinase and the rSWD*Pm*2, and three different concentrations of rSWD*Pm*2 were used. For subtilisin A: 18 nM of subtilisin A; 0, 0.11, 0.22, 0.44 and 0.88 mM *N*-benzoyl-Phe-Val-Arg-*p*-nitroanilide; and 0, 8.125, 16.25 and 32.5 nM of rSWD*Pm*2 were used. The reactions were made into a total volume of 100 μl using 50 mM Tris–HCl, pH 8.0 and initiated by the addition of proteinase.

After incubating at 30 °C for 15 min, they were arrested with 50 μl of 50% (v/v) acetic acid. The absorbance of *p*-nitroaniline was measured at 405 nm. The amount of *p*-nitroaniline was calculated using a millimolar extinction coefficient (1 cm) of 9.96. The activities were calculated as nmole of *p*-nitroaniline/min and were plotted against the concentrations of substrates as a substrate saturation curve and a Lineweaver–Burk plot. The apparent Michaelis–Menten K_M's at different concentrations of inhibitor and V_{max} were determined. The apparent K_M's were replotted against the concentrations of inhibitor for the calculation of inhibition constant (K_i).

Results

Characterization of SWD cDNAs

The cDNA sequences encoding the SWD proteins were searched from the *P. monodon* EST database (http://pmonodon.biotec.or.th) [28] and 50 clones homologous to the cDNAs of SWD-containing proteins were identified. These cDNA sequences were clustered into three different types, namely SWD*Pm*1, SWD*Pm*2 and SWD*Pm*3. The SWD*Pm*1 (17 clones) and SWD*Pm*3 (1 clone) were identified from the hemocyte cDNA library of unchallenged shrimp, whilst SWD*Pm*2 (32 clones) were detected in the cDNA libraries of unchallenged and both WSSV- and heat-challenged shrimp. The nucleotide sequence of each SWD*Pm* EST cluster was reblasted against the data in the GenBank. The BLASTX results showed that SWD*Pm*1, SWD*Pm*2 and SWD*Pm*3 significantly matched with the SWD cDNA from *P. monodon* (AY464465) previously reported by Jiménez-Vega et al. [21] with 96%, 86% and 46% identity, respectively. The obtained cDNA sequences of SWD*Pm*1 (EU623979), SWD*Pm*2 (EU623980) and SWD*Pm*3 (EU623981) consisted of 416, 427 and 438 bp including a predicted open reading frame of 279, 282 and 240 bp, respectively, encoding a polypeptide of 92, 93 and 80 amino acids, respectively. The characteristics of the three SWD*Pm* including predicted molecular mass of the mature proteins and their isoelectric point (p*I*) are shown in Table 2.

Sequence comparison and phylogenetic tree analysis

Via a search of the Simple Modular Architecture Research Tool (SMART) database, the deduced amino acid sequences of SWD*Pm*1, SWD*Pm*2 and SWD*Pm*3 proteins showed typical characters of the shrimp SWD proteins that contained a Pro-Arg region and a SWD with eight cysteine residues forming a 4-DSC domain (Figure 1A). The results from ClustalW pairwise comparison revealed that the deduced amino acids of SWD*Pm*1 (EU623979) in the present study were identical to those of the SWD protein of *P. monodon* (AY464465) except for the replacements of S_3 to N, R_{35} to Q and N_{79} to R. Thus the high sequence identity between SWD*Pm*1 and *P. monodon* SWD proteins suggested that they are probably allelic forms of the same gene.

The deduced amino acid sequence of SWD*Pm*2 and SWD*Pm*3 shared 86% and 45% amino acid identity to SWD*Pm*1, respectively. Although they contain a highly conserved signal peptide and a WAP domain with eight conserved cysteine residues, the low sequence identity in the absence of shuffled domains, large indels or C-terminal sequence divergence suggests that SWD*Pm*2 and SWD*Pm*3 are probably encoded from different SWD genes rather than are products of differential splicing or ±frameshifts.

To determine the relationship of the SWD*Pm* proteins with other shrimp SWD proteins and the known WAP domain containing proteins including crustins and secretory leukocyte protease inhibitors (SLPIs) from several crustaceans, a phylogenetic tree was constructed using the NJ distance-based method to compare the amino acid sequences of the WAP domain. The unrooted tree obtained indicates that SWD*Pm*1 and SWD*Pm*2 protein sequences are more closely related to the other shrimp SWD proteins, while SWD*Pm*3 diverted from the shrimp SWD proteins and was grouped within the cluster of WAP domain of SLPI from various shrimp species. Nevertheless, the phylogenetic tree indicated that the shrimp SWD proteins were more closely related to WAP domain of shrimp SLPI than crustins (Figure 1B).

Gene expression in different tissues

Tissue-specific mRNA expression was assessed by RT-PCR. The amplification yielded amplicons of 181, 212 and 215 bp of gene-specific cDNA fragments from SWD*Pm*1, SWD*Pm*2 and SWD*Pm*3, respectively. The internal control, EF-1α mRNA, was detected in all the selected tissues (Figure 2A) and was used to normalize the SWD*Pm* expression levels. SWD*Pm*1 and SWD*Pm*3 transcripts were specifically expressed in hemoctyes at high and low relative expression levels, respectively, whilst the SWD*Pm*2 transcript was abundantly expressed in hemoctyes as well but was also

Table 2 Characteristics of the *P. monodon* SWD*Pm* (SWD*Pm*1, SWD*Pm*2 and SWD*Pm*3) cDNA transcripts and predicted protein products.

Accession no.:	SWD*Pm*1 EU623979	SWD*Pm*2 EU623980	SWD*Pm*3 EU623981
Full-length sequence (bp)	416	427	438
ORF (bp)	279	282	240
Signal sequence (aa)	24	24	24
Mature protein (aa)	68	69	56
Molecular weight (kDa)	7.29	7.38	6.23
Isoelectric point (p*I*)	8.07	8.29	8.28

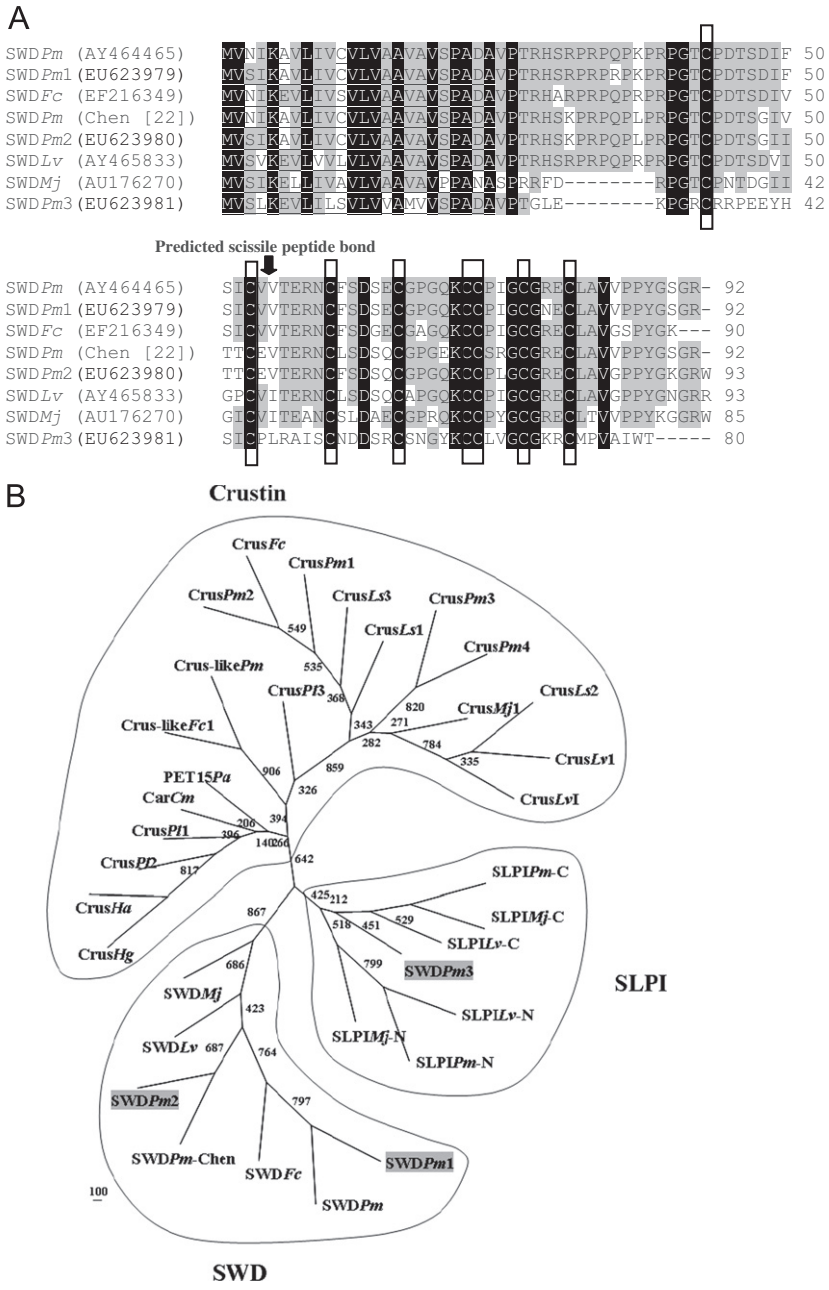

Figure 1 Sequence analysis of SWD*Pm* genes. (A) Multiple alignment of deduced amino acid sequence of SWD cDNAs of various shrimp species. Black and gray highlights indicate complete conservation and conservation in three or more species, respectively. Arrow indicates the predicted scissile peptide bond. The eight conserved cysteine residues are boxed. (B) Bootstrapped neighbor-joining tree of WAP domain containing proteins from crustaceans: SWD proteins from *P. monodon* (SWD*Pm*1-3, EU623979, EU623980 and EU623981, respectively; SWD*Pm*, AY464465; SWD*Pm*; Chen [22]), *L. vannamei* (SWD*Lv*, AY465833), *F. chinensis* (SWD*Fc*, XP623150) and *M. japonicus* (SWD*Mj*, XP623150); Crustins from *P. monodon* (Crus-like*Pm*, EF654658; Crus*Pm*1, CF415873; Crus*Pm*2, BI018072; Crus*Pm*3, BI018073; Crus*Pm*4, CF415873), *M. japonicus* (Crus*Mj*1, AB121740), *L. vannamei* (Crus*Lv*1, AF430071; Crus*LvI*, AY488492), *L. setiferus* (Crus*Ls*1, AF430077; Crus*Ls*2, AF430078; Crus*Ls*3, AF430079), *F. chinensis* (Crus-like*Fc*1, DQ097703; Crus*Fc*, AY871268), *C. maenas* (Car*Cm*, AJ237947), *H. americanus* (Crus*Ha*, CN853187), *H. gammarus* (Crus*Hg*, CAH10349), *Pacifastacus leniusculus* (Crus*Pl*1, EF523612; Crus*Pl*2, EF523613; Crus*Pl*3, EF523614), *Panulirus argus* (PET15 *Pa*, AAQ15293); Secretory leukocyte protease inhibitors (SLPIs) from *M. japonicus* (SLPI*Mj*, ABW88999), *L. vannamei* (SLPI*Lv*; [41]) and *P. monodon* (SLPI*Pm*, BI784457). The three bootstrapped neighbor-joining tree indicate the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original deduced protein sequences.

detected to a lesser extent in the hepatopancreas and gill (Figure 2A). No SWD*Pm* transcripts were detected in the lymphoid organ, intestine and heart. The result demonstrates that *P. monodon* hemocytes are likely to be the main site of SWD*Pm* transcript expression for all three detected isoforms.

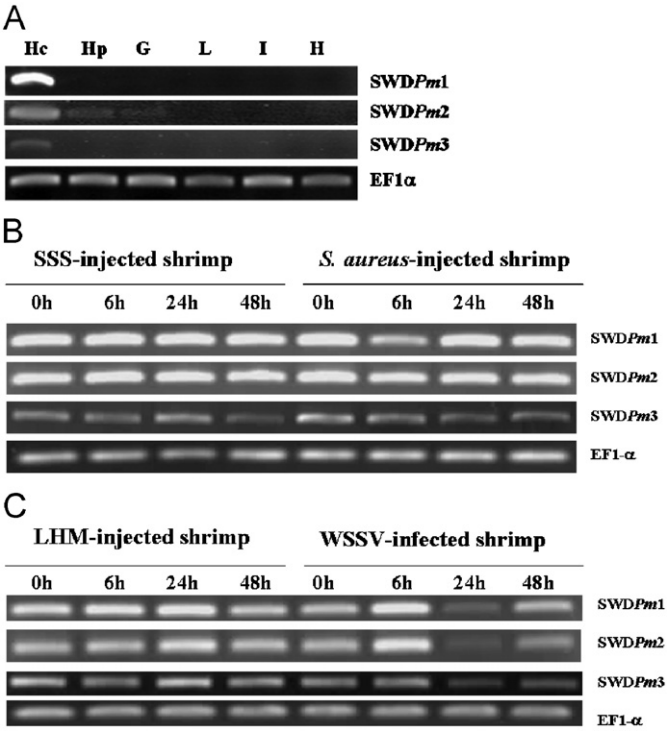

Figure 2 (A) Tissue-specific expression of SWD*Pm*1, SWD*Pm2* and SWD*Pm*3 by RT-PCR analysis: Hemocytes (Hc); hepatopancreas (Hp); lymphoid (L); gill (G); intestine (I); heart (H). EF-1α was used as a control housekeeping gene control to indicate and standardize the amount of cDNA template in each of the various tissues. A representative experiment of two independent experiments is shown. (B) Time course analysis of SWD*Pm* mRNAs expression in hemocytes after challenge with formalin-inactivated *S. aureus*, or viable WSSV virions. For each time point (0, 6, 24 and 48 h) the cDNAs from three individual shrimp were pooled and used as templates for the second stage-specific amplification of the RT-PCR. Changes in mRNA expression were compared with the control shrimp which were injected with SSS solution or LHM. EF-1α was used as a control housekeeping gene control to indicate and standardize the amount of cDNA template in each of the various reactions. A representative experiment of two independent experiments is shown.

Expression of the SWD*Pm* genes after bacterial and viral challenge

The expression pattern of SWD*Pm*1, SWD*Pm*2 and SWD*Pm*3 mRNAs in the hemocytes of shrimp challenged with the Gram-positive bacteria, *S. aureus* and with the virus, WSSV, was determined by a semi-quantitative RT-PCR and the data are summarized in Figure 2B, respectively. Compared to the control group injected with only SSS solution, expression of SWD*Pm*1 rapidly decreased within 6 h post-bacterial challenge, and then returned to non-stimulated levels by 24 h and remained so at 48 h. No detectable change in the transcription levels of SWD*Pm*2 and SWD*Pm*3 transcripts was evident in the bacterial injected shrimp, compared with the control, at least at these tested time points (Figure 2B). In WSSV-injected shrimp, the expressions of SWD*Pm*1 and SWD*Pm*2 transcripts were potentially strongly upregulated at 6 h post-viral challenge compared to that of the control shrimp injected with LHM. However, by 24 h post-challenge SWD*Pm*1 and SWD*Pm*2 transcripts were dramatically decreased to below control levels suggesting transient strong down-regulation, since by 48 h post-challenge transcript levels had returned back to normal expression levels. For SWD*Pm*3, no early (6 h) induction was noticed but by 24 h a decrease in the relative transcript expression level was observed with some, but not full, recovery by 48 h post-challenge.

Genomic organization

The genome organization of SWD*Pm*2, the probable major isoform (32 clones of 50 SWD*Pm* ESTs) was further investigated by PCR-based cloning and sequencing of the encoding region of *P. monodon* genomic DNA. Sequence analysis of the potential SWD*Pm*2 genomic DNA (EU623982) revealed the presence of three exons (87, 165 and 27 bp) interrupted by two introns (900 and 606 bp) within the 1811 bp genomic sequence (Figure 3). The sequences at the exon/intron boundaries followed the GT–AG rule. The first exon covers some of the 5′ untranslated region (21 bp), the signal peptide coding region (24 aa) and part of N-terminus of the mature peptide (5 aa). The second exon includes the proline-arginine rich domain and an SWD of the mature peptide. The third exon is composed of the terminal C-terminus (9 aa) of the mature peptide and some of the 3′ UTR (stop codon and 2 bp).

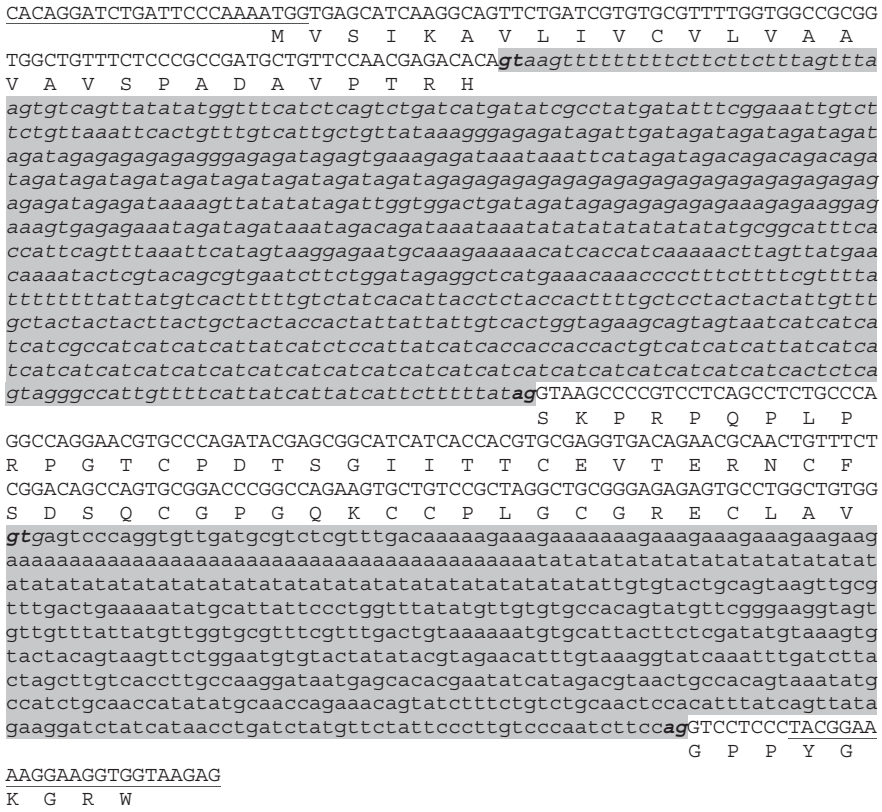

Figure 3 Genomic structure of the cloned and sequenced *P. monodon* SWD*Pm*2 gene region (EU623982). Predicted coding nucleotides and deduced amino acids of each exon are capitalized. Introns are shaded gray and illustrated with lower case letters. Binding sites for the PCR primers used for amplification are underlined. Bold italic letters indicate the predicted intron dinucleotide acceptor and donor sites for RNA splicing.

Expression and purification of the recombinant SWD*Pm*2

In order to better understand the biological role of SWD*Pm*2, the recombinant protein was expressed in *E. coli*. The DNA fragment encoding a chimera between rSWD*Pm*2 mature protein (no signal protein) with a hexa His-tag sequence at the N-terminus was obtained by PCR (Figure 4). The confirmed recombinant plasmid was transformed into *E. coli* Rosetta (DE3) pLysS. After induction, the Coomassie brilliant blue staining of the gels revealed the induction of an approximately 8 kDa protein (Figure 5). The protein was detected after 1 h of IPTG induction and gradually increased over the 4 h assayed. After sonication, both the supernatant and pellet fractions were analyzed by 15% (w/v) SDS-PAGE, which showed that target protein with an expected molecular weight around 8 kDa was only expressed in inclusion bodies not in the soluble fraction (data not shown). Under the induction condition of 1 mM IPTG for 4 h at 37 °C, a large amount of inclusion bodies was obtained. The inclusion body pellet fraction was solubilized in lysis buffer containing 8 M urea and further purified by Ni-NTA affinity chromatography. The purity of the rSWD*Pm*2 was examined on 15% (w/v) SDS-PAGE. A major protein band of expected molecular mass, corresponding to the predicted rSWD*Pm*2 protein, was successfully detected in the elution fraction and its molecular mass determination by MALDI-TOF mass spectrometry revealed a mass of ~8398 Da, in good agreement with that expected from the predicted amino acid sequence (Figure 5) and with no other signs of significant contamination.

Antimicrobial activity

The antimicrobial activity of the purified rSWD*Pm*2 protein was assessed using a liquid growth inhibition assay and was found to exhibit a strong antimicrobial activity against the Gram-positive bacteria *A. viridans* and *M. luteus*, with an MIC value of 1.56–6.25 μM (Table 3), and a lower activity against *S. aureus* and *B. megaterium* (MIC value of 12.5–50 μM). However, no activity against *S. haemolyticus* upto 50 μM was noted, nor against any of the five species of Gram-negative bacteria examined up to the highest tested concentration of 50 μM (Table 3).

Serine proteinase inhibitory assay

The function of SWD*Pm*2 as a proteinase inhibitor was investigated using purified rSWD*Pm*2 protein. The inhibitory effects on the hydrolysis of synthetic chromogenic substrates by the serine proteinases trypsin, chymotrypsin, elastase and subtilisin A, were examined. The remaining activities of proteinases were determined and plotted against the concentrations of the inhibitors (Figure 6A). The rSWD*Pm*2 exhibited strong inhibition against subtilisin

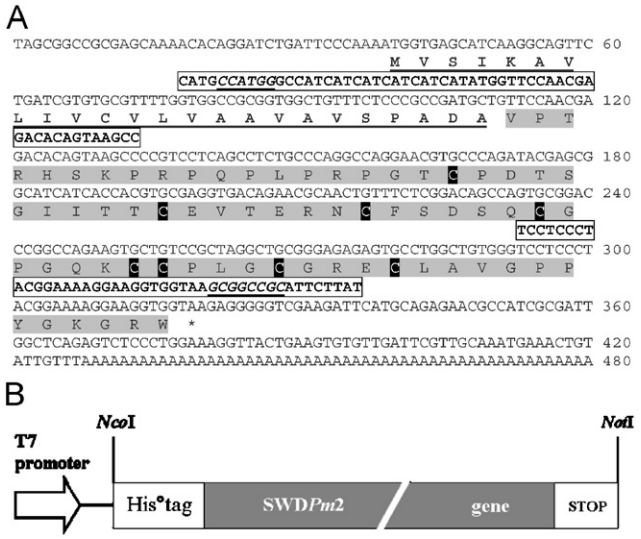

Figure 4 Construction of recombinant SWD*Pm*2/pET-28b(+) expression vector. (A) The full-length nucleotide (above) and predicted amino acid (below) sequences of SWD*Pm*2 cDNA from *Penaeus monodon*. The putative signal peptide is in bold and underlined. The primers used for PCR amplification are boxed. The mature protein is shown by gray boxes. The six conserved cysteine residues of the WAP domain are in black boxes. The restriction sites in the primers are in italic and underlined. (B) Schematic representation of the SWD*Pm*2 gene in which the *Nco*I–*Not*I SWD*Pm*2 PCR fragment (shaded) was cloned in-frame into the expression vector downstream of the hexa His-Tag.

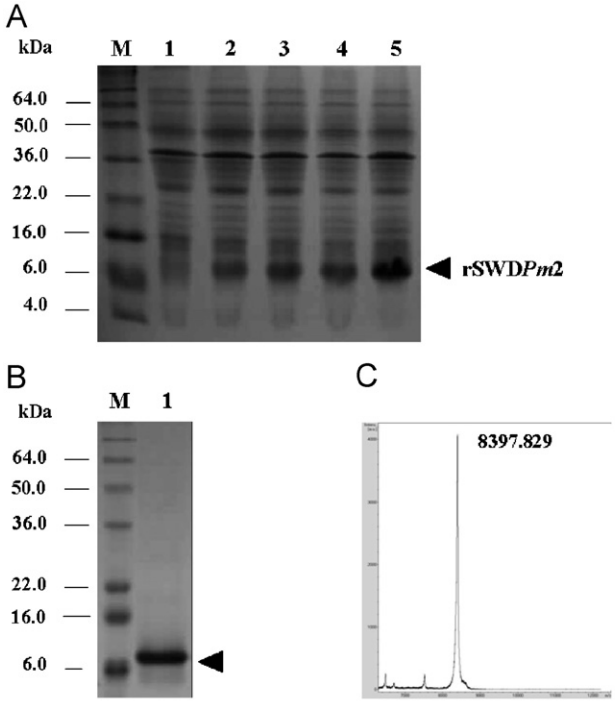

Figure 5 SDS-PAGE analysis to examine expression of rSWD*Pm*2 (A) under induction with 1.0 mM IPTG for 0, 1, 2, 3 and 4 h (lanes 1–5) in the crude extract of *E. coli* Rosetta (DE3) pLysS. Lane M is the protein standard ladder. Molecular weight determination of the purified rSWD*Pm*2 by 15% (w/v) SDS-PAGE (B, lane 1) and MALDI-TOF spectra (C).

Table 3 Antimicrobial activity of purified rSWD*Pm*2 peptide.

Microorganisms	MIC (μM)
Gram-positive bacteria	
Staphylococcus aureus	25–50
Staphylococcus haemolyticus	>50
Aerococcus viridans	3.125–6.25
Bacillus megaterium	12.5–25
Micrococcus luteus	1.56–3.125
Gram-negative bacteria	
Salmonella thyphimurium	>50
Escherichia coli 363	>50
Enterobacter cloacea	>50
Vibrio harveyi	>50
Klebsiella pneumoniae	>50

MIC values are expressed as the interval of concentration [a]–[b], where [a] is the highest concentration tested at which microbial growth can be observed and [b] is the lowest concentration that causes 100% growth inhibition.

A, but no detectable inhibition of the hydrolysis of *N*-benzoyl-Phe-Val-Arg-*p*-nitroanilide by trypsin, *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide by chymotrypsin and *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide by elastase was observed even with an inhibitor concentration of more than 10 times that of the proteinase.

Determination of inhibition constant

The equilibrium dissociation constant K_i for the interaction of subtilisin A with rSWD*Pm*2 was determined directly by adding the substrate to an equilibrium mixture of proteinase and inhibitor, and measuring the rate of release of the reaction product. The Lineweaver–Burk plots were constructed (Figure 6B), and the K_M and V_{max} were determined to be 0.15×10^{-3} M and 0.85 nmole/min, respectively. After replotting the apparent K_M's against the inhibitor concentrations, the K_i value was determined to be 1.98×10^{-9} M for subtilisin A, under the assay conditions.

Discussion

In this study, we report the identification of the cDNAs encoding three different SWD-containing proteins (SWD*Pm*1, SWD*Pm*2 and SWD*Pm*3) from the black tiger shrimp, *P. monodon*. The nucleotide and deduced amino acid sequences of the SWD*Pm* showed high sequence similarity to known shrimp SWD proteins by the BLAST programs. They contain a highly conserved signal peptide followed by a proline-arginine rich region at the N-terminus and a similar 4-DSC in the WAP motif towards the C-terminus. This domain organization has been described as distinctive and characteristic of shrimp SWD proteins which were recently designated as type III crustin by Smith et al. [18].

The cDNAs encoding the SWD-containing proteins were first identified from the hemocytes of *P. monodon* and the white shrimp, *L. vannamei* [21]. Homology searches showed

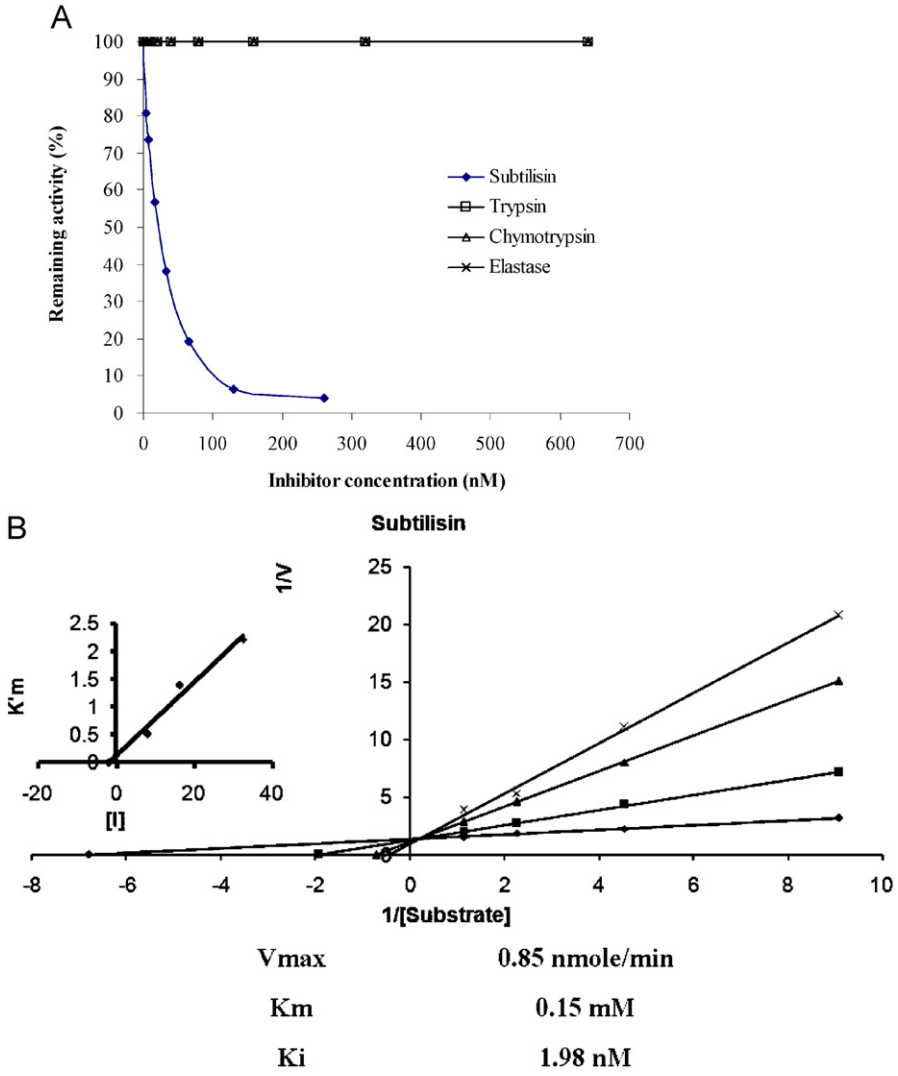

Figure 6 (A) The inhibitory activity of purified rSWD*Pm*2 on subtilisin A and three other serine proteinases. The remaining proteolytic activity on the appropriate synthetic chromogenic substrate (see methods) was calculated and plotted against concentration. A representative experiment of two independent experiments is shown. (B) Lineweaver–Burk plot of subtilisin A activity at different concentrations of rSWD*Pm*2: 0 (♦), 8.125 (■), 16.25 (▲) and 32.5 (×) nM. The plot of apparent K_M's (K'_M) against the concentration of the inhibitor (rSWD*Pm*2) is shown in the inset for the determination of K_i. The values of V_{max}, K_M and K_i are indicated underneath the graphs. Each data point was an average of two independent experiments.

a high sequence similarity of the SWD*Pm* with the SWD proteins from *L. vannamei* (59–90%) and *P. monodon* (58–98%), respectively, as well as high similarity (57–94%) with the sequence described as *P. monodon* chelonianin [22]. The high amino acid sequence identity (96%) of SWD*Pm*1 and SWD proteins from *P. monodon* [21] suggests that they might be simple allelic variants encoded by a single polymorphic gene whilst, in contrast, SWD*Pm*2 and SWD*Pm*3 are new isoforms of SWD proteins from *P. monodon*. Moreover, the distance-based phylogenetic tree generated for the WAP amino acid sequences of the SWD*Pm* and other crustacean proteins containing the WAP domain indicated that the WAP domain of SWD*Pm*3 is distinct from SWD*Pm*1 and SWD*Pm*2 and closer to those of shrimp SLPI proteins.

Tissue-specific expression analysis in adult *P. monodon* shrimp revealed that all three SWD*Pm* transcripts were mainly expressed in hemocytes, an expression profile that is similar to that of the SWD gene of *L. vannamei* [21]. The result indicated that the hemocyte is likely to be a main site for SWD*Pm*1-3 synthesis. The immune reactions of shrimp and crustaceans are predominantly achieved through circulating hemocytes and that many immune factors are stored in hemocytes or released into plasma from hemocytes [1], this is not an unexpected result if the SWD*Pm*1-3 gene products have an immune function, and the potential evidence for this is discussed below.

The response of SWD*Pm*1-3 expression to infection with the Gram-positive bacteria *S. aureus* and to viral (WSSV) challenge was investigated. The transcript expression of SWD*Pm*1 showed an early response with rapid down-regulation by 6 h after injection with *S. aureus* whilst, however, that for SWD*Pm*2 and SWD*Pm*3 did not significantly change. Previously, Jiménez-Vega et al. [21] demonstrated that SWD transcript in *L. vannamei*, infected with the

Gram-negative bacteria *Vibrio alginolyticus*, and also displayed an early response with a significant transcript level increase at 3–6 h after injection. In the European lobster, *Homarus gammarus* the expression level of crustin was down-regulated after challenge with the Gram-negative bacteria *Listonella anguillarum* but up-regulated after inoculation with the Gram-positive bacteria *A. viridans* var. *homari* [29]. Clearly, at a simple level the pattern of expression of crustin family genes is not consistent and the higher level of complexity might not only depend on whether the response is to a Gram-positive or Gram-negative bacteria, or virus type, but also the pathogenicity of the microorganisms [18], and may also have coevolved (e.g. control region) in different species with different evolutionary exposure to pathogens leading to species-specific responses.

Of particular interest here is the response in the expression of SWD*Pm*1-3 to WSSV challenge. The expression of SWD*Pm*1 and SWD*Pm*2 mRNAs showed a significant early and late biphasic response with marked up-regulation of transcript expression at 6 h after WSSV injection relative to mock infections followed by the transient down-regulated regulation at 24 h of all three SWD*Pm* transcripts which all had returned to normal levels at 48 h. The results indicated that SWD*Pm*1 and SWD*Pm*2 are highly responsive to WSSV stimulation. A similar expression pattern has been observed in the SLPI-like protein (named double-WAP domain) of the kurma prawn, *Marsupenaeus japonicus*, which rapidly increased in transcript levels during the early phase (6 h) after WSSV infection [30]. Difference in expression profiles of shrimp SWD mRNA between bacterial and WSSV challenges may be due to different regulatory mechanisms responding to bacteria (Gram-positive or -negative) or virus infection in shrimp, and as such is likely to be ecologically influenced and relevant.

The organization of the genomic region encompassing the SWD*Pm*2 gene sequence revealed that the gene (1811 bp) is composed of three exons interrupted by two introns. When comparing the SWD*Pm*2 gene obtained from this study with the previously reported SWD gene in *P. monodon* [23], the sizes of the first intron were markedly different. The length of intron 1 in SWD*Pm*2 (900 bp) was approximately 4-fold smaller than of the corresponding intron in the SWD gene. Indeed, both introns are widely different at the sequence level and, for example, reveal no shared microsatellites (abundant in the two introns of SWD*Pm*2). Moreover, analysis of the exon coding sequences suggested that the two isoforms are probably encoded from different genes and that they do not arise as a result of alternative exon splicing of the exon or frame shifts of the same gene.

Despite the report of the sequences of several shrimp SWD proteins, no biological function has yet been ascribed to the proteins. The WAP domain has been described in proteins with diverse functions [31] including antiproteinase and antimicrobial activities [19,32]. To elucidate the biological function of the *P. monodon* SWD proteins, recombinant SWD*Pm*2 protein was expressed and analyzed for antimicrobial and proteinase inhibitory activities, revealing a significant antibacterial activity against four out of five Gram-positive bacteria (*A. viridans*, *M. luteus*, *S. aureus* and *B. megaterium*), but did not show activity against all five assayed Gram-negative bacteria. In agreement, crustins, which are well-characterized WAP proteins found in various species of crustaceans, are mainly active against Gram-positive bacteria [18,25,33–35].

Members of the WAP domain protein family, including SLPI and elafin, have been shown to function as serine proteinase inhibitors and perhaps to control pathogen proteinases [19]. Therefore, the proteinase inhibitory activity of SWD*Pm*2 against various serine proteinases was examined. The results showed that rSWD*Pm*2 strongly inhibited subtilisin A with a K_i of 1.98×10^{-9} M, but was not active against trypsin, chymotrypsin and elastase. This function is similar to that of a WAP domain (AWP IV) at the C-terminus of AWAK (Avian WAP domain- and Kunitz domain-containing) protein which functions as an inhibitor of serine proteinases including the microbial subtilisin A and proteinase K. However, no significant inhibition of elastase from pig leukocytes was observed [36]. SLPI inhibits a variety of proteinases, including neutrophil elastase and cathepsin G, trypsin, chymotrypsin and chymase. Elafin was shown to be a specific serine proteinase inhibitor of elastases from neutrophil and pancreatic origin and neutrophil protease 3 [37,38] but could not inhibit trypsin, plasmin, α-chymotrypsin and cathepsin G [38]. SLPI and elafin bind tightly to their targets with K_i values in the 10^{-9}–10^{-10} molar range [31]. Here, the derived K_i value of rSWD*Pm*2 against subtilisin A indicates that SWD*Pm*2 is potentially a potent inhibitor of subtilisin A and that the primary functions are likely to include antimicrobial action and also to inhibit the bacterial proteinase to limit microbial infection and pathogenesis.

The proteinase inhibitor activity of the WAP domain is reported to be characterized by the presence of a methionine (Met) residue at the scissile peptide bond adjacent to the second cysteine in the 4-DSC [31]. In crustins, the Met residues have been substituted by cationic and hydrophobic amino acids and an antibacterial activity has been proposed instead [18]. In agreement, the WAP domain-containing proteins that are not proteinase inhibitors, including mouse SWAM1 and SWAM2 whose Met is replaced by phenylalanine and arginine, respectively [32], and omwaprin from snake venom with Met to lysine [39], also have been shown to have antibacterial activity.

In this light it is interesting that SWD*Pm*2 has Val substituted for Met at the predicted scissile peptide bond, similar to that found in the C-terminal WAP domain (AWP IV) of an AWAK protein. AWP IV does not have the typical primary contact region characteristic for proteinase inhibitors in the WAP domain, but still exhibits proteinase inhibitor activity [36]. In the case of SLPI, the first WAP domain with a scissile peptide bond between L_{19} and R_{20} lacks the proteinase inhibitor activity but displays antibacterial activity, whereas the second WAP domain with a scissile peptide bond between L_{72} and M_{73} has potent proteinase inhibitor activity but shows very low antibacterial activity [40].

Our results clearly demonstrate for the first time that, *in vitro*, rSWD*Pm*2, and thus likely the *P. monodon* shrimp SWD protein (SWD*Pm*2) as well, exhibits antimicrobial activity against Gram-positive bacteria and a potent proteinase inhibitory capacity towards subtilisin A. In shrimp, the AMPs and the serine proteinase inhibitors play important roles in the innate immune system in response to pathogenic bacterial infection. The presence of SWD*Pm*2

transcripts in hemocyte and its protein functions as an anti-Gram-positive bacteria and antiproteinase suggested that it is a potent immune effector to protect the shrimp from pathogen infection.

Acknowledgments

This work was supported by grants from (i) the Thailand Research Fund (TRF) awarded to Piti Amparyup, (ii) from the Commission on Higher Education and (iii) Thailand National Center for Genetic Engineering and Biotechnology (BIOTEC).

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