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First identification and characterization of a triple WAP domain containing protein in *Procambarus clarkii* provides new insights into the classification and evolution of WAP proteins in crustacean

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ABSTRACT

Whey acidic protein domain (WAPD) is a usual motif in crustaceans, and is found mainly in the immune-related proteins. In the present study, a protein containing three tandem WAPDs was identified in red swamp crayfish *Procambarus clarkii* and designated as PcTWD. This is the first report of a protein of such domain architecture in crustaceans. Introducing the WAPDs of PcTWD into phylogenetic analysis led to the classification of crustacean WAP proteins into classical crustins and proteins containing solely WAPDs. PcTWD was widely expressed in multiple tissues, including hemocytes, gills, hepatopancreas, heart, stomach and intestine. Its expression could be significantly induced by *Staphylococcus aureus* or *Aeromonas hydrophila* challenge. Knockdown PcTWD expression by RNAi suppressed host resistance against *A. hydrophila*, while exogenous recombinant PcTWD could enhance the host immunity. The three WAPDs showed a labor division. The first two domains were responsible for the protease inhibitory activity, and the third domain contributed to the antimicrobial activity. Thus PcTWD was found as an important protein in crayfish antibacterial immunity.

1. Introduction

WAP (whey acidic protein) is the most abundant protein in the milk serum of some mammals, including mice, rat and rabbit among others [1,2]. The typical characteristic of WAP is the four-disulfide core (4DSC) in a conserved arrangement, which is also called the WAP domain (WAPD) [3]. This structure is similar to that of the domains of serine protease inhibitor. Comprised of approximately 40–50 amino acids with eight highly conserved cysteine residues, WAPDs occur not only in mammals, but also in many other non-milk proteins from both vertebrates and invertebrates [4]. Stabilized with an overall conserved tertiary structure formed by the eight cysteine residues, WAPD containing proteins exert different functions, perhaps due to the variation of the stretch between adjacent cysteine residues, or due to the variation of extra region besides of WAPD.

In mammals, several WAPD containing proteins were found as the molecular marker of some cancers [5]. These proteins also participate in multiple physiological processes mainly based on the protease inhibitory activity against cathepsin G, trypsin, elastase, etc. As the protease inhibitors, WAPD containing proteins could inhibit the epithelial cells proliferation and reduce the breast cancer progression [6,7]. Numerous WAPD proteins were also identified in lower vertebrates and invertebrates, though most of the proteins were not functionally investigated. Due to the medical and economic significance of these species, some WAPD protein from reptiles, mollusks and crustaceans were studied using recombinant or native proteins. For example, waprins were found present in snake venom [8]. This family possessed antimicrobial abilities, and could kill Gram-positive bacteria by disrupting the cell membrane [9]. Abalone perlwapins contained three tandem WAPDs, and were found involved in shell formation by binding mineral crystals and inhibiting calcium deposition in shell matrix [10].

The WAPD proteins in crustaceans may be the mostly well studied among the invertebrate homologs, due to the aquaculture importance of shrimp, crab, lobster and crayfish. The first member of this family was originally identified from the crab *Carcinus maenas* and designated as carcinin [11]. Later, many homologs of this protein were identified in decapod crustaceans, and these proteins were generally named as crustins. Till now, crustin family could be divided into four sub-groups by a typical classification approach. Type I crusins were characterized with the presence of a Cys-rich region between the signal sequence and

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Table 1
Primers used in this study.

Primers	Sequence (5′-3′)	Tm (°C)	GC (%)	PCR efficiency (%)
(q)RT-PCR				
PcTWDRTF	CCAACAACAGCAATCGCC	56.3	56	96.0
PcTWDRTR	ACACAAAACAGAACCTCCCG	58.4	50	
β-actinRTF	AAACTTTCAACACTCCCGCTATG	60.9	43	98.2
β-actinRTR	CGAACGATTTCTCGCTCTGC	60.5	55	
EF1αRTF	CCACAAAGGCAGGTGAAAAGG	61.2	52	100.6
EF1αRTR	ATTGGGTGAACCAAGCAGGG	60.5	55	
RNAi				
PcTWDiF	GCGTAATACGACTCACTATAGGATCAAGATGACACCCCTAA	76.9	44	
PcTWDiR	GCGTAATACGACTCACTATAGGCACTGGTTCCTAAACACAA	76.9	44	
GFPiF	GCGTAATACGACTCACTATAGGTGGTCCCAATTCTCGTGGAAC	79.9	49	
GFPiR	GCGTAATACGACTCACTATAGGCTTGAAGTTGACCTTGATGCC	79.1	47	
Recombinant expression				
PcTWDExF	CGCGGATCCGTCTCTCCTCCTTTCATCCC	76	62	
PcTWDExR	CCGCTCGAGTTACATACATTCCCTGCCGCA	74.9	57	

WAPD, and type II crusins contained an additional Gly-rich region before the Cys-rich region. Differently, type III crustins, which were also referred as single WAP domain (SWD) proteins, contained a Pro-Arg-rich region between signal sequence and WAPD, while the so-called type IV crustins contained two WAPDs [4]. However, some new classification modes of crustin family were raised. For example, a recent review classified crutsins into three distinct groups based on: 1) the characteristics of Gly-rich region, and 2) the F and A distances located within the WAPD. Compared to the classical approach, this approach introduced the characteristics of the WAPD as an index for classification [12].

Aquaculture of red swamp crayfish *Procambarus clarkii* have increased these years in China. However, emerging disease threatened the crayfish aquaculture greatly. Studying the crayfish immunity would help to develop new strategies of disease prevention and control. Through the transcriptomic analysis aiming to screen the pathogen-inducible genes, a protein containing three tandem WAPDs were identified and named as PcTWD. To the best of our knowledge, this is the first discovery of a protein containing solely three WAPDs in crustaceans. Its function in crayfish antibacterial immunity was characterized. Moreover, the identification of this triple WAPD containing protein might provide new insights into the classification and evolution of WAPD containing proteins in crustacean.

2. Materials and methods

2.1. Animals

Healthy red swamp crayfish with a weight of 10–15g each were collected from an aquaculture farm in Xuyi, Jiangsu, China. The crayfish were cultured in room temperature and fed with commercial fish food daily for about two weeks before experiment. During the culture before experiment, the animals with typical unhealthy syndromes, such as slow moving and reacting and little food intake were excluded. All crayfish used in the experiment were randomly selected.

2.2. Immune stimulation and sample collection

Staphylococcus aureus and *Aeromonas hydrophila* were maintained in our lab. After overnight culture, the bacteria were collected by centrifugation at 6,000 rpm for 5 min. After washing with PBS, the bacteria were suspended in PBS and the bacterial suspension was used as the inoculum. For immune stimulation, about 3×10^5 colony forming unit (CFU) were injected intramuscularly into the fourth abdominal segment of crayfish, with equal volume of PBS as the control. At specific time points after injection, the animals were placed on ice for 15 min before bleeding and sampling. The crayfish hemolymph was quickly collected into cold anticoagulant solution (0.14 M NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid, 10 mM EDTA, pH 4.6) [13] and centrifuged at $800 \times g$ for 10 min to isolate the hemocytes pellet. The total RNAs were then extracted with TRIzol (Invitrogen) according to the manufacturer's instructions. At least five animals were used to prepare each sample.

2.3. Identification and characterization of PcTWD sequence

Total RNAs from the hemocytes of healthy and *A. hydrophila*-stimulated crayfish were sent to the BGI Company (Shenzhen, China) for transcriptome sequencing to identify the immune inducible genes. A fragment encoding a full open reading frame which encoded a protein of a signal peptide and three tandem WAPDs was selected and this gene was designated as PcTWD. Signal sequence was predicted using SignalP 4.1 (cbs.dtu.dk/services/SignalP/), and the domain architecture was predicted using online SMART server (smart.embl-heidelberg.de/). Multiple alignment was performed by MEGA 6.0 and presented by the GENDOC software. Neighbor-joining phylogenetic tree was also built using MEGA 6.0.

2.4. Expression profiles analysis

First strand cDNA was synthesized using the BioTeke Reverse Transcriptase Kit (Beijing, China), according to the manufacturer's instructions. The distribution of the PcTWD transcripts was analyzed by RT-PCR, using a pair of specific primers (PcTWDRTF/R, Table 1). β-actin was amplified with the primers β-actinRTF/R (Table 1) as the reference. PCR was performed with conventional procedure. The expression of PcTWD in hemocytes at specific time point after *A. hydrophila* challenge was evaluated by real-time RT-PCR with the CFX96 Real-time System (Bio-Rad, Hercules, CA, USA) and the iQ SYBR Green Supermix (Bio-Rad). The procedure was set as: 94 °C for 3 min; followed by 40 cycles of 94 °C for 10 s and 60 °C for 1 min; and then melting from 65 °C to 95 °C. Both β-actin and elongation factor 1-α (EF1α, EF1αRTF/R) were selected as the internal references for real-time RT-PCR. The data was calculated using the $2^{-\Delta\Delta}$Ct method, and normalized to untreated sample. Three independent repeats were performed and results represented the mean ± SD.

2.5. RNAi

A partial PcTWD cDNA fragment was amplified by PCR with primers linked with the T7 promoter (PcTWDiF/R, Table 1) and used as the template to produce double-stranded RNA with an *in vitro* T7 transcription kit (Takara, Dalian, China). GFP dsRNA was generated in the same way using the primers listed in Table 1 (GFPiF/R) as the

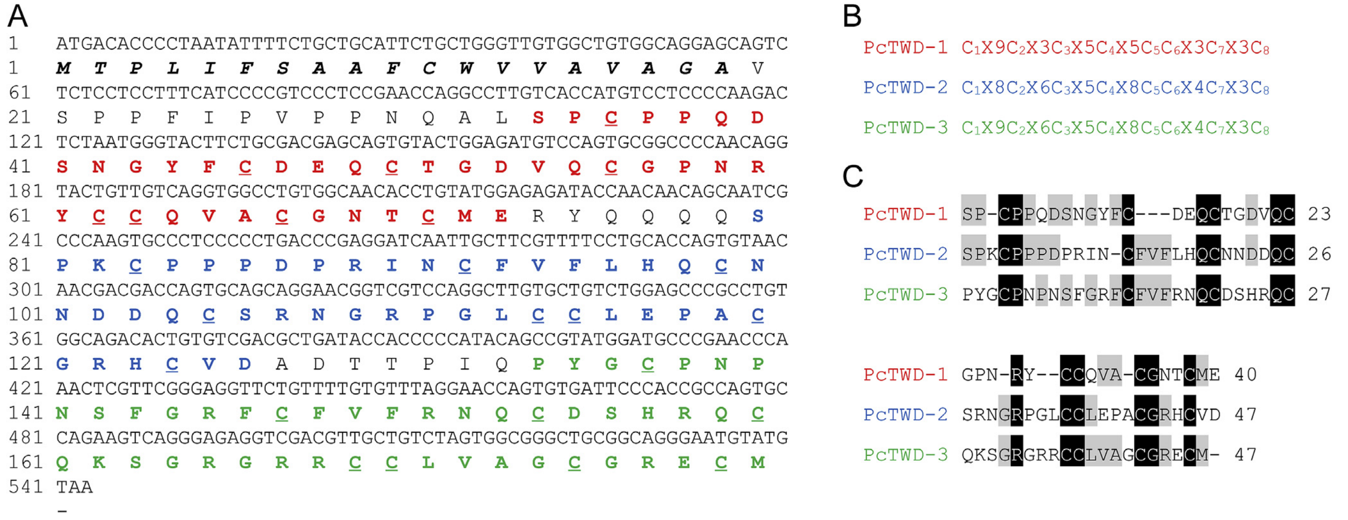

Fig. 1. Sequence analysis for PcTWD. (A) The open reading frame of PcTWD. The sequence was translated and analyzed by SMART server. The nucleotides and residues were numberd at left. Signal peptide was in bold and italic. Three WAPDs were in red, blue and green respectively. The conserved cysteine residues were underlined. (B) The arrangement of the eight cysteine residues of each WAPDs. (C) Alignment of the sequences of three WAPDs. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

control. To determine the RNAi efficiency, each animal was injected into the fourth abdominal segment intramuscularly with a series of dose of PcTWD dsRNA (10, 20, 50 μg) or GFP dsRNA (50 μg). The interference efficiency was detected 24 h after injection using qRT-PCR, using β-actin as the internal reference gene. Experiments were performed in triple repeats independently. For each test, at least five crayfish were used for each injection.

2.6. Survival analysis

After validating that PcTWD expression could be silenced by 50 μg of dsRNA injection, 30 crayfish were injected with 50 μg of PcTWD dsRNA, and another 30 crayfish were injected with 50 μg of GFP dsRNA as the control. The crayfish was then infected with 10^6 CFU of *A. hydrophila* 24 h after dsRNA injection. The survival rate of crayfish in each group was measured, and finally statistical analysis was conducted using GraphPad software.

2.7. Recombinant expression and purification

A pair of primers (PcTWDExF/R, Table 1) were used to amplify the fragment encoding the mature PcTWD. The fragment was ligated into pET32a(+) vector. The recombinant plasmid was transformed into *Escherichia coli* Rosseta (DE3) cells. Protein expression was induced with 0.5 mM of isopropyl-Β-ᴅ-thiogalactopyranoside (IPTG) at 37 °C for 6 h. The recombinant protein was expressed in soluble, and was purified by Ni-NTA affinity chromatography. For the protein which would be applied *in vivo*, the column was washed deeply with 0.1% Triton X-114 (Sigma) before final elution to remove the endotoxin [14]. A plain pET32a(+) vector was used simultaneously to express a tag for control.

2.8. Peptide synthesis

The peptides were commercially synthesized by GenScript Company (Nanjing, China). The N-terminus of the peptide was acetylated, and the C-terminus was amidated.

2.9. Protease inhibition assay

The inhibitory ability of PcTWD against Proteinase K (Sigma) was investigated using N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (AAPF, Sigma) as the substrate. Proteinase K and AAPF was dissolved to a concentration of 0.1 mg/ml and 1 mg/ml in 50 mM Tris-HCl (pH 8.0). The inhibitor was dissolved and diluted to a series of concentrations. The inhibitor solution (90 μl) was incubated with 1 μl of protease solution at 30 °C for 10 min, and then placed on ice immediately. AAPF solution (15 μl) was then added. The mixture was incubated at 30 °C for another 15 min. The reaction was terminated by adding 50 μl of 50% acetic acid. The absorbance at 405 nm was detected to monitor the formation of p-nitroanilide. The remaining protease activity was then determined. BSA was used as the control of inhibitors. The assays were performed trice independently, and the results represented mean ± SD.

2.10. Antimicrobial ability assay

A. hydrophila and *S. aureus* were cultured overnight, and diluted with a poor medium (1% NaCl, 0.5% tryptone) to a concentration of 10^8 CFU/ml. Bacteria suspension (20 μl) was incubated with protein solution (20 μl), and poor medium was added to a final volume of 200 μl. The mixture was maintained at room temperature for 24 h. The absorbance at 620 nm was detected to monitor the growth of bacteria. Three independent repeats were peformed, and the results were expressed as mean ± SD.

2.11. Statistical analysis

Most results in current study were analyzed by the Stutent's *t*-test, and the significance was accepted with $p < 0.05$. For the survival assay, the data were analyzed by the Log-rank (Mantel-Cox) test using GraphPad Prism.

3. Result

3.1. Identification and characterization of PcTWD sequence

Through a transcriptome sequencing of *A. hydrophila* infected crayfish hemocytes, a cDNA encoding three tandem WAPDs was identified. This gene was named as PcTWD (GenBank accession number MN296711). The open reading frame of PcTWD was 543 bp, encoding a polypeptide of 180 amino acids. A signal peptide sequence of 19 residues was located at the N-terminus of the peptide by SignalP prediction. The mature peptide comprised of three WAPDs. The first WAPD contained 38 residues, and was shorter than the other two WAPDs. The residues amounts of the second domain and the third domain were 44 and 45, respectively (Fig. 1A). Each domain contained the eight conserved cysteines which would form four disulfide bonds to stabilize the overall structure of WAPD. The first WAPD adopted the typical arrangement of cysteine residues (C1–C8) as proposed in vertebrate WAPs (C1-Xn-C2-Xn-C3-X5-C4-X5-C5-C6-X3/5-C7-X3/4-C8). However, the numbers of adjacent residues of C4–C5 and C6–C7 of the latter two WAPDs were both eight and four, respectively (Fig. 1B and C). The variation of the adjacent region might influence the structure and function of WAPDs. The molecular weight and isoelectric point of the mature PcTWD were 17.85 kDa and 7.77, respectively.

3.2. Phylogenetic analysis of PcTWD and crustacean WAPD proteins

To reveal the possible evolutionary relationship between PcTWD and other crustacean WAPD proteins, a neighbor-joining phylogenetic analysis was performed. Since these WAPD proteins varied in both the domain number and the extra region besides of WAPD, and such variation would, at least to a certain degree, influence the clustering when performing the phylogenetic analysis. Thus the WAPDs of these proteins were selected to evaluate their phylogenetic relationship. As shown in Fig. 2, the crustacean WAPDs could be divided into two major clusters. One cluster included the WAPDs from type I, type II and type III crustins. The other cluster included the domains from those proteins which contained solely WAPDs. This suggested that these two families were distinct and evolved separately. Though crustins were characterized by the extra region besides of the WAPD, their WAPDs were already distinct from those from the other family. Thus a model for the classification and possible evolution of the crustacean WAPD proteins was proposed (Fig. 3). The two families were originated from different ancient WAPDs. For the crustin family, the ancient WAPD acquired some extra region during evolution. Acquisition of P/R rich region or Cys-rich region generated two groups, one was the current type-III crustins, and the other was the combination of type I and II crustins. Then some member of type I crustins acquired Gly-rich region and evolved into type II crustins. For the other family which contained solely WAPDs, duplication might be the major cause for the appearance of DWD and TWD proteins.

3.3. Expression profiles of PcTWD

The expression profiles of PcTWD were studied to check whether it

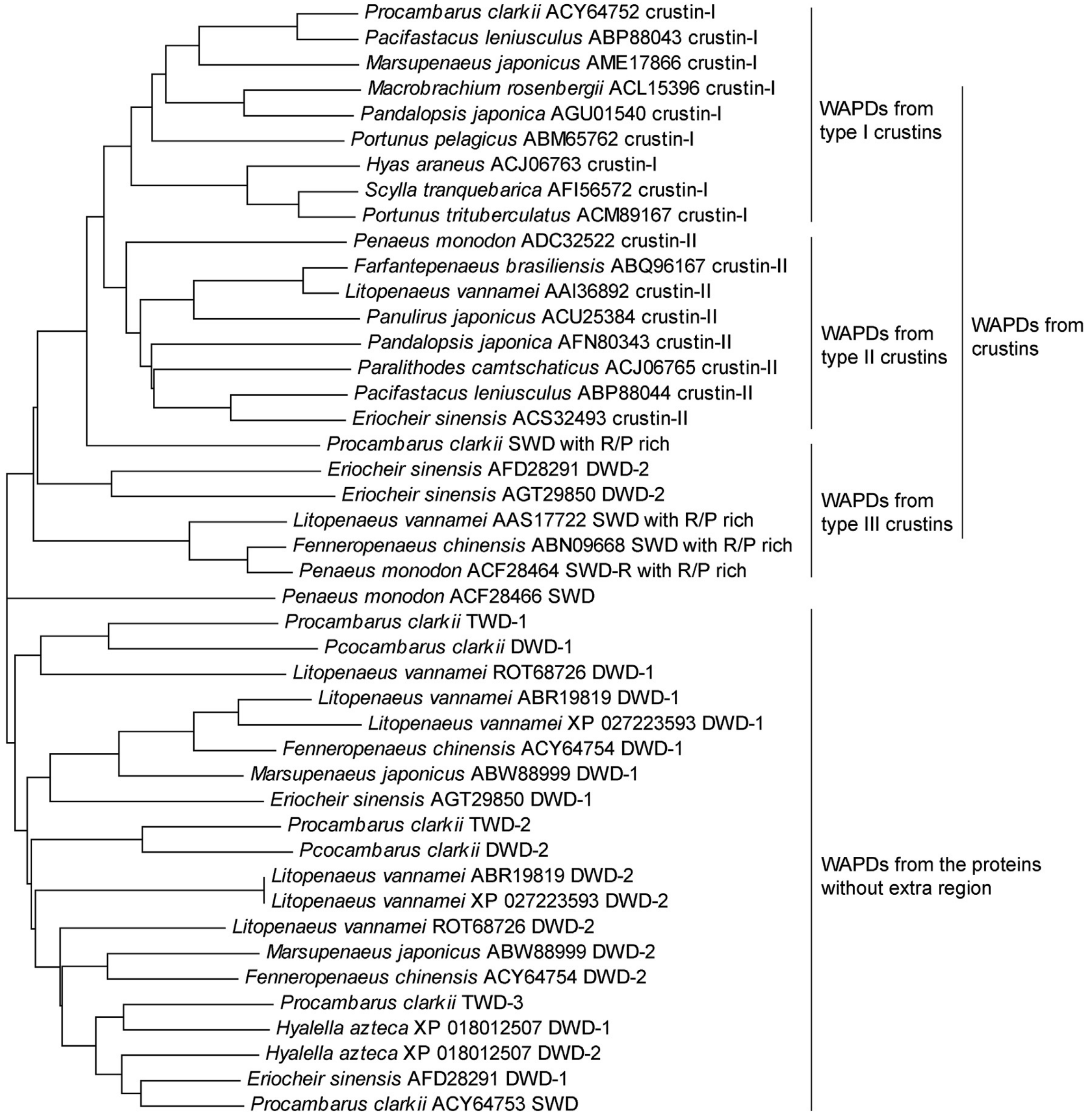

Fig. 2. Neighbor-joining phylogenetic analysis of the WAPDs from crustaceans. MEGA6 was used to build the tree with bootstrap of 1000.

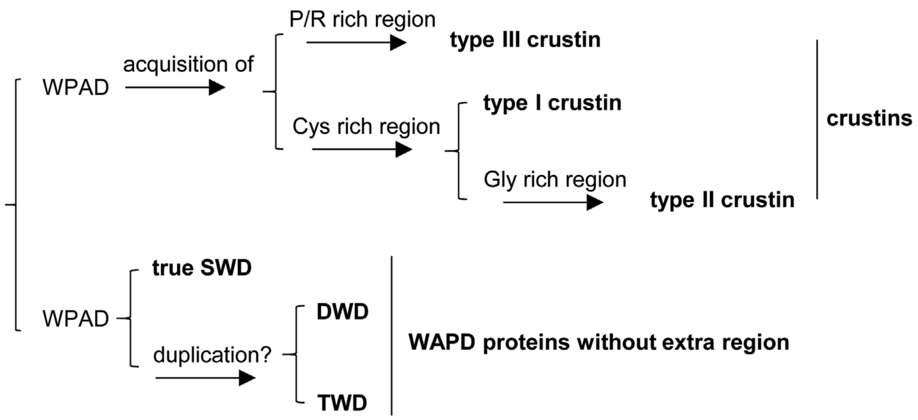

Fig. 3. A possible mode for the classification and evolution of crustacean WAPD proteins.

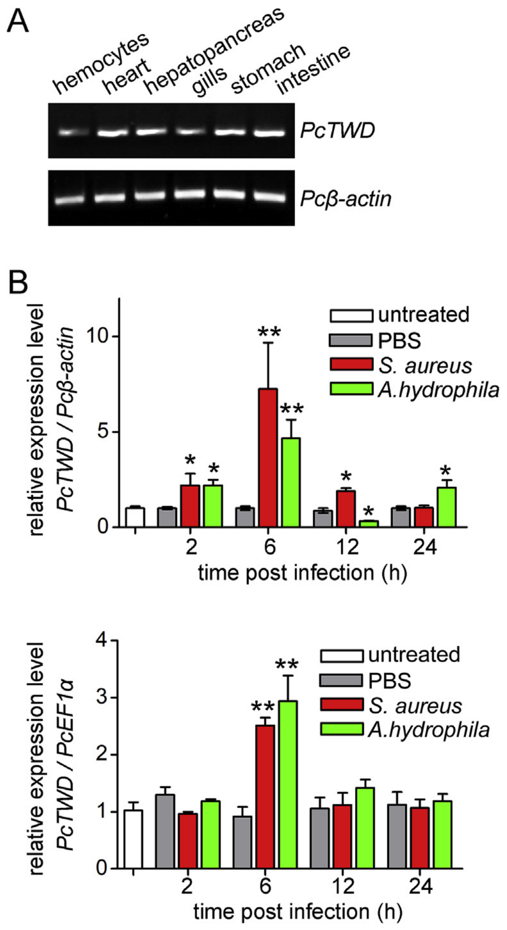

Fig. 4. Expression profiles of PcTWD. (A) Tissue distribution of PcTWD transcripts. RNAs were from healthy crayfish, and the expression was detected by RT-PCR with β-actin as the internal reference. Each sample was originated from at least five crayfish. Shown data were the representative of two independent experiments. (B) Expression level of PcTWD in hemocytes after bacterial infection. qPCR was performed to study the expression of PcTWD. Expression level was normalized to that in the untreated crayfish. Data showed the mean ± SD from three independent experiments. $^{**}p < 0.01$, $^{*}p < 0.05$, calculated by Student's *t*-test. Independent repeats were performed in triplicate with at least five animals used for each sample.

was involved in the innate immune response of crayfish. As shown in Fig. 4A, PcTWD transcripts were ubiquitously detected in all six selected tissues, including hemocytes, heart, hepatopancreas, gills, stomach and intestine. The wide distribution of PcTWD suggested that it might be a key participator in crayfish immunity. To determine whether PcTWD was indeed inducible by bacterial challenge, its expression after *S. aureus* and *A. hydrophila* stimulation was detected in hemocytes. The results showed that both Gram-positive bacteria and Gram-negative bacteria could induce the expression of PcTWD in a similar manner. The significant induction reached to a peak at 6 h post infection, and then gradually decreased from 12 h to 24 h post infection (Fig. 4B). The obvious induction of PcTWD expression supported that PcTWD was deeply involved in the crayfish immune response against bacterial infection.

3.4. Function of PcTWD in antibacterial response

To determine the specific function of PcTWD in the antibacterial response, RNAi was performed to knock down PcTWD expression and to check whether this would influence crayfish immunity. As shown in Fig. 5A, PcTWD dsRNA application could significantly suppress the expression of PcTWD. The remaining expression level after RNAi was about 30% of that in in control sample. After validating that PcTWD expression could be knocked down, *A. hydrophila* infection was performed in the dsRNA-treated crayfish. The results showed that PcTWD knockdown significantly suppressed the immunity of crayfish against *A. hydrophila*. The survival rate of PcTWD knockdown crayfish after infection was lower than that of the control group. All tested animals died at 4 d after infection, while about a half of control crayfish was still

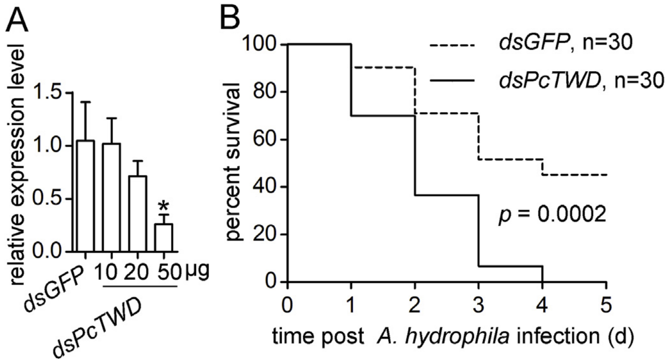

Fig. 5. Function of PcTWD in antibacterial immunity analyzed by RNAi. (A) RNAi efficiency of PcTWD in crayfish hemocytes. Crayfish was injected with 50 μg of PcTWD dsRNA, or equal amounts of GFP dsRNA as control. RNAi efficiency was studied by qRT-PCR. At least five crayfish were used for each injection. Data were representative of two independent repeats. $^{*}p < 0.05$, analyzed by Student's *t*-test. (B) Protective role of PcTWD against *A. hydrophila* infection. *A. hydrophila* (10^6 CFU) was injected into PcTWD silenced crayfish to determine the survival rates. Thirty animals were used for each treatment. Data were analyzed by Log-rank (Mantel-Cox) test by GraphPad Prism.

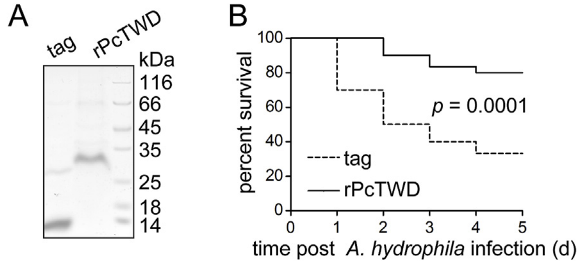

Fig. 6. Function of PcTWD in antibacterial immunity analyzed by over-expression. (A) Purified rPcTWD and the tag. Proteins were expressed in *E. coli* cells, and purified by affinity chromatography. (B) Protective role of rPcTWD against *A. hydrophila*. Crayfish was pre-injected with 2 μg of rPcTWD, and then *A. hydrophila* (10^6 CFU). Thirty crayfish were used for each group, and the results were analyzed by Log-rank (Mantel-Cox) test using GraphPad Prism.

alive (Fig. 5B). This suggested that PcTWD was important for crayfish to resist *A. hydrophila* infection.

To validate the discovery obtained from RNAi assay, an "over-expression" like test was performed by introducing the exogenous recombinant PcTWD into crayfish. As shown in Fig. 6A, recombinant PcTWD was expressed in bacteria in soluble form and purified. rPcTWD application obviously enhanced crayfish resistance against *A. hydrophila* infection. The survival rate of rPcTWD-treated group was always higher than that of the control group. The survival percent of the control group was less than 40% at 5 d after infection, while more than 80% animals were alive in the experimental group at that time (Fig. 6B). Thus the above results confirmed the significance of PcTWD in host immunity to protect crayfish from pathogenic *A. hydrophila* infection.

3.5. Protease inhibitory activity and antimicrobial activity of PcTWD

Previous reports had suggested that WAPD possessed protease inhibitory activity or antimicrobial activity. Thus whether PcTWD possessed such two activities was studied. As shown in Fig. 7A, rPcTWD exhibited inhibitory ability against protease K. The inhibition efficiency was as high as about 50%, compared to the control group. Since PcTWD contained three tandem WAPDs, these domains were solely chemically synthesized to make clear the contribution of each domain in the protease inhibition. The results showed that the first two WAPDs contributed to the protease inhibition while the last WAPD did not exhibit such an activity (Fig. 7B).

In contrast, the last domain possessed direct antimicrobial activity against both *S. aureus* and *A. hydrophila*, determined by a liquid bacteria growth inhibitory assay. Differently, the first domain did not exhibit any antimicrobial activity, while the second domain could slightly inhibit bacterial growth (Fig. 8). These results suggested that the three domains made different contributions to the full protein, and these contributions together made PcTWD an important member in crayfish antibacterial immunity.

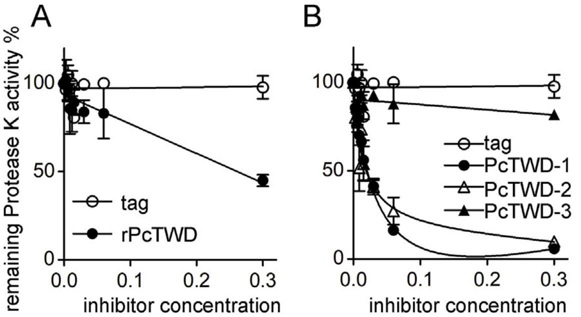

Fig. 7. Protease inhibitory activity of rPcTWD (A) and three separate WAPDs (B). Proteinase K and AAPF was dissolved to a concentration of 0.1 mg/ml and 1 mg/ml with 50 mM Tris-HCl (pH 8.0). The inhibitors were dissolved to a series of concentrations. The inhibitor (90 μl) was incubated with 1 μl of protease at 30 °C for 10min. AAPF (15 μl) was then added to the mixture. After another incubation at 30 °C for 15 min. The reaction was terminated to detect the absorbance at 405 nm. The remaining protease activity was then calculated.

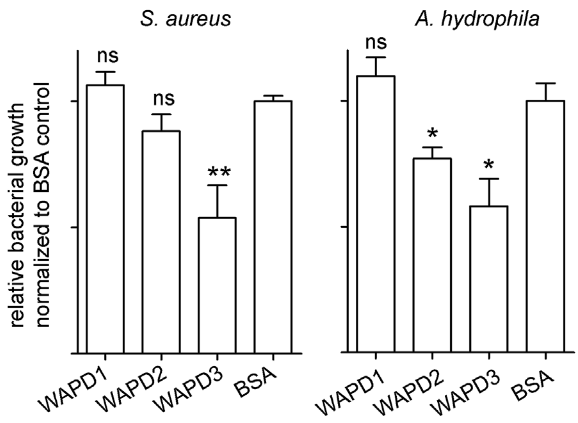

Fig. 8. Antimicrobial ability of three separate domains of PcTWD. Bacterial suspension (10^8 CFU/ml, 20 μl) was incubated with protein solution (20 μl). Medium was added to a final volume of 200 μl. The mixture was maintained at room temperature for 24 h. The absorbance at 600 nm was detected to represent the bacterial growth. Data was normalized to the control group.

4. Discussion

In the present study, a protein contained three tandem WAPDs was identified in *P. clarkii*. This was the first identification of a protein with triple WAPDs in crustaceans, though a lot of proteins with one or two WAPDs had been cloned and characterized in shrimp, crayfish and crab among others. Until now, such unique domain arrangement was only found in abalones and fish. For example, perlwapin was identified in green lip abalone *Haliotis laevigata* and the donkey ear abalone *H. arsinina*. It was involved in shell formation regulation by suppressing the deposition of calcium in the shell matrix [10,15]. Another perlwapin like protein which also contained three WAPDs from Atlantic salmon *Salmo salar* was found expressed in the head kindey which was an inflammation closely related organ, suggesting its possible involvement in fish immunity [16]. Though these three proteins shared the same domain composition, the sequence similarity among them was low (data not shown), suggesting that they might not share the same origin. Nevertheless, the presence of these proteins in fish, abalone and crayfish suggested that such domain arrangement might not be accidental.

The identification of PcTWD provided a good marker for the classification of WAPD proteins in crustacean. A majority of previous studies separated the crustacean WAPD proteins into four subgroups, and named all of them as crustins [4]. However, increasing studies had found that the proteins which contained solely WAPDs were distinctive from those contained some additional region (cysteine-rich region and glycine-rich region for instance) aside of the WAPD when performing the phylogenetic analysis [17]. Actually these analysis were conducted using the complete sequence. The presence of the extra region aside from the WAPD, at least to a certain degree, would certainly influence the phylogenetic clustering. Thus the finding of a tripe WAPD containing protein enriched the analyzing scope. Then we used WAPDs only to build the phylogenetic tree in this study to exclude the bias caused by the extra region. And the results again supported that the domains from the proteins solely containing WAPDs and from the type I/II/III crustins were also clustered distinctly. It can be proposed that these two family evolved separately. Based on this observation, crustin family could be divided into only three subgroups which were type I, type II and type III. The other proteins contained solely the WAPD could be regarded as a parallel family.

Among the crustacean WAPD proteins, PcTWD was unique not only due to the domain arrangement, but also because of its wide distribution in multiple tissues. Generally, most crustacean WAPD proteins were primarily expressed in hemocytes. For example, the expression level of PcDWD in hemocytes was much higher than in other tissues [17]. Hemocytes were the major immune-related cells for crustaceans, and the high expression and storage of WAPDs proteins in hemocytes ensured that these proteins could be rapidly released to the circulating hemolymph upon the degranulation of hemocytes after pathogen infection. It was generally accepted that the low expression of these protein in other tissues might origin from the infiltrating hemocytes in these vascularized tissues [18]. Thus these genes could be regarded as hemocytes specific. However, this might not be the case for those WAPD proteins whose expression in hemocytes was much lower than in other tissues. For example, the expression level in gills of a DWD protein from *Eriocheir sinensis* was more than 10 folds higher than that in hemocytes. The high expression in gills suggested that this DWD might functioned in a different manner from those hemocytes specific genes [19]. Among the crustacean WAPD proteins, the one with the most similar distribution manner with PcTWD was the DWD protein from *P. monodon*. PmDWD transcripts were detected highly in multiple tissues, including hemocytes, epipodite, gills, intestine, heart, and hepatopancreas [20]. Such wide distribution of PcTWD and PmDWD might be an evidence for their full participance in host immunity throughout the whole body. The obvious induction of PcTWD expression after bacterial infection also supported the significance of PcTWD in the host antibacterial immunity.

The function of PcTWD in antibacterial response was confirmed by the survival assay. Silencing its expression using RNAi suppressed the host resistance against *A. hydrophila*, and administration of exogenous recombinant PcTWD could lower the mortality caused by *A. hydrophila* infection. The protective role of PcTWD might originate from its two kinds of biological activities including the protease inhibitory ability and the antimicrobial ability. These two features had been proven as the two principle manners for WAPD proteins to participate in host antibacterial immunity [12]. However, these two abilities might not be exclusively separated. By inhibiting the proteases secreted by invading bacteria, WAPD proteins could suppress, on one hand the bacteria virulence, or on the other hand their nutrient acquisition which would led to the limitation of bacterial growth. Also some previous studies found that WAPD protein could directly interact with the bacterial cell wall components and disrupt the bacteria integrity [9]. In this case the antimicrobial ability of WAPD protein was independent of the protease inhibitory ability. For example, mammalian SLPIs, which contained two WAPDs, possessed both protease inhibitory activity and bacterial growth inhibitory activity. These two activities were relied on the two separate domains, respectively [21]. Here, labor division was also found among the three WAPDs of PcTWD. As analyzed, the first two WAPDs were responsible for the protease inhibitory activity while the third one made major contribution to the antimicrobial activity. The biochemical and structural basis of the labor division needed further study to reveal.

5. Conclusion

A triple WAPDs containing protein PcTWD was identified and characterized in *P. clarkii*. This was the first finding of a protein with such domain architecture in crustacean. PcTWD played an important role in crayfish antibacterial immunity possibly through its protease inhibitory activity and antimicrobial activity. Moreover, the discovery of this protein with three tandem WAPDs provided new insight into the classification and evolution of crustacean WAPD proteins.

Declarations of interest

None.

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