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Characterization of a double WAP domain-containing protein from the red swamp crayfish *Procambarus clarkii*

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ABSTRACT

Crustaceans express multiple whey acidic protein (WAP) domain containing proteins which are components of host immunity. In the present study, a new double WAP domain containing protein was identified from red swamp crayfish *Procambarus clarkii*, designated *Pc*-DWD. The ORF is 387 bp, encoding 128 amino acids consisting of signal peptide of 18 residues, and two tandem WAP domains of 38 and 44 residues. Multiple alignment indicates the presence of conserved motifs in both WAP domains, and phylogenetic analysis shows that *Pc*-DWD is a new member of the type-IV crustin family. *Pc*-DWD transcripts were found most abundantly in hemocytes, gills, intestine and heart, and induced by *Vibrio anguillarum, Staphylococcus aureus* and white spot syndrome virus challenge. RNAi knockdown of *Pc*-DWD expression led to increased expression of white spot syndrome virus genes and increased crayfish mortality after virus infection. Recombinant *Pc*-DWD exhibited strong protease inhibitory activity towards commercial subtilicin A and protease K. *Pc*-DWD inhibited the crude proteases from *V. anguillarum* and *S. aureus* cultures and from the crayfish tissue extracts. We infer that *Pc*-DWD acts in crayfish bacterial and viral immunity.

1. Introduction

Initially identified in the whey acidic protein (WAP), the most abundant protein in the milk serum of certain mammals, WAP domains (WAPD) occur in many non-milk proteins in almost all metazoans [1,2]. WAPD contains approximately 50 amino acid residues, and is characterized by eight conserved cysteine residues to form a four-disulfide core (4DSC). A proposed arrangement of the cysteine residues (C1-C8) in vertebrate WAPs is C1-Xn-C2-Xn-C3-X5-C4-X5-C5-C6-X3/5-C7-X3/4-C8 [3], although this arrangement is variable. Along with the accumulation of newly identified WAPD containing proteins (WAPDPs), especially in invertebrates, variability in the stretch between adjacent cysteines increases [4,5]. Nonetheless, the conserved cysteines support a stable tertiary structure which is essential for diverse activities of WAPDPs.

In addition to the serum components, some small secretory proteins with WAPD act in growth and tissue differentiation by regulating the epithelial cell proliferation and extracellular matrix remodeling [6,7]. With protease inhibitory activity, these proteins can prevent elastase-type serine proteases from degrading laminin and suppress MAPK signaling [8]. Through a similar biochemical mechanism, some WAPDPs can affect the progression of certain cancers [9]. Some WAPDPs have antibacterial activity [10]. For example, two members of the anti-leukoprotease family, the secretory leucocyte proteinase inhibitor and elafin, are active against bacteria, fungi or virus *in vitro* and act in inflammation *in vivo* [11].

WAPD proteins are also widely expressed in other animals, including numerous invertebrates [12]. Several WAPD families have been characterized, including waprins, Ku-wap-fusins and chelonianin from reptiles, perlwapsins from molluscs and crustins from crustaceans. These WAPD proteins vary in domain architecture and physiological function. Wapsins containing a signal peptide and a sole WAPD are present in snake venom. They exhibited antimicrobial activities, mostly due to the WAPD [13,14]. Pearlwapsins consist of signal peptide and three tandem WAPDs, and function in abalone shell formation [15]. The crustacean WAPD proteins are crustins [4]. The first crustin,

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Table 1
Primers used in this study.

Primers	Sequences 5′-3′
RealF	TGTTGTTTGGGGTTGTCGT
RealR	GAGTGTGCTGGTATCTGGTCA
18SF	TCTTCTTAGAGGGATTAGCGG
18SR	AAGGGGATTGAACGGGTTA
VP28F	AGCTCCAACACCTCCTCCTTCA
VP28R	TTACTCGGTCTCAGTGCCAGA
IE1F	GACTCTACAAATCTCTTTGCCA
IE1R	CTACCTTTGCACCAATTGCTAG
iF	TGGGGTTGTCGTCATCAC
iR	TGCTCTTCACCTGCTGCTA
GFPiF	GCGTAATACGACTCACTATAGGTGGTCCCAATTCTCGTGGAAC
GFPiR	GCGTAATACGACTCACTATAGGCTTGAAGTTGACCTTGATGCC
ExF	TACTCAGAATTCGCAGATCCTTCACCCGACACA
ExR	TACTCACTCGAGTTAGTGTTGTGTTGAAGTGTCA

carcinin, was isolated based on antimicrobial activity from *Carcinus maenas* hemocytes [16]. Many crustins were identified from shrimp, crab, lobster and crayfish [4,7]. All crustins contain an N-terminal signal peptide and a C-terminal WAPD of about 40–50 residues. Four sub-groups are characterized by the inter part between two regions. The special region is a cysteine-rich domain for type-I and type-II crustins with an extra glycine rich region before the cysteine-rich domain. Type-III crustins feature a region rich in proline and arginine residues, while type-IVs contain another WAPD [4]. Similar to the vertebrate homologs, these crustins have antimicrobial and protease-inhibitory activities.

Red swamp crayfish, *Procambarus clarkii,* is a food animal with a wide global distribution. It is susceptible to pathogens, including bacteria, fungi and viruses, which seriously challenge crustacean aquaculture. It follows that continued research into the immunobiology of *P. clarkii* and other invertebrate aquaculture systems is necessary. We are working to meet this global-scale need by discovery and analysis of new anti-microbial proteins. A random sequencing of a *P. clarkii* cDNA library revealed a protein containing two WAPDs, termed *Pc*-DWD. Its expression pattern following pathogen challenge was investigated. Here, we report on the outcomes of our investigation.

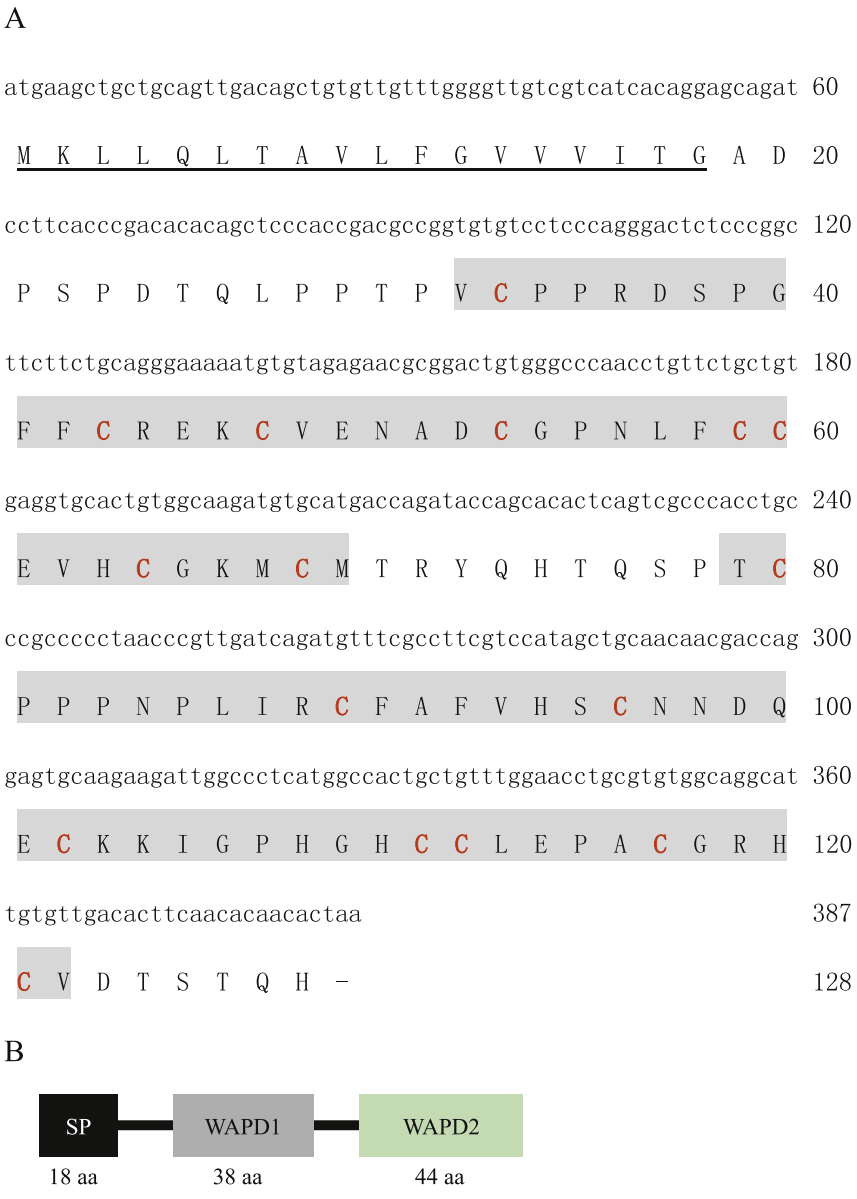

Fig. 1. The sequence information of *Pc*-DWD. (A) The cDNA and deduced amino acid sequences of *Pc*-DWD. The signal peptide is underlined. The two WAPDs are shown in gray background. The conserved cysteine residues are shown in red. (B) The sequence model of *Pc*-WAPD. SP stands for signal peptide. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)