Recombinant expression and antimicrobial activity characterisation of three crustin proteins from the crayfish *Pacifastacus leniusculus*

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**Summary**

Aquatic decapod crustaceans possess only an innate immunity to protect them against pathogenic invasions. A major component of this immune system is a group of molecules known as antimicrobial peptides (AMPs), which are cationic molecules less than 10kDa and that display a broad spectrum of antimicrobial activity towards gram-positive and gram-negative prokaryotes, but are not toxic to their eukaryotic hosts. AMPs form amphipathic structures which allow them to interact with the cell membranes of bacteria, where they cause structural disruptions and increase permeability that eventually destroy the pathogens. Several AMPs have now been isolated from aquatic decapods such as the shore crab *Carcinus maenas* and the Pacific white shrimp *Litopenaeus vannamei* and characterised analytically, and they were found to possess in common a whey-acidic-protein (WAP) domain that is the site of serine proteinase inhibitory activity. Of these AMPs, three penaeidins from *L. vannamei*, and an 11.5kDa crustin from *C. maenas* have been experimentally proven to exhibit antimicrobial activity. This project was aimed at recombinantly expressing three crustin cDNAs from the crayfish *Pacifastacus leniusculus* using the pGEX 4T-1 vector which tags the crustin protein with glutathione-S-transferase (GST), and testing the proteins for antimicrobial activity and proteinase-inhibitory activity. All three crustin proteins were found to be partially insoluble due to a high cysteine content, so only the most soluble protein of the three, crustin 1, was cultured on a large scale and purified for use in the tests for activity. Cleavage of the GST tag off the crustin protein was performed using thrombin protease, but yields of the crustin protein was very low compared to the GST tag due to degradation of the unstable “mature” protein. Consequently, tag-containing recombinant crustin protein was used in the tests for activity instead. However, there was no antimicrobial activity detected against both gram-negative and gram-positive bacteria, nor was there any exhibition of proteinase-inhibitory activity by the GST-tagged crustin protein. This absence of activity by the recombinant protein may be attributed to several factors, including the presence of the GST tag which may inhibit proper protein folding and thereby function, as well as low concentrations of the protein used in the activity tests. The nature of these crustacean AMPs and their instabilities pose difficulties in the tests for their activity, but even though comparative analysis of the proteins against other more well-researched AMPs offers an alternative method of gaining insight into their properties, experimental testing will be important in determining the spectrum of bacteria they act against.

**Introduction**

Aquatic invertebrates, such as crustaceans, do not possess an adaptive memory-dependent immunity that is mediated by clonally derived antibodies and T-lymphocytes (1). Instead, they are solely dependent on their innate immune system for protection against pathogens (2). This crucial first line of defence is a nonadaptive cellular and humoral mechanism involving antimicrobial peptides (AMPs), hemocytes, melanization, lectin production, the release of oxygen radicals and the pro-phenoloxidase cascade (2,3,4).

AMPs are a major component of innate immunity in crustaceans. They are defined as molecules exhibiting antimicrobial activities and which are usually less than 10 kDa in mass. The small size enables the peptides to be easily synthesized without requiring specialised cells or tissues, and they are able to respond quickly and efficiently to invading microorganisms when they are released by circulating hemocytes at the sites of pathogenic entry. This ability to act with sufficient immediacy also stems from the peptides’ broad
spectrum of activity against prokaryotes without requiring cellular or humoral memory, and their lack of toxicity towards eukaryotic cells (1,2,3,5,6).

The majority of AMPs are cationic and form amphipathic structures consisting of a hydrophobic and hydrophilic surface. The mechanism of action of these AMPs therefore involves the disruption of bacterial membranes through an increase in membrane permeability mediated by the formation of membrane pores, or the disruption of membrane structure induced by the interaction of the peptides’ cationic charges with the positively-charged membranes and consequent displacement of membrane lipids (1,2,7). In aquatic invertebrates, AMPs are widely found in tissues like the gut and respiratory organs, since these are major sites of potential pathogenic invasions (1,2).

The first crustacean AMP to be isolated and partially sequenced was a 6.5kDa proline-rich cationic AMP from the shore crab *Carcinus maenas*, and it was found to exhibit antimicrobial activity against both gram-negative and gram-positive bacteria (6,8). Following in the footsteps of this discovery was the characterization of AMPs called penaeidins from the hemolymph of the Pacific white shrimp *Litopenaeus vannamei*. These peptides exhibit both antifungal and antimicrobial activity. This antimicrobial activity is specific for gram-positive bacteria, but do not have a strong response towards gram-negative bacteria (9). Also widely distributed within the family of decapod crustaceans are the AMPs called crustins (8). The first crustin purified was an 11.5kDa cysteine-rich peptide with specific gram-positive antimicrobial activity. It was isolated from the granular hemocytes of *C. maenas*, but has been found to be biochemically and functionally distinct from the 6.5kDa proline-rich AMP, it also contains a whey-acidic-protein (WAP) domain (1,3). A WAP domain is comprised of eight-cysteine residues that form a conserved four-disulphide core of approximately 50 amino acids (8). Proteins containing WAP domain motifs possess serine proteinase inhibitory properties, which are considered to be fundamental to the antimicrobial activities of AMPs (3,5).

AMPs present themselves as promising alternatives to antibiotics in the face of increased antibiotic-resistance developed by pathogenic bacteria. Aquatic decapod crustaceans are hugely important key resources to fisheries and aquaculture around the world, but they are susceptible to incapacitating microbial diseases and are regarded as vectors for parasites and pathogens, which are harmful to fishes and humans. Invertebrates are abundant sources of AMPs, and decapod crustaceans provide an ample source of hemolymph that is required for experimental work (5). Research into the AMPs of crustaceans will shed further light on possible prevention and treatment of those aquatic diseases, since much is already known concerning the biochemical processes underlying aquatic invertebrate innate cellular immunity.

In the paper by Brockton et al., the 11.5kDa crustin isolated from the hemolymph of *C. maenas* was characterised at the gene, transcription and protein level (10). Unfortunately, the antimicrobial activity of this peptide was not tested due to difficulties in obtaining pure samples of the AMP from the recombinant proteins. In fact, current literatures regarding crustacean immunity appear to have putatively assigned antimicrobial activity to crustin proteins based on their abundance in the hemolymph, and based on both DNA sequence and predicted structural similarities to existing AMPs. Only one paper has experimentally succeeded in proving the antimicrobial activity of a crustin believed to be an AMP. This was the work of Relf et al. (5), whereby the 11.5kDa protein isolated from the haemocytes of the shore crab *C. maenas* was found to be active only against aquatic gram-positive bacteria. The
majority of research work carried out on crustacean AMP has dealt with the isolation and characterization of the proteins, but have not experimentally determined the antimicrobial nature of them, either from not carrying out the tests, or failing to achieve positive results from the tests.

The work undertaken in this project loosely mirrored the work done by Brockton et al., and was aimed at recombinantly expressing three different crustins cDNAs obtained from the crayfish *Pacifastacus leniusculus* and characterising them for antimicrobial activities against a range of gram-positive and gram-negative bacteria. The procedure used for detecting antimicrobial activity was the radial diffusion method. The test works by applying the test sample to a well punched into an agar containing bacteria and nutrients conducive to their growth. Antimicrobial activity of the sample is determined through the presence or absence of clear zones that do not contain bacteria colonies surrounding the test sample well. If clear zones are observed, it implies that the test sample was able to inhibit the growth of bacteria. Conversely, if there is no clear zone present, it suggests that the sample does not possess any antimicrobial activity.

**Results**

**Genes**

Three crustin cDNAs, namely crustin 1, crustin 2 and crustin 3 were analysed with MacVector 7.2.2 (Accelrys) to determine their gene sizes and to calculate their respective protein molecular weights (Table 1).

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<th>crustin 1</th>
<th>crustin 2</th>
<th>crustin 3</th>
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<td>size of gene (bp)</td>
<td>309</td>
<td>281</td>
<td>417</td>
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<tr>
<td>Molecular weight of protein (kDa)</td>
<td>11.7</td>
<td>10.6</td>
<td>14.5</td>
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**Polymerase Chain Reaction (PCR) and Electrophoresis**

The three crustin cDNAs were amplified by PCR and the subsequent DNA samples were verified using gel electrophoresis by comparing the resulting DNA bands with a base-pair (bp) ladder to approximate their sizes (Figure 1).
Figure 1. Image of gel electrophoresis of crustin 1, crustin 2 and crustin 3 genes. Two samples of each gene were amplified by PCR and 7 µl of each sample was loaded onto and run through a 1% agarose-gel to determine their purity and to ascertain their identities via their DNA sizes. The DNA size of each band corresponds to the size of each cDNA including their respective cloning primers. Lanes 1, 4 and 8 contain negative controls; lanes 2 and 3 contain crustin 1; lanes 5 and 6 contain crustin 2; in lane 7 is the 100-bp DNA ladder and in lanes 9 and 10 is crustin 3.

Transformation with TOP10 cells and TOPO vectors

Each crustin gene was successfully inserted into TOPO vectors, transformed into TOP10 competent cells and cultured on suitable agar plates. The recombinant plasmids were purified from the cells thereafter and then sent for DNA sequencing. The results of the sequencing were poor since there was a high level of noise that rendered the DNA sequences unreliable, but the sequences of the starting cDNAs were confirmed nonetheless. The recombinant vector was also cut with the EcoRI restriction enzyme, which is expected to cleave the vector at both ends of the gene insert, and run through a gel. The image obtained from the electrophoresis showed that the crustin genes were successfully recombined into the TOPO vectors.

Recombinant protein expression, solubility and purification

The three crustin genes were recombined into pGEX 4T-1 vectors and transformed into the competent BL21 cells for protein expression. The expression of the glutathione-S-transferase (GST)-tagged proteins was successful for all the three crustins when compared against the empty pGEX 4T-1 vector not containing any foreign gene inserts. Their respective solubilities were assessed by running the protein samples through a sodium dodecyl sulfate polyacrylamide gel electrophoresis SDS-PAGE) (Figure 2). The elucidation of protein solubility is essential towards determining the volume of cell culture to prepare in order to obtain sufficient yields of the recombinant protein for further tests.

All three crustin proteins were soluble to some extent. Crustin 1 was found to have the highest solubility of the three crustins, and hence was the only protein chosen for large-scale recombinant protein expression and purification. Differing concentrations of isopropyl β-D-1-thiogalactopyranoside (IPTG) used to induce protein-expression during cell culture did not affect the amount of protein yielded, since crustin 1 proteins induced under conditions of
0.1mM IPTG and 1mM IPTG produced equally thick bands in the SDS-PAGE gel. Thereafter, recombinant protein expression in cell cultures was achieved using 1mM IPTG.

Figure 2. Protein solubilities of crustin 1, crustin 2 and crustin 3. Lane 1 contains the marker; lane 2 contains the empty pGEX 4T-1 vector (not containing foreign gene inserts); in lane 3 is crustin 1 proteins cultured in 1mM IPTG and in lanes 4, 5 and 6 are crustin 1, crustin 2 and crustin 3 respectively, cultured in 0.1mM IPTG. The sizes of the respective protein bands observed consist of the molecular weight of the crustin protein and its 26kDa GST tag.

Crustin 1 recombinant proteins were purified using GSTrap™ HP 1 ml columns, and the protein samples collected were verified by running them through an SDS-PAGE (Figure 3). It was observed that in the 1st ml of purified eluates collected during the purification process, there was the highest concentration of GST-tagged crustin 1 proteins, but also a significant presence of contaminant proteins with lower molecular weights than the recombinant protein (Figure 3). The amount of these contaminant proteins decreases substantially, or is absent in subsequent eluates collected. To verify whether these contaminant proteins were native proteins from the BL21 competent cells used, BL21 cells were cultured in 500 ml of 1x Luria Bertani (LB) broth and incubated overnight at 37°C with ample aeration. The culture was then treated according to the protocol used for ‘Recombinant protein expression’ and ‘Protein purification’. The 1st ml of eluted BL21 cell proteins collected from the purification process was run through an SDS-PAGE against eluate samples of the purified recombinant crustin 1 proteins (Figure 4). The contaminant proteins that were found in the same eluate as the purified recombinant crustin 1 proteins were not observed in the eluted proteins from the BL21 competent cells. Therefore, it is most possible that the contaminant proteins were degraded crustin 1 proteins that were tagged to GST.
Figure 3. SDS-PAGE gel image of expressed and purified GST-tagged crustin 1. Lane 1 contains the marker; lane 2 the empty pGEX 4T-1 vector; lane 3 the crustin 1 recombinant protein culture before purification; lanes 4, 5, 6 respectively contain the 1st, 2nd and 3rd ml of eluate from a single purification session, and in lanes 7, 8, 9 are respectively the 1st, 2nd and 3rd ml of eluate from a different purification session. However, the two separate purifications were performed using the same cell culture. There is a significant presence of contaminant proteins in the 1st ml of eluate, but these were either greatly reduced, or absent in the subsequent eluates.

Figure 4. SDS-PAGE gel image of eluate from protein expression culture of BL21 cells not containing pGEX 4T-1 vectors. Lane 1 contains the marker; lanes 2 and 3 contain the flow-through effluents from sample application to the purification column, which were collected from two separate purifications performed on the same culture of recombinant crustin 1; lanes 4 and 5 respectively contain the 1st and 2nd ml of eluate from the BL21 cell culture; in lanes 6 and 7 are the 1st ml of eluate from two separate purifications performed on the same culture of recombinant crustin 1. There are some faint protein bands observed in lane 4, but they do not correspond to the bands caused by contaminant proteins in the eluate of the recombinant crustin 1 protein in lanes 6 and 7. There are no bands observed in lane 5.
**GST tag cleavage**

Thrombin protease was used to cleave the GST-tag from the purified recombinant crustin protein in order to obtain pure samples of the crustin 1 protein for further tests. Due to expected low concentrations of protein yields, precipitation of the cleaved products was carried out in order to further concentrate the proteins for the subsequent SDS-PAGE performed to check for the presence and yields of the GST-tag and the crustin 1 protein. Unfortunately, it was noted that the time allowed for precipitation was very long and may have resulted in the destruction and loss of some proteins.

The results showed that there was a significantly low yield of crustin 1 protein compared to the amount of GST after cleavage at room temperature for 6 hours and 10 hours, and this was observable by running the sample containing both the cleaved crustin 1 protein and GST-tag through a SDS-PAGE. There was a faintly visible protein band corresponding to crustin 1 observed for the recombinant protein sample cut at 10 hours, but none observed when cleavage time was 6 hours (Figure 5). Optimization of cleavage conditions was subsequently attempted by varying the length of cutting time and the temperature. Nevertheless, crustin 1 yield could not be significantly improved and the concentration of protein obtained was deemed too low for proceeding on to antimicrobial activity tests.

![SDS-PAGE gel image of cleaved GST-tagged crustin 1 eluate](image.png)

*Figure 5. SDS-PAGE gel image of cleaved GST-tagged crustin 1 eluate. In lane 1 is the marker; lanes 2 and 3 contain the 1st and 2nd ml of eluate from the same cell culture respectively, while lanes 4 and 5 contain the cleaved recombinant protein after 6 hrs and 10 hours at room temperature respectively. Clearly visible bands corresponding to the 26kDa GST are detected in the lanes containing the cleaved protein. A very faint band from the crustin 1 protein is barely detectable in lane 5, but there is no such visible band in lane 4. The crustin 1 protein band was, however, present in lane 4 during early destaining of the SDS-PAGE gel, but is no longer visible due to over-destaining.*

Consequently, the tests for antimicrobial activity was carried out using recombinant crustin 1 proteins tagged with GST instead, since the concentrations of the purified recombinant proteins obtained were much higher than the concentration of pure crustin 1 proteins yielded after cleavage and are thus more substantial for test usage.
Tests for antimicrobial activity

The radial diffusion method was performed to test the proteins for antimicrobial activity, since it is relatively easy to carry out, does not require excessive amount of the test samples, and also allows easy visualization of the clear zones and measurements of their sizes. Two variations of the test were carried out using several different species of gram-positive and gram-negative bacteria.

The protein concentration of the GST-tagged crustin 1 sample used in the first radial diffusion method was ~ 0.484 (A280). There was ample colony growth in all the agar plates, but no clear zone observed around the wells containing the crustin 1 protein sample for all the different bacteria tested, indicating an absence of antimicrobial activity in the crustin 1 protein. The negative control and positive controls produced expected results. There was no zone of clearing around the wells containing the negative controls (1x phosphate-buffered saline (PBS) buffer), but clear zones of varying sizes were observed around the antibiotic wells in all the agars containing the different bacteria.

A repetition of the test for antimicrobial activity following a modified model of the radial-diffusion method according to Lehrer et al. was performed (11). Staining of the agar gels with diluted Coomassie brilliant blue for easier visualization of the bacteria colony yielded poor results. Distinct blue-stained colonies were observable for the Staphylococcus aureus agar gel, and there were large clear zones around the three antibiotic wells containing ampicillin, kanamycin and streptomycin, but not around the well containing the crustin 1 test protein (concentration ~ 0.6 mg/ml). It would thus seem that antimicrobial activity was absent in the test sample.

The agar gels containing the other bacteria were stained-blue, but there were no distinctly visible bacteria colonies observed, and no obvious zones of clearing seen around the antibiotic wells. It is most probable that there was no bacterial growth at all in these plates, perhaps due to the choice of culture agar used. No clear zones were observed around the test sample wells either. However, it is not possible to draw conclusions regarding antimicrobial activity from this observation, since there was an absence of bacteria growth in these agar gels in the first place.

Test for proteinase-inhibiting activity

The test for proteinase-inhibiting activity involved the use of elastase, its chromogenic peptide substrate which produces a yellow colouration when acted upon by the enzyme, and the crustin 1 protein as a potential proteinase-inhibitor.

Colour changes were observed in all the enzymatic assays, including the controls and the test assays containing the recombinant crustin 1 sample. However, the colour intensity and time taken for the colour changes to be observed was similar for the control and the assays containing different concentrations of recombinant crustin 1 protein in the tests carried out using 1 µl of elastase, and 3x and 10x diluted elastase. The use of diluted samples of the enzyme was aimed at countering its highly active nature, and to allow for the visualization of differing rates of colour changes in the assays that may imply some initial proteinase-inhibitory action on elastase at the beginning of the reaction, if any. Consequently, it is most likely that the recombinant crustin 1 protein did not exhibit any experimentally detectable proteinase-inhibiting activity. Due to the lack of observable differences in the colour
intensities of the assays, the absorbance values of the assays at 405 nm as quantitative measures of proteinase activity was not measured.

**Discussion**

Both the crustin 1 and crustin 2 proteins from the crayfish *P. leniusculus* were analytically found to have a high proline and cysteine content relative to other amino acids. However, although crustin 3 also possesses a significantly high proportion of proline and cysteine, glycine was found to be the amino acid with the highest presence. Because no structural or functional analysis was carried out on these three crustin proteins, it is not possible to affirm the relative importance of the different amino acids. Nevertheless, it may be deduced that the contrasting hydrophobicity of cysteine against proline and glycine structures the amphipathic attribute of crustin as an AMP (12,13). Current literature on crustacean AMPs may also suggest the putative roles of the proline, cysteine and glycine amino acids (2).

The number of cysteine molecules in all three crustin proteins was found to be twelve after analytical translation of their respective gene sequences. This is similar to the identification of twelve cysteine residues at the carboxy-terminal regions of AMPs from the kuruma prawn *M. japonicus*, the white shrimp *L. vannamei* and the shore crab *C. maenas*. These carboxy-terminal regions have conserved sequences suggestive of proteinase inhibitory activity (3). It is thus likely that the twelve cysteine amino acids in crustins 1, 2 and 3 form 6 disulphide bridges within which contains a possible WAP-domain signature composed of 4 disulphide bridges that is believed to be essential for antimicrobial activity (10).

Solubility tests performed on purified crustins determined that the proteins were only partially soluble, with crustin 1 deemed to be the most soluble of the three. The partially insoluble nature of these proteins might be due to the rich presence of the highly hydrophobic polar amino acid cysteine (13), but further analysis need to be undertaken in order to determine the reasons for the different solubilities amongst the three crustins. A large-scale 2-litre culture of crustin 1 was performed to overcome the loss of proteins due to insolubility, and to have eventual concentrations of purified GST-tagged crustin 1 that are sufficiently high for subsequent tests.

The purified samples of recombinant crustin proteins were not completely pure. Contaminant proteins of molecular weights lighter than the GST-tagged protein were observed to be present in SDS-PAGE gels. Further procedures involving the comparison of the purified crustin protein samples with samples of BL21 *E. coli* native cell proteins which have been passed through the purification column, confirmed that the contaminant proteins were not bacterial proteins from the expression cells. Instead, it is highly probable that the contaminant proteins, judging by their molecular weights, are crustin 1 proteins which have been degraded from the carboxy-terminal end and that are still tagged to GST. Further tests involving the construction of a Western blot and the detection of GST-tagged proteins using GST-antibodies can be performed to validate the identities of these contaminant proteins. The presence of these carboxy-terminal degraded GST-tagged proteins may be attributed to the action of proteinase derived from the BL21 expression cells, as no proteinase inhibitor was used during the purification process.

The SDS-PAGE gel image of the GST-tag and the crustin 1 protein after thrombin protease cleavage of the recombinant protein shows a significantly higher yield of the GST-tag than that of crustin 1, which is considerably low. It was expected that the yield of GST and the
crustin 1 protein would be approximately similar, since they are present in a 1:1 ratio in the recombinant protein. The bands observed for the crustin 1 protein after cleavage are however, not visible due to excessive de-staining of the gel. Faint bands corresponding to the very low yields of crustin 1 were observed early during the destaining process, and could be made visible by staining the gel again. Unfortunately, this was not done.

According to Brock et al. (10), the cleavage of the leader sequences off AMPs results in rapidly degraded “mature” protein sequences, and this seems a likely explanation for the observations from the SDS-PAGE gel of the cleaved recombinant protein, where there is a low yield of crustin 1. Nevertheless, the experimental conditions for the GST-tag cleavage such as cleavage time and temperature can also be further optimized to maximise crustin yield by overcoming the possibility that not all the recombinant proteins were cleaved. Perhaps precautions may also be designed to retard the rate of “mature” crustin peptide degradation, so as to obtain a crustin yield that is as high as possible.

Since there was insufficient crustin 1 obtained for further usage in protein activity tests after recombinant protein cleavage, the antimicrobial activity tests and proteinase-inhibitor tests were attempted using recombinant crustin 1 tagged to GST. In spite of this, there was no antimicrobial activity detected against either gram-negative or gram-positive bacteria, and the peptides did no exhibit inhibition towards proteinase either. Several possibilities may be presented to explain this lack of activity by crustin 1. Degradation of the recombinant protein might have occurred during the extended period of time it was maintained at 4°C in between tests, thereby leading to a loss of antimicrobial activity. The presence of the GST tag on the N-terminal of the crustin protein may also prevent it from folding into its physiologically functional conformation, thereby rendering the protein non-functional. In the tests for antimicrobial activity, the concentration of the recombinant protein added to the sample wells may have been insufficiently high to detect any inhibitory actions on bacterial growth.

In the paper by Jiravanichpaisal et al. (14), the expression of genes encoding for the same three crustins used in this project was investigated through in vivo inoculation of the crayfish P. leniusculus with bacteria. The results of this investigation provide clues as to the types of bacteria each crustin protein may exhibit antimicrobial activity towards. It was found that crustin protein production was induced in response to both gram-negative and gram-positive bacteria; the crustin 2 gene was believed to be constitutively and highly expressed whereas there was transcriptional upregulation the crustin 3 gene when the crayfish was immunologically challenged with a non-pathogenic gram-positive bacterium. Therefore, it would be reasonable to expect the three crustin proteins to exhibit in vitro antimicrobial activity to the same kinds of bacteria they respond to in vivo.

Current problems hindering effective experimental forays into the testing of AMPs for antimicrobial activity include inefficient isolation of the proteins in the laboratory, mainly due to the amino acid composition of AMPs that render them insoluble to some extent. Nevertheless, attempts should be made to experimentally characterise any antimicrobial properties, if present, of so-called ‘AMPs’, despite the current reliance on the deducing of their activity through analysis against existing well-investigated AMPs. Better understanding of the properties of AMPs, such as their structures and relative stabilities, may also aid in the optimisation and designing of experimental methods that are more capable of isolating and characterising them in the laboratory.
**Materials and Methods**

**cDNA-clones**

The Comparative Animal Physiology department provided the three antimicrobial peptide cDNAs, namely crustin 1, crustin 2 and crustin 3, isolated from the crayfish *Pacifastacus leniusculus*, and their respective forward and reverse primers. The DNA sequences of each crustin cDNA, as well as their relevant 15 base-pairs long primers were also known. Each gene was translated using the program MacVector 7.2.2 (Accelrys) in order to elucidate their base-pair lengths and the molecular weights of their proteins.

**Vectors and Hosts**

Two different vectors were utilized in plasmid recombination and transformation, cell culture, and subsequent recombinant plasmid isolation and purification. These were the TOPO® vector (Invitrogen), and the pGEX-4T-1 vector which tags the recombinant protein with glutathione-S-transferase (GST) (Amersham Biosciences AB). The host cells used for transformation included One Shot® TOP10 Chemically Competent *Escherichia coli* (Invitrogen) and competent *E. coli* BL21 (Novagen).

**Bacteria**

Antimicrobial activity was tested using both gram-positive and gram-negative bacteria. The gram-positive bacteria used was *Staphylococcus aureus*; the gram-negative bacteria used include *Escherichia coli* and the following bacteria derived from the crayfish *P. leniusculus* – two species of *Aeromomas hydrophila* isolated from the hemolymph and hepatopancreas, *Chryseobacterium sp*, *Pseudomonas anguilliseptica*, *Acinetobactor sp.*, two species of *Pseudomonas* both isolated from the hemolymph and *Obesumbacterium proteus*.

**Polymerase Chain Reaction (PCR)**

PCR amplification (98°C for 30s, 30 x [98° for 10s, 56°C for 30s, 72°C for 30s] and 72°C for 10 mins) was carried out on the three crustin genes using Phusion™ High-Fidelity DNA Polymerase (Finnzymes) according to the protocol supplied by the manufacturer.

**Gel Electrophoresis**

DNA samples were verified and analysed by running them against a 100 base-pair ladder through 1% agarose gel using appropriate voltages for a suitable amount of time. (The running voltage and time varies depending on the size of the gel, and on how well a separation is desired for the DNA samples).

**TOP10 cells with TOPO vectors**

To purify the crustin genes from the PCR samples, the QIAquick PCR purification kit (Qiagen Ltd.) was used according to the protocol provided by the manufacturers, except for the elution of DNA, where double distilled water was used instead of the recommended elution mediums. The purified crustin genes were transformed into TOP10 cells following procedures for chemical transformation given by the suppliers. Plating of the transformation samples was carried out on Luria Bertani (LB) ampicillin (0.1 mg/ml) agar plates containing
X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside), and culturing took place overnight at 37°C, after which a single colony for each respective crustin gene was transferred to LB ampicillin broth and further cultured overnight in a shaking incubator at 37°C. Recombinant TOPO vectors containing the crustin genes were isolated and purified using the Sigma GenElute Plasmid Miniprep kit (Sigma-Aldrich), following the instructions provided, but DNA elution was carried out with double-distilled water instead of the recommended elution mediums. The crustin genes were then sent to Rudbeck Laboratory for sequencing. *EcoRI* was also used to cut the recombinant TOPO vectors (1 hr, 37°C), and gel electrophoresis was subsequently carried out to check for the presence of the crustin genes.

**BL21 cells with pGEX 4T-1 vectors**

pGEX 4T-1 vectors chemically transformed into TOP10 cells were purified using the Sigma GenElute Plasmid Miniprep kit. Restriction enzyme cutting was carried out on the vectors and crustin gene inserts using *BamHI* and *XhoI* in K Buffer (37°C for 4 hours) with additional units of restriction enzymes added after 2 hours. To purify these cut DNA, the samples underwent gel electrophoresis using 1% agarose gel at a suitable voltage. The DNA bands corresponding to the linearized vector and all three crustin inserts were cut out for use with the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences) following the manufacturer’s instructions, except that the elution medium used was 1x DNA dilution buffer (Roche Rapid DNA ligation kit, Roche Applied Science) instead of the recommended elution buffer, and the pGEX 4T-1 vectors and crustin gene inserts were subsequently ligated together using the Roche Rapid DNA ligation kit following the protocol provided by the supplier. Finally, the recombinant plasmids were transformed into competent BL21 cells following the procedures for TOP10 cell transformation.

**Recombinant protein expression**

Small-scale cultures of 15ml were prepared for each crustin protein, whereby 5 colonies of recombinant plasmid containing a crustin gene were added to LB ampicillin (0.1 mg/ml) broth and allowed to grow at room temperature with ample aeration until OD$_{600}$ ~ 0.6-0.8. *Isopropyl-β-D-thiogalactopyranoside* (IPTG) was added to concentrations of 0.1mM and 1mM, and the culture then incubated at room temperature for a further 4 hours. Following this, the cultures were centrifuged (16 060 x g, 10 min, 4°C) and the pellets obtained were resuspended in 1x lysis buffer (50mM NaH$_2$PO$_4$, 300mM NaCl, 1mM 2-methacryl coenzyme A, pH 8.0). Sonification was then carried out (pulsed at 60% duty cycle for 20 s, on ice for 1 min, 5 cycles), and the samples were subsequently centrifuged again (16 060 x g, 10 min, 4°C). The resultant supernatant was then used to test for the solubility of each of the three crustin proteins.

Seed stock cultures of transformed BL21 cells containing crustin 1 recombinant proteins were prepared in LB ampicillin broth using several colonies (~ 5-7) obtained from the agar plate cultures. These were grown at 37°C in a shaking incubator until OD$_{600}$ ~ 0.6-0.8.

A large-scale culture of 2 litres of LB ampicillin broth was also prepared for only the crustin 1 protein, wherein seed stock was added to the total culture volume in the ratio of 1:100, then incubated at 37°C until OD$_{600}$ ~ 0.6-0.8 was once again attained before IPTG was added to 1mM concentration in order to induce over-expression of the proteins. The culture was then allowed to grow at room temperature with sufficient aeration overnight, after which it was
centrifuged (4000 x g, 20 min, 4°C) with the resulting pellets resuspended in 1x phosphate-buffered saline (PBS) buffer (140mM NaCl, 2.7mM KCl, 10mM Na2HPO4, 1.8mM KH2PO4, pH 7.3) and sonified (pulsed at 60% duty cycle for 20 s, on ice for 1 min, 5 cycles). The samples were then centrifuged again (5939 x g, 30 min, 4°C), and the supernatant retained for protein purification.

**Protein Solubility**

1x sodium dodecyl sulfate (SDS) loading buffer containing dithiothreitol (DTT) to a concentration of 0.1M was added to the respective supernatants obtained, and the samples were heated at 95°C (~ 5 min) before being run through a SDS polyacrylamide gel electrophoresis (PAGE) (10% separating gel, 4% stacking gel). Bacteria transformed with empty pGEX 4T-1 vectors not containing any recombinant gene was used as a negative control. The vector was transformed into BL21 competent cells and treatment of the transformed cells carrying the empty vector was in the same manner as detailed for the small-scale culture of the three crustins.

**Protein Purification**

GSTrap™ HP 1 ml columns (Amersham Biosciences) were used for purifying the GST-tagged proteins, according to the instructions provided by the manufacturer. The samples were passed through the column three times by collecting the effluent after each pass-through and running it through the column again. Elution of the GST-tagged proteins proceeded 1 ml at a time, such that the column was kept at 4°C for 20 minutes before eluting the 1 ml eluate. Protein concentrations in the eluates collected were measured using the BioPhotometer (6131, Eppendorf). To verify the presence of the desired GST-tagged proteins, sample volumes of the eluates were mixed with 1x SDS loading buffer (0.1M DTT), heated at 95°C (~ 3 min) and then ran through a SDS-PAGE (10% separating gel, 4% separating gel). Samples purified in a similar way from BL21 cells transformed with empty pGEX 4T-1 vector not containing any recombinant gene was used as a control.

**GST-tag cleavage**

To cleave the GST tags off the recombinant crustin 1 proteins, thrombin protease (1 unit/µl) (Amersham Bioscience) was added to the eluted samples (1 unit/100 µg GST fusion protein) and allowed to stand at room temperature for 6, 10 and 12 hours, as well as 2 hours at 30°C. The HiTrap™ Benzamidine FF (high sub) 1 ml column (Amersham Bioscience AB, Uppsala, Sweden) was used to remove the thrombin protease after each of the time period.

The cleaved proteins were precipitated by adding 10 µl of 100% trichloroacetic acid (TCA) for every 100 µl of sample, and allowed to stand overnight at 4°C. However, this duration of precipitation time has been noted to be too long which may result in potential protein loss. Therefore, it is recommended that precipitation be allowed to proceed for 30 min on ice. Thereafter, it was centrifuged (16 060 x g, 15 min, 4°C) and the pellet obtained was re-dissolved in cold acetone. This was then followed by another round of centrifuge under the same conditions as before, after which the supernatant was discarded and the pellet was air-dried at room temperature. The dried pellet was then dissolved in 1x SDS loading buffer (0.1M DTT) and then heated at 95°C (~ 5 min) before running the protein sample through an
SDS-PAGE (15% separating gel, 4% stacking gel) to check for the presence of GST and the crustin 1 protein.

Buffer exchange

Buffer exchange was performed on two samples of eluates purified from the same cell culture using two different methods.

To substitute the elution buffer containing the desired recombinant proteins with 1x PBS buffer (140mM NaCl, 2.7mM KCl, 10mM Na₂HPO₄, 1.8mM KH₂PO₄, pH 7.3), the protein sample was dialysed using a Spectra/Por® Membrane (MWCO: 3,500, Spectrum Laboratories Inc.) in 1x PBS buffer overnight, according to a sample volume to buffer volume ratio of 1:2000. The final protein concentration of the sample was measured using a BioPhotometer.

Buffer exchange was also achieved using a Protein Desalting Spin Column (Pierce) according to the instructions provided by the supplier. The sample was subsequently concentrated using a Microcon YM-3 Centrifugal Filter Device (NMWL: 3,000, Millipore AB) according to the protocol supplied by the manufacturer, and spun at 4°C at the recommended g-force until the desired volume of concentrated recombinant crustin 1 protein sample was obtained. The final protein concentration was measured using the Bradford protein assay.

Tests for antimicrobial activity

The radial diffusion method was utilized to assess the antimicrobial activity of the GST-tagged crustin 1 recombinant protein. The bacterial cultures were adjusted to an OD₆₀₀ value of 0.1, and added to 10 ml of liquified trypsin soy agar (TSA) in the ratio of 1: 1000, which was then plated and allowed to solidify. Five wells were punched in each agar plate for the control (1x PBS buffer), the positive controls ampicillin (50mg/ml), kanamycin (50mg/ml) and streptomycin (50mg/ml), and the GST-tagged crustin 1 protein sample. 15 µl of each was added to the respective wells. The plates containing E.coli and S. aureus were incubated overnight at 37°C, while the plates containing all the crayfish-derived bacteria were cultured overnight at 30°C.

An alternative treatment of the radial-diffusion method according to Lehrer et al. (11) was also carried out. (However, standard circular laboratory Petri dishes were used for plating the agars, instead of the square Petri dish used by the paper’s authors). Five wells were punched in each agar plate for the negative control, the positive controls and the recombinant crustin 1 protein sample, and filled with 5 µl of each respectively. Only the bacteria E. coli, S. aureus, the species of Acinetobacter isolated from the crayfish hemolymph, A. hydrophila and P. anguilliseptica were used in this assay.

Test for proteinase-inhibiting activity

Proteinase-inhibiting activity was assayed using the enzyme elastase from human leukocytes (Sigma). The lyophilized powdered elastase was dissolved in double distilled water to a concentration of 0.2 µg/µl. The chromogenic peptide substrate used was MeOSuc-Ala-Ala-Pro-Val-pNA (Sigma).
Recombinant crustin 1 was added to 1 µl of elastase enzyme in 1:1, 1:5, 1:10, 1:20, 1:50 and 1:100 molar ratios, and each assay was incubated with 100 µl of 0.1 M Tris/HCl pH 8 for 10 min at 30°C, after which 50 µl of 2mM chromogenic peptide substrate was added. The reaction was allowed to proceed at 30°C until colour changes were observed in the assay. The control experiments carried out in parallel with the assays did not contain the recombinant crustin protein. 200 µl of 50% acetic acid should be used to terminate the enzymatic reactions, and absorbance readings should be carried out at 405 nm.

The assay was also repeated using 3 x and 10 x diluted solutions of elastase enzyme, but with the same amounts of recombinant proteins as used before.

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References

Appendix

DNA sequence of crustin 1

CGATCACCACCTTTCCGTCCACTCTCGTCTCCTAGGCCTAAAGTGGACATTCCAG
GCTGTGTCACACACTGTCAGGTCAAGGACAAACGCGGTCTCTTTCTACTGCTGCTCGA
GAGGAACTACTATGTGACAGGACAAACGCGGTCTCTTTCTACTGCTGCTGCTACG
CAAGGATGACAGGACACTGCGCCAGTGCTGAGAATGTGCTGCTACTCCTCGCAAA
CCACCAGCTCATCTGCGCAATCAGAGGAGATCGTAACTGATATCTGGA

Primer for crustin 1

Forward: ATAGGATCCCGATCAACACCTTTCCG
Reverse: ATACTCGAGTCTCCTGAGTT

DNA sequence of crustin 2

CACATCTCCCAGCCCGCCGCGGAGGGCTGCAACTACTACTGCAAGAAGGCTGAA
GGTCCTAAACAAAGGGCTTAACACTACTGCTGCGCAGCGGAGTATACATCCCCGCTGAAG
CGGGAAGAAAGCAGCTGGGTTATTACCGCCGCTCCTGAACTACGCAAGAAGGCTGAA
TTCCCAAGACACACTCAAGGTTGCGCCCAATGAAGTTGACCCCTGCAACACCTGAG
AGTTGTTGCTGACACCTGCCTGCAACTCACCTGCAGAGCTCATTCTCAAACTAG

Primer for crustin 2

Forward: ATAGGATCCACATCTCTCCCGCCCGCCG
Reverse: ATACTCGAGCTAGTTAATATAGAAGTG

DNA sequence of crustin 3

CAGAACACTAACACCAACCAACTGACTGATCGGAGGGCTTTTGCAAGGCCGGAGTGGA
CTTCTTCCAGGACCTGCAATCAGGAGGCCGAATTAGGATTCTCCAGGAGGTGTAATCC
TTCCGGAAGCTTCCAGGAGGTATACAGTGGTGAATCTCATCAATAGGTTT
AGACTGCAAGGGAGAAGCCTCAGTGCTCAGACTGGCAACTGTGAGATACATACCT
CCGTAACCCCGTCAACCGTGCTCTACTATTTACGCAACAGAGATAAAAACTCGCTAC
AAGTGTCTCTCCTGACCCGATTTGTCAGACACCCTGACTCCCGGCGCCGCTCG
AGTGCTATACTGACAACGACTGCGGACCCCTCGGATAATGTTGCTGCGAGCGCCTG
TCTCGACACACTACGTCTCGAACACCTGCGCGTAA

Primer for crustin 3

Forward: ATAGGATCCAGAACAACAACTACACC
Reverse: ATACTCGAGTTACGCGAGGGTGTTGCA