รายงานวิจัยฉบับสมบูรณ์

โครงการ การศึกษาโครงสร้างและหน้าที่ของ Crustin*Pm*1 และ Crustin*Pm*7 จากกุ้งกุลาดำ

Penaeus monodon

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พฤษภาคม 2554

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ศูนย์เชี่ยวชาญเฉพาะทางด้านอณูชีววิทยาและจีโนมกุ้ง ภาควิชาชีวเคมี คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

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สนับสนุนโดยสำนักงานคณะกรรมการอุดมศึกษา และสำนักงานกองทุนสนับสนุนการวิจัย

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บทคัดย่อ

(Thai) **การศึกษาโครงสร้างและหน้าที่ของ Crustin*Pm*1 และ Crustin*Pm*7 จากกุ้งกุลาดำ *Penaeus monodon***

ในงานวิจัยก่อนหน้านี้มีการค้นพบครัสติน (crustin) หลายไอโซฟอร์มจากกุ้งกุลาดำ *Penaeus monodon* ครัสตินเป็นเปปไทด์ต้านจุลชีพ ที่มีประจุสุทธิเป็นบวก และประกอบไปด้วยซีสเตอีน (cysteine) เป็นจำนวนมาก โดยซีสเตอีนดังกล่าวเป็นองค์ประกอบของ single whey acidic protein (WAP) โดเมน ที่อยู่บริเวณ C-terminus ของครัสติน ครัสตินนั้นมีฤทธิ์ต้านแบคทีเรียทั้งแกรมบวก และแกรมลบ ในงานวิจัยนี้ คณะผู้วิจัยได้ศึกษาสมบัติการจับและฤทธิ์การต้านจุลชีพของ crustin*Pm*1 และ crustin*Pm*7 ซึ่งเป็นไอโซฟอร์มที่พบมากในเม็ดเลือดของกุ้งกุลาดำ มีรายงานว่า crustin*Pm*1 มีฤทธิ์ยับยั้งแบคทีเรีย แกรมบวกเท่านั้น ในขณะที่ crustin*Pm*7 สามารถยับยั้งแบคทีเรียได้ทั้งแกรมบวก และแกรมลบ จากการศึกษาการจับของครัสตินกับเซลล์แบคทีเรีย พบว่า ครัสตินทั้งสองไอโซฟอร์มสามารถจับกับแบคทีเรียได้ ทั้งแกรมบวก และแกรมลบ ผลของ Enzyme-linked immunosorbent (ELISA) assay แสดงให้เห็นว่าครัสตินสามารถจับกับ lipoteichoic acid (LTA) และ lipopolysaccharide (LPS) ซึ่งเป็นองค์ประกอบของผนังเซลล์ แบบ Posittive cooperative โดยมีค่า Hill slope (H) มากกว่า 2 ซึ่งบ่งชี้ว่า ครัสติน 2 โมเลกุล เข้าจับกับ LTA หรือ LPS 1 โมเลกุล นอกจากนี้ ครัสตินทั้งสองไอโซฟอร์มยังสามารถเหนี่ยวนำให้เกิด bacterial agglutination และการเปลี่ยนแปลงสมบัติของเยื่อหุ้มเซลล์ชั้นใน (Inner membrane permeabilization) ของ *Escherichia coli* ได้อีกด้วย ภาพจาก Scanning Electron Microscopy (SEM) แสดงให้เห็นการเปลี่ยนแปลงบนผิวเซลล์ของ *Staphylococcus aureus, Vibrio harveyi* และ *E. coli* เมื่อบ่มแบคทีเรียดังกล่าวกับ crustin*Pm*7 ในขณะที่ crustin*Pm*1 สามารถทำให้เกิดการเปลี่ยนแปลงบนผิวเซลล์ของ *S. aureus* และ *E. coli* เท่านั้น ซึ่งผลดังกล่าว สอดคล้องกับฤทธิ์การต้านจุลชีพของ crustin*Pm*1 ซึ่งไม่มีฤทธิ์ต่อ *V. harveyi* จากการศึกษาทั้งหมด คาดว่า การทำให้เกิด bacterial agglutination และการเปลี่ยนแปลงบนผนังเซลล์แบคทีเรียเป็นกลไลที่สำคัญของครัสติน ในการยับยั้งแบคทีเรีย จากการหาสภาวะที่เหมาะสมในการตกผลึกครัสติน พบว่าสามารถตกผลึก crustin*Pm*1 ใน 0.2 M Lithium citrate tribasic tetrahydrate, 20% PEG 3350, pH 8.4 ได้ อย่างไรก็ตาม ยังไม่พบสภาวะที่เหมาะสมในการตกผลึก crustin*Pm*7

คำหลัก: Antimicrobial peptides, Bacterial agglutination, Crystallization, Crustin, Inner membrane permeabilization, lipopolysaccharide (LPS), lipoteichoic acid (LTA), *Penaeus monodon*

Abstract

(English) **Structural and Functional Study of Crustin*Pm1* and Crustin*Pm7* from the Black Tiger Shrimp *Penaeus monodon***

Several isoforms of crustin have been identified in the black tiger shrimp *Penaeus monodon*. These cationic cysteine-rich antimicrobial peptides contain a single whey acidic protein (WAP) domain at the C-terminus and exhibit antimicrobial activity against both Gram-positive and Gram-negative bacteria. In this work, we investigate the binding properties and antimicrobial actions of crustin*Pm*1 and crustin*Pm*7, the two most abundant crustin isoforms found in the haemocyte of *P. monodon*. Previously, crustin*Pm*1 showed strong inhibition against Gram-positive bacteria, whilst crustin*Pm*7 acted against both Gram-positive and Gram-negative bacteria. A binding study showed that both crustins can bind to Gram-positive and Gram-negative bacterial cells. Enzyme-linked immunosorbent (ELISA) assay suggested that crustins bind to the cell wall components, lipoteichoic acid (LTA) and lipopolysaccharide (LPS) with positive cooperativity of Hill slope (H) > 2. This indicates that at least two molecules of crustins interact with one LTA or LPS molecule. In addition, both crustins can induce bacterial agglutination and cause inner membrane permeabilization in *Escherichia coli.* Scanning Electron Microscopy (SEM) revealed the remarkable change on the cell surface of *Staphylococcus aureus, Vibrio harveyi* and *E. coli* after the bacteria were treated with the recombinant crustin*Pm*7. Meanwhile, crustin*Pm*1 can cause a visible change on the cell surface of *S. aureus* and *E. coli* only. This is in agreement with the fact that crustin*Pm*1 has shown no antimicrobial activity against *V. harveyi*. It is likely that the antimicrobial activity of crustins mainly relies on their ability to agglutinate bacterial cells and to disrupt the physiochemical properties of bacterial surface. Crystallization experiments were also carried out. Recombinant crustin*Pm*1 was crystallized in 0.2 M Lithium citrate tribasic tetrahydrate, 20% PEG 3350, pH 8.4. However, no crytallization condition for rcrustin*Pm*7 was found.

Keywords: Antimicrobial peptides, Bacterial agglutination, Crystallization, Crustin, Inner membrane permeabilization, lipopolysaccharide (LPS), lipoteichoic acid (LTA), *Penaeus monodon*

Executive Summary

(English) **Structural and Functional Study of Crustin*Pm1* and Crustin*Pm7* from the Black Tiger Shrimp *Penaeus monodon***

(Thai) **การศึกษาโครงสร้างและหน้าที่ของ Crustin*Pm1* และ Crustin*Pm7* จากกุ้งกุลาดำ *Penaeus monodon***

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1. Merit of the Proposed Research

The black tiger shrimp *Penaeus monodon* has long been valuable asset of Thai economic. However, in the past years, the black tiger shrimp farming has been down significantly due to concerns on production cost and diseases. To revive the black tiger shrimp industry, new technology to help increase the *Penaeus monodon*'s survival rate and lower its production cost is unavoidably required.

Like most invertebrates, shrimps lack a true adaptive immune system and rely on innate immune responses, including the production of anitimibrobial proteins. Various antimicrobial peptides have been identified in shrimps such as penaeidins, anti-lipopolysaccharide factors (ALFs), lysozymes and crustins (Tassanakajon et al). Antimicrobial activity of crustins from *Penaeus monodon* has previously been examined; however, binding of such molecules to bacteria and cell components has not yet been studied in details. In this work, we proposed to investigate the biological roles of crustins based on binding and immune-challenge experiments and crystallography technique. Crustin*Pm1* exhibits antibacterial activity against only Gram-positive bacteria (Supungul et al, 2008). On the contrary, crustin*Pm7* inhibits both Gram-positive and Gram-negative bacteria including *Vibrio harveyi*, a major pathogenic in shrimp aquaculture (Amparyup et al, 2008). In view of this, it is possible that these two isoforms of crustins may be subject to different mechanisms. This work has revealed the mechanism of action of crustins and crystallization condition of crustin*Pm1*. Clearly, knowledge of shrimp immunity is essential for establishing the strategies for disease controls in shrimp aquaculture. In Thailand, shrimp export is one of the most important commodities, in term of value. In this aspect, this work would be a real asset in shrimp industry and contribute towards the Thai economy no less.

2. Objectives

2.1 To express and purify the recombinant crustin*Pm*1 (rcrustin*Pm*1) and crustin*Pm*7 (rcrustin*Pm*7)

2.2 To examine the binding ability of crustin*Pm1* and crustin*Pm7* to bacteria and cell components

2.3 To investigate bacterial agglutination induced by rcrustin*Pm*1 and rcrustin*Pm*7

2.4 To examine Inner membrane permealization of *Escherichia coli* strain MG1655

2.5 To examine physical changes on cell surface of bacteria treated with rcrustin*Pm*1 and rcrustin*Pm*7

2.6 To determine the influence of cell components stimulation on crustin*Pm*1 and crustin*Pm*7 transcription

2.7 To crystallize rcrustin*Pm*1 and rcrustin*Pm*7 for structure determination

3. Research Methodology

3.1 Expression and purification of the recombinant crustion*Pm1* and crustin*Pm7*

Crustin*Pm1* and Crustin*Pm7* were purified as described in Supungul et al, 2008 and Amparyup et al, 2008 with some modifications. In brief, the *E.coli* BL21 containing the recombinant crustin plasmid was grown at 37 ^{o}C in LB medium plus ampicillin until they reach an OD_{600} of 0.6. Expression of crustin*Pm*1 and crustin*Pm*7 was then induced by addition of IPTG and cells were harvested by centrifugation after 4-5 hrs induction. Since rcrustin*Pm*1 and rcrustin*Pm*7 were expressed in the inclusion bodies, it was necessary to solubilized the inclusion bodies in a denaturing solution prior to purification. rCrustins were then purified using a Ni-NTA agarose column; and the eluted protein was dialyzed against a refolding solution. Gel filtration was carried out for protein samples subjected to crystallization. The concentration of purified protein was determined by bicinchoninic acid (BCA) protein

assay (Pierce). Western blotting was also performed to identify the His-tagged rcrustins using a primary anti-His antibody (GE-Healthcare).

To ensure that the purified rcrustin*Pm*1 and rcrustin*Pm*7 are active, the antimicrobial activity of purified rcrustin*Pm*1 and rcrustin*Pm*7 was determined by liquid growth inhibition assay (Destoumieux et al, 1999) using the Gram-positive bacteria *Staphylococcus aureus* and Gram-negative bacteria *Escherichia coli* 363. The mid-logarithmic growth phase cultures of bacteria were diluted with poor broth (1% bactotryptone, 0.5% NaCl, pH 7.5) to an OD_{600} of 0.001. These diluted bacteria cultures (100 μl) were then mixed with either the two-fold serially diluted recombinant protein samples or 50 mM MES buffer pH 5.8 (negative control) in a 96-well plate. After vigorous shaking overnight, bacterial growth was measured at OD_{600}. The minimum inhibitory concentration (MIC) value was shown as the range between the highest concentration of the protein where bacterial growth was observed and the lowest concentration that caused 100% of inhibition bacterial growth.

3.2 Binding properties of crustin*Pm1* and crustin*Pm7*

Bacterial binding assay was carried out in order to clarify whether rcrustin*Pm*1 and rcrustin*Pm*7 bind to bacterial cells in different manners. The binding properties of rcrustin*Pm*1 and rcrustin*Pm*7 to Gram-positive bacterial *Staphylococcus aureus, Stapphylococcus haemolyticus, Micrococcus luteus and Bacillus Megterium* and Gram-negative bacterial *Eirwenia carotovara, Enterobacter cloacae, Escherichia coli* 363*, Escherichia coli* MG 1655 and *Vibrio harveyi* 1526 were investigated. Experiments were set up as described by Lee & Soderhall, 2001 with some modification (Lee & Soderhall, 2001). Briefly, bacteria cells were resuspended in MES buffer pH 5.8 and then incubated with 5 μg of purified rcrustin*Pm*1 and rcrustin*Pm*7 at 4 °C for 35 minutes. Bacterial cells incubated with MES buffer pH 5.8 were used as negative control. In addition, binding experiments of *B. megaterium* and *E. coli 363* to purified recombinant ALF*Pm3* (Somboonwiwat et al, 2008) and to purified recombinant type-I Lyzosyme were carried out as positive and negative control, respectively (Supungul et al, 2010). After incubation, cells were pelleted and the supernatant was removed. Bacteria pellets were then resuspended and washed three times with resuspension buffer (MES buffer pH 5.8). Bound protein was finally eluted with 1×SDS-PAGE sample loading buffer. Supernatant (unbound protein), washed

fractions (unbound/weakly bound protein), eluted fractions (bound protein) and cell pellets (strongly bound protein) were analysed by SDS-PAGE and Western blot. Western blot analysis was carried out using anti-Histidine tag monoclonal antibody (GE-healthcare).

Quantitative binding assay of purified crustin*Pm*s to cell wall components lipopolysaccharide (LPS) from *E. coli* serotype 0111:B4 (Sigma) and lipoteichoic acid (LTA) from *S. aureus* (Sigma) were performed by an enzyme-linked immunosorbent assay (ELISA) using anti-histidine tag monoclonal antibody. Microtiter plates were coated with 100 µl (30 µg/ml) of LPS or LTA. Excess LPS or LTA were washed out with PBS-T (PBS containing 0.1% (v/v) Tween™-20). The plate was blocked with 5% (w/v) BSA in PBS-T prior to incubation with various amounts of purified rcrustin*Pm*1 and rcrustin*Pm*7 overnight at room temperature. The plate was washed with PBS-T twice and 100 µl anti-histidine tag monoclonal antibody in PBS-T 1:5,000 (v/v) was added. A hundred microlitre of alkaline phosphatase-conjugated secondary antibody against IgG in PBS-T 1:10,000 (v/v) was applied to the plate, followed by washing step and addition of 50 µl *p*-nitrophenylphosphate. The reaction was terminated by addition of 0.4 M NaOH. The plate was incubated for 15 min and then analyzed in microtiter plate reader (FLUOstar OPTIMA Microreader, SMG Labtechnology) at 405 nm. The apparent dissociation constants (K_d) and the maximum binding (A_{max}) parameters were calculated with GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, Calofornia, USA, using nonlinearly fitting as one site – Specific biding with Hill slope (equation 1).

$$Y = (B_{max} .[X]^h)/((K_d)^h + [X]^h) \quad \text{(Eq.1)}$$

3.3 Bacterial agglutination assay

Bacterial agglutination was studied in 96-wells round bottom microtiterplates and visualized by microscope. Nine bacterial strains including four Gram-positive bacteria, *S. aureus, S. haemolyticus, M. luteus and B. Megterium* and five Gram-negative bacteria *E. carotovara, E. cloacae, E. coli*363*, E. coli* MG 1655 and *V. harveyi*1526, were used to investigate the agglutination properties of rcrustin*Pm*1 and rcrustin*Pm*7. Bacteria cultures (1.5 ml) of optimal density (OD) at 600 nm $>$ 1 were pelleted and resuspended in agglutination buffer (50 mM MES buffer pH 5.8 containing 100 mM NaCl and 10 mM $CaCl_2$) three times. Fifty microlitre aliquot of resuspended bacteria were then transferred into 96-wells

round bottom plates. The rcrustin*Pm*1 and rcrustin*Pm*7 proteins were diluted into agglutination mixture, in order to obtain the final concentration of protein 200 µg/ml in each well. Self-or auto agglutination was observed in the well with no proteins. The mixture was incubated for 16 h at 22°C without shaking. After agglutination or sedimentation, 5 µl of samples were taken from the bottom of the well and transferred to a microscope slide. Bacterial samples were then Gram- stained and examined by light microscope.

3.4 Inner membrane permeabilization

Lactose permease deficient *E. coli* strain MG1655 (lacY: Tn10dKan) was used to test crustin*Pm*s' ability to induce inner membrane permeabilization. *E. coli* MG1655 was grown in LB medium containing 0.1 mM IPTG. When OD_{600} of bacteria cultures reached 0.4-0.6, they were mixed with either rcrustin*Pm*1 or rcrustin*Pm*7 (100 µM). A hundred and fifty microliters aliquots of bacteria culture were taken out at 0, 1, 3, 5, 8 and 16 h. The samples at each time point were centrifuged at 8000×g and the supernatants were stored at -20°C.

Supernatant fractions were subjected to β-galactosidase activity assay. The samples were incubated with 6 µM O-nitrophenyl-β-D-galactopyranoside (ONPG) at 30 °C for 5 min. The reaction was stopped by adding 100 µl of 2 M sodium carbonate into the reation mixture. The absorbance at 405 nm was measured by microtiter plate reader (BMG Labtech). For control and negative control reactions, MES buffer, pH 5.8 and bovine serum albumin (BSA) were added into bacteria culture, respectively.

3.5 Scanning Electron Microscopy (SEM)

S. aureus, *E. coli* MG1655 and *V. harveyi* were used in this experiment. Bacteria were grown overnight in LB media or TSB media in case of *V. harveyi*. The bacterial culture were inoculated into fresh medium and incubated at 37°C for *S. aureus* and *E. coli* MG1655, 30°C for *V. harveyi* with shaking until they reached exponential phase. Five mililitre of bacteria culture were collected by centrifugation, washed twice and re-suspended in MES buffer, pH 5.8. Bacterial samples were then incubated with 10-fold over the MIC value of rcrustin*Pm*1 or rcrustin*Pm*7 for 1 h and then pelleted by centrifugation. The specimens were fixed in 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer for 1-2 h and washed three times with 0.1 M phosphate buffer prior to dehydration through a graded series of ethanols. After critical point drying, samples were mounted on 1 cm stubs and were gold-coated using an

Ion sputter coater (Balzers, model SCD 040). Images were taken from each specimen using JEOL scanning electron microscopy, model JSM-5410LV.

3.6 Expression profile of crustin*Pm1* and crustin*Pm7* in hemocytes after LPS and LTA stimulation

Study of influence of bacterial cell wall components (LPS and LTA) on crustin transcription in haemocytes was carried out. LPS and LTA (0.5 μg per g of shrimp) were injected into three-month-old *P. monodon* shrimps. Saline-injected shrimps were regard as control groups. Three shrimps from each group (LPS, LTA, saline) were collected at 0, 6, 12, 24, 48 hr after injection. Hemolymph was collected for total RNA isolation and the level of crustin*Pm1* and crustin*Pm7* transcription was determined by RT-PCR using specific primers.

3.7 Crystallization of rcrustin*Pm*1 and rcrustin*Pm*7

Along with biochemical data, attempts to determine crystal structure of crustin were carried out. Crystallization screening of rcrustin*Pm*1 and rcrustin*Pm*7 was performed in 96-well plates using crystallization screen kits from Hampton research (Crystal Screen 1&2™, Index™, PEG/Ion 1&2™, SaltRX™) and Emerald Biosystems (Wizard III & IV). Crystallisation trays were incubated at either 20°C or 4°C and were observed at day 1, 3, 5, 7 and every week up to 2 months. The promising crystallization conditions were then optimized in 24-well plates. Quality of crystals was determined by X-ray diffraction.

4. Results

4.1 Expression and purification of the recombinant crustion*Pm1* and crustin*Pm7*

The recombinant crustin*Pm*1 and crustin*Pm*7 were expressed in inclusion bodies and purified under denaturing condition using a Ni-NTA affinity column chromatography. The protein was eluted stepwise with the denaturing solution containing 150 mM imidazole. SDS–PAGE analysis eluted fractions showed a major protein with an approximate size of 14.7 kDa and 12.8 kDa of *r*crustin*Pm*1 and *r*crustin*Pm*7, respectively (Figure 1A). Both recombinant crustin proteins were identified by Western immunoblotting using anti-His antibody (Figure 1B). Two minor bands were observed on SDS-PAGE even though proteins were treated with β-mercaptomethanol and heated. It is likely that they are dimer

and trimer forms of crustin since the protein contain 12 cysteine residues and the reducing condition in SDS–PAGE may not reduce all disulphide linkages.

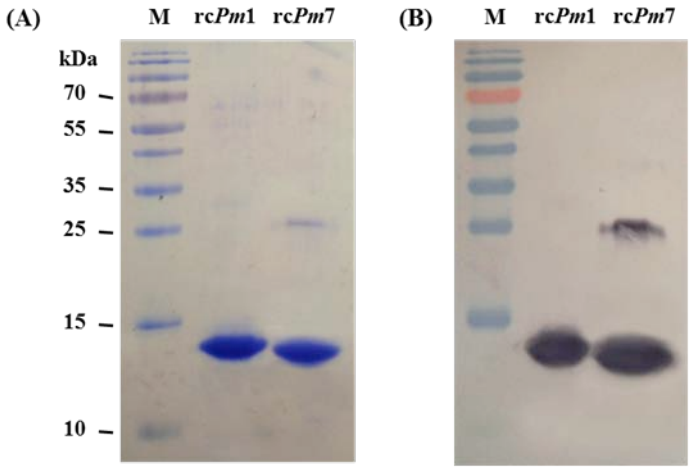

Figure 1 15% SDS–PAGE (A) and Western blotting (B) analysis of the purified *r*crustin*Pm*1 (rc*Pm*1) and *r*crustin*Pm*7 (rc*Pm*7).

To confirm antimicrobial activity of purified rcrustin*Pm*1 and rcrustin*Pm*7, Gram-positive bacteria *Staphylococcus aureus* and Gram-negative bacteria *Escherichia coli* 363 were used for minimal inhibitory concentration (MIC) determination. Both crustins exhibited antimicrobial activity against *S. aureus* with MIC value of 2.5-10 μM for rcrustin*Pm*1 and 2-8 μM for rcrustin*Pm*7. On the contrary, only *r*crustin*Pm*7 exhibited significant antimicrobial activity against *E. coli* 363 (2-5 μM). These results correspond to the antimicrobial activity of rcrustin*Pm*1 and rcrustin*Pm*7 reported previously (Amparyup et al, 2008; Supungul et al, 2008).

4.2 Binding properties of crustin*Pm1* and crustin*Pm7*

First, control experiment was carried out by incubating of *S. aureus* with rcrustin*Pm*7 (sample), ALF*Pm*3 (positive control), type-I Lysozyme (negative control) or MES pH 5.8 buffer. The Silver-stained SDS-PAGE showed that rcrustin*Pm*7 was mostly found in pellet fraction (P) in similar manner to ALF*Pm*3 (Figure 2). Type-I Lyzosyme, meanwhile, was found in supernatant fraction. This indicated that rcrustin*Pm*7 bound to *S. aureus* cells.

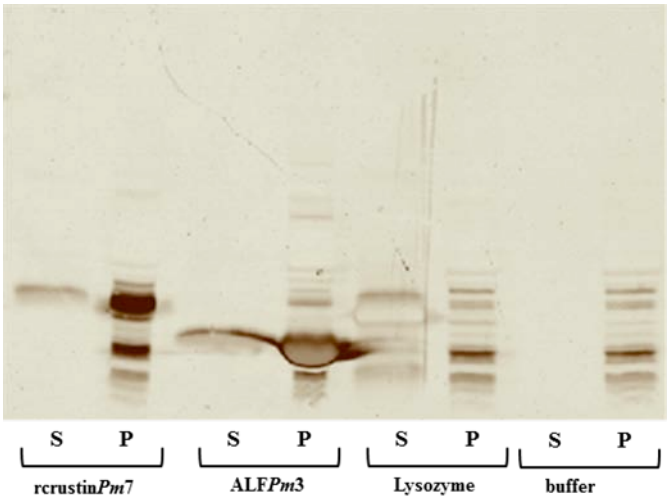

Figure 2 Silver-stained SDS-PAGE of *S. aureus* mixed with rcrustin*Pm*7, ALF*Pm*3, type-I Lysozyme or MES buffer pH 5.8. (Lane S: Supernatant fractions; Lane P: Pellet fractions)

Bacterial binding properties of rcrustin*Pm*1 and rcrustin*Pm*7 were then investigated by incubating Gram-positive (*Stapylococcus aureus*, *Staphylococcus haemolyticus*, *Bacillus megaterium*, *Micrococcus luteus*) and Gram-negative bacteria (*Enterobacter cloacae*, *Escherichia coli* 363*, Escherichia coli* MG1655, *Vibrio harveyi* 1526) with rcrustin*Pm*1 and rcrustin*Pm*7, pelleted and washed three times prior to elution. As shown in Figure 3, rcrustin*Pm*1 and rcrustin*Pm*7 were mainly found in elution fractions, whilst small amounts were present in pellet fractions. This indicates that rcrustin*Pm*1 and rcrustin*Pm*7 bind to both Gram-positive and Gram-negative bacteria.

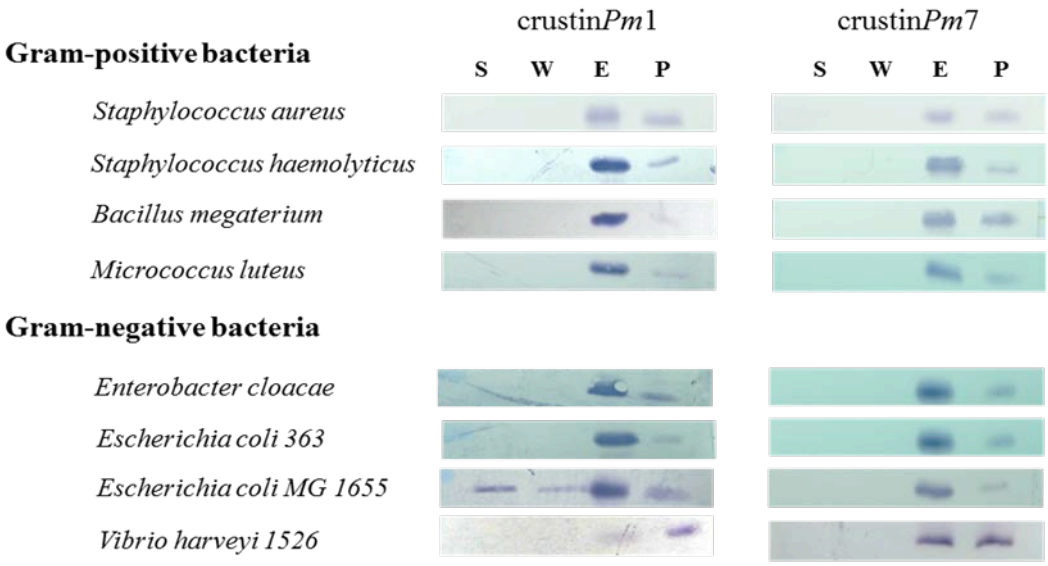

Figure 3 Binding of *r*crustin*Pm*1 and *r*crustin*Pm*7 to bacterial cells. (Lane S: Supernatant fraction; Lane W: Washed fraction; Lane E: Elution fraction; Lane P: Pellet fraction.

Enzyme-linked immunosorbent assay (ELISA) was carried out to quantitatively measure the binding, if any, of *r*crustin*Pm*1 and *r*crustin*Pm*7 to immobilized LPS and LTA. The data revealed that rcrustin*Pm*1 and rcrustin*Pm*7 binds to both LPS and LTA in a concentration-dependent and saturated manner. Nonlinear regression analysis with a One site : Specific binding with Hill slope model showed that the *r*crustin*Pm*s bound to LPS and LTA with an apparent dissociation constant (K_d) of crustin*Pm*1 as 6.204×10^{-6} (LPS) and 5.710×10^{-7} (LTA) M with Hill slope (H) = 2.513 (LPS) and 2.033 (LTA) (Figure 4A) and of crustin*Pm*7 as 2.205×10^{-7} (LPS) and 2.072×10^{-7} (LTA) M with Hill slope (H) = 2.147 (LPS) and 2.059 (LTA) (Figure 4B). (crutin*Pm*1, R^2 = 0.9816 and 0.9829 for LPS and LTA; crustin*Pm*7 R^2 = 0.9849 and 0.9888 for LPS and LTA)

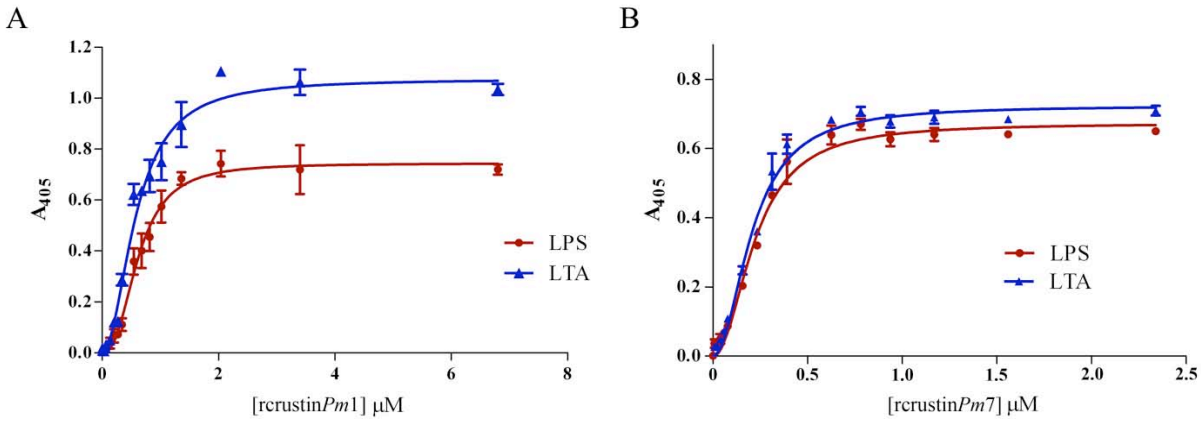

Figure 4 Quantitative binding of *r*crustin*Pm*1 (A) and *r*crustin*Pm*7 (B) to immobilized LPS and LTA. The microtiter plates were coated with 3 μg of LPS (red) or LTA (blue) and incubated with increasing amounts of *r*custin*Pm*s.

4.3 Bacterial agglutination assay

Bacterial binding properties of *r*crustin*Pm*1 and *r*crustin*Pm*7 were tested in soluble phase by monitoring bacterial agglutination properties. Agglutination was analyzed visually in 96-wells round bottom plate. When agglutination occurred, a large solid aggregates were visible microscopically (Figure 5).

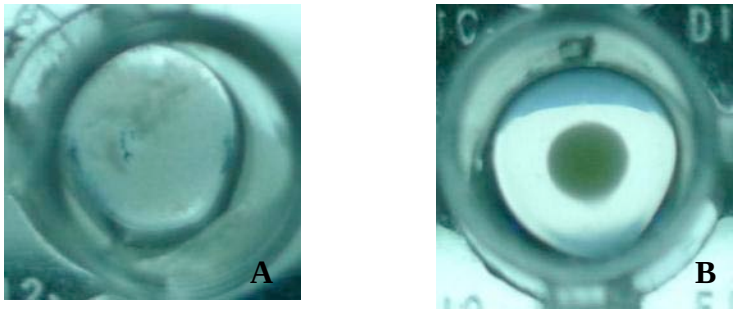

Figure 5 Pattern of bacterial agglutination in 96-wells round bottom plate. When agglutination was occurred, bacterial cell was aggregated and glazed around the round bottom plate (A), when there was no bacterial agglutination, bacterial cells were precipitated in the middle of the well (B).

Several Gram-positive (*Staphylococus aureus, Staphylococcus haemolyticus, Microciccus luteus and Bacillus Megterium*) and Gram-negative bacteria **(***Eirwinia carotovara, Enterobacter cloacae, Escherichia coli*363*, Escherichia coli* MG 1655 and *Vibrio harveyi*1526) were incubated with rcrustin*Pm*1 and rcrustin*Pm*7. Among these, agglutination of Gram-positive bacteria, *S. aureus, M. luteus* and *B. Megterium*, and Gram-negative bacteria, *E. coli*363 and *E. coli* MG 1655 were observed post treatment with rcurstin*Pm*1 (Figure 6). Similar results were found with rcrustin*Pm*7 treatment, except in the case of *V. harveyi* where rcrustin*Pm*7 could induce bacterial agglutination but rcrustin*Pm*1 could not (Figure 6). It should be noted that no bacterial agglutination was observed after incubating rcrustin*Pm*1 and rcrustin*Pm*7 with *E. cloacae* or *E. carotovara*.

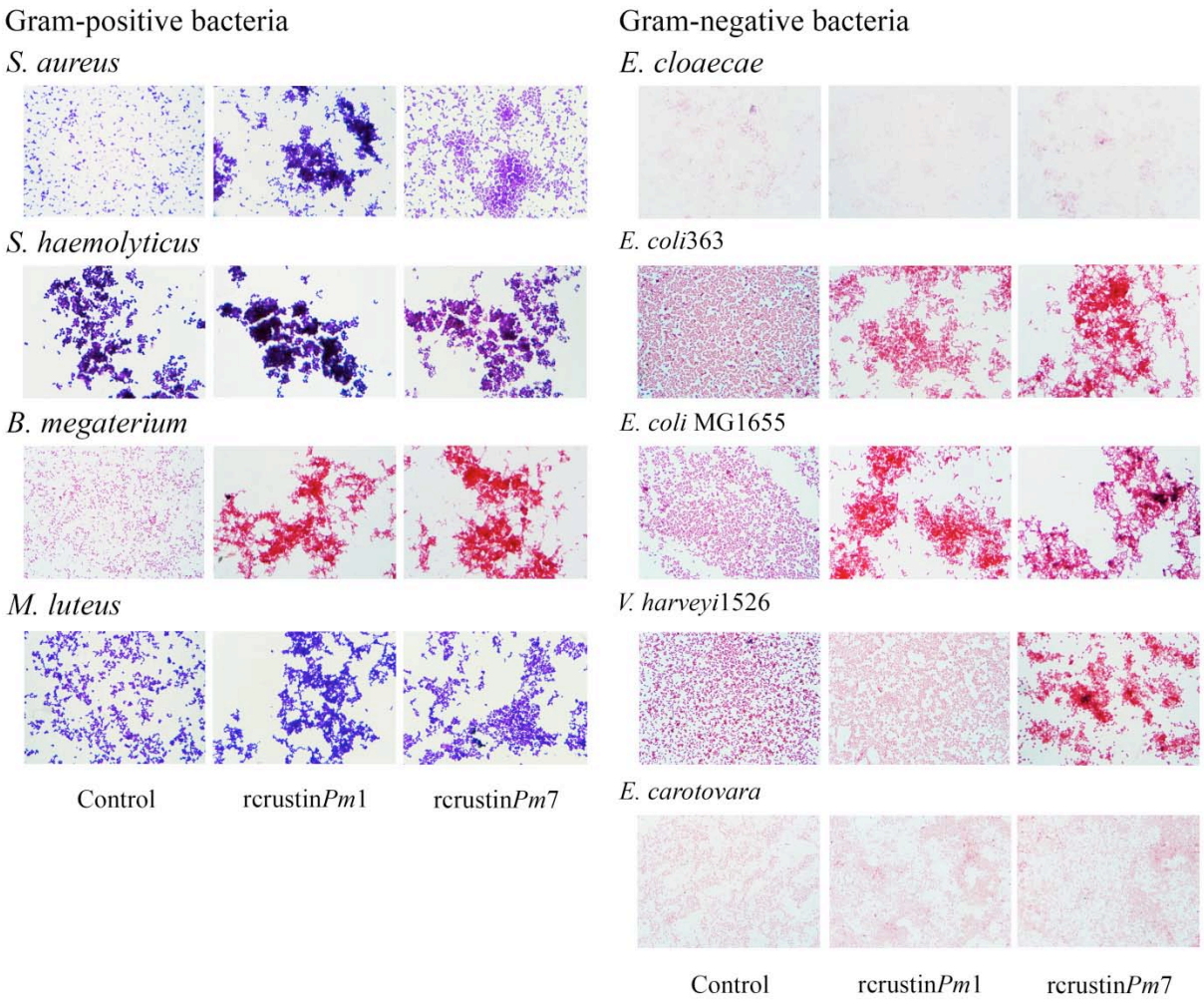

Figure 6 Bacterial agglutination by rcrustin*Pm*1 and rcrustin*Pm*7. The bacteria were mixed and incubated with 200 µg/ml of rcrustin*Pm*1 and rcrustin*Pm*7 as described in Materials and methods. Bacterial agglutination was observed in Gram-stained under light microscope.

4.4 Inner membrane permeabilization

Inner membrane permeabilization of *E. coli* strain MG1655 lacY: Tn10dKan was determined by measuring its β-galactosidase activity after incubation with rcrustin*Pm*1 or rcrustin*Pm*7. β-galactosidase is located in the cytosol and hence it will be detected when the bacteria cell membrance is permeabilized. β-galactosidase activity was assayed by mixing supernatant bacteria culture at each incubation time points with O-nitrophenyl-β-D-galactopyranoside (ONPG). When the β-galactosidase cleaves ONPG, O-nitrophenol is released. This compound is yellow color and absorb light at 405 nm.

Figure 7 shows a plot of detected β-galactosidase activity against incubation time of rcrustin*Pm*1 and rcrustin*Pm*7 with *E. coli* strain MG1655. It is clear that rcrustin*Pm*7 can induce inner membrane permeabilization of *E. coli* strain MG1655 as β-galactosidase activity significantly increase over time. In contrast, small change in β-galactosidase activity was observed when incubating rcrustin*Pm*1 with *E. coli* strain MG1655. This indicated that rcrustin*Pm*7 has higher ability to induce inner membrane permeabilization of *E. coli* MG1655 than rcrustin*Pm*1.

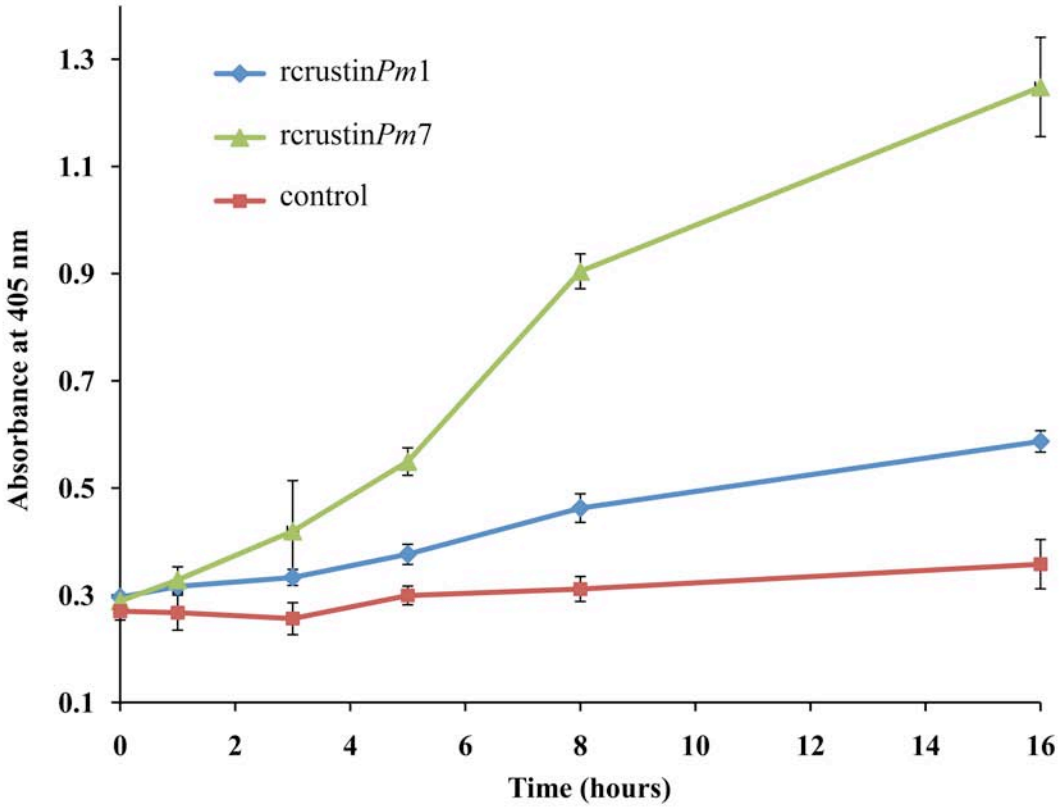

Figure 7 A plot of β-galactosidase activity of *E. coli* MG1655 over incubation time. Recombinant crustin*Pm*1 (blue) and crustin*Pm*7 (green) induced inner membrane permeabilization of *E. coli* strain MG1655, in comparison to control (red).

4.5 Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) was used to observe the cells of Gram-positive bacteria *S. aureus*, Gram-negative bacteria *E. coli* MG1655 and *V. harveyi* after exposure to rcrustin*Pm*1 or rcrustin*Pm*7. Compared with the control, *S. aureus* cells treated with rcrustin*Pm*1 and rcrustin*Pm*7

appeared to be rougher, distorted and covered with cell debris (Figure 8). They were also clumped together, showing bacterial agglutination. Similar changes were found on *E. coli* MG1655 cells incubated with rcrustin*Pm*1 and rcrustin*Pm*7. Both rcrustin*Pm*s can induce bleb formation on *E. coli* MG1655 cells; and in the case of rcrustin*Pm*7 treatment, it is likely that rcrustin*Pm*7 causes cell fusion which may lead to agglutination of *E. coli* MG1655 (Figure 8). Unlike *S. aureus* and *E. coli* MG1655 cells, *V. harveyi* cells treated with rcrustin*Pm*1 showed no change in cell appearance. However, exposure of rcrustin*Pm*7 caused heavy damage and pore formation on the cell surface of *V. harveyi* (Figure 8).

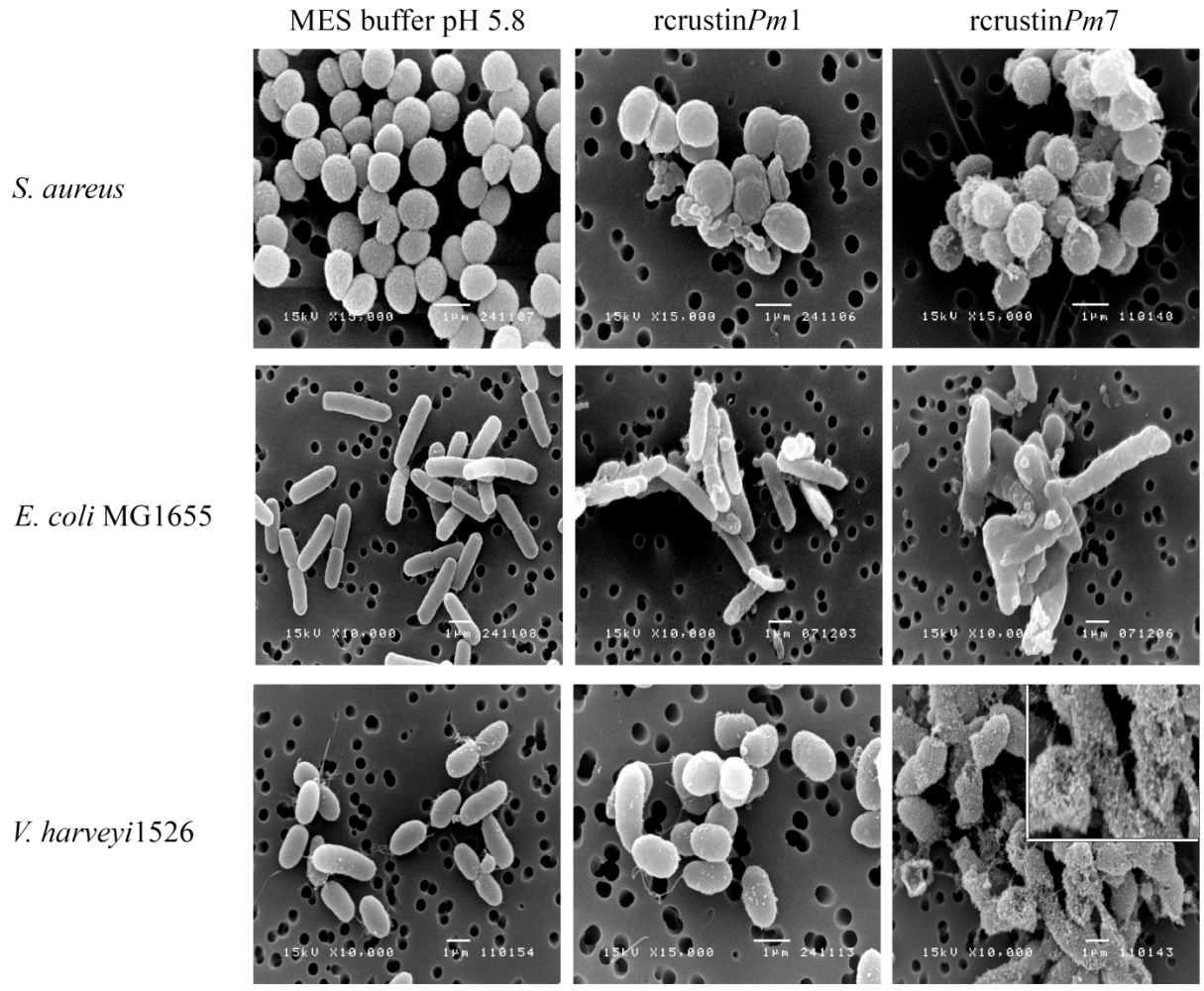

Figure 8 SEM image of the bacterial cells after treated with rcrustin*Pm*1 and rcrustin*Pm*7. *S. aureus*, *E. coli* and *V. harveyi* were incubated with the amount of rcrustin*Pm*1 and rcrustin*Pm*7 at 10-fold higher than their MIC values for 1 h. The control samples were incubated with 50 mM MES buffer pH 5.8. The inset figure of *V. harveyi* 1526 shows pores on the bacterial cells.

4.6 Expression profile of crustin*Pm1* and crustin*Pm7* in hemocytes after LPS and LTA stimulation

LPS, LTA (0.5 μg per g of shrimp) and saline (control) were injected into three-month-old *P. monodon* shrimps. Three shrimps from each group (LPS, LTA, saline) were collected at 0, 6, 12, 24, 48 hr after injection. Hemolymph was collected for total RNA isolation and the level of crustin*Pm1* and crustin*Pm7* transcription was determined by RT-PCR. Figure 9A showed that LTA stimulates transcription of *crustinPm1* at 6 hr. The levels of *crustinPm1* transcription at 12, 24 and 48 hr after LTA injection were similar to that of saline-injected shrimps. Meanwhile, no significant change in *crustinPm1* expression was observed with LPS-injected shrimps, in comparison with control group. In contrast, expression of *crustinPm7* was up-regulated at 6 hr after LTA injection and remained 1.5 fold higher than those in control group at 12, 24 and 48 hr post-injection (Figure 9B). Similar to crustin*Pm*1 results, LPS seems to have no effect on *crustinPm7* expression.

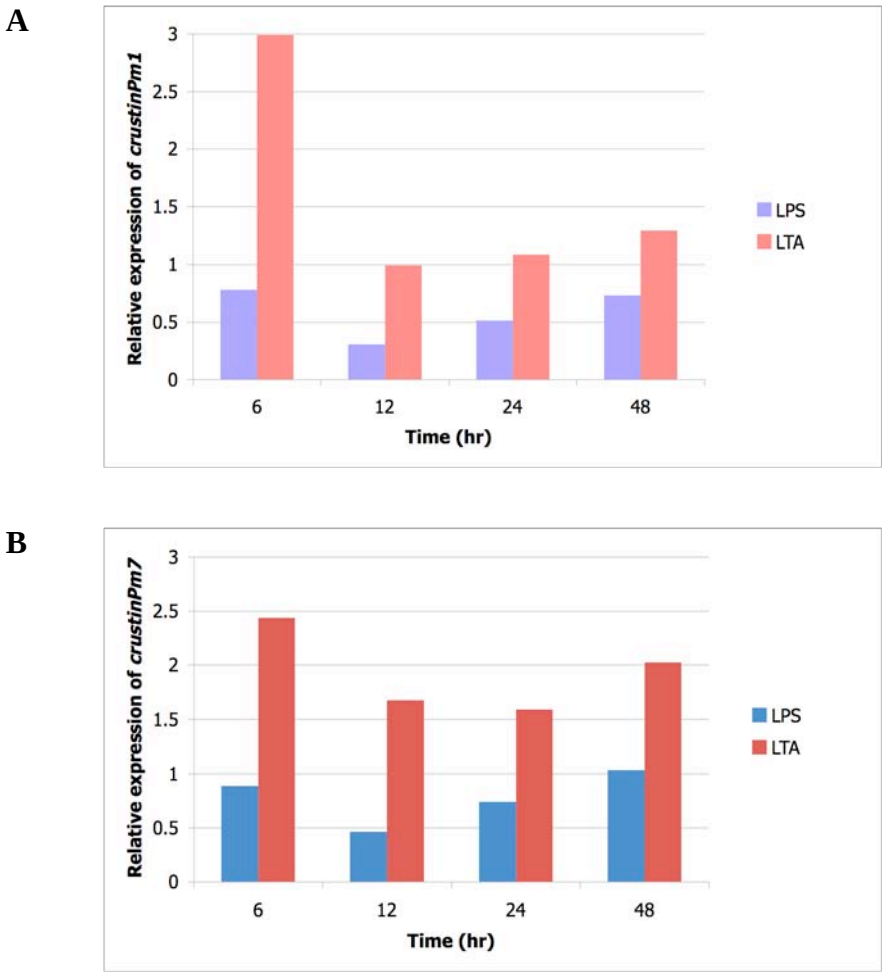

Figure 9 Expression of crustin*Pm*1 and crustin*Pm*7 mRNA after LPS and LTA injection.

4.7 Crystallization of rcrustin*Pm*1 and rcrustin*Pm*7

Crystallization of rcrustin*Pm*1 and rcrustin*Pm*7 were carried out in 96-well plates using crystallization screening kits from Hampton research (Crystal Screen 1&2™, Index™, PEG/Ion 1&2™, SaltRX™) and Emerald Biosystems (Wizard III & IV). Crystals of rcrustin*Pm*1 appears in 0.2 M Lithium citrate tribasic tetrahydrate, 20% PEG 3350, pH 8.4 (Figure 10). This crystallization condition was then optimized using additive screen and scale up in 24-well plates. rCrustin*Pm*1 crystal was tested on in-house X-ray source but show no diffraction. So far, no crystallization condition for rcrustin*Pm*7 has been found.

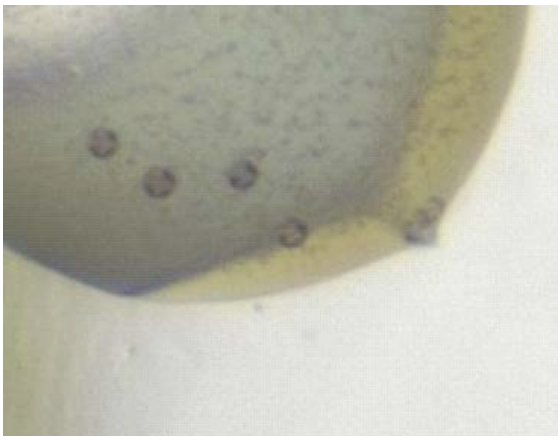

Figure 10 Crystals of rcrustin*Pm*1. Crystals appeared in 0.2 M Lithium citrate tribasic tetrahydrate, 20% PEG 3350, pH 8.4

5. Discussion

In previous studies, rcrustin*Pm*1 showed antimicrobial activity against Gram-positive bacteria (Supungul et al, 2008) while rcrustin*Pm*7 could inhibit both Gram-positive and Gram-negative bacteria (Amparyup et al, 2008). In this work, we investigated modes of action of rcrustin*Pm*1 and rcrustin*Pm*7 that may correlate with their antimicrobial activity, expression profile of crustin*Pm1* and crustin*Pm7* in hemocytes after LPS and LTA stimulation and crystallization of rcrustin*Pm*1 and rcrustin*Pm*7.

It is possible that rcrustin*Pm*1 lacks antimicrobial activity against Gram-negative bacteria because it may not be able to bind to Gram-negative bacteria. To test this hypothesis, rcrustin*Pm*1 and rcrustin*Pm*7 were expressed in inclusion bodies of *E. coli* strain Rosetta (DE3) and purified under denaturing conditions. The recombinant proteins were refolded and tested for their antimicrobial activity. Both rcrustin*Pm*1 and rcrustin*Pm*7 exhibited antimicrobial activity against *S. aureus* and *E. coli* 363, with MIC values closed to those published in Supungul et al., 2008 and Amparyup et al., 2008. As predicted, rcrustin*Pm*7, which has antimicrobial activity against both Gram-positive and Gram-negative bacteria, can bind to both types of bacteria. However, rcrustin*Pm*1, which lacks significant antimicrobial activity against Gram-negative bacteria, can also bind to both Gram-positive and Gram-negative bacteria (Figure 3). Therefore it appears that the antimicrobial activity of crustins does not correlate with their binding ability to bacterial cells.

Crustins carry a net positive charge and may adopt a β-sheet globular structure stabilized by intramolecular disulfide bonds (Smith et al, 2008). Crustins may interact with bacterial cell membranes through electrostatic attraction, and subsequently integrate into lipid bilayer, causing membrane pore formation or extensive membrane rupture (Brogden, 2005). Thus, binding of crustins to anionic LPS and LTA may be crucial for their antimicrobial activity. Quantitative binding assays showed that both rcrustin*Pm*1 and rcrustin*Pm*7 bound LTA and LPS, cell wall components of Gram-positive and Gram-negative bacteria respectively, in a sigmoidal manner (Figure 4). Nonlinear regression analysis with a One site : Specific binding with Hill slope model suggested that rcrustin*Pm*1 bind more tightly to LTA than to LPS (K_d = 6.204×10^{-6} M for LPS; K_d = 5.710×10^{-7} M for LTA). This is in agreement with the fact that rcrustin*Pm*1 exhibits high antimicrobial activity against Gram-positive bacteria but has no significant activity against Gram-negative bacteria (Amparyup et al, 2008; Supungul et al, 2008). In contrast, rcrustin*Pm*7 bind LPS and LTA with similar affinities (K_d = 2.205×10^{-7} M for LPS; K_d = 2.072×10^{-7} M for LTA). In general, antimicrobial peptides must be attracted to bacterial surfaces before performing antimicrobial actions. It is possible that crustins may have to bind LPS or LTA tightly with a K_d in 10^{-7} M range in order to provide such electrostatic attraction. In a comparison with rALF*Pm3*, crustin binds to LPS and LTA with 10-fold lower affinity (rALF*Pm3*; K_d = 1.26 x 10^{-8} M for LPS and K_d = 1.34 x 10^{-8} M

for LTA) (Somboonwiwat et al, 2008). Not surprisingly, rALP*Pm3* has stronger broad antimicrobial activity than crustins (Somboonwiwat et al, 2005).

According to the Hill slope model, both rcrustin*Pm*1 and rcrustin*Pm*7 exhibit positive cooperativity of H > 2 (Figure 4), indicating that rcrustin*Pms* form complexes with LPS or LTA with a 2:1 stoichiometry. Crustin binds to LPS in a similar manner as Factor C from the horseshoe crab (Tan et al, 2000). A high positive cooperativity and multiple LPS binding sites make Factor C, one of the clotting factors in endotoxin activated coagulation cascade, highly sensitive to LPS. The positive cooperativity in LPS and LTA may somehow play an important role in antimicrobial activity of crustin.

Adsorption of cationic agents onto the bacterial cell membrane neutralizes and reverses the surface charge of bacteria (Dyar & Ordal, 1946). This may result in bacterial agglutination (Avi-Dor & Yaniv, 1953). Both rcrustin*Pm*1 and rcrustin*Pm*7 can induce bacterial agglutination in some bacteria (Figure 6). It is likely that the antimicrobial activity of crustins is correlated with their ability to induce bacterial agglutination (Table 1). For example, rcrustin*Pm*1 can induce bacterial agglutination and exhibits antimicrobial activity against Gram-positive bacteria, *S. aureus*, *M. luteus* and *B. megaterium*; however, it neither inhibits growth nor induces agglutination against Gram-negative bacteria *V. harveyi.* In contrast, rcrustin*Pm*7 can induce bacterial agglutination and showed antimicrobial activity against *V. harveyi*. Since crustins are mainly found in haemocytes of shrimps, it is possible that bacterial agglutination occurs to prevent bacteria from spreading in the blood system.

SEM images also confirm bacterial agglutination induced by rcrustin*Pm*1 and rcrustin*Pm*7. *E.coli* MG1655 treated with rcrustin*Pm*7 were fused together, forming a large clump of cells (Figure 6). Similar phenomena were also observed in the cells of *S. aureus* when treated with rcrustin*Pm*1 or rcrustin*Pm*7, demonstrating clearly that crustin*Pm*s mediate bacterial agglutination.

Table 1 Summary of bacterial agglutination and antimicrobial activity of rcrustin*Pm*1 and rcrustin*Pm*7.

Bacteria strain	Bacterial agglutination [1]		MIC value (uM) [2]	
	Crustin *Pm*1	Crustin *Pm*7	Crustin *Pm*1	Crustin *Pm*7
Gram-positive bacteria	+	+	3.13-6.25	5-10
Staphylococcus aureus	S/A	S/A	50-100	2.5-5
Staphylococcus haemolyticus	+	+	6.25-12.50	1.25-2.5
Bacillus megaterium	+	+	25-50	2.5-5
Micrococcus luteus				
Gram-negative bacteria				
Enterobacter cloacae	-	-	NA	NA
Escherichia coli 363	+	+	50-100	2.5-5
Escherichia coli MG1655	+	+	50-100	2-5
Vibrio harveyi 1526	-	+	NA	2.5-5
Eirwinia carotovara	-	-	NA	ND

[1] S/A, Self/Auto agglutination; -, No agglutination; +, Agglutination.

[2] MIC values are from Supungul et al., 2008 and Amparyup et al., 2008

NA, Not active at 80 mM; ND, No data

Agglutination involves lattice formation. Attachment of crustin onto bacterial cells may sensitized them to form the lattice that constitutes agglutination. To form a lattice, crustin molecules need to crosslink bacterial cells. A quantitative binding study showed that at least two molecules of crustins interact with one molecule of LTA or LPS. This positive cooperative binding fits well with a lattice formation mechanism where, presumably, crustin molecules presumably bridge adjacent LTA or LPS molecules, causing bacterial agglutination. Based on positive cooperative binding and the correlation between the antimicrobial activity and bacterial agglutination properties of crustin, we propose that bacterial agglutination is probably a major mode of action for the antimicrobial activity of crustin.

It is evident that both rcrustin*Pm*s can disrupt the outer membranes of bacterial cells (Figure 7). Surface blebbing is clearly shown in *S. aureus* cells treated with rcrustin*Pm*1 and rcrustin*Pm*7. While rcrustin*Pm*7 causes severe damage and pore formation on cell surface of *V. harveyi*. Consistent with its antimicrobial activity against *V. harveyi*, rcrustin*Pm*1 causes no change on the surface of *V. harveyi* cells.

After outer membrane disruption, crustin may enter bacterial cells and approach the inner membrane. Inner membrane permeabilization can cause loss of metabolites and eventually lysis of the bacterial cell. Clearly, both rcrustin*Pm*1 and rcrustin*Pm*7 can permeabilize the inner membrane of *E. coli* MG1655 (Figure 8). A higher degree of inner membrane permeabilization was observed in *E.coli* MG1655 treated with rcrustin*Pm*7, when compared to rcrustin*Pm1* treatment. This is consistent with the fact that rcrustin*Pm*7 has high antimicrobial activity against *E. coli* MG1655, but rcrustin*Pm1* showed very low antimicrobial activity against *E. coli.*

Expression profile of crustin*Pm*1 and crustin*Pm*7 in haemocytes showed that both crustin*Pm*1 and crustin*Pm*7 can be stimulated by LTA (Figure 9). *CrustinPm1* gene was up-regulated at 6 hr after LTA injection and dropped to normal level (compared to control group) at 12, 24 and 48 hr post-injection. Expression of crustin*Pm*7 was also highest at 6 hr after LTA injection and stayed above normal level (1.5 fold) at 12, 24 and 48 hr post-injection. However, no significant change in crustin*Pm*1 and crustin*Pm*7 expression was observed after LPS injection. However, it was reported that expression level of crustin*Pm*7 increased more than 5-fold at 24 post-challenge with *V. harveyi* and returned to normal level within 72 hr (Amparyup et al, 2008). It is possible that other components of Gram-negative bacteria, not LPS, trigger crustin*Pm*7 expression.

Crustin*Pm*1 was crystallized in 0.2 M Lithium citrate tribasic tetrahydrate, 20% PEG 3350, pH 8.4 (Figure 10). However, they showed no diffraction at in-house X-ray source. It is possible that crustin*Pm*1 crystals were too small and further crystal optimization is needed. No crystallization condition of rcrustin*Pm*7 was found. This may due to non-homogeneity of rcrustin*Pm*7 sample. We need to find new expression system and purification procedure for rcrustin*Pm*7.

6. Conclusion

rCrustin*Pm*1 and rcrustin*Pm*7 can both bind to Gram-positive and Gram-negative bacteria, even though rcrustin*Pm*1 showed no significant antimicrobial activity against Gram-negative bacteria. Both crustins also bind the cell wall components, LPS and LTA with a 2:1 stoichiometry in a positive cooperative manner. In addition, both proteins can cause bacterial agglutination, outer membrane disruption and inner membrane permeabilization. It is likely that the antimicrobial activity of crustins mainly relies on their ability to agglutinate bacteria cells and to disrupt the physiochemical properties of bacterial surface. Expression profile of crustin*Pm1* and crustin*Pm7* in hemocytes after LPS and LTA stimulation suggested that both *crustinPm1* and *crustinPm7* genes respond to LTA, not LPS. Crustin*Pm*1 crystals appeared in 0.2 M Lithium citrate tribasic tetrahydrate, 20% PEG 3350, pH 8.4 but they showed no diffraction using in-house X-ray source. So far, crystallization conditions for rcrustin*Pm*7 have not been found.

7. Output

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

   Krusong, K., Pasapong, P., Supungul, P., Tassanakajon, A. A comparative study of antimicrobial properties of crustinPm1 and crustinPm7 from the black tiger shrimp *Penaeus mondon*. (submitted)

2. การนำผลงานวิจัยไปใช้ประโยชน์
   - เชิงสาธารณะ (มีเครือข่ายความร่วมมือ)
   - เชิงวิชาการ (สร้างนักวิจัยใหม่)
3. อื่นๆ (เช่น หนังสือ การจดสิทธิบัตร)
   - เสนอผลงาน (โปสเตอร์) และผลงานตีพิมพ์ใน Proceeding

   Binding properties of crustin*Pm*1 and crustin*Pm*7 from the black tiger shrimp *Penaeus monodon.* The 22nd Annual meeting of the Thai Society for Biotechnology. “International conference on biotechnology for healthy living”. Prince of Songkla University, Trang Campus, Thailand. October 20-22rd. p.1291-1297.

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A Comparative Study of Antimicrobial Properties of Crustin*Pm*1 and Crustin*Pm*7 from the Black Tiger Shrimp *Penaeus monodon*

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Abbreviations LPS, lipopolysaccharide; LTA, lipoteichoic acid; *Pm*, *Penaeus monodon*

Abstract

Several isoforms of crustin have been identified in the black tiger shrimp *Penaeus monodon*. These cationic cysteine-rich antimicrobial peptides contain a single whey acidic protein (WAP) domain at the C-terminus and exhibit antimicrobial activity against both Gram-positive and Gram-negative bacteria. In this paper, we investigate the binding properties and antimicrobial actions of crustin*Pm*1 and crustin*Pm*7, the two most abundant crustin isoforms found in the haemocyte of *P. monodon*. Previously, crustin*Pm*1 showed strong inhibition against Gram-positive bacteria, whilst crustin*Pm*7 acted against both Gram-positive and Gram-negative bacteria. A binding study showed that both crustins can bind to Gram-positive and Gram-negative bacterial cells. Enzyme-linked immunosorbent (ELISA) assay suggested that crustins bind to the cell wall components, lipoteichoic acid (LTA) and lipopolysaccharide (LPS) with positive cooperativity of Hill slope (H) > 2. This indicates that at least two molecules of crustins interact with one LTA or LPS molecule. In addition, both crustins can induce bacterial agglutination and cause inner membrane permeabilization in *Escherichia coli*. Scanning Electron Microscopy (SEM) revealed the remarkable change on the cell surface of *Staphylococcus aureus, Vibrio harveyi* and *E. coli* after the bacteria were treated with the recombinant crustin*Pm*7. Meanwhile, crustin*Pm*1 can cause a visible change on the cell surface of *S. aureus* and *E. coli* only. This is in agreement with the fact that crustin*Pm*1 has shown no antimicrobial activity against *V. harveyi*. It is likely that the antimicrobial activity of crustins mainly relies on their ability to agglutinate bacterial cells and to disrupt the physiochemical properties of bacterial surface.

Introduction

Crustins are crustacean cationic antimicrobial peptides with a single whey acidic protein (WAP) domain at the C-terminus (Smith et al., 2008). Crustins have been identified from various crustaceans (Hauton et al., 2006; Zhang et al., 2007), including several shrimp species e.g. *Penaeus monodon* (Amparyup et al., 2008b; Chen et al., 2005; Chen et al., 2004; Jimenez-Vega et al., 2004; Supungul et al., 2004; Supungul et al., 2008; Vatanavicharn et al., 2009), *Litopenaeus setiferus* (Bartlett et al., 2002), *Litopenaeus vannamei* (Bartlett et al., 2002; Jimenez-Vega et al., 2004; Vargas-Albores et al., 2004) and *Fenneropenaeus chinensis* (Jia et al., 2008; Zhang et al., 2007). Smith et al, 2008 classified crustins into three main types (Types I-III) based on their domain arrangement (Smith et al., 2008). All types of crustins possess a signal sequence at the N-terminus and the WAP domain at the C-terminus. In general, the WAP domain consists of 50 amino acid residues with eight cysteine residues at defined positions. These cysteine residues generate four intracellular disulfide bonds creating a tightly packed structure. The WAP domain has also been found in proteins with diverse functions, including protease inhibition (Sallenave, 2000) and antimicrobial activity (Shugars, 1999; Wiedow et al., 1998).

Type I crustins contain a cysteine-rich region between the signal sequence and the WAP domain. These types of crustins are mainly found in crabs, lobsters and crayfish (Brockton et al., 2007; Christie et al., 2007; Hauton et al., 2006; Jiravanichpaisal et al., 2007; Stoss et al., 2004). Type II crustins, meanwhile, have a signal sequence followed by a glycine-rich region, a cysteine-rich region and the WAP domain. Most crustins from shrimp belong to this group. Unlike Types I and II, Type III crustins have no cysteine-rich domain. Instead, they possess a proline-

arginine-rich domain between the signal sequence and the WAP domain. Type III crustins are generally called single-whey domain (SWD) proteins and are reported in *Penaeus monodon* (Amparyup et al., 2008a), *Litopenaeus vannamei* (Jimenez-Vega et al., 2004), *Marsupenaeus japonicus* (Rojtinnakorn et al., 2002) *and Fenneropenaeus chinensis* (Jia et al., 2008).

Several Type II crustins have been identified in the black tiger shrimp *P. monodon* (Amparyup et al., 2008b; Supungul et al., 2008; Vatanavicharn et al., 2009). They possess a signal peptide, a glycine-rich region and 12 conserved cysteine residues containing a single WAP domain at the C-terminus (Figure 1). Amino acid sequence analysis revealed high-sequence diversity within *P. monodon* Type II crustins. They vary greatly in length and have different amino acid sequences at the signal peptide and the inter-cysteine segments between a cysteine-rich region and a single WAP domain. The WAP domain itself, however, is highly conserved with the consensus pattern: $CX_6CX_{8-13}CX_2DX_2CX_{4-5}KCCX_3CX_5C$. Among these five isoforms, crustin*Pm*1 and crustin*Pm*7 are the two most abundant isoforms in the haemocytes. Crustin*Pm*1 exhibits antibacterial activity against Gram-positive bacteria (Supungul et al., 2008). Crustin*Pm*7, or so-called crustin-like*Pm*; however, has antimicrobial activity against both Gram-positive and Gram-negative bacteria (Amparyup et al., 2008b). These findings are particularly interesting, since these two crustins may possess different antimicrobial modes of action.

To date, the antimicrobial mechanism of action of crustin remains unknown. In this paper, we investigate the binding properties of crustin*Pm*1 and crustin*Pm*7 to bacterial cells and the cell wall components, lipoteichoic acid (LTA) and lipopolysaccharide (LPS). Bacterial agglutination and inner membrane

permeabilization by crustins were also studied. Scanning Electron Microscopy (SEM) was used to observe any physiological changes occurring on the surface of bacterial cells after incubation with crustins.

Materials and Methods

2.1 Expression and purification of recombinant crustinPm1 and crustinPm7

Expression and purification of rcrustin*Pm*1 and rcrustin*Pm*7 were carried out as described previously (Amparyup et al, 2008b; Supungul et al, 2008) with some modifications. In brief, *crustinPm1* and *crustinPm7* genes in pET-28b were expressed in *Escherichia coli* strain Rosetta (DE3). After isopropyl-β-D-thio-galactoside (IPTG) induction (1mM), the cells were harvested and resuspended in 1xPBS buffer, pH 7.4 (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4). The cell suspension was sonicated and inclusion bodies were collected by centrifugation at 5000 x g for 10 mins at 4 °C. The inclusion bodies were solubilized in a denaturing solution containing 50 mM Tris-HCl, pH 8.0, 8 M urea and 20 mM imidazole. rCrustin*Pm*1 and rcustin*Pm*7 were purified through a Ni-NTA agarose column under denaturing conditions and eluted stepwise with a denaturing solution containing 150 mM imidazole. Protein fractions were then analyzed by 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The purified protein was dialyzed against a dialysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 10% glycerol, 1 mM oxidized glutathione, 0.1 mM reduced glutathione and urea). Urea was gradually removed from protein solution by reducing the urea concentration in 2 M steps (6 M→4 M→2M). At the final step, the purified protein was dialysed in 50 mM MES pH 5.8 buffer and any remaining precipitant was removed by centrifugation. The concentrations of purified rcrustin*Pm*1 and rcustin*Pm*7 were

determined by bicinchoninic acid (BCA) protein assay (Pierce). Western blotting was also performed to identify the His-tagged rcrustins using a primary anti-His antibody (GE-Healthcare) (Supungul et al., 2008).

2.2 Antimicrobial activity assay

The antimicrobial activity of each purified proteins, rcrustin*Pm*1 and rcustin*Pm*7, was determined by a liquid growth inhibition assay (Destoumieux et al., 1999) using the Gram-positive bacteria *Staphylococcus aureus* and Gram-negative bacteria *Escherichia coli* 363. The mid-logarithmic growth phase cultures of bacteria were diluted with poor broth (1% bactotryptone, 0.5% NaCl, pH 7.5) to an OD_{600} of 0.001. These diluted bacteria cultures (100 µl) were then mixed with either the two-fold serially diluted recombinant protein samples or 50 mM MES buffer pH 5.8 (negative control) in a 96-well plate. After vigorous shaking overnight, bacterial growth was measured at OD_{600}. The minimum inhibitory concentration (MIC) value was shown as the range between the highest concentration of the protein where bacterial growth was observed and the lowest concentration that caused 100% of inhibition bacterial growth.

2.3 Bacterial cell binding assay

The binding properties of rcrustin*Pm*1 and rcrustin*Pm*7 to the Gram-positive bacteria *Staphylococcus aureus, Stapphylococcus haemolyticus, Micrococcus luteus and Bacillus Megaterium* and the Gram-negative bacteria *Eirwenia carotovara, Enterobacter cloacae, Escherichia coli* 363, *Escherichia coli* MG 1655 and *Vibrio harveyi* 1526 were investigated. Experiments were set up as described by Lee and Soderhall, 2001 with some modifications. In brief, 3 ml of logarithmic phase bacteria cultures were pelleted and resuspended in 50 mM MES buffer pH 5.8. The cell suspensions were incubated with 5 µg of purified rcrustin*Pm*1 or rcrustin*Pm*7, or 50

mM MES buffer, pH 5.8 (negative control), for 35 mins at 4 °C. In addition, the purified recombinant type-I lysozyme and the purified recombinant anti-lipopolysaccharide factor (rALF*Pm3*) were mixed with the cell suspensions and used as negative and positive controls, respectively. After incubation, the cells were pelleted, resuspended and washed three times with 50 mM MES buffer pH 5.8. Bound protein was finally eluted with 1×SDS-PAGE sample loading buffer. The supernatants (unbound protein), wash fractions (unbound/weakly bound protein), elution fractions (bound protein) and cell pellets (strongly bound protein) were analysed by SDS-PAGE (silver staining) and Western blot using a primary anti-His antibody.

2.4 LPS and LTA binding assay

Quantitative binding of purified rcrustin*Pm*1 and rcrustin*Pm*7 to cell wall components, LPS from *E. coli* serotype 0111:B4 (Sigma) and LTA from *S. aureus* (Sigma) were performed by an enzyme-linked immunosorbent assay (ELISA) using anti-His monoclonal antibody. First, a microtiter plate was coated with 100 µl of either LPS (30 µg/ml) or LTA (30 µg/ml). Excess LPS or LTA were washed out with PBS-T (PBS containing 0.1% (v/v) Tween™-20). The plate was blocked with 5% (w/v) BSA in PBS-T prior to incubation with various amounts of purified rcrustin*Pm*1 and rcrustin*Pm*7 and overnight incubation at room temperature. The plate was washed twice with PBS-T before adding 100 µl of anti-His monoclonal antibody (GE-healthcare) in PBS-T 1:5,000 (v/v) was added. One hundred microliter of alkaline phosphatase-conjugated secondary antibody against IgG in PBS-T 1:10,000 (v/v) was applied to the plate, followed by washing and the addition of 50 µl *p*-nitrophenylphosphate. The reaction was terminated by the addition of 0.4 M NaOH. The plate was incubated for 15 mins and then analysed in microtiter plate reader

(FLUOstar OPTIMA Microreader, SMG Labtechnology) at 405 nm. The recorded absorbance was plotted against the protein concentration and the data were fitted to the nonlinearly fitting as one site – Specific binding with Hill slope equation (Equation 1) using GraphPad Prism version 4.0 (GraphPad Software, San Diego, California, USA),

$$Y = (B_{max} .[X]^h)/((K_d)^h + [X]^h) \text{ (Eq.1)}$$

where Y is the recorded absorbance, B_{max} is the maximum specific binding (absorbance), [X] is the protein concentration, h is the Hill slope and K_d is the apparent dissociation constant.

2.5 Bacterial agglutination assay

Nine bacterial strains including four Gram-positive bacteria, *S. aureus, S. haemolyticus, M. luteus and B. Megterium* and five Gram-negative bacteria *E. carotovara, E. cloacae, E. coli*363, *E. coli* MG 1655 and *V. harveyi*1526, were used in a bacterial agglutination assay. Approximately 1.5 ml of bacteria cultures (OD_{600} > 1) were centrifuged at 8000×*g* for 10 mins. The pellets were resuspended and washed three times with agglutination buffer (50 mM MES buffer pH 5.8 containing 100 mM NaCl and 10 mM $CaCl_2$). Fifty microliters of bacterial suspension were mixed with the final concentration of 200 µg/ml rerustin*Pm*1 or rerustin*Pm*7 in a 96-well round bottomed plate. A negative control for self- or auto-agglutination was set up by mixing the resuspended bacteria with 50 mM MES buffer pH 5.8. The microtiter plate was then incubated for 16 hours at 22°C without shaking. Five microliters of sedimented bacteria were taken from the bottom of the well and transferred to a

microscope slide. Bacterial samples were Gram-stained prior to examination by light microscopy.

2.6 Inner membrane permeabilization assay

An inner membrane permeabilization assay was performed by measuring the β-galactosidase activity of *E. coli* strain MG1655 lacY:Tn10dKan (Steinberg & Lehrer, 1997) after its incubation with rcrustin*Pm*1 and rcrustin*Pm*7. Lactose permease deficient *E. coli* strain MG1655 was a kind gift from Dr. Javaregowda Nagaraju (Centre for DNA Fingerprinting and Diagnostics, Hyderabad, India). The logarithmic phase of *E. coli* MG1655 cultures, grown in LB broth containing 0.1 mM IPTG, were incubated with either 100 μM rcrustin*Pm*1 or rcrustin*Pm*7 at 37 °C. As a negative control, cell cultures were incubated with 50 mM MES buffer, pH 5.8. One hundred and fifty microliter aliquots of the bacterial cultures were taken after 0, 1, 3, 5, 8 and 16 hours, pelleted and supernatants stored at -20°C. β-galactosidase activity was assayed by incubating supernatants with 6 μM O-nitrophenyl-β-D-galactopyranoside (ONPG) for 5 mins at 30 °C. Reactions were stopped by adding 2 M sodium carbonate, and absorbance at 405 nm (A_{405}) was measured by microtiter plate reader (BMG Labtech).

2.7 Scanning Electron Microscope (SEM)

Five mililiters of the logarithmic phase of *S. aureus*, *E. coli* MG1655 and *V. harveyi* cultures were collected by centrifugation, washing twice and re-suspension in 50 mM MES buffer, pH 5.8. The bacterial samples were incubated with either rcrustin*Pm*1 or rcrustin*Pm*7 at a concentration 10-fold greater than their MIC value concentration for 1 hr. They were subsequently fixed in 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer for 2 hrs, washed three times with 0.1 M phosphate buffer and dehydrated through a graded ethanol series. After critical point drying, samples were

mounted on 1 cm stubs and gold-coated using an Ion sputter coater (Balzers, model SCD 040). The specimens were then observed with a JEOL scanning electron microscope (model JSM-5410LV).

Results

3.1 Protein expression, purification, refolding and antimicrobial activity test

The rcrustin*Pm*1 and rcrustin*Pm*7 were predominantly expressed in inclusion bodies in *E. coli* strain Rosetta (DE3) (data not shown). Both recombinant crustins were purified under denaturing condition using a Ni-NTA affinity chromatography and then refolded to their native state. Purified rcrustin*Pm*1 and rcrustin*Pm*7 were identified by Western blotting using an anti-His antibody (data not shown).

To confirm the presence of rcrustin*Pm*1 and rcrustin*Pm*7 in their active form, antimicrobial activity tests of purified rcrustin*Pm*s against Gram-positive bacteria *S. aureus* and Gram-negative bacteria *E. coli* 363 were carried out. Both purified rcrustin*Pm*s showed high antimicrobial activity against *S. aureus* with MIC value of 2.5-10 µM for *r*crustin*Pm*1 and 2-8 µM for *r*crustin*Pm*7. On the contrary, only *r*crustin*Pm*7 exhibited significant antimicrobial activity against *E. coli* 363 (2-5 µM). These results correspond to the antimicrobial activity of rcrustin*Pm*1 and rcrustin*Pm*7 reported previously (Supungul et al., 2008; Amparyup et al., 2008b)

3.2 Bacterial binding

Bacterial binding properties of rcrustin*Pm*1 and rcrustin*Pm*7 were investigated using several Gram-positive (*Stapylococcus aureus*, *Staphylococcus haemolyticus*, *Bacillus megaterium*, *Micrococcus luteus*) and Gram-negative bacteria (*Enterobacter cloacae*, *Escherichia coli* 363, *Escherichia coli* MG1655, *Vibrio harveyi* 1526). The bacterial cells were incubated with rcrustin*Pm*1 and rcrustin*Pm*7, pelleted and washed

three times prior to elution. As shown in Figure 2, rcrustin*Pm*1 and rcrustin*Pm*7 were mainly found in elution fractions, whilst small amounts were present in pellet fractions. rCrustin*Pm*1 and rcrustin*Pm*7 were not observed in supernatant or wash fractions, indicating that rcrustin*Pm*1 and rcrustin*Pm*7 bind to both Gram-positive and Gram-negative bacteria.

It is worth noting that a control experiment was also used to confirm binding of rcrustin*Pm*1 and rcrustin*Pm*7 to bacterial cells. It demonstrated that rcrustin*Pm*1 and rcrustin*Pm*7 do not precipitate in 50 mM MES buffer pH 5.8 (data not shown). In addition, both rcrustin*Pms* were found in bacterial cell pellets, which resemble that observed with ALF*Pm3* (used as positive control). Type-I lysozyme (used as negative control), meanwhile, was not present in a bacterial cell fraction, but was present in a soluble fraction (data not shown). These results suggest that the presence of rcrustin*Pm*1 and rcrustin*Pm*7 in bacterial cell pellets is due to the binding of both proteins to bacterial cells, rather than protein precipitation.

3.3 LTA and LPS binding

Since *r*crustin*Pm*1 and *r*crustin*Pm*7 can bind to both Gram-positive and Gram-negative bacteria, their binding properties to cell surface components, LPS and LTA, were analyzed further by ELISA. LPS and LTA are cell wall components found in Gram-negative and Gram-positive bacteria, respectively. Quantitative binding of *r*crustin*Pm*1 and *r*crustin*Pm*7 to LPS and LTA were obtained by titrating increasing concentrations of each protein into a fixed concentration of LPS or LTA. Binding parameters were determined by plotting the observed A_{405} against protein concentration and fitting the data to nonlinear regression as one site – Specific binding with Hill slope equation (Equation 1 in Materials and Methods).

Both rcrustin*Pm*1 and rcrustin*Pm*7 produced a sigmoidal binding curve with LPS and LTA. Nonlinear regression analysis with a One site : Specific binding with Hill slope model showed that rcrustin*Pm*1 bound to LPS and LTA with an apparent dissociation constant (K_d) of 6.204×10^{-6} M with Hill slope (H) of 2.513 for LPS and a K_d of 5.710×10^{-7} M with H of 2.033 for LTA (Figure 3A). This demonstrated that rcrustin*Pm*1 binds tightly to LPS and LTA with K_d in the μM range. It also showed that rcrustin*Pm*1 bound LPS 1 order of magnitude more weakly, compared with LTA. In addition, Hill slopes (H) of 2.513 (R^2=0.9816) for LPS and 2.033 (R^2=0.9829) for LTA suggest positive cooperativity between at least two rcrustin*Pm*1 molecules and a single molecule of LPS or LTA.

Similar results were also observed with rcrustin*Pm*7. The protein binds tightly to both LPS and LTA with similar K_ds of 2.205×10^{-7} M and 2.072×10^{-7} M for LPS and LTA, respectively (Figure 3B). Hill slopes for LPS and LTA were 2.147 (R^2=0.9849) and 2.059 (R^2=0.9888), respectively, indicating a cooperative interaction between at least two molecules of rcrustin*Pm*7 and one molecule of LTA or LPS.

3.4 Bacterial agglutination

To investigate whether rcrustin*Pm*1 and rcrustin*Pm*7 can cause bacterial agglutination, several Gram-positive (*Staphylococus aureus, Staphylococcus haemolyticus, Microciccus luteus and Bacillus Megterium*) and Gram-negative bacteria (*Eirwinia carotovara, Enterobacter cloacae, Escherichia coli*363, *Escherichia coli* MG 1655 and *Vibrio harveyi*1526) were incubated with rcrustin*Pm*1 and rcrustin*Pm*7. Among these, agglutination of Gram-positive bacteria, *S. aureus, M. luteus* and *B. Megterium*, and Gram-negative bacteria, *E. coli*363 and *E. coli* MG 1655 were observed post treatment with rcurstin*Pm*1 (Figure 4). Similar results were found with rcrustin*Pm*7 treatment, except in the case of *V. harveyi* where rcrustin*Pm*7

could induce bacterial agglutination but rcrustin*Pm*1 could not (Figure 4). It should be noted that no bacterial agglutination was observed after incubating rcrustin*Pm*1 and rcrustin*Pm*7 with *E. cloacae* or *E. carotovara.*

3.5 Inner membrane permeabilization

Inner membrane permeabilization of *E. coli* strain MG1655 lacY: Tn10dKan was determined by measuring its β-galactosidase activity after incubation with rcrustin*Pm*1 or rcrustin*Pm*7. Since β-galactosidase is located in the cytosol, it will be detected when membrane permeabilization occurs. A plot of β-galactosidase activity (A_{405}) against incubation time represents inner membrane permeabilization activity of rcrustin*Pm*1 and rcrustin*Pm*7.

As seen in Figure 5, β-galactosidase activities of *E. coli* MG1655 samples when treated with rcrustin*Pm*7 greatly increased over the incubation period. A major change in β-galactosidase activity was observed after 5 hours incubation. In contrast, β-galactosidase activities of samples treated with rcrustin*Pm1* gradually increased. The total change in A_{405} of rcrustin*Pm1* samples was much smaller than that of rcrustin*Pm7*. It is clear that rcrustin*Pm*7 has higher membrane-permeabilizing activity against *E. coli* MG1655 than rcrustin*Pm*1.

3.6 Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) was used to observe the cells of Gram-positive bacteria *S. aureus*, Gram-negative bacteria *E. coli* MG1655 and *V. harveyi* after exposure to rcrustin*Pm*1 or rcrustin*Pm*7. Compared with the control, *S. aureus* cells treated with rcrustin*Pm*1 and rcrustin*Pm*7 appeared to be rougher, distorted and covered with cell debris (Figure 6). They were also clumped together, showing bacterial agglutination. Similar changes were found on *E. coli* MG1655 cells

incubated with rcrustin*Pm*1 and rcrustin*Pm*7. Both rcrustin*Pm*s can induce bleb formation on *E. coli* MG1655 cells; and in the case of rcrustin*Pm*7 treatment, it is likely that rcrustin*Pm*7 causes cell fusion which may lead to agglutination of *E. coli* MG1655 (Figure 6). Unlike *S. aureus* and *E. coli* MG1655 cells, *V. harveyi* cells treated with rcrustin*Pm*1 showed no change in cell appearance. However, exposure of rcrustin*Pm*7 caused heavy damage and pore formation on the cell surface of *V. harveyi* (Figure 6).

Discussion

In previous studies, rcrustin*Pm*1 showed antimicrobial activity against Gram-positive bacteria (Supungul et al., 2008) while rcrustin*Pm*7 could inhibit both Gram-positive and Gram-negative bacteria (Amparyup et al., 2008b). Amino acid sequence alignment of crustin*Pm*s revealed a conserved primary structure of crustin*Pm*1 and crustin*Pm*7, a signal peptide, a glycine-rich region and 12 conserved cysteine residues containing a single WAP domain at the C-terminus (Figure 1). In this paper, we investigated modes of action of rcrustin*Pm*1 and rcrustin*Pm*7 that may correlate with their antimicrobial activity.

It is possible that rcrustin*Pm*1 lacks antimicrobial activity against Gram-negative bacteria because it may not be able to bind to Gram-negative bacteria. To test this hypothesis, rcrustin*Pm*1 and rcrustin*Pm*7 were expressed in inclusion bodies of *E. coli* strain Rosetta (DE3) and purified under denaturing conditions. The recombinant proteins were refolded and tested for their antimicrobial activity. Both rcrustin*Pm*1 and rcrustin*Pm*7 exhibited antimicrobial activity against *S. aureus* and *E. coli* 363, with MIC values closed to those published in Supungul et al., 2008 and Amparyup et al., 2008. As predicted, rcrustin*Pm*7, which has antimicrobial activity against both Gram-positive and Gram-negative bacteria, can bind to both types of

bacteria. However, rcrustin*Pm*1, which lacks significant antimicrobial activity against Gram-negative bacteria, can also bind to both Gram-positive and Gram-negative bacteria (Figure 2). Therefore it appears that the antimicrobial activity of crustins does not correlate with their binding ability to bacterial cells.

Crustins carry a net positive charge and may adopt a β-sheet globular structure stabilized by intramolecular disulfide bonds (Smith et al., 2008). Crustins may interact with bacterial cell membranes through electrostatic attraction, and subsequently integrate into lipid bilayer, causing membrane pore formation or extensive membrane rupture (Brogden, 2005). Thus, binding of crustins to anionic LPS and LTA may be crucial for their antimicrobial activity. Quantitative binding assays showed that both rcrustin*Pm*1 and rcrustin*Pm*7 bound LTA and LPS, cell wall components of Gram-positive and Gram-negative bacteria respectively, in a sigmoidal manner (Figure 3). Nonlinear regression analysis with a One site : Specific binding with Hill slope model suggested that rcrustin*Pm*1 bind more tightly to LTA than to LPS ($K_d = 6.204\times10^{-6}$ M for LPS; $K_d = 5.710\times10^{-7}$ M for LTA). This is in agreement with the fact that rcrustin*Pm*1 exhibits high antimicrobial activity against Gram-positive bacteria but has no significant activity against Gram-negative bacteria (Amparyup et al., 2008b; Supungul et al., 2008). In contrast, rcrustin*Pm*7 bind LPS and LTA with similar affinities ($K_d = 2.205\times10^{-7}$ M for LPS; $K_d = 2.072\times10^{-7}$ M for LTA). In general, antimicrobial peptides must be attracted to bacterial surfaces before performing antimicrobial actions. It is possible that crustins may have to bind LPS or LTA tightly with a K_d in 10^{-7} M range in order to provide such electrostatic attraction. In a comparison with rALF*Pm3*, crustin binds to LPS and LTA with 10-fold lower affinity (rALF*Pm3*; $K_d = 1.26 \times 10^{-8}$ M for LPS and $K_d = 1.34 \times 10^{-8}$ M for LTA)

(Somboonwiwat et al., 2008). Not surprisingly, rALP*Pm3* has stronger broad antimicrobial activity than crustins (Somboonwiwat et al., 2005).

According to the Hill slope model, both rcrustin*Pm*1 and rcrustin*Pm*7 exhibit positive cooperativity of H > 2 (Figure 3), indicating that rcrustin*Pms* form complexes with LPS or LTA with a 2:1 stoichiometry. Crustin binds to LPS in a similar manner as Factor C from the horseshoe crab (Tan et al., 2000). A high positive cooperativity and multiple LPS binding sites make Factor C, one of the clotting factors in endotoxin activated coagulation cascade, highly sensitive to LPS. The positive cooperativity in LPS and LTA may somehow play an important role in antimicrobial activity of crustin.

Adsorption of cationic agents onto the bacterial cell membrane neutralizes and reverses the surface charge of bacteria (Dyar & Ordal, 1946). This may result in bacterial agglutination (Avi-Dor & Yaniv, 1953). Both rcrustin*Pm*1 and rcrustin*Pm*7 can induce bacterial agglutination in some bacteria (Figure 4). It is likely that the antimicrobial activity of crustins is correlated with their ability to induce bacterial agglutination (Table 1). For example, rcrustin*Pm*1 can induce bacterial agglutination and exhibits antimicrobial activity against Gram-positive bacteria, *S. aureus*, *M. luteus* and *B. megaterium*; however, it neither inhibits growth nor induces agglutination against Gram-negative bacteria *V. harveyi*. In contrast, rcrustin*Pm*7 can induce bacterial agglutination and showed antimicrobial activity against *V. harveyi*. Since crustins are mainly found in haemocytes of shrimps, it is possible that bacterial agglutination occurs to prevent bacteria from spreading in the blood system.

SEM images also confirm bacterial agglutination induced by rcrustin*Pm*1 and rcrustin*Pm*7. *E.coli* MG1655 treated with rcrustin*Pm*7 were fused together, forming a large clump of cells (Figure 6). Similar phenomena were also observed in the cells of

S. aureus when treated with rcrustin*Pm*1 or rcrustin*Pm*7, demonstrating clearly that crustin*Pm*s mediate bacterial agglutination.

Agglutination involves lattice formation. Attachment of crustin onto bacterial cells may sensitized them to form the lattice that constitutes agglutination. To form a lattice, crustin molecules need to crosslink bacterial cells. A quantitative binding study showed that at least two molecules of crustins interact with one molecule of LTA or LPS. This positive cooperative binding fits well with a lattice formation mechanism where, presumably, crustin molecules presumably bridge adjacent LTA or LPS molecules, causing bacterial agglutination. Based on positive cooperative binding and the correlation between the antimicrobial activity and bacterial agglutination properties of crustin, we propose that bacterial agglutination is probably a major mode of action for the antimicrobial activity of crustin.

It is evident that both rcrustin*Pm*s can disrupt the outer membranes of bacterial cells (Figure 6). Surface blebbing is clearly shown in *S. aureus* cells treated with rcrustin*Pm*1 and rcrustin*Pm*7. While rcrustin*Pm*7 causes severe damage and pore formation on cell surface of *V. harveyi*. Consistent with its antimicrobial activity against *V. harveyi*, rcrustin*Pm*1 causes no change on the surface of *V. harveyi* cells.

After outer membrane disruption, crustin may enter bacterial cells and approach the inner membrane. Inner membrane permeabilization can cause loss of metabolites and eventually lysis of the bacterial cell. Clearly, both rcrustin*Pm*1 and rcrustin*Pm*7 can permeabilize the inner membrane of *E. coli* MG1655 (Figure 5). A higher degree of inner membrane permeabilization was observed in *E.coli* MG1655 treated with rcrustin*Pm*7, when compared to rcrustin*Pm1* treatment. This is consistent with the fact that rcrustin*Pm*7 has high antimicrobial activity against *E. coli* MG1655, but rcrustin*Pm1* showed very low antimicrobial activity against *E. coli*.

In conclusion, rcrustin*Pm*1 and rcrustin*Pm*7 can both bind to Gram-positive and Gram-negative bacteria, even though rcrustin*Pm*1 showed no significant antimicrobial activity against Gram-negative bacteria. In addition, both crustins bind the cell wall components, LPS and LTA with a 2:1 stoichiometry in a positive cooperative manner. Both proteins can also cause bacterial agglutination, outer membrane disruption and inner membrane permeabilization. We propose that the antimicrobial activity of crustins mainly relies on their ability to agglutinate bacteria cells and to disrupt the physiochemical properties of bacterial surface.

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Figure legends

Figure 1 Comparison of *Penaeus monodon* crustin amino acid sequences.

(*) indicates amino acid identify and (.) and (:) indicate amino acid similarity. The signal peptides, glycine rich regions, cysteine rich regions and WAP domains are indicated by gray boxes. The bolds letters correspond to the 12-conserved cysteines. Access number, crustin*Pm*1(FJ686014), crustin*Pm*4 (FJ686015), crustin*Pm*5 (FJ380049), crustin*Pm*6 (GH717910), crustin*Pm*7 (EF654658).

Figure 2 Binding of rcrustin*Pm*1 and rcrustin*Pm*7 to bacterial cells. *Staphylococcus aureus*, *Staphylococcus haemolyticus*, *Bacillus megaterium*, *Micrococcus luteus*, *Enterobacter cloacae*, *Escherichia coli* 363, *Escherichia coli* MG1655 and *Vibrio harveyi* 1526 were incubated with rcrustin*Pm*1 and rcrustin*Pm*7 as described in Materials and methods. Supernatant fractions (S), wash fractions (W), elution fractions (E) and pellet fractions (P) were analyzed by Western blot using anti-histidine tag monoclonal antibody.

Figure 3 Quantitative binding of rcrustin*Pm*1 (A) and rcrustin*Pm*7 (B) to immobilized LPS and LTA. The microtiter plates coated with 3 µg of LPS (●) or LTA (▲) were incubated with increasing amounts of either rcrustin*Pm*1 (A) or rcrustin*Pm*7 (B). Bound rcrustins were detected by anti-histidine tag monoclonal antibody. The data are shown with the best fit to a nonlinear fit as one site – specific binding with Hill slope. The data are given the best fit values as followed: A, data for rcrustin*Pm*1, K_d for LPS = 6.204×10^{-6} M with Hill slope (H) = 2.513, K_d for LTA =

5.710×10^{-7} M with H = 2.033; B, data for rcrustin*Pm*7, K_d for LPS = 2.205×10^{-7} M with H = 2.147, K_d for LTA = 2.072×10^{-7} M with H = 2.059.

Figure 4 Bacterial agglutination by rcrustin*Pm*1 and rcrustin*Pm*7. The bacteria were mixed and incubated with 200 µg/ml of rcrustin*Pm*1 and rcrustin*Pm*7 as described in Materials and methods. Bacterial agglutination was observed in Gram-stained under light microscope.

Figure 5 Kinetics of inner membrane permeabilization of *E. coli* strain MG1655 by rcrustin*Pm*1 and rcrustin*Pm*7. The inner membrane permeabilization was determined by measuring the β-galactosidase activity of *E. coli* sample incubated with 100 µM rcrustin*Pm*1 and rcrustin*Pm*7. The absorbance at 405 nm, which is proportional to the β-galactosidase activity, is plotted against the incubation time of bacterial samples with rcrustin*Pm*1 (◆) and rcrustin*Pm*7 (▲). The bacteria sample incubated with 50 mM MES pH 5.8 was used as control (■).

Figure 6 SEM image of the bacterial cells after treated with rcrustin*Pm*1 and rcrustin*Pm*7. *S. aureus*, *E. coli* and *V. harveyi* were incubated with the amount of rcrustin*Pm*1 and rcrustin*Pm*7 at 10-fold higher than their MIC values for 1 h. The control samples were incubated with 50 mM MES buffer pH 5.8. The inset figure of *V. harveyi* 1526 shows pores on the bacterial cells.

Figure

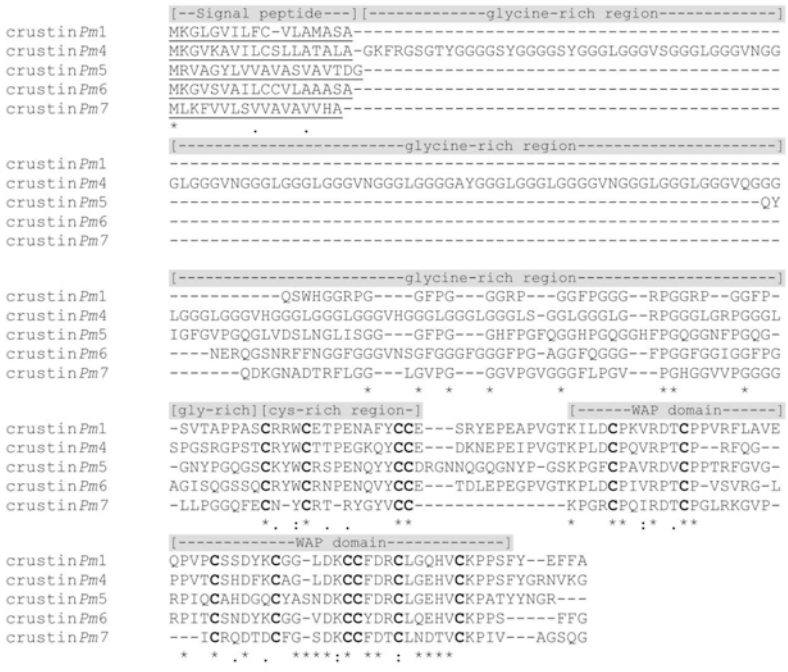

Figure 1

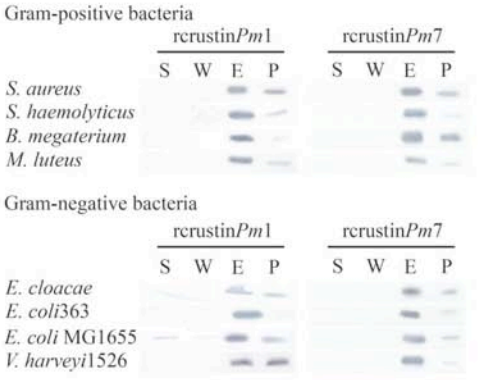
Gram-positive bacteria
rcrustinPm1
rcrustinPm7
S W E P
S W E P
S. aureus
S. haemolyticus
B. megaterium
M. luteus
Gram-negative bacteria
rcrustinPm1
rcrustinPm7
S W E P
S W E P
E. cloacae
E. coli363
E. coli MG1655
V. harveyi1526

Figure 2

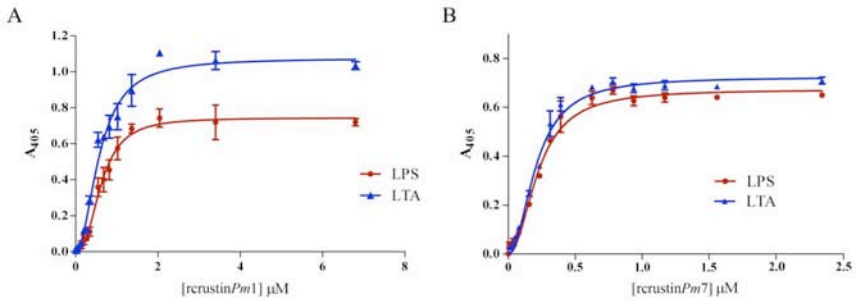

Figure 3

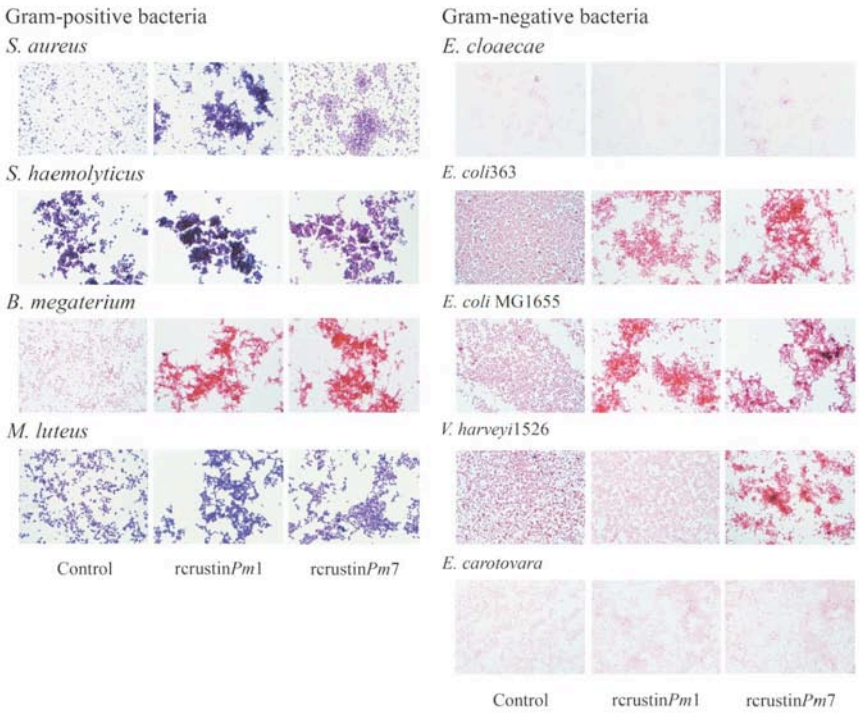
Gram-positive bacteria
S. aureus
S. haemolyticus
B. megaterium
M. luteus
Control
rcrustinPm1
rcrustinPm7
Gram-negative bacteria
E. cloaecae
E. coli363
E. coli MG1655
V. harveyi1526
E. carotovara
Control
rcrustinPm1
rcrustinPm7

Figure 4

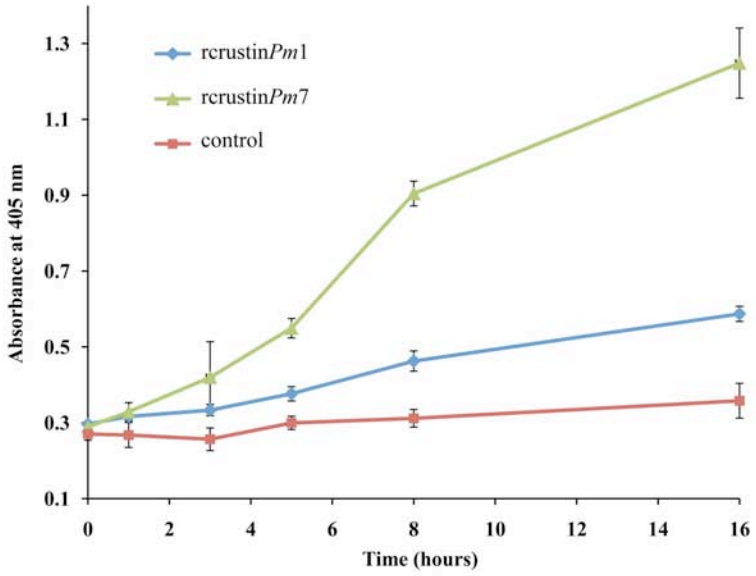
rcrustinPm1
rcrustinPm7
control
Absorbance at 405 nm
Time (hours)
1.3
1.1
0.9
0.7
0.5
0.3
0.1
0
2
4
6
8
10
12
14
16

Figure 5

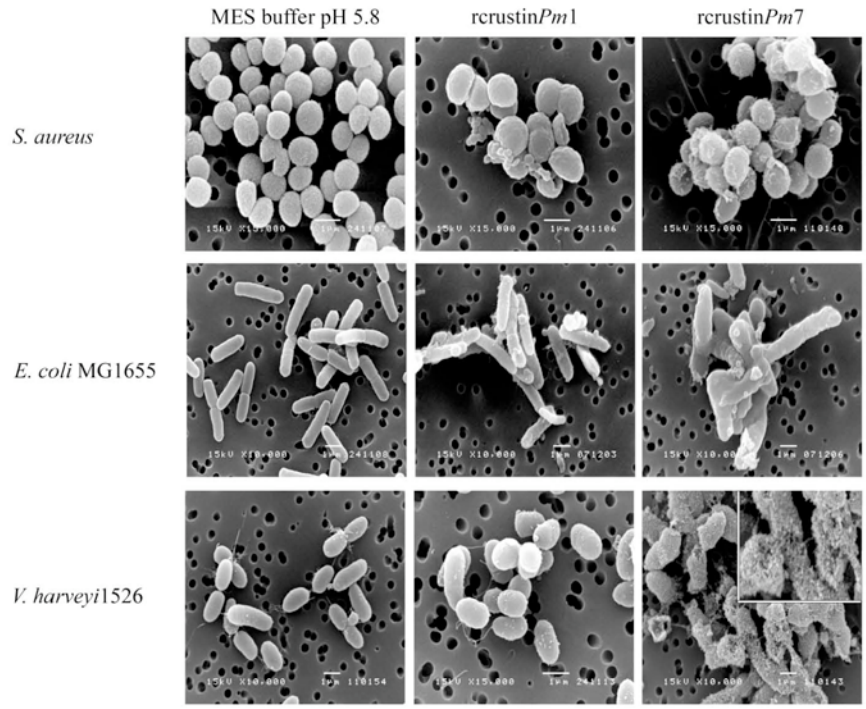
MES buffer pH 5.8
rcrustin*Pm*1
rcrustin*Pm*7
S. aureus
E. coli MG1655
*V. harveyi*1526

Figure 6

Table

Table 1 Summary of bacterial agglutination and antimicrobial activity of rcrustin*Pm*1 and rcrustin*Pm*7.

Bacteria strain	Bacterial agglutination [1]		MIC value (uM) [2]	
	Crustin *Pm*1	Crustin *Pm*7	Crustin *Pm*1	Crustin *Pm*7
Gram-positive bacteria				
Staphylococcus aureus	+	+	3.13-6.25	5-10
Staphylococcus haemolyticus	S/A	S/A	50-100	2.5-5
Bacillus megaterium	+	+	6.25-12.50	1.25-2.5
Micrococcus luteus	+	+	25-50	2.5-5
Gram-negative bacteria				
Enterobacter cloacae	-	-	NA	NA
Escherichia coli 363	+	+	50-100	2.5-5
Escherichia coli MG1655	+	+	50-100	2-5
Vibrio harveyi 1526	-	+	NA	2.5-5
Eirwinia carotovara	-	-	NA	ND

[1] S/A, Self/Auto agglutination; -, No agglutination; +, Agglutination.

[2] MIC values are from Supungul et al., 2008 and Amparyup et al., 2008

NA, Not active at 80 μM; ND, No data