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Bacterial Overexpression of Recombinant Heteroscorpine-1 (rHS-1), a Toxin from *Heterometrus laoticus* Scorpion Venom: Trends for Antibacterial Application and Antivenom Production

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Abstract Heteroscorpine-1 (HS-1) was identified as a member of the scorpine family. HS-1 shows insecticidal activities, exhibiting a low median lethal dose (LD_{50}) in mealworm (*Tenebrio molitor* L.) and inhibitory activities against *Bacillus subtilis, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa*. In this study, a recombinant HS-1 (rHS-1) was produced by overexpression in *E. coli*. A large yield

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of product was obtained. The structure of purified rHS-1 was confirmed through mass spectrometry. Both anti-crude venom and anti-rHS-1 antibodies specifically recognized rHS-1, suggesting its structural similarity. Reactivated rHS-1 caused roughening and blebbing of bacterial cell surfaces. It showed higher activity than that of pre-refolded protein. Antisera raised against a partially purified and mis- or unfolded peptide can inhibit relevant bioactivity.

Keywords *Heterometrus laoticus* · Heteroscorpine-1 · Scorpion venom · Toxin · Potassium channel blocker

Introduction

Many snakes inject at least 1 ml of venom in each bite, but a dose of less than 20 μl of scorpion venom will produce comparable effects on the physiology of prey and enemies. Thus, there is a high potential that scorpion venom will be a source of natural products with novel properties. Toxins are the most remarkable peptides in scorpion venoms. These toxins are mainly classified as ion channel blockers. Some toxins are predicted to have anti-microorganism and insecticidal effects (Diego-García et al. 2008; Possani et al. 1999, 2000).

The scorpines comprise a group of proteins identified as potassium channel blockers with antimicrobial activities (Conde et al. 2000; Zhu and Tytgat 2004). The first scorpine was discovered in *Pandinus imperator* (African emperor scorpion) venom, with inhibitory activities against *Bacillus subtilis* and *Klebsiella pneumoniae* as well as against the ookinete and gamete stages of *Plasmodium berghei* malaria (Conde et al. 2000). Opiscorpine, the second scorpine, is reported in African *Opistophthalmus carinatus* scorpion venom. It shows activity against the plant fungus *Fusarium oxysporum* (Zhu and Tytgat 2004). Heteroscorpine-1 (HS-1) is the next member, discovered in the venom of *Heterometrus laoticus*, a black giant forest scorpion most commonly found in the northeastern area of Thailand (Uawonggul et al. 2006). In terms of biological activities, native HS-1 significantly inhibits the growth of at least three types of bacteria, including *B. subtilis*, *K. pneumoniae,* and *Pseudomonas aeruginosa* (Uawonggul et al. 2007). However, limited availability makes further characterization impossible.

Recently, scorpion toxins have been routinely expressed in various sources, such as *E. coli*, yeast, and insect cell lines (Banerjee et al. 2006; Cao et al. 2003; Johnson et al. 2000; Li et al. 2006; Mao et al. 2007). Expression of these proteins with high efficiencies is useful for biochemical and pharmacological studies. We previously reported that HS-1 was the peptide identified as a K^+ channel blocker. Therefore, in this study, bacterial overexpression of recombinant HS-1 (rHS-1) was successfully achieved. A plasmid with a *HS-1* gene was constructed using a pGEX-6P-3 vector with the fusion part of glutathione S-transferase (GST). The rHS-1 protein was purified, reactivated, and characterized for its bioactivities. Anti-rHS-1 antibody was produced and used to neutralize the activity of crude venom.

Materials and Methods

Gene Construction

Two primers were used for the polymerase chain reaction (PCR). The forward primer (5′-GGATCCGGATGGATTAATGAAGAGAAGATACAAAAG-3′) contained a *Bam*HI site (underlined) upstream from the Gly codon GGA. The reverse primer (5′-CTCGAGGTCCCCCTTTGGCTGCAATTA-3′) had a *Xho*I site (underlined) 18 residues downstream from the stop codon TAA (Fig. 1). PCR was performed with a 94°C, 5 min initial denaturing step, followed by 30 cycles of 30 s of denaturation at 94°C, 1 min of annealing at 55°C, and 1 min of elongation at 72°C. Final elongation was at 72°C for 7 min. For construction, pDrive (Qiagen) was primarily used for ligation with the 261-bp PCR products using its U overhang properties for cloning in *E. coli*.

Gene Expression and Purification

For expression, the pDrive vector was cut with *Bam*HI and *Xho*I. The digested product was resolved by electrophoresis and further gel-purified using a gel extraction kit (Qiagen). The eluent was inserted into the pGEX-6P-3 vector (4900 bp, Pharmacia Biotech, Fig. 1), previously cut with *Bam*HI and *Xho*I,

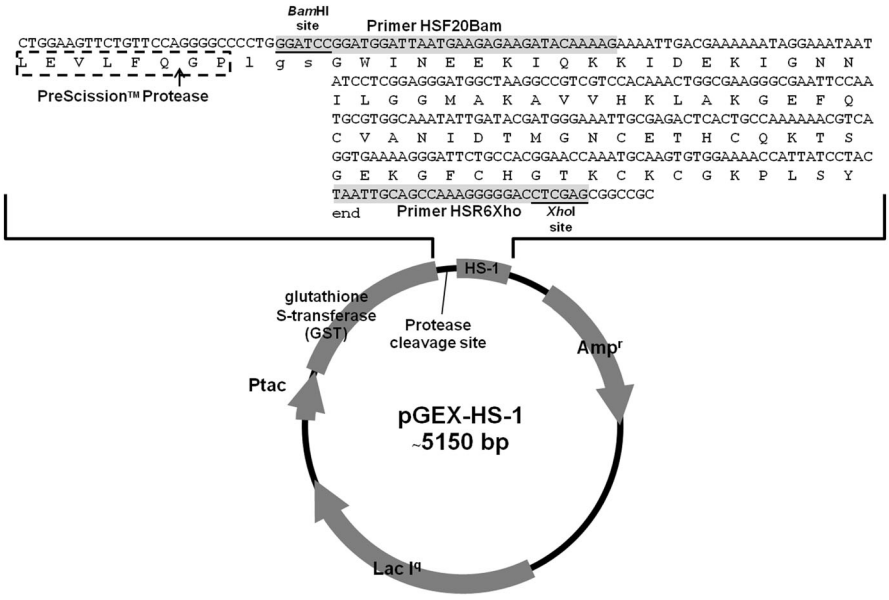

Fig. 1 HS-1 expression vector construction using pGEX-6P-3 vector. The product of PCR using forward (HSF20Bam) and reverse (HSR6Xho) primers was inserted into the pGEX 6P-3 vector at the *Bam*HI and *Xho*I cleavage sites (*underlined*). The HS-1 amino acid sequence is shown in *capital letters*. The protease cleavage site is shown in a *dashed box*. *Gray shading* indicates the sequences of primers used

resulting in a pGEX-HS-1 plasmid 5150 bp in size. The plasmid was transformed into *E. coli* JM109 competent cells. After overnight incubation, a single colony from a transformation plate was inoculated in 20 ml LB broth with ampicillin selection. After shaking at 120 rpm at 28°C overnight, a second inoculation for medium-scale preparation was started using 500 ml of cells with an OD_{600} of approximately 0.05. After shaking at 37°C at a rate of 250 rpm, until the OD_{600} had reached 0.5–0.6, cells were induced by the addition of 0.2 mM isopropyl β-D-thiogalactopyranoside (IPTG) followed by continuous agitation at 210 rpm for 5 h at 37°C. Cells were pelleted by centrifugation at 4000*g* for 10 min at 4°C. The pellet was washed in 10 ml sonication buffer (50 mM sodium phosphate pH 7.8 and 300 mM NaCl) and spun at 4000*g* for 4 min at 4°C. The cell pellet was collected and washed in 10 ml precooled NETN buffer (100 mM NaCl, 20 mM Tris–HCl pH 8.0, 1 mM EDTA, and 2% NP-40) and disrupted by sonication on ice. After centrifugation at 10,000*g* for 20 min at 4°C, the supernatant was incubated with 1 ml of 50% slurry glutathione–Sepharose 4B beads (GE Healthcare) at 4°C with rocking for 60 min to purify the recombinant protein. Beads with immobilized recombinant protein were washed with 20 ml NETN buffer. The matrix was then washed and equilibrated with 10 volumes (10 ml) of cleavage buffer (50 mM Tris–HCl pH 7.2, 150 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol) at 5°C. After removing the residual buffer, the GST tag was cleaved from the fusion protein using 80 U/ml PreScission Protease (GE Healthcare) in 1 ml bead slurry in 100 µl cleavage buffer with gentle suspension at 5°C for 4 h. The beads were briefly spun (500*g*, 5 min), and the supernatant containing recombinant protein was collected. GST and GST-uncut protein were removed by repeated incubation with glutathione–Sepharose beads. Protein purity was finally evaluated by subjecting 5 µl of solution to Tris-tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Tris-tricine SDS-PAGE

The method used here followed a published protocol (Schagger and von Jagow 1987) with slight modification. Samples were mixed with an equal volume of 2× SDS sample buffer (125 mM Tris pH 6.8, 25% glycerol, 4% SDS, 0.008% BPB, and 10% 2-mercaptoethanol). Before resolving at 90 V, the upper reservoir was filled with cathode buffer pH 8.5 (100 mM Tris, 100 mM tricine, and 0.1% SDS), and the lower with the anode buffer (200 mM Tris pH 8.8).

Two-Dimensional PAGE

Immobilized pH gradient (IPG)-SDS-PAGE was performed as previously described (Prajanban et al. 2012). The sample was separated in the first dimension on a 7 cm immobilized pH gradient strip (pH 3–11). Then, the strip was rehydrated for 12 h and focused for 9250 total V × h using the Ettan IPGphor (GE Healthcare Bio-Sciences). Next, the strip was washed in SDS equilibration buffer (50 mM Tris–HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, and 0.002% bromophenol blue) containing 2% dithiothreitol followed by a buffer containing 5% iodoacetamide. The second resolution dimension consisted of 15% SDS-PAGE. Proteins were

stained with Coomassie Brilliant Blue G-250. Excised spots were analyzed for their peptide mass fingerprints using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry at the Genome Institute, National Science and Technology Development Agency, Pathumthani, Thailand.

Refolding Process

Samples were resuspended in denaturation buffer (50 mM Tris–HCl pH 8.0, 5 M guanidine HCl, and 5 mM EDTA) and incubated on ice for 1 h (Junn et al. 2005). The protein solubilized in the denaturation buffer was slowly diluted 1:10 using renaturation buffer (50 mM Tris–HCl pH 8.0, 1% Triton X-100, 8 mM reduced glutathione (GSH), 1 mM oxidized glutathione (GSSG), 0.5 mM PMSF, and 0.3 M arginine). The soluble fractions were then desalted using a PD-10 desalting column (GE Healthcare). Proteins were separated by 16.5% Tris-tricine SDS-PAGE.

Antibacterial Assay and Scanning Electron Microscopy

Diameters of inhibition zones were measured after employing the disc diffusion method using Mueller–Hinton agar plates at 37°C (Uawonggul et al. 2007). Bacterial cells from the inhibition zone were fixed in 2.5% glutaraldehyde overnight before coating with gold and observing under scanning electron microscope (LEO 1450VP, LEO Electron Microscope).

Cross-reactivity

Mice were subcutaneously injected with antigen previously mixed with complete Freund's adjuvant at 1:1 (v/v). Boosts were mixed with antigen at 1:1 (v/v) with incomplete Freund's adjuvant at 2-week intervals. Three days after the fourth boost, antiserum was analyzed via ELISA and Western immunoblotting. For ELISA, immobilized antigen on a polystyrene plate was incubated with antiserum and detected indirectly with alkali phosphatase-conjugated anti-mouse IgG antibody. The substrate in the final step was *p*-nitrophenyl phosphate with 405 nm spectroscopic detection. For Western immunoblotting, proteins separated by SDS-PAGE were transferred to nitrocellulose membranes. The resulting antigen on the membrane was incubated with antiserum and alkali phosphatase-conjugated anti-mouse IgG antibody. A substrate kit was used to detect the resulting bands.

Bioassay and Neutralization Assay

The median lethal dose (LD_{50}) of rHS-1 was determined by sample injection into mealworms (*Tenebrio molitor* L., 0.10 ± 0.01 g body weight). Control animals were injected with PBS buffer, pH 7.4. Those without any movement after 30 min of injection were considered dead. For the antibody neutralization assay, the median paralytic dose (PD_{50}) was detected in crickets (*Gryllus* sp., 0.30 ± 0.03 g body weight). Ten microliters of the respective solution, venom and venom pretreated with anti-crude venom and anti-rHS-1 antibodies, was injected in animals

abdominally. Crude venom incubated with anti-Vibrio Ogawa antibody was used as a control. Venom-injected crickets that could not turn over from a dorsal-upright position within 10 min were considered to be paralyzed.

Results

HS-1 Gene Characterization

A previous study discovered the partial amino acid sequence of native HS-1 by MALDI-TOF tandem mass spectroscopy analysis, revealing a high similarity score to scorpine (Uawonggul et al. 2007). Consecutive rounds of PCR using genomic DNA as a template with seven pairs of generated and nongenerated primers, designed from scorpine family sequences, revealed the full sequence of the *HS-1* gene (accession no. P0C2F4 = protein name > sp|P0C2F4|). After deduction, 76 amino acid residues were present in the mature protein, with Gly as the first. Three disulfide bonds consisting of six cysteine residues were programmatically predicted, similar to other scorpine protein family members (Zhu and Tytgat 2004). No significant difference between the mass theoretically determined (8293.07 Da) and empirically determined mass by mass spectrometry (8293.496 Da) was observed, suggesting no additions, deletions, or any post-expression processes affected the primary structure.

HS-1 Gene Expression and Purification

Following protein expression resulting from IPTG induction, whole-cell lysates were resolved in Tris-tricine SDS-PAGE, and one major band at approximately 34 kDa clearly appeared (Fig. 2). This band size corresponded to the expected size of GST-HS-1 recombinant protein. Recombinant protein was then cut with PreScission Protease and purified, and a single band of purified rHS-1 protein at approximately 8.3 kDa was obtained (Fig. 3). Protein quantitation using Bradford dye staining indicated a protein concentration of 1 μg/μl. Ultimately, 200 μg of HS-1 per 500 ml initial growth was obtained, suggesting a large amount of HS-1 protein expression throughout the procedure. Fortunately, the expression product was completely soluble in aqueous solution, and no inclusion bodies were detected.

Reactivation of rHS-1

Since the soluble rHS-1 was not correctly folded to obtain bioactivity from this peptide, a refolding method was performed using the reduced and oxidized glutathione system. After slow exposure to glutathione and removal of contaminants by dialysis, SDS-PAGE showed refolded HS-1 as a major band at approximately 8 kDa, in agreement with that of rHS-1 (Fig. 4). An evaluation for immunological reactivity revealed that anti-crude venom and anti-rHS-1 antibodies specifically recognized the refolded HS-1 (Fig. 5), suggesting that no

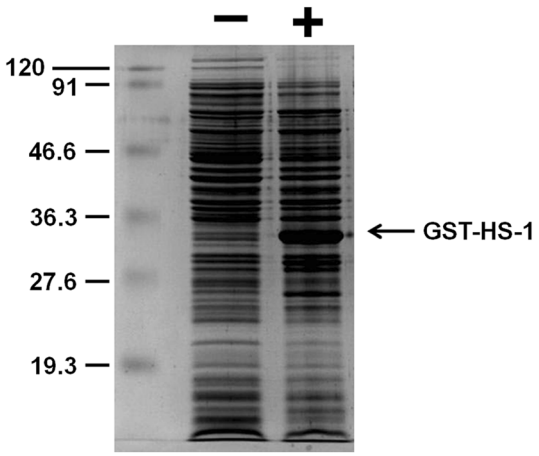

Fig. 2 Heterologous bacterial expression of recombinant HS-1. After resolving by 13.5% Tris–glycine SDS-PAGE, the protein bands were visualized by 0.1% CBB R-250. *Lane* −, before IPTG induction. *Lane* +, after IPTG induction. *Left* Band size in kilodaltons

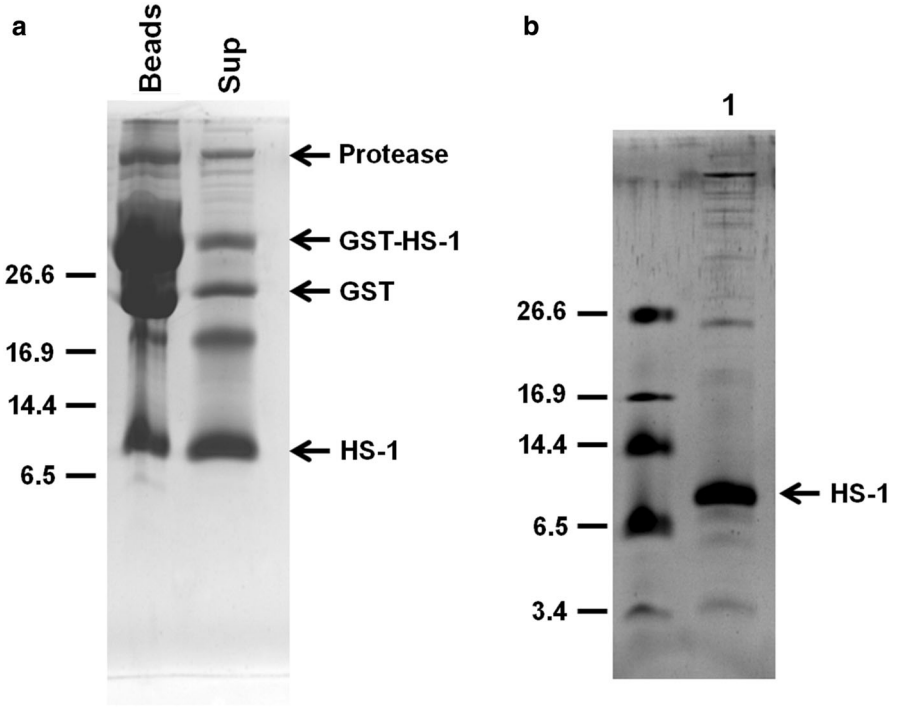

Fig. 3 Purification of the rHS-1 peptide from *E. coli*. (**a**) *Lane Beads,* GST-HS-1 binding to glutathione–Sepharose beads. *Lane Sup*, GST-HS-1 from beads was cleaved with PreScission protease and resolved by 16.5% Tris-tricine SDS-PAGE. (**b**) The supernatant from **a** was further incubated with the beads to remove GST and GST-HS-1. *Arrow* indicates cleaved rHS-1

significant structural changes occurred during the refolding process. However, a poor recovery (less than 1 μg) of refolded HS-1 was obtained from an initial 200 μg of rHS-1.

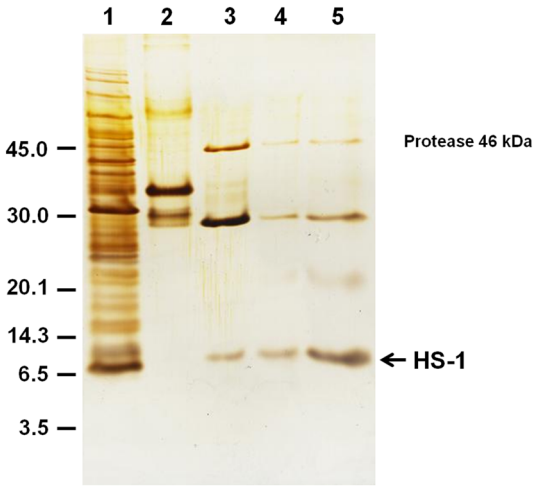

Fig. 4 SDS-PAGE analysis of refolded recombinant HS-1. After purification, proteins were resolved by 16.5% Tris-tricine SDS-PAGE and then stained by silver nitrate solution. *Lane 1* unbound material; *2* bound material on beads before digestion with PreScission enzyme; *3* beads after cleavage; *4* supernatant after cutting; *5* refolded HS-1 fraction after refolding

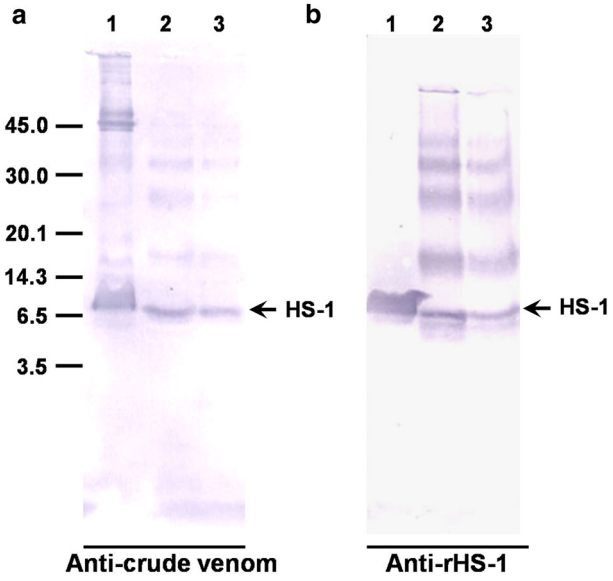

Fig. 5 Cross-reactivity analysis of recombinant HS-1 by Western blotting. Proteins were probed with anti-crude venom (**a**) and anti-rHS-1 antibodies (**b**) with 1:100 dilutions of each antiserum. *Lane 1* crude scorpion venom; *2* unfolded rHS-1; *3* refolded rHS-1. *Arrows* indicate HS-1

However, refolded rHS-1 seemed to have activity. For insecticidal activity, the LD_{50} of refolded HS-1 was approximately 52 μg/g body weight of mealworm, significantly lower in value (higher in activity) than that of the pre-refolded protein (Table 1). The refolded protein also exhibited clear zones of inhibition when exposed to the three types of HS-1-susceptible bacteria (Table 2).

Recombinant HS-1 Identification and Characterization

A proteomic approach was used to verify purified rHS-1. After being subjected to 2D-PAGE, a major spot corresponding to HS-1 was analyzed (Fig. 6). Peptide mass fingerprinting of that protein revealed three peptides corresponding to a partial HS-1 sequence covering around 52% of the mature protein (Table 3). Two sequences, KIDEKIGNNILGGMAKA and KGEFQCVANIDTMGNCETHCQKT, collectively represent 40 of the 76 residues of the full-length mature sequence. This result indicated that rHS-1 and HS-1 were the same protein.

Table 1 Median lethal doses of crude scorpion venom, recombinant HS-1, and refolded HS-1 in mealworms (*Tenebrio molitor*)

Peptide	LD_{50} (μg/g body weight)
Crude scorpion venom	44.67 ± 1.54
Recombinant HS-1	>150.0
Refolded HS-1	52.50 ± 3.53

Table 2 Antibacterial activities of crude scorpion venom, recombinant HS-1, and refolded HS-1 against three bacteria by disc diffusion method (clear zone presented in millimeters)

Bacterium	Control	Crude venom	rHS-1	Refolded HS-1
Bacillus subtilis	0.00	7.00 ± 0.00	0.00	3.67 ± 0.58
Klebsiella pneumonia	0.00	13.83 ± 0.29	0.00	2.17 ± 0.29
Pseudomonas aeruginosa	0.00	8.33 ± 0.58	0.00	3.50 ± 0.50

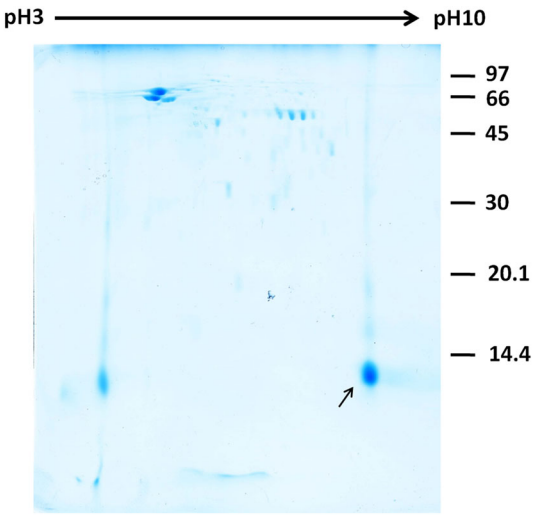

Fig. 6 Two-dimensional PAGE analysis of purified recombinant HS-1. In the first dimension (IEF), 100 μg of protein was loaded on a 7-cm IPG strip (pH 3–11). In the second dimension, 15% SDS-PAGE was used. Proteins were visualized by colloidal Coomassie Brilliant Blue G-250. *Arrow* indicates spot identified by MALDI-TOF analysis. *Right* Band size in kilodaltons

Table 3 Identification of recombinant HS-1

Matched peptide	Range of mature protein coverage	XC score[a]
KIGNNILGGMAKA	15–27	82
KIDEKIGNNILGGMAKA	11–27	26
KGEFQCVANIDTMGNCETHCQKT	34–56	55

[a] Obtained after peptide mass fingerprint analysis. Purified protein was confirmed to be HS-1

Cross-reactions of Recombinant HS-1

To determine cross-reactivity of rHS-1 with native HS-1, anti-crude venom and anti-rHS-1 antibodies were produced by subcutaneous injection of either scorpion crude venom or purified rHS-1 into mice. ELISA showed that the antibodies had titres of $1{:}10^4$ against their antigens. Western immunoblotting revealed the specificity of the antibodies to their antigens (Fig. 5). rHS-1 was recognized by both antibodies (Fig. 5). It is interesting that anti-rHS-1 and anti-crude venom antibodies specifically bound to native HS-1 in scorpion crude venom and refolded HS-1, respectively (Fig. 5). These results suggest that the epitopes of the native and the recombinant proteins are still conserved.

Effects of Recombinant HS-1 on Bacterial Cell Morphology

Anti-microorganism peptides normally act by affecting bacterial cell walls (Pata et al. 2011). The cellular morphologies of *B. subtilis, K. pneumoniae,* and *P. aeruginosa* were examined by scanning electron microscopy following treatment with crude venom or refolded HS-1 (Fig. 7). Untreated bacterial cells had normal and smooth cell surfaces. In contrast, such bacterial cells treated with crude venom and refolded HS-1 showed apparent changes in their morphology. Roughening and blebbing of the bacterial surfaces were both readily observable.

Antibody-Mediated Neutralization of Venom Toxicity

The possibility of neutralizing the toxicity of crude scorpion venom by anti-rHS-1 antibody was investigated by PD_{50} determination in crickets (Table 4). A significant increase in the PD_{50} value of crude venom was observed (34.00 μg/g body weight) after animals were exposed to crude venom pretreated with anti-rHS-1 antibody, compared with those injected with untreated crude venom (11.13 μg/g body weight) or control antibody (anti-Vibrio Ogawa antibody, 11.40 μg/g body weight). This result suggests that the antibody was able to neutralize the toxic effects of crude scorpion venom. Therefore, anti-rHS-1 antibody may have broader implications, such as in the development of antivenom.

Discussion

This study outlines the preparation of HS-1 on a large scale and introduces two points of interest: (1) reactivated rHS-1 may be used for therapeutic purposes,

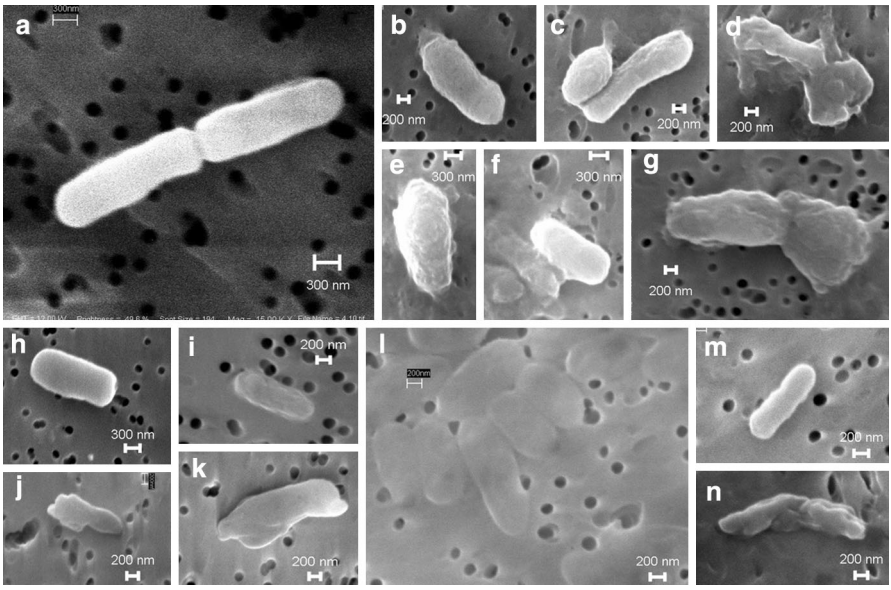

Fig. 7 Effect of recombinant HS-1 on bacterial phenotypes observed using scanning electron microscopy. *Bacillus subtilis* (**a**–**g**), *Klebsiella pneumoniae* (**h**–**k**), and *Pseudomonas aeruginosa* (**l**–**n**) were observed. Cells that received no treatments (**a**, **h**, **l**) or were treated with *Heterometrus laoticus* scorpion crude venom (**b**–**d**, **i**, **m**) or refolded HS-1 (**e**–**g**, **j**–**k**, **n**) in the disc diffusion procedure were prepared for examination under scanning electron microscope. Protein concentration: crude venom 5.31 μg/μl; refolded HS-1 0.72 μg/μl. *Bars* indicate sizes in nanometers

Table 4 Neutralization assay against anti-crude venom and anti-recombinant HS-1 antibodies in crickets (*Gryllus* sp.)

Treatment	PD_{50} (μg/g body weight)
Crude venom	11.13 ± 0.23
Crude venom incubated with anti-crude venom antibody	92.50 ± 13.43
Crude venom incubated with anti-rHS-1 antibody	34.00 ± 5.65
Crude venom incubated with anti-Vibrio Ogawa antibody	11.40 ± 0.84

especially for its antimicrobial activity, and (2) an antibody against nontoxic (unfolded) rHS-1 may be employed for antivenom production in the future.

Although one member of the scorpine protein family has been successfully expressed in a eukaryotic system with the intention of being expressed in the targeted host (*Anopheles gambiae* cells), no overexpression was obtained in the host (Carballar-Lejarazú et al. 2008). As potent antiplasmodial peptides, scorpines have been expressed in entomophatogenic fungi (Fang et al. 2011) or symbiotic bacteria (Wang et al. 2012). A recombinant scorpine-like peptide, Ev37, has been reported to block the kv1.3 channel without cytolytic activity (Feng et al. 2013). Expression of HS-1 in this study, however, showed at least two unique characteristics when compared with other scorpine family members: no inclusion bodies were detected,

and the recombinant protein was able to recover its activity in just one iteration of the GSH/GSSG refolding process.

Technically, the yield of HS-1 decreased after induction for more than 5 h (data not shown), similar to the expression of BmTXKβ in *E. coli* (Cao et al. 2003). Extended periods of induction of BmTXKβ at high temperatures cause a reduction in expression yield, suggesting that the recombinant fusion protein in the bacterial cytoplasm or in the medium is degraded by intracellular enzymes. BmTXKβ was finally successfully expressed in *E. coli* at 30°C, whereas *E. coli* containing the *hs-1* gene grew slower at 30°C than 37°C, causing slower induction (Guatimosim et al. 2000). Furthermore, fusion HS-1 was cut before being purified. The cleavage enzyme, PreScission Protease, was never directly removed; however, no contaminant bands appeared upon SDS-PAGE analysis, indicating that the protease has no significant impact in any other assays or characterizations in further steps.

Although the mass fingerprint result from mass spectrometry helped to confirm the identity of rHS-1, the rHS-1 completely lost activity. The PD_{50} value was relatively high in an insecticidal assay. This result is not entirely surprising, as gene expression of eukaryotic products in a prokaryotic system always encounters the problem of improper post-translational modifications, especially disulfide bond misformation (Johnson et al. 2000), resulting in defective conformational changes. HS-1 contains three predicted disulfide bridges involving six cysteine residues toward the C terminus (Uawonggul et al. 2007). For recombinant CssII, eight cysteines compose four disulfide bridges. The number of possible structures is 105, whereas the number for HS-1 is 15, with a relatively lower possibility of structural variation. Thus, HS-1 showed a tendency to be easier to recover.

A reduced and oxidized glutathione system was selected for the refolding procedure in a suitable buffer to recover HS-1 activity. This system is believed to promote disulfide bond formation by NADPH-mediated GSH/GSSG intercalation. HS-1 activity was apparently recovered by this process. The rHS-1 as well as the refolded rHS-1 ran to the same position in SDS-PAGE analysis. Cross-reactions using antibodies against crude venom and refolded rHS-1 were observed. Both antibodies perfectly recognized HS-1, suggesting no major changes in its epitope structures. Refolded HS-1 showed activity in mealworms, crickets, and bacteria, although that activity was less than that of crude venom. Bacteria treated with crude venom or refolded HS-1 exhibited blebbing in the cell membrane, in agreement with the unusual bacterial cell membrane morphology observed previously (Uawonggul et al. 2007). These results confirm that the refolding process recovers the rHS-1 activity. The three-dimensional conformation of rHS-1 might change after the refolding process, which may affect its antimicrobial activity, possibly mediated by the carboxyl-terminal segment composed of the typical Cys pattern of invertebrate defensin (Zhu and Tytgat 2004). Refolded HS-1, however, exhibited lower activities than previously reported for purified HS-1 (Uawonggul et al. 2007), suggesting that rHS-1 molecules were only partially structurally recovered (Estrada et al. 2007). The anti-rHS-1 antibody was able to neutralize the toxicity of crude venom. This implied the cross-reactivity of anti-rHS-1 antibody with native HS-1, confirming that they have similar structures (at least at the primary structure level). The folding

of their structures at higher levels (secondary and/or tertiary structures) may be different.

Recent proteomic and transcriptomic analysis has revealed that scorpion venoms are composed of dozens or even hundreds of distinct protein components (Rodriguez de la Vega et al. 2010). In *H. laoticus* venom, the 2D-PAGE profile with Coomassie Brilliant Blue staining revealed at least 50 spots (Incamnoi et al. 2013). Though crude venom is clearly composed of many active components, neutralization of only HS-1 was able to reduce total venom activity by nearly one-third $\{1 - [(92.50 - 34.00)/92.50] = 0.37\}$ (Table 4). This result corresponds to that of Pg8 expression, the toxin from the scorpion *Parabuthus granulates* (García-Gómez et al. 2009). When recombinant Pg8 was used for immunization, its antiserum completely neutralized Pg8's toxic effect. In that instance, only one component in the venom was inhibited by antibody conjugation, but the toxicity of the venom was significantly reduced. This suggests that HS-1 may contribute the majority of scorpion venom's toxicity, as well.

This study presents a solution to at least four of these challenges: (1) rHS-1 was produced by overexpression without any toxic effects to bacterial cells. There was no stop or delay during expression, and a large yield of product was obtained. (2) Anti-HS-1 antibody shows high neutralizing efficiency against crude venom by reducing the LD_{50} value by one-third, while the anti-crude venom antibody displayed little effect on the LD_{50} value. An antibody against this one component is predicted to reduce symptoms of scorpion envenomation significantly. (3) An antiserum raised against a partially purified and mis- or unfolded peptide can actually inhibit a relevant bioactivity (the paralysis assays). (4) Allergenic reactions among scorpion victims are predicted to be lower after treatment with anti-rHS-1 antiserum than with anti-crude venom antibody or any antibody cocktails currently used because the number of antigenic determinants (epitopes) of HS-1 must be much less than those of crude venom. These findings may aid in antivenom production, which has emerged as a major need, especially in Third World countries where scorpion envenomation remains a serious health problem.

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