Processing of an Antibacterial Peptide from Hemocyanin of the Freshwater Crayfish *Pacifastacus leniusculus*

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An antibacterial peptide with 16 amino acid residues was found in plasma of the freshwater crayfish, *Pacifastacus leniusculus*. This peptide, designated astacidin 1, was purified by cation-exchange column chromatography and reverse-phase high performance liquid chromatography. Astacidin 1 has a broad range of antibacterial activity, and it inhibits growth of both Gram-positive and Gram-negative bacteria. The primary sequence of astacidin 1 was FKVQNHGQVVKIFHH- COOH. The molecular mass was 1945.2 Da, and no carbohydrate-linked amino acid residues could be found by mass spectrometry. A synthetic astacidin 1 resulted in similar activity as the authentic astacidin 1 against Gram-positive bacteria, whereas it had less or no activity against Gram-negative bacteria. Three amino-terminal-truncated synthetic peptides were made; they all showed low activity, suggesting that the amino-terminal part of astacidin 1 contributes to the antibacterial activity. The structure of astacidin 1 based on the CD results showed that it has a β-sheet structure in citric acid buffer at pH 4, 6, and 8. Cloning of astacidin 1 shows that it is the carboxyl-terminal part of crayfish hemocyanin and that astacidin 1 is produced by a proteolytic cleavage from hemocyanin under acidic conditions. The processing and release of astacidin 1 from hemocyanin is enhanced when crayfish are injected with lipopolysaccharide or glucan.

Antimicrobial peptides have become recognized as important components of the nonspecific host defense or innate immune system in a variety of organisms ranging from plants and insects to animals, including molluscs, crustaceans, amphibians, birds, fish, mammals, and humans (1–3). The primary structures of antimicrobial peptides with positively charged and hydrophobic amino acids are highly diverse, yet their secondary structures share a common feature of amphipathicity, and many of these peptides are membrane-active by ion-channel formation or carpet effect (4, 5). Although they exhibit great structural diversity, they are often divided into four major groups according to composition and secondary structural patterns. The first group has an antiparallel β-sheet structure containing three disulfide bridges, and these defensin peptides can be divided into two subgroups according to their structure. The mammalian defensins have a triple-stranded β-sheet structure (6), whereas insect defensins form two-stranded β-sheets with a flanking α-helix (7). Although all defensins contain three disulfide bonds, the mammalian and insect defensins show different three-dimensional structures. Cecropin and magainin family peptides contain linear peptides forming α-helices and are deprived of cysteine residues. This group generally has a random coil structure in aqueous solution and can penetrate bacterial membranes and disrupt the membrane structure by ion channel formation (8–10). A third group of peptides has a loop structure containing one or more cysteine residues such as bactenecin, and the fourth group comprises peptides with an over-representation of specific amino acids, such as proline, arginine (11–13), and glycine residues (14, 15) or tryptophan-rich peptide (16). The proline-rich peptides are present in insects, crustaceans, and mammals. However, until now, no glycine-rich molecules have been reported in mammals.

The Toll signaling pathway is involved in regulating dorsal-ventral polarity in developing embryos and synthesis of antimicrobial peptides in *Drosophila*. Antimicrobial peptides synthesized in the fat body are secreted into the hemolymph. One role of the Toll pathway in *Drosophila* immune response is to activate the synthesis of these peptides after fungal or Gram-positive bacterial infection (17), whereas the immune deficiency pathway is involved in producing peptides aimed at Gram-negative bacteria in *Drosophila*.

Several antimicrobial peptides have been characterized from insects and chelicerates, and only a few peptides have been reported from crustaceans such as the shore crab *Carcinus maenas* (18) and the shrimp *Penaeus vannamei* (19, 20). Here we present the isolation, biochemical characterization, and synthesis of a new antimicrobial peptide, astacidin 1, from plasma of the freshwater crayfish, *Pacifastacus leniusculus*.

**EXPERIMENTAL PROCEDURES**

**Animals**—Freshwater crayfish, *P. leniusculus*, were purchased from Berga Krafodling, Södermanland, Sweden and were maintained in tanks with aerated water at 10 °C. Only intermolt crayfish were used in this study.

**Purification of Antibacterial Peptides**—Hemolymph was prepared by collecting blood from 400 crayfish in anticoagulant buffer (0.14 M NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid, 10 mM EDTA, pH 4.6) (21). After centrifugation at 4 °C and 800 × g for 10 min, the plasma was removed from hemocytes and stored at −80 °C until further analysis. For purification of antibacterial proteins from plasma, the frozen samples were thawed and trifluoroacetic acid was added to a final concentration of 0.1%. After incubation at 4 °C for 12 h, the sample...
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was centrifuged at 16,000 \( \times g \) for 20 min, and the supernatant was diluted twice with 0.05% trifluoroacetic acid water and directly subjected to C-18 reverse-phase chromatography (ϕ 2.7 × 9 cm, Waters) equilibrated with 0.05% trifluoroacetic acid water. The sample was eluted with a step gradient of 20, 50, and 80% acetonitrile containing 0.05% trifluoroacetic acid, and then 50% acetonitrile elution fraction was applied to the second C-18 column using 0–50% acetonitrile/water/0.05% trifluoroacetic acid as a linear gradient. For the next purification step, Mono S cationic ion exchange chromatography was performed using an FPLC system (Amersham Biosciences). The absorbed proteins were eluted with a linear gradient of 0–1 M NaCl containing 10 mM sodium phosphate buffer, pH 6.0. The sample was then further purified to homogeneity by reverse-phase HPLC (Amersham Biosciences smart chromatography system) on C-18 column using acetonitrile/water/0.05% trifluoroacetic acid gradients of 0–60% acetonitrile in 60 min at a flow rate of 100 \( \mu \)l/min. Ultraviolet absorption was monitored at 280, 254, and 214 nm. The eluted peak fractions were vacuum-dried and used for assay of antibacterial activity and determination of amino acid sequences.

**Determination of Amino Acid Sequence and Mass Analysis**—The homogeneous purified peptide was identified based on Edman sequence analysis using an Applied Biosystem 476A automated amino acid sequencer. For mass analysis and for confirming amino acid sequences, MALDI-TOF-MS\(^1\) was performed in a Q-tof tandem mass spectrometer (Micromass, Manchester, UK) equipped with nonspray interphase.

**Interpretation of mass spectra** was done by using the MassLynx (Micromass) suite of software programs.

**Peptide Synthesis**—An amidated 16-residue antibacterial peptide and three different truncated peptides were synthesized by the solid phase method (22). The molecular masses of the synthetic peptides were determined with MALDI mass spectra.

**Acid-urea PAGE**—The purity of the authentic and synthetic peptides was checked with 20% acetic acid-urea polyacrylamide gel electrophoresis followed by Coomassie staining for peptides as described by Selsted and Becker (23). A low molecular mass calibration kit for electrophoresis (Amersham Biosciences) was used, containing rabbit muscle phosphorylase b (94 kDa), bovine serum albumin (67 kDa), egg white ovalbumin (43 kDa), soybean trypsin inhibitor (20.1 kDa), bovine milk \( \alpha \)-lactalbumin (14.4 kDa), and aprotinin (6.5 kDa). A synthetic peptide of astacin 1 (1.9 kDa) was also used.

**Assay of Antibacterial Activity**—During the purification procedure, the antimicrobial activities of samples were monitored by a radial diffusion using *Bacillus megaterium* BM11 and *Escherichia coli* D21 as test organisms as described by Lehrer et al. (24). Briefly, a 10-mL culture of bacterial cells in mid-logarithmic phase was subject to centrifugation at 900 \( \times g \) for 5 min, washed with 10 mM sodium phosphate buffer, pH 7.4, and then resuspended in 10 mL of the same buffer. One hundred \( \mu \)L of bacterial solution containing 1 \( \times 10^9 \) colony-forming units was added to 10 mL of previously autoclaved agar (10 mM sodium phosphate, pH 7.4, 1% (w/v) LB medium, 1% (w/v) agarose, 0.02% (v/v) Tween 20), and the mixture was poured into a petri dish. Peptide samples were added directly to 3-mm wells made on the solidified underlayer agar. After incubation for 3 h at 37 °C, the plates were overlaid with 10 mL of sterile agar containing a double-strength (6% (w/v)) solution of LB and 1% agarose, and then they were incubated for 12–24 h at 30–37 °C. The minimal inhibitory concentration was determined using the same method and tested against several species of Gram-negative and Gram-positive bacteria. The lowest concentration of the antibacterial peptide that showed visible suppression of growth was defined as the minimal inhibitory concentration.

A liquid growth inhibition assay was performed as described in Lee et al. (25). Bacteria grown in LB medium (peptone 10 g, yeast extract 5 g, NaCl 5 g, glucose 1 g (distilled water 1 liter) was collected in the exponential phase of growth and resuspended with phosphate-buffered saline, pH 6.0, at a density of 1 \( \times 10^8 \) cells/mL. Samples were suspended in 200 \( \mu \)L of 0.2% (w/v) bovine serum albumin and then incubated in 190 \( \mu \)L of LB medium with 10 \( \mu \)L of bacterial suspension and shaking for 3 h at 37 °C. The optical density at 650 nm was measured on each sample.

**cDNA Cloning and Nucleotide Sequencing of Astacin 1**—The cDNA library was screened with 5\( \times \)\(^{-32}\)P\]-labeled mixed probe (AT/C/T/TT/ACAC/CT/TC/GCC/A/G/T/G/T/G/A/T/CT/TCTG/GAC; I is inosine), which was designed according to the following amino acid sequence of astacin 1, VQNQHGQVVKIFHH-COOH. For the initial screening, ~120,000 recombinants of crayfish hepatopancreas Act 11 cDNA library were used. The membranes were prehybridized at 65 °C for 1 h in 5\( \times \) SSC (750 mM NaCl, 75 mM Na-citrate, pH 7.0), 5\% Denhardt’s solution (100 mM Denhardt’s solution is 2% (w/v) bovine serum albumin, N.A, not assayed.

**Table I**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Peptide</th>
<th>Amino acid sequence</th>
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<tbody>
<tr>
<td><strong>Table II</strong></td>
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<tr>
<td><strong>Minimal inhibition concentration of astacin 1 and synthetic peptides</strong></td>
<td></td>
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<tr>
<td>Peptide Amino acid sequence</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SP-1 (1–16)</td>
<td>SP-2 (15)</td>
</tr>
<tr>
<td>Shigella flexneri ATCC 203</td>
<td>15 ( \mu )M</td>
<td>205 ( \mu )M</td>
</tr>
<tr>
<td>Proteus vulgaris OX19 ATCC 6380</td>
<td>&gt;20 ( \mu )M</td>
<td>&gt;1 ( \mu )M</td>
</tr>
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<td>Escherichia coli D21</td>
<td>15 ( \mu )M</td>
<td>410 ( \mu )M</td>
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<tr>
<td>Pseudomonas aeruginosa OT 97</td>
<td>&gt;20 ( \mu )M</td>
<td>617 ( \mu )M</td>
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<tr>
<td>Bacillus megaterium B11</td>
<td>1.9 ( \mu )M</td>
<td>1.95 ( \mu )M</td>
</tr>
<tr>
<td>Bacillus subtilis ATCC 6633</td>
<td>15 ( \mu )M</td>
<td>20 ( \mu )M</td>
</tr>
<tr>
<td>Staphylococcus aureus Cowan 1</td>
<td>&gt;20 ( \mu )M</td>
<td>&gt;1 ( \mu )M</td>
</tr>
<tr>
<td>Staphylococcus aureus SC-1</td>
<td>&gt;20 ( \mu )M</td>
<td>94 ( \mu )M</td>
</tr>
<tr>
<td>Micrococcus luteus M1.11</td>
<td>12.8 ( \mu )M</td>
<td>23 ( \mu )M</td>
</tr>
</tbody>
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\(^{1}\) The abbreviations used are: MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; CD, circular dichroism; LPS, lipopolysaccharide; HPLC, high performance liquid chromatography; LB, Luria-Bertani.
2% (w/v) Ficoll, and 2% (w/v) polyvinylpyrrolidone), 100 /H9262
mg/ml salmon sperm DNA, and 0.5% SDS. The membranes were then hybridized at
65 °C for 12 h in the same solution of prehybridization. After the second
screening, positive clones were amplified with PCR using a pair of
gt 11-specific primers (GGATTGGTGGCGACGACT and GCTT-
ATGCTCTGTA). PCR conditions were 94 °C for 45 s, 55 °C for
30 s, and 72 °C for 2 min carried out for 30 cycles. The largest PCR
product was subcloned into TOPO cloning vector (Invitrogen). The
plasmids were released according to the instructions of the manufac-
turer (Sigma). For confirming the size of plasmids, the insert was
digested out by the restriction enzyme
EcoRI and then run on 1%
agarose gel. It was sequenced with an Applied Biosystems PRISM dye
terminator cycle sequencing ready reaction kit (PerkinElmer Life Sci-
ences). The cDNA sequence was analyzed with MacVector 6.5.1. soft-
ware (Kodak). The nucleotide and the deduced amino acid sequences
were compared using the BLAST program (National Center for Biotech-
nology Information, Bethesda, MD).

Processing of Astacidin 1 from Hemocyanin
—
To test the production
of astacidin 1 in crayfish, plasma was separated from hemocytes, di-
luted twice with anticoagulant buffer or CAC buffer (10 mM sodium
cacodylate, 5 mM CaCl2, pH 7.0), and treated with trifluoroacetic acid at
a final concentration of 0.1%. Plasma (14 mg/ml) was incubated for
different time intervals, such as 0.5, 1, 2, 3, 4, and 5 days, at 4 °C. As a
control, plasma was treated with trifluoroacetic acid and then immedi-
ately centrifuged without any incubation. Each sample was centrifuged
at 16,000 × g for 20 min, and the resulting supernatant was subjected
to SEP-PAK chromatography. The samples were eluted with 80% ace-
tonitrile containing 0.05% trifluoroacetic acid and then vacuum dried.

Immunization of Crayfish for Production of Astacidin 1—Ten cray-
fish were injected with 100 /H9262
mg of LPS (E. coli serotype 055:B5; Sigma)
or laminarin (∼1–3-glucan; Sigma) dissolved in 100 /H9262
ml of distilled
water. After incubation of injected crayfish for 6 h in water at 16 °C,
both plasma and hemocyanin were diluted twice with CAC buffer and treated with trifluoroacetic acid at a final concentration of
0.1% and then further incubated for 12 h at 4 °C. The samples were
centrifuged at 16,000 × g for 20 min, and the resulting supernatant was
digested out by the restriction enzyme EcoRI and then run on 1%
agarose gel. It was sequenced with an Applied Biosystems PRISM dye
terminator cycle sequencing ready reaction kit (PerkinElmer Life Sci-
ences). The cDNA sequence was analyzed with MacVector 6.5.1. soft-
ware (Kodak). The nucleotide and the deduced amino acid sequences
were compared using the BLAST program (National Center for Biotech-
nology Information, Bethesda, MD).
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RESULTS

Purification of Astacidin 1—An antibacterial peptide, astacidin 1, was purified from crayfish plasma by adding the acidified plasma solution to C-18 reverse-phase column chromatography. The eluted fractions were assayed for their antibacterial activity against two bacterial strains, *B. megaterium* BM11 and *E. coli* D21. The fractions containing antibacterial activity were collected, vacuum dried, and subjected to Mono S cation exchange column chromatography for further purification of astacidin 1. Most proteins did not bind to the cation exchange column. Finally, the antibacterial peptide was purified to homogeneity by reverse-phase HPLC. The purity of samples was monitored by 20% acid-urea PAGE (Fig. 1A), because astacidin 1 could not be detected in 20% SDS-PAGE (Fig. 6A).

Determination of Primary and Secondary Structures—The new antibacterial peptide from crayfish plasma was analyzed for its primary structure by Edman degradation and MALDI-TOF-MS. Astacidin 1 consists of 16 amino acid residues with the sequence FKVQNQHGQVVKIFHHC. The mass of astacidin 1 determined by MALDI-TOF-MS was 1945.2 Da, which matched the sequence FKVQNQHGQVVKIFHHC. The peptide does not contain carbohydrate-linked amino acid residues or cysteine residues.

For determination of the secondary structure of astacidin 1, CD spectra were determined in citric acid buffer at different pH values as well as at different temperatures. No significant changes in CD spectra were observed under various temperature conditions. However, the CD spectra showed that 40–53.8% of the molecule has a β-sheet structure at pH 4, 6, and 8. Moreover, the addition of up to 80% (v/v) acetonitrile to astacidin 1 results in a 27% β-sheet structure content (data not shown). The predicted secondary structure of astacidin 1 based on CD data is shown Fig. 1B. Two putative β-sheet structures in each terminus of astacidin 1 may help to disturb the cell wall and membrane of bacteria, as is known from several other antimicrobial peptides.

Antibacterial Activity Spectrum of Authentic and Synthetic Astacidin 1—To fully characterize the biochemical properties of astacidin 1, we performed solid-phase synthesis of the 16-amino acid peptide and three different amino-terminal-truncated peptides, designated SP-1 to -4 (Table I). The purity of the synthetic peptides was confirmed by 20% acid-urea PAGE (Fig. 1C). The synthetic peptide SP-1 had similar antibacterial activity as the authentic native astacidin 1 against Gram-positive bacteria such as *B. megaterium* BM11, *Bacillus subtilis* ATCC 6633, and *Micrococcus luteus* MI 11, whereas the synthetic peptide had lower antibacterial activity toward Gram-negative bacteria. The amino-terminal-truncated peptides had much lower antibacterial activity than the complete synthetic 16-amino acid peptide (Table II). This result indicates that the amino-terminal amino acids contribute to the antibacterial activity. The difference in antibacterial activity between native and synthetic astacidin 1 may be because of the solubility of the synthetic peptide because the synthetic peptide is less soluble than the native peptide in water. Therefore, the synthetic peptide was dissolved in water containing 0.05% trifluoroacetic acid. In this solution with low pH, the secondary structure of the synthetic peptide is changed from a random coil to a β-sheet according to our CD data, which might have affected the antibacterial activity against Gram-negative bacteria. Contamination with other antibacterial peptides in the native astacidin 1 can be excluded, because the amino acid sequence and homogeneity of native astacidin 1 used for these experiments were determined by MALDI-TOF-MS.

Circular Dichroism Measurements—All CD spectra were obtained by JASCO-720 spectropolarimeter. Cellular path length was 1 mm. The concentration of stock solution of protein was determined by bicinchoninic acid assay (27). The stock solution was diluted to 50–100 μg/ml in appropriate buffers. All the experiments were carried out at 25 °C. Scan speed was set with 10 or 20 nm/min. The scan was carried out three times and averaged to the mean value. The contents of secondary structure were calculated using the method of Yang (28).
To reveal whether astacidin 1 is processed.

Induction of Astacidin 1 Production by Injection of LPS or Glucan—To reveal whether astacidin 1 is processed.

The plasma (14 mg/ml) was prepared in anticoagulant buffer and was treated with trifluoroacetic acid in a time-dependent manner. After SEP-PAK chromatography, 50 μg of protein was subjected to 20% acid-urea PAGE. The arrow shows produced astacidin 1.

Cloning and Nucleotide Sequence Analysis of Astacidin 1—We obtained a positive clone from a crayfish hepatopancreas cDNA library. The amino acid sequence of astacidin 1 was used to design and synthesize degenerate primers. Using the 5'-[γ-^32P]ATP labeling method, specific DNA fragments representing astacidin 1 were amplified. The largest clone was shown to code for the complete amino acid sequence of astacidin 1. The nucleotide sequence and deduced amino acid sequence are shown in Fig. 2. The cDNA has an open reading frame of 1,980 nucleotides corresponding to a 660-amino acid residue, and this sequence turns out to be a hemocyanin. The underlined amino acid sequences of the cDNA perfectly match the amino acid sequences of astacidin 1, and a termination codon directly follows astacidin 1 sequence. This indicates that the isolated antibacterial peptide corresponds to the carboxyl terminus of hemocyanin. The first 17 amino acid residues of hemocyanin form a typical signal sequence. Therefore, hemocyanin of crayfish consists of a 660-amino acid residue with a calculated molecular mass of the protein portion of 75,316 Da and an estimated pI of 5.47. Hemocyanin is a blue copper-containing oxygen-transporting molecule and is the predominant protein in the plasma of many crustaceans. The six histidine residues with open circles in Fig. 2 are essential for binding the two oxygen-binding copper atoms in hemocyanin, and they are conserved in all hemocyanins as well as in invertebrate prophenoloxidases (29–31).

Comparisons of the deduced amino acid sequence of hemocyanin cDNA with shrimp hemocyanin and crayfish Prophenoloxidase shows 58 and 33% identity, respectively (Fig. 3). It also has high similarity with other hemocyanins such as hemocyanin α-subunit of Homarus americanus (AJ272095) and hemocyanin subunits 1 (AJ344361), 2 (AJ344362), and 3 (AJ344363) of Palinurus vulgaris.

Processing of Astacidin 1 from Crayfish Hemocyanin and Induction of Astacidin 1 Production by Injection of LPS or Glucan in Crayfish—To reveal whether astacidin 1 is processed from hemocyanin, plasma was incubated under acidic condition in a time-dependent manner using the anticoagulant buffer or CAC buffer. The processing of astacidin 1 from hemocyanin was detectable after 12 h of incubation under acidic condition, and it further increased up to 5 days of incubation (Fig. 4). This processing occurs under neutral pH such as in the CAC buffer with a final concentration of 2.5 mM CaCl₂. However, higher CaCl₂ concentration, i.e., more than 2.5 mM, prohibited this processing (data not shown).

To evaluate whether a proteinase is involved in this process,
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the prophenoloxidase activation system (29, 30), the clotting system (32), and the synthesis of antibacterial peptides (33). The result in this study shows that the carboxyl-terminal part of crayfish hemocyanin is processed by a cysteine-like protease and this processing is up-regulated by LPS or glucan treatment to produce a biologically active antimicrobial peptide, astacidin 1.

DISCUSSION

The cells of invertebrates and mammals produce various antimicrobial substances that act as endogenous antibiotics or disinfectants. Most antimicrobial peptides consist of fewer than 100 amino acids; these peptides are amphipathic, carry a net positive charge, and manifest a well defined α-helical or β-sheet structure in membrane-like environments. Expression of antimicrobial peptides can be constitutive, inducible, or both; several reviews of this topic have appeared in recent years (2, 33–36). Although there are extensive studies on antimicrobial proteins as important immune molecules in various animals, a few antibacterial proteins have been characterized from crustaceans (18, 19).

Here we describe the molecular and functional characterization of a novel peptide with a broad-spectrum antibacterial activity from the hemolymph of the freshwater crayfish, P. leniusculus, which we have named astacidin 1. The antibacterial molecule was purified to homogeneity and is fully characterized at the level of its primary and secondary structure by a combination of reverse-phase chromatography, cation exchange chromatography, MALDI-TOF-MS, and CD spectrum. Astacidin 1 consists of 16 amino acid residues with no cysteine, a strong cationic property, and a β-sheet structure based on CD spectrum, which is likely to be important for its antibacterial activity. Another antibacterial peptide, thanatin from the bug Podisus maculiventris (37), shows a structure similar to that of astacidin 1. Thanatine is a 21-amino acid residue peptide containing two cysteine residues that form an internal disulfide bridge. This peptide has two β-sheet stranded sheets (five residues each), which are held together by a single disulfide bridge. Such an antiparallel two-stranded β-sheet structure is also found in brevinins from frog (38), protegrins from porcine leukocytes (39), and tachyplesins isolated from the horseshoe crab, Tachypleus tridentatus (40). However, there is no sequence homology between astacidin 1 and these peptides including thanatin. Three truncated synthetic peptides were made to elucidate the minimal structure required for antibacterial activity. The amino-terminal-truncated synthetic peptide had less antibacterial activity than authentic astacidin 1, suggesting that the amino-terminal amino acids are important for antibacterial activity.

Many antimicrobial peptides are derived from larger precursors, and processing and generation of antibacterial peptides have been reported from several species. For example, in amphipods, buforin I from the stomach gland cells of the Asian toad Bufo bufo is generated by a pepsin-mediated processing of the cytoplasmic histone H2A (41). In mice, the precursor α-defensin is cleaved by metalloproteinase, a matripsin to produce α-defensin (42), and human defensin-5 is also processed by paneth cell trypsin (43). The organization and processing of peptides from one large precursor molecule is an efficient way to synthesize different effector molecules and amplify the antibacterial response. Interestingly, astacidin 1 is released from the carboxyl-terminal part of crayfish hemocyanin by a cysteine-like protease and is up-regulated by injection of LPS or glucan. The LPS injection results in the generation of other proteins than astacidin 1, whereas glucan injection mainly leads to production of astacidin 1. Recently, three kinds of small antimicrobial peptides were reported from shrimp, which could also be produced from the carboxyl-terminal part of hemocyanin (44). The production of these peptides can be enhanced by exposure to LPS. The small antimicrobial peptide with a molecular mass of 2.7 kDa named PvHct purified from Peneaus vannamei has a similar size to astacidin 1 (Fig. 3), whereas two other peptides, PsHct 1 and PsHct 2 purified from Peneaus stylirostris, are large (7.9 kDa and 8.3 kDa, respectively), but none of them has any homology to astacidin 1.

Hemocyanin is an interesting molecule that serves as an oxygen carrier for many chelicerates and crustaceans. In a recent study, hemocyanins were suggested to have phenoloxidase activity after proteolytic cleavage at the amino-terminal part of hemocyanins in chelicerates such as the spider, Eurytelma californicum (45, 46), and the horseshoe crab, Tachypleus tridentatus (47). Several physicochemical properties of hemocyanins are very similar to those of phenoloxidase (EC 1.14.18.1) (48, 49). Phenoloxidase is an efficient immune molecule for non-self-recognition and is the terminal compound of the so-called prophenoloxidase activating system that is involved in immune reaction such as melanin production, cell adhesion, encapsulation, and phagocytosis as well as sclerotization of the arthropod cuticle (29). It is expressed in hemocytes without a signal peptide and synthesized as a zymogen that is activated by a proteolytic cleavage of an amino-terminal peptide. In contrast, hemocyanin is produced in hepatopancreas and then released to plasma. Evolution seems to have developed a double function of hemocyanin in the chelicerates (50). Under normal conditions the hemocyanin has a function as an oxygen carrier, but it may be converted to phenoloxidase after infection to prevent microbial invasion. The amino acid sequence of crayfish hemocyanin reveals high homology with shrimp hemocyanin and crayfish prophenoloxidase, but there is no homology in the carboxyl-terminal part (Fig. 3). Therefore, only crayfish hemocyanin, and not crayfish prophenoloxidase, can produce and release astacidin 1. In this study, we report that crustacean hemocyanin can be processed by a cysteine-like protease, most likely from a lysosomal organelle, to generate an antimicrobial peptide under acidic condition and that this production can be further enhanced by injecting LPS and glucan into the animal.

REFERENCES
