

A Novel Big Defensin Identified in Horseshoe Crab Hemocytes: Isolation, Amino Acid Sequence, and Antibacterial Activity¹

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Hemocytes of the horseshoe crab (*limulus*) contain a family of arthropodous peptide antibiotics, termed the tachyplesin family, and antibacterial protein, called anti-LPS factor, of which the former is located in the small (S) granules and the latter in the large (L) granules of the hemocytes. In our ongoing studies on granular components, we have identified here a novel defensin-like substance present in both L- and S-granules. This substance strongly inhibits the growth of Gram-negative and -positive bacteria, and fungi, such as *Candida albicans*. The isolated substance, tentatively termed "big defensin," consists of 79 amino acid residues, of which the COOH-terminal 37 residues have a sequence similar to those of mammalian neutrophil-derived defensins, especially rat defensin. Characterization of the disulfide motif in big defensin indicated that the disulfide array is identical to that of β -defensins from bovine neutrophils. One clear structural difference is that the *limulus* hemocyte-derived big defensin has an extension of the NH₂-terminal hydrophobic sequence with 35 amino acid residues followed by the COOH-terminal cationic defensin portion. This amphipathic nature of big defensin seems likely to be associated with its potent antibacterial activity. Furthermore, antibacterial activities of the NH₂-terminal hydrophobic region and the COOH-terminal defensin portion separated by tryptic digestion are significantly different: the former displays a more potent activity against Gram-positive bacteria, whereas the latter is more potent against Gram-negative bacteria. Big defensin, therefore, may prove to represent a new class of defensin family possessing two functional domains with different antimicrobial activities.

Key words: antibacterial activity, big defensin, hemocytes, horseshoe crab, primary structure.

Circulating hemolymph in invertebrates contains biologically active substances, such as antibacterial peptides, lectins, complements, and cell agglutinins, all of which contribute to a self-defense system against invaders (1-3). Among them, a wide variety of oligopeptides and polypeptides has been identified in many invertebrates and all seem to be major defense molecules with antibacterial and antifungal activities (4-6).

In horseshoe crab (*limulus*), the hemocytes contain at least two polypeptides, anti-lipopolysaccharide (LPS) factor (7, 8) and tachyplesin (9) or polyphemusin (10), both of which neutralize a variety of LPS activities and inhibit significantly the growth of Gram-negative and -positive

bacteria and fungi (9, 10). The cytoplasm of this hemocyte is filled with two types of granules, larger (L) but less dense and smaller (S) but dense, as seen on electron micrographs (11, 12). Of these antibacterial substances, anti-LPS factor is located in the L-granules, together with several clotting factors (13) and protease inhibitors (14, 15), whereas tachyplesin peptides are exclusively concentrated in the S-granules (12). In particular, the concentration of tachyplesins in hemocytes is extremely high, with approximately 10 mg being present in the hemolymph of an individual horseshoe crab. These peptides may contribute to host defense of *limulus* (14). In our ongoing studies on these granular components, we have identified a third antibacterial polypeptide, tentatively termed "big defensin," present in both L- and S-granules of hemocytes.

We describe here the isolation, amino acid sequence and antibacterial activities of this novel big defensin.

EXPERIMENTAL PROCEDURES

Materials—Horseshoe crabs (*Tachypleus tridentatus*) collected in Imazu Bay, Fukuoka Prefecture, were maintained in the laboratory aquarium. Hemolymph plasma and

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Abbreviations: CFU, colony forming unit; HPLC, high performance liquid chromatography; LPS, lipopolysaccharide; NP, neutrophil peptide; PTH, phenylthiohydantoin; Page, polyacrylamide gel electrophoresis; Pe, S-pyridylethyl; SDS, sodium dodecyl sulfate.

hemocytes were collected from horseshoe crabs by the method previously reported (9). About 50 g (wet weight) of hemocytes was suspended in 200 ml of 20 mM Tris-HCl buffer (pH 8.0) containing 50 mM NaCl, homogenized in a physcotron (Nihon-Seimitsu, Kyoto), then centrifuged at 8,000 rpm for 30 min in a Hitachi 20 PR-52 centrifuge. The supernatant was removed and the precipitate was washed twice with 200 ml of the same buffer. The hemocyte debris thus obtained was suspended in 100 ml of 30% acetic acid, homogenized, and centrifuged at 8,000 rpm for 30 min. The pellet was extracted twice with 100 ml of 30% acetic acid. Finally, the acid-extract was concentrated by lyophilization and used as the source for isolation of antibacterial substances. Zymogen factor C (16), coagulogen (17), tachypleasin (9), and anti-LPS factor (18) were purified as described. Sephadex G-50 and an electrophoresis calibration kit were obtained from Pharmacia LKB Biotechnology, Uppsala. Endoproteinase Asp-N from Boehringer Mannheim, lysyl endopeptidase from Wako Pure Chemical, Tokyo, and thermolysin from Sigma Chemical, St. Louis, MO, were used. Cosmosil 5C4-300, YMC-Pack C4 and YMC S-5 120A ODS, and Chemcosorb 5-ODS-H were purchased from Nacalai Tesque, Kyoto, YMC-CO., Kyoto, and Chemco Scientific, Tokyo, respectively. All other chemicals were of analytical grade or of the highest quality commercially available.

Assay for Big Defensin—The samples were assayed for their inhibitory effect on LPS-mediated activation of limulus zymogen factor C (9). During HPLC purification, 10 μ l of each peak was assayed, as described (10).

SDS-Polyacrylamide Gel Electrophoresis (PAGE)—SDS-PAGE was performed in 10–20% gradient slab gels according to the method of Laemmli (19). The gels were stained with Coomassie Brilliant Blue R-250.

Reduction, S-Alkylation, and Proteolytic Digestions—For amino acid sequence analysis, the samples were reduced in the presence of 4 M urea and S-alkylated with iodoacetamide (7). The S-alkylated sample (10–20 nmol) was digested with lysyl endopeptidase (E/S = 1/200, w/w) in 0.4 M Tris-HCl, pH 8.5, at 37°C for 6 h. The same sample (20 nmol) was also digested with thermolysin (E/S = 1/50, w/w) in 0.1 M ammonium formate, pH 7.5, containing 4 mM CaCl₂ and 4 M urea, at 37°C for 6 h. A large peptide (2.2 nmol) derived from the lysyl endopeptidase digest was further fragmented with endoproteinase Asp-N (E/S = 1/50, w/w) in 0.1 M Tris-HCl, pH 8.0, containing 2 M urea, at 37°C for 34 h. To identify the COOH-terminal end, the S-alkylated sample (20 nmol) was digested with carboxypeptidase B (E/S = 1/200, mol/mol) in 50 mM Tris-HCl, pH 8.0, at 37°C for 20, 60, and 180 min, and the resulting free amino acids were analyzed using a Hitachi amino acid analyzer (10).

Separation of Big Defensin-Derived Peptides, and Sequencing and Amino Acid Analysis—Peptides were separated by reversed-phase HPLC using a Chemcosorb 5-ODS-H (2.1 \times 150 mm) column with a linear gradient of 0–80% acetonitrile in 0.1% trifluoroacetic acid for 60 min at a flow rate of 0.2 ml/min. The effluent was monitored at 214 nm. Amino acid analyses of peptide samples were performed as described (10), using a PICO-TAG system (Waters, Millipore, Milford, MA). The sequence analysis was carried out using an Applied Biosystems 477A or 473A gas-phase sequencer (10).

Assignments of Disulfide Bridges in Big Defensin—The position of disulfide linkages in big defensin was determined by analyzing the peptides derived from proteolytic digest of the intact sample. The sample (100 μ g) was dissolved in 80 μ l of 50 mM Tris-HCl, pH 6.5, and digested with proteinase K (E/S = 1/100, w/w) at 37°C for 41 h. The peptides generated were separated by reversed-phase HPLC on a YMC-Pack C4 column (4.6 \times 150 mm) with a linear gradient of 0–80% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.5 ml/min. Absorbance was monitored at 280 nm. The major peptide (peak 1) eluted from this column was further digested with thermolysin (E/S = 1/2, w/w) in 0.2 M ammonium acetate, pH 6.5, containing 1 mM CaCl₂, at 60°C for 3.5 h. The digest was separated by reversed phase HPLC on YMC S-5 120A ODS (4.6 \times 150 mm), under the same conditions as described above, and the peptides isolated were used for amino acid and sequence analyses (20).

IonSpray Mass Spectrometry—Positive IonSpray mass spectrometry spectra were obtained on an AP-I-III IonSpray mass spectrometer (Sciex) equipped with an ion-spray, collision cell, and array detector. Sample was placed on the tip of a stainless steel probe, mixed with a liquid matrix [a mixture of 0.5% HCOOH and 50% CH₃CN (w/w)] and measured, as described (21).

Antimicrobial Activity—The following bacteria were used for determination of the antimicrobial activity: *Salmonella typhimurium* LT2 (smooth), *Salmonella minnesota* R595 (Re mutant), *Escherichia coli* 09: K39 (K⁻ strains), *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Candida albicans*. All bacteria were grown in Tryptosoy broth (Eiken, Tokyo). *S. typhimurium* LT2 (smooth), *S. minnesota* R595 (Re), *E. coli* 09: K39 (K⁻), and *K. pneumoniae* were plated on nutrient agar (Eiken). *C. albicans* was plated on guanofracin-Sabouraud agar plates. Bacterial cultures were collected at the logarithmic phase of growth, washed twice with an isotonic or a hypotonic phosphate-buffered saline (PBS; pH 7.0), and adjusted to a final concentration of 5×10^8 to 1×10^9 cells per ml. To 450 μ l of bacterial suspension, 50 μ l of peptide was added and the mixture was incubated at 37°C for 1 h; 100 μ l of the reaction mixture was then plated onto the agar plate. After 24 h of incubation at 37°C, the number of colony forming units (CFU) was counted. As a control experiment, PBS was added to the bacterial suspension and the mixture was incubated for 1 h, plated on agar, and cultured. For some experiments, the percentage of the control CFU was determined.

LPS-Binding Assay—One milliliter of 1% sheep erythrocytes was sensitized and mixed with 0.2 ml of Re-LPS (*S. minnesota* R595) solution (100 μ g/ml), and the mixture was incubated at 37°C for 30 min; this was followed by washing with PBS. Then 50 μ l of a 1.0% suspension of sensitized erythrocytes was mixed with 50 μ l of a twofold serial dilution of samples in a U-bottom microtiter plate, and the mixture was incubated at 37°C for 1 h. The activity was expressed as the minimum agglutinating concentration (22).

Antiserum and Immunoblotting—An antiserum against big defensin was raised in rabbits. The intact protein (200 μ g) was emulsified in a synthetic adjuvant, TiterMax (Vaxcel, GA) and given intradermally. After 4 wk the rabbits were given 200 μ g of the antigen in the same

adjuvant, as a booster. Blood was drawn 3 wk after the second injection and serum was stored at -80°C . For immunoblotting, gels of SDS-PAGE were transferred to nitrocellulose membranes, using an electroblot apparatus (Bio-Rad) at 40 V for 18 h. The membranes were then treated with the antiserum and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG, as described (23). Immunoreactive proteins attached to membranes were visualized after horseradish peroxidase reduction of 4-chloro-1-naphthol.

RESULTS

Isolation of Big Defensin from Horseshoe Crab Hemocytes—The acid extract prepared from *T. tridentatus* hemocyte debris was first fractionated on a Sephadex G-50 column (Fig. 1). Big defensin, which was detected by its inhibitory activity against the LPS-mediated limulus factor C activation (9), was concentrated in the third peak, indicated by a solid bar. These fractions (tube Nos. 60 to 70) were collected, concentrated by lyophilization, and dissolved in 5 ml of 10% acetic acid. Part of the sample was injected onto a Cosmosil 5C4-300 column. Elution was performed with a linear gradient of acetonitrile containing 0.1% trifluoroacetic acid for 30 min at the flow rate of 0.5 ml/min. As shown in Fig. 2, seven peaks were separated and the last major peak indicated by a solid bar had inhibitory activity against zymogen factor C activation mediated by LPS (data not shown). These fractions were collected and concentrated by lyophilization. The yield of big defensin thus prepared was about 4 mg from 50 g of hemocytes (wet weight). The purified big defensin gave a single band (M_r 9,000) on SDS-PAGE under reducing or non-reducing conditions (Fig. 3).

Amino Acid Composition and Sequence Analysis of Big Defensin—Table I shows amino acid composition of the isolated big defensin. It had a high content of alanine, arginine, and serine/valine, while methionine and hexosamines were not detected. The overall composition of big defensin was closely consistent with the sum of the entire sequence established, as shown in Fig. 4. The overall strategy used to prove the entire structure of big defensin was determination of the sequence of a complete set of

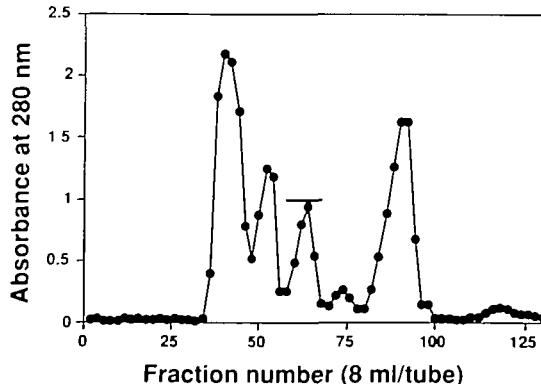


Fig. 1. Gel filtration of an acid-extract obtained from horseshoe crab (*T. tridentatus*) hemocyte debris on a Sephadex G-50 column. The extract (25 ml) was applied to the column (3 \times 90 cm) and eluted with 30% acetic acid at a flow rate of 40 ml/h. The fractions indicated by a solid bar were collected.

peptides (K-1 and K-2) generated by cleavage at a lysyl bond, then the alignment of these peptides with overlaps from other digests. An overlapping peptide (TH-1) was obtained by cleavage of whole protein at an arginyl bond with thermolysin digestion, and each residue of the sequences was identified twice by Edman degradation, using the S-alkylated derivative and the peptide (K-2/N-1) obtained from the digests of lysyl endopeptidase and endoproteinase Asp-N. These results yielded the entire sequence of big defensin consisting of 79 residues.

To confirm the COOH-terminal end of intact protein, the S-alkylated sample was digested with carboxypeptidase B under the conditions described under "EXPERIMENTAL PROCEDURES." Only an arginine was released after 20 min of incubation, which was consistent with the COOH-terminal arginine identified in the peptide K-1 (Fig. 4). The isolated intact big defensin was also subjected to an Ion-Sprey mass spectrometry to determine if there was a modified amino acid residue. The results indicated that the observed mass value of big defensin (8,629.89) was completely consistent with the theoretical value (8,629.90) calculated from the amino acid sequence, and suggested the lack of modified residues in the material (data not shown).

Assignment of Disulfide Linkages in Big Defensin—Big defensin contains six cysteines, but no carboxymethylcysteine was found on amino acid analysis after treatment of intact big defensin with iodoacetamide in the absence of dithiothreitol, thereby indicating the presence of three disulfide linkages in the material.

The intact protein was digested with a combination of proteinase K and thermolysin and two disulfide-containing peptides (peptides 1 and 2) were isolated, as described under "EXPERIMENTAL PROCEDURES." Amino acid analysis after performic acid oxidation showed that peptide 2 contained Trp⁵¹-Cys⁶²-Arg⁶³ and Val⁶⁹-Cys⁷⁰ (data not

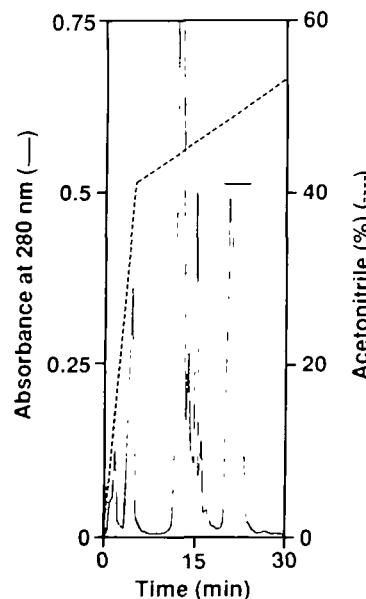


Fig. 2. Separation of big defensin by HPLC. The sample obtained by gel filtration (Fig. 1) was applied to a Cosmosil 5C4-300 column (4.6 \times 150 mm) and eluted at a flow rate of 0.5 ml/min with a linear gradient of acetonitrile containing 0.1% trifluoroacetic acid. The fractions indicated by a solid bar were collected.

shown), indicating a disulfide bond of Cys⁵²-Cys⁷⁰. On the other hand, peptide 1 was composed of three chains linked by disulfide bonds, Ser⁴⁴-Cys⁴⁵, Ser⁵⁴-Lys⁵⁵-Cys⁶⁶, and Phe⁷⁴-Cys⁷⁵-Cys⁷⁶-Arg⁷⁷. Sequence analysis of peptide 1 of 1.5 nmol revealed the complete sequence, Ser (yield of PTH amino acid; 290 pmol) and Phe (730 pmol) at the first cycle, Lys (695 pmol) and Cystine (not quantitated) at the second, Cystine (not quantitated) at the third, and Arg (150 pmol) at the fourth. These data clearly indicated that the remaining two disulfide bonds were Cys⁴⁵-Cys⁷⁵ and Cys⁶⁶-Cys⁷⁶.

Figure 5 shows charge distribution, hydrophathy profile, and predicted secondary structure for big defensin, in addition to the sequence similarity between the limulus defensin portion and a rat neutrophil-derived defensin (24). The NH₂-terminal region of big defensin consisting of 35 residues was highly hydrophobic, followed by positively

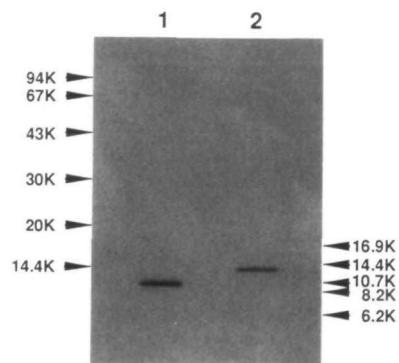


Fig. 3. SDS-PAGE of the isolated big defensin. The purified big defensin was subjected to SDS-PAGE in the presence (lane 1) and absence (lane 2) of 2-mercaptoethanol. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250.

TABLE I. Amino acid composition of big defensin.

Amino acid	Analysis*	Sequence
	(Residues/molecule)	
Asp	6.4	2
Asn		4
Thr	3.1	3
Ser	7.1	7
Glu	1.5	1
Gln		0
Pro	3.4	3
Gly	6.3	6
Ala	12.5	12
Cys	4.9 ^b	6
Val	7.2 ^c	7
Met		0
Ile	3.8 ^c	4
Leu	3.0	3
Tyr	6.0	6
Phe	2.0	2
Lys	1.0	1
His	2.2	2
Trp	1.9 ^d	2
Arg	8.0	8
Total	80.3	79

* Extrapolated or average values estimated from 24, 48, and 72 h hydrolysates. ^bDetermined as cysteic acid after performic acid oxidations. ^cTaken from the values of a 72 h hydrolysate. ^dEstimated on a 24 h hydrolysate with 3 M mercaptoethanesulfonic acid.

charged residues corresponding to the COOH-terminal defensin portion, thereby indicating that the entire molecule had an extremely amphipathic nature. On the other hand, a significant sequence similarity of big defensin with rat NP-2 defensin was noted, especially in the COOH-terminal cationic portion. This portion exhibited up to 50% homology in the aligned regions, and gaps were considered as substitutions, regardless of length (Fig. 5). These results suggest that the limulus big defensin is a member of a superfamily that is structurally related to the mammalian neutrophil-derived defensins so far identified in human, rabbit, and rat (24).

Biological Activity of Big Defensin—Figure 6 shows the inhibitory activity of big defensin against LPS-mediated activation of limulus factor C zymogen. The inhibitory activity was also compared with those of tachyplesin (9) and the anti-LPS factor (7). The potency of this activity was evaluated from the dose-response curve of these substances against zymogen factor C activation mediated by LPS. The effective dose for 50% inhibition was calculated to be 110 nM for big defensin, whereas those for tachyplesin and anti-LPS factor were 9 and 12 nM, respectively, in good agreement with reported data (9). These results suggest that neutralization of LPS activity by big defensin is much weaker than events seen in tachyplesin and anti-LPS factor.

The 50% inhibitory concentration (IC_{50}) of big defensin for growth of various bacteria was also determined using the microplate culture method (24). As summarized in Table II, big defensin had potent antimicrobial activity toward various bacteria, especially under hypotonic conditions. Big defensin showed almost the same potency and inhibitory effect on growth of Gram-negative (*Escherichia* and *Salmonella*) and Gram-positive bacteria (*Staphylococcus*). Furthermore, the growth of fungi, such as *Candida albicans*, was also inhibited by big defensin, indicating that its inhibitory spectrum is analogous to that of tachyplesin (9, 10).

During preparation of big defensin-derived peptides for sequencing analysis, we found that the protein was cleaved only at Arg³⁷ with trypsin in 0.2 M Tris-HCl, pH 6.5 (E/S =

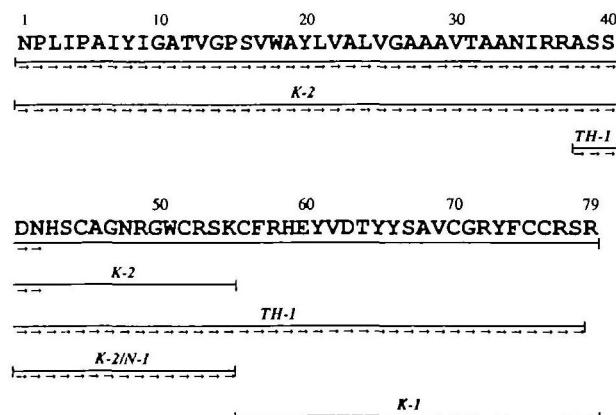


Fig. 4. The entire amino acid sequence of big defensin isolated from horseshoe crab (*T. tridentatus*) hemocytes. Residues identified by a gas-phase peptide sequencer are indicated by arrows. K, lysyl endopeptidase-digested peptides; TH, thermolysin-digested peptides; N, endoproteinase Asp N-digested peptide.

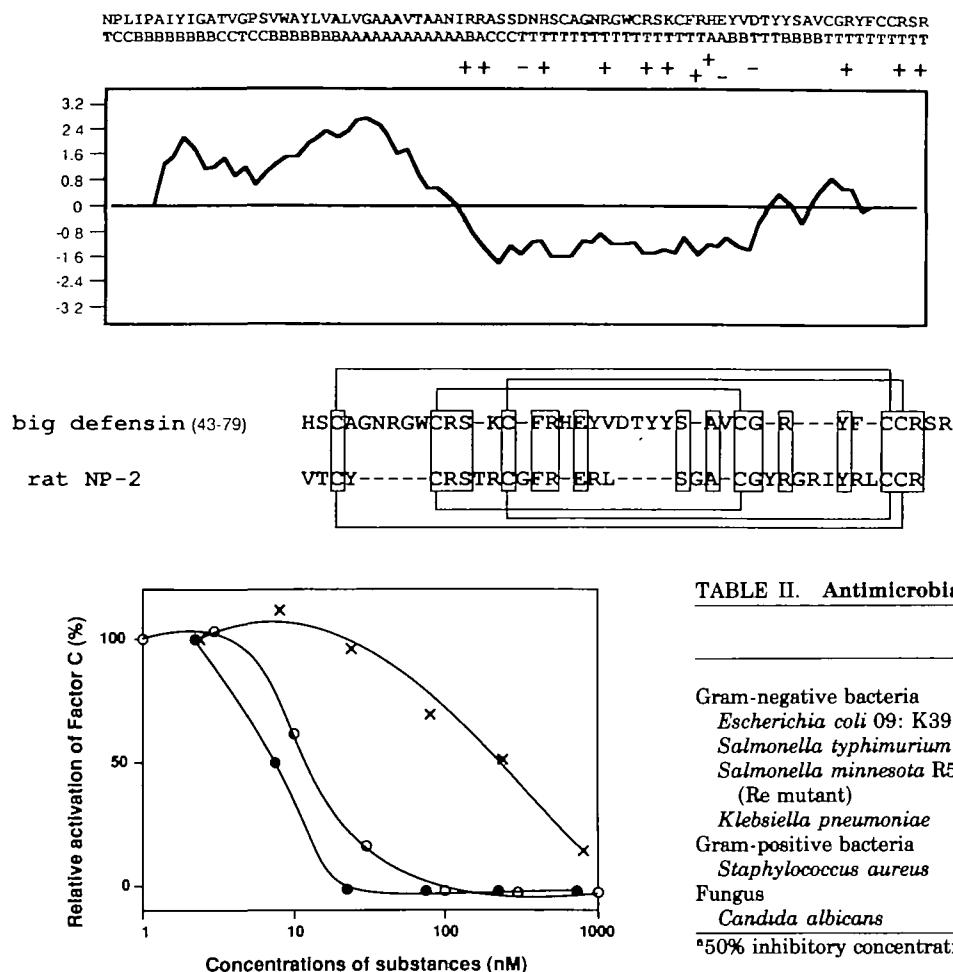


Fig. 5. Charge distribution, secondary structure prediction, and hydropathy profile of limulus big defensin and its sequence similarity with rat NP-2 defensin. Charged residues are indicated by + or - below the sequence (top). In the predicted structure (middle line), the residues are shown in α -helical (A), β -sheet (B), coil (C), and β -turn (T) conformational states (36). In the hydropathy profile, each hydropathic index (ordinate) is the mean value of 9 successive residues. The sequence similarities between big defensin and rat NP-2 defensin are shown at the bottom. Gaps (---) are inserted to increase similarity and identical residues are boxed.

Fig. 6. Inhibitory activity of big defensin against the LPS-mediated activation of limulus factor C zymogen. LPS ($0.2 \mu\text{g}/\text{ml}$) was preincubated with various concentrations (0.005– $1 \mu\text{M}$) of big defensin (x), tachypleasin (○), or anti-LPS factor (●), in a total volume of $100 \mu\text{l}$ of 50 mM Tris-HCl buffer, pH 8.0, containing human serum albumin ($0.5 \text{ mg}/\text{ml}$). Then, $100 \mu\text{l}$ of factor C zymogen ($0.3 \mu\text{g}/\text{ml}$) was added and the factor C activation was assayed as described (9). The relative activation of zymogen factor C was expressed, taking the amidase activity of factor C generated in the absence of tachypleasin as 100%.

1/50, w/w). The resulting NH₂- and COOH-terminal fragments containing the hydrophobic region and the "classical" defensin domain, respectively, were separated by reversed-phased HPLC on a YMC-Pack C4 column (4.5 \times 150 mm) (data not shown). Figure 7 shows antibacterial activity of big defensin and its tryptic derivatives against *S. typhimurium* LT2 (S) and *Staphylococcus* fragment and the COOH-terminal cationic fragment had significant antibacterial activities. It was of particular interest that the NH₂-terminal portion of big defensin shows higher antibacterial activity than the COOH-terminal portion against Gram-positive bacteria (*Staphylococcus*), while their potential activities against Gram-negative bacteria (*Salmonella*) were reversed, suggesting a functional difference may exist between the two regions.

The erythrocyte-agglutinating activity, so-called LPS-binding activity, of big defensin was tested using sheep erythrocytes sensitized with *S. minnesota* Re-LPS (22). As

TABLE II. Antimicrobial activity of limulus big defensin.

	Antimicrobial activity	
	Isotonic (IC ₅₀ ^a ; $\mu\text{g}/\text{ml}$)	Hypotonic
Gram-negative bacteria		
<i>Escherichia coli</i> 09: K39 (K ⁻ strain)	5	2.5
<i>Salmonella typhimurium</i> LT2 (S)	20	<1.3
<i>Salmonella minnesota</i> R595 (Re mutant)	1.3	<0.6
<i>Klebsiella pneumoniae</i>	>10	<1.3
Gram-positive bacteria		
<i>Staphylococcus aureus</i>	<2.5	<2.5
Fungus		
<i>Candida albicans</i>	>20	10–20

^a50% inhibitory concentration.

shown in Table III, the LPS-binding activity of various substances tested was highest in tachypleasin (9) as expected, and the activity of big defensin was also significant, as compared with that of anti-LPS factor (18). In contrast, the NH₂- and COOH-terminal fragments, in addition to the S-alkylated big defensin, showed weak LPS-binding activ-

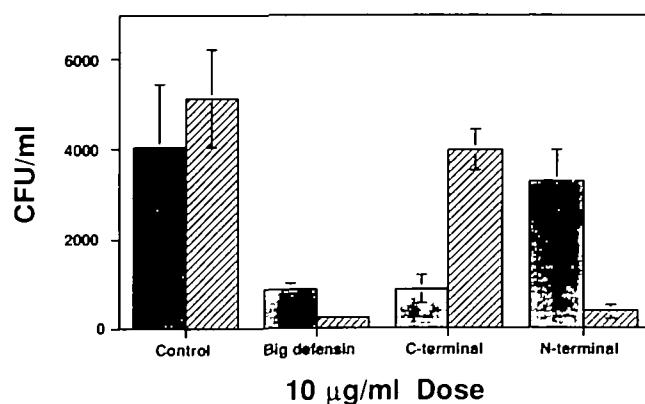


Fig. 7. Antibacterial activity of limulus big defensin and its derivatives. The antibacterial activity against *Salmonella typhimurium* LT2 (S), ■, and *Staphylococcus aureus*, □, is shown as colony forming units (CFU) relative to that for the PBS-treated control. The standard deviations in the figure were calculated from the data with three experiments.

TABLE III. LPS-binding activities of limulus big defensin and its derivatives.

	LPS-binding activity*
	($\mu\text{g}/\text{ml}$)
Big defensin	6.3
Its NH ₂ -terminal fragment	50
Its COOH-terminal fragment	>50
S-Alkylated big defensin	25
Tachyplesin	3.2
Anti-LPS factor	12.5

*Minimum agglutinating concentration of *Salmonella minnesota* Re-LPS-sensitized sheep erythrocytes.

ity, indicating that the native conformation of the entire molecule is required for its binding with LPS.

Subcellular Localization—When we analyzed the NH₂-terminal sequence, NPLIPAIYIGATVGPSV, of big defensin, the molecule proved to be almost identical to that of protein components contained in both L- and S-granules of hemocytes, named L9 and S5, as previously reported (12). The identities of big defensin with L9 and S5 were further supported by amino acid compositions (12). The existence of big defensin in both granules was also confirmed by immunoblotting analysis, using anti-big defensin antiserum (Fig. 8). These data suggest that big defensin is located in both L- and S-granules, but isoforms of big defensin may exist in L- or in S-granules.

DISCUSSION

The big defensin we identified appears to be identical to granular components L9 and S5, previously found in both small and large granules, since the NH₂-terminal sequence up to 17 residues and the amino acid compositions are indistinguishable from those of L9 and S5 (12). Both granules are secreted from hemocytes by stimulation with LPS (11). Thus, big defensin is apparently released through exocytosis on LPS stimulation.

The isolated big defensin consists of a total of 79 amino acid residues, in which the COOH-terminal region composed of 37 amino acids resembles rat NP-2 defensin (24). Limulus big defensin, however, is distinct from the mammalian defensins in molecular size, the latter of which commonly have 29–34 amino acid residues in common (24, 25). It is noteworthy that the disulfide motif in big defensin is identical to that of β -defensins from bovine neutrophils (26, 27) but not to that of classical defensins including rat NP-2 defensin. Furthermore, the structural organization of limulus big defensin differs markedly from those of insect defensins not only in disulfide bridge locations but also in the molecular size. Insect defensins isolated from various species (2), such as *Phormia terraenovae* (28) and *Sarcophaga peregrina* (5, 6), are cationic, 34 to 43 residue peptides, all containing six cysteines present in three intramolecular disulfide bridges. Therefore, the overall structure of limulus big defensin is unique in consisting of a polypeptide with the NH₂-terminal extension. A new isoform of defensin has also been found in bovine tracheal mucosa (29, 30) and in paneth cells of the human small intestine (31, 32). The size, basic charge and three intramolecular disulfide bonds of these mature defensins are similar to those isolated from mammalian circulating phagocytic cells previously mentioned. On the other hand,

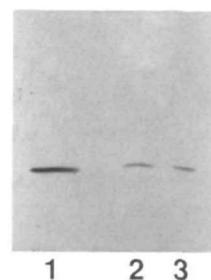


Fig. 8. Immunoblotting of L- and S-granules with antibody against big defensin. Lane 1, purified big defensin ($0.2 \mu\text{g}$); lane 2, small granules ($4 \mu\text{g}$); lane 3, large granules ($4 \mu\text{g}$).

limulus big defensin isolated here is clearly distinct in molecular size from these isoforms. All this evidence suggests the existence of an additional defensin family with a high molecular weight, in vertebrates and invertebrates.

Based on the hydropathy profile for big defensin, this molecule may have a highly amphipathic nature, since there is high hydrophobicity in the NH₂-terminal region and clustering of cationic residues in the COOH-terminal region (Fig. 5). This amphipathic character may be closely related to its biological activity, as many amphipathic peptides and proteins express a cytolytic and an antibacterial activity (33–35). Big defensin shows strong antibacterial activity against not only Gram-negative and -positive bacteria but also fungi (Table II). This broad antimicrobial spectrum of big defensin is also present in mammalian and insect defensins (24). A noteworthy characteristic of limulus big defensin in the antibacterial activity is that there is a functional difference between the NH₂-terminal portion corresponding to residues 1 to 37 and the COOH-terminal defensin portion corresponding to residues 38 to 79. Although intact big defensin has antibacterial activities against both *S. typhimurium* and *Staphylococcus aureus*, the NH₂-terminal hydrophobic fragment is more effective than the COOH-terminal defensin fragment against Gram-positive bacteria (Table II). In contrast, the COOH-terminal defensin fragment shows a more potent activity than the NH₂-terminal hydrophobic fragment against Gram-negative bacteria. These different antibacterial potencies may be critical for host defense. There is, nevertheless, the possibility that the antibacterial activity of big defensin against both Gram-positive and -negative bacteria may have been expressed after cleavage at the -Arg³⁷-Ala³⁸-bond, by proteinases secreted from invading microbes. As limulus big defensin is susceptible to thermolysin, this possibility is likely.

In conclusion, this report is apparently the first to demonstrate the existence of a novel type of big defensin with 79 amino acid residues in the limulus hemocyte granules. The purified big defensin has antibacterial activity *in vitro* against Gram-negative and -positive bacteria, in addition to antifungal activity, thereby indicating a broad spectrum of the activity. Therefore, it may serve synergistically as a defense molecule against invading microbes, together with the anti-LPS factor (7, 18) and tachyplesins (9, 10), all of which are probably secreted into extracellular fluid, in the presence of an invader.

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REFERENCES

- Hoffmann, J.A. and Hetru, C. (1992) Insect defensins: Inducible antibacterial peptides. *Immunol. Today* **13**, 411-415
- Cociancich, S., Bulet, P., Hetru, C., and Hoffmann, J.A. (1994) The inducible antibacterial peptides of insects. *Parasitol. Today* **10**, 132-139
- Boman, H.G. (1991) Antibacterial peptides: Key components needed in immunity. *Cell* **65**, 205-207
- Boman, H.G. and Hultmark, D. (1987) Cell-free immunity in insects. *Annu. Rev. Microbiol.* **41**, 103-126
- Ando, K., Okada, M., and Natori, S. (1987) Purification of sarcotoxin II, antibacterial proteins of *Sarcophaga peregrina* (flesh fly) larvae. *Biochemistry* **26**, 226-230
- Matsuyama, K. and Natori, S. (1988) Purification of three antibacterial proteins from the culture medium of NIH-Sape-4, an embryonic cell line of *Sarcophaga peregrina*. *J. Biol. Chem.* **263**, 17112-17116
- Stone, K.L., LoPresti, M.B., Crawford, J.M., DeAngelis, R., and Williams, K.R. (1989) *A Practical Guide to Protein and Peptide Purification for Microsequencing* (Matsudaira, P.T., ed.) pp. 31-47, Academic Press, San Diego, CA
- Muta, T., Miyata, T., Tokunaga, F., Nakamura, T., and Iwanaga, S. (1987) Primary structure of anti-lipopolysaccharide factor from American horseshoe crab, *Limulus polyphemus*. *J. Biochem.* **101**, 1321-1330
- Nakamura, T., Furunaka, H., Miyata, T., Tokunaga, F., Muta, T., Iwanaga, S., Niwa, M., Takao, T., and Shimonishi, Y. (1988) Tachyplesin, a class of antimicrobial peptide from the hemocytes of the horseshoe crab (*Tachypleus tridentatus*). *J. Biol. Chem.* **263**, 16709-16713
- Miyata, T., Tokunaga, F., Yoneya, T., Yoshikawa, K., Iwanaga, S., Niwa, M., Takao, T., and Shimonishi, Y. (1989) Antimicrobial peptides, isolated from horseshoe crab hemocytes, tachyplesin II, and polyphemusins I and II: Chemical structures and biological activity. *J. Biochem.* **106**, 663-668
- Toh, Y., Mizutani, A., Tokunaga, F., Muta, T., and Iwanaga, S. (1991) Morphology of the granular hemocytes of the Japanese horseshoe crab *Tachypleus tridentatus* and immunocytochemical localization of clotting factors and antimicrobial substances. *Cell Tissue Res.* **266**, 137-147
- Shigenaga, T., Takayenoki, Y., Kawasaki, S., Seki, N., Muta, T., Toh, Y., Ito, A., and Iwanaga, S. (1993) Separation of large and small granules from horseshoe crab (*Tachypleus tridentatus*) hemocytes and characterization of their components. *J. Biochem.* **114**, 307-316
- Iwanaga, S., Miyata, T., Tokunaga, F., and Muta, T. (1992) Molecular mechanism of hemolymph clotting system in *Limulus*. *Thrombosis Res.* **68**, 1-32
- Iwanaga, S., Muta, T., Shigenaga, T., Miura, Y., Seki, N., Saito, T., and Kawabata, S. (1994) Role of hemocyte-derived granular components in invertebrate defense. *Ann. N.Y. Acad. Sci.* **712**, 102-116
- Miura, Y., Kawabata, S., and Iwanaga, S. (1994) A *Limulus* intracellular coagulation inhibitor with characteristics of the serpin superfamily. *J. Biol. Chem.* **269**, 542-547
- Muta, T., Tokunaga, F., Nakamura, T., Morita, T., and Iwanaga, S. (1993) *Limulus* clotting factor C: Lipopolysaccharide-sensitive serine protease zymogen in *Methods in Enzymology* (Laszlo, L. and Mann, K.G., eds.) Vol. 223, pp. 336-345, Academic Press, New York
- Nakamura, S., Iwanaga, S., Harada, T., and Niwa, M. (1976) A clottable protein (coagulogen) from amoebocyte lysate of Japanese horseshoe crab *Tachypleus tridentatus*. *J. Biochem.* **80**, 1011-1021
- Morita, T., Ohtsubo, S., Nakamura, T., Tanaka, S., Iwanaga, S., Ohashi, K., and Niwa, M. (1985) Isolation and biological activities of limulus anticoagulant (anti-LPS factor) which interacts with lipopolysaccharide (LPS). *J. Biochem.* **97**, 1611-1620
- Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
- Muta, T., Hashimoto, R., Miyata, T., Nishimura, H., Toh, Y., and Iwanaga, S. (1990) Proclotting enzyme from horseshoe crab hemocytes. *J. Biol. Chem.* **265**, 22426-22433
- Takao, T., Gonzalez, J., Yoshidome, K., Sato, K., Asada, T., Kammei, Y., and Shimonishi, Y. (1993) Automatic precursor-ion switching in a four-sector tandem mass spectrometer and its application to acquisition of the MS/MS product ions derived from a partially ¹⁸O-labeled peptide for their facile assignments. *Anal. Chem.* **65**, 2394-2399
- Lerrick, J.W., Hirata, M., Shimomura, Y., Yoshida, M., Zheng, H., Zhong, J., and Wright, S.C. (1993) Antimicrobial activity of rabbit CAP18-derived peptides. *Antimicrob. Agents Chemother.* **37**, 2534-2539
- Harlow, E. and Lane, D. (1989) *Antibodies: A Laboratory Manual*, pp. 471-510, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Lehrer, R.I., Ganz, T., and Selsted, M.E. (1991) Defensins: Endogenous antibiotic peptides of animal cells. *Cell* **64**, 229-230
- Selsted, M.E. and Harwig, S.S.L. (1989) Determination of the disulfide array in the human defensin HNP-2. *J. Biol. Chem.* **264**, 4003-4007
- Selsted, M.E., Tang, Y.-Q., Morris, W.L., McGuire, P.A., Novotny, M.J., Smith, W., Henschen, A.H., and Cullor, J.S. (1993) Purification, primary structure, and antibacterial activities of β -defensins, a new family of antimicrobial peptides from bovine neutrophils. *J. Biol. Chem.* **268**, 6641-6648
- Tang, Y.-Q. and Selsted, M.E. (1993) Characterization of the disulfide motif in BNBD-12, an antimicrobial β -defensin peptide from bovine neutrophils. *J. Biol. Chem.* **268**, 6649-6653
- Lambert, J., Keppi, E., Dimarcq, J.-L., Wicker, C., Reichhart, J.-M., Dunbar, B., Lepage, P., Dorsselaer, A.V., Hoffmann, J., Fothergill, J., and Hoffmann, D. (1989) Insect immunity: Isolation from immune blood of the dipteran *Phormia terraenovae* of two insect antibacterial peptides with sequence homology to rabbit lung macrophage bactericidal peptides. *Proc. Natl. Acad. Sci. USA* **86**, 262-266
- Diamond, G., Zasloff, M., Eck, H., Brasseur, M., Maloy, W.E., and Bevins, C.L. (1991) Tracheal antimicrobial peptide, a cysteine-rich peptide from mammalian tracheal mucosa: Peptide isolation and cloning of a cDNA. *Proc. Natl. Acad. Sci. USA* **88**, 3952-3956
- Diamond, G., Jones, D.E., and Bevins, C.L. (1993) Airway epithelial cells are the site of expression of a mammalian antimicrobial peptide gene. *Proc. Natl. Acad. Sci. USA* **90**, 4596-4600
- Jones, D.E. and Bevins, C.L. (1992) Paneth cells of the human small intestine express an antimicrobial peptide gene. *J. Biol. Chem.* **267**, 23216-23225
- Ouellette, A.J., Miller, S.I., Henschen, A.H., and Selsted, M.E. (1992) Purification and primary structure of murine cryptdin-I, a paneth cell defensin. *FEBS Lett.* **304**, 146-148
- Strom, D.R., Rosenthal, K.S., and Swanson, P.E. (1977) Polymyxin and related peptide antibiotics. *Annu. Rev. Biochem.* **46**, 723-763
- Kaiser, E.T. and Kezdy, F.J. (1984) Amphiphilic secondary structure: Design of peptide hormones. *Science* **223**, 249-255
- Fujii, G., Selsted, M.E., and Eisenberg, D. (1993) Defensins promote fusion and lysis of negatively charged membranes. *Protein Sci.* **2**, 1301-1312
- Garnier, J., Osguthorpe, D.J., and Robson, B. (1978) Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. *J. Mol. Biol.* **120**, 97-120

BIOTECHNOLOGY**Gene and Protein Engineering**

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CORRECTIONS

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CORRECTIONS**Vol. 115, No. 4 (1994)**

In the paper entitled "The Delayed Glucocorticoid-Responsive and Hepatoma Cell-Selective Enhancer of the Rat Arginase Gene Is Located around Intron 7" by Tomomi Gotoh, Yougo Haraguchi, Masaki Takiguchi, and Masataka Mori (pp. 778-788), the nucleotide sequence in Fig. 6C (page 783) contained three errors as follows.

- (1) An additional T should be in between T at position 93 and A at position 94 of the upper strand.
- (2) Additional AACCC should be inserted in between G at position 175 and A at position 176 of the upper strand.
- (3) A at position 230 of the upper strand should be deleted.

The correction (2) led to change of the nucleotide sequence for footprint area IV and for the corresponding gel shift probe in Fig. 7A. Using corrected probe IV, reexaminations for experiments in Figs. 7 to 9 were done. Obtained results, however, were concordant with conclusions of the paper. Presumably, since the change was limited to the 5' end portion of the footprint area, it did not affect protein-binding activity of the gel shift probe. The revised nucleotide sequence has been deposited in the DDBJ/EMBL/GenBank databases.