

Callinectin, an Antibacterial Peptide from Blue Crab, *Callinectes sapidus*, Hemocytes

Lester Khoo, David W. Robinette, and Edward J. Noga*

North Carolina State University, College of Veterinary Medicine, 4700 Hillsborough Street, Raleigh, NC 27606, USA

Abstract. This paper describes the isolation of an approximately 3.7 kDa, basic, antibacterial peptide (designated callinectin), which represents the major antibiotic activity in blue crab, *Callinectes sapidus*, hemocytes. A single-step purification using low-pressure cation-exchange chromatography yielded a highly purified (>95%) peptide. Purity was confirmed by C₄ reverse-phase high-performance liquid chromatography (RP-HPLC), native gel electrophoresis, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), capillary electrophoresis, and mass spectral analysis. The partial amino acid sequence obtained via Edman degradation revealed no significant homology to other reported peptides in the Basic Local Alignment Search Tool (BLAST) program database.

Key words: antibacterial peptide, blue crab, non-specific immunity

INTRODUCTION

Crustaceans, like other invertebrates, lack a specific immune response system and depend entirely on nonspecific antimicrobial defenses such as phagocytosis, encapsulation, and nonspecific defense molecules. This is unlike the vertebrate immune system, which is characterized by its finely discriminative ability, that involves among other components, lymphocytes, which impart both specificity and memory (Marchalonis and Schluter 1990; Humfreys and Reinherz 1994; Ratcliffe 1989). For example, the humoral aspect of invertebrate immunity relies entirely on nonspecific defense molecules rather than the sophisticated immunoglobulin system that is the foundation of vertebrate hu-

moral activity. Nonspecific molecules in the crustacean arsenal include phenoloxidase, bactericidins, and lectins (Takahashi et al. 1995; Schapiro 1975).

Nonspecific molecules, specifically peptide antibiotics, have been well documented in other noncrustacean invertebrates. These include cecropins (Boman 1994; Hultmark et al. 1980; Steiner et al. 1981; Faye et al. 1975; Okada and Natori 1984, 1985; Matsumoto et al. 1986), sapaceins (insect defensins) (Matsumaya and Natori 1988), attacins (Hultmark et al. 1983), tachypleins, polyphemusins (Pistole and Graf 1986; Nakamura et al. 1988; Miyata et al. 1989), and anti-LPS factor (ALF) (Morita et al. 1985; Muta et al. 1990). Several of these polypeptides are inducible (attacins and cecropin) (Hultmark et al. 1980; Kockum et al. 1984). Besides being integral to immunity, some of these molecules have other significant biological activities such as binding endotoxin (tachyplein and ALF) (Miyata et al. 1989; Morita et al. 1985), functioning as digestive enzymes (lysozymes) (Daffre et al. 1994), or in insect development

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Lester Khoo's present address is Mississippi State University, College of Veterinary Medicine, Delta Research and Extension Center, P.O. Box 197, Stoneville, MS 38776, USA

*Corresponding author. Fax: 919-513-6336; e-mail: ed_noga@ncsu.edu

(sapeccins) (Komano et al. 1991). Others, such as cecropin, are not limited to invertebrates but have been isolated from mammals (Lee et al. 1989).

The blue crab, *Callinectes sapidus*, is a decapod crustacean of the brachyuran family, Portunidae. Like many other decapod crustaceans (e.g., lobsters, shore crabs, and penaeid shrimp) (Stewart and Zwicker 1972; Chisolm and Smith 1992; Schnapp et al. 1996; Noga et al. 1996a,b), the blue crab has broad-spectrum antibacterial activity in its hemolymph that constitutes part of its nonspecific defenses (Noga et al. 1994). Initial investigations revealed that this antibacterial activity was proteinaceous (inactivated by proteolysis) and found mainly within the hemocytes (Noga et al. 1996a,b). This paper describes the isolation and characterization of the peptide responsible for the majority of this activity.

MATERIAL AND METHODS

Initial studies indicated that the antibacterial activity could be inactivated by purified lipopolysaccharides (LPS) (Khoo and Noga, unpublished data). Thus, glassware and tubing for all experiments were treated to eliminate endotoxin by washing with 1.0 N NaOH and rinsing with copious amounts of pyrogen-free water. All buffers were made using pyrogen-free water; these buffers were verified to be endotoxin-free using the chromogenic *Limulus* amoebocyte lysate test (QCL 1000, Biowhittaker, Walkersville, MD, USA) according to the manufacturer's instructions. All Tris buffers (TB) were 0.05 M and pH 7.2, unless stated otherwise. All ammonium acetate buffers (AAB) were pH 5.15. Protein concentration during different purification steps was determined using the DotMetric assay (Geno Technologies Inc, St. Louis, MO, USA) according to the manufacturer's instructions.

Polypeptide Isolation

Presumptively healthy, live, adult, intermolt crabs were obtained from a local seafood supplier and were chilled at 4–8°C for at least 2.5 h. All walking/swimming appendages (all pereopods excluding the cheliped) were surface sterilized (70% ethanol), dried, and segments were amputated through the intraarthrodial joint at the level of the mero-podite and carpodite (third and fourth proximal segments) with alcohol-sterilized scissors. Free-flowing hemolymph (approximately 5–8 ml per crab) was collected via gravity into a sterile, 15-ml polypropylene tube containing 1 ml of

cold, 10 mM caffeine with 3% NaCl, gently mixed, and centrifuged at 1000g for 15 min at 4°C. The plasma was decanted and the resulting hemocyte pellet was harvested. Pooled pellets were reconstituted in approximately one twentieth the original volume of hemolymph with sterile TB, frozen at –70°C for 3–12 h, thawed, and triturated using a glass tissue homogenizer. The lysate was brought up to one tenth its original hemolymph volume with TB, centrifuged at 1000g for 20 min at 4°C, and the supernate sequentially filtered through 0.45- μ m and 0.22- μ m filters (Acrodisc, Gelman Sciences, Ann Arbor, MI, USA). This 10 \times hemocyte lysate was stored at –70°C until used for purification or testing.

The basic (cationic or positively charged) nature of the antibacterial polypeptide was initially determined using Waters Acell Plus QMA and CM Sep-pak cartridges (Waters Millipore, Marlborough, MA, USA). Cartridges were pre-conditioned with 5 ml deionized water. One milliliter of 10 \times hemocyte lysate was loaded onto the QMA cartridge, washed with 5 ml of TB, and sequentially eluted with 5 ml each of 0.05 M TB of pH 8.0 and 9.0. Similarly, 1 ml of lyophilized 10 \times hemocyte lysate was reconstituted with 1 ml of 0.1 M AAB and loaded onto the preconditioned CM Sep-pak cartridge, washed with 5 ml of 0.1 M AAB, and eluted with 5 ml of 1.0 M AAB. The flow-through, wash, and elution solutions were tested using a spot assay (see below) after being lyophilized and reconstituted in 20 μ l of TB.

For large-scale separations, carboxymethyl cellulose (CM52 medium) (Whatman, Hillsboro, OR, USA) that had been preequilibrated with 1.0 M AAB was packed into a 1.6-cm \times 20-cm glass column (Pharmacia Fine Chemicals, Uppsala, Sweden). The column was then reequilibrated with 0.1 M AAB. Five milliliters of 10 \times hemocyte lysate was lyophilized, reconstituted in 500 μ l of 0.1 M AAB, and loaded onto the column. The column was washed with 200 ml of the same buffer and then a 0.1–1.0 M linear gradient of AAB was run at a flow rate of 1.0 ml/min. The eluate was monitored at 280 nm (Pharmacia UV-M monitor, Uppsala, Sweden). Fractions (3 ml each) were lyophilized and reconstituted with 50 μ l TB and tested (spot assay). Fractions with antibacterial activity were pooled and stored at –70°C.

Pooled material from one CM52 run was reconstituted in 100 μ l TB, brought up to 2 ml in 0.02 M AAB and loaded onto a C₄ reverse-phase column (Delta Pak, 5 μ m, 300 Å , 3.9 \times 150 mm) (Waters Millipore). A 15-min, 0.5 ml/min, isocratic flow of 7% acetonitrile in 0.02 M AAB was followed by a 45-min linear gradient up to 70% acetonitrile in

0.02 M AAB. The latter was then maintained isocratically for another 15 min. The eluate was monitored at 280 nm using a Waters 487 UV detector. One milliliter fractions were collected, lyophilized, reconstituted in 10 μ l of TB, and tested for antibacterial activity via the spot assay.

Antibacterial Assays

The spot assay was modified from Zasloff (1987). Briefly, an overnight (18–20 hr) culture of *Escherichia coli* D31 grown at 37°C in trypticase soy broth with 1% NaCl (TSB/NaCl) was washed three times with cold, phosphate-buffered saline (pH 7.2) after centrifuging at 1000g for 10 min at 4°C. Bacterial suspension [1 ml adjusted to an OD₅₇₀ of 0.1 (10⁸ CFU/ml)] and 1 ml of streptomycin sulfate (10 mg/ml, Sigma Chemical Company, St. Louis, MO, USA; final concentration 100 μ g/ml) was added to an autoclaved agarose medium [1.57 g of low EEO agarose, 0.5 g of NaCl, 20.7 ml of 5 \times Luria Bertaini (LB) broth, 20.7 ml of 1 M phosphate buffer (pH 6.8), and 58.6 ml of distilled, deionized water] that had been cooled to about 45–48°C. The agarose was poured into gridded Petri dishes and refrigerated at 4°C until used. For routine monitoring of antibacterial activity during purification steps, 5 μ l of each test sample was pipetted onto a bacterial plate, and the plate was examined for clear zones in the bacterial lawn after 18–20 h of incubation at 37°C. Results were read as diameter of clearing and estimated amount of clearing (i.e., complete: 100%; partial < 100% to 25%; and trace <25% clearing).

For quantitation of antibacterial activity, we used the method of Hultmark et al., where lethal concentration (LC) was determined by adding serial, twofold dilutions of calinectin in TB to 2-mm-diameter wells of a D31 agarose plate. Clearing zone diameters were then measured after incubation at 37°C for 18 h. Cecropin A (Sigma #C-9421) was used as a positive control.

Polypeptide Analysis

SDS-PAGE of CM52 and RP-HPLC-purified material was carried out using precast Tris-tricine gels (16% polyacrylamide with a 4% stacking gel) according to the manufacturer's instructions (BioRad, Hercules, CA, USA) in the BioRad Mini-Protean II apparatus at 100 V for approximately 1.5 h using a running buffer of 0.10 M tricine and 0.1% SDS. The tracking dye in the sample buffer was 0.04% Coomassie blue (G-250). Gels were stained with Coomassie brilliant blue (R-250) in 7% acetic acid and destained in 7% acetic acid.

The native PAGE method was an adaptation of Gabriel (1971) and Hultmark et al. (1980). An acidic native gel with a discontinuous buffer system consisting of 10% polyacrylamide resolving gel (pH 4.3) and a 4% stacker (pH 6.8) was prepared using a 30% acrylamide–bisacrylamide solution (BioRad) without SDS, and electrophoresed in the BioRad Mini-Protean II apparatus. Methyl green was used as the tracking dye; electrophoresis [100 V for 1 h in wet ice bath with pre-chilled (4°C) running buffer] was carried out towards the cathode. Half the gel was stained and destained as described for the SDS-PAGE gels. The other half of the gel was soaked for 20 min with gentle agitation in 150 ml of filter-sterilized (0.22 μ m) gel wash solution consisting of 80 ml of 5 \times LB broth, 80 ml of 1 M phosphate buffer (pH 6.7), 4 ml of 20% glucose solution, 236 ml of deionized water, and 4 ml of streptomycin sulfate (10 mg/ml). The excess gel wash solution was removed, and the gel was placed on a bacterial plate as prepared for the spot assay. After incubation for 18–20 h at 37°C, it was examined for clear zones in the bacterial lawn.

Capillary electrophoresis of CM52-purified material was carried out on a 50- μ m \times 57-cm Beckman fused silica column connected to a Beckman Pace 2100 (Fullerton, CA, USA). Electrophoresis was run at 21 kV using sodium phosphate buffer (0.05 M, pH 2.5) and monitored at 214 nm.

Amino acid sequencing was performed via Edman chemistry on an Applied Biosystems 477A pulsed-liquid protein sequencer (Foster City, CA, USA). The derivatized phenylthiohydantoin (PTH) amino acids were analyzed with an on-line, microbore Applied Biosystems 120A HPLC. The partial (26 amino acids) sequence thus obtained was subjected to homology search using the Basic Local Alignment Search Tool (BLAST) program (version 1.4) (Altschul et al. 1990) from the National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health. The “blastp” portion of the program was used to compare the sequence with known sequences in the database.

Mass spectrometry was performed on multiple samples (both CM52- and RP-HPLC-purified) using the matrix-assisted Laser Desorption method on a PerSeptive Biosystems Voyager workstation (Framingham, MA, USA).

RESULTS

The 10 \times hemocyte lysate preparations had protein concentrations of 8–11 mg/ml, with detectable antibacterial activity

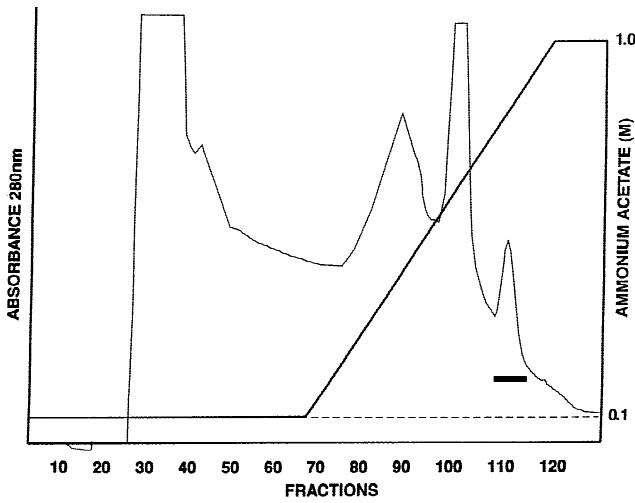


Fig. 1. Chromatogram of low pressure cation exchange (CM52) chromatography of hemocyte lysate; bar indicates peak with antibacterial activity.

(trace) at a 1:32 dilution using *E. coli* D31. Multiple freeze-thaw cycles resulted in slight loss of activity, especially at lower pH (pH 4) (data not shown). Heating to 100°C for as little as 1 min totally inactivated the activity, but there was no loss if incubated at 37°C for 30 min.

Isolation of Callinectin

The basic nature of the antibacterial activity was identified by its lack of binding at pH 7.2 to the anion exchange medium (QMA cartridge) with activity detected only in the flow-through and wash, but not the eluate. The activity bound to the cation exchange matrix (CM cartridge) and was only eluted by a higher molarity buffer. Large-scale purifications were thus performed with a weak cation exchange material, CM52. Antibacterial activity was consistently eluted from the column at approximately 85–90% of the gradient (0.85–0.9 M ammonium acetate) as a single peak (last peak) (Fig. 1). About 40 µg of protein was obtained from this peak. Trace activity was detected at a dilution with a protein concentration of approximately 0.003 mg/ml (about a 100-fold increase in specific activity compared to the 10× hemocyte lysate). Relative purity as judged by C_4 RP-HPLC (Fig. 2) and SDS-PAGE (Fig. 3) ranged from 80 to 90%. Greater than 90% purity was consistently achieved by pooling the latter two thirds of the peak (Fig. 5 below). In some replicates, a much weaker antibacterial activity was detected earlier in the gradient (0.3–0.4 M ammonium acetate); no attempt was made to purify this activity.

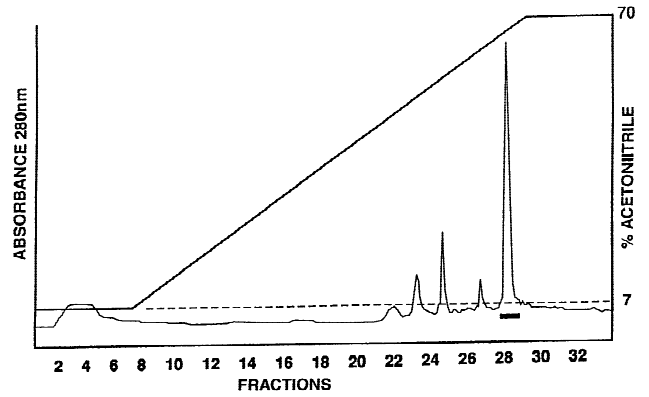


Fig. 2. Chromatogram of RP-HPLC of CM52-purified peptide; bar indicates peak with antibacterial activity.

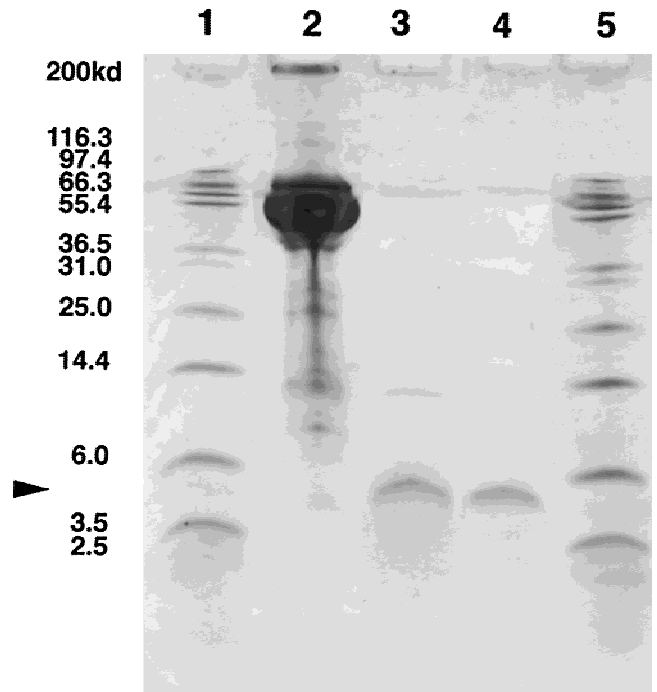


Fig. 3. Tris-tricine SDS-PAGE-Coomassie brilliant blue stain. Lanes 1 and 5, molecular weight markers (Novex Mark 12 wide range protein standard, Novell Experimental Technology, San Diego, CA, USA); lane 2, hemocyte lysate; lane 3, CM52-purified peptide; lane 4, RP-HPLC purified peptide (8 µg); arrowhead indicates the band corresponding to callinectin.

On the RP-HPLC column, antibacterial activity eluted with the major peak (approximately 40 µg protein) at 80–90% of the gradient (56–63% acetonitrile). Its antibacterial activity was much weaker than the activity loaded onto the column.

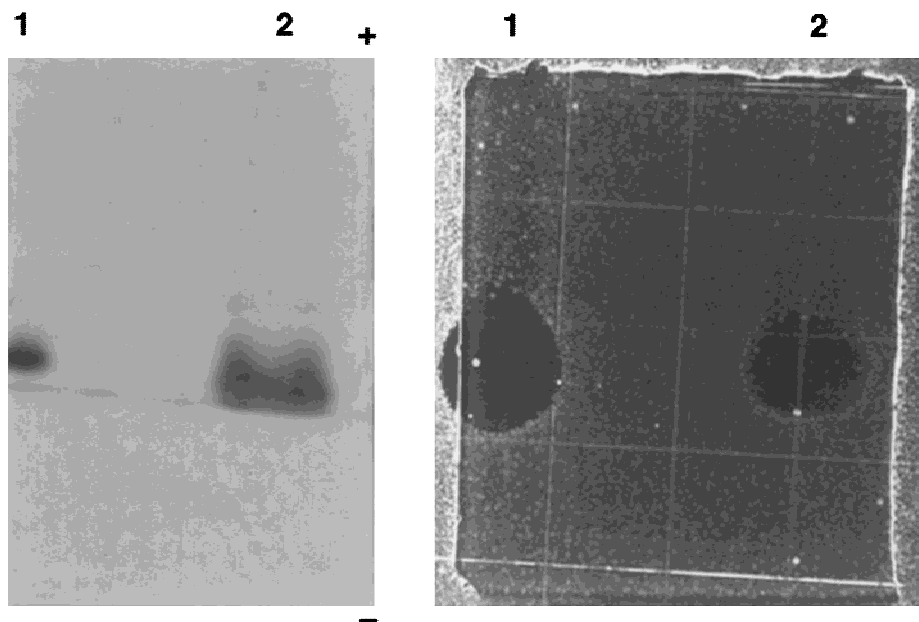


Fig. 4. Native gel of pure callinectin. **A:** Gel stained with Coomassie brilliant blue; lane 1, cecropin (2 µg); lane 2, CM52-purified peptide (10 µg). Note single bands in each lane. **B:** Gel overlaid on a bacterial lawn; Duplicate lanes were run using the same gel as in A except that only 0.4 µg of cecropin was used in lane 1. Note clear zones in bacterial lawn corresponding to the bands in A.

Biochemical Analysis of Callinectin

The multiple, poorly resolved bands present in the 10× hemocyte lysate were reduced to two distinct bands in the CM52-purified material (Fig. 3): a major band at about 5.0 kDa and a weaker staining band at 12.4 kDa. Only one band (approximately 5.0 kDa) was evident with the RP-HPLC-purified material. With the native gel acid PAGE, a single band was present in the lanes containing cecropin and CM52-purified material (Fig. 4A). These bands matched the clear zones in the bacterial lawn on the corresponding half of the gel tested for antibacterial activity (Fig. 4B).

Only one peak was evident in the chromatogram from the high-performance capillary electrophoresis performed on CM52-purified material (Fig. 5). Mass spectrometry of the purified material yielded a single peak with a molecular mass of 3698.9 ± 2.1 Da (mean of seven determinations, data not shown). Amino acid sequencing of different runs of either CM52-purified material or RP-HPLC-purified material yielded similar results. The N-terminal sequence WNSNXXFHVGRPPVVGVRPGXVXFRAP (X = undetermined possibly due to modifications) was detectable to residue 26. No significant homology with any known polypeptide was detected using the BLAST program.

Antibacterial Activity

The lethal concentration of callinectin against *Escherichia coli* D31 was 1.44 µM, compared to 0.29 µM for cecropin A.

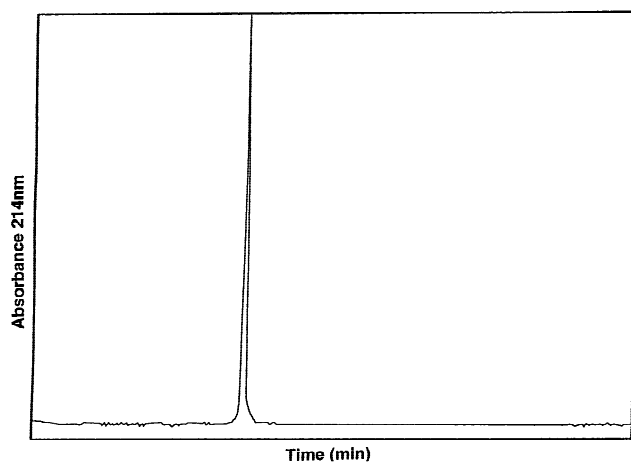


Fig. 5. High-performance capillary electrophoresis of CM52-purified material (latter two thirds of the peak with activity from Fig. 1). Note single peak indicating >95% purity.

The latter value is similar to that reported previously for cecropin A (Hultmark et al. 1980).

DISCUSSION

The list of antibacterial peptides that have been isolated from both invertebrates and vertebrates has expanded dramatically in the past decade (Hetru et al. 1994). This is in part due to recognition of the fundamental importance of

these peptides to antimicrobial defenses of phylogenetically diverse groups of animals. In crustaceans, antibacterial peptides have also been isolated from the granular hemocytes of shore crabs (*Carcinus maenas*) (Chisholm and Smith 1992; Schnapp et al. 1996) and the hemolymph of penaeid shrimp (Destomieux et al. 1997). The shore crab activity is related to bactenecin, an antibiotic first identified from bovine leukocytes (Schnapp et al. 1996). Penaeidins, isolated from the hemolymph of *Penaeus vannamei*, represent a new class of cystine-containing peptide antibiotic (Destomieux et al. 1997).

Callinectin from blue crab hemocytes was purified using its basic (cationic) nature. This property is shared by many antibacterial peptides and proteins, including cecropins (Bowman and Hultmark 1987), sapaceins (Hetru et al. 1994), and tachyplesins (Muta et al. 1990), as well as magainins (Zasloff 1987; Westerhoff et al. 1989) and bactericidal/permeability-increasing protein (BPI) (Weiss 1990), the latter two from *Xenopus* and the neutrophils of mammals, respectively. It is thought that this basic nature is integral to the antibacterial function by allowing them to interact with bacterial membranes and perhaps form a transmembrane channel that causes cell lysis (Weiss 1990; Zasloff 1994; Weiss et al. 1983).

The cationic nature of BPI was exploited in its purification. It was bound to bacteria and then eluted with divalent cations (Mannion et al. 1989). We attempted to use this procedure to purify callinectin but we were unable to elute it from *E. coli* D31 using high concentrations of either divalent or monovalent cations. The adsorbed antibacterial activity could only be eluted using 75–100% dimethyl sulfoxide (DMSO) or 1.0 N NaOH. These experiments led us to discover the apparent lipopolysaccharide (LPS) -binding property of callinectin as evidenced by the loss of hemocyte lysate antibacterial activity after incubation with LPS (Khoo and Noga, unpublished data).

The elution profile of the C₄ RP-HPLC chromatogram, as well as the amino acid sequence, indicates that callinectin is hydrophobic. Hydrophobicity may be important in its function as an antibacterial peptide by allowing it to interact with bacterial membrane phospholipids. The majority (12 of 26) of the sequenced residues are amino acids with non-polar R groups, which contributes to the hydrophobicity of the peptide. These amino acids often occur in short stretches and are most often interrupted by single amino acids with uncharged polar R groups or those with positively charged (basic) polar R groups. Proline is the most abundant of the nonpolar amino acids (4 of 26) in the

partial sequence obtained. However, these residues are not arranged in the typical motifs of the known proline-rich antimicrobial peptide sequences (Hetru et al. 1994). Of significance is the apparent dearth of amino acids with negatively charged (acidic) polar R groups.

Purified callinectin was active against *E. coli* D31. We have previously shown that blue crab hemolymph has potent, broad-spectrum, antibacterial activity against many gram-negative organisms, including vibrios and aeromonads. This activity is found exclusively in the hemocytes (Noga et al. 1996a,b). Callinectin's predominance as an antibacterial factor in blue crab hemocytes suggests that it plays a major role in blue crab immunity. We have previously demonstrated that the antibacterial activity of blue crab hemolymph is severely depressed in polluted waters (Noga et al. 1994); thus, callinectin may provide a useful biomarker for health assessment of coastal ecosystems.

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