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# Penaeidins, a New Family of Antimicrobial Peptides Isolated from the Shrimp *Penaeus vannamei* (Decapoda)*

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**We report here the isolation of three members of a new family of antimicrobial peptides from the hemolymph of shrimps *Penaeus vannamei* in which immune response has not been experimentally induced. The three molecules display antimicrobial activity against fungi and bacteria with a predominant activity against Gram-positive bacteria. The complete sequences of these peptides were determined by a combination of enzymatic cleavages, Edman degradation, mass spectrometry, and cDNA cloning using a hemocyte cDNA library. The mature molecules (50 and 62 residues) are characterized by an $NH_2$-terminal domain rich in proline residues and a COOH-terminal domain containing three intramolecular disulfide bridges. One of these molecules is post-translationally modified by a pyroglutamic acid at the first position. Comparison of the data obtained from the cDNA clones and mass spectrometry showed that two of these peptides are probably COOH-terminally amidated by elimination of a glycine residue. These molecules with no evident homology to other hitherto described antimicrobial peptides were named penaeidins.**

Living in an aquatic environment rich in microorganisms, crustaceans have developed effective systems for detecting and eliminating noxious microorganisms. The defense mechanisms, largely based on the activity of the blood cells, include encapsulation, phagocytosis and associated oxygen-dependent microbicidal mechanisms (1), the prophenoloxidase activating system leading to melanization, and hemolymph coagulation, a rapid and powerful system that prevents blood loss upon wounding and participates in the engulfment of invading microorganisms (2). In the horseshoe crab (Chelicerata, Merostomata), the oldest existent marine arthropod, the hemocytes respond to a bacterial endotoxin activation by cell adhesion and degranulation. The released granule-specific proteins include clotting factors essential for hemolymph coagulation, lectins, and a large number of antimicrobial substances (for review see Ref. 3). In insects, the synthesis of potent antimicrobial peptides or polypeptides induced upon injury is a major and important component of the humoral innate host defense (4). Surprisingly, in crustaceans, the role of antimicrobial peptides in the survival against invading microorganisms has hardly been studied. Until now, bactericidal activities have only been demonstrated in the hemocytes of very few crustaceans (5). Three constitutive hemocytic proteins have been isolated to date in the shore crab, *Carcinus maenas*, and one of these, a 6.5-kDa antibacterial peptide, has been partially characterized (6).

Antimicrobial peptides are widespread in the living kingdom, and a large number of these molecules have been isolated from vertebrates and invertebrates (reviewed by Hetru *et al.* (7)) as well as from plants (8). For the time being and for convenience, these antimicrobial peptides are tentatively classified into four distinct groups based on amino acid sequences, secondary structures, and functional similarities: (i) linear basic peptides forming amphipathic $\alpha$-helices including the cecropins, the first antimicrobial peptide isolated from insect hemolymph (for review see Ref. 9); (ii) peptides with one to six intramolecular disulfide bridges including the arthropod defensins (10), antifungal peptides from *Drosophila*, drosomycin (11) and metchnikowin (12), thanatin from *Podisus* (13), tachyplesin, big defensin and tachycitin from limulus (14–16), and other cysteine-rich antimicrobial peptides isolated from a scorpion (17) and from a bivalve mollusk (18, 19); (iii) proline-rich peptides, among them the apidaecins and abaecins from Hymenoptera (20, 21) and drosocin from *Drosophila* hemolymph (22); (iv) glycine-rich antimicrobial peptides or polypeptides (9–30 kDa) such as the attacins (23), diptericin (24) and sarcotoxins (25). The mode of action, the broad activity, the molecular diversity, and the noncytotoxicity of all these circulating antimicrobial peptides make them very attractive as therapeutic agents for pharmaceutical or agricultural applications (26, 27).

The cultivation of penaeid shrimp is a worldwide economically important activity especially in intertropical developed and developing countries. However, this industry is now suffering serious problems linked to infectious diseases (28, 29), which cause a decrease in growth in shrimp production resulting in vast economic losses. In this context, the control of diseases has become a priority in terms of research in immunology and genetics to insure the long term survival of shrimp

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*The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) Y14925, Y14926, Y14927, and Y14928 and to the Swiss-prot Data Base with accession numbers P81056, P81057, P81058, P81059, and P81060.*

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aquaculture. Therefore, we have undertaken the isolation of antimicrobial peptides in the tropical shrimp *Penaeus vannamei*.

We report here, for the first time in a crustacean, the biochemical characterization, the antimicrobial activities, and the cDNA cloning of three antimicrobial peptides purified to homogeneity from the hemolymph of *P. vannamei* that have not been experimentally infected. These peptides, with molecular masses ranging from 5.5 to 6.6 kDa, are characterized by an over-representation of proline residues in their $NH_2$-terminal domain and by 6 cysteine residues engaged in three intramolecular disulfide bridges concentrated in their COOH-terminal domain. One of these molecules is unusual in that the $NH_2$ and COOH termini are blocked by a pyroglutamic acid residue and an amidation, respectively. These peptides, which cannot be associated to groups hitherto described, were named penaeidins, after the genus *Penaeus*.

## MATERIALS AND METHODS

### *Animals and Hemolymph Collection*

Juvenile white leg shrimp, *P. vannamei* (Penaeidae, Decapoda) were obtained from an intensive shrimp farm in the province of Guayas, Ecuador. A total of 225 ml of hemolymph from five hundred animals (weight ranging from 10 to 30 g) was collected from the ventral sinus located at the base of the first abdominal segment, under 1/10 volume of anticoagulant buffer (10% sodium citrate, pH 7) supplemented with 200 $\mu$M phenylthiourea as a melanization inhibitor and 40 $\mu$g/ml aprotinin as a protease inhibitor. The hemolymph was then centrifuged at 700 × *g* at 4 °C for 15 min to remove the blood cells. Plasma (cell-free hemolymph) and hemocytes were separately frozen at −70 °C until use.

### *Peptide Extraction*

*Plasma Sample*—The plasma was first diluted (1:1 v/v) with MilliQ water and further (1:1 v/v) with 0.1% trifluoroacetic acid. The pH was then brought to 3.9 with 1 M HCl in an ice-cold water bath under gentle stirring for 1 h. Two successive centrifugations (8000 × *g*, 20 min, 4 °C) were performed to clarify the supernatant, which was kept in an ice-cold water bath at 4 °C until use.

*Hemocyte Sample*—After thawing, the hemocytes were homogenized using a Dounce apparatus (maximum, 152 $\mu$m; minimum, 76 $\mu$m) in 50 mM Tris buffer, pH 8.7, containing 50 mM NaCl. After centrifugation (8000 × *g*, 20 min, 4 °C), the supernatant (cytosolic fraction) was acidified to pH 3.6 by the addition of 1 M HCl and kept without freezing at 4 °C until further purification. The pellet containing cellular organelles was extracted in 2 M acetic acid by sonication (3 × 30 s) at medium power (Branson Ultrasons, Annemasse, France) in an ice-cold water bath. Debris was eliminated by centrifugation (8000 × *g*, 20 min, 4 °C), and the organelle acid extract was kept at 4 °C until use.

### *Solid Phase Extraction Prepurification*

The plasmatic fraction and the cellular cytosolic and organelle acid extracts were separately loaded onto 35 cc Sep-Pak $C_{18}$ Vac cartridges (10 g, Waters Associates) equilibrated in acidified water (0.05% trifluoroacetic acid). After washing with acidified water, three stepwise elutions were performed with successively 5, 40, and 80% acetonitrile in acidified water. The different fractions obtained were lyophilized and reconstituted with MilliQ water before subjection to reversed-phase HPLC.[1]

### *HPLC Purification*

*Step 1: Reversed-phase HPLC*—The 40% Sep-Pak fractions were subjected to reversed-phase chromatography on an Aquapore RP300 $C_8$ column (4.6 × 220 mm, Brownlee™) equilibrated in acidified water (0.05% trifluoroacetic acid). Separation of the 40% Sep-Pak fractions was performed with a linear gradient of 2–60% acetonitrile in acidified water over 80 min (0.72% acetonitrile/min) at a flow rate of 1 ml/min. Fractions were hand collected, dried under vacuum (Speed-Vac, Savant), reconstituted in MilliQ water, and tested for antimicrobial activity as described below.

*Step 2: Size Exclusion Chromatography*—Reversed-phase fractions showing the antimicrobial activity were further purified by size exclusion chromatography using two serially linked HPLC columns (Ultraspherogel SEC 3000 and SEC 2000 columns, 7.5 × 300 mm, Beckman) protected by a precolumn (Ultraspherogel SEC, 7.5 × 40 mm, Beckman). Elution was performed under isocratic conditions with 30% acetonitrile in acidified water (0.05% trifluoroacetic acid) at a flow rate of 0.5 ml/min. Fractions were hand collected and treated as above.

*Step 3: Reversed-phase Chromatography*—Different gradients were used for this third purification step of peptides 1–3. Peptides 1 and 2 were purified on the same reversed-phase column as in Step 1 at a controlled temperature of 35 °C with a linear biphasic gradient of 2–21% acetonitrile in acidified water (0.05% trifluoroacetic acid) over 10 min (1.9% acetonitrile/min) and of 21–35% over 50 min (0.28% acetonitrile/min) at a flow rate of 0.25 ml/min. Peptide 3 was purified with a linear biphasic gradient of 2–23% acetonitrile in acidified water over 10 min (2.1% acetonitrile/min) and of 23–37% over 50 min (0.28% acetonitrile/min) at a flow rate of 0.25 ml/min at 35 °C.

*Step 4: Final Purification Steps*—The last purification steps for peptides 1–3 were performed on a narrow bore $C_{18}$ reversed-phase column (Delta Pak HPI $C_{18}$, 2 × 150 mm, Waters Associates) at 40 °C at a flow rate of 0.25 ml/min using the biphasic gradients described above in Step 3.

All HPLC purification steps at room temperature were carried out on a Beckman Gold HPLC system equipped with a Beckman 168 photodiode array detector. For the HPLC purifications under controlled temperature, a Waters HPLC system (Waters 626 pump) attached to a tunable absorbance detector (Waters 486) was used. Column effluent was monitored by its UV absorption at 225 nm. Fractions corresponding to absorbance peaks were hand collected in polypropylene tubes (Microsorb 75 × 12 mm, Nunc immunotubes), concentrated under vacuum (Savant), and reconstituted in MilliQ water (Millipore™) before antimicrobial activity was tested.

### *Capillary Zone Electrophoresis*

Peptide purity was ascertained by capillary zone electrophoresis. Analysis was performed on 2 nl of fractions using a 270A-HT electrophoresis system (Applied Biosystems, Inc.) equipped with a fused silica capillary (length, 72 cm; internal diameter, 50 $\mu$m). Electrophoresis was monitored at 30 °C in 20 mM citrate buffer, pH 2.5, at 20 kV. Capillary effluent was detected by its absorbance at 200 nm.

### *Reduction and S-Pyridylethylation*

Purified peptides were subjected to reduction and alkylation using the procedures already described (30). Briefly, the peptide (1–2 nmol) was dissolved in 40 $\mu$l of 0.5 M Tris HCl containing 2 mM EDTA and 6 M guanidine hydrochloride, pH 7.5, to which 2 $\mu$l of 2.2 M dithiotreitol were added. The samples were incubated under oxygen-free conditions for 1 h at 45 °C. 2 $\mu$l of freshly distilled 4-vinylpyridine (Aldrich) were added, and incubation was continued for 10 min at 45 °C under $N_2$ to prevent oxidation. The *S*-pyridylethylated peptide was desalted on an Aquapore RP300 $C_8$ column (220 × 4.6 mm, Brownlee™) using a linear gradient of 2–60% acetonitrile in acidified water (0.05% trifluoroacetic acid) over 120 min (0.48% acetonitrile/min) at a flow rate of 1 ml/min.

### *Enzymatic Cleavage*

*Trypsin and α-Chymotrypsin Treatments*—Native and *S*-pyridylethylated peptides (5 $\mu$g) were subjected individually to trypsin and α-chymotrypsin treatments (Boehringer Mannheim). Trypsin and chymotrypsin hydrolysis were performed at an enzyme/substrate ratio of 1:20 (w/w) in a 40-$\mu$l reaction containing 0.1 M Tris-HCl at pH 8.5 and in a 50-$\mu$l reaction containing 100 mM Tris-HCl at pH 7.5 and 10 mM $CaCl_2$, respectively. Incubations were carried out for 16 h at 37 and 25 °C for trypsin and α-chymotrypsin treatments, respectively. The reactions were stopped by acidification with 0.1% trifluoroacetic acid. Peptidic fragments were separated on a Delta Pak HPI $C_{18}$ column (2 × 150 mm, Waters Associates) and eluted with a linear gradient of 2–80% acetonitrile in acidified water over 120 min (0.65% acetonitrile/min) at a flow rate of 0.25 ml/min.

*Arginyl Endopeptidase Treatment*—The *S*-pyridylethylated peptide (5 $\mu$g) was treated with arginyl endopeptidase (Takara, Otsu) at an enzyme/substrate ratio of 1:100 (w/w) in a 20-$\mu$l reaction containing 10 mM Tris-HCl at pH 8.0 and 0.01% Tween 20. Incubation was performed for 16 h at 37 °C. Peptidic fragments were separated following the procedure described above.

[1] The abbreviations used are: HPLC, high performance liquid chromatography; MALDI-TOF-MS, matrix-assisted laser desorption/ionization-time of flight-mass spectrometry; nanoES-MS-MS, nanoelectrospray ionization tandem mass spectrometry; MIC, minimal inhibitory concentration; PCR, polymerase chain reaction; ORF, open reading frame; MES, 4-morpholinoethanesulfonic acid; ACTH, adrenocorticotropic hormone.

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*Thermolysin Treatment*—10 μg of each native and *S*-pyridylethylated peptides were separately treated with thermolysin (EC 3.4.24.27) from *Bacillus thermoproteolyticus* (Boehringer Mannheim) for 1 h at 37 °C at an enzyme/substrate ratio of 1:2 (w/w) in 0.1 M MES at pH 6.5 supplemented with 2 mM $CaCl_2$. The digestion was stopped by adding 50 μl of 1% trifluoroacetic acid. The peptides generated by protease hydrolysis were separated by reversed-phase HPLC for further characterization by MALDI-TOF-MS and microsequencing by Edman degradation.

### *Mass Measurement by MALDI-MS*

*Instrumentation*—This study was carried out on a Bruker (Bremen) BIFLEX™ matrix-assisted laser desorption time-of-flight mass spectrometer equipped with SCOUT™ High Resolution Optics, an X-Y multisample probe, a gridless reflector, and the HIMAS™ linear detector. This instrument has a maximum acceleration potential of 30 kV and may be operated in either linear or reflector mode. Ionization was accomplished with the 337 nm beam from a nitrogen laser with a repetition rate of 3 Hz. The output signal of the detector was digitized at a sampling rate of 250 MHz in the linear mode using a 1 GHz digital oscilloscope. A camera mounted on a microscope allowed the inspection of the sample crystallization homogeneity before measurement. All spectra were obtained in the linear positive ion mode and externally calibrated with a mixture of three standard peptides (angiotensin II, ACTH 18–39, and bovine insulin with $MH^+$ at *m/z* 1047.2, 2466.1, and 5734.6, respectively).

*Sample Preparation*—Purified peptides or enzymatically derived fragments (1 μl) were deposited on a thin layer of α-cyano-4-hydroxycinnamic acid crystals made by fast evaporation of a saturated solution in acetone (31, 32). The droplets were allowed to dry under gentle vacuum before introduction into the mass spectrometer.

### *Sequencing by Nanoelectrospray Tandem Mass Spectrometry*

*Instrumentation*—The nanoelectrospray (nanoES) experiments were done on a triple quadrupole Bio-Q mass spectrometer, upgraded by the manufacturer so that the source and the quadrupoles had Quattro II performances (Micromass Ltd. UK, Altrincham). The conventional electrospray probe was modified so that a glass capillary similar to that described by Wilm and Mann (33) could be positioned at about 2 mm from the first cone of the electrospray source. The source was used without counter electrode, and the drying gas heated at 50 °C was nitrogen. The glass capillary and extracting cone voltages were 900 and 50 V, respectively. Electrical contact between the probe tip and the metallized glass capillary (long needle type glass capillaries purchased from the Protein Analysis Company, Odense M) was made by using a graphite cone inside the Swagelok union instead of the customary brushing of an organic solution of graphite (33) giving interference ions in the low *m/z* range. Before connecting the glass capillary into the mass spectrometer, it was opened by briefly touching a metal capillary (0.5-mm inner diameter × 150 mm) connected to a vacuum source. The opened glass capillary was then washed by applying a $N_2$ pressure to reduce contamination by impurities in the metal layer. After loading the sample solution at a concentration of 1 pmol/μl in acetonitrile/water containing 1% formic acid, the glass capillary was inserted in the MS source, and static air pressure was applied to give a flow rate of approximately 20 nl/min, which allowed a stable signal recording for up to 3 h.

*Nanoelectrospray Tandem Mass Spectrometry*—In a first approach, the parent ion (*m/z* = 520.1) produced at a low cone voltage ($V_c$ = 30 V) was selected in the first quadrupole mass analyzer and fragmented by collision-induced dissociation with argon gas at $4.5 \times 10^{-2}$ Pa at 40 V. To gain additional structural information, MS-MS experiments on fragments generated by source collision of the parent ion at *m/z* 520 with an extracting cone voltage of 100 V were performed using 60 and 80 V in the collision cell. The resulting fragment ions were named according to Roepstorff and Fohlman's nomenclature (34). The quadrupole analyzers were calibrated by using the multiply charged ions from a separate acquisition of horse heart myoglobin (16951.5 Da).

*Microsequence Analysis*—Native, *S*-pyridylethylated peptides and peptidic fragments were subjected to Edman degradation on a pulse liquid automatic sequenator (Applied Biosystems, model 473A).

### *Antimicrobial Assays*

*Microorganisms*—The microbial strains used to determine antimicrobial activities during the purification steps were those used in previous studies (11, 35): *Micrococcus luteus* (Gram-positive strain), *Escherichia coli* D31 (Gram-negative strain), and *Neurospora crassa* as a filamentous fungus. The marine fungus *Fusarium oxysporum*, pathogenic for penaeid shrimp (gift from Dr. Alain Vey, INRA, St. Christol-les-Alès, France) was used to complete the activity spectrum.

*Antibacterial Assay*—After each step of purification, an aliquot of each eluted fraction reconstituted in MilliQ water was tested by the liquid growth inhibition assay already described (22). Briefly, 10-μl aliquots from each test fraction were incubated in microtiter plates with 100 μl of a suspension of a midlogarithmic phase culture of bacteria (*E. coli* D31 or *M. luteus*) at a starting optical density of $A_{600}$ = 0.001 in Poor-Broth nutrient medium (1% bactotryptone, 0.5% NaCl, w/v). Bacterial growth was assayed by measurement of the optical density at $A_{600}$ after a 24-h incubation at 30 °C.

An identical procedure was used to determine the minimal inhibitory concentration (MIC) of the molecules on the previously described bacterial strains. The MIC values are expressed as intervals of concentration (*a–b*), where *a* is the highest concentration at which bacteria are growing and *b* is the lowest concentration that causes 100% of growth inhibition (36).

*Bacteriostatic Assay*—A midlogarithmic phase culture of *M. luteus* in Poor-Broth nutrient medium was incubated at 30 °C in the presence of the antimicrobial peptides of interest or water (control). The final concentration of the molecules to be tested was eight times over the MIC value. 20-μl aliquots were removed at different time intervals and plated on nutrient agar. The number of colony-forming units was determined after 24 h at 37 °C.

*Antifungal Assay*—Antifungal activity was monitored against *N. crassa* and *F. oxysporum* as described previously (11, 13) by a liquid growth inhibition assay. Briefly, 80 μl of fungal spores (final concentration, $10^4$ spores/ml) suspended in potato dextrose broth (Difco) at half-strength supplemented with tetracycline (10 μg/ml) and cefotaxim (100 μg/ml) were added to 10 μl of fractions in microtitration plates. The final volume was brought to 100 μl by the addition of 10 μl of water. Growth inhibition can be observed microscopically after a 24-h incubation at 25 °C in the dark and measured by the increase in optical density (at 600 nm) after 48 h.

### *Penaeidin-specific DNA Probe and Screening of cDNA Library*

Poly(A)$^+$ RNA from juvenile shrimp hemocytes harvested 6 and 12 h after a bacterial challenge were used to construct a cDNA library in the ZAP Express vector (Stratagene, La Jolla, CA) following the manufacturer's instructions.

Reverse transcription and polymerase chain reaction (PCR) were used to prepare a DNA probe corresponding to the P3 peptide (see "Results") isolated from *P. vannamei*. From the peptide sequence obtained by Edman degradation, a degenerate oligonucleotide probe pool corresponding to the residues 38–44 of the mature molecule was designed by back translation: 5′-GGIAT(A/T/C)(A/T)(G/C)ITT(C/T)(A/T)(G/C)ICA(A/G)GC-3′ (see Fig. 4*A*). 3 μg of total hemocyte RNA were submitted to reverse transcription using the Ready-to-Go You-prime first-strand beads kit (Pharmacia Biotech Inc., Uppsala, Sweden) with a 18-base poly(dT) oligonucleotide as primer. One-fifth of the reaction was directly used as a template for polymerase chain reaction with the degenerate pool primers and the poly(dT) oligonucleotide. PCR was performed with five cycles consisting of 1 min at 94 °C, 1 min at 37 °C, and 1 min at 72 °C and 35 cycles consisting of 1 min at 94 °C, 1 min at 50 °C, and 1 min at 72 °C in 1.5 mM $MgCl_2$ and 1 μM primers.

The resulting 497-base pair fragment corresponding to a fragment of P3 cDNA was sequenced and a 440-base pair subfragment, for the most part consisting of the 3′-untranslated region, was generated by *Bsa*AI enzymatic hydrolysis (see Fig. 4*A*). This fragment was cloned into a pBluescript vector (Stratagene). It was labeled by random priming using the Ready-to-Go DNA labeling kit (Pharmacia) and used to screen 500,000 plaques from the cDNA library transferred to Hybond-N filter membranes (Amersham Corp.). High stringency hybridizations were carried out overnight at 65 °C in 5 × Denhardt's solution, 5 × SSPE (1 × SSPE = 150 mM NaCl, 1.25 mM EDTA, 10 mM sodium phosphate, pH 7.4), 0.1% SDS, 100 μg/ml salmon sperm DNA. The filters were washed in a solution of 0.5 × SSPE containing 0.1% SDS at 65 °C followed by autoradiography. A secondary screening was performed to purify the positive plaques. Phagemids were obtained by *in vivo* excision according to the manufacturer's instructions and sequenced on both strands.

A screening at low stringency was performed to isolate other members of the family. A probe was generated by PCR on a P3 cDNA clone with 5′-GTGTACAAGGGCGGTTACACG-3′ as the upstream primer and 5′-CAACAGGTTGTCAAGCGAGGT-3′ as the downstream primer. The amplified fragment consisted mainly of the P3 open reading frame (ORF). Radiolabeling and hybridization were identical to those pre-

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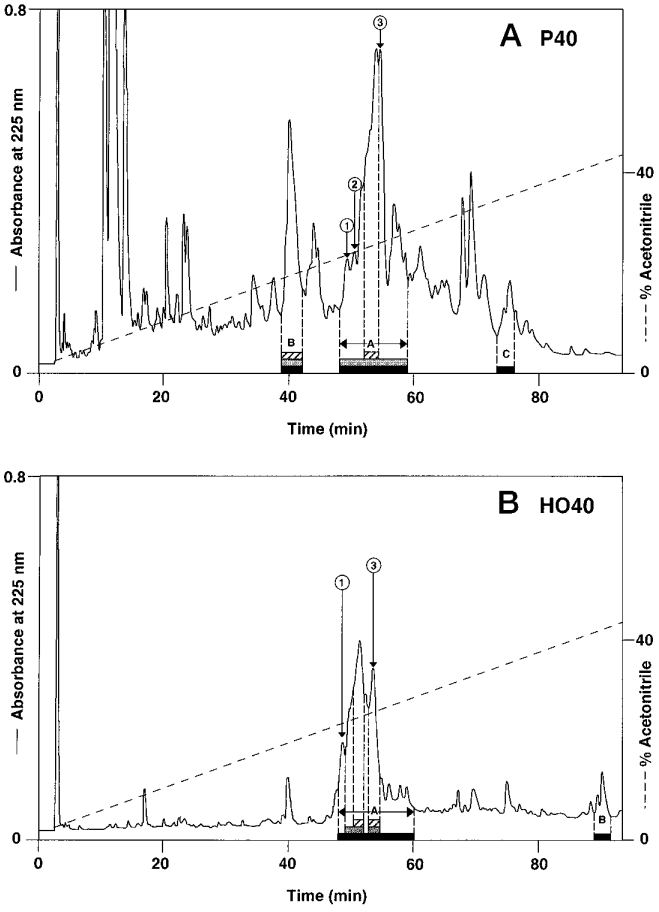


FIG. 1. **Reversed-phase HPLC of two acidic extracts obtained from plasma (*A*) and hemocytes (*B*) of experimentally uninfected *P. vannamei*.** The fractions eluted with 40% acetonitrile in acidified water from Sep-Pak $C_{18}$ cartridges (P40 and HO40) were analyzed on an Aquapore RP300 $C_8$ column using a 2–60% linear gradient (*dashed line*) of acidified acetonitrile over 80 min (0.72% acetonitrile/min) at a flow rate of 1 ml/min. Absorbance was monitored at 225 nm (*solid line*). Antimicrobial activity against *E. coli* D31 (*hatched rectangles*), *M. luteus* (*black shaded rectangles*), and *N. crassa* (*gray shaded rectangles*) was measured by liquid growth inhibition assays. The regions displaying antimicrobial activity are indicated by letters (*A*, *B*, and *C*). Fractions containing the peptides characterized in this study are indicated by *arrows* (*1*, *2*, and *3*).

viously described with the exception of a reduced hybridization temperature (50 °C).

## RESULTS

*Isolation of Antimicrobial Peptides from P. vannamei Hemolymph*—Antimicrobial peptides were purified from about 225 ml of hemolymph prepared from *P. vannamei* shrimps collected in an intensive culture farm and that had not been experimentally bacteria-challenged. Three different acid extracts were prepared, one from plasma (cell free hemolymph; P) and two from hemocytes: cytosolic (HC) and organelle-rich (cellular debris; HO) fractions (see "Materials and Methods"). The three extracts were directly applied to Sep-Pak $C_{18}$ cartridges. Elutions were successively performed with 5, 40, and 80% solutions of acetonitrile in acidified water. We focused our attention mainly on the 40% Sep-Pak fraction issued from the plasmatic extract, from the hemocyte organelles, and from the cytosolic fraction referred to as P40, HO40, and HC40, respectively. The three samples were further fractionated by reversed-phase HPLC using a linear gradient of 2–60% of acetonitrile over 80 min (0.72% acetonitrile/min). All the fractions were assayed for their activities against two bacterial strains (Gram-positive *M. luteus* and Gram-negative *E. coli* D31) and against the filamentous fungus *N. crassa*. No activity was recorded for any of the eluted fractions issued from chromatography of the HC40 fraction. On the other hand, fractions eluted from P40 (Fig. 1*A*) and HO40 (Fig. 1*B*) samples presented antimicrobial activity and shared a common active zone (P40A and HO40A) corresponding to fractions eluted between 47 and 60 min (26–29% of acetonitrile), which displayed activities against the two bacterial strains and the fungus. Two additional antimicrobial regions were obtained after reversed-phase chromatography of the plasmatic fraction: (i) P40B, which is eluted around 40 min (21–22% of acetonitrile), contains molecules active against the three microorganisms tested, and (ii) P40C, which corresponds to molecules eluted at approximately 75 min (38–39% of acetonitrile), exhibits activity exclusively against *M. luteus*. Activity was not detected in either of these two regions for HO40; however, activity was found against *M. luteus* in another set of fractions eluted at a retention time around 90 min (45–46% of acetonitrile) (HO40B; Fig. 1*B*).

The present study concerns exclusively the purification of the antimicrobial molecules found in the P40A and HO40A fractions, the two main zones of activity. We chose to concentrate in particular on the P40A fractions. The P40A fractions 1, 2, and 3 (Fig. 1*A*) were subjected to four successive purification steps consisting of size exclusion by HPLC followed by three different reversed-phase HPLC steps (see "Materials and Methods"). The antimicrobial activities against the three test microorganisms (*M. luteus*, *E. coli* D31, and *N. crassa*) were monitored during the different steps on aliquots of purified fractions. Finally, three peptides P1, P2, and P3 corresponding to the fractions 1, 2, and 3, respectively, were purified to homogeneity as monitored by capillary zone electrophoresis (data not shown) and submitted to further chemical characterization.

*Primary Structure Determination of Three Antimicrobial Peptides*—The three peptides purified from plasma were separately analyzed for their primary structure. Mass measurement by MALDI-MS on the closely eluted molecules, P1 and P2, showed very similar masses at 5484.8 and 5520.0 Da (data not shown), respectively. P3 presented a higher mass at 6617.4 Da (data not shown).

Following the mass spectrometry measurements, P1 (5484.8 Da) was subjected to Edman degradation. The following partial 31-residue proline-rich $NH_2$-terminal sequence was obtained: Tyr-Arg-Gly-Gly-Tyr-Thr-Gly-Pro-Ile-Pro-Arg-Pro-Pro-Pro-Ile-Gly-Arg-Pro-Pro-Leu-Arg-Leu-Val-Val-Xaa-Ala-Xaa-Tyr-Arg-Leu-Ser. No phenylthiohydantoin signals were obtained in positions 25 and 27. To gain additional information about the P1 sequence, the molecule was subjected sequentially to trypsin and α-chymotrypsin treatments. Trypsin cleavage generated a molecule at 5163.8 Da, which corresponds to P1 missing the dipeptide Tyr-Arg, and then α-chymotrypsin treatment on the 5163.8-Da molecule generated a 4888-Da peptide by removal of the $NH_2$-terminal tripeptide Gly-Gly-Tyr. No additional internal cleavage was observed by MALDI-TOF-MS. To get additional structural information on the COOH-terminal part, an aliquot of the peptide was subjected to reduction and *S*-pyridylethylation. First, the *S*-pyridylethylated peptide was subjected to MALDI-TOF-MS measurement. The mass obtained (6122.3 Da) was 637.5 Da greater than the mass of the native peptide (5484.8 Da), which corresponds to the presence of 6 alkylated cysteines. In addition, the *S*-pyridylethylated peptide P1 was treated with trypsin, α-chymotrypsin, and arginyl endopeptidase. The resulting peptides were analyzed by MALDI-TOF-MS, and the selected fragments were sequenced by Edman degradation. The sequences from overlapping fragments were compared, and from this we were able to deduce the entire amino acid sequence of P1 (Fig. 2). Comparison of the mass

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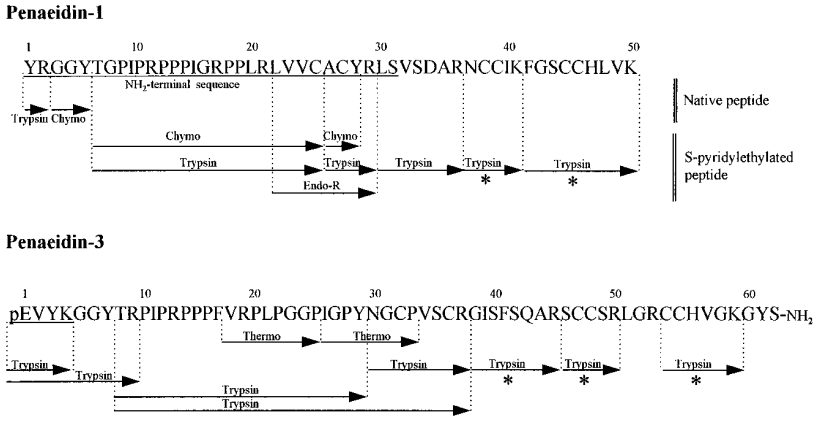


FIG. 2. **Amino acid sequences of penaeidin-1 and 3 isolated from the hemolymph of *P. vannamei*.** The *arrows* indicate the sequences of the fragments obtained after trypsin, α-chymotrypsin (*Chymo*), arginyl endopeptidase (*Endo-R*), and thermolysin (*Thermo*) treatments. For penaeidin-1, the enzymatic treatments were performed on the native and *S*-pyridylethylated peptide. The *underlined* amino acids correspond to direct $NH_2$-terminal sequencing on native penaeidin-1. The penaeidin-3 *underlined* sequence was obtained by nanoES-MS-MS, and *pE* stands for a pyroglutamic acid. *Asterisks* indicate peptidic fragments that could not be positioned in the molecules by a biochemical approach (no overlap).

calculated from the primary structure (5485.6 Da) with the mass measured by mass spectrometry (5484.8 Da) suggested first that we had the full sequence of the molecule and second that the six cysteines were forming three intramolecular disulfide bridges. However, the assignment of the COOH-terminal sequence was not possible using this biochemical approach because no overlapping fragments were obtained in this region (Fig. 2). From our results, P1 is a 50-residue peptide of which 7 of the first 19 amino acids are proline. In the COOH-terminal domain, there are 6 cysteines engaged in three intramolecular disulfide bridges, with 4 of the cysteines occurring in two doublets. In addition, this molecule is particularly rich in basic residues (5 arginines and 2 lysines) distributed all along the peptide, giving a calculated pI of 9.34. Searches in the SWISS-PROT protein data base revealed no significant sequence similarity to other antimicrobial peptides. For this reason, this molecule, which appears to be novel, was named penaeidin-1 after the genus *Penaeus*.

Only a partial sequence of 21 residues could be determined by Edman degradation for the P2 peptide (5520 Da). This $NH_2$-terminal sequence differed from penaeidin-1 at one position, leucine 20 in P1 being replaced by a phenylalanine in P2. As there was not sufficient material for further structural characterization by protease cleavage, we only performed reduction and *S*-pyridylethylation on P2. After this treatment, a mass increase of 635.6 Da suggested the presence of 6 cysteines in the remaining COOH-terminal part of the molecule. This peptide, which apparently belongs to the same family as penaeidin-1 was named penaeidin-2.

The third 6617.4-Da antimicrobial peptide (P3) that we have purified to homogeneity was initially subjected to sequencing by Edman degradation at a same quantity as for P1 and P2. Unfortunately, no phenylthiohydantoin signal was detected, suggesting that the molecule was $NH_2$-terminally blocked. *S*-Pyridylethylation followed by MALDI-TOF mass spectrometry analysis, demonstrated the presence of 6 cysteines as deduced from the mass variation (636 Da) observed between the native and the *S*-pyridylethylated peptide. To gain structural information, the *S*-pyridylethylated peptide was further submitted to cleavage, first with trypsin and then with thermolysin. All the fragments purified by reversed-phase HPLC were analyzed by mass spectrometry and Edman degradation (Fig. 2). One of the fragments from the tryptic digest, a fragment of 519 Da, was resistant to Edman degradation, suggesting that it corresponded to the P3 $NH_2$-terminal segment.

The $NH_2$-terminally blocked peptide with $MH^+$ at *m/z* 520 has been shown to be pure by reversed-phase HPLC, MALDI-TOF-MS, and ES-MS analysis (data not shown). Due to the relatively low picomolar amount of peptide, tandem mass spectrometry was performed using nanoES. It was thus possible to

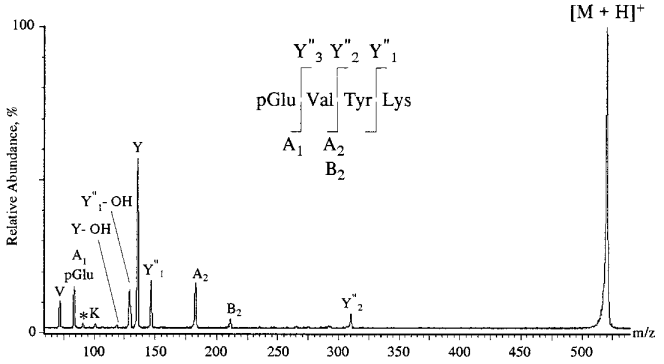


FIG. 3. **Daughter ions observed by nanoES-MS-MS of the molecular ion (*m/z* = 520) of the $NH_2$-terminally blocked tryptic peptide of the penaeidin-3.** The fragmentation data shown here, combined with other mass-spectral data (Table I), allowed deduction of the pGlu-Val-Tyr-Lys sequence.

extend the measuring time to about 3 h. Source fragmentation and MS-MS on the molecular ion *m/z* 520 yielded a series of ions and fragments, which allowed us to propose the following sequence: pGlu-Val-Tyr-Lys (Fig. 3).

To confirm this proposed sequence, a series of MS-MS experiments were performed on several first generation fragment ions. These first generation fragment ions, produced in the source, were successively selected by the first quadrupole and submitted to collision by the collision cell, and the fragments were analyzed with the second quadrupole. Results are presented in Table I, and all the second generation fragment ions observed were in agreement with the sequence proposed above, such as the ion at *m/z* 409, which yielded the Val-Tyr-Lys sequence (ions: $Y''_1$, $B_4$, $Y''_2$, $B''_3$, $B_3$, $A_3$, $Y''_3$, $I_3$, $I_4$, $I_2$, and $A_2$), and the ion at *m/z* 183, which corresponded to the sequence pGlu-Val (ions: $A_2$, $A_1$ and $I_2$) (Table I). This MS-MS analysis clearly established that the $NH_2$-terminal amino acid of P3 is a pyroglutamic acid.

From the results of the above experiments, a $NH_2$-terminal amino acid sequence of 37 residues could be unambiguously established for P3 (Fig. 2). However, three additional tryptic fragments belonging to the COOH-terminal domain: Gly-Ile-Ser-Phe-Ser-Gln-Ala-Arg, Ser-Cys-Cys-Ser-Arg, and Cys-Cys-His-Val-Gly-Lys could not be ordered. Moreover, the mass calculated from all the sequenced fragments was not in agreement with the mass measured by MALDI-MS, indicating that some amino acids were missing. P3 clearly belongs to the same family as penaeidin-1 and -2 and was therefore named penaeidin-3. However, penaeidin-3 is longer than the two other peptides and is post-translationally modified by cyclization of the $NH_2$-terminal residue to a pyroglutamic acid.

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TABLE I

*Amino acid sequence deduced from the nanoES-MS-MS study of the $NH_2$-terminally blocked tryptic fragment of penaeidin-3*

MS, molecular ion ($m/z$ = 520) and its fragments generated in source by high cone voltage. MS-MS, daughter ions observed by MS-MS for the molecular ion ($m/z$ = 520) and for the fragment ions generated in source ($m/z$ = 409, 310, and 183). The fragments were named according to Roepstorff and Fohlman's nomenclature (34), and the related ion masses observed for Lys and Tyr were named according to that of Falick *et al.* (37).

| MS | pGlu-Val-Tyr-Lys | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 520.1 [pGlu Val Tyr Lys + H]$^+$ | | 409.1 [Val Tyr Lys + H]$^+$ | | 310.1 [Tyr Lys + H]$^+$ | | 182.9 [pGlu Val + H]$^+$ | |
| MS-MS | 520.4 | $Y''_4$ | 409.2 | $Y''_3$ | 310.2 | $Y''_2$ | 182.9 | $A_2$ |
| | 310.3 | $Y''_2$ | 390.7 | $B_4$ | 147.2 | $Y''_1$ | 84.2 | $A_1$ |
| | 211.2 | $B_2$ | 310.3 | $Y''_2$ | 136.0 | $I_3$ | 72.1 | $I_2$ |
| | 183.0 | $A_2$ | 265.2 | $B''_3$ | 129.3 | Related ion mass of Lys | | |
| | 146.8 | $Y''_1$ | 262.9 | $B_3$ | 118.7 | $I_3$-OH | | |
| | 135.9 | $I_3$ | 235.0 | $A_3$ | 107.0 | Related ion mass of Tyr | | |
| | 129.3 | $C''_1$ | 147.0 | $Y''_1$ | 101.2 | $I_4$ | | |
| | 118.7 | $I_3$-OH | 135.8 | $I_3$ | 91.0 | Related ion mass of Tyr | | |
| | 100.9 | $I_4$ | 100.7 | $I_4$ | 84.0 | Related ion mass of Lys | | |
| | 90.9 | Related ion mass of Tyr | 83.7 | $I_4$-$NH_2$ | | | | |
| | 84.0 | $A_1$, $I_1$ | 71.9 | $A_2$, $I_2$ | | | | |
| | 72.0 | $I_2$ | | | | | | |

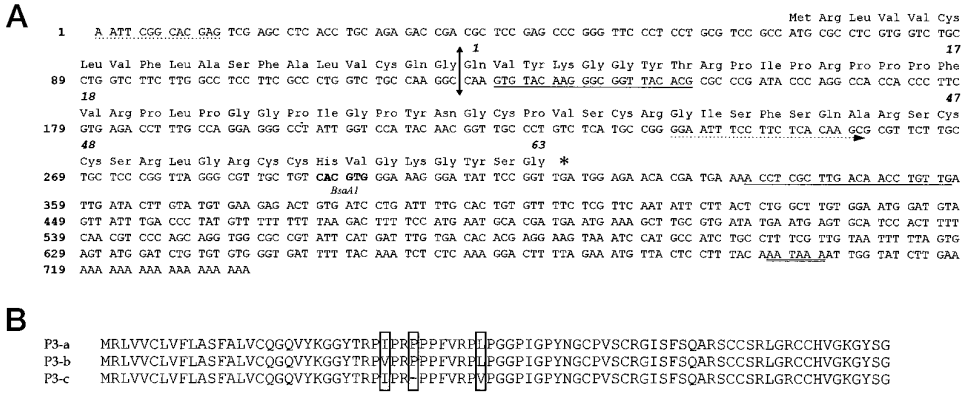


FIG. 4. *A*, nucleotide sequence of a penaeidin-3 cDNA clone from the shrimp *P. vannamei*. The deduced amino acid sequence of the ORF is shown *above* the nucleotide sequence. An *asterisk* indicates the stop codon. A polyadenylation signal is *double-underlined*. The *double-headed arrow* indicates the putative cleavage site by a signal peptidase. A *dotted arrow* indicates the position of the degenerate probe pool used in reverse transcription-PCR experiments to generate a probe for penaeidin-3 cDNA isolation. A *Bsa*AI restriction site used in cloning of a probe for penaeidin-3 cDNA isolation is in *boldface type*. The *underlined* sequences correspond to the primers used to generate a probe for penaeidin-2 cDNA isolation. The *Eco*RI linker used in construction of the cDNA library is indicated by a *dotted line*. *B*, alignment of the deduced amino acid sequences of the three penaeidin-3 cDNA clones isolated. Sequence differences are in *boxes*. The *dash* represents a gap at the indicated position.

Finally, we have demonstrated using the same strategy that the antimicrobial peptides present in the HO40A region (Fig. 1*B*) purified from an acid extract of the organelle-rich fraction of hemocytes were identical to penaeidin-1 and -3.

*Cloning of cDNAs Encoding Penaeidin-3*—To fully identify the amino acid sequences of the different penaeidins, we have prepared a size-selected cDNA library from the hemocytes of bacteria-challenged *P. vannamei*. To isolate the penaeidin-3 cDNA, degenerate oligonucleotides corresponding to the segment composed of residues 38–44 of the mature peptide were designed and used in reverse transcription-PCR experiments with a poly(dT) oligonucleotide on RNA extracted from bacteria-challenged shrimp hemocytes. A 497-base pair PCR fragment was identified by sequencing to be a P3 cDNA fragment consisting of the end of the ORF and the 3′-untranslated region. The 3′-untranslated region, which was supposed to be less conserved among the three peptides, was cloned and used to screen the hemocyte cDNA library in an attempt to isolate the P3 cDNA. Among the 161 hybridization-positive clones obtained, four were sequenced. One of them contained an ORF encoding an 82-amino acid sequence (P3-a) starting with a methionine codon and ending with a stop codon (Fig. 4*A*). The deduced amino acid sequence begins with a 19-residue signal peptide rich in hydrophobic amino acids. The cleavage site for signal peptidase is most likely located after the glycine residue preceding the glutamine at position 1 as predicted by the SignalP VI.1 software (38). This signal peptide is directly COOH-terminally flanked by a 63-amino acid sequence starting with a glutamine residue and ending with a glycine residue. This sequence clearly confirmed the partial sequences of penaeidin-3 obtained by the biochemical methods discussed above and allowed us to unambiguously establish the complete penaeidin-3 primary structure. Assuming that the mature peptide started with a pyroglutamic acid (cyclization of the glutamine residue) (39, 40), as observed by nanoES-MS-MS experiments, the mass calculated from the deduced amino acid sequence was 56.4 Da greater than the measured mass (6617.4 Da). This observation strongly suggests that penaeidin-3 can be COOH-terminally amidated by elimination of a glycine residue.

Among the three other clones sequenced, two different deduced amino acid sequences (P3-b and P3-c; Fig. 4*B*) were identified. P3-b differed from P3-a by the replacement of an isoleucine at position 30 in P3-a by a valine in P3-b, whereas P3-c lacked proline at position 33 compared with P3-a and P3-b. In addition, the leucine 40 of P3-a and P3-b was replaced by a valine residue in P3-c.

*Cloning of a cDNA Encoding Penaeidin-2*—Another screening was performed on the same filters using the p3-a ORF as a probe under less stringent hybridization conditions (50 °C). This DNA fragment corresponds to the penaeidin-3 coding re-

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                                                                                                                                                                Met Arg Leu Val Val
  1  AAT TCG GCA CGA GCT CCC TCT AGC CTC ACC TGC AGA GAC CGA CGC TCC GAG CCC GGG TTC CCT CCT GCG TCC GCC ATG CGC CTC GTG GTC
                                                                      1                                                                                                                                14
     Cys Leu Val Phe Leu Ala Ser Phe Ala Leu Val Cys Gln Gly Glu Ala Tyr Arg Gly Gly Tyr Thr Gly Pro Ile Pro Arg Pro Pro Pro
 91  TGC CTG GTC TTC TTG GCC TCC TTC GCC CTG GTC TGC CAA GGC GAA GCG TAC AGG GGC GGT TAC ACA GGC CCG ATA CCC AGG CCA CCA CCC
     15                                                                                                                                                                                                44
     Ile Gly Arg Pro Pro Phe Arg Pro Val Cys Asn Ala Cys Tyr Arg Leu Ser Val Ser Asp Ala Arg Asn Cys Cys Ile Lys Phe Gly Ser
181  ATT GGA AGA CCA CCG TTC AGA CCT GTT TGC AAT GCA TGC TAC AGA CTT TCC GTC TCA GAT GCT CGC AAT TGC TGC ATC AAG TTC GGA AGC
     45                          51
     Cys Cys His Leu Val Lys Gly  *
271  TGT TGT CAC TTA GTA AAA GGA TAA AGA AAT TGA CGG AGA AGA CAA TGG AAA CCT GGC TTG ACA ACT TGT TAA TTA ATA CTC ATA TGT GAA
361  GAG ATT GCA ACC CTG ATT TGT GTC AAG GAT GTG GGT ATT TCG TCT ATC CAT CGC TAA AGA TTC TTC CAT GAA TGT ATG ATG AAG GAA AGT
451  GCA TGT GTG TAA GTA TGT ATG TAT GTG CTT ACA GGT ATT TGT TGC ATT AAG TGT CCG TGT ATT TAG GAT CTG CAA CAC ACG AGG AAG AGA
541  ATA TTT GCC ACT TGC CAT TTA TTT CAG TTT CTG TAA GTG TGG ATC TGT GAG AGG TTG GTG TTG ACA GAT CTC TCT TTT ACA AAT AAA TTT
631  GAT ATC TGT GAA AAA AAA AAA AAA AAA A
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FIG. 5. **Nucleotide sequence of a penaeidin-2 cDNA clone from the shrimp *P. vannamei*.** The deduced amino acid sequence of the ORF is shown *above* the nucleotide sequence. An *asterisk* indicates the stop codon. A polyadenylation signal is *double-underlined*. The *double-headed arrow* indicates the putative cleavage site by a signal peptidase. The *Eco*RI linker used in construction of the cDNA library is indicated by a *dotted line*.

gion for which the translated sequence is strongly conserved among the three purified peptides. Positive clones that had not appeared in the previous screening were further studied, and eight of them were subjected to plaque purification. Only one of these clones differed from the clone encoding P3-a as determined by the pattern of fragments after hydrolysis by restriction enzymes and was therefore sequenced. The deduced amino acid sequence obtained was highly homologous to that of penaeidin-1 and -2, and the COOH-terminal domain was fully identical to the sequence established for penaeidin-1 by Edman degradation. From the deduced amino acid sequence, we were able to order the two tryptic fragments of penaeidin-1, which could not be placed after the biochemical analysis (Fig. 2). However, the presence of a phenylalanine residue at position 20 in the mature peptide (Fig. 5) was in favor of penaeidin-2 cDNA. This was confirmed by comparison of the mass values. Indeed, the mass calculated for the deduced amino acid sequence (5575.6 Da) was 55.6 Da (mass of a glycine residue) greater than the mass measured for penaeidin-2. These data strongly suggest that penaeidin-2 consists of a COOH-terminal domain fully identical to that of penaeidin-1, with an extra amino acid (glycine residue) probably involved in the COOH-terminal amidation of the mature peptide. From cDNA cloning data, penaeidin-2 is processed from a precursor molecule with a 21-residue pre-region identical to the penaeidin-3 signal peptide with the addition of 2 residues (Glu-Ala) immediately preceding the observed cleavage site (Fig. 5). This cleavage site was predicted by SignalP VI.1 software with an additional potential site, predicted at position −3 before the Tyr-1 residue. This alternate cleavage site corresponds to that observed in penaeidin-3 maturation.

*Antimicrobial Activity and Bacteriostatic Assay of Penaeidin-3*—In liquid growth inhibition assays, the purified penaeidin-3 had marked activity against *M. luteus* (MIC = 0.6–2.5 μM) and was moderately active against *E. coli* 363 (MIC > 5 μM). Penaeidin-3 was also found to be active against the two filamentous fungi tested: *N. crassa* (phytopathogen) and the penaeid shrimp pathogen *F. oxysporum* (MIC > 5 μM).

When penaeidin-3 was incubated at various time intervals with *M. luteus* at 18 μM, a concentration 8-fold higher than the MIC value, no growth of the bacteria was observed after a 24-h incubation (Table II) compared with a control experiment. Moreover, the number of colony forming units remained constant at the different incubation times tested, suggesting that penaeidin-3 does not kill *M. luteus* but rather inhibits its growth by a bacteriostatic effect.

TABLE II
*Bacteriostatic effect of penaeidin-3 on M. luteus*

Penaeidin-3 at a final concentration of 18 μM or water (control) was added to an exponential growth phase culture of *M. luteus*. Aliquots were removed at various times, and the number of colony forming units/ml (cfu/ml) was determined after an overnight incubation on LB agar plates at 37 °C.

| Time of incubation | Control | Penaeidin-3 |
|---|---|---|
| | *$10^4$ cfu/ml* | |
| 1 min | 3.85 | 4.30 |
| 30 min | 4.40 | 4.45 |
| 2 h | 6.70 | 3.15 |
| 4 h | 9.45 | 4.60 |
| 7 h | 51.50 | 4.50 |
| 24 h | >1000 | 5.60 |

## DISCUSSION

We report here the first isolation and full characterization of antimicrobial peptides from a crustacean (Decapoda). These peptides (penaeidin-1, -2, and -3) were purified from the plasma and hemocytes of experimentally uninfected shrimp *P. vannamei* (Penaeidae), which were obtained from an intensive Ecuadorian shrimp farm. Among the isolated peptides, three were purified to homogeneity and fully characterized at the level of their amino acid sequences, using a combination of reversed-phase chromatography, Edman degradation, and mass spectrometry (MALDI and nanoES). In addition, cDNA clones encoding two of the three antimicrobial peptides were isolated by screening a cDNA library prepared from hemocytes collected from bacteria challenged *P. vannamei*. Analysis of the deduced amino acid sequences revealed that the mature peptides are processed from precursor molecules, which have highly conserved signal peptides at their $NH_2$ termini. From the cDNA sequences, we were able to demonstrate that penaeidin-2 and -3 are extended by a glycine residue, which is probably eliminated by COOH-terminal amidation of these two molecules.

In shrimp, hemocytes have been demonstrated to be a key element of the defense system. As with other crustacean in the Decapoda order, hemocytes are involved in different immune responses such as the prophenoloxidase-activating system or the clotting reaction, which is mediated by the release of a hemocytic transglutaminase (2, 41, 42). From our studies and the preliminary results obtained from the shore crab, *C. maenas* (6), it can be inferred that hemocytes in Decapoda also participate in the production and storage of antimicrobial peptides. Our data show that the blood cells of the shrimp *P. vannamei* are a site of production and storage of the antimicrobial peptides isolated because they were found in the acid extract of a hemocytic organelle-rich fraction. Moreover, the cDNAs for these peptides were isolated from a library constructed with hemocyte mRNA. This implies that these antimicrobial molecules are processed from mRNA to active compounds within the hemocytes. This is also observed in the horseshoe crab *Tachypleus tridentatus* (Chelicerata). In this arthropod, the hemocytes are extremely sensitive to microbial substances such as lipopolysaccharides and β-glucans. Upon stimulation, the hemocytes degranulate and release into the extracellular fluid a series of substances involved in immune defense, including several antimicrobial peptides such as tachyplesins (14), big defensin (15), or tachycitin (16). A similar

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penaeidin-1     YRGGYTGPIPRPPPIGRPPLR-----LVVC--ACYRLSVSDARNCCIKFGSCCHLVK
penaeidin-2     YRGGYTGPIPRPPPIGRPPFR-------PVC-NACYRLSVSDARNCCIKFGSCCHLVK*
penaeidin-3  pE VYKGGYTRPIPRPPPFVRPLPGGPIGPYNGCPVSCRGISFSQARSCCSRLGRCCHVGKGYS*
```

FIG. 6. **Sequence comparison of penaeidin-1, -2, and -3 from *P. vannamei*.** The full penaeidin sequences obtained by a biochemical approach and completed by the cDNA cloning data were aligned. Gaps were introduced to optimize the alignment. Cysteines are in *boldface type*. Identical residues and conservative replacements are in *boxes*. *Asterisks* indicate COOH-terminal amidation, and *pE* stands for pyroglutamic acid.

mechanism is likely to occur in shrimp as suggested by the presence of antimicrobial peptides in the plasma of the animals used for this study. Indeed, although the animals were not infected experimentally, we can assume that they were subjected during their capture and intense manipulation to stress conditions leading to a hemocytic activation and partial degranulation. We do not know whether production of these molecules is induced upon infection or whether, as for the horseshoe crab, the peptides are stored in the hemocytes and released upon infection by hemocytic activation and partial degranulation. Further studies investigating the transcription profiles of penaeidins following microbial infection will address this question.

Penaeidin-1,-2 and -3 share many general characteristics with other antimicrobial peptides. They are cationic peptides with positive net charges of 7 for penaeidin-1 and -2 and 8 for penaeidin-3, containing 50 (penaeidin-1 and -2) and 62 residues (penaeidin-3). Their calculated isoelectric points vary from 9.34 for penaeidin-1 and -2 to 9.84 for penaeidin-3. In contrast to penaeidin-1 and -2, penaeidin-3 is $NH_2$-terminally blocked by a pyroglutamic acid. Identical $NH_2$-terminal blocking amino acids have already been observed in other antimicrobial peptides such as hymenoptaecin (36) or some bovine $\beta$-defensins (43). The analysis of penaeidin-2 and -3 cDNAs showed the presence of a glycine codon at final position in the ORF. However, the experimentally determined masses clearly indicate that the glycine residue is eliminated in the mature peptide. Therefore, we can assume that the two peptides are COOH-terminally amidated. Because no cDNA clone has been sequenced for penaeidin-1, we do not yet have any conclusive evidence about the possible amidation of the COOH terminus of the molecule. Such a COOH-terminal amidation has also been observed in other marine invertebrate antimicrobial peptides such as the tachyplesins from *T. tridentatus* (14) and their *Limulus polyphemus* analogues, the polyphemusins (44). This modification has also been described in the insect cecropins (45), and in vertebrate antimicrobial peptides (*e.g.* magainins) (46), where it was shown to be functionally important by increasing antimicrobial activity compared with the same peptides, which have a free carboxyl group.

The overall structure of the three peptides isolated in *P. vannamei*, is unique in that it consists of a $NH_2$-terminal domain rich in proline residues and a cysteine-rich COOH-terminal region. The three penaeidins are composed of a proline-rich $NH_2$-terminal domain and a COOH-terminal domain containing 6 cysteines engaged in the formation of three intramolecular disulfide bridges. 4 of the 6 cysteines are organized in two doublets separated by 5 residues. The central-most cysteines are separated by 1, 2, or 3 residues in penaeidin-1, -2, and -3, respectively (Fig. 6). Penaeidins contain the same number of cysteines as the arthropod and mammalian defensins, the $\beta$-defensins (for review see Ref. 7), or the *T. tridentatus* big defensin (15). However, the cysteine motif in penaeidins has no significant homology with those found in any of the molecules mentioned above. For example, the cysteine stabilized $\alpha\beta$ motif characteristic of insect and plant defensins (47–49) as well as some scorpion toxins (50), which stabilizes an $\alpha$-helix on a $\beta$-sheet through two disulfide bridges (48), is not found in the penaeidins. Moreover, as the penaeidin disulfide bridge positions are still unknown, we cannot predict the three-dimensional structure by homology with any of the antimicrobial peptides whose structures have been characterized to date. These striking features led to the creation of a novel family, the penaeidins, after the genus *Penaeus*, in which the molecules are characterized by post-translational modifications (COOH-terminal amidation and/or $NH_2$-terminal cyclization by a pyroglutamic acid) and the presence of three intramolecular disulfide bridges, features that confer to the peptides a high stability as observed by their high resistance to proteolysis.

During preliminary HPLC purification steps, shrimp hemolymph was shown to display various zones with bactericidal or bacteriostatic activities (data not shown) corresponding to cationic molecules (peptidic or nonpeptidic substances), which will be the subject of further studies. Among these, the penaeidins alone were isolated and characterized. They are essentially active against Gram-positive bacteria and are much less active against the Gram-negative bacteria tested. Like apidaecins (20), penaeidins were found to have a bacteriostatic effect at the concentrations tested. This suggests that penaeidins do not display a lytic activity on bacteria but rather interfere with cell propagation. Penaeidins were also shown to display activity against the fungal test strain *N. crassa* and against a fungus *F. oxysporum* shown to be pathogenic for shrimp as other members of the genus *Fusarium* (51, 52). Unfortunately, the quantities of all the peptides obtained in the present study were insufficient to conduct a more exhaustive antimicrobial activity spectrum. In this respect the production of recombinant penaeidins will be of special interest. In addition, it will be very important to analyze the activity of the peptides under conditions that more closely resemble the physiological parameters of shrimp, *i.e.* pH and salt, because several antimicrobial peptides have been shown to be salt-sensitive (53). Our tests were performed under standard conditions that do not correspond to the osmolarity of shrimp extracellular fluid or intracellular pH ranges. It will also be of great interest to check the activity of the penaeidins against a variety of pathogens such as the fungi *Lagenidium sp.* (54), which infects the larvae, and different bacterial species such as *V. harveyi* (55) or *V. penaeicidae* (56), which are associated with shrimp diseases, and against viruses that represent the most serious pathogens for shrimp (29).

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