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

(Received for publication, July 8, 1997, and in revised form, September 11, 1997)

Delphine Destoumieux‡, Philippe Bulet§, Damarys Loewi, Alain Van Dorsselaer, Jenny Rodriguez**, and Evelyne Bachère‡ ‡

From the ‡Institut Français de Recherche et d’Exploitation de la Mer/CNRS, Unité Mixte de Recherche 219, “Défense et Résistance chez les Invertébrés Marins,” Université de Montpellier 2, CC 80, 34095 Montpellier, France, the §Institut de Biologie Moléculaire et Cellulaire, Unité Propre de Recherche 9022, CNRS, “Réponse Immunitaire et Développement chez les Insectes,” 15 rue René Descartes, 67084 Strasbourg Cedex, France, the ‡Laboratoire de Spectrométrie de Masse Bio-Organique, Unité de Recherche Associée 31, CNRS-Université Louis Pasteur, Faculté de Chimie, 1 rue Blaise Pascal, 67008 Strasbourg Cedex, France, and the **Centro Nacional de Acuicultura e Investigaciones Marinas-Escuela Superior Politécnica del Litoral, Guayaquil, Ecuador

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 NH₂-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 pyrogulamic 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 microbialic mechanisms (1), the prophologidase 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 (Chelicerrata, 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 hemocyte 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 α-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), thannatin from Podisus (13), tachyplein, 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 other 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
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 penaei-<ref>1</ref>ns, 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 100 volume of anticoagulant buffer (10% sodium citrate, pH 7) supplemented with 200 µM phenylthiourea as a melanization inhibitor and 40 µg/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 separated by centrifuging at 7000 × g at 4 °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 until use.

**Hemocyte Sample**—After thawing, the hemocytes were homogenized using a Dounce apparatus (maximum, 152 ml) in 50 mM Tris buffer, pH 8.5, containing 50 mM NaCl. After centrifugation (8000 × g, 20 min, 4 °C), the supernatant (cytosolic fraction) was acidified to pH 3.9 with 1 M HCl until further purification. The pellet containing cellular organelles was then sonicated (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 organellar acid extract was kept at 4 °C until use.

**Solid Phase Extraction Prepurification**

The plasmatic fraction and the cellular cytosolic and organellar 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 subjecting to reversed-phase HPLC and 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 (Ultra- spherogel SEC 30000 and SEC 2000 column, 7.5 × 300 mm, Beckman) 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 from 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$_8$ 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 photo diode 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 (Savant), and reconstituted in MilliQ water (Miliapore) before antimicrobial activity was tested.

**Capillary Zone Electrophoresis**

Peptide purity was ascertained by capillary zone electrophoresis. Analysis was performed on 2 ml of fractions using a 270A-HT electrophoresis system (Agilent Bioanaysis, Inc.) equipped with a fused silica capillary (length, 72 cm; internal diameter, 50 µm). 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-Pyridylethylolation**

Purified peptides were subjected to reduction and alkylation using the procedures already described (30). Briefly, the peptide (1–2 nmol) was dissolved in 40 µl of 0.5 M Tris HCl containing 2 mM EDTA and 6 mM guanidine hydrochloride, pH 7.5, to which 2 µl of 2.2 M dithiothreitol were added. The samples were incubated under oxygen-free conditions for 1 h at 45 °C. 2 µl 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 2–80% 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 a-Chymotrypsin Treatments**—Native and S-pyridylethylated peptides (5 µg) were subjected individually to trypsin and a-chymotrypsin treatments (Boehringer Mannheim). Trypsin and chymotrypsin hydrolyses were performed at an enzyme/substrate ratio of 1/20 (w/w) in a 40-µl reaction containing 0.1 ml Tris-HCl at pH 8.5 and in a 50-µl 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 a-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 (0.85% acetonitrile/min) at a flow rate of 0.25 ml/min.

**Arginyl Endopeptidase Treatment**—The S-pyridylethylated peptide (5 µg) was treated with arginyl endopeptidase (Takara, Otsu) at an enzyme/substrate ratio of 1:100 (w/w) in a 20-µl 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.
**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 CaCl₂. The digestion was stopped by adding 50 μl of 0.5 M trichloroacetic acid. The precipitated hydrolysates 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 multisoloscope. A camera mounted on a microscope allowed the inspection of the sample crystalization 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.

**Sequence by Nanoelectrospray Tandem Mass Spectrometry**

**Instrumentation**—The nanoelectrospray (nanoES) experiments were done on a triple quadrupole Bio-Q mass spectrometer, upgraded by the manufacturer with SCOUT™ High Resolution Optics, and equipped with SCOUT™ linear time 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 open glass capillary was then washed by applying a N₂ 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 (Vc = 30 V) was selected in the first quadrupole mass analyzer and fragmented by collision-induced dissociation with argon gas at 4.5 × 10⁻⁶ Pa at 40 V. To gain additional structural information, MS-MS experiments were 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 peptic 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, 55). *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. Christolles-Ales, 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 agar diffusion method 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 A₅₆₀ = 0.001 in Poor-Broth nutrient medium (1% bacitracine, 0.5% NaCl, w/v). Bacterial growth was assayed by measurement of the optical density at A₅₆₀ 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, 10⁶ 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 microlitration 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 was constructed that is complementary to the residues 38–44 of the mature molecule was designed by back translation: 5’-GGIAATATCT/CAT/G(C/T)TT/CAT/A/TT/GC/ICA/A/GC-3’ (see Fig. 4A). 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 mixture 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 MgCl₂ 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 for penaeid shrimp (gift from Dr. Alain Vey, INRA, St. Christolles-Ales, France) was used to complete the activity spectrum.

**Results**—Isolated from *Platylus* sp. (Penaeus japonicus), Penaeidins are a family of peptides, having antimicrobial, antifungal, and antibacterial effects. The chemical structures of these peptides have been described, and their antifungal, antibacterial, and antihelminthic activity have been demonstrated in vivo (36). The present work, by using MALDI-MS for the determination of the molecular mass of the isolated peptides, demonstrates that the most active Penaeidins are P1 and P2.
Antimicrobial peptides were purified from about 225 ml of hemolymph prepared from Penaeus vannamei shrimps collected in an intensive culture farm and that had not been experimentally bacteria-challenged. Three different acid extracts were directly applied to Sep-Pak C18 cartridges. Elu-

brisch; HO) fractions (see "Materials and Methods"). The three different fractions were further fractionated by reversed-phase HPLC using a linear gradient of 2–60% of acetonitrile over 80 min (0.72% ace-

tonitrile/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).

RESULTS

Isolation of Antimicrobial Peptides from P. vannamei Hemo-

lymph—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 C18 cartridges. Elu-

tions were successively performed with 5, 40, and 80% solu-
tions of acetonitrile in acidified water. We focused our attention mainly on the 40% Sep-Pak fraction issued from the plasmaic 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 filamen-
tous 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. 1A) and HO40 (Fig. 1B) samples presented antimicrobial activity and shared a common active zone (P40A and HO40A) correspond-
ing 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 ace-

tonitrile), 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. 1B).

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 concen-

trate in particular on the P40A fractions. The P40A fractions 1, 2, and 3 (Fig. 1A) 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 sepa-

rately analyzed for their primary structure. Mass measure-

ment 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 NH2-terminal sequence was obtained: Tyr-Arg-Gly-Gly-Tyr-Thr-Gly-Pro-Ile-Pro-Arg-Pro-Pro-Ile-Gly-Pro-Pro-Leu-Leu-Val-Val-Xaa-Ala-Xaa-Tyr-Arg-Leu-Ser. No phenylthiodyantoin signals were obtained in positions 25 and 27. To gain additional information about the P1 sequence, the molecule was subjected sequentially to trypsin and a-chymotrypsin treatments. Trypsin cleavage generated a molecule at 5163.8 Da, which corresponds to P1 missing the dipeptide Tyr-Arg, and then a-chymotrypsin treatment on the 5163.8-Da molecule generated a 4888-Da peptide by removal of the NH2-terminal tripeptide Gly-Gly-Tyr. No additional internal cleavage was observed by MALDI-TOF-MS. To get addi-

tional structural information on the COOH-terminal part, an aliquot of the peptide was subjected to reduction and S-pyri-
dylethylation. First, the S-pyridylethylated peptide was sub-

jected to MALDI-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, a-chymotrypsin, and arginyl endopeptidase. The resulting peptides were analyzed by MALDI-

TOF-MS, and the selected fragments were sequenced by Ed-
man 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).
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 after the genus Penaeus, has been shown to be pure by reversed-phase HPLC, MALDI-MS, and MS-MS analysis using nanoES. It was thus possible to 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$^0_1$, B$_0$, Y$^0_2$, B$_3$, B$_4$, A$_0$, Y$^0_3$, I$_3$, I$_4$, and A$_2$), and the ion at m/z 183, which corresponded to the sequence pGlu-Val (ions: A$_2$, A$_3$, and I$_3$) (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-Ser-Ser-Arg, and Cys-Cys-His-Val-Gly-Lys could not be order. 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.
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.

m/z molecular ion (of hemocytes were identical to penaeidin-1 and -3. B) purified from an acid extract of the organelle-rich fraction prepared a size-selected cDNA library from the hemocytes of

Finally, we have demonstrated using the same strategy that the antimicrobial peptides present in the HO40A region (Fig. 1B) 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 *P. vannamei* primary structure. Assuming that the mature peptide

**TABLE I**

| MS | 520.1 | 409.1 | 310.1 | 182.9 |
|----|-------|-------|-------|-------|
| [pGlu Val-Tyr-Lys + H]^+ | [Val Tyr-Lys + H]^+ | [Tyr-Lys + H]^+ | [Glu Val + H]^+ |
| MS-MS | 520.4 | 409.2 | 310.2 | 182.9 |
| 310.3 | 390.7 | B^+ | 147.2 | 84.2 |
| 211.2 | B_2 | 310.3 | Y^+ | 136.0 |
| 183.0 | A_1 | 265.2 | B^+ | 129.3 |
| 146.8 | Y | 262.9 | B_3 | 118.7 |
| 135.9 | C_1 | 255.0 | A_3 | 107.0 |
| 120.3 | C_2 | 147.0 | Y | 101.2 |
| 118.7 | I_3-OH | 135.8 | I_1 | 91.0 |
| 100.9 | I_4 | 100.7 | I_4 | 84.0 |
| 90.9 | Related ion mass of Tyr | 83.7 | I_3-NH_2 | |
| 84.0 | A_1, I | 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 *dotted arrow* indicates the putative cleavage site by a signal peptidase. A *dotted line* represents a gap at the indicated position.

**Penaeidins, Crustacean Antimicrobial Peptides**

**Cloning of cDNAs Encoding Penaeidin-2**—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-2 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 *P. vannamei* primary structure. Assuming that the mature peptide

**Cloning of a cDNA Encoding Penaeidin-2**—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-2 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 *P. vannamei* primary structure. Assuming that the mature peptide

**Cloning of a cDNA Encoding Penaeidin-2**—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-2 cDNA, degenerate oligonucleotides corresponding to the segment composed of residues 38–44 of the mature peptide were designed

**Cloning of a cDNA Encoding Penaeidin-2**—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-2 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 *P. vannamei* primary structure. Assuming that the mature peptide

**Cloning of a cDNA Encoding Penaeidin-2**—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-2 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 *P. vannamei* primary structure. Assuming that the mature peptide

**Cloning of a cDNA Encoding Penaeidin-2**—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-2 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 *P. vannamei* primary structure. Assuming that the mature peptide

**Cloning of a cDNA Encoding Penaeidin-2**—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-2 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 *P. vannamei* primary structure. Assuming that the mature peptide

**Cloning of a cDNA Encoding Penaeidin-2**—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-2 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 *P. vannamei* primary structure. Assuming that the mature peptide

**Cloning of a cDNA Encoding Penaeidin-2**—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-2 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 *P. vannamei* primary structure. Assuming that the mature peptide
Penaeidins, Crustacean Antimicrobial Peptides

**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 EcoRI linker used in construction of the cDNA library is indicated by a *dotted line*.

**TABLE II**

*) 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 |
|--------------------|---------|-------------|
| 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        |

For the text, please refer to the original document.
Penaeidins, Crustacean Antimicrobial Peptides

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. Cysteine 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 NH2-terminally blocked by a pyroglutamic acid. Identical NH2-terminal blocking amino acids have already been observed in other antimicrobial peptides such as hymenoptaecin (36) or some bovine β-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 tachylinesins from *T. tridentatus* (14) and their *Limulus polyphemus* analogues, the polypheumsins (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 NH2-terminal domain rich in proline residues and a cysteine-rich COOH-termini-

REFERENCES

1. Bachere, E., Mialhe, E., and Rodriguez, J. (1995) *Fish Shellfish Immunol.* 5, 597–612
2. Soderhall, K., Cerenius, L., and Johansson, M. W. (1996) in *New Directions in Invertebrate Immunology* (Soderhall, K., Iwanaga, S., and Vasta, G. R., eds) pp. 229–253, SOS Publications, Fair Haven, NJ
3. Iwanaga, S., Miuta, T., Shigenaga, T., Miura, Y., Seki, N., Saito, T., and Kawahata, S. (1994) *Ann. N. Y. Acad. Sci.* 712, 102–116
4. Hoffmann, J. A., Reichhart, J.-M., and Hetru, C. (1996) *Curr. Opin. Immunol.* 8, 13–23
5. Chisholm, J. R. S., and Smith, V. J. (1992) *J. Mar. Biol. Assoc. U. K.* 72, 529–542
6. Schnapp, D., Kemp, G. D., and Smith, V. J. (1996) *Eur. J. Biochem.* 240, 532–539
7. Hetru, C., Bulet, P., Cociancich, S., Dimarqu, J.-M., Hoffmann, D., and Hoffmann, J. A. (1994) in *Phylogenetic Perspectives in Immunity: The Insect Host Defense* (Hoffmann, J. A., Janeway, C. A., and Natori J. S., eds) pp. 43–66, CRC Press, Boca Raton, Fl.
