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Immunology

Insect immunity: Isolation from immune blood of the dipteran *Phormia terranovae* of two insect antibacterial peptides with sequence homology to rabbit lung macrophage bactericidal peptides

(insect defensins/Gram-positive bacteria/amino acid sequence/fast atom bombardment mass spectrometry)

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ABSTRACT **We have isolated from the hemolymph of immunized larvae of the dipteran insect *Phormia terranovae* two peptides that are selectively active against Gram-positive bacteria. They are positively charged peptides of 40 residues containing three intramolecular disulfide bridges and differ from one another by only a single amino acid. These peptides are neither functionally nor structurally related to any known insect immune response peptides but show significant homology to microbicidal cationic peptides from mammalian granulocytes (defensins). We propose the name "insect defensins" for these insect antibiotic peptides.**

Insects of various orders have been shown conclusively to build up an immune response after a first bacterial challenge or after an injury of the integument (reviewed in refs. 1 and 2). In lepidopterans, several antibacterial peptides are synthesized during this response: cecropins (4-kDa basic peptides; refs. 3 and 4), attacins (20- to 22-kDa basic or acidic proteins; ref. 5), and lysozyme (6). From the dipteran *Phormia terranovae*, we have recently isolated and identified a family of 8-kDa peptides, which we have named diptericins (7). They appear in the immune hemolymph of larvae together with cecropin-like (7) and other as-yet-unidentified antibacterial peptides. The immune hemolymph of this species does not contain lysozyme, a rather ubiquitous enzyme active on Gram-positive bacteria. However, we have repeatedly observed that this immune hemolymph contains a potent anti-Gram-positive activity that is clearly not attributable to diptericins, which are active on Gram-negative cells only (7). We have now isolated and characterized two 4-kDa peptides responsible for anti-Gram-positive activity of immune hemolymph of *P. terranovae*: they are 40-residue peptides differing by one single amino acid, as described in this paper.

MATERIALS AND METHODS

Bacterial Strains. The bacterial strains were gifts from the following colleagues: *Escherichia coli* D31 (streptomycin resistant) and *Enterobacter cloacae* β12 (nalidixic acid resistant), from H. Boman (University of Stockholm); *E. coli* 7624, *Pseudomonas aeruginosa* 76110, *Staphylococcus aureus* 7625, and *Bacillus subtilis* 6633, from Y. Piémont (University of Strasbourg); and *Bacillus thuringiensis* Berliner 1715 (streptomycin-resistant and rifampicin-resistant strains). *Bacillus megaterium* MA, *B. subtilis* (strains S3, Mo201, QB122, and QB935), and *E. coli* BZB 1011, from M. Lecadet, J. Millet, A. Klier, C. Campelli, and A. Pugsley (Pasteur Institute, Paris). *B. thuringiensis* 53137 and *Micrococcus luteus* A270 were obtained from the Pasteur Institute Collection, Paris. All strains were usually grown in Bertani's rich nutrient medium.

Immunization. Third-instar wandering larvae of *P. terranovae* were injured with thin needles soaked in a logarithmic-phase culture of *E. cloacae* β12. Hemolymph was collected after 24 hr by pricking the anterior part of the larvae, after which the body was gently squeezed to press out a drop (10–15 μl) of hemolymph, which was recovered in a precooled sterile plastic tube containing aprotinin (Sigma). No contamination by gut contents occurred with this method. The hemolymph from 3000 larvae was pooled per experiment and centrifuged at 36,000 × *g* for 20 min at 4°C. The cell-free supernatant, referred to as plasma, was deep-frozen (−70°C) until further use.

Antibacterial Assay. Assay conditions were essentially those described by Hultmark *et al.* (8). Sterile Petri dishes (diameter, 9 cm) received 7.5 ml of melted agar in buffered nutrient medium (Difco; pH 7.2) containing ≈2 × 10^5 logarithmic-phase cells of a given bacterial strain. Wells (2-mm diameter) were cut into the freshly poured plates after the solidification of the agar. Each well received a 2-μl sample of the liquid suspected to contain antibacterial molecules. The plates were incubated for 24 hr at 37°C, and the diameters of the clear zones were recorded, after subtraction of the well diameter.

Lysis Assay. Dried cells of *Micrococcus lysodeikticus* (=*M. luteus*; Sigma) were suspended at a concentration of 0.025% in 0.1 M ammonium acetate (pH 6.8), giving an absorbance of 0.9 at 600 nm. The presence of lysozyme-like activity was monitored at 20°C by recording loss of absorbance. Reference activity was from hen egg-white lysozyme (Merck).

Purification of Antibacterial Molecules. *Step I: Heat treatment.* In each experiment, some 30 ml of plasma of *P. terranovae* were collected from 3000 third-instar larvae and diluted as follows: 1 volume of 1 M acetic acid and 2 volumes of 40 mM ammonium acetate to 3 volumes of plasma. The mixture was then submitted to heat treatment (4 min in a boiling water bath) with constant stirring. The precipitate was removed and washed with 40 mM ammonium acetate adjusted to pH 6.8, and a final volume of 140 ml of supernatant was obtained, which will be referred to as the "heat-step supernatant."

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Step II: Cation-exchange chromatography. The heat-step supernatant was applied to a column (2.5 × 18 cm) of CM-Trisacryl (IBF/LKB) equilibrated with 40 mM ammonium acetate (pH 6.8). After the column was rinsed with 2000 ml of buffer, the molecules retained by the exchanger were eluted at the same pH with 1600 ml of a linear gradient of 40–500 mM ammonium acetate. Fractions (8 ml) were collected at a flow rate of 80 ml/hr during the exclusion step and of 40 ml/hr during the gradient step. Ultraviolet absorption was monitored at 280 nm. The antibacterial activity was assayed on a 2-μl aliquot of each fraction.

Step III: Sep-Pak cartridge fractionation. The active fractions corresponding to a peak containing anti-*M. luteus* activity detected in the preceding step were pooled and applied to a Sep-Pak cartridge (C_{18} Waters). Stepwise elution was performed with increasing proportions of acetonitrile in water acidified with 0.1% trifluoroacetic acid. Antibacterial activity was monitored on aliquots of the fractions that had been vacuum-dried to remove acetonitrile.

Step IV: Reversed-phase HPLC. The active fractions from the preceding step were freeze-dried, and the residue was dissolved in 100 μl of 0.11% trifluoroacetic acid in water and subjected to reversed-phase HPLC on a Bakerbond C_{18} WP column (0.46 × 25 cm). Elution was performed with a linear gradient of acetonitrile in water acidified with 0.1% trifluoroacetic acid. The flow rate was 1 ml/min, and 0.5-ml fractions were collected. Ultraviolet absorption was monitored at 210 nm, and antibacterial activity was determined on 2-μl aliquots of the fractions.

Reduction and Pyridylethylation. Reduction and pyridylethylation of cysteine residues was carried out in the vapor phase (9) by using tributylphosphine, 4-vinylpyridine, and pyridine in an evacuated tube at 60°C for 2 hr, with the sample deposited on a glass-fiber disc, which subsequently could be placed directly in the sequencer or dried in a small tube that could be subjected to vapor-phase hydrolysis before amino acid analysis.

Amino Acid Analysis. After the addition of 400 pmol of norleucine as internal standard and drying, samples for analysis were hydrolyzed in the vapor of 6 M HCl at 110°C for 20 hr *in vacuo*. After hydrolysis, amino acids were converted to their phenylthiocarbamyl derivatives by using phenylisothiocyanate essentially as described by the Waters Picotag manual. Phenylthiocarbamyl derivatives were then separated by reversed-phase HPLC on a Picotag column (0.39 × 15 cm; equilibrated with 0.14 M acetate buffer, pH 6.25/6% acetonitrile) by using a gradient of acetonitrile.

Carboxypeptidase Digestion. Carboxypeptidase digestion was done with carboxypeptidase Y (10) for 0, 1, 2, 4, 8, and 20 hr with norleucine as internal standard and was followed by amino acid analysis. One aliquot was hydrolyzed to determine the total amount of antibacterial peptide used.

Primary Structure Determination. Amino acid sequence was determined on the native and derivatized peptide by automatic sequential Edman degradation using an Applied Biosystems model 470A gas-phase sequencer equipped with a model 120 on-line analyzer for the characterization of the phenylthiohydantoin derivatives by reversed-phase HPLC. Detection was at 254 nm. Amounts of phenylthiohydantoin derivatives were quantified by comparing peak areas to those of the corresponding standards.

Mass Spectrometry. Positive mass spectra were obtained on a VG ZAB-SE double-focusing instrument (mass range, 15 kDa at 8-keV ion energy) and recorded on a VG 11–250 data system. Ionization of the sample was performed with ≈1 μA of 30-keV-energy cesium ions from the cesium ion gun normally fitted by VG Analytical. The high-resolution spectra were generated by voltage scanning over 300 Da, but at 4000 resolution. The scan rate was chosen in such a way that each scan took 15–20 s in the high- and low-resolution modes. In all cases, several scans were summed by operating the data system as a multichannel analyzer, generally over a total acquisition time of 1–2 min. Mass calibration was carried out with cesium iodide clusters from a separate introduction of cesium iodide. The total time to acquire and process the data was 10–15 min per sample. The underivatized peptides were dissolved in 5% (vol/vol) acetic acid at a concentration of ≈10 μg/μl. The matrix was 1-thioglycerol containing 1% trifluoroacetic acid. Typically 1 μl of matrix was deposited on the target and then 1 μl of the peptide solution was added and mixed with the matrix by using the tip of the needle. The conventional data system supplied by the manufacturer was used for processing the results. Multichannel analyzer data were smoothed and submitted to peak detection by the standard routine of the data system. Calibration and mass measurement of the processed data were done by using spectra generated by cesium iodide.

RESULTS

Appearance of Anti-*M. luteus* Activity in the Hemolymph of *P. terranovae* Larvae After Immunization. In a pilot experiment, 3000 third-instar larvae of *P. terranovae* were immunized, and the hemolymph was collected after 24 hr. The cell-free plasma contained significant anti-*M. luteus* activity and was subjected to heat treatment under acidic conditions, which did not result in loss of activity in the supernatant (data not shown). No anti-*M. luteus* activity was monitored under the same conditions in plasma from normal larvae of the same age. Additional experiments showed that the anti-*M. luteus* activity was completely abolished by protease treatment (data not shown), indicating that the molecules responsible for this activity are peptides.

Isolation of Two Anti-*M. luteus* Peptides (Peptides A and B) from Immune Hemolymph of *P. terranovae*. The supernatant from heat-treated plasma was subjected to cation-exchange chromatography under conditions as described, and aliquots of the eluted fractions were tested against *M. luteus* and also against *E. coli* D31 as in previous studies (7, 11). The results (Fig. 1) show the presence of two well-defined peaks of anti-*M. luteus* activity (A and B), together with the previously reported five major peaks of anti-*E. coli* activity. After an intermediary step on a Sep-Pak cartridge, the anti-*M. luteus* substances of peaks A and B were further purified by reversed-phase HPLC. Apparently pure substances were recovered, as judged by UV monitoring at 210 nm (Fig. 2 gives an example for peptide A), and the yields were as follows: 60 μg of peptide A and 25 μg of peptide B from, respectively, 180 and 70 ml of immune hemolymph.

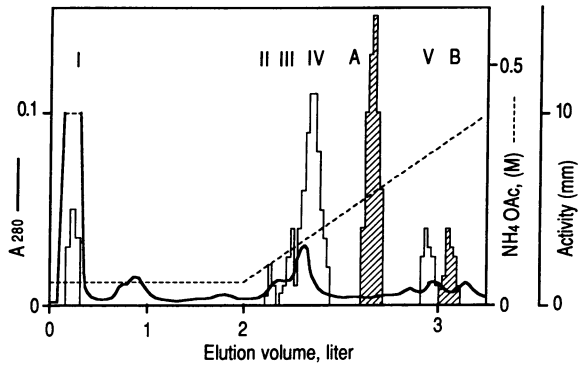

FIG. 1. Cation-exchange chromatography. Plasma (30 ml) from immune larvae of *P. terranovae* was heat-treated and applied on a CM-Trisacryl column (2.5 × 18 cm). Seven peaks containing antibacterial activity were separated: I–V [named according to our previous studies (7, 11)], five peaks with anti-*E. coli* activity (empty columns); and A and B, two peaks containing anti-*M. luteus* activity (hatched columns).

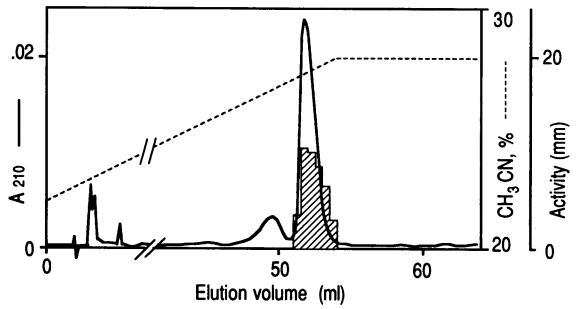

FIG. 2. Reversed-phase HPLC. After an intermediary step on a Sep-Pak cartridge, the active material from peak A of Fig. 1 was subjected to HPLC separation (Bakerbond C_{18} WP column, 0.46 × 25 cm) and yielded a well-defined peak of anti-*M. luteus* activity (hatched columns). The elution was performed in 65 min with a linear gradient of 22–28% acetonitrile.

Amino Acid Composition of Peptide A. Table 1 shows that there is a high glycine and cysteine content but no proline, methionine, phenylalanine, or glutamic acid residues.

Primary Structure Determination of Peptides A and B. Automatic Edman degradation was performed on 300 pmol of peptide A and 2 nmol of peptide B with repetitive yields of 95% and 93%, respectively. Both sequences finished abruptly at residue 40 (Fig. 3). All but one of the aspartic residues found by amino acid analysis after acid hydrolysis are actually in the amide form. This results in a high net positive charge for the molecule, which is consistent with the ion-exchange behavior. The carboxypeptidase analysis performed on peptide A showed very little, suggesting the possibility that the molecule has a blocked or inaccessible C terminus. With the native molecules of peptides A and B, no phenylthiohydantoin derivative was identified at positions 3, 16, 20, 30, 36, and 38, but after reduction and pyridylethylation of peptide A, the phenylthiohydantoin derivative of *S*-pyridylethylcysteine was obtained in good yield in these positions. If one assumes that the blanks in peptide B correspond also to cysteines (see the results below from mass spectrometry), the sequences of peptides A and B are identical except for the replacement of glycine-32 in A by arginine-32 in B.

Mass Spectrometry of Peptides A and B. The average molecular weights of peptides A and B were measured at a resolution of 1000. Under these conditions, the protonated molecular ion appears as a hump whose centroid corresponds to the average molecular weight of the pseudo-molecular ion—i.e., to its chemical mass (12, 13). For both peptides, a wide scan over the mass range of 1–5 kDa produced spectra that were characterized by two intense peaks (Fig. 4; see also

Table 1. Amino acid (AA) composition of peptide A

AA	Residue per mol a*	b†	AA	Residue per mol a*	b†
Asx	4.5	5	Tyr	1.0	1
Glx	0	0	Val	1.9	2
Ser	2.1	2	Met	0.3	0
Gly	7.1	7	Cys	5.4	6
His	2.0	2	Ile	1.0	1
Arg	3.2	3	Leu	4.1	4
Thr	1.9	2	Phe	0	0
Ala	3.9	4	Lys	1.1	1
Pro	0	0			

Cysteine was identified as the *S*-pyridylethyl derivative. No tryptophan residue has been found by sequencing.
*Average of two amino acid analyses.
†Composition expected from sequencing.

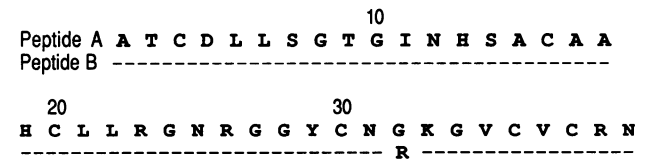

FIG. 3. Amino acid sequences (in one-letter code) of peptides A and B. For peptide A, cysteine was identified as the *S*-pyridylethyl derivative: its presence in peptide B was confirmed by amino acid analysis and mass spectrometry. The dashed line indicates identical residues.

Fig. 6). In Fig. 4 the peaks of m/z = 4061.22 and 2031.06 are interpreted respectively as the unresolved singly charged ($[MH]^+$) and doubly charged ($[MH_2]^{2+}$) molecular ion clusters corresponding to average molecular masses of 4060.22 and 4060.12. These values are in good agreement with the calculated one (4060.60) based on the sequence data, assuming that the six cysteine residues form three disulfide bridges and that no amino acid is modified by a functional group. The molecular peak $[MH]^+$ of a minor impurity (<10%) is visible at m/z = 3699.46 (and at m/z = 1850.12 for $[MH_2]^{2+}$), corresponding to a molecular weight of 3698.3 Da. This shows that mass spectrometry is capable, in some cases, of detecting minor impurities in a fraction eluted from reversed-phase chromatography as a single peak. This impurity was not detected during the Edman degradation. The small peak at m/z = 4167.71 is interpreted as an adduct with the matrix $[M + H + \text{thioglycerol}]^+$ rather than an impurity; this is very common in fast atom bombardment mass spectroscopy and confirms that the peak at m/z = 4061.22 is a molecular ion.

Peptide A was further characterized by measuring the molecular weight of the monoisotopic ion. At a resolution of 4000, the different isotopic ions were visible (Fig. 5). The experimental isotopic pattern was superimposable on that which was expected and allowed the measurement of the protonated monoisotopic molecular ion at m/z = 4058.90, a value that was in excellent agreement with the expected one (4058.81).

The spectrum of peptide B (Fig. 6) is similar to that of peptide A. The average molecular weight deduced from the protonated molecular ion $[MH]^+$ at m/z = 4160.16 is 4159.16 Da, which is in agreement with the value of 4159.74 Da expected from the Edman degradation data. $[MH_2]^{2+}$ at m/z = 2080.38 confirms the identification of peak m/z = 4160.16 as the molecular ion. The peaks at m/z = 4196.85 and at m/z = 4258.15 probably correspond to impurities.

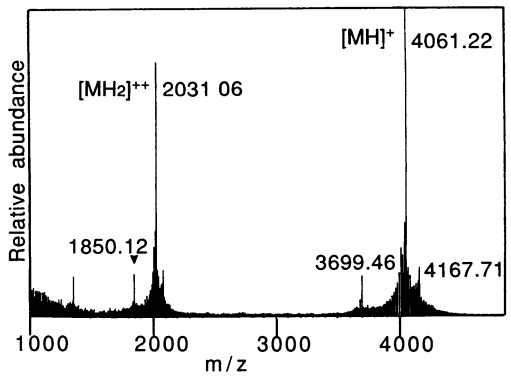

FIG. 4. Wide scan (1000–5000 Da) at resolution 1000 of peptide A. The two major peaks correspond to singly charged $[MH]^+$ at m/z = 4061.22 and to doubly charged $[MH_2]^{2+}$ at m/z = 2031.06. An adduct with thioglycerol is visible at m/z = 4167.71, confirming that peak 4061.22 is a molecular ion. A minor impurity is visible at m/z = 3699.46, with the doubly charged ion at m/z = 1850.12.

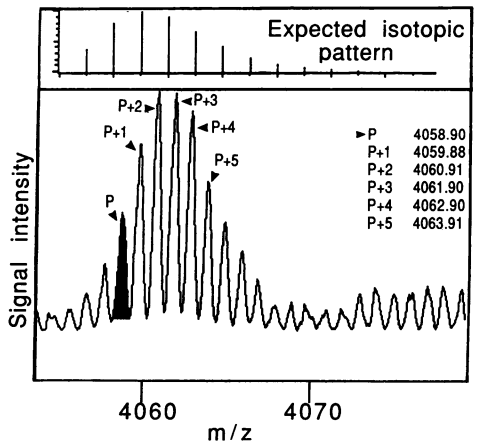

FIG. 5. Determination of the isotopic pattern of peptide A by using a resolution of 4000 in the narrow scan mode. A voltage scan (*Lower*) was performed to cover the range of the two cesium iodide clusters (4030.05 and 4289.86 Da). The comparison with the expected isotopic pattern (*Upper*), which is calculated from the elemental composition of the peptide, allows the identification of the monoisotopic peak P (darkened) measured at m/z = 4058.90.

Mode of Action of Anti-*M. luteus* Peptide A from Immune Hemolymph of *P. terranovae*. Pure peptide A (800 ng) was tested against all of the bacterial strains listed in *Materials and Methods*. Activity was monitored only against Gram-positive cells, *M. luteus* and *B. megaterium* being the most sensitive species. Lower, but clear-cut activity was recorded against five strains of *B. subtilis*. *S. aureus* was poorly sensitive, as were three strains of *B. thuringiensis*.

Fig. 7 shows that a 1-hr contact with peptide A (0.5 μM) is sufficient to kill growing or resting cells of *M. luteus*.

Lysozyme activity, conventionally tested by measuring the decrease in optical density of a suspension of *M. luteus* dried cells, was not detected with peptide A up to a concentration of 10 μM, whereas hen egg-white lysozyme was already active at a concentration as low as 30 nM. This indicates that peptide A is not a lysozyme and that its target is probably not the cell wall. During incubation of *M. luteus* with 0.5 μM pure peptide A, a rapid (15 mn) lysis occurred (monitored under optical microscopy) only with protoplasts and not with intact cells, indicating that the target of peptide A is probably the cytoplasmic membrane.

DISCUSSION

The two peptides isolated in this study from immune hemolymph of *P. terranovae* larvae exhibit selective activity against Gram-positive bacteria. They are neither structurally nor functionally related to lysozyme, which plays a major role in the immune reactions in other insect orders—e.g., in Lepidoptera (6, 14) and Orthoptera (15, 16). As mentioned earlier, we have been unable to detect in normal or immune hemolymph of *P. terranovae* any molecules related to lysozyme. It is tempting to assume that in this species the apparent absence of lysozyme is compensated in the defense against Gram-positive bacteria by peptides A and B. The presence in immune hemolymph of nonlysozymic peptides selectively active against Gram-positive bacteria has not been reported for other insects; this has prompted us to characterize these peptides in some detail.

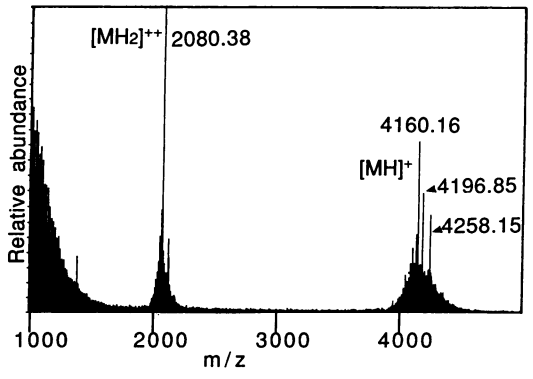

FIG. 6. Wide scan (1000–5000 Da) at resolution 1000 of peptide B. The two major peaks correspond to singly charged $[MH]^+$ at m/z = 4160.16 and to doubly charged $[MH_2]^{2+}$ at m/z = 2080.38. An average molecular weight of 4159.16 Da is deduced from the $[MH]^+$ peak. Impurities are visible at m/z = 4196.85 and 4258.15.

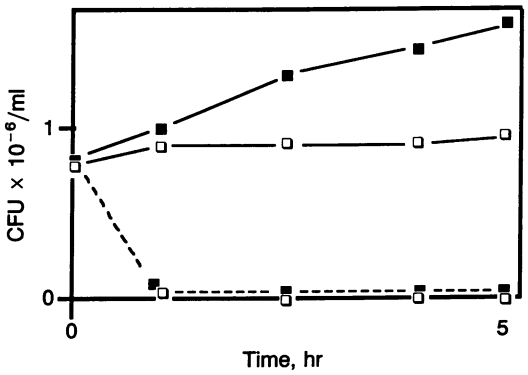

FIG. 7. Cells from an exponential-growth-phase culture of *M. luteus* ($OD_{600} \approx 0.1$) were washed, resuspended either in rich medium (■) or in insect saline (130 mM NaCl/5 mM KCl/1 mM $CaCl_2$) (□), and incubated at 37°C with shaking. Peptide A (0.5 μM) was added at time 0 (---) or replaced by an equivalent volume of distilled water in control experiments (—). Aliquots were removed at time intervals indicated on the abscissa and were plated on nutrient agar to determine the number of colony forming units (CFU) after an overnight incubation at 37°C.

The structural studies performed on peptide A show that it is 40 residues long, and the evidence from amino acid sequencing indicates a positive net charge as expected from the ion-exchange chromatography. Of special interest is the direct measurement of the molecular mass of peptide A (4061.2): the comparison with the mass given by the amino acid sequence (4060.6) indicates that peptide A is not post-translationally enzymically modified (e.g., glycosylated or phosphorylated) and that the six cysteines are involved in three disulfide bridges.

The results obtained by Edman degradation and mass spectrometry of peptide B conclusively show that it differs from peptide A only by the replacement of a glycine residue by an arginine residue in position 32. Indeed, the value obtained by direct measurement of the molecular mass of peptide B matches that deduced from Edman degradation of the native peptide, if one assumes that all six undetermined amino acids of peptide B correspond to cysteines and that they form three disulfide bridges in peptide B as in peptide A. The difference in molecular weights between peptides A and B (99 Da) reflects the replacement of a glycine residue (57 Da) by an arginine residue (156 Da). This replacement also accounts for the difference in charge and explains why peptide B is retained more strongly than peptide A by the cation-exchange resin. At the DNA level, this change could result from a single base change. Since the two peptides have been isolated from hemolymph obtained from several thousand larvae, there is a distinct possibility of genetic polymorphism.

The two molecules isolated and characterized in this study noticeably differ from all other peptides that have been described so far from the immune blood of insects. In particular, the presence of three disulfide bridges suggests

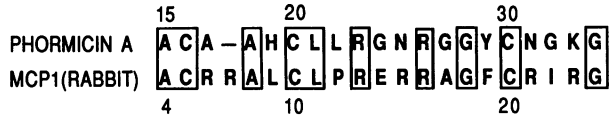

FIG. 8. Comparison of the amino acid sequences (one-letter code) of peptide A and MCP1 from rabbit lung macrophages. Homology between the two peptides was found by computer search, and the most homologous regions are aligned. Numbers indicate amino acid positions in both proteins. Identical residues are boxed.

that peptides A and B have compact globular forms: this feature has no counterpart in any of the other known insect immune response peptides.

A computer search of protein sequence data banks has revealed that there is substantial homology with two microbicidal cationic peptides (MCP) isolated from rabbit lung macrophages (17): 10 of 21 residues are identical, and several replacements are conservative (Fig. 8). These rabbit lung macrophage peptides are single-chain cationic peptides of 33 amino acid residues, containing three intramolecular disulfide bridges and differing only by the substitution of an arginine for a leucine at position 13. They are active against certain Gram-positive bacteria and fungi. The presence of small cationic antimicrobial peptides has also been reported from rabbit heterophil granulocytes (18) and from human neutrophils (19), where they were referred to as defensins. Therefore, we propose the name of "insect defensins" for the peptides characterized in the present study. It has been suggested that small, basic peptides could be widespread among the cells involved in the immune response of various mammalian species. To our knowledge, a sequence homology between molecules of the immune system of insects and mammals (apart from the ubiquitous lysozymes) has not been reported before. We suggest that small cationic peptides with disulfide bridges are ancestral bactericidal molecules that have been essentially conserved during evolution.

We are indebted to Mrs. Annie Meunier for excellent assistance with HPLC separations to immunopeptides, to Ian Davidson for amino acid analyses, to Daniel Guignier for the computer search of the National Biomedical Research Foundation and the Swiss Protein Data Bases, and to Drs. John Collins and Andrew Coulson, who carried out a homology search on the Edinburgh AMT DAP. We are grateful for the kind cooperation of Dr. Rudolf van den Broek and Mr. Patrick Kelly of Applied Biosystems for assistance in the identification of the cysteine residues.

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