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Purification and characterization of a scorpion defensin, a 4kDa antibacterial peptide presenting structural similarities with insect defensins and scorpion toxins

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Insect defensins are a group of inducible small-sized antibacterial peptides with three intramolecular disulfide bridges. NMR studies have recently shown that they share striking structural similarities with scorpion toxins. We have investigated in a scorpion species, *Leiurus quinquestriatus*, the potential presence of antibacterial molecules and report the isolation and structural characterization of a novel insect defensin homologue, which we refer to as scorpion defensin. This peptide shows a remarkably high degree of sequence homology with a defensin recently characterized in a species belonging to the ancient insect order of the Odonata with which it defines a novel ancient subclass of defensins. The scorpion defensin has in common with the scorpion toxins a consensus sequence Cys-[...]-Cys-Xaa-Xaa-Xaa-Cys -[...]-Gly-Xaa-Cys-[...]-Cys-Xaa-Cys present in all scorpion toxins characterized so far.

Insect defensins form a group of 4kDa inducible antibacterial cationic peptides which have been isolated from a variety of insect species belonging to the orders of the Odonata, Diptera, Hymenoptera and Coleoptera (1). These peptides show some sequence similarities with defensins, a group of 3-4 kDa antimicrobial cationic peptides present in neutrophils and macrophages from various mammalian species, including humans (2). Both insect and mammalian defensins have characteristically six cysteines engaged in three intramolecular disulfide bridges. The connectivity of the cysteines is however different between the insect and mammalian peptides and recent 3-D structure determinations by NMR studies (3-7) cast doubt on the possibility that these molecules might be homologous, i.e. derive from a common ancestral microbicidal peptide (see 1 and below). Indeed, the insect defensins consist of three distinct domains : an N-terminal loop, an α–helix and a C-terminal antiparallel β–sheet. The loop is linked by one of the disulfide bridges to the first strand of the β–sheet, whereas the α–helix is stabilized via the two other bridges to the second strand of the β–sheet. In contrast, mammalian defensins consist of β–sheets and lack an α–helix (5-7).

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It has recently been highlighted that insect defensins share striking structural similarities with scorpion toxins (8). These toxins have in common a structural motif formed of a small C-terminal antiparallel triple-stranded β–sheet linked to an α–helix by two disulfide bridges and to an N-terminally extended fragment by a third bridge (9). This structural motif is always found in association with the following consensus sequence (8) : Cys1-[...]-Cys2-Xaa-Xaa-Xaa-Cys3-[...]-Gly-Xaa-Cys4-[...]-Cys5-Xaa-Cys6, where Cys1, in the N-terminally extended fragment, is linked to Cys4 in the second strand of the sheet, and Cys2 and Cys3 in the α–helix are respectively linked to Cys5 and Cys6 in the third strand of the sheet. As shown in Fig 1, the same consensus sequence is present in insect defensins.

The observation that insect defensins and scorpion toxins share significant structural similarities has prompted the hypothesis that they could derive from a common ancestor. We ask in the present study whether the same organism, i.e. a scorpion, produces both types of molecules. We have investigated in *Leiurius quinquestriatus*, from which several venom toxins were initially discovered (10,11), the potential presence of an insect defensin homologue. We now report the isolation from normal and bacteria-challenged blood of scorpion of a new insect defensin homologue which we will refer to as *scorpion defensin*. It shows a remarkably high degree of sequence similarity with a defensin recently characterized in the ancient insect order of the Odonata [class of the Paleoptera](12) and has the consensus sequence typical for scorpion toxins (see above).

MATERIALS AND METHODS

Bacterial Strains and Media. Escherichia coli D31 (streptomycin resistant) was a gift from Prof. H.G. Boman (University of Stockholm) and *Micrococcus luteus* A270 was from the Pasteur Institute Collection, Paris. They were grown on Luria-Bertani's rich nutrient medium.

Animals and Hemolymph. The study was performed using 35 individuals of *Leiurus quinquestriatus (Arachnidae, Scorpionidae, Buthidae)* from Saudi Arabia. These scorpions were at different stages of development and their weights ranged from 0.5 to 4.8 g. Scorpions (20 individuals) were injected with a heat killed suspension of both *M. luteus* and *E. coli* bacteria; each individual receiving an injection of 2.10^6 bacteria of each species in a volume of 10 µl per gram of body weight. A group of 15 individuals received no injection of bacteria (unchallenged scorpions). The hemolymph of the scorpions was collected one week after injection. Volumes of 20-400 µl, depending on the weight, were punctured from each animal. The hemolymph was pooled for each group (bacteria-challenged and unchallenged) and immediately centrifuged at 70,000g for 50 min at 4°C. The pellet was discarded and the supernatant was frozen at -30°C until use.

Purification of the Antibacterial Peptide. Step I: Sep-Pak Prepurification - The cell-free hemolymph (supernatant, see above) was diluted (vol/vol) with acidified water (0.1% TFA) and loaded onto a Sep-Pak C_{18} cartridge. After washing with 5 ml of acidified water (0.05% TFA), elutions were performed with solutions of 10%, 40% and 80% acetonitrile in acidified water (0.05% TFA). All fractions were concentrated in a vacuum-centrifuge (Savant) to remove the organic solvent and TFA and reconstituted with MilliQ water and the presence of antibacterial activity was detected with the plate growth inhibition assay as described in.12.

Step II: Reversed-phase HPLC - The 40% fraction was taken up in 200 µl of 2% acetonitrile in acidified water and applied on an Aquapore OD 300 C_{18} column (250 x 4.6 mm, Brownlee Associates) equilibrated with 2% acetonitrile in acidified water. Elution was performed with a linear gradient of 2-52% of acetonitrile in acidified water over 90 min at a flow rate of 1 ml/min. The column effluent was monitored by absorbance at 225 nm and by the plate growth inhibition assay.

Step III: Final Purification - The fractions that contained the antibacterial activity were applied on an Aquapore RP 300 C_8 column (250 x 4.6 mm, Brownlee Associates). The column was developped with a gradient of 10-60% acetonitrile in acidified water over 90 min at a flow rate of 1 ml/min. Antibacterial activity was detected as above. All HPLC purifications were performed with a Beckman Gold HPLC system equiped with a photodiode array detector Beckman 168.

Microsequence Analysis. Automated Edman degradation of peptide in their native form and after reaction with 4-vinyl pyridine and detection of phenylthiohydantoin derivatives were performed on a pulse liquid automatic sequenator (Applied Biosystems, model 473 A).

Enzymatic Digestions and Separation of Products of Digestion. Endoproteinase Asn digestion: 3.5 nmol of pure peptide were treated with Endo-N (Takara) at a peptide/enzyme ratio of 10 to 1 (0.04 mU in 100 µl) for 17h at 37°C in 20 mM sodium buffer, 0.01% Tween 20, 1mM DTT, 1 mM EDTA, pH 5.0. The digestion was stopped by adding 100 µl of water containing 0.05% TFA. The peptide mixture produced by enzymatic digestion was applied on an Aquapore OD 300 C_{18} column (250 x 4.6 mm, Brownlee Associates) equilibrated with 2% acetonitrile in acidified water. Elution was performed with a linear gradient of 2-80% of acetonitrile in acidified water over 120 min at a flow rate of 0.8 ml/min.

Mass Spectrometry. The average masses of the peptide were measured as earlier reported (12).

RESULTS

Hemolymph was collected from bacteria-challenged scorpions (see Materials and Methods) and was subjected to centrifugation to remove the hemocytes. The presence of antibacterial activity was tested on an aliquot of the supernatant by the plate growth inhibition assay using *M. luteus* and *E. coli* D31 as test organisms. A marked anti-*M. luteus* activity was observed, whereas no activity was apparent against *E. coli*. When hemolymph was collected from normal, unchallenged, scorpions and processed as above, anti-*M. luteus* activity was also monitored, indicating that bacterial challenge is not mandatory for the appearance of antibacterial activity in the hemolymph. As for hemolymph of bacteria-injected scorpions, that of normal individuals did not exhibit anti-*E. coli* activity.

Given that injecting scorpions is a risky procedure and that normal hemolymph contains anti-*M. luteus* activity, we preferred to start the purification of antibacterial molecules from blood of unchallenged animals. Cell-free hemolymph (1,7 ml/fifteen individuals) was first filtered through a Sep-Pak C_{18} cartridge which was stepwise eluted with various concentrations of acetonitrile in acidified water. Again the antibacterial activity was monitored by the plate growth inhibition assay (test orgasnism : *M. luteus*) and it appeared that all the detectable activity was eluted in the 40% acetonitrile fraction. The eluate was next applied to a reversed-phase HPLC column and eluted with a linear gradient of acetonitrile in acidified water. Anti-*M. luteus* activity was observed in a single absorption peak (eluting at 27% of acetonitrile in acidified water). No other fraction contained detectable antibacterial activity.

The active compounds were further purified by a two-step purification procedure (see Materials and Methods). An apparently pure substance was recovered, as judged by UV monitoring at 225 nm. An estimated 16 µg of pure antibacterial peptide were obtained from 1.7 ml of normal hemolymph.

The purified antibacterial peptide was subjected to pyridylethylation and a partial amino acid sequence was obtained with 250 pmoles of peptide. The sequence was as follows Xaa-Phe-Gly-Cys-Pro-Leu-Asn-Gln-Gly-Ala-Cys-His-Arg-His-Cys-Arg-Ser-Ile-Arg-Xaa-Xaa-Gly-Gly-Tyr-Cys-Ala... To gain complete information on the sequence, we used Endoproteinase Asn to cleave the peptide between Asn and Gln; in the present situation, this cleavage gave the additional advantage of eliminating in one of the fragments the Cys4/Pro5 doublet which is not favourable for sequencing by automated Edman degradation. A total of 3.5 nmol of pure peptide were subjected to this enzyme and two fragments were separated by reversed-phase HPLC and

sequenced by Edman degradation. One fragment corresponded to the seven N-terminal residues identified above and a second fragment of 31 residues gave the COOH-terminal sequence of the peptide. The combined data lead to conclude that the complete sequence is Gly-Phe-Gly-Cys-Pro-Leu-Asn-Gln-Gly-Ala-Cys-His-Arg-His-Cys-Arg-Ser-Ile-Arg-Arg-Arg-Gly-Gly-Tyr-Cys-Ala-Gly-Phe-Phe-Lys-Gln-Thr-Cys-Thr-Cys-Tyr-Arg-Asn. This result was corroborated by mass measurement of the intact peptide which yielded a molecular mass of m/z = 4322.3 which is in good agreement with the average isotopic mass (MH^+) = 4321.0 calculated from the sequence data assuming that the six cysteines are engaged in three intramolecular disulfide bridges. The calculated pI of the molecule is 9.5.

In an additional series of purifications, hemolymph from bacteria-challenged scorpions was processed as above and yielded an apparently pure anti-*M. luteus* peptide with a chromatographical behaviour identical to that isolated from normal, untreated scorpions. A partial amino-acid sequence of this peptide on 18 residues gave a sequence identical to that of peptide isolated from the hemolymph of normal scorpions.

DISCUSSION

The results presented in this study show, in the first line, that blood from normal scorpions contains an antibacterial activity which is accounted for by the presence of a small-sized cationic peptide with high sequence similarity to insect defensins. We do not exclude at present that scorpion blood contains other antibacterial peptides, but can be affirmative that in our extraction procedures, the insect defensin homologue is the predominant antibacterial peptide. In contrast to the situation observed in insects, the antibacterial activity is present in unchallenged scorpions suggesting that the gene encoding scorpion defensin is constitutively expressed.

The sequence data which we have presented here call for two sets of comparisons:

(1) Scorpion defensin / insect defensins.

Up-to-date, the sequences of seven insect defensins have been published (Fig. 1). They have in common a significant number of residues and namely the cysteine array.

It is of interest to note that the scorpion defensin has a high degree of similarity with the insect defensin from Odonata. Indeed, the two molecules have identical lengths and twenty six

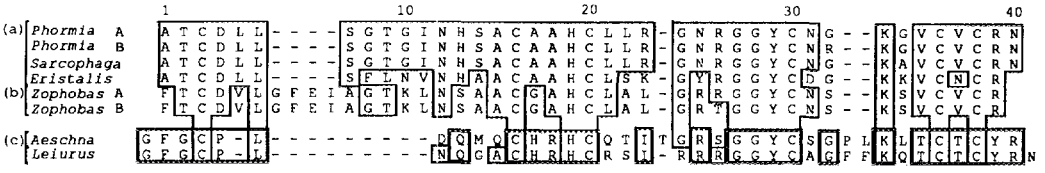

Fig. 1. Alignment of the sequence of the scorpion defensin with the sequences of insect defensins (1). The defensins were characterized from species belonging to three insect orders : (a) Diptera, (b) Coleoptera and (c) Odonata. The scorpion defensin is closely related to the *Aeschna* defensin (c). Bars indicate gaps to optimize the alignment. Identical amino acids are boxed. The bolded boxes represent the identical residues between *Aeschna* and scorpion defensins.

out of the thirty eight amino-acid residues are identical. In addition, five of the non-identical residues are conservative replacements. Assuming that the conserved presence of the six cysteines, and namely the consensus motif Cys-[...]-Cys-Xaa-Xaa-Xaa-Cys -[...]-Gly-Xaa-Cys-[...]-Cys-Xaa-Cys, allows for a similar 3-D structure in the scorpion defensin, an essential difference with the dipteran and colepteran defensins appears to pertain to the length of the N-terminal loop which is shorter by six residues in the scorpion and Odonata defensins. In summary, the Odonata defensin is structurally closer to the scorpion defensin than to the defensins reported from the more recent insect orders. *Leiurus* and *Aeschna* defensins represent obviously an ancient subclass of defensins.

(2) Scorpion defensin / scorpion toxins.

Scorpion toxins are considerably more heterogeneous than insect defensins (Table I). The primary structures of the toxins belonging to different families share little similarities. For example, toxins acting on mammalian K^+ channels have 35-40 amino acids and three disulfide bridges while those acting on mammalian or insect Na^+ channels have 60-70 amino acids and four disulfide bridges.

The only conserved amino acids between toxins and defensins are those of the consensus sequence presented in the Introduction. In addition, most long-chain Na^+ channel-acting toxins posses the Gly-Tyr-Cys sequence present in all hitherto published defensin sequences.

In conclusion, our data show that scorpions known to produce channel-blocking toxins also produce antibacterial defensins. The latter are believed to act via membrane-permeabilisation of Gram-positive bacteria (14) but have not been reported so far to act on the membranes of eucaryotic cells. The existence of an extended consensus sequence and the similarities in 3-D structures already reported for both types of peptides (3, 4, 9) have prompted the hypothesis that they derive from a common ancestor and our report on the presence of defensin in *Leiurus* lends additional credibility to this hypothesis. It will be interesting to investigate in detail the structure-activity relationships of these molecules and to understand the

Table I : Comparison of the sequence of *Leiurus quinquestriatus* scorpion defensin with the sequences of scorpion toxins. Alignment of the scorpion toxins is from (8) and (13). **A.** Scorpion toxins acting on Na^+ channels, *Aah* i : *Androctonus australis hector* insectoxin, *Aah* t : *Androctonus australis hector* toxin II, *CsE* v 3 : *Centruroides sculpturatus Ewing* variant 3, *Be* t I5A : *Buthus eupeus* toxin I5A. **B.** Scorpion toxins acting on K^+ channels; *Lqh* ch : *Leiurus quinquestriatus hebroeus* charybdotoxin, *Lqh* ch-2 : *Leiurus quinquestriatus hebroeus* charybdotoxin-2, *Lqh* sc : *Leiurus quinquestriatus hebroeus* scyllatoxin, *Bt* ib : *Butus tamulus* iberiotoxin, *Cn* no : *Centruroides noxius* noxiutoxin. **C.** Scorpion defensin; *Lqh* def : *Leiurus quinquestriatus hebroeus* defensin.

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A
Aah i  KKNGYAVDSS-GKAPECLL-----SHYCNNQCTKVH-YADKGYCCL-----LSCYCFGLNDDKKVLEISDTRKSYCDTTIIN
Aah t  VKDGYIVDDV-NCTYFCGR-----NAYCNEECTKL--KGESGYCQWASPYGNACYCYKLPDHVRTKGPGRCH
CsE v 3 KEGYLVKKSDGCKYGCLKLGE--NEGCDTECKAKNQGGSYGYCYA-----FACWCEGLPESTPTYPLPNKSC
Be t  I5A          MCMPCFTTDPNMAKKCRDCCG------GNGKCFG-----PQCLCNR
B
Lqh ch      ZFT------NVSCT-----TSKECWSVCQRLHNTS-RGKCMN-----KKCRCYS
Lqh ch-2    ZFT------QESCT-----ASNQCWSICKRLHNTN-RGKCMN-----KKCRCYS
Lqh sc      ZFT------DVDCS-----VSKECWSVCKDLFGVD-RGKCMN-----GKCRCYQ
Bt ib       TII------NVKCT-----SPKQCSKPCKELYGSSAGAKCMN-----GKCKCYNN
Cn no                AFCN------LRMCQLSCRSLG---LLGKCIG-----DKCECVKH
C
Lqh def             GFGCP----LNQGACHRHCRSIRRR--GGYCAG--FFKQTCTCYRN
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reasons which make one type active against procaryotes and the other group channel-blockers in eucaryotic cells.

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