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Purification and Primary Structure of Ceratotoxin A and B, Two Antibacterial Peptides from the Female Reproductive Accessory Glands of the Medfly *Ceratitis capitata* (Insecta:Diptera)

DANIELA MARCHINI,* PIERO C. GIORDANO,† REINOUT AMONS,‡ LUIGI F. BERNINI,† ROMANO DALLAI*§

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In the present article we report the purification and the amino acid sequence of two antibacterial peptides present in the secretion of the female reproductive accessory glands of the dipteran insect *Ceratitis capitata*. Both peptides consist of 29 amino acid residues, are heat stable, strongly basic and differ from each other for the substitution of two amino acids. Their primary sequence and predicted secondary structure are related to other families of peptides known to have lytic and/or antibacterial activity. We propose the name ceratotoxins (from *Ceratitis*) for these antibacterial peptides.

Antibacterial peptides Amino acid sequence *Ceratitis capitata* Ceratotoxin Insect immunity

INTRODUCTION

The insect immune system reacts against invading microorganisms with the recruitment of at least six different types of haemocytes and/or with a humoral response (see reviews by Götz and Boman, 1985; Ratcliffe *et al.*, 1985; Dunn, 1986; Boman and Hultmark, 1987; Boman *et al.*, 1991). Insect humoral immunity depends on the production of a number of antibacterial proteins or peptides extensively studied in recent years, namely (in Lepidoptera, Diptera and Hymenoptera): cecropins (4 kDa mol. wt), attacins (20 kDa mol. wt), diptericins (9 kDa mol. wt), defensins and sapecins (4 kDa mol. wt), apidaecins (2 kDa mol. wt) and lysozyme (14 kDa mol. wt). The appearance of antibacterial peptides in the haemolymph is experimentally induced by the injection of bacteria (Whitcomb *et al.*, 1974; Chadwick, 1975) or by injury (Postlethwait *et al.*, 1988). Lysozyme represents an exception, since it can be found in insects' haemolymph also in the absence of induction (Flyg *et al.*, 1987; Boman and Hultmark, 1987). Only a few data have appeared in the literature about defence factors present in compartments other than haemolymph (Mohrig and Messner, 1968; Rupp and Spence, 1985; Russell and Dunn, 1990; Samakovlis *et al.*, 1991). One of them, melittin, the bee venom toxin effective against bacteria and eukaryotic cells (Boman and Hultmark, 1987; Boman *et al.*, 1989), is produced as a component of a gland secretion (Bousquet *et al.*, 1979). Recently we demonstrated the non-induced antibacterial properties of the secretion (AGF) from the female reproductive accessory glands of *Ceratitis capitata* (Marchini *et al.*, 1991).

In this paper we report the purification and the amino acid sequence of two peptides isolated from an AGF fraction containing a potent activity against a number of gram-negative and gram-positive bacterial strains.

MATERIALS AND METHODS

Bacterial strains and media

The test organism for antibacterial activity was *E. coli* LE 392, cultured in NZYM medium (Sambrook *et al.*, 1989). Freeze dried cells of *M. luteus* (Sigma), suspended in 100 mM Na-phosphate buffer pH 6.8, were used in the bacteriolytic assay. Agarose (FMC) or agar (DIFCO) were added respectively at the concentration of 0.7% when solid medium was required.

*Department of Evolutionary Biology, Siena University, I-53100 Siena, Italy.
†Department of Human Genetics, Leiden University, 2300 RA Leiden, The Netherlands.
‡Department of Medical Biochemistry, Leiden University, 2300 RA Leiden, The Netherlands.
§Author for correspondence.

Insects and preparation of accessory gland secretion fluid (AGF)

Adult (5–20 day old) females of *C. capitata*, reared in standard laboratory conditions, were dissected in order to recover AGF as previously described (Marchini *et al.*, 1989). The secretion fluid, collected in 100 mM Na-phosphate buffer at pH 6.8–7.2, was stored at −20°C after freeze-drying.

Protein assay

The protein concentration of the AGF was determined according to Bradford (1976), using as standard bovine serum albumin (BSA).

Assays for antibacterial activity

The inhibition zone assay was determined essentially as described by Faye and Wyatt (1980). Fractions recovered from chromatographic separations of AGF were lyophilized, redissolved in 2 μl 100 mM Na-phosphate buffer pH 6.8 and introduced in wells (2 mm dia) punched into NZYM agarose plates (1 mm thickness) containing a 0.1% suspension of *E. coli* (O.D. = 0.5 at 600 nm). The lysozyme-like activity was assayed at pH 6.8 using 100 mM Na-phosphate/0.7% agar plates containing 1 mg/ml dried *M. luteus*. The samples were applied on wells as described above and the plates incubated overnight at 37°C. Ampicillin (0.5–0.75 μg) and hen's egg lysozyme (5 μg) (both purchased from Sigma) were used as control.

Purification of the antibacterial fractions

Step 1. Gel filtration—a batch of AGF from 1000 accessory glands (750 μg total protein) in 200 μl 0.5 M Na-phosphate buffer pH 7 was loaded onto a Superose 12 HR 10/30 column (Pharmacia) equilibrated with 100 mM ammonium acetate buffer pH 6.6 and fitted in a standard FPLC apparatus (Pharmacia). An aliquot (1/10) of each eluted fraction was tested for the activity against *E. coli* and *M. luteus*. The molecular size was determined applying the same chromatographic conditions using as markers BSA (68 kDa), chymotrypsinogen A (25 kDa), hen egg white lysozyme (14.8 kDa) and bovine insulin (5.75 kDa).

Step 2. First cation exchange chromatography—the fractions from the Superose 12 chromatography eluting at a retention volume compatible with a molecular weight of 15.5 kDa and active against *E. coli* and *M. luteus*, were diluted up to 3.6 ml with distilled water to reach a concentration of 50 mM ammonium acetate buffer pH 6.6. This volume was loaded onto a column of CM–Sepharose fast flow (Pharmacia) packed into a standard cartridge HR 5/10 and fitted in the FPLC apparatus. The sample was eluted with a discontinuous gradient of 65 ml [Fig. 1(B)] starting from 10 mM ammonium acetate pH 6.6 (phase A) up to 1 M ammonium acetate pH 6.6 (phase B). An aliquot (1/9) of each fraction was tested against *E. coli* and *M. luteus*.

Step 3. Second cation exchange chromatography—the fractions from step 2, active against *E. coli*, were lyophilized, redissolved in 100 μl buffer A, reloaded on the same CM–Sepharose column and eluted with a linear, less steep gradient from 10 mM ammonium acetate pH 6.6 to 0.3 M ammonium acetate pH 6.6 [Fig. 1(C)]. An aliquot (1/8) of each fraction was then assayed against *E. coli*.

Step 4. A purified fraction from step 3 was run again on a Superose 12 column using an elution buffer containing 154 mM NaCl and 10 mM Na-phosphate buffer, pH 6.7 [Fig. 1(D)]. The eluate was monitored at 214 nm.

Step 5. Reversed-phase HPLC—two tubes of the anti-*E. coli* active fraction from the second CM–Sepharose chromatography were lyophilized, suspended in 100 μl 0.125% TFA in water and applied to a reversed-phase C-8 column (LiChrospher RP-8 5 μm 75/4, Merck) fitted in a standard Beckman HPLC apparatus. The solvents used for the chromatography were: 0.125% trifluoroacetic acid in water (phase A) and 30 vol phase A + 70 vol acetonitrile (phase B). The sample was eluted in 60 ml solvent, within a linear gradient ranging from 20 to 80% phase B in phase A, at a flow rate of 1 ml/min. The eluate was monitored at 220 nm. The fractions corresponding to the peaks were tested against *E. coli* and a small amount used for the amino acid sequence determination.

Primary structure determination

The amino acid sequence was determined in the presence of polybrene (Serva) with an Applied Biosystem 475 A Protein Sequencer, equipped on line with a 120 A PTH Analyzer (Amons, 1987).

FIGURE 1. (*Caption on facing page.*) (A)–(E). Purification of ceratotoxin A and B. (A) Superose 12 FPLC separation of AGF (750 μg total protein). Elution buffer, 100 mM ammonium acetate pH 6.6; flow rate, 0.5 ml/min; fractions, 0.5 ml. Only a few fractions show anti-*E. coli* activity (solid blocks) whereas activity against *M. luteus* (open blocks) is broadly distributed. (B) First CM–Sepharose FPLC separation. Sample: anti *E. coli*/*M. luteus* fractions from step 1. A discontinuous gradient was applied (4 ml isocratic, from 0 to 10% B in 6 ml, from 10 to 30% B in 25 ml, from 35 to 100% B in 25 ml). Phase A, 10 mM ammonium acetate buffer pH 6.6; phase B, 1 M ammonium acetate pH 6.6. Flow rate, 0.5 ml/min. Fractions, 1 ml. Anti-*E. coli* activity, solid blocks; anti-*M. luteus* activity, open blocks. (C) Second CM–Sepharose FPLC separation. Sample, anti-*E. coli* fractions from step 2. Phases, flow rate and fraction volumes are identical to the previous chromatography. A linear gradient going from 0 to 30% in 70 ml was applied to elute the sample. Fraction Nos 54–62 are active against *E. coli* (solid blocks). (D) Superose 12 FPLC gel filtration of an aliquot of tube No. 60 from step 3. The major peak corresponds to a molecular size of 14 kDa. The eluate is monitored at 214 nm. (E) RP–C8 HPLC separation of the fraction Nos 59 and 61 from step 3. The two peaks correspond to ceratotoxin A and B.

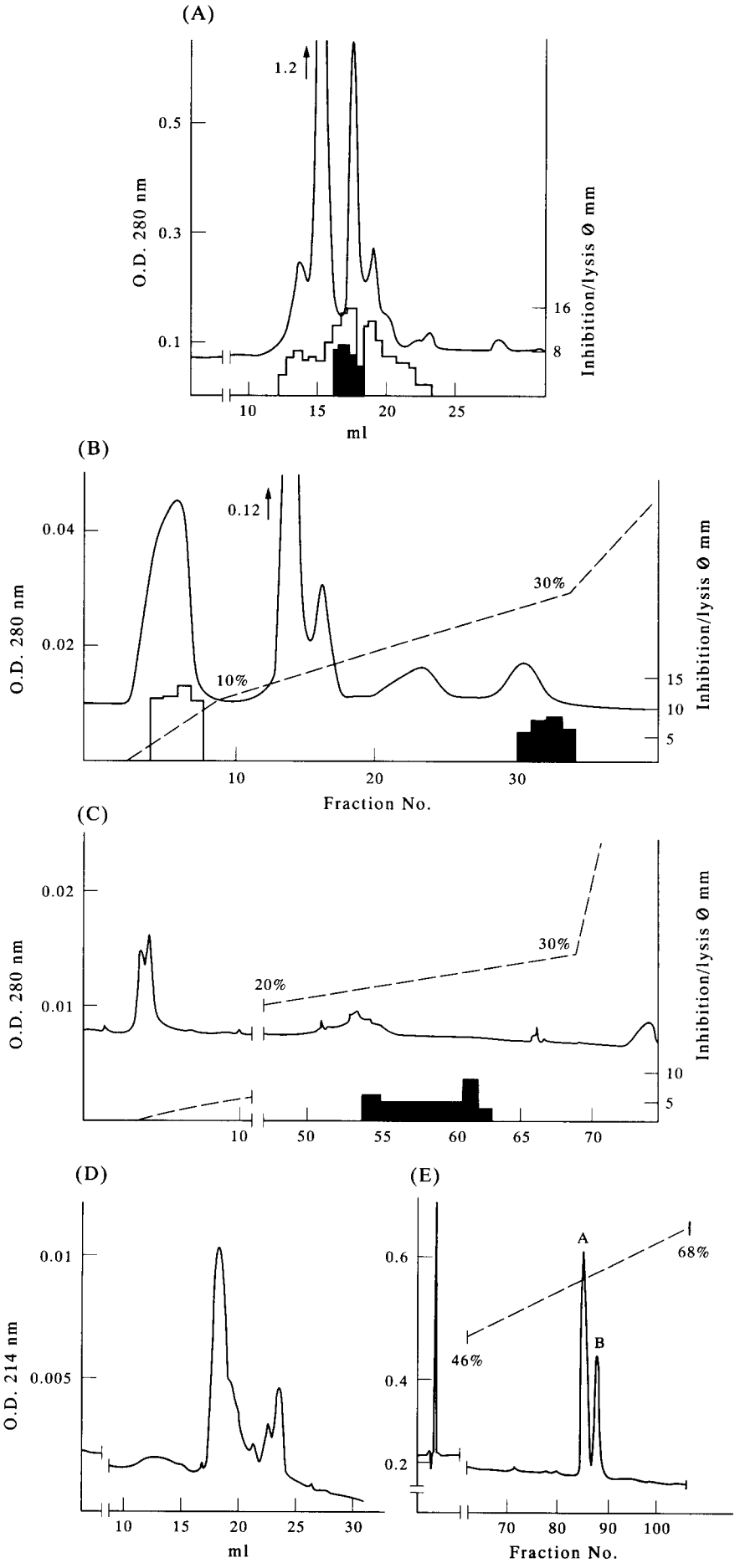

Fig. 1. (*See facing page.*)

Heat treatment

Purified anti-*E. coli* fractions from the second CM–Sepharose chromatography were submitted to heat treatment to test their stability. The samples, in 100 mM Na-phosphate buffer pH 6.8, were transferred, in aliquots from 10 to 100 μl, from an ice bath to a boiling water bath, incubated for 5 min and brought back to 0°C. The tubes were centrifuged at 10,000 *g* for 3 min and the supernatants tested against *E. coli*. Appropriate control samples from the same batch of the heat treated fractions were also assayed.

Bacteriolytic assay

E. coli cells collected in the exponential phase of growth were centrifuged and suspended in ice cold 100 mM Na-phosphate buffer pH 6.8 to give an O.D. of 0.2–0.3 at 600 nm. A heat treated aliquot (20 μl) of purified peptides obtained from step 3 was added to 980 μl of bacterial suspension. In the control tube an equal amount of buffer was added. The samples were mixed, incubated at 37°C and the absorbance at 600 nm was monitored at regular intervals for 40 min. According to Hultmark *et al.* (1980), one lytic activity unit (U) is defined as the amount of factor giving 50% reduction of absorbance at 600 nm after 30 min at 37°C.

Haemolytic assay

The experiments were performed under sterile conditions essentially as described by Boman *et al.* (1989): plastic Petri dishes (9 cm dia) were overlayed with a mixture containing 0.7% agarose, 3.5% BSA and 3% washed erythrocytes in 154 mM phosphate buffer pH 7.3. Samples (3 μl) of heat treated purified peptides from step 3 of increasing concentration were applied to wells punched into the solidified mixture. Plates were incubated at 37°C for 12 h. At the same time samples from the same batch was incubated against *E. coli*, as previously described to compare the extent of haemolytic and antibacterial activity.

Computer analysis

Computer analysis of sequence data (secondary structure, helicalwheel, sequence comparison, isoelectric point) was performed using the University of Wisconsin GCG package (Devereux *et al.*, 1984).

RESULTS

Isolation of two anti-E. coli peptides (ceratotoxin A and B) from the accessory gland secretion fluid

We started the purification of the anti-*E. coli* activity of AGF with a gel filtration step which, in previous experiments (Marchini *et al.*, 1991), led to the identification of such activity in a fraction corresponding to a molecular size of 15.5 kDa. As shown in Fig. 1(A), we detected in the eluate two different antibacterial activities: anti-*M. luteus*, found in a broad peak including twenty fractions and anti-*E. coli*, limited to a few fractions active against *M. luteus* as well. In order to further purify and resolve the anti-*E. coli* from the anti-*M. luteus* activity, we used a mild cation exchanger (CM–Sepharose) because of the irreversible adsorption of the anti-*E. coli* factor observed after chromatography on a sulphonated ion exchanger (Mono S) (Marchini *et al.*, 1991). Two well detectable and non-overlapping antibacterial activities were eluted this time from CM–Sepharose [Fig. 1(B)], the anti-*E. coli* fraction being clearly more basic than the peak active against *M. luteus*. From this step onward, our investigation dealt exclusively with the anti-*E. coli* protein.

Since the anti-*E. coli* fraction obtained from the second purification step was definitely contaminated by another protein(s), the active material was submitted to a second round of CM–Sepharose chromatography. The experiment was carried out with a smoother molarity gradient and resulted in the elution of an antibacterial peak giving no absorption at 280 nm [Fig. 1(C)]. We assumed that either the protein had an extremely high specific activity or that, more probably, lacked aromatic amino acids absorbing at that wavelength. In order to decide between the two alternatives, the material obtained from the second CM–Sepharose column was again submitted to gel filtration on Superose 12 and monitored at 214 nm. Figure 1(D) shows the outcome of this experiment: a major peak corresponding to a molecular weight of 14 kDa, and three minor peaks of about 5, 3.5 and 2.6 kDa respectively. The suspected absence of tyrosin and tryptophan was later confirmed by the analysis of the purified factor. The anti-*E. coli* fractions from step 3 were further purified by reversed-phase HPLC. The chromatogram [Fig. 1(E)], monitored at 220 nm, shows two sharp, well resolved peaks having an area ratio of *ca.* 2:1. The major and minor fraction will be henceforth indicated as ceratotoxin A and B respectively. Both peaks exhibited an essentially equivalent, strong, specific anti-*E. coli* activity.

Primary structure determination of ceratotoxin A and B

Automatic Edman degradation was performed at two different purification stages. The first analysis, on a fraction of the second CM–Sepharose chromatography (step 3), yielded a clean sequence of 29 amino acids with two possible substitutions at position 6 and 19. The second, carried out on both peaks from the reversed-phase HPLC purification (step 4), confirmed the first results and allowed the complete characterization of ceratotoxin A and B.

Table 1 shows the sequence of two almost identical peptides of 2.87 and 2.86 kDa respectively, differing from each other for the substitution of two hydrophobic

TABLE 1. The amino acid sequences are reported in one-letter code. Dashed lines indicate identical residues

	Primary structure of ceratotoxin A and B
Residue No.	1 10 20 29
Ceratotoxin A	SIGSALKKALPVAKKIGKIALPIAKAALP
Ceratotoxin B	-----F----------A----------

aminoacids (leucine and isoleucine) at position 6 and 19 of ceratotoxin A with two similarly apolar residues (phenylalanine and alanine) at the corresponding positions of ceratotoxin B. Acidic amino acids, cysteine, methionine, tyrosine and tryptophan are absent; basic amino acids are represented by six lysine residues which confer to these peptides a (calculated) isoelectric point of 11.49.

Heat stability

The anti-*E. coli* purified fractions from the second CM–Sepharose retained the original activity after heat treatment (not shown).

Bacteriolytic activity

As shown in Fig. 2, a heat treated, purified sample had a strong lytic action against *E. coli*, corresponding to *ca.* 0.5 U/nmol peptide.

Haemolytic activity

In order to establish whether the lytic activity was also directed against eukaryotic cells, human erythrocytes were tested with purified, heat treated peptides.

We have observed that the minimal amount of pure ceratotoxin able to produce a lysis halo, under the conditions described in Materials and Methods, was at least eight times higher than the amount of pure ceratotoxin needed to obtain an antibacterial halo (Fig. 3).

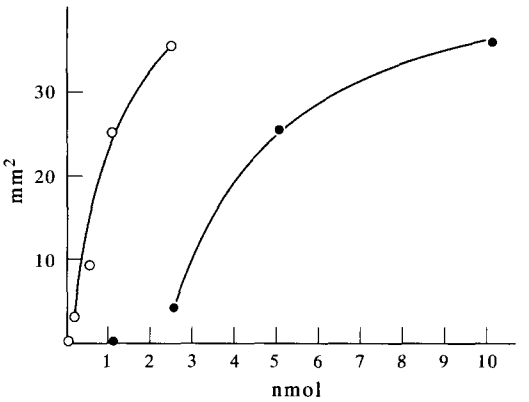

FIGURE 3. Anti *E. coli* activity (open circles) and haemolytic activity (solid circles) of purified fractions from the second CM–Sepharose. Both activities are expressed in areas of inhibition/lysis respectively vs peptide concentration.

DISCUSSION

In this work we report the purification and the primary structure of ceratotoxin A and B, two antibacterial peptides present in the female reproductive accessory gland secretion of the dipteran *Ceratitis capitata.*

Ceratotoxin A and B are closely related peptides, stable at 100°C and strongly basic, as it is shown by their behaviour on ion exchangers and can be deduced by the amino acid composition. The determination of the primary structure has revealed that, assuming an α-helix folding, both ceratotoxins are perfect amphiphilic peptides with all the solvent-accessible, polar residues lying on an area parallel to the α-helix axis and occupying, on the helicalwheel projection (Fig. 4), one-third of the helix surface. The helicalwheel projection describes the relative position of aminoacids with respect to the axis of a continuous α-helix having 3.6 residues per turn; it does not predict the secondary structure of the peptide.

The calculated molecular weight of ceratotoxin A and B is 2.87 and 2.86 kDa, respectively. When redissolved in phosphate buffer at physiological pH after the reversed-phase chromatography they regain the biological activity and the molecular size of the native fraction. We assume, therefore, that the molecular form found in the gland secretion consists of a polymer apparently made up of four to six subunits. Molecular sieving is not an appropriate method for assessing exactly the number of subunits in a very small polymer. That the aggregation of ceratotoxin molecules does occur in physiological conditions is suggested, however, by the existence of

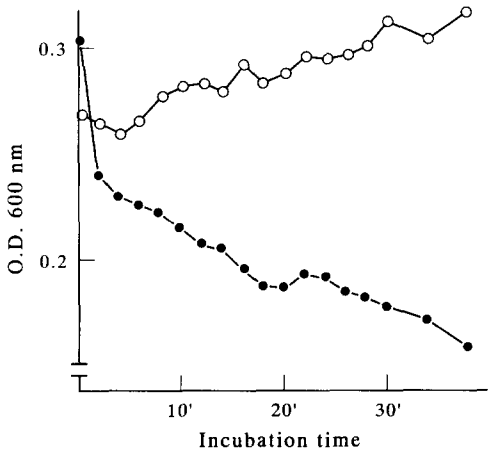

FIGURE 2. Bacteriolytic activity of a heat treated aliquot (1.7 nmol) from the second CM–Sepharose (solid circles), in comparison to the control (open circles). *E. coli* suspension was in 100 mM Na-phosphate buffer pH 6.8. Incubation was carried out at 37°C.

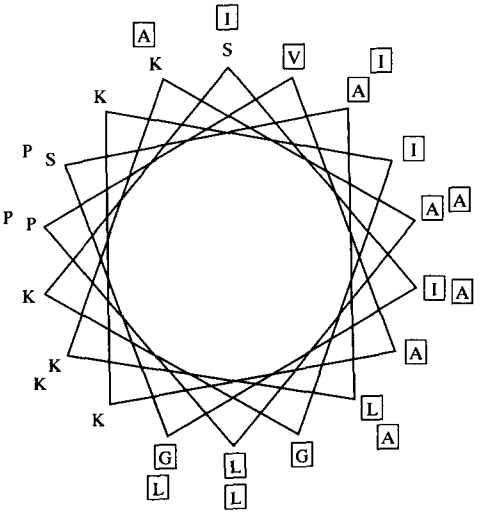

FIGURE 4. Helicalwheel of ceratotoxin A peptide. Hydrophobic residues are indicated by square symbols.

active fractions showing on Superose 12 different molecular sizes (see later also). Furthermore the co-elution with another protein(s) of a larger size can be excluded because the peptide exhibit the same chromatographic behaviour in the crude extract and after complete purification. Presumably the subunits are assembled in such a way to expose the charged and polar residues in the external, aqueous environment while the apolar side chains are packed in the interior of the structure. Such a spatial arrangement would also explain the solubility in water of these peptides which are composed prevalently of hydrophobic amino acids. The polymerization of four amphiphilic subunits has been demonstrated by X-ray crystallographic analysis in the case of melittin, the toxic component of bee venom (Terwilliger and Eisenberg, 1982). The second run of purified ceratotoxin on Superose 12 revealed the presence of different aggregation forms ranging from about 14 to 3 kDa. It is unlikely that the fractions having a size lower than 14 kDa represent degradation products due to proteolysis, since these fractions, as well as a low molecular weight peak described in a previous work (Marchini *et al.*, 1991), retained the inhibitory activity against *E. coli*.

The heat stability, high isoelectric point, the low molecular weight (in the range of 2–4 kDa) and, in many instances, the amphiphilic structure are properties common to other insect antibacterial peptides as cecropins (including sarcotoxin I), apidaecins and defensins (Hultmark *et al.*, 1980, 1982; Okada and Natori, 1983; Casteels *et al.*, 1989; Matsuyama and Natori, 1988; Lambert *et al.*, 1989). As melittin and cecropins (Boman *et al.*, 1989; Hultmark *et al.*, 1980; Steiner *et al.*, 1981), the ceratotoxins are lytic, act against growing and non-growing bacteria and their activity is directed against gram-negative and -positive bacterial strains, whereas insect defensins are active preferentially on gram-positive bacteria (Matsuyama and Natori, 1988; Lambert *et al.*, 1989) and apidaecins and diptericins are active only against growing gram-negative bacteria (Casteels *et al.*, 1989; Dimarcq *et al.*, 1988, 1990). These properties are shared also with peptides isolated from the stomach and the skin of *Xenopus laevis* (Zasloff, 1987; Moore *et al.*, 1991), from mammalian cells (Lee *et al.*, 1989; Selsted *et al.*, 1985a, b; Selsted and Harwig, 1987) and a number of antibiotics (alamethicin, gramicidin S) (Fox and Richards, 1982).

TABLE 2. Ceratotoxin A from *C. capitata* (present work); CPF (RP-HPLC fraction No. 67) and magainin II from *Xenopus laevis* (Moore *et al.*, 1991; Zasloff, 1987); cecropin A from *Drosophila* (Boman *et al.*, 1991); melittin from *Apis mellifera* (Habermann and Jentsch, 1967). Capital letters indicate identical residues present in at least three peptides. Letters underlined indicate identical residues present in ceratotoxin and at least one other peptide

Alignment of the amino acid sequences of some antibacterial peptides of different origin	
Ceratotoxin	s IGsaLkKaLpvAkKiGki aLpiAk aalp
CPF	GfGs fLgKaLkaAlKiGan aLGgAp Qq
Cecropin	gwlk kIGkk i e r v gqh t r da t iq gLGi Aq Qaanvaatar
Melittin	GIGavL-KvLtt g l pa l i swi k r k r Qq
Magainin	GIGk fL----hsAkKfGka f vGe i mNs

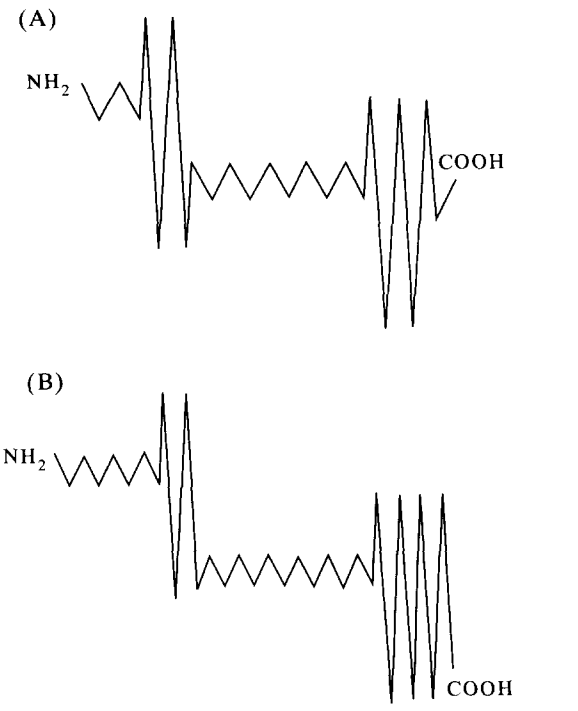

FIGURE 5. (A) and (B). Predicted secondary structure (Chou and Fasman, 1978) of ceratotoxin A and cecropin, respectively.

The amphiphilic nature of the α-helix is most probably responsible for the antibacterial and lytic properties of ceratotoxins, in analogy with other antimicrobial peptides.

A computer search of a protein sequences data bank has indicated consistent homologies between ceratotoxins and other amphiphilic peptides of different origins (Table 2). The greatest homology is displayed in comparison with one of the caerulein precursor factor (CPF) peptides from *Xenopus laevis* (Moore *et al.*, 1991) with a percentage of similarity/identity of 55.5%/48%. Among insect antimicrobial peptides strong homologies are observed in comparison with cecropin and melittin (Table 2).

A plot of the secondary structure of ceratotoxin A, predicted according to Chou and Fasman (1978), shows two domains separated by a region of lower helical content having proline and glycine at position 11 and 17, respectively [Fig. 5(A)]. This plot is very similar to that obtained with the same method for cecropin [Fig. 5(B)], which is made up (Steiner *et al.*, 1988) of two helical domains linked by a more flexible hinge also containing proline and glycine.

The homology of ceratotoxin with melittin is not confined only to their primary structure but involves also the ability to lyse mammalian red blood cells (Boman *et al.*, 1989; Schmidt, 1982). Our data on the lytic and antimicrobial activities of ceratotoxin is consistent with the data reported for melittin (Boman *et al.*, 1989). Furthermore, ceratotoxin and melittin are produced in

organs sharing the same evolutionary origin. Both peptides are not induced, but naturally secreted by a reproductive gland in *Ceratitis capitata* (Dallai *et al.*, 1985, 1988) and by a venom gland (Owen and Bridges, 1974), which is a modified reproductive gland (Adiyodi and Adiyodi, 1975; Leopold, 1984) in the honey bee.

Probably the original function of the secretion from reproductive accessory glands consists in maintaining aseptic conditions during fertilization and/or protecting eggs and early larvae from pathogenic organisms present in the external environment. In this sense it is of interest the recent finding in the *Drosophila* male ejaculatory duct of an antibacterial peptide that could be transferred into the female at mating (Samakovlis *et al.*, 1991).

The two forms of ceratotoxin are not likely to be the expression of two different alleles at a single locus. The insects used throughout the investigation derive from a small, inbred population reared in our laboratory for many years and should have a low average heterozygosity. Most probably peptides A and B are expressed by duplicated genes; the presence of the motif K X A L P repeated three times along the sequence indicates the recurrence of duplications in the DNA coding for the peptides.

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