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Effects on electrophoretic mobility and antibacterial spectrum of removal of two residues from synthetic sarcotoxin IA and addition of the same residues to cecropin B

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Cecropin B and cecropin IA (sarcotoxin IA) are 35- and 39-residue antibacterial peptides from a silk moth and a meat fly, respectively. Using solid phase synthesis we have made these peptides as well as two 37-residue analogs, one containing a deletion of leucine and lysine (residues 2a and 2b) as compared to cecropin IA, the other containing an insertion of leucine and lysine at the corresponding place in cecropin B. This addition and removal of a lysine residue did not cause the expected change in electrophoretic mobility. When tested for antibacterial spectra, the insertion analog was found to be as active as the parent compound while the deletion analog had lost most of its antibacterial capacity. In addition it was shown that the C-terminal amide contributes to the broad spectrum properties of the cecropins.

Cecropin B; Sarcotoxin IA; Solid-phase synthesis; C-terminal amide effect; Antibacterial peptide; Insect immunity

1. INTRODUCTION

Many insects respond to a bacterial infection or an injury by the production of a potent broad spectrum antibacterial activity in the hemolymph (review [1]). In the Cecropia moth, *Hyalophora cecropia*, the main components responsible for the activity are cecropins A, B and D [2,3]. These peptides are 37, 35 and 36 amino acid residues long, respectively, and they have strongly basic N-terminal parts and hydrophobic C-terminal regions ending with α-amide groups. We have previously described the synthesis of cecropins A [4] and B [5] as well as a number of analogs of the A-form [6]. Part of this work shows the importance of a tryptophan in position 2 and this is also a conserved residue in all eight cecropins so far sequenced [1].

Cecropin IA from the meat fly *Sarcophaga peregrina* (also referred to as 'sarcotoxin IA') consists of 39 amino acid residues and was originally reported to have a free C-terminal carboxyl group [7]. If cecropin B and cecropin IA are aligned from the N-terminus, Trp-2 is the only amino acid shared within the first 15 residues. However, fig.1 shows that if residues 2a and 2b are considered as a deletion in cecropin B or an insertion in cecropin IA, 7 of the first 15 residues are shared, or 10 if one considers conservative replacements. We therefore decided to synthesize the parent compounds and two cecropin analogs with 37 residues: analog 1 with the sequence of cecropin IA with residues 2a and 2b deleted and analog 2 with the structure of cecropin B with the dipeptide Leu-Lys inserted at positions 2a and 2b (using cecropin B numbering, fig.1). At the same time we wanted to: (i) replace Trp-2 by the corresponding D-amino acid, and (ii) study the effect of an amidation of the C-terminal residue. All peptides made were compared for electrophoretic mobility at acidic pH and for antibacterial activity. When our syntheses were completed Natori's group reported that

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	12 2a2b 5 10 15 20 25 30 35
Analog 1:	GW KIGKKIERVGQHTRDATIQGLGIAQQAANVAATAR
S.p. Cecropin IA:	GW L K KIGKKIERVGQHTRDATIQGLGIAQQAANVAATAR
H.c. Cecropin B:	KW KVFKKIEKMGRNIRNGIVKAGPAIAVLGEAKAL
Analog 2:	KW L K KVFKKIEKMGRNIRNGIVKAGPAIAVLGEAKAL

Fig.1. Sequences for cecropin IA from *Sarcophaga peregrina* (S.p.) and cecropin B from *Hyalophora cecropia* (H.c.) flanked by two analogs. Analog 1 contains the sequence of S.p. cecropin IA with L-2a and K-2b deleted, while in analog 2 these two residues were inserted in the same places in the sequence of H.c. cecropin B. The numbering refers to cecropin B. All natural cecropins are amidated at the C-termini. Analog 1 and cecropin IA were made with and without the amide.

cecropin IA from *Sarcophaga* does have an amidated C-terminal residue [8].

2. MATERIALS AND METHODS

2.1. *Solid phase synthesis*

The methods used for the synthesis of peptides were as described [4–6]. The deletion and insertion peptides and their respective parent compounds were single-chain syntheses until residue 3 where the peptide-resins were divided into two parts and either two or four more residues were added. Purification was by HPLC on μBondapak C_{18} reverse-phase column and elution with a linear gradient of 20–40% acetonitrile [5]. All synthetic peptides gave amino acid analysis in agreement with theoretical expectations. For both analogs the sequence of the first 14 residues was confirmed using a gas phase sequenator (Applied Biosystems 470A). Cecropin A-OH was produced from a synthetic gene for a fusion protein and kindly obtained from Ingene, Santa Monica, CA.

2.2. *Antibacterial assays*

For each peptide and for each test bacterium 3 μl of a series of dilutions were placed in small wells in thin agar plates seeded with test bacteria as described [9]. After an overnight incubation at 30°C the inhibition zones were recorded and the lethal concentration of each peptide (LC, the lowest concentration that inhibits growth) was calculated as described [9]. Since large inhibition zones give low LC values, such values are more accurate than high LC values which come from small zones.

2.3. *Acidic electrophoresis*

Separations were performed at pH 4.3 in 15% polyacrylamide gels (acidic PAGE) with dimensions 178 × 2.7 × 138 mm using the buffers described [10]. At pH 1.7 we used as gel buffer 0.135 M HCl with 0.9 M KCl and an electrophoresis buffer containing 0.035 M β-alanine with 0.07 M HCl. Relative mobility (R_B) was calculated using cecropin B as standard.

3. RESULTS AND DISCUSSION

3.1. *Polyacrylamide gel electrophoresis*

Acidic PAGE is known to discriminate between different cecropins [3] and in this investigation the method was originally applied as a test of purity. It was then discovered that the addition and removal of normally charged groups in some cases gave unexpected results. Since we wanted as many examples as possible on charge differences and since cecropin A was available with a C-terminal amide and with a free carboxyl group these compounds were also included. Fig.2 shows that the 8 chemically synthesized peptides gave one well-defined band at pH 4.3. The exception was cecropin A-OH which gave an additional weaker band with a faster mobility. For each compound the relative mobility (R_B) was compared to the predicted charge (table 1). In addition, in order to estimate the effect of an uncharged C-terminal carboxyl group a PAGE was also run at pH 1.7. Table 1 shows that the difference between a charged and an uncharged carboxyl group produced an R_B difference of 0.10–0.13. In the three cases where C-terminals COOH and $COHN_2$ could be compared (lanes 4 vs 5, 8 vs 9 and 10 vs 11 in fig.1) there was approximately the same difference in R_B as expected (0.10–0.14).

At acidic pH the addition and removal of a lysine residue should produce a full charge difference. It was therefore expected that analog 1 would show a reduced mobility compared to cecropin IA (sarcotoxin IA) and that analog 2 would be significantly faster than cecropin B. However, when Leu-Lys was added to cecropin B the increase in R_B at pH 4.3 (1 vs 2 in fig.2) was only 0.01, while the two examples of removal of this dipeptide (8 vs 10 and 9 vs 11) decreased R_B with only 0.04 and 0.05. At pH 1.7 the differences seen were even smaller (table 1). From these results we must conclude that at least some of the lysine

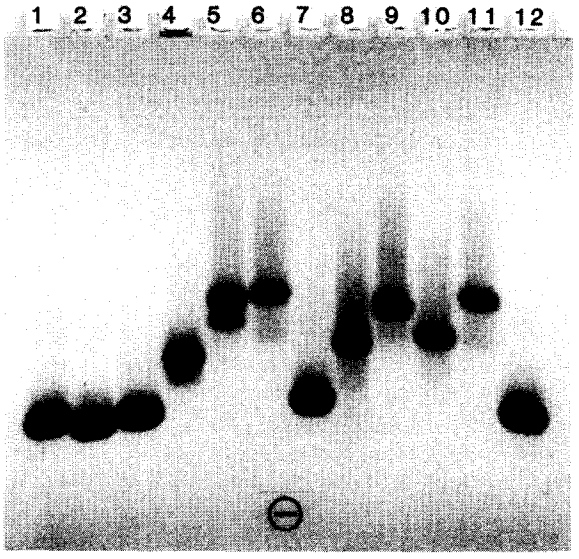

Fig.2. PAGE at pH 4.3 of different cecropins and some analogs. The experiment was run for 8 h at 200 V using the conditions specified [10]. Staining was with Coomassie brilliant blue R.250. The following samples were applied in lanes: 1, cecropin B; 2, analog 2; 3, [D-Trp^2]cecropin B; 4, cecropin A; 5, cecropin A-OH; 6, analog 1-OH; 7, cecropin B; 8, cecropin IA; 9, cecropin IA-OH; 10, analog 1; 11, analog 1-OH; 12, cecropin B.

groups in the cecropins are not protonated as expected. We have no reason to believe that these results are due to impurities or incorrect peptide structures.

3.2. *Antibacterial activities*

We have previously selected *Escherichia coli* D21, *Pseudomonas aeruginosa* OT97, *Bacillus megaterium* Bm11 and *Micrococcus luteus* Ml11 as sitable test organisms for assay of the broad spectrum antibacterial activity typical of the cecropins [1]. These four bacteria were used in plate assays to determine the LC values for the peptides studied by acidic PAGE.

The results which are given in table 1 allow a number of conclusions. First, cecropin A and cecropin B from *H. cecropia* were 4–7-times more active than cecropin IA (sarcotoxin IA) when tested on *B. megaterium* and more than 10-times more active on *M. luteus*, while activities were comparable on the two other bacteria. Since *S. peregrina* is a meat fly and *H. cecropia* is a leaf eater, it is possible that the differences observed could reflect significant differences in the natural flora to which the two insects are adapted.

Secondly, the replacements of L-Trp-2 with its D-form gave a small but probably significant increase in all four LC values. Similar results have previously been observed for other peptides where a D-isomer has modified the conformation of the peptide or improved the stability toward enzymatic degradation (review [11]).

Thirdly, the amidation of the C-terminal carboxyl group was tested in three cases, for cecropin A, for cecropin IA (sarcotoxin IA) and for analog 1. In two of the cases the activity against *E. coli* was unaffected, but in the case of analog 1 a 3-fold increase was observed. For the three other test bacteria (strains OT97, Bm11, and MI11) the amidated form was 3–4-times more active than the corresponding compound with a free carboxyl group. For cecropin A the differences were larger but these results should be judged with some caution because of the electrophoretic heterogeneity. The overall conclusion is that the amidation of the carboxyl group increases the broad spectrum properties of the cecropins and therefore must provide an increased survival value for the insects. Likewise, amidation of the C-terminus has been found in all sequenced cecropins [1].

Replacement of an anionic C-terminal carboxyl group of a peptide by an uncharged amide was predicted to increase the helix dipole [12] and was experimentally observed to increase the helicity of both ribonuclease S-peptide [13] and several glucagon analogs [14]. Therefore, the helicity of these cecropin amides would be expected to be higher than the acids and, since an amphipathic helix has been implicated in cecropin action, higher antibacterial activity might have been expected.

Thirdly, analog 2 (cecropin B with the dipeptide Leu-Lys inserted in positions 2a and 2b, fig.1) showed the same antibacterial activity as the parent compound. In contrast, analog 1 (in which the dipeptide Leu-Lys was removed from sarcotoxin IA) showed a 10-fold decrease in activity against *E. coli* and activity was fully abolished against the three other test bacteria. Similarly, analog 1 with a free carboxyl group was 30-fold less active against *E. coli* than sarcotoxin-OH and completely inactive against the other three test organisms.

We have previously discussed the importance of amphipathic helices for the antibacterial activity of the cecropins [6]. However, the first 15 residues of

Table 1

Electrophoretic mobilities and antibacterial spectra of different cecropins and analogs

Compound	Residues	Predicted charge	Relative mobility[a]		Lethal concentration (μM)			
			pH 4.3	pH 1.7	*E. coli* D21	*P.aeruginosa* OT97	*B.megaterium* Bm11	*M. luteus* M111
Cecropin B	35	8+	1.00	1.00	0.6	1.5	1.0	1.2
Analog 2	37	9+	1.01	1.02	1.0	1.1	1.0	0.9
[D-Trp^2]cecropin B	35	8+	0.98		0.5	0.7	0.6	0.5
Cecropin A	37	7+	0.86	0.88	0.4	2.6	0.6	1.4
Cecropin A-OH	37	6+	0.72	0.82	0.5	8	11	18
Cecropin IA	39	7+	0.85	0.85	0.5	2.6	4.2	18
Cecropin IA-OH	39	6+	0.75	0.86	0.5	10	19	34
Analog 1	37	6+	0.82	0.84	4.8	>96	>96	>96
Analog 1-OH	37	5+	0.71	0.84	15	>161	>161	>161

[a] The relative mobilities at pH 4.3 are the mean values from two experiments. The differences observed were in three cases <0.01, in one case 0.01, in three cases 0.02 and in one case 0.03. The faster band in cecropin A-OH had R_B values of 0.77 and 0.88 at pH 4.3 and 1.7, respectively

both analog 1 and analog 2 can form amphipathic helices. Thus, disruption of such structures can hardly explain the difference in activity between the two analogs. We have also tried other alternatives to align the sequences in fig.1, but in no way have we found an explanation for the differences between the two analogs. We must therefore conclude that the dramatic difference recorded between two equally long cecropin analogs was unexpected and must reflect an unknown structure-function relation of importance.

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