TOXIN III FROM *ANEMONIA SULCATA*: PRIMARY STRUCTURE

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1. Introduction

Neurotoxins are very important tools for the analysis of molecular aspects of nerve conduction and transmission. Toxic molecules already available for study of molecular aspects of conduction include:

(i) Tetrodotoxin and saxitoxin, which are highly specific for blocking the Na^+ channel in most axons [1,2].

(ii) Veratridine and batrachotoxin which depolarize nerve or muscle membranes by a selective increase in the resting sodium permeability [3,4].

(iii) Scorpion neurotoxin, a miniprotein which affects the closing of the Na^+ channel and the opening of the K^+ channel [5,6].

A series of neurotoxins was recently isolated in the pure form from the sea anemone *Anemonia sulcata* [7]. The sequence of one of these neurotoxins, ATX_{II}, has now been established. It is a miniprotein comprising only 47 amino acids (5000 mol. wt) crosslinked by three disulfide bridges [8]. This neurotoxin has been described as a useful tool for the analysis of the molecular aspects of nerve conduction [9] and of the coupling between excitation and secretion in synapses [10]. It also displays a strong cardiotoxic action [11].

Among the polypeptide neurotoxins isolated from *Anemonia sulcata*, one, which has been called toxin III, has a particularly low mol. wt approx. 2700, which corresponds to a sequence of less than 30 amino acids [7]. It is the primary structure of this toxin that we report in this paper.

2. Materials and methods

Toxin III was purified according to [7]. Carboxypeptidases A (EC 3.4.12.2) and B (EC 3.4.12.3) treated with diisopropylphosphofluoridate and α-chymotrypsin (EC 3.4.12.1) were purchased from Worthington. Papain (EC 3.4.22.2) and aminopeptidase M (EC 3.4.11.2) were from Sigma, imidodipeptidase (EC 3.4.13.9) from Miles Lab. Sephadex G-25 was obtained from Pharmacia and Biogel P4 from Biorad Labs. Dithioerythritol, iodoacetic acid and sequanal grade dimethylbenzylamine came from Pierce Chemical C. *N*-Ethylmorpholine purum grade, tryptamine puriss were purchased from Fluka and the solution of ampholine (40% w/w, pH 3–10) from LKB *p*-toluene sulfonic acid, RS grade was from Carlo Erba.

2.1. *Toxin isoelectric point determination*

Electrofocusing of the toxin was carried out as described in the LKB instructions manual (1801-E.0.1). The dense buffer was made up of 7.5 ml 10% solution of ampholine and 28 g sucrose diluted to 60 ml with distilled water. The less dense solution in which the toxin (200 nmol) was dissolved constituted 2.5 ml of

the previous ampholine solution and 1 g sucrose. The electrofocusing was for 20 h at 500 V. The column was eluted in 4.0 ml fractions. The pH of each fraction was measured so as $A_{280\,nm}$.

2.2. *S-Carboxymethylation*

After reduction of the half-cystine residues by dithioerythritol (60-fold excess over disulfide bonds) under nitrogen in 0.25 M Tris buffer with 5 M guanidine, pH 8.60, at 40°C, for 20 h, *S*-carboxymethylation was carried out as described [12] but in the buffer used for reduction. The modified protein (RCM-toxin) was recovered by gel filtration on a Sephadex G-25 column (3 × 75 cm) equilibrated in 0.1 M ammonium bicarbonate, at pH 8.60.

2.3. *α-Chymotrypsin digestion*

One μmol RCM-toxin was digested by α-chymotrypsin in 1 ml 0.2 M ammonium bicarbonate, pH 8.0, at 37°C, for 12 h, with a final enzyme to substrate ratio of 5% (w/w): 1% enzyme was added to initiate the digestion, then four additions of the same amount of enzyme were made at 1 h intervals. The digest was directly applied on a column of Biogel P4 (2 × 200 cm) equilibrated in 0.1 M ammonium acetate buffer, at pH 8.60.

2.4. *Peptides amino acid compositions*

Amino acid compositions of peptides were determined after 20 h hydrolysis in 5.7 N HCl, at 110°C. The determination of tryptophan residues was performed according to [13], the hydrolysis being achieved by the *p*-toluene sulfonic acid in the presence of tryptamine for 20 h, at 110°C, in vacuum-sealed tubes. Some peptides have been completely hydrolyzed enzymatically by the procedure [14]. The peptides (about 100 μg) were first hydrolyzed by papain (5 μg) in ammonium acetate buffer (0.05 M, pH 5.35) during 2 h at 37°C. After lyophilisation, the residue was submitted to the action of aminopeptidase M (150 μg) in presence of imidodipeptidase (10 μg) in trimethylamine/acetic acid buffer, pH 8.2. In both cases, hydrolysis were achieved in presence of mercaptoethanol (5 μl 1 : 32 solution in water). Amino acid analysis was performed on a Beckman model 120 C Analyzer.

2.5. *Automatic sequential degradation*

Sequential Edman type degradations were achieved in a protein Sequencer PS 100 Socosi (Saint Maur 94100, France). The peptide program was used in presence of hake parvalbumin (0.4 ml solution at 5 mg/ml) as described [15]. PTH amino acids were identified by gas chromatography [16] with a Beckman GC-65 apparatus and by thin-layer chromatography (TLC) on silica gel plates [17,18] and on polyamide double-faced sheets [19]. Furthermore acidic conversions of phenylthiohydantoins derivatives into free amino acids were performed with 5.7 N HCl in presence of 0.1% $SnCl_2$, for 4 h, at 150°C [20] and the products analyzed on the Beckman 120 C. The arginine derivative was also revealed by Sakagushi's reaction [21] on paper strips.

2.6. *C-Terminal determination*

The RCM toxin (100 nmol) was dissolved in 0.2 M *N*-ethylmorpholine buffer, pH 8.0 and digested by carboxypeptidase A, at 35°C, with 5% enzyme (w/w). In a second experiment, after 6 h digestion by carboxypeptidase A, carboxypeptidase B was added in an enzyme to substrate ratio of 1/25 on a molar basis. In both cases, aliquots of digest were removed after 15, 30, 60 and 360 min, acidified with 1.0 N HCl (0.1 ml), freeze dried and subjected to amino acid analysis.

3. Results and discussion

The automated Edman degradation of the RCM toxin (300 nmol) allowed us to determine directly the sequence of amino acids till to the lysine at position 26, except for the two positions 2 and 23 (see fig.1) which remained ambiguous due to low yields. All the amino acid derivatives were determined by at least two of the identification procedures described in Materials and methods.

The chymotryptic peptides were obtained after chromatography of the RCM toxin digest on a Biogel P4 column (fig.2). The different fractions were analyzed for their amino acid compositions (see table 1) and C_I (200 nmol), C_{III} (170 nmol) and C_V (200 nmol) have been sequenced by the automated Edman degradation procedure.

Results are shown also on fig.1. The identification by TLC at positions 2 and 23 of the serine residues as

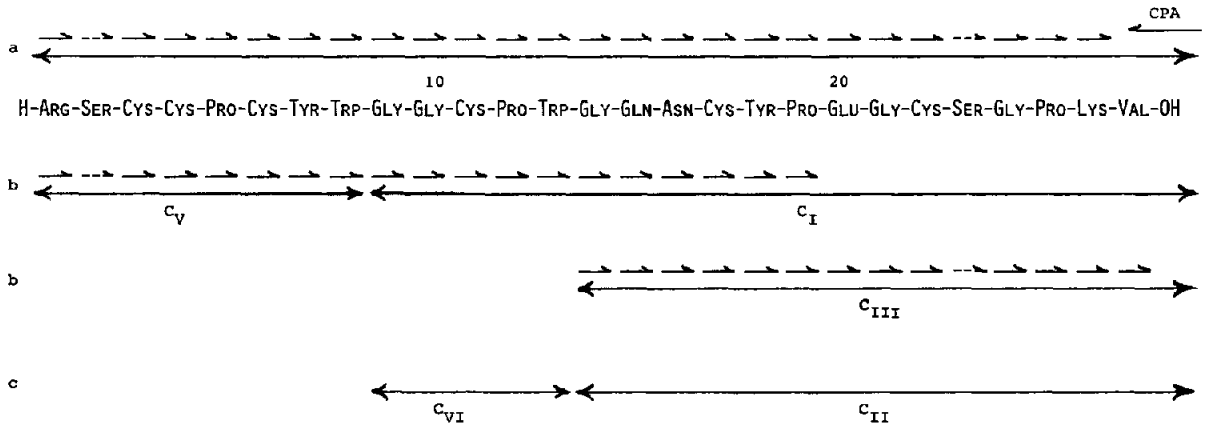

Fig.1. Amino-acid sequences of toxin III of *Anemonia sulcata*. Horizontal arrows (⟶) indicate the positions determined by Edman degradation: (a) on the complete protein; (b) on chymotryptic peptides. The dotted arrows (– – ➛) express some ambiguity in the direct identification. (CPA) shows the amino-acid released by action of carboxypeptidase A on RCM toxin. (c) Peptides identified by amino acid analysis.

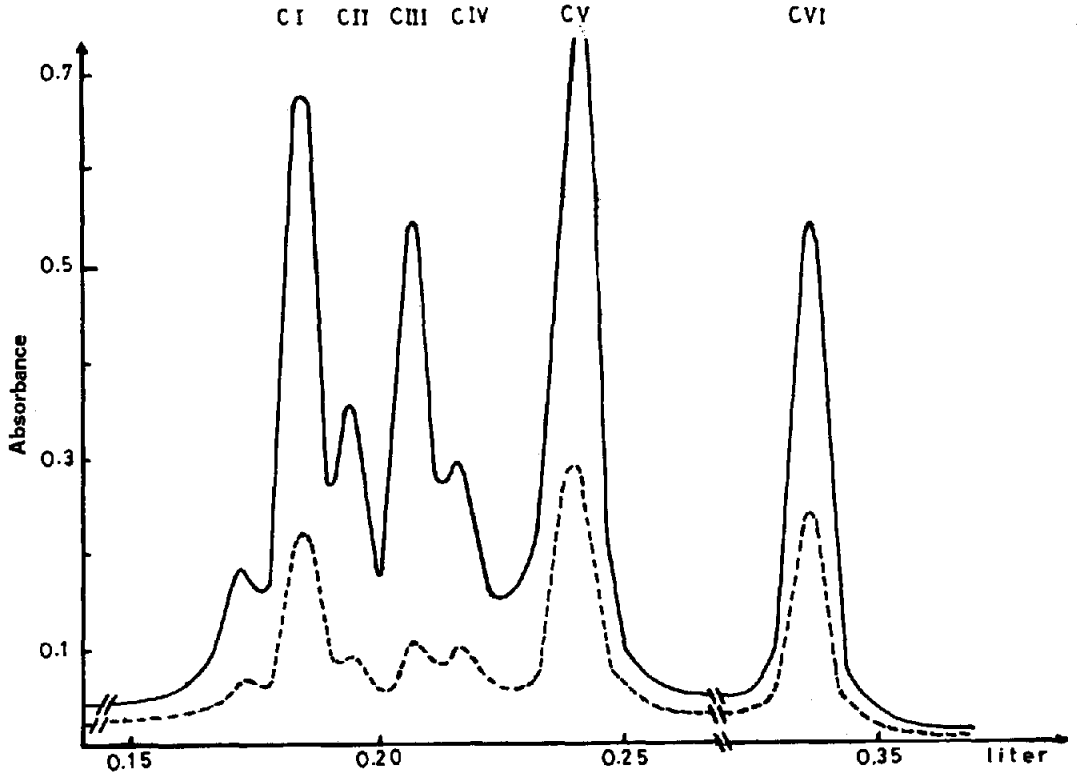

Fig.2. Elution pattern of the chymotryptic digest of RCM toxin on a Biogel P4 column (2 × 200 cm) equilibrated in 0.1 M ammonium acetate buffer, pH 8.6, flow rate 12 ml/h. Full line, $A_{230\,nm}$. Dotted line, $A_{280\,nm}$.

Table 1
Amino acid compositions of the RCM toxin and of the chymotryptic fragments

Amino acids	RCM toxin		C_I		C_{II}		C_{III}		C_V		C_{VI}	
Aspartic acid	1.1	(1)	1.1	(1)	1.0	(1)	1.0	(1)				
Threonine												
Serine	1.7	(2)	1.0	(1)	1.0	(1)	1.1	(1)	1.2	(1)		
Glutamic acid	2.0	(2)	2.0	(2)	2.0	(2)	2.1	(2)				
Proline	4.2	(4)	2.8	(3)	2.3	(2)	2.2	(2)	1.2	(1)	0.9	(1)
Glycine	4.9	(5)	4.8	(5)	3.1	(3)	2.9	(3)			2.0	(2)
Alanine												
CM-cysteine	5.5	(6)	2.9	(3)	2.2	(2)	2.0	(2)	3.0	(3)	1.0	(1)
Valine	1.0	(1)	1.0	(1)	1.0	(1)	1.0	(1)				
Methionine												
Isoleucine												
Leucine												
Tyrosine	1.7	(2)	0.9	(1)	0.8	(1)	0.9	(1)	1.0	(1)		
Phenylalanine												
Histidine												
Lysine	1.0	(1)	1.0	(1)	0.9	(1)	1.0	(1)				
Arginine	1.2	(1)							0.9	(1)		
Tryptophan	2.0[a]	(2)	+[b]	(1)					+[b]	(1)	+[b]	(1)
Total		(27)		(19)		(14)		(14)		(8)		(5)
Sequence positions of peptides			9–27		14–27		14–27		1–8		9–13	
Yield (%)			22		16		40		63		34	

[a] Tryptophan determination according [13]
[b] Ehrlich's reaction

PTH derivatives remained slightly ambiguous; however acidic conversions of these derivatives showed the presence of serine. Finally the amino acid compositions of C_{III} and C_V, both after acidic and enzymatic hydrolysis, clearly stated the presence of one residue of serine in each of these peptides that could only be placed at the above mentioned positions. According to amino acid composition, fraction C_{VI} should correspond to positions 9–13. The amino acid compositions of fractions C_{II} and C_{III} are identical. Therefore, we assumed the same location in the sequence for these two peptides, although no satisfactory explanation has been found with regard to their different elution on Biogel P4. The action of carboxypeptidase A and B showed only the release of 1 Val/mol toxin. Lysine at position 26 was not released probably due to the presence of proline at position 25.

The amino acid sequence of toxin III of *Anemonia sulcata*, as shown on fig.1, is in complete agreement with the amino acid composition of the RCM toxin (table 1) and consistent with the isoelectric point of the native toxin, estimated to be near 8.1 by electrofocusing.

The remarkable features of the toxin III sequence are the following:

(i) The toxin is only 27 amino acids long and rich in disulfide bridges.

(ii) Eight amino acids are not represented at all in this toxin sequence, Met, Leu, Ile, Phe, Thr, His, Asp and Ala.

(iii) Aromatic residues are abundant (2 Tyr, 2 Trp) as well as glycine residues which nearly make up 20% of the total amino acid content.

(iv) There are three main regions in the sequence, the N-terminal dipeptide which is very polar, the sequence from residues 3–14 which is predomi-

nantly hydrophobic and the C-terminal part of the molecule which has again a polar character.

There is no evident extensive sequence homology between toxin II, the 47 amino acids long toxin and toxin III sequenced in this paper. The only common sequence is Gly–Cys–Pro which is in positions 10–13 in toxin III and in positions 26–28 in toxin II [7]. It is of interest to remark that this sequence is in both cases in a very hydrophobic region of the molecule (region 21–31 in toxin II). This hydrophobic region containing both in toxin II and in toxin III two tryptophan which sit on each side of the common sequence Gly–Cys–Pro. Although toxin II and toxin III appear to have considerable differences in primary structure, their mode of action on crayfish nerves does not exhibit such discrepancies. Toxin III similarly to toxin II [9] considerably prolongs the action potential of crayfish nerves at concentrations as low as 10^{-8} M (Cavey, M. T., Romey, G. and Lazdunski, M., in preparation), its main effect being the prevention of the closing step of the sodium channel. However toxin III displays a veratridin-like depolarizing action which is more accentuated than that observed for toxin II at low concentrations. Nevertheless the presently available pharmacological results show that although the two toxins have very different sequences, they have essentially the same type of mode of action. Until now important sequence homologies have been found within the class of snake neurotoxins [22] or within the class of scorpion neurotoxins [23]. The situation seems to be different within the class of the sea-anemone toxins (which, as a matter of fact, have no homology with snake or scorpion neurotoxins).

The small size of toxin III from *Anemonia sulcata* makes it particularly interesting for structure–fuction analysis using the peptide synthesis approach. Toxin III is after apamin (18 amino acids and 2 disulfide bridges), the smallest polypeptidic neurotoxin actually known.

Just as this paper was ready for submission, the complete amino-acid sequence of the same toxin was published [24]; the determination of this sequence was performed using Edman degradation in solid-phase. All the results were in agreement with those exposed here, except for residues 22 and 23, the sequence Ser–Cys in place of Cys–Ser. We determined these positions on the whole RCM toxin and confirmed

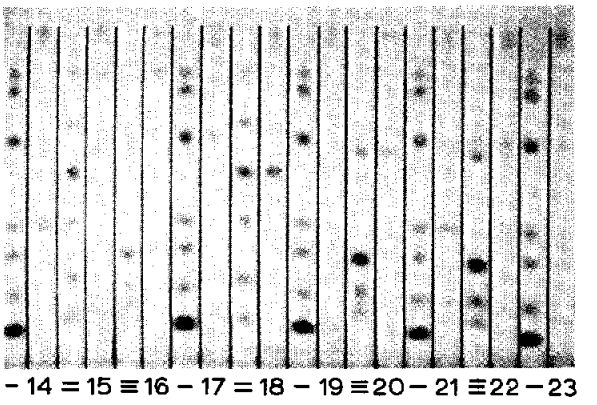

Fig.3. Thin-layer chromatography on silica gel plates (Edman solvent H) of the PTH amino acids obtained from the sequencing of peptide C_{III} (residues 14–27 in the sequence of the toxin). Reference mixtures are indicated by horizontal bars: (–), (=) (≡) as described [17,18].

by the sequencing of the chymotryptic peptide C_{III}, as shown on fig.3. This clearly demonstrates the presence of a residue of carboxymethylcysteine in position 22.

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