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THE AMINO ACID SEQUENCE OF TOXIN V FROM ANEMONIA SULCATA

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INTRODUCTION : Sea anemone toxins have become very useful tools to study the voltage-dependent Na^+ channel in nerve, cardiac and muscle cells (1-4).

Four different neurotoxins - AS_I, AS_{II}, AS_{III} and AS_V - have been isolated in the pure form from Anemonia sulcata (5,6). These toxins are polypeptides consisting of 46, 47, 27 and 46 amino acid residues respectively. Although they are all specific for the voltage-dependent Na^+ channel, they display a marked difference in their toxicity for different animal species. Some are more active on crustaceans, others on mammals (6). Three of the four toxins - AS_I, AS_{II} and AS_{III} - have already been sequenced (7-10). AS_V has been discovered more recently (6); it is the most toxic of the four polypeptides when injected to mice and the most useful one in studies dealing with the Na^+ channel. The present paper reports the primary structure of AS_V.

MATERIALS AND METHODS : Toxin V was purified according to (6). S-carboxymethylation was made according to (11) with minor modifications as follows : 1.3 mg toxin V was reduced with 1.5 µl β-mercaptoethanol in 250 µl of a 0.36 M Tris-HCl buffer at pH 8.6 containing 8 M urea and 0.2% ethylenediamine-tetraacetate at 37°C under N_2 atmosphere. After 4 hr 22 µmol of ^{14}C iodoacetic acid (1.14 µCi, New England Nuclear Co. and Serva Feinbiochemica) were added and the mixture was incubated further for 2 hr at 37°C. The CM-toxin was recovered by gel filtration on a Bio-Gel P_2 column (1 x 70 cm) equilibrated with 70% formic acid.

Trypsin digestion - 200 nmol of CM-toxin V was digested with trypsin (50/1 : w/w, Worthington Biochemicals) in a 0.1 M pyridine-collidine acetate buffer at pH 8.2 at 37°C for 12 hr. Peptide separation was carried out by gel filtration on a Sephadex G25 column (1 x 100 cm) equilibrated in a 0.1 M pyridine-acetate buffer at pH 7.0. Peptide elution was monitored by radioactivity and by amino acid analysis after hydrolysis.

Amino acid analysis - The toxin or peptides were hydrolysed in a mixture of CF_3CO_2H/HCl (1:2) at 166°C between 25 to 50 min (12). For cystine analysis, the toxin was oxidized with performic acid (13) and hydrolysed. For tryptophane analysis, hydrolysis was made with 3 N mercaptoethanesulfonic acid according to (14) using a microscale modification (Maeda, Scheffler & Tsugita, unpublished data). Amino acid analysis was performed on a Durrum D500 analyser, set to a sensitivity of 2.5 nmol of amino acids.

Abbreviations : Tris : tris(hydroxymethyl)aminomethane; CM : carboxymethyl; AS_I : toxin I from the sea anemone Anemonia sulcata; AS_{II} : toxin II from the sea anemone Anemonia sulcata; AS_{III} : toxin III from the sea anemone Anemonia sulcata; AS_V : toxin V from the sea anemone Anemonia sulcata; AX_I : toxin I from the sea anemone Anthopleura xanthogrammica. Enzymes : carboxypeptidase A (EC.3.4.17.1), carboxypeptidase B (EC.3.4.17.2), carboxypeptidase P (EC.3.4.16.1), trypsin from bovine pancreas (EC.3.4.21.4).

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Table I - Amino acid composition of toxin V and of the tryptic fragments

	ASV		T(1-14)	T(15-36)	T(15-46)
	25'	50'	25'	25'	25'
Asp	4.19	3.99 (4)	1.82 (2)	2.05 (2)	1.90 (2)
Thr	1.71	1.81 (2)	- (0)	0.91 (1)	2.05 (2)
Ser	4.32	3.80 (4)	1.85 (2)	2.22 (2)	2.03 (2)
Pro	3.36	3.45 (3.5)	1.48 (2)	0.97 (1)	1.75 (1.5)
Gly	7.50	7.50 (7.5)	2.02 (2)	3.92 (4)	5.9 (5.5)
Ala	0.99	0.98 (1)	- (0)	1.00 (1)	1.00 (1)
Cys	5.85	(6)			
Val	1.60	1.85 (2)	0.79 (2)	- (0)	- (0)
Ile	1.22	1.54 (2)	- (0)	0.50 (1)	1.48 (2)
Leu	3.51	3.67 (4)	1.00 (1)	1.93 (3)	2.01 (3)
His	1.57	1.75 (2)	- (0)	0.88 (1)	1.96 (2)
Lys	4.06	3.87 (4)	- (0)	1.82 (2)	3.65 (4)
Arg	0.96	1.05 (1)	0.60 (1)	- (0)	- (0)
Trp	2.51	(3)			

Numbers in parentheses were deduced from the sequence studies.

N-terminal analysis - 120 nmol of the ^{14}C CM-toxin V were first boiled in 0.5% sodium dodecylsulfate at 100°C for 5 min and then transferred to the spinning cup of a Beckman sequenator 890C (15) equipped with an autoconverter (P6 from Sequamat). We used the normal 0.1 M quadrol program from Beckman with double coupling in the first step. The thiazolinone amino acids were converted into phenylthiohydantoin amino acids with a mixture of acetylchloride: methanol (1:7, v/v). All reagents for the sequenator were from Beckman, except ethylacetate and butylchloride from Pierce and dithioerythritol from Sigma. Polybrene (3 mg) from Pierce was used as a carrier (16) and washed 3 times by processing the normal protein program. Dithioerythritol was added to the solvents as reported in (17). The resulting phenylthiohydantoin amino acids were analysed by high pressure chromatography (Pye Unicam LC_3) on a silica gel column (Si 100 5 m from Serva). The solvents were dichloroethane/methanol (250: 1.4, v/v) for the derivatives of apolar amino acids and dichloroethane/dimethylsulfoxide/methanol/acetic acid/water (250:8:8:2:0.75 by volume) for those of the polar amino acids (18). Derivatives of arginine and histidine were identified by thin layer chromatography on polyamides sheets (F.1700, Scheider & Schüll) (19) with a modified solvent : 0.75% pyridine acetate pH 4.25 (20). ^{14}C labelled CM-cystine was detected with a scintillation counter Beckman LS 8100.

C-terminal analysis - 5 µg of carboxypeptidases were added to 1-2 nmol of the CM-toxin or peptides, and incubated at 37°C for 6 hr (21). The digestion buffers were 0.1 N pyridine-collidine acetate, pH 8.5 for carboxypeptidases A and B, and 0.1 N pyridine-formate, pH 2.5 or pH 5.5 for carboxypeptidase P (Takara-Shuzo, Japan). Carboxypeptidase A treated with diisopropylfluorophosphate was from Worthington Biochemicals, carboxypeptidase B (Sigma) was treated with diisopropylfluorophosphate to completely remove endopeptidase activity.

RESULTS : The amino acid composition of the toxin is given in Table 1. Carboxypeptidase P digestion on the CM-toxin revealed the C-terminal sequence. Digestions at different pH resulted into different extents of digestion as shown in Fig. 1. Both carboxypeptidases A and B failed to digest.

Using CM-AS_V, the automated Edman degradation provided the entire sequence of the 46 residues (Fig. 1). ^{14}C CM-cystine was found at positions 4, 6, 27,

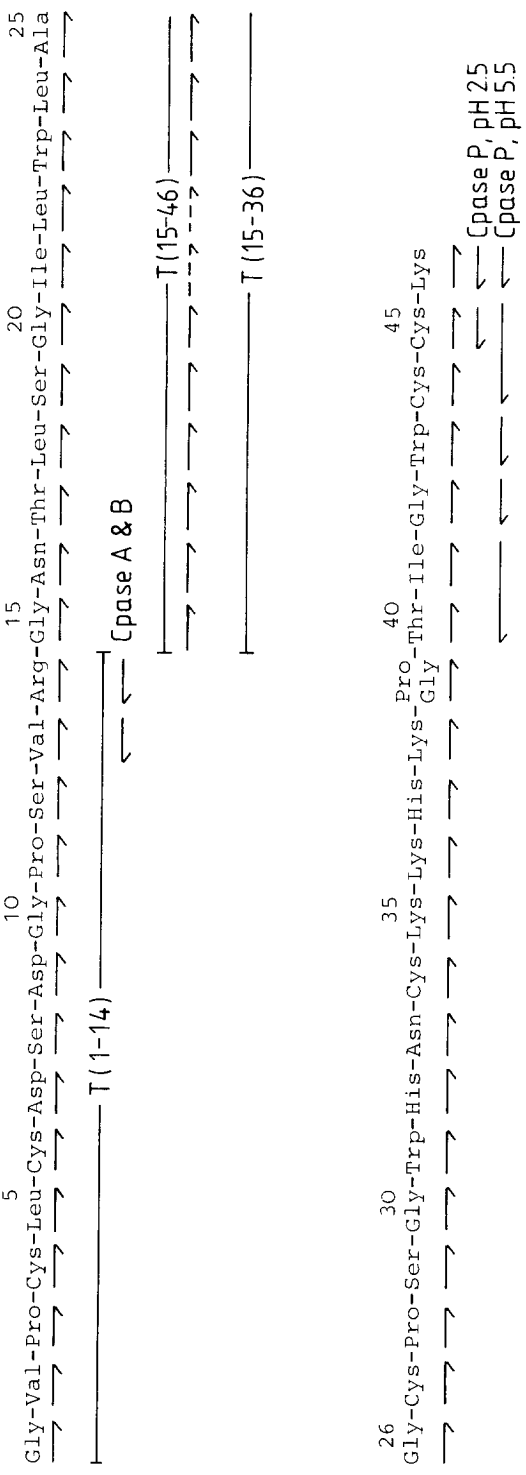


Figure 1. Amino acid sequence of toxin V from Anemonia sulcata

Table II - Determination of the position of ^{14}C cystine residues[a] in ASV sequence

Step	2	3	4	5	6	7	25	26	27	28	29	33	34	35	36	43	44	45	46	47
Counts	28	51	1676	208	1499	256	74	72	439	231	86	58	314[b]	193	123	82	160	183	116	111

[a] 1/10 of the volume of each step from the sequenator was analysed with a liquid scintillation system.

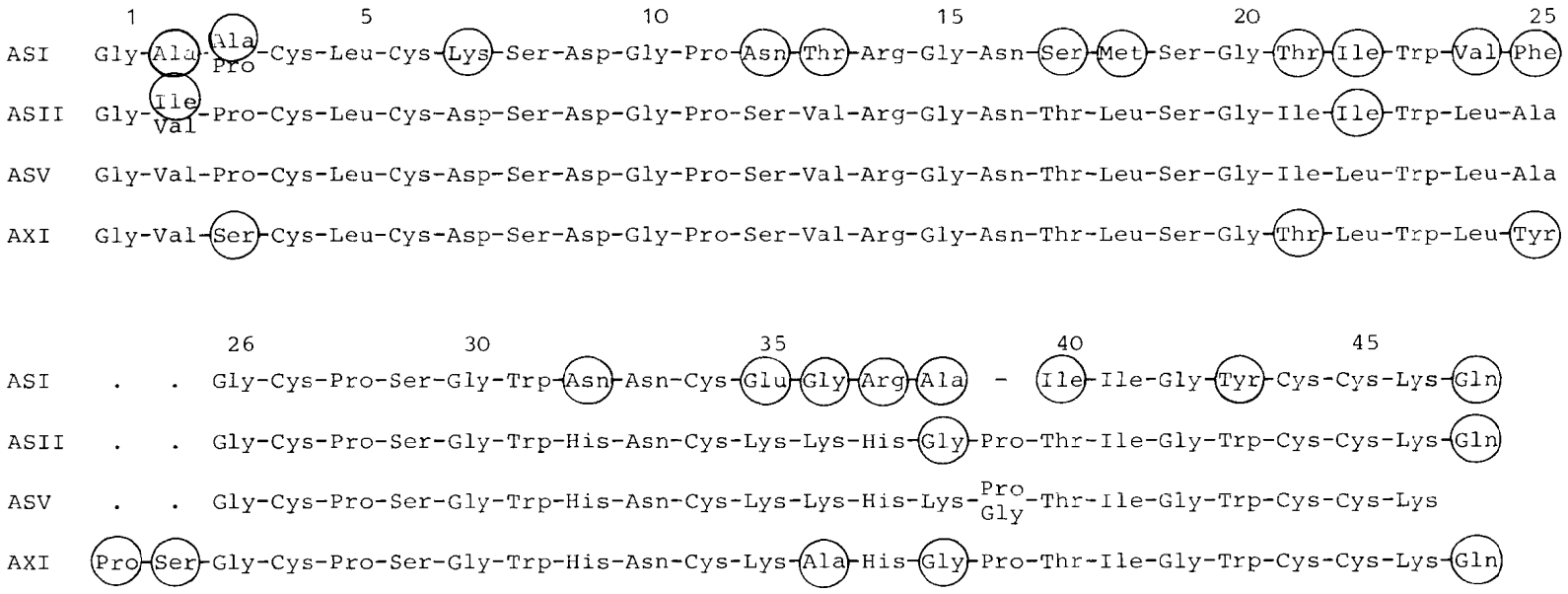
[b] Calculated from these values (taking a background of 70 counts) the yield of the sequenator for one step is about 97% (We recovered 15.2% of the expected counts after 29 steps which corresponds to a loss of 2.9% per step).

34, 44 and 45 (Table II). The other phenylthiohydantoin amino acids were determined as described in Materials and Methods. The sequence was established unambiguously for all the steps, except for step 39. In this step, both proline and glycine were found in approximately equal amounts indicating a microheterogeneity. The average recovery for each step was 97%. However, we found noticeable overlappings (about 10%) from step 29 and a further increase of the overlappings (20%) from step 40 to the end of the sequence. This can be explained by the presence of proline residues in positions 28 and 39. To confirm the sequence obtained by the sequenator, CM-AS_V was digested with trypsin and the resulting peptides were separated by gel filtration on Sephadex G25. Three main peaks were recovered. The first peak contained the N-terminal peptide T(1-14), the second the middle part T(15-36) and the last one the C-terminal peptide T(15-46) which overlaps with T(15-36). Amino acid compositions of these peptides are given in Table I. Peptide compositions predicted by the sequence (indicated in parentheses) were in agreement with the values observed, except for valine, proline and arginine in T(1-14) and for isoleucine and leucine in both T(15-36) and T(15-46). These low values found experimentally are due to difficult cleavage of valyl and isoleucyl peptide bonds during hydrolysis (12). Peptide T(1-14) was digested with carboxypeptidases A and B and confirmed the C-terminal sequence to be Val-Arg. C-terminal sequence of peptides T(15-36) and T(15-46) were confirmed using carboxypeptidase P. Peptide T(15-46) (50 nmol) was sequenced with the automatic sequenator and the sequence found is shown in Fig. 1. Amino acid compositions and partial sequences of these tryptic peptides coincided with the sequence of the toxin directly established by the sequenator.

DISCUSSION : The main features of the amino acid sequence of toxin V are the following : (i) the absence of 5 very common amino acids : methionine, tyrosine, phenylalanine, glutamic acid and glutamine, (ii) a high content of tryptophan and cystein, 3 Trp and 6 Cys for 46 residues and (iii) the presence of 3 hydrophobic regions, the N-terminal region (2-6), the middle part (21-28) and the C-terminal region (41-45) of the toxin sequence. Like the other sea anemone toxins, AS_V is a protein with an hydrophobic character (63% hydrophobic amino acid) (22). Comparative sequences of toxins I, II and V from Anemonia sulcata and of toxin I (AX_I) from Anthopleura xanthogrammica (23) are presented in Fig. 2. Toxin V shows 65% identical residues with toxin I, 90% with AX_I and 95% with toxin II.

There is an extensive homology between toxins II and V (Fig. 2). The main differences observed are : (i) a conservative mutation in position 22 between isoleucine (AS_{II}) and leucine (AS_V), (ii) the loss of glutamine in the C-terminal position without alteration of the rest of the C-terminal sequence, and (iii) the replacement in the position 38 of a glycine residue (AS_{II}) by a lysine residue (AS_V). It is interesting to remark that such small changes in sequence result into fairly larger differences in biological activity. AS_V is five times more toxic than AS_{II} on mice, it binds better to the Na^+ channel of rat brain synaptosomes and is more than 10 times more active than AS_{II} in stimulating $^{22}Na^+$ uptake by neuroblastoma cells (6).

The more extensive differences in sequence between AS_V and AS_I are related to very extensive differences in the toxicity of these peptides for mice. Although the two toxins have similar types of toxicities towards crabs, the toxicity towards mice is nearly absent for AS_I whereas it is high for AS_V (6). There is at least a 1000 times differences in the potencies of AS_I and AS_V ($AS_V \gg AS_I$) for the stimulation of $^{22}Na^+$ uptake by neuroblastoma cells. Differences in sequence that can be responsible for the decrease of toxicity towards mice appear in Fig. 2. It will be necessary to sequence other sea anemone toxins that are active on crustaceans but not on mammals, like AS_I, to delineate exactly what are the minimum sequence requirements in anemone



The encircled amino acids are different from those of ASV.

Figure 2. Homologies in the amino acid sequences from different sea anemone toxins: Toxins ASI, ASII, ASV from Anemonia Sulcata and toxin AXI from Anthopleura Xanthogrammica

toxins that are responsible for the animal species selectivity of the toxins. It already appears that important changes in sequence in the 35-40 region are present in AS_I as compared to other 3 toxins that are active on mammals. Arginine 14 which is known to be essential for the binding of AS_{II} to the Na^+ channel in synaptosomes (24) is present in the structure of all toxins sequenced up till now.

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