Purification and Chemical and Biological Characterizations of Seven Toxins from the Mexican Scorpion, *Centruroides suffusus suffusus*  

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Seven polypeptides highly toxic to mice were isolated from the venom of the scorpion, *Centruroides suffusus suffusus* (Css), and their chemical and toxic properties were characterized. It was shown that the most active toxins by intracerebroventricular injection are less active when injected subcutaneously. The complete amino acid sequence (66 residues) of toxin II (Css II) has been determined. The C-terminal end is amidated as found for most other scorpion toxins. Css II is a β-type toxin, previously used to define the binding site for activation of the sodium channel. Using rat brain synaptosomes, we demonstrated that all Css toxins compete with 125I-Css I1 to bind to site 4 and should be considered as β-scorpion toxins. Specific binding parameters for Css VI, one of the most active toxins, were determined: \( K_D = 100 \) pm; capacity in binding sites, 2.2 pmol of toxin/mg of synaptosomal protein. Css VI was shown to inhibit γ-aminobutyric acid uptake by synaptosomes; \( K_0.5 = 100 \) pm, which agrees with its \( K_D \). Competition experiments between the sevenCss toxins and 125I-Css II for antiserum raised against Css II demonstrated that all these toxins have common antigenic properties.

Scorpion toxins which selectively bind with high affinity to voltage-dependent sodium channels have proved to be valuable tools for the identification and characterization of these ionophoric structures. Scorpion toxins constitute a family of basic minipeptides made of a single chain of 60–70 amino acid residues cross-linked by four disulfide bridges (1). They alter the normal functioning of voltage-dependent nerve and muscle sodium channels. Among toxins which are active in mice, two groups have been defined according to differences in their binding properties and in their pharmacological activities (2, 3). The first group (α-toxins) is composed of toxins isolated mainly from venom derived from scorpions living in the "Old World." These α-toxins slow the inactivation of the sodium channel, and their binding to the receptor at site 3 decreases with depolarization of the membrane (4, 5). Toxins of the second group (β-toxins) are obtained from venoms of American scorpions (6–9). They modify the sodium channel activation process and bind to a different receptor site (site 4). Their binding affinity is independent of the membrane potential in synaptosomes (3).

The present work deals with an extensive study of toxins lethal to mice which are present in the venom of *Centruroides suffusus suffusus*, a scorpion living in the area of Durango in Mexico. Seven Css1 toxins have been purified and characterized. One of them,Css II, has been entirely sequenced here. It was the first β-toxin to be used to demonstrate the existence of the fourth receptor site on the sodium channel (2). Specific binding parameters of Css VI, one of the most active toxins in the venom, were defined on rat brain synaptosomal fractions. The antigenic properties of these polypeptides were studied extensively.

**MATERIALS AND METHODS**

**RESULTS**

Purification of *Centruroides suffusus suffusus* Toxins—The different venom fractions obtained during the purification process are described in the flow chart given in Fig. 1. The elution patterns of the chromatographic steps can be found in the Miniprint Section (Figs. 2–5). Css toxins were numbered according to retention time on cationic exchangers. Fig. 6 shows polyacrylamide gel electrophoresis of major toxins compared with the venom's water extract. Table I gives quantitative data related to purification. When scorpion telsons were used instead of venom as starting material, the results were slightly different: only Css II was recovered from fraction R1, but with a lower yield (60% of that obtained with the venom), and Css I could only be detected. In this case, fraction R2 was not further purified. Toxicity tests were performed on mice both by subcutaneous and intracerebroventricular injections. The results show that the toxins are much more active by intracerebroventricular injection: about 100 times more active with Css I–III and VII; about 1000–1500 times with Css IV–VI. One protein, P1, inactive in mice, was also obtained in a pure form. Its amino acid composition is given in Table II together with those of the Css toxins. Css I and II have the same amino acid composition, and P1 is closely related to toxins. Isoelectric focusing (not shown) indicates that both Css I and II have a pI value approaching 9, Css I being slightly more acidic than Css II. The most likely explanation:

1. The abbreviations used are: Css, *Centruroides suffusus suffusus*; AAh, *Androctonus australis* Hector; GABA, γ-aminobutyric acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

2. Portions of this paper (including "Materials and Methods," "Sequence Determination," Figs. 2–7, and additional references) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-2867, cite the authors, and include a check or money order for $9.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

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Characterizations of Toxins from a Mexican Scorpion Venom

4453

Effects of Css Toxins on Binding of $^{125}$I-AaH to Anti-Css II IgGs—Fig. 9 gives dose-response curves obtained when adding different homologous Css toxins to an equimolar mixture of $^{125}$I-Css II and anti-Css II IgGs. Concentrations of Css toxins which allow the half-maximum effect to be attained are given in Table II. It appears that Css II has high affinity for its specific antibodies ($K_D = 0.17 \text{ nM}$). The shape of the displacement curve is noticeable when Css I is used: at first, it follows the standard curve and then deviates, but complete competition is still possible. It would be interesting to determine the amino acid sequence of Css I to check for degradation which could affect some but not all antigenic regions of this protein.

The common antigenic behavior of these beta-toxins may also reflect a high degree of sequence homology.

Characterization of Css Toxin Binding to Rat Brain Synap-

tosomal Fraction—On the basis of their binding properties to rat brain synaptosomes and the different mechanisms which act on the sodium channels of excitable membranes, the existence of two types of scorpion toxins, alpha- and beta-toxins, has been demonstrated (2, 3). These two types of scorpion toxins are represented by Androctonus australis Hector (AaH) II (alpha) and Css II (beta), respectively. Css toxins have been tested on rat brain synaptosomal fractions in two sets of displacement experiments using either radioiodinated AaH II or Css II. Css toxins are unable to compete with $^{125}$I-AaH II for site 3 on the sodium channel, even with concentrations up to 10 $\mu$M (data not shown). On the other hand, all Css toxins behave like beta-toxins because they cause $^{125}$I-Css II to be completely displaced from site 4 (Fig. 10). The $K_D$ values calculated from these displacement experiments are given in Table II: several toxins (i.e., Css IV–VII) were more efficient than Css II in displacing $^{125}$I-Css II from its site. Finally, the venom extract was used in competition experiments with $^{125}$I-AaH for site 3, and concentrations up to 10 mg/ml were ineffective. In addition, the binding properties of Css VI were studied on the rat brain synaptosomal fraction: the Scatchard plot is linear and indicates that $^{125}$I-Css VI binds to a single class of noninteracting binding sites (data not shown). Four independent experiments gave an average $K_D$ of 100 $\pm$ 10 $\mu$M and a site capacity of 2.2 $\pm$ 0.2 pmol/mg of protein. This specific binding has been further correlated with the inhibitory action of Css VI on GABA uptake by rat brain synaptosomal fractions. The dose-response curve of this effect is given in Fig. 11: the value of $K_D$ (100 $\text{pm}$) corresponds well to the binding characteristics of Css VI. As already shown with Css II (3, 10), this inhibition of GABA uptake is probably due to a change in the potential-dependent sodium channel activity induced by beta-toxin binding at site 4.

**DISCUSSION**

Using 15 g of C. suffusus suffusus venom, seven toxins which are highly active in mice were obtained in a pure state as evidenced by ion-exchange chromatography (Miniprint Section, Figs. 4 and 5) and polyacrylamide gel electrophoresis (Fig. 6). The toxicity of the seven Css toxins accounts roughly for 40% of the venom toxicity regardless of the injection method. We believe this nonquantitative yield is the consequence of cumulative losses occurring during the numerous purification procedures. Lethality was tested on mice throughout; and consequently, only the so-called "mammal toxins," i.e., those proteins toxic in mice, were detected and purified. Many different toxins have also been found in other Centruroides venoms, i.e., Centruroides sculpturatus Ewing (6) and Centruroides nocxius (7). One may ask whether this can be attributed to the high number of animals milked or to a single animal's capacity to synthesize several toxins, or to a combination of these two factors. This represents a general problem with pooled scorpion venoms (11).

The amino acid sequence of Css II as well as preliminary results on Css I and other Css toxins indicate that, as with other scorpion toxins, Css toxins are single chain miniproteins with 60–66 residues cross-linked by four disulfide bridges (Table II). This is evident in the amino acid sequence of Css II given in this paper and also by experiments in progress in our laboratory (3) on the sequence of other Css toxins. Amino acid compositions (Table II) show a high content in aromatic and basic amino acids, which is common to all scorpion toxins. Css toxins have several characteristics: one or, more generally, two amino acids are lacking, and the aspartic acid/glutamic

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3Sampieri, F., Becchi, G., and Rochat, H., experiments in progress.
Characterizations of Toxins from a Mexican Scorpion Venom

Table I

Quantitative data concerning the purification and etiological characterization of Css toxins

| Toxins    | Yield in weight of toxins from crude venom | sc LD₅₀ | Yield in toxicity from crude venom | icv LD₅₀ | Yield in toxicity from crude venom | Radiomunoassay | Radioreceptor assay |
|-----------|-------------------------------------------|--------|----------------------------------|----------|----------------------------------|---------------|-------------------|
|           |                                            | µg/kg mouse |                                  | µg/kg mouse |                                  | Kₐ₅          | Kᵦ                 |
| Crude venom | 100.0                                      | 600     | 100.0                              | 4.25      | 100.0                              | 37.0          | 3.30               |
| Cas I     | 0.11                                        | 100     | 1.8                               | 0.60      | 0.7                               | 0.30         | 10.00             |
| Cas II    | 1.20                                        | 25      | 37.0                              | 0.25      | 20.4                              | 0.17         | 3.30               |
| Cas III   | 0.04                                        | 85      | 0.3                               | 0.60      | 0.3                               | 1.50         | 3.00               |
| Cas IV    | 0.14                                        | 115     | 0.9                               | 0.12      | 4.9                               | 0.79         | 1.13               |
| Cas V     | 0.02                                        | 130     | 0.1                               | 0.08      | 1.0                               | 1.20         | 0.30               |
| Cas VI    | 0.09                                        | 85      | 0.7                               | 0.06      | 6.4                               | 1.00         | 0.08               |
| Cas VII   | 0.07                                        | 115     | 0.8                               | 0.50      | 0.6                               | 5.00         | 1.13               |
| P1        | 0.11                                        | 12,500  |                                   | 30.00     |                                   | 38.00        | 330.00            |
| Total     |                                            | 1.75    |                                   | 41.6      |                                   | 34.3         |                   |

*After subcutaneous injection.
*After intracerebroventricular injection.

Table II

Amino acid composition of C. sulfus sulfus toxins

| Residue | Toxin I | Toxin II | Toxin III | Toxin IV | Toxin V | Toxin VI | Toxin VII | P1 |
|---------|---------|----------|-----------|----------|---------|----------|-----------|----|
| Asp     | 5.04    | 5.03     | 5.87      | 4.86     | 5.85    | 5.70     | 6.68      | 6.09 |
| Thr     | 3.08    | 3.06     | 2.80      | 2.89     | 2.93    | 3.08     | 2.63      | 2.39 |
| Ser     | 4.03    | 4.15     | 1.67      | 1.90     | 1.70    | 1.70     | 1.10      | 1.62 |
| Glu     | 6.89    | 7.01     | 6.24      | 5.87     | 4.45    | 6.84     | 4.10      | 4.48 |
| Pro     | 2.17    | 2.12     | 2.38      | 2.09     | 2.41    | 2.32     | 2.63      | 3.18 |
| Gly     | 6.07    | 6.12     | 7.90      | 6.70     | 8.44    | 7.68     | 8.57      | 7.92 |
| Ala     | 3.05    | 3.13     | 2.08      | 2.01     | 3.48    | 2.12     | 3.78      | 0    |
| Cys     | 7.92    | 7.85     | 5.94      | 5.60     | 6.38    | 7.23     | 6.42      | 5.51 |
| Val     | 2.93    | 3.09     | 2.38      | 2.10     | 2.15    | 2.06     | 2.19      | 1.84 |
| Met     | 0       | 0        | 0         | 0        | 0       | 0        | 0         | 0   |
| Ile     | 0       | 0        | 0         | 0        | 0       | 0        | 0         | 0   |
| Leu     | 5.60    | 5.86     | 4.81      | 4.55     | 4.65    | 3.78     | 5.06      | 3.65 |
| Tyr     | 6.57    | 6.95     | 5.46      | 5.62     | 7.03    | 5.77     | 7.23      | 4.22 |
| Phe     | 1.06    | 1.07     | 2.99      | 3.00     | 1.28    | 3.26     | 1.16      | 2.13 |
| His     | 1.05    | 1.00     | 1.32      | 1.22     | 1.95    | 1.55     | 2.10      | 1.98 |
| Lys     | 1.21    | 1.01     | 4.86      | 4.16     | 5.95    | 7.23     | 5.71      | 6.34 |
| Arg     | 1.11    | 1.07     | 1.94      | 1.89     | 2.00    | 1.19     | 1.97      | 1.01 |
| Trp     | 1.92    | 1.80     | 1.77      | 1.97     | 1.84    | 2.06     | 2.18      | 2.73 |
| Total   | 66      | 66       | 63        | 60       | 64-67   | 64-65    | 67        | 58-60 |

*ΩM X 10⁻³ | 19.50   | 20.44    | 19.00    | 19.51    | 19.82   | 21.90    | 22.20     |

The complete amino acid sequence of Css II was determined using standard procedures, but with two objectives: to use automated Edman degradation extensively and to avoid where possible peptide purification procedures. Css II, like many other scorpion toxins (1), was found to be amidated at the C-terminal end. The sequence of Css I will have to be determined in order to ascertain whether Css I and II differ in one (or more) amidated groups.

The primary structures of Css II and other scorpion toxins are compared in Fig. 12. The sequences are arranged such that the cysteine residues are aligned and deletions are introduced to maximize homology. These amino acid deletions occur in positions which are different in a and b-toxins. a-Toxins are characterized by two deletions, the first (at least 20) of 3 amino acid residues) begins at position 20, whereas the second (generally of 2 or 3 amino acid residues) is located at position 69. The beginning of a deletion at position 57 replaces the proline found in all other a- and b-toxins. Glutamine (position 32) and histidine (position 57) replace lysine and glycine residues present in other b-toxins, respectively. Despite these modifications, the amino acid sequence for Css II seems to comply to the general sequence proposed for scorpion toxins (24).
Characterizations of Toxins from a Mexican Scorpion Venom

FIG. 8. Amino acid sequence of toxin II of C. suffusus suffusus. A, automatic sequencing of 500 nmol of S-methylated Cas II; B, automatic sequencing of the tryptic digest of the 1200 nmol of S-pyridylethylated, NH$_2$-succinylated Cas II; C, automatic sequencing of peptide T36-63; D, automatic sequencing of the Staphylococcus aureus protease digest of Cas 11; E, automatic sequencing of peptide T54-66; F, automatic sequencing of peptide C63-66; ---, not determined.

FIG. 9. Effect of Cas I-VII and P1 on an interaction of $^{125}$I-labeled Cas II with anti-Cas II IgGs. The standard curve was based on Cas II (O): 0.2 nM $^{125}$I-Cas II and 0.2 nM anti-Cas II IgGs (final concentrations). For Cas I (€), Cas III (€), Cas IV (§), Cas V (¹), Cas VI (Ç), Cas VII (Δ), and P1 (W), incubation conditions were the same as for the standard curve.

FIG. 10. Competitive experiments between $^{125}$I-Cas II and Cas toxins. The rat brain synaptosomal fraction (0.4 mg/ml) was incubated 30 min at 37 °C in the following medium: 140 mM choline chloride, 5.4 mM KCl, 25 mM Hepes, 10 mM glucose, 0.25% bovine serum albumin, Tris base to obtain pH 7.2, in the presence of 0.1 nM $^{125}$I-Cas II and increasing concentrations of native Cas I (€), Cas II (O), Cas III (€), Cas IV (§), Cas V (¹), Cas VI (Ç), Cas VII (Δ), and P1 (W). $B_o$ is the binding of $^{125}$I-Cas II in the absence of native toxin, and $B$ is the binding in the presence of the indicated concentrations of native toxin. Nonspecific binding has been subtracted.

All Cas toxins react either with anti-Cas II serum or with purified anti-Cas II IgGs, which indicates that they all are antigenically homologous to Cas II. These results may lead to the development of an efficient serotherapy against Centruroides scorpio. Although there is no evidence that each of the 120,000 animals used was able to synthesize the seven toxins, the fact that complete cross-reactive has been obtained (Fig. 9) seems to indicate that, regardless of the Cas venom batch used, an efficient antisera will be obtained. The situation is quite different with $\alpha$-toxins whose polymorphism is well documented (i.e. Androctonus, Buthus, and Leirus genus (11, 25)). These $\alpha$-toxins have been classified into four groups according to amino acid sequence homologies and antigenic group determinations (1, 26-28). Moreover, this polymorphism, which exists with toxins present in individual venoms from A. australis Hector (29), poses a problem for the development of an efficient and general serotherapy. In other respects, the possibility of generating antibodies which neutralize $\alpha$H II (an $\alpha$-toxin) using synthetic peptides which correspond to definite sequence portions of AaH II (30) has recently been demonstrated. This technique will be extended to Cas toxins in the near future.

Unlike scorpion $\alpha$-toxins which bind to site 3 on the sodium channel, Cas II has been shown to bind to site 4 (2) and thus is considered as the first toxin of the $\beta$-toxin group. This binding has been further correlated with a pharmacological effect on the voltage-dependent sodium channel activation (3). We attempted to define the ($\alpha$- or $\beta$-) type for each of the...
Characterization of Toxins from a Mexican Scorpion Venom

seven purified Cs toxins. This was accomplished using competition experiments with labeled \( \alpha \) and \( \beta \)-toxins, i.e. \( ^{125}\text{I}-\text{AaH II} \) or \( ^{125}\text{I}-\text{Cs II} \). The results clearly indicate that the seven Cs toxins are \( \beta \)-type toxins. Furthermore, we found no competition when Cs venom extract was used with \( ^{125}\text{I}-\text{AaH} \) (\( \alpha \)-toxin). These data strongly support the idea that no \( \alpha \)-type toxin is present in the Cs venom. The characteristics of Cs venom are therefore different from those of \( \text{C. serrulatus} \) venom where both \( \alpha \) and \( \beta \)-toxins are present (31) and also perhaps from the characteristics of \( \text{C. sculpturatus Evil} \), although contradictory results have been published concerning its toxicity (32-34).

Cis I is less efficient than Cis II in displacing \( ^{125}\text{I}-\text{Cis} \), whereas the other toxins, Cis III-VII, are relatively more active. In fact, the most efficient toxins in displacing \( ^{125}\text{I}-\text{Cis} \) from rat brain synaptosomes are those which are the most active in lethality tests performed by intracerebroventricular injection. This is significant because it indicates that when one is looking for toxins which are highly active in the central nervous system, it is best to perform intracerebroventricular injection in order to monitor the purification process. For toxins active in the peripheral nervous system, the subcutaneous method is perhaps better suited. This affinity difference may be caused by differences in sodium channels present in the central and peripheral nervous systems (35-37). Cis VII does not fit this scheme. Indeed, its calculated \( K_d \) is similar to \( K_d \) values for Cis IV and VI, whereas its LD\(_{50} \) (intracerebroventricular) is close to the values obtained with Cis I-III (Table II). This discrepancy has not yet been clearly explained. However, one may suppose that the affinity of the toxin for its binding site results from chemical interactions between a region of the toxin and a site of its target; whereas the pharmacological effect related to toxicity may result from an interaction, not restricted to the binding site, between toxin and the sodium channel.

The number of sites for Cis VI (2.2 pmol/mg of synapticosomal protein) is about twice that found for Cis II on the same starting material (2). In the case of Cis II, this difference can be explained by the fact that the number of sites depends on pH: it is twice as high at pH 6 as at pH 7.2 (38). We did not find such a pH dependence with Cis VI.

Cis II is known to inhibit the rate of GABA uptake and to increase the rate of GABA release by synaptosomes (3). This is also true for Cis VI whose inhibition \( K_d \) corresponds to the binding affinity of this toxin with the sodium channel. These data confirm the previously presented hypothesis (10) that this effect on GABA can be used for testing \( \beta \)-toxin activity on synaptosomes.

In addition to the seven Cs toxins, one protein (P1) was purified and its amino acid composition was found to be closely related to those of toxins I-VII (Table II). This protein was tested for toxicity and its ability to compete with \( ^{125}\text{I}-\text{Cis} \) in binding experiments, either with anti-Cis II IgGs or synaptosomes (Figs. 9 and 10 and Table II). This protein can be considered either as a toxin with low activity or as a nonactive analogue contaminated by traces (less than 1\% monomer) of a very active toxin. In either case, the determination of its primary structure will be of great interest for a better understanding of structure-activity relationships in \( \beta \)-scorpion toxins.

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Additional references are found on p. 4459.
Characterizations of Toxins from a Mexican Scorpion Venom

Figure 2: Gel filtration on Sephadex G-50 in 0.1 M ammonium acetate buffer, pH 6.5, at a column 4 x 20 cm. Flow rate: 20 ml/hr, the material submitted to fractionation is either (A) the water extract of 2 g of crude venom or (B) the crude venom acetate extract of 1588 samples of A. m. mexicanorum. (A) gel filtration with previous treatment with Sephadex G-50 of 2 (from A). Four columns of 3.5 x 100 cm connected in series. Flow rate: 95 ml/hr, vertical arrows and related numbers indicate start of the consecutives elutions. The fractions corresponding to the full line of the diagrams were pooled as indicated, the material was then lyophilized and the samples were dissolved in 10 ml of buffer. (B) gel filtration with previous treatment with Sephadex G-50 of 0.3 (from A). Four columns of 3.5 x 100 cm connected in series. Flow rate: 95 ml/hr, vertical arrows and related numbers indicate start of the consecutives elutions. The fractions corresponding to the full line of the diagrams were pooled as indicated, the material was then lyophilized and the samples were dissolved in 10 ml of buffer.

Figure 3: Chromatography on DEAE-Sephadex A-50 in 0.1 M ammonium acetate buffer, pH 6.5, of the fractions A (A) obtained from 6 g of venom of the Fraction C (B) from 15 g of venom. 94% of the material was eluted with 0.1 M of sodium acetate (pH 5.5). The fractions corresponding to the full line of the diagrams were pooled as indicated, the material was then lyophilized and the samples were dissolved in 10 ml of buffer.

Figure 7: Chromatography on Sephadex G-50 (A) in 0.1 M ammonium acetate buffer, pH 6.5, of Fraction A obtained from 10 g of venom. 91% of the material was eluted with 0.1 M of sodium acetate. The fractions corresponding to the full line of the diagrams were pooled as indicated, the material was then lyophilized and the samples were dissolved in 10 ml of buffer.
Characterizations of Toxins from a Mexican Scorpion Venom

From the amino acid composition or chymotryptic peptide C G 48-52 (see below), the difficulty of identifying these three residues could be explained by the fact that thin layer chromatography did not allow a clear separation of the testa-dialdehyde and arginyl carboxylic acid dipeptides. In accordance with trypsin specificity the only lysine residue of peptide T 19-24 was placed in position 64. In another experiment, peptide T 19-24 (50 nmoles) was first succinylated and then hydrolyzed by thioglycolic acid. The hydrolysate was directly submitted to amino acid sequencings and the sequence from glycine-38 up to isoleucine-45 was identified indicating that only the glutamyl-glycine bond (residues 38-40) was breaking in this experiment. A first run (50 nmoles, three cycles) gave the following sequence: isoleucine, glutamate and a third methyl ester derivative (Tyr-Asp-Leu), according to the peptide amino acid composition, could be derived from arginine or asparagine dipeptide. In a second run (10 nmoles) the material remaining in the sequencer after two cycles of degradation was recovered and identified as asparaginyl dipeptide in the amino acid analyzer. This result might support the idea that the methionine residue of Tyr-Leu-Thr-Pro in the trypsin-purified crude venom extract might correspond to the sequence of this experiment. The presence of this C-terminal asparagine dipeptide could explain the peptide results obtained when using carboxypeptidase B and 8 and hydrazide-lysine to determine the C-terminal residue of the molecule. Another run (10 nmoles of the methyl ester derivative) was made. The amino acid sequence obtained from the C-terminal dipeptide, but also constituting an overlapping sequence between peptides T 39-43 and T 36-40, a new purification step using carboxypeptidase-lysine was necessary to obtain two other chymotryptic peptides. The amino acid composition of these peptides (Table 1) and their cluster sequence on the venom (Fig. 7a) showed that these two peptides (C 33-38 and C 40-47, respectively) could only be respectively serine and 5-carboxymethyllysine.

The automatic sequencing run was performed according to the usual conditions. The automatic sequence was in accordance with the amino acid sequence of the first 26 residues except for stage 10, 18 and 19 where no derivative was obtained these positions being occupied by half-lysine residues as we conclude that the sequence in C 1-26 have identical C-terminal sequences.

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Figure 7 Gel filtration on Biogel P4 of the tryptic digest of 100 nmoles of 5-carboxymethyllysine-C 2-36. The column (1 x 200 cm) was eluted with 0.1 M sodium phosphate buffer pH 7.0, at a rate of 1 ml/hr; absorbance at 230 nm (-) and absorbance at 280 nm (0) pure trypsin peptides.