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# Crotoxin, a Phospholipase $A_2$ Neurotoxin from the South American Rattlesnake *Crotalus durissus terrificus*: Purification of Several Isoforms and Comparison of Their Molecular Structure and of Their Biological Activities†

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ABSTRACT: Crotoxin, the major toxin of the venom of the South American rattlesnake *Crotalus durissus terrificus* is a mixture of several isoforms that differ slightly in their molecular structure. The toxin consists of two nonidentical subunits: a basic and weakly toxic phospholipase $A_2$, component B, and an acidic and nontoxic subunit, component A. In the present investigation, we have used fast-performance liquid chromatography (FPLC) on anionic and cationic exchange columns to purify isoforms of both crotoxin subunits. Two component A isoforms and four component B isoforms were obtained in a homogeneous state, and their purity was verified by isoelectric focusing in polyacrylamide gels. The amino acid composition of the purified component A and component B isoforms was in good agreement with the protein sequences determined previously with mixtures of isoforms. The amino acid compositions indicated that for both crotoxin components the isoforms differed only by the replacement of few amino acid residues. Eight crotoxin complexes have been prepared in a homogeneous state by reassociation of pure component A and component B isoforms. The quantitative comparison of enzymatic and pharmacological properties of the reconstituted crotoxins indicated that the two component A isoforms had identical properties, whereas the four component B isoforms fell in two classes: crotoxin complexes formed with component B isoforms of the first class were enzymatically less active and pharmacologically more potent than those obtained with component B isoforms of the second class.

Crotoxin, the main toxic component of the South American rattlesnake *Crotalus durissus terrificus* was the first animal neurotoxin to be purified and crystallized, half a century ago (Slotta & Fraenkel-Conrat, 1938a). Early investigations also showed that crotoxin possessed a phospholipase activity (Slotta & Fraenkel-Conrat, 1938b, 1939). Crotoxin exerts its pathophysiological action by blocking neuromuscular transmission (Vital-Brazil & Excell, 1970); its effects are primarily presynaptic, causing a typical triphasic modification of neurotransmitter release from nerve terminals (depression, facilitation, and final blockade) (Hawgood & Smith, 1977; Chang & Lee, 1977; Hawgood & Santana de Sa, 1979). Similar effects are observed with other snake neurotoxins that possess phospholipase $A_2$ activity, such as β-bungarotoxin (Chang et al., 1973; Strong et al., 1976; Abe et al., 1977), notexin (Harris et al., 1973), taipoxin (Kamenskaya & Thesleff, 1974;

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Cull-Candy et al., 1976), and caudoxin (Lee et al., 1982). In addition to its presynaptic effects, crotoxin also acts postsynaptically by blocking the response to acetylcholine through a stabilization of the acetylcholine receptor in an inactive conformational state (Vital-Brazil, 1966; Hanley, 1978; Bon et al., 1979).

Further investigations revealed that crotoxin is made up of two different subunits, component A and component B, the latter carrying the enzymatic activity of the toxin [Breithaupt et al., 1971; Hendon & Fraenkel-Conrat, 1971; for a review, see Breithaupt (1976)]. Component B is a single polypeptide chain of 122 amino acids, and its sequence is similar to that of other phospholipases $A_2$ from mammalian pancreas or from snake venoms (Fraenkel-Conrat et al., 1980; Aird et al., 1986). Component A consists of three polypeptide chains, linked by seven disulfide bridges (Breithaupt et al., 1974); their amino acid sequences are homologous to various parts of nontoxic phospholipase $A_2$ from crotalid venoms, suggesting that component A derives from a phospholipase precursor molecule by limited proteolysis (Aird et al., 1985).

The two nonidentical subunits of crotoxin behave in a synergistic manner: component B alone is weakly toxic and acts at neuromuscular junctions like the whole toxin, although larger doses are required to cause the same effect; component A is nontoxic by itself and has no pharmacological action, but applied in combination with component B, component A enhances the pharmacological efficacy and, in particular, the lethal potency of component B (Breithaupt et al., 1971; Hendon & Fraenkel-Conrat, 1971; Hawgood & Smith, 1977; Chang & Lee, 1977; Bon et al., 1979). The two crotoxin subunits are tightly associated in a one to one complex and can be separated only in the presence of 6 M urea or below pH 2 (Rübsamen et al., 1971; Horst et al., 1972). However, upon interaction with membranes, crotoxin splits into its subunits, component A being released in solution (Jeng et al., 1978; Bon et al., 1979). The isolated phospholipase subunit, component B, adsorbs to membranes in a nonsaturable manner (Jeng et al., 1978; Bon et al., 1979). In the presence of component A, however, component B binds to a limited number of high-affinity binding sites present on synaptic membranes (Bon et al., 1979). Component A is thus able to direct component B to the neuromuscular junction, thereby considerably enhancing the latter's lethal potency.

Crotoxin, as purified from *C. durissus terrificus* venom by low-pressure gel filtration and ion-exchange chromatography (Hendon & Fraenkel-Conrat, 1971), is in fact a mixture of several proteins, having very similar molecular structures and pharmacological properties but differing in their chromatographic properties (Hendon et al., 1970; Breithaupt et al., 1974; Wu et al., 1983) and electrophoretic mobility (Aird & Kaiser, 1985). Several of these molecular variants, crotoxin isoforms or isotoxins (Hendon et al., 1970; Breithaupt et al., 1975), may be present in individual venoms, suggesting that they derive from the expression of several isogenes in the same snake (Faure & Bon, 1987).

In the present investigation, we purified several crotoxin isoforms to a homogeneous state by FPLC[1] and analyzed their chemical, enzymatic, and pharmacological similarities and differences. We observed that the crotoxin variants resulted from the association of isoforms of both crotoxin components A and B. The amino acid composition of the purified isoforms indicated that they differed by a small number of amino acid residues. In some cases, these substitutions resulted in slight modifications in the enzymatic and pharmacological properties of the toxin: some crotoxin isoforms were less toxic and enzymatically more active than others, and these properties were dependent upon the nature of the component B subunit.

## Experimental Procedures

*Animals.* Male Swiss mice, 3 weeks old (18–20 g), were obtained from the Ferme de Rennemoulin, Institut Pasteur (Paris, France).

*Materials.* The venom from *Crotalus durissus terrificus* was purchased from the Instituto Butantan (Sao-Paulo, SP, Brazil). Sephadex G-25 (medium) and G-75 (superfine) beads, prepacked Mono-Q HR5/5, Mono-Q HR16/10, and Mono-S HR5/5 columns, and equipment for fast-performance liquid chromatography (FPLC) (pumps P-500, programmer GP-250, detector UV-1, recorder REC-492, and fraction collector FRAC-100) were from Pharmacia Fine Chemicals (Uppsala, Sweden). DEAE-cellulose DE-52 was purchased from Whatman Biochemicals Ltd. (Springfield Mill, U.K.). Ingredients for polyacrylamide gels (PAG) were from Serva (Heidelberg, FRG); Ampholines pH 4–6 for isoelectric focusing were from LKB (Bromma, Sweden); calibration kits for p*I* determination were from Pharmacia Fine Chemicals (Uppsala, Sweden), and Coomassie Brilliant Blue R250 was from Sigma (St. Louis, MO). Isoelectric focusing was performed with a LKB Multiphor apparatus (Bromma, Sweden), equipped with a power supply and a cooling device. The fluorescent compound for determination of phospholipase activity, 1-palmitoyl-2-(10-pyrenyldecanoyl)-*sn*-glycero-3-phosphatidic acid monomethyl ester (pyrene-PA-monomethyl) was from KSV Chemicals (Helsinki, Finland), and fatty acid free bovine serum albumin was from Sigma Chemical Co. (St. Louis, MO). All other chemicals and solvents were of the highest available purity, from Merck A.G. (Darmstad, FRG) or Prolabo (Paris, France).

*Isolation of Crotoxin.* Crotoxin was isolated by gel filtration on a Sephadex G-75 column (Hendon & Fraenkel-Conrat, 1971) as follows: *C. durissus terrificus* venom (1–2 g) was dissolved in 30–50 mL of 100 mM ammonium formate, pH 3.0, containing 100 mM NaCl by gentle stirring at 4 °C for 15 h (overnight). The solution was centrifuged 30 min at 5000 rpm (4000*g*) in a Sorvall GSA rotor. The supernatant was removed and kept at 4 °C; the small pellet was resuspended in 10 mL of ammonium formate buffer and centrifuged as before. The two supernatants were combined (crude extract), and the pellet, which never represented more than 10% of the dried venom and which represented less than 3% of its lethal potency, was discarded. The crude extract was applied to a Sephadex G-75 column, previously equilibrated with 100 mM ammonium formate, pH 3.0, containing 100 mM NaCl. Protein elution was followed by measuring the absorption of the eluant at 280 nm. Crotoxin was identified by measuring its phospholipase activity with a colorimetric assay and its lethal potency ($LD_{100}$) by intravenous injection in mice. It was concentrated by lyophilization and dialyzed against large volumes of 50 mM sodium phosphate, pH 6.5.

Crotoxin was further fractionated by FPLC on an anion-exchange column, Mono-Q HR16/10. Samples of 50 mL containing 90–130 mg of crotoxin were applied with a Superloop device to a preparative Mono-Q column, previously equilibrated with 50 mM sodium phosphate buffer, pH 6.5. The elution was performed in 35 min, at a flow rate of 8 mL/min, with (i) 40 mL of sodium phosphate buffer, (ii) 160

[1] Abbreviations: FPLC, fast-performance liquid chromatography; pyrene-PA-monomethyl, 1-palmitoyl-2-(10-pyrenyldecanoyl)-*sn*-glycero-3-phosphatidic acid monomethyl ester; TCA, trichloroacetic acid; PAG, polyacrylamide gel; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

mL of a linear NaCl gradient from 0 to 0.3 M in sodium phosphate buffer, (iii) 24 mL of a linear NaCl gradient from 0.3 to 1 M in sodium phosphate buffer, and (iv) 16 mL of 1 M NaCl in sodium phosphate buffer. Five crotoxin fractions containing various crotoxin isoforms were obtained and designated fraction I to fraction V. They were concentrated by lyophilization, desalted by dialysis against large volumes of deionized water, and lyophilized again. The dried powder was kept at −18 °C for up to 6 months.

*Isolation of Crotoxin Subunits.* The crotoxin subunits, component A and component B, were isolated by ion-exchange chromatography in the presence of 6 M urea (Hendon & Fraenkel-Conrat, 1971). The crotoxin isoforms of fraction II (168 mg) and of fraction IV (13 mg) were dissolved in small volumes (5 and 1 mL, respectively) of 6 M urea buffered at pH 6.5 with 50 mM sodium phosphate. They were incubated at room temperature (about 20 °C) for 90 min and applied to DEAE-cellulose DE-52 columns (2 × 25 cm for fraction II and 1 × 12 cm for fraction IV), previously equilibrated with 6 M urea in 50 mM sodium phosphate, pH 6.5. Component B, which does not bind to the anion-exchange column, was eluted in the equilibration buffer. Component A was eluted with 6 M urea and 0.4 M NaCl in 50 mM sodium phosphate, pH 6.5. The two isolated crotoxin components were desalted by extensive dialysis against deionized water (fraction II) or by gel filtration on Sephadex G-25 columns (1.5 × 5 cm), equilibrated with 50 mM ammonium acetate, pH 4.7.

The isolated component A isoforms of fraction II and of fraction IV were purified by FPLC on anion-exchange columns Mono-Q HR16/10 or HR5/5. When chromatography was performed on a Mono-Q HR16/10 column (fraction II), the procedure used was that described for the purification of crotoxin. When chromatography was carried out on a Mono-Q HR5/5 column (fraction IV), the column was equilibrated with 50 mM sodium phosphate, pH 6.5, and the elution was performed at a flow rate of 1 mL/min with (i) 5 mL of 50 mM sodium phosphate buffer containing 0.1 M NaCl, (ii) 20 mL of a linear NaCl gradient from 0.1 to 0.4 M in sodium phosphate buffer, and (iii) 3 mL of a linear NaCl gradient from 0.4 to 1 M in sodium phosphate buffer. In both cases the major protein peaks were collected, concentrated by lyophilization, and desalted by dialysis against deionized water or by gel filtration on Sephadex G-25 columns (1.5 × 5 cm) equilibrated with deionized water.

The isolated component B isoforms of fraction II and of fraction IV were purified by FPLC by cation-exchange chromatography on a Mono-S HR5/5 column. The samples were dissolved in 2 mL of 50 mM sodium phosphate, pH 6.5, and injected on the Mono-S column previously equilibrated with sodium phosphate buffer. Elution was carried out at a flow rate of 1 mL/min with (i) 3 mL of equilibration solution, (ii) 40 mL of a linear gradient from 0 to 1 M NaCl and from 0 to 6 M urea in 50 mM sodium phosphate, pH 6.5, and (iii) 3 mL of 1 M NaCl and 6 M urea in sodium phosphate buffer. Fractions of 0.8 mL were collected. The major protein peaks containing the purified isoforms of component B were desalted by gel filtration on Sephadex G-25 columns (1.5 × 5 cm), equilibrated with 100 mM ammonium acetate, pH 4.7. The purified isoforms of component A or component B were stored frozen at −18 °C.

*Reconstitution of Crotoxin Isoforms.* Pure crotoxin isoforms were prepared by mixing purified isoforms of crotoxin subunits at a molar ratio of component A/component B of 1.2/1.0. Their homogeneity was checked by isoelectric focusing on PAG and by FPLC on anion-exchange column Mono-Q HR5/5, elution being achieved at a flow rate of 1 mL/min with (i) 5 mL of the equilibration buffer (50 mM sodium phosphate, pH 6.5), (ii) 20 mL of a linear NaCl gradient from 0 to 0.3 M in sodium phosphate buffer, (iii) 3 mL of a linear NaCl gradient from 0.3 to 1 M in sodium phosphate buffer, and (iv) 2 mL of 1 M NaCl in sodium phosphate buffer.

*Phospholipase Assay.* Phospholipase $A_2$ activity was measured spectrophotometrically with fluorescent substrates (pyrene-containing phospholipids) (Hendrickson & Rauk, 1981; Thurên et al., 1983) as modified by Radvanyi, Jordan, Russo-Marie, and Bon (unpublished experiments). Briefly, 10 μL of 10-pyrene-PA-monomethyl (0.2 mM in ethanol) was added to 1 mL of 50 mM Tris-HCl, pH 7.5, containing 100 mM NaCl, 1 mM EDTA, 0.1% serum albumin, and 6 mM $CaCl_2$. The solution was introduced in a spectrofluorometer and excited at 345 nm, the fluorescence emission being recorded at 398 nm. The blank was monitored for a few minutes, and the enzymatic reaction was initiated by addition of the phospholipase $A_2$. Alternatively, the pH-stat method of Desnuelle et al. (1955) was used according to the procedure described by Lôbo de Araûjo and Radvanyi (1987). For chromatographic fractions, fast estimations were obtained by a colorimetric assay (Lôbo de Araûjo & Radvanyi, 1987).

*Isoelectric Focusing.* Isoelectric focusing was carried out on disposable LKB Ampholine PAG plates pH 3.5–9.5 or on freshly prepared PAG plates. The latter (10 × 24 cm; 2 mm thick) were obtained by the polymerization of 60 mL of a solution containing 8% acrylamide, 0.21% bis(acrylamide), 5.5% sucrose, 5% LKB Ampholine pH 4–6, and 60 μL of 10% ammonium persulfate. Electrode paper strips were moistened with 1 M $H_3PO_4$ (anode) and 1 M NaOH (cathode). The PAG plate was refrigerated at 10 ± 2 °C, and a pH gradient was preformed by applying current at a constant power of 25 W, for 30 min. Samples of 10 μL (2 mg/mL) were applied at the center of the plate, and isoelectric focusing was carried out at 25-W constant power for 1 h. The voltage increased during the electric focalization to reach a value of about 2000 V at equilibrium. After isoelectric focusing, the plate was fixed for 45 min in 11.5% trichloroacetic acid (TCA)/3.47% sulfosalicylic acid. The plate was washed in a destaining solution (3 × 15 min; 25% ethanol, 8% acetic acid), stained overnight with 0.12% Coomassie Brilliant Blue in 25% ethanol/8% acetic acid, and finally destained by several washings with the destaining solution.

*Determination of Protein Concentration.* The protein concentration of crotoxin and of its isolated subunits was determined spectrophotometrically by recording their ultraviolet absorption spectrum with a Kontron Uvikon SP800 spectrophotometer. The following molar extinction coefficients and molecular weights were used: $E_{278}$ = 41 000 $M^{-1}$ $cm^{-1}$ and $M_r$ 24 000 for crotoxin; $E_{276}$ = 9500 $M^{-1}$ $cm^{-1}$ and $M_r$ 9500 for isolated component A; $E_{278}$ = 29 000 $M^{-1}$ $cm^{-1}$ and $M_r$ 14 500 for isolated component B.

*Amino Acid Analysis.* Amino acid analyses were performed with Biotronik amino acid analyzer LC 5001 according to a single-column procedure (Hummel, 1959). Samples containing 30–50 μg of protein were hydrolyzed in vacuo in 0.1 mL of 6 N HCl for 15, 48, and 72 h at 110 °C (Spackmann et al., 1958). For the exact determination of tyrosine, hydrolysis was carried out for 15 h in the presence of 0.2% phenol. Methionine was determined as methionine sulfone after performic acid oxidation according to Hirs (1967). Half-cystine was estimated as cysteic acid after performic oxidation. The ratio of tyrosine to tryptophan was calculated by the method of Beaven

Table I: Enzymatic Activity and Lethal Potency of Isolated and Reconstituted Crotoxin Isoforms[a]

| fractions | I | II | | | | | III | IV | V | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| protein (mg) | 95 | 223 | | | | | 78 | 29 | 11 | | |
| $PLA_2$ activity ($\mu$mol $min^{-1}$ $mg^{-1}$) | 13 | 12 | | | | | 13 | 32 | 28 | | |
| $LD_{50}$ ($\mu$g/kg) | 55 | 49 | | | | | 45 | 210 | 155 | | |
| component B | | CBII | CBIIa | CBIIb | CBIIc | CBIId | | CBIV | | | |
| protein (mg) | | 48 | 0.5 | 14 | 6.2 | 13 | | 8.3 | | | |
| $PLA_2$ activity ($\mu$mol $min^{-1}$ $mg^{-1}$) | | 32 | 24 | 47 | 16 | 22 | | 38 | | | |
| crotoxin | | CAIICBII | CAIICBIIa | CAIICBIIb | CAIICBIIc | CAIICBIId | | CAIVCBIV | | CAIICBIV | CAIVCBIIb |
| $PLA_2$ activity ($\mu$mol $min^{-1}$ $mg^{-1}$) | | 11 | 30 | 17 | 4.6 | 4 | | 33 | | 42 | 13 |
| $LD_{50}$ ($\mu$g/kg) | | 60 | 160 | 70 | 85 | 65 | | 170 | | 240 | 65 |
| $pI$ | | | 4.9 | 5.17 | 5.1 | 5.25 | | | | | 5.08 |

[a] Protein concentrations were determined spectrophotometrically. Phospholipase activity was measured with a fluorometric assay. Lethal potency ($LD_{50}$), expressed as micrograms of protein injected per kilogram of body weight, was estimated by intravenous injection in mice. The various fractions were labeled as indicated in Figure 1. Reconstituted crotoxin isoforms were obtained by mixing the isolated isoforms at a molar ratio of 1.2 component A to 1.0 component B. $pI$s were determined by isoelectric focusing, as described in Figure 5B.

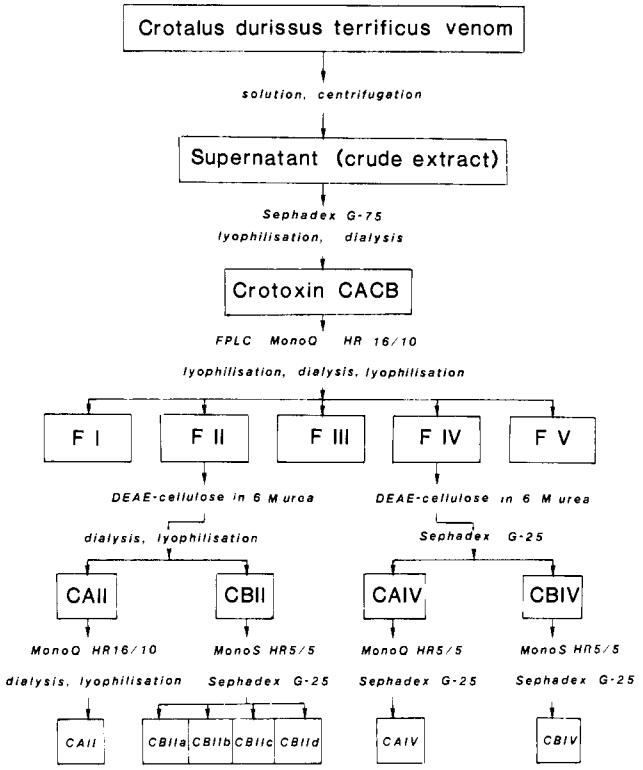


FIGURE 1: Flow diagram of the purification of isoforms of crotoxin subunits.

and Holliday (1952) by recording the absorption spectrum of the protein in 0.1 M NaOH. Three to eight independent amino acid determinations were performed for each sample.

*Lethal Potency.* Lethality assays were performed with male Swiss mice by intravenous injection of 0.2 mL of the tested solution per 20 g of body weight. The $LD_{50}$ was calculated by the method of Spearman-Kärber, three mice being injected with each fraction, in doses that differed by a factor of 1.414 ($\sqrt{2}$). Rapid estimations were also carried out by injecting one mouse with doses that increased by a factor of 2.

## RESULTS

*Fractionation of Crotoxin.* Figure 1 summarizes the method used for the isolation of crotoxin from the venom of *C. durissus terrificus*. Dried venom was dissolved in ammonium formate, pH 3.0, and centrifuged at low speed in order to eliminate the insoluble material. The supernatant (crude extract) was first submitted to gel filtration on a Sephadex G-75 column (Hendon & Fraenkel-Conrat, 1971). The elution pattern is shown in Figure 2. A major protein peak containing almost

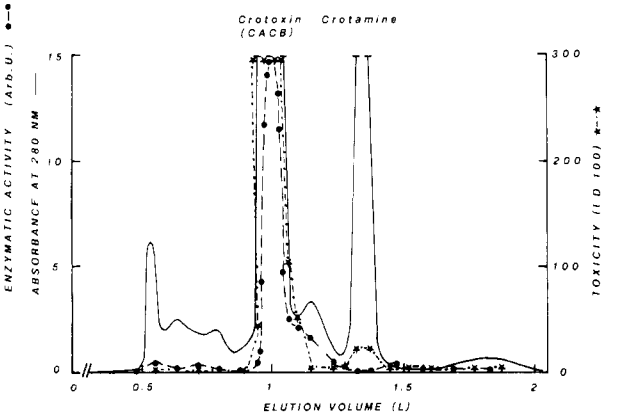


FIGURE 2: Gel filtration of *C. durissus terrificus* venom on Sephadex G-75. The crude extract obtained from 1.3 g of *C. durissus terrificus* venom (50 mL) was applied to a Sephadex G-75 column (5 × 100 cm) previously equilibrated with 100 mM ammonium formate, pH 3.0, containing 100 mM NaCl. The elution was carried out at a flow rate of 18 mL/h. Fractions of 8 mL were collected, and their absorbance (—) was measured at 280 nm. The phospholipase activity (●) was determined by a colorimetric assay. The lethal potency (★) was estimated as $LD_{100}$ by intravenous injection into mice.

all the phospholipase $A_2$ activity of the venom and at least 90% of its lethal potency elutes with an apparent $M_r$ of 24 000 ± 2000. This fraction consists mainly of crotoxin (CACB) (Hendon & Fraenkel-Conrat, 1971). The fraction that elutes in the void volume of the column is nontoxic (Figure 2) and was observed to contain convulxin (Prado-Franceschi & Vital-Brazil, 1969; Vargaftig et al., 1980), a platelet-aggregating glycoprotein (Marlas et al., 1983). The last fraction, which eluted as a polypeptide of apparent $M_r$ of 5000 ± 1000, consisted of a single basic protein which corresponds to crotamine (Laure, 1975): it is toxic when injected intravenously at high doses ($DL_{50}$ 2.3 mg/kg) and possesses the pharmacological properties of this toxin (Cheymol et al., 1971) as tested by C.-C. Chang (personal communication).

The fraction containing crotoxin (CACB) was pooled as indicated in Figure 2, concentrated by lyophilization, and dialyzed against a large volume of 50 mM sodium phosphate, pH 6.5. Crotoxin was further purified by FPLC on an anion-exchange column, Mono-Q HR16/10 (Figure 3A). Five protein fractions (FI to FV) were eluted with a NaCl gradient. They were further characterized by determining (i) their phospholipase $A_2$ activity (Table I), (ii) their lethal potency ($LD_{50}$) by intravenous injection into mice (Table I), and (iii) their amino acid composition after acid hydrolysis (Table II). The three major fractions (FI, FII, and FIII) which represented together about 90% of the material applied to the FPLC

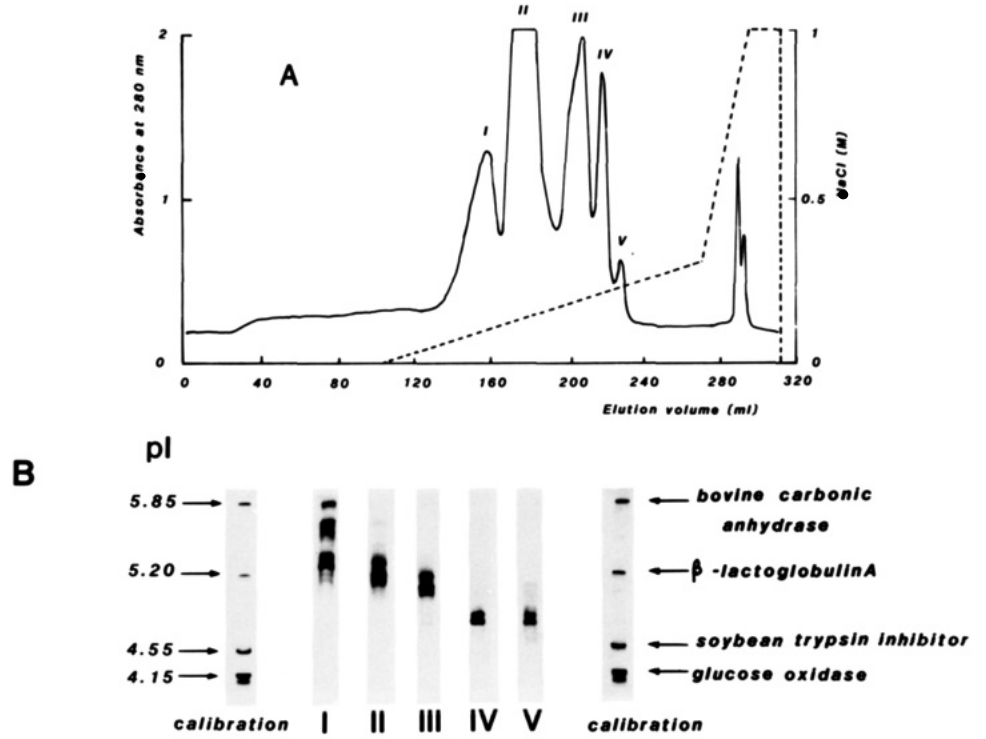


FIGURE 3: Fractionation of crotoxin by preparative ion-exchange chromatography on a Mono-Q HR16/10 column. (A) A total of 97 mg of purified crotoxin was dissolved in 50 mL of 50 mM sodium phosphate buffer, pH 6.5, and applied with a Superloop loading system to a preparative Mono-Q HR16/10 column. The elution was performed at a flow rate of 8 mL/min with a NaCl gradient in sodium phosphate buffer. The absorbance of the eluant was monitored at 280 nm, and 5-mL fractions were collected. (B) Samples of 10 μL of the indicated fractions I–V (2 mg/mL) were applied to the center of the isoelectric focusing gel and focused with a pH gradient (Ampholines pH 4–6). Proteins were stained with Coomassie Brilliant Blue. The pH gradient was calibrated with the following standards: (a) bovine carbonic anhydrase, p*I* 5.85; (b) β-lactoglobulin, p*I* 5.20; (c) soybean trypsin inhibitor, p*I* 4.55; (d) glucose oxidase, p*I* 4.15.

Table II: Amino Acid Composition of Crotoxin Fractions and of Isoforms of Crotoxin Subunits[a]

| amino acid | crotoxin | | | | | | component A | | | component B | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CACB[b] | I | II | III | IV | V | CA[b] | CAII | CAIV | CB[b] | CBIIa | CBIIb | CBIIc | CBIId | CBIV |
| Cys | 28 | 28.2 | 29.8 | 27.4 | 29.3 | 28.7 | 14 | 13.3 (0.4) | 13.3 (0.5) | 14 | nd | 14.5 (0.6) | 14.1 (0.5) | 13.8 (0.6) | 13.5 (0.7) |
| Asx | 18–20 | 20.9 | 22.3 | 22.4 | 22.9 | 21.6 | 9–11 | 11.8 (0.3) | 12.6 (0.3) | 9 | 9.5 (0.5) | 10.0 (0.4) | 8.9 (0.5) | 9.3 (0.4) | 9.9 (0.3) |
| Thr[c] | 11 | 10.6 | 11.1 | 11.1 | 10.8 | 11.5 | 4 | 3.8 (0.2) | 4.8 (0.2) | 7 | 7.0 (0.4) | 6.8 (0.5) | 6.7 (0.2) | 6.9 (0.4) | 6.8 (0.1) |
| Ser[c] | 11 | 11.6 | 12.3 | 11.6 | 12.8 | 13.2 | 5 | 5.3 (0.2) | 5.2 (0.1) | 6 | 7.0 (0.5) | 5.7 (0.1) | 5.7 (0.5) | 5.7 (0.4) | 6.4 (0.3) |
| Glx | 21–23 | 22.6 | 23.0 | 23.0 | 24.1 | 22.9 | 9–13 | 14.0 (0.2) | 13.7 (0.4) | 8–9 | 9.9 (0.6) | 9.1 (0.2) | 8.0 (0.3) | 8.4 (0.2) | 10.8 (0.4) |
| Pro | 9 | 5.7 | 5.6 | 5.2 | 6.5 | 5.2 | 5 | 4.6 (0.1) | 4.6 (0.3) | 4 | 3.3 (0.2) | 3.7 (0.1) | 4.3 (0.2) | 3.7 (0.1) | 3.6 (0.2) |
| Gly | 21 | 21.9 | 22.0 | 21.6 | 20.7 | 20.8 | 10 | 9.9 (0.2) | 9.5 (0.3) | 11 | 9.7 (0.2) | 10.8 (0.1) | 10.7 (0.1) | 11.3 (0.1) | 9.0 (0.2) |
| Ala | 12 | 13.0 | 13.3 | 13.3 | 12.7 | 12.6 | 6 | 6.7 (0.0) | 6.4 (0.0) | 6 | 6.1 (0.0) | 6.2 (0.0) | 6.2 (0.0) | 6.3 (0.0) | 6.0 (0.0) |
| Val | 3 | 4.4 | 4.2 | 4.3 | 5.0 | 4.7 | 1 | 1.2 (0.0) | 1.3 (0.2) | 2 | 2.8 (0.2) | 2.9 (0.1) | 2.2 (0.3) | 2.1 (0.1) | 2.9 (0.1) |
| Met | 3 | 3.0 | 3.1 | 3.0 | 2.9 | 2.7 | 1 | 1.0 (0.2) | 1.0 (0.2) | 2 | nd | 1.8 (0.2) | 1.7 (0.2) | 1.8 (0.1) | 1.7 (0.2) |
| Ile | 7 | 7.3 | 7.7 | 7.7 | 6.9 | 7.0 | 2 | 3.1 (0.2) | 2.9 (0.2) | 5 | 3.8 (0.2) | 3.9 (0.1) | 4.8 (0.1) | 4.8 (0.0) | 3.7 (0.2) |
| Leu | 7 | 6.6 | 6.9 | 7.0 | 6.7 | 6.8 | 1 | 1.1 (0.1) | 1.0 (0.1) | 6 | 5.7 (0.2) | 6.0 (0.1) | 6.0 (0.1) | 6.0 (0.1) | 5.8 (0.2) |
| Tyr | 14 | 13.1 | 10.5 | 13.1 | 10.8 | 12.8 | 3 | 3.0 (0.3) | 3.0 (0.3) | 11 | 9.5 (0.3) | 10.0 (0.1) | 10.7 (0.2) | 10.8 (0.1) | 10.1 (0.1) |
| Phe | 10 | 9.9 | 9.4 | 9.0 | 9.0 | 9.2 | 4 | 3.0 (0.3) | 3.0 (0.2) | 6 | 5.9 (0.1) | 6.2 (0.1) | 6.2 (0.1) | 6.1 (0.1) | 6.1 (0.1) |
| His | 3 | 4.5 | 5.1 | 5.4 | 3.9 | 5.8 | 1 | 1.6 (0.1) | 1.6 (0.3) | 2 | 3.9 (0.5) | 3.1 (0.5) | 3.3 (0.4) | 3.2 (0.2) | 2.7 (0.1) |
| Lys | 10–12 | 12.2 | 12.7 | 12.4 | 12.3 | 12.1 | 2 | 2.1 (0.0) | 2.0 (0.0) | 8–10 | 10.3 (0.8) | 10.5 (0.4) | 11.0 (0.3) | 10.8 (0.3) | 9.8 (0.4) |
| Arg | 11–14 | 12.2 | 11.6 | 11.1 | 12.4 | 11.5 | 2 | 2.4 (0.1) | 2.4 (0.1) | 9–12 | 9.9 (0.7) | 8.8 (0.1) | 8.9 (0.5) | 8.3 (0.4) | 8.9 (0.6) |
| Trp | 4 | nd | nd | nd | nd | nd | 1 | nd | nd | 3 | nd | 3.1 (0.3) | nd | 3.0 (0.3) | nd |

[a] The amino acid composition is expressed as moles of residue per mole of crotoxin or of crotoxin component, assuming $M_r$ of 24 000, 9500, and 14 500 for respectively crotoxin, component A, and component B. Three to eight independent analyses were carried out for each determination. Numbers in parentheses are the standard deviations. nd, not determined. [b] The amino acid composition was calculated from the sequence data, as determined by Aird et al. (1985, 1986). [c] The values were extrapolated at zero time of hydrolysis.

column possessed similar enzymatic activities (12–13 μmol of fluorescent fatty acid liberated per minute per milligram of protein) and a high lethal potency ($LD_{50}$ 50 μg/kg) (Table I). The two minor fractions (FIV and FV) which represented 6.7 and 2.5%, respectively, of the total protein material applied to the column were less toxic ($LD_{50}$ around 200 μg/kg) and possessed a higher phospholipase activity (30 μmol $min^{-1}$ $mg^{-1}$) (Table I). The amino acid compositions of all fractions (Table II) were extremely similar and resembled that deduced from the protein sequence of crotoxin, determined by Aird et al. (1985, 1986), indicating that they all correspond to isoforms of crotoxin, as suggested in an earlier study (Faure & Bon, 1987). However, when submitted to isoelectric focusing with a pH 4–6 gradient, the fractions purified by FPLC (FI to FV) were found to be heterogeneous and to contain up to five constituents, with p*I* ranging from 4.9 to 5.7 (Figure 3B). These fractions are therefore in fact mixtures of crotoxin isoforms.

*Purification of Isoforms of Crotoxin Subunits.* Since the crotoxin fractions purified by FPLC consisted of several isoforms, we decided to isolate the two crotoxin components A and B (Hendon & Fraenkel-Conrat, 1971; Rübsamen et al.,

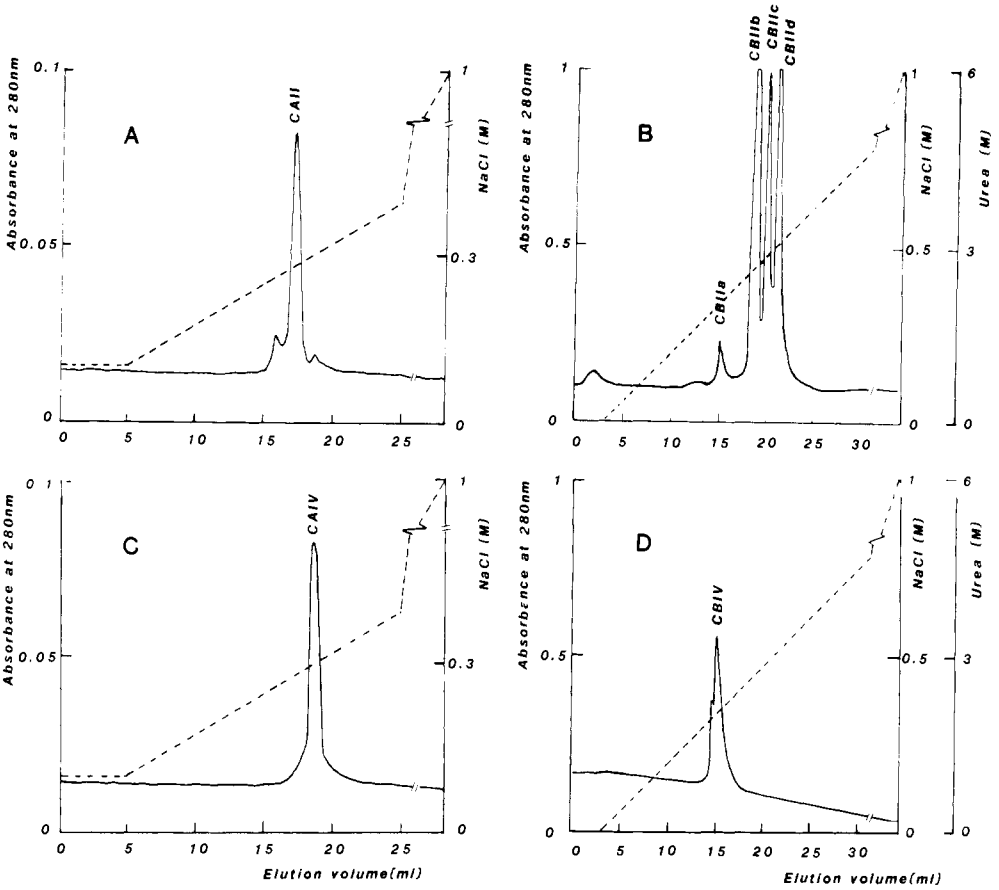


FIGURE 4: Purification of isoforms of component A and component B from isolated crotoxin fractions. (A and C) A total of 100 $\mu$g of component A isolated from crotoxin of fraction II (A) and fraction IV (C) was chromatographed on an anion-exchange column, Mono-Q HR5/5. Elution was achieved at a flow rate of 1 mL/min with a linear NaCl gradient in 50 mM sodium phosphate buffer, pH 6.5. (B and D) A total of 1.5 mg of component B isolated from crotoxin of fraction II (B) and fraction IV (D) was dissolved in 2 mL of 50 mM sodium phosphate, pH 6.5, and injected in the FPLC cation-exchange column, Mono-S HR5/5. The elution was carried out at a flow rate of 1 mL/min with a gradient of NaCl (0–1 M) and urea (0–6 M) in 50 mM sodium phosphate, pH 6.5.

1971; Breithaupt et al., 1974, 1975; Breithaupt, 1976) and to purify some of the isoforms of isolated crotoxin subunits (Figure 1). We studied FII and FIV, because they were the most abundant fractions which differed in their lethal potency (Table I). Component A and component B were separated by ion-exchange chromatography on a DEAE-cellulose column, in the presence of 6 M urea (Hendon & Fraenkel-Conrat, 1971). They were renatured by removal of urea by extensive dialysis against desionized water or by gel filtration on a Sephadex G-25 column.

When further fractionated by FPLC on an ion-exchange Mono-Q HR5/5 column (Figure 1), component A obtained from FII was found to consist of one major isoform that eluted from the Mono-Q column at 285 ± 5 mM NaCl and two minor constituents that eluted at lower and higher ionic strength (Figure 4A). Component A from FIV was homogeneous and eluted at a concentration of NaCl of 300 ± 5 mM (Figure 4C). In both cases, the two major isoforms of component A were further purified on a preparative scale by FPLC on Mono-Q HR16/10 or Mono-Q HR5/5 columns, and their homogeneity was checked by analytical chromatography on a Mono-Q HR5/5 column. The two purified isoforms of component A (CAII and CAIV) were different since, when mixed and chromatographed together on a Mono-Q column, they eluted as two distinct peaks, at 285 and 300 mM NaCl, respectively (results not shown). The amino acid compositions of the two purified component A isoforms were in good agreement with the protein sequence data reported by Aird et al. (1985) with the exceptions of Glx, Ile, and Phe, whose proportions differ by one amino acid residue per mole of component A (Table II). Furthermore, two differences were also noticed between the two component A isoforms: compared to CAII, CAIV possesses two additional residues, one threonine and one aspartic acid or asparagine (Table II). This latter additional residue might be an aspartic acid since the two component A isoforms differ in their chromatographic properties on ion-exchange columns, CAIV being more negatively charged than CAII (Figure 4A,C). One might also consider the possibility of replacements of aspartic acid and/or glutamic acid for respectively asparagine and/or glutamine to take into account the difference in the electric charge of the two component A isoforms.

Component B isoforms were fractionated by FPLC on a cation-exchange column, Mono-S HR5/5 (Figure 1), elution being achieved with a procedure involving a linear gradient of urea (from 0 to 6 M) and NaCl (from 0 to 1 M). Component B isolated from crotoxin of fraction II (Figure 1) was found to consist of three major isoforms (CBIIb, CBIIc, and CBIId) and one minor species, CBIIa (Figure 4B). These four isoforms of component B were purified on a preparative scale by the same procedure. The homogeneity of the isolated component B isoforms was checked by FPLC under the same conditions (results not shown). Component B from crotoxin of FIV eluted as a single peak with a shoulder, indicating that it consisted of two molecular forms, CBIVa and CBIVb, which have not been separated (Figure 4D). Furthermore, when the elution patterns obtained with isolated component B isoforms were compared, CBIIa appeared similar to CBIVa (Figure 4B,D). The phospholipase $A_2$ activity of the purified isoforms of component B was determined with a fluorescent assay

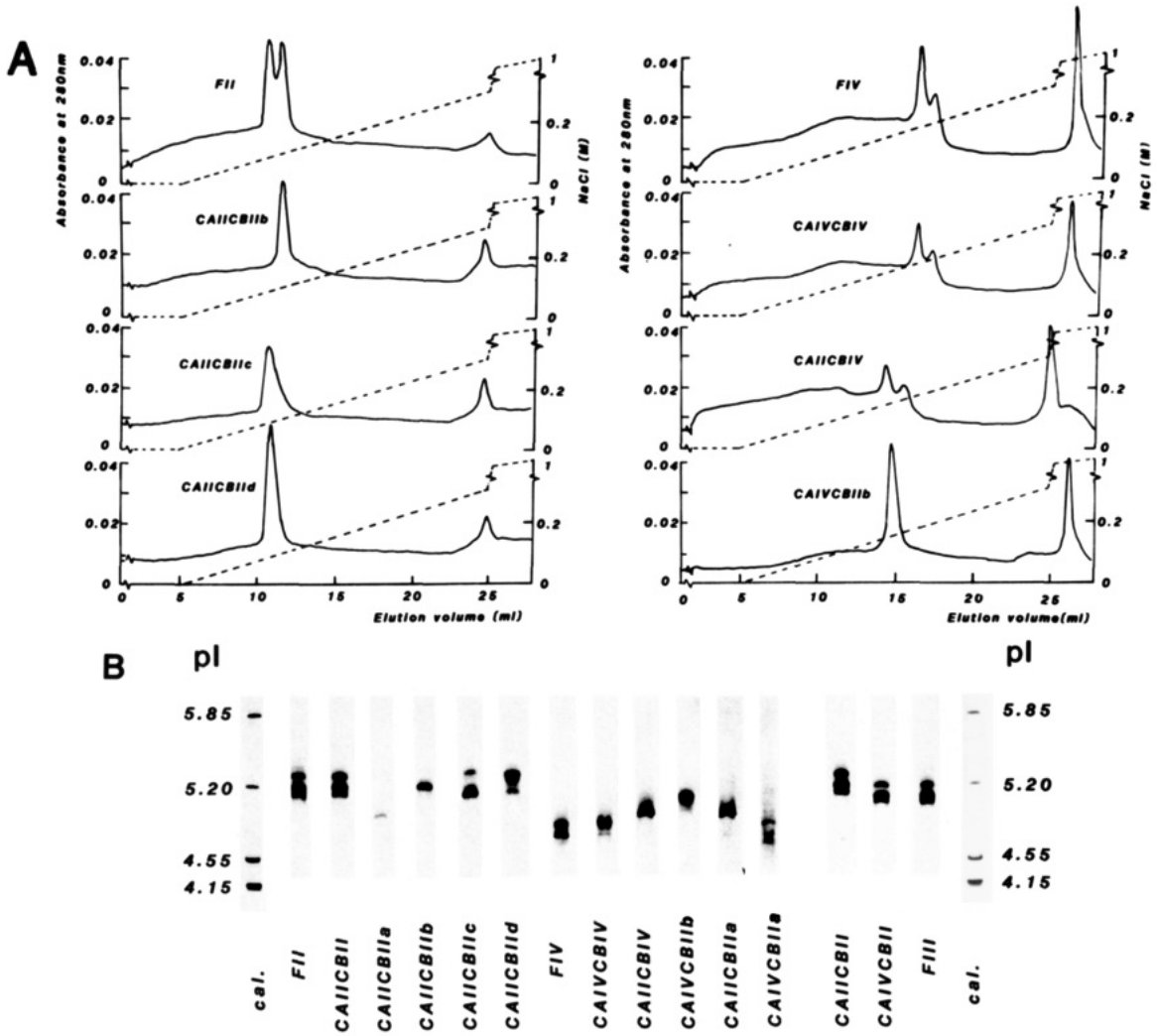


FIGURE 5: Reconstitution of crotoxin isoforms with purified isoforms of isolated subunits. Reconstituted crotoxin was prepared by mixing the indicated isoforms of crotoxin subunits at a molar ratio of 1.2 component A to 1 component B. (A) Samples of 200 μL containing 80–150 μg of native or reconstituted crotoxin were chromatographed on a Mono-Q HR5/5 column, elution being achieved with two consecutive NaCl gradients in 50 mM sodium phosphate, pH 6.5. (B) Samples of 20 μg (10 μL) of the indicated native or reconstituted crotoxin isoforms were electrofocused with Ampholine pH 4–6. Proteins were stained with Coomassie Brilliant Blue. The pH calibration was achieved with protein standards as in Figure 3B.

(Table I). CBIIb and CBIV showed slightly higher specific activities than the other components.

Table II shows the amino acid composition of the four purified isoforms of component B (CBIIa, CBIIb, CBIIc, and CBIId) and of the mixture of the two component B isoforms, CBIVa and CBIVb. As expected, these compositions are in agreement with that deduced from the protein sequence determined by Aird et al. (1986) for component B. However, we observed minor but significant differences among the various isoforms. We found (i) 11 Lys instead of 8–10 in the case of CBIIc and CBIId, (ii) 8 Arg instead of 9–12 in CBIId, (iii) 10 Asp + Asn instead of 9 in CBIIb, (iv) 10 Glu + Gln instead of 8–9 in CBIIa, (v) 3 Pro instead of 4 in CBIIa, (vi) 10 Gly instead of 11 in CBIIa, (vii) 3 Val instead of 2 in CBIIa and CBIIb, (viii) 4 Ile instead of 5 in CBIIa and CBIIb, and (ix) 10 Tyr instead of 11 in CBIIa and CBIIb. These differences may be simply explained by the fact that the sequence was determined for a mixture of component B isoforms. In agreement with this hypothesis, Fraenkel-Conrat et al. (1980) reported variants of Ser/His, Val/Ile, Leu/Tyr, and Glx/Gly, respectively, at positions 1, 18, 27, and 107. Similarly, Aird et al. (1986) mentioned that several amino acids exist at the same position in the sequence: Lys and Arg were both found at positions 37 and 69, and either Gln, Glu, or Arg was determined at position 33, depending upon the source of the venom. The amino acid compositions that we determined for purified component B isoforms of crotoxin further indicate that (i) the compositions of the two isoforms CBIIc and CBIId were very similar and agreed closely with the composition calculated from the sequence determined by Aird et al. (1986) and (ii) the isoforms CBIIb and CBIIa as well as the mixture CBIVa–CBIVb appeared more different, with four and seven amino acid changes (Table II).

*Preparation of Crotoxin Isoforms.* Crotoxin isoforms were prepared by the reassociation of stoichiometric amounts of isolated component A and component B isoforms. The reconstituted crotoxin isoforms were identified by FPLC on an anion-exchange column, Mono-Q (Figure 5A), and by isoelectric focusing (Figure 5B). For example, crotoxin isoforms of fraction II were obtained by combining the major component A isoform found in this fraction, CAII, with the component B isoforms CBIIa, CBIIb, CBIIc, and CBIId (Figure 1). When submitted to FPLC on a Mono-Q column, each of these reconstituted crotoxin isoforms eluted as a single and symmetrical peak (Figure 5A). On the other hand, when fraction II was rechromatographed, it eluted as a double peak, the first one corresponding to two crotoxin isoforms, CAIICBIIc and CAIICBIId, and the second one to a single species,

CAIICBIIb. The minor crotoxin isoform of fraction II, CAIICBIIa, eluted at a higher ionic strength (results not shown). When submitted to isoelectric focusing, fraction II was found to consist of three major components with p*I*s of 5.25, 5.17, and 5.10 and of a minor one with a p*I* of 4.90. Isoelectric focusing of the purified crotoxin isoforms showed that these bands correspond respectively to CAIICBIId, CAIICBIIb, CAIICBIIc, and CAIICBIIa (Figure 5B and Table I). In addition, the isoelectric focusing patterns obtained with the reconstituted crotoxin isoforms further demonstrated that the purified isoforms of crotoxin components A and B were homogeneous, with the exception of CBIIc and CBIId, which showed cross-contamination (Figure 5B).

A similar reconstitution study performed with the isolated crotoxin components A and B purified from fraction IV confirmed that CBIV is composed of two components whereas component A of CAIV appeared to be homogeneous. Further, the latter is different from CAII since CAIVCBIIb was eluted from the Mono-Q column as a single and symmetrical peak, at a higher ionic strength than CAIICBIIb (Figure 5A). The p*I* values of these complexes were also different, respectively 5.08 and 5.17 (Figure 5B and Table I). As expected, isoelectric focusing confirmed the heterogeneity of CBIV and further showed that CBIVa was identical with CBIIa since one of the two bands of CAIICBIV and of CAIVCBIV focused at the p*I*s of CAIICBIIa and CAIVCBIIa, 4.90 and 4.20, respectively (Figure 5B). Furthermore, other reconstituted crotoxin isoforms were found to correspond to isoforms preexisting in *C. durissus terrificus* venom. For example, fraction III was found to contain the single component A isoform of fraction IV, CAIV, combined with the four component B isoforms, CBIIa, CBIIb, CBIIc, and CBIId, derived from fraction II (Figure 5B).

The enzymatic and toxic properties of pure crotoxin isoforms prepared by reconstitution were investigated by measuring their phospholipase $A_2$ activity and their lethal potency ($LD_{50}$) (Table I). Although all crotoxin isoforms have similar properties, we observed slight but significant quantitative differences (Table I). In particular, CAIICBIIb, CAIICBIIc, CAIICBIId, and CAIVCBIIb possessed a high lethal potency and a low phospholipase $A_2$ activity, whereas CAIICBIIa, CAIVCBIV, and CAIICBIV were less potent and enzymatically more active. These observations indicate that the two component A isoforms that we purified have identical functional properties since replacing CAII by CAIV in the crotoxin complex did not alter either its enzymatic activity or its lethal potency (Table I). They also suggest that component B isoforms belong to at least two classes, crotoxin complexes prepared with isoforms of the first class (CBIIb, CBIIc, CBIId) being more toxic and having lower phospholipase $A_2$ activity than those obtained with isoforms of the second class (CBIIa and CBIV) (Table I).

## Discussion

Crotoxin has been reported to possess several molecular variants or isoforms (Hendon et al., 1970; Breithaupt et al., 1974; Wu et al., 1983; Faure & Bon 1987). In order to analyze these components, we isolated about 0.5 g of the toxin from a single batch of *C. durissus terrificus* venom that we obtained from the Instituto Butantan (Sao-Paulo, Brazil), by gel filtration on Sephadex G-75. Crotoxin was further fractionated by FPLC on cation- and anion-exchange columns, after separation of the two toxin subunits. We observed the presence of several variants of both subunits, differing in their chromatographic or electrophoretic properties. We purified two isoforms of component A and four isoforms of component B in a homogeneous state, which produced eight crotoxin isoforms by recombination of these isolated subunits.

The two component A isoforms, CAII and CAIV, appeared to be very similar in their molecular structure, and their amino acid composition was close to that deduced from the protein sequence determined by Aird et al. (1985). CAII differs from CAIV by one or two additional residues, threonine and/or aspartic acid or asparagine. The chromatographic and electrophoretic properties indicate that CAIV possesses at least one negative charge more than CAII. Since component A is constituted of three polypeptides, cross-linked by seven disulfide bridges and resulting from the limited proteolysis of a single chain precursor, one may therefore suggest that one of the polypeptides of CAIV possesses additional residues, threonine and/or aspartic acid. This interpretation is in good agreement with the observation of Breithaupt et al. (1974), who reported the isolation of two chromatographic variants in one of the three polypeptides of component A, peptide 23–61, according to the protein sequence (Aird et al., 1985). It is therefore possible that the two component A isoforms that we isolated here differ by the presence in CAIV of additional residues, threonine and/or aspartic acid, at one extremity of this peptide. According to this interpretation, one would expect that the two component A isoforms are the consequence of slightly different posttranscriptional processes rather than the expression of different genes or isogenes. Alternatively, the difference in the electric charge of the two component A isoforms might also be due to the replacement of asparagine and/or glutamine residues by aspartic and/or glutamic acid residues. In this case, they would derive from different genes (isogenes), as previously proposed (Faure & Bon, 1987). A comparison of the protein sequences of the two isoforms will give a definitive answer to these questions.

Although the amino acid compositions of the four component B isoforms that we purified were in general agreement with that deduced from the protein sequences (Fraenkel-Conrat et al., 1980; Aird et al., 1986), there were slight but significant variations in the different isoforms. These differences might be simply explained by the fact that the protein sequence was determined with a mixture of component B isoforms. Fraenkel-Conrat et al. (1980) and Aird et al. (1986) in fact mentioned allelic variations at positions 1, 18, 27, 33, 37, 69, and 106 [numbering according to Aird et al. (1986)], which correspond to the amino acid residues that differed in the purified component B isoforms. The compositions of CBIIc and CBIId were very close to the composition deduced from the sequence (Aird et al., 1986), with only one amino acid difference, while those of CBIIb and CBIIa were more different, with respectively four and seven changes.

Pure crotoxin isoforms were prepared by recombination of the isolated subunits. This procedure allowed us to reconstitute the four isoforms of crotoxin that were present in fraction II purified by FPLC on a Mono-Q column. Furthermore, by recombination of the purified component B isoforms of one FPLC fraction (fraction II) with the purified component A isoform of another FPLC fraction (fraction IV), it has been possible to recreate the main crotoxin isoforms from another FPLC fraction (fraction III). This demonstrates that the various crotoxin isoforms of the *C. durissus terrificus* venom are derived from the random association of isoforms of the two subunits. In the present investigation we purified two different component A isoforms and four different component B isoforms and therefore obtained eight different crotoxin isoforms, representing the major crotoxin isoforms in the venom batch that we studied.

The functional properties of the various crotoxin isoforms are similar, as expected on the basis of the similarity of their molecular structure. The phospholipase and the lethal potency of the reconstituted complexes were found to vary, slightly but significantly, according to the nature of component B but not according to the nature of component A. Thus the various isoforms of component A appear to be functionally identical, while those of component B fall in two classes. The first class (CBIIb, CBIIc, and CBIId) forms crotoxin complexes with a low enzymatic activity and a high lethal potency, whereas the second class (CBIIa and CBIV) forms enzymatically more active but less toxic crotoxin complexes. Since the isolated component B isoforms of the two classes did not differ significantly in their phospholipase activity when tested in the absence of component A, we suggest that the differences are due to the interactions between the two subunits in the crotoxin complex. Since the dissociation of the crotoxin complex is a major feature of the synergistic action of its subunits (Jeng et al., 1978; Bon et al., 1979), it is tempting to propose that the two classes of component B isoforms differ in the stability of the complex formed with component A.

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