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ISOLATION AND CHARACTERIZATION OF THREE PHOSPHOLIPASES A FROM THE CROTOXIN COMPLEX

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Summary

1. Three phospholipases A (phosphatide acyl-hydrolase, EC 3.1.1.4) have been isolated from the crotoxin complex, the main toxic compound of the *Crotalus durissus terrificus* venom.

2. Two basic phospholipases A were highly purified from the crotoxin complex by single chromatography on carboxymethyl cellulose. The yields were 10% and 38% (w/w), respectively. They showed no differences with regard to isoelectric point, enzymatic activity, immunological properties, and toxicity. One acidic phospholipase A, purified to a final yield of 1—3% by chromatography on carboxymethyl cellulose, gel filtration on Sephadex G-50, and chromatography on DEAE-cellulose, was found to have one third of the specific enzymatic activity of the basic enzymes. The acidic phospholipase A was nontoxic and antigenically different from the basic enzymes.

3. Crotapotin, an acidic peptide of the crotoxin complex (31% yield, w/w), potentiated the toxicity and inhibited the enzymatic activity of the basic phospholipase A isoenzymes, but did not interact with the acidic phospholipase A.

4. The purified enzymes were homogeneous with respect to cellogel electrophoresis, polyacrylamide gel electrophoresis, dodecyl sulfate-gel electrophoresis, immunoelectrophoresis, and isoelectric focusing.

5. The molecular weights of the three phospholipases were found to be in the same range as determined by gel filtration in 6 M guanidine · HCl (14 500) and dodecyl sulfate-gel electrophoresis (15 800). The isoelectric points of these enzymes were at 9.7 and 4.8 for the first two and the third, respectively.

6. The amino acid compositions of the three *Crotalus* phospholipases were rather similar. The acidic enzyme contained more acidic instead of basic amino

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acid residues. The two methionine residues of each phospholipase were found to be positioned nearby the NH_2- and the C-terminal of the protein chains. A third methionine residue was demonstrated in the acidic phospholipase A. Fingerprint maps of the basic enzymes showed only slight differences.

7. NH_2- and C-terminal sequence analyses indicated a striking homology between the three *Crotalus* phospholipase A isoenzymes and several phospholipases from other sources.

Introduction

Crotoxin is the main toxic compound of the *Crotalus durissus terrificus* (Brazilian rattlesnake) venom [1]. Crotoxin with its high toxicity and low phospholipid splitting activity was considered to be a molecular complex of the acidic peptide crotapotin and basic phospholipases A [2—7]. Crotapotin lacks the toxicity and enzymatic activity of crotoxin, but by interacting with the weakly toxic *Crotalus* phospholipase A potentiates the toxicity and inhibits the enzymatic activity of the basic phospholipase A to the original level of crotoxin [2—5].

Other naturally occurring polypeptides also inhibiting the enzymatic action of phospholipase A have been isolated from *Naja naja* [8] and from *Bothrops neuwiedii* venom [9]. These phospholipases, however, were by far less toxic than the basic enzyme from *Crotalus durissus terrificus.*

Toxic phospholipases A were obtained from *Vipera berus* [10,11], *Naja naja atra* [12] and *Naja nigricollis* [13] venom. To the best of our knowledge only the basic *Crotalus* phospholipases were found to occur as a salt-like complex together with a peptide, which potentiates the toxicity of the enzyme.

The present paper reports the preparation and characterization of three highly purified phospholipases A from the venom of *Crotalus durissus terrificus.* One of the two basic *Crotalus* phospholipases A has already been described [2—7,14]. The striking homology of the primary structure of the three *Crotalus* phospholipases with other phospholipases [15—19] is pointed out.

Materials and Methods

Materials

Crotoxin was prepared from *Crotalus durissus terrificus* venom (Dr Bücherl, Instituto Butantan, São Paulo; Miami Serpentarium LOT Number CTOL, Miami, Fla.) according to Slotta and Fraenkel-Conrat [1] with slight modifications described by Neumann and Habermann [20]. The anti-*Crotalus* serum Y 138 was a gift from Dr Bücherl.

The following marker proteins were used: ovalbumin (M_r = 43 000 and 86 000), hemoglobin (bovine, M_r = 16 125, 32 250, and 64 500), soybean trypsin inhibitor (M_r = 21 500), β-lactoglobulin (M_r = 18 360), myoglobin (horse, M_r = 16 950), lysozyme (egg white, M_r = 14 310), and insulin (bovine, M_r = 5730) from Serva, Heidelberg; lactate dehydrogenase (M_r = 36 000) from Sigma, St. Louis; hemoglobin (human, M_r = 15 500) own preparation; cytochrome *c* (horse heart, M_r = 12 300 and 24 600) from Boehringer Mannheim,

and Kunitz trypsin inhibitor (M_r = 6520) from Bayer, Leverkusen. The M_r-values were taken from Dayhoff [21].

Carboxymethyl cellulose (CM 32, Whatman), DEAE-cellulose (DE 32, Whatman) and the Sephadex charges (Pharmacia) were prepared for use according to the instructions of the manufacturers. Guanidine hydrochloride (Roth, Karlsruhe) was purified according to Nozaki and Tanford [22]. Cellulose acetate sheets (Cellogel), TPCK-trypsin (bovine, treated with *p*-tosyl-L-phenylalanine-chlormethylketone), fluorescamine (Fluram, p.a.), 5-dimethylamino-1-naphthalinesulfonylchloride (dansylchloride), agarose, 2-iodoacetamide, acrylamide, *N,N'*-methylenebisacrylamide, and *t*.-butyl hypochlorite were obtained from Serva, Heidelberg. Leucine aminopeptidase was from Boehringer, Mannheim. Ampholine (pH 3—10, 9—11) was from LKB (Stockholm). Trifluoroacetic acid, 2-mercaptoethanol, Coomassie brilliant blue R 250, amidoschwarz 10 B, sorbitol p.a., platinochloric acid ($H_2PtCl_6 \cdot 6H_2O$), ninhydrin, α-nitroso-β-naphtol, *p*-dimethyl-aminobenzaldehyde and phenanthrene quinone were from Merck, Darmstadt. Cyanogen bromide, p.a., and phenylisothiocyanate were from Fluka, Buchs. Micropolyamide layers (DC Fertigfolien F 1700) and papers for fingerprint (49 × 58 cm; 2043 B) were from Schleicher and Schüll, Dassel. All other substances were of analytical grade.

Methods

Isolation procedures. The two basic enzymes were obtained as homogeneous proteins by chromatography of the crotoxin complex on carboxymethyl cellulose as described by Rübsamen et al. [4]. The acidic phospholipase A containing fractions were rechromatographed on carboxymethyl cellulose by use of a flat gradient from 0.1 to 1.5 M ammonium formate buffer, pH 3.5. Further purification was done by gel filtration on Sephadex G-50 in 10% acetic acid. The final purification was performed by chromatography on DEAE-cellulose with a linear gradient from 0.05 to 1.5 M ammonium formate buffer, pH 4.5.

The acidic *crotalus* phospholipase A could be isolated by the same procedures from the so-called crotamine-enriched fraction [20].

Electrophoretic methods. Cellogel electrophoresis was performed with 0.06 M sodium barbital/acetate buffer, pH 8.5. 2 V per cm were applied for 1 h. Staining with amidoschwarz (0.5%) in methanol/water/acetic acid (5 : 4 : 1, by vol).

Polyacrylamide gel electrophoresis was carried out at pH 8.3 and 2.3 according to Maurer [23]. The gel cylinders were scanned at 280 nm in a Gilford recording densitometer before staining with Coomassie blue [24].

Dodecyl sulfate-gel electrophoresis was performed according to Shapiro et al. [25] with some modifications [26,27].

Immunoelectrophoresis was carried out in 0.8% agarose gel using 0.05 M sodium acetate buffer, pH 5.0. Electrophoresis was done with 4 mA per microscope slide for 1 h, followed by immunodiffusion for 24 h and staining with 0.5% amidoschwarz.

Isoelectric focusing. Isoelectric focusing experiments in polyacrylamide gel slides were performed according to Awdeh [28] and Leaback and Rutter

[29] with modifications already published in a previous paper [7].

Focusing in a narrow-range pH gradient was performed with Ampholine pH 9—11 stabilized by 12 ml of a sorbitol gradient in a small U-tube (0.9 cm internal diameter).

Determination of the molecular weights. The molecular weights of the phospholipases were determined by gel filtration on Sephadex G-100 in the presence of 6 M guanidine · HCl [30] and by dodecyl sulfate-gel electrophoresis.

Determination of phospholipase A activity. Phospholipase A activity was screened by the egg yolk coagulation test [31]. Purified phospholipase A was assayed by potentiometric titration with 0.05 M NaOH using 3 ml of egg yolk/0.85% NaCl (1 : 12, by vol.) — 0.1 M $CaCl_2$ as substrate [32]. The specific activity was determined as μequiv. $OH^- \cdot min^{-1} \cdot mg^{-1}$ phospholipase A at 40° C and pH 8.0.

Toxicity determination. Mice (NMRI, Hannover; 20—30 g) were injected intravenously with various doses of the material, dissolved in 0.1 ml of 0.85% NaCl per 10 g body weight. Deaths were recorded at 24 h.

Protein determination. Protein content was determined by ultraviolet absorbance at 280 nm, by modified Folin reaction [33] or by the use of fluorescamine [34].

Amino acid analyses. All samples were hydrolyzed with 6 M HCl (suprapur) in evacuated sealed tubes at 110° C for 24 h or 72 h and then analyzed on a Unichrom Analyzer (Beckman, München) or on the Biotronik Analyzer LD 6000. The serine values were extrapolated to zero time. The tryptophan content was determined spectrophotometrically [35].

Absorbance spectra. The absorbance spectra were determined in a Unicam SP-8000 ultraviolet recording spectrophotometer.

Chemical fragmentation. Cyanogen bromide cleavage at the methionine residues was performed as described by Steers et al. [36].

Cleavage of disulfide bonds. The disulfide bridges were reduced and carboxamidomethylated according to Hirs [37].

Enzymic digestions. Tryptic digestion of maleylated [38] proteins was performed for 14 h in 0.1 M ammonium bicarbonate, pH 8.2 at 40° C using an enzyme:protein ratio of 1 : 50. Demaleylation occurred at pH 3.0 for 48 h at 40° C.

Some peptide fragments were completely hydrolyzed by the use of leucine aminopeptidase [39] followed by amino acid analyses in order to determine the amide groups in these fragments.

Fingerprint maps of the tryptic peptides. Fingerprints were obtained by descending paper chromatography with *n*-butanol/pyridine/water/acetic acid (150 : 100 : 120 : 30, by vol; pH 5.2) followed by high-voltage electrophoresis with pyridine/formic acid/water (1 : 20 : 279, by vol; pH 1.8) in the second dimension.

30 V per cm and about 80 mA were applied for 1 h. The following color reactions were used: (1) Ninhydrin followed by the reaction for tyrosine [40]; (2) Chlorination for detection of peptide bonds [41]; (3) Itano's reaction for arginine [42]; (4) Platinic iodide for sulfur compounds [43]; (5) Ehrlich's reagent for tryptophan [44] subsequent to buffered ninhydrin.

Isolation of the peptides. The cyanogen bromide fragments were separated by gel filtration on Sephadex G-50. The fragments resulting from sulfitolysis were separated by gel filtration on Sephadex G-25 (medium).

The cyanogen bromide fragments of the acidic phospholipase A were finally purified by chromatography on carboxymethyl cellulose using a gradient of 0.1 to 2.0 M ammonium formate buffer, pH 3.5, per 500 ml.

Determination of the N-terminal sequence. The N-terminal sequences of the native phospholipases were determined by aid of the modified Dansyl-Edman method [45]. Dansyl-Edman degradation [46,47] of the N-terminal cyanogen bromide fragments was followed by chromatography on micropolyamide layers using the solvent systems of Woods and Wang [48]. Gln and Asn were identified by amino acid analyses after complete enzymatic hydrolysis of the N-terminal peptides by leucine aminopeptidase. The C-terminal sequences of the two basic phospholipases were determined by dansyl-Edman degradations of the C-terminal cyanogen bromide fragments.

Results

Occurrence of isoenzymes in the crotoxin complex

The yields of the different components of the crotoxin complex are listed in Table I. Two basic phospholipases (fractions V and VI, Fig. 1A) were obtained with high yields from the crotoxin prepared from *Crotalus durissus terrificus* venoms of the Instituto Butantan (São Paulo). The content of fraction VI varied from 30% to 40%. Fraction V lacked in some crotoxin batches [4], but reached in other samples 20%. In contrast one atypical crotoxin complex prepared from a *Crotalus durissus terrificus* venom of the Miami Serpentarium contained only 2% of a basic phospholipase A (Fig. 1B). This atypical crotoxin was difficult to solubilize (only 11% solubility in 0.85% NaCl instead of the usual 85%) and showed an atypical pattern in the disc gel electrophoresis. The toxicity of the soluble portion of the Miami crotoxin was relatively low (0.27 mg/kg mice, intravenously, instead of 0.11 mg/kg). The main component of the atypical crotoxin was an unidentified fraction with a low content in half-cystines.

A considerable amount of an acidic phospholipase A could be isolated from the Miami crotoxin (Fig. 1B). The same type of acidic phospholipase A

TABLE I

COMPOSITION OF THE NORMAL AND OF AN ATYPICAL CROTOXIN

Typical crotoxin		Yield (%)	Atypical crotoxin		Yield (%)
			not adsorbed protein		53
I, II	crotapotin	31	I	crotapotin	1.2
	acidic phospholipase A	1.1		acidic phospholipase A	2.8
III	intermediate fraction	2.8	III	intermediate fraction	1.5
IV	crotamine	4.5	IVa,b	crotamine	3.8
V	basic phospholipase A	10			
VI	basic phospholipase A	38	VI	basic phospholipase A	2.2
	Total recovery	87.4		Total recovery	64.5

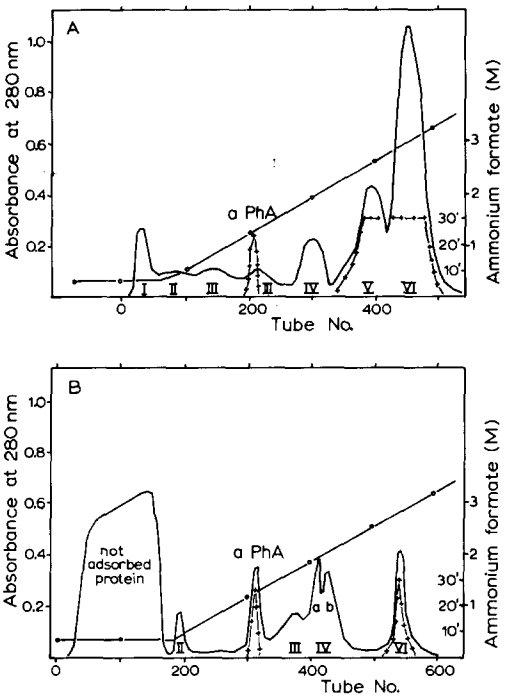

Fig. 1. Chromatography of typical crotoxin (A) and an atypical crotoxin (B) on carboxymethyl cellulose. 800 mg crotoxin were applied to 5 × 14 cm CM-cellulose and eluted by a gradient of 0.1 to 3 M ammonium formate buffer, pH 3.5, with a flow rate of 10 ml per 10 min and fraction. The effluent was monitored at 280 nm (———). Phospholipase activity was tested by checking egg yolk coagulation time (+———+; right ordinate). Molarity (o———o). Notation of the fractions is listed in Table I. aPhA, acidic phospholipase A.

was detected in the intermediate fraction III of the Brazilian crotoxin (Fig. 1A).

Similar amounts of the acidic phospholipase A were detected in the crotamine-enriched fraction obtained from the crude venom during the preparation of the crotoxin complex [20]. The highly purified acidic phospholipase A accounted for 2% of this crotamine fraction.

Purity of the phospholipase A isoenzymes

The two basic *Crotalus* phospholipases A, prepared by single chromatography on CM-cellulose, were homogeneous with respect to their behaviour on cellogel electrophoresis, polyacrylamide gel electrophoresis, dodecyl sulfate-gel electrophoresis, immunoelectrophoresis, and isoelectric focusing. These two phospholipases could not be distinguished from one another by means of either polyacrylamide gel electrophoresis or dodecyl sulfate-gel electrophoresis or isoelectric focusing. Both enzymes showed the same immunological properties with respect to immunodiffusion and immunoelectrophoresis. The two basic phospholipases appeared as well defined peaks when applied to a second carboxymethyl cellulose column; fraction V was eluted again at the ionic strength of 1.6—1.8 M ammonium formate, and fraction VI reappeared at 1.8—2.1 M.

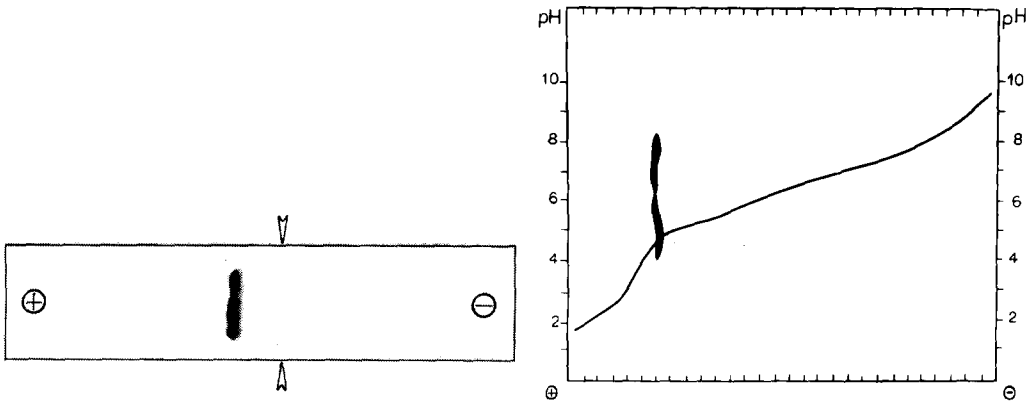

Fig. 2. Electrophoresis of the acidic *Crotalus* phospholipase A on a cellulose acetate foil. 60 μg of the protein were applied at the starting line (arrow). The protein migrated to the anode at pH 8.5. 2 V per cm for 1 h. Staining with amidoschwarz.

Fig. 3. Isoelectric focusing of two acidic phospholipases A in a polyacrylamide gel plate (12 × 10 × 0.2 cm; T = 3.5%, C = 5%; 2% ampholine, pH 3—10). 0.1 mg of the enzymes were applied onto the gel surface by the use of filter papers (1.5 × 0.5 cm). The acidic phospholipases have been isolated from the typical crotoxin (above) and from an atypical crotoxin (below). 12 h focusing time with a constant voltage of 150 V. The pH gradient (———) measured in gel eluates has been superimposed on the pixture.

The acidic phospholipase A was purified to homogeneity as demonstrated by electrophoresis on cellulose acetate sheets (Fig. 2), by polyacrylamide gel electrophoresis, dodecyl sulfate-gel electrophoresis, immunoelectrophoresis, and isoelectric focusing in polyacrylamide gel plates (Fig. 3). The highly purified enzyme appeared as a single peak in further ion exchange chromatographies and gel filtration experiments.

Isoelectric points

No difference could be shown between the two basic enzymes with regard to their isoelectric points. Both proteins were focused in a very sharp zone at pH 9.71 using a linear pH gradient from 8.0 to 10.5, stabilized by a sorbitol density gradient. In the polyacrylamide gel plate the adsorption of the basic proteins was very strong and by it both phospholipases appeared as broad bands at pH 9.5—9.8. The isoelectric point of the acidic phospholipase A was 4.8 (Fig. 3).

Molecular weights

The molecular weights of the three *Crotalus* phospholipases were found to be about 16 000 as shown by gel filtration on Sephadex G-100 in 6 M guanidine · HCl and by dodecyl sulfate-gel electrophoresis (Fig. 4).

Immunological behaviour

The two basic phospholipases showed immunological identity, whereas the acidic enzyme was antigenically different (Fig. 5).

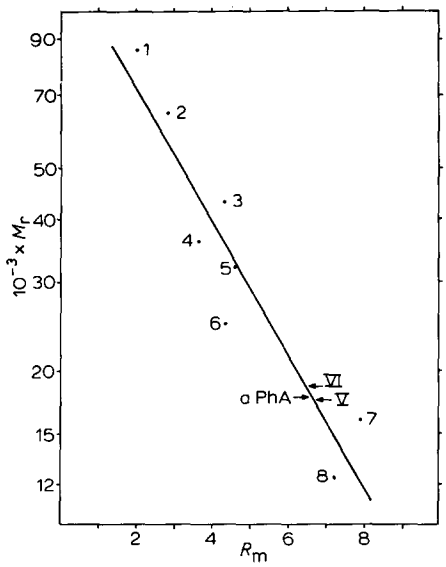

Fig. 4. Molecular weight determination by sodium dodecyl sulfate gel electrophoresis with the modified method of Laemmli [26,27]. R_m is the electrophoretic mobility relative to bromphenol blue. Semilog plot of the molecular weight of marker proteins against R_m. (•) R_m values of the marker proteins in the 15% gel. ↑ indicate the points of intersection of the R_m values of the acidic phospholipase A (aPhA) and the basic phospholipase fractions V and VI with the regression line log M_r = 5.12–1.31 R_m (method of least squares). The marker proteins used were ovalbumin dimer (1), hemoglobin (2), ovalbumin monomer (3), lactate dehydrogenase (4), hemoglobin, two chains (5), cytochrome *c* dimer (6), hemoglobin, one chain (7), and cytochrome *c* monomer (8).

Enzymatic activity

The basic enzymes have specific activities of about 200 μequiv. · min^{-1} · mg^{-1} protein, using egg yolk suspensions with 0.1 M $CaCl_2$ as substrate at 40°C and pH 8.0. The specific enzymatic activity of the acidic phospholipase A was

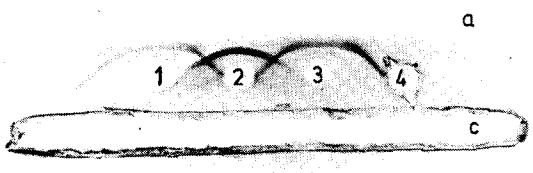

Fig. 5. Immunodiffusion of the *Crotalus* phospholipases against anti-crotalus serum. 30 μg acidic phospholipase A (1; isolated from typical crotoxin), a mixture of 0.5 μg of each of the basic *Crotalus* phospholipases (2), 30 μg acidic phospholipase (3; obtained from the atypical crotoxin), and 0.1 μg of each of the basic enzymes (4) were allowed to diffuse for 24 h against 50 μl of the anti-*Crotalus* serum (a) and 50 μl of control serum (c; rabbit, undiluted). Staining with amidoschwarz.

TABLE II

TOXICITY AND ENZYMATIC ACTIVITY OF THE *CROTALUS* PHOSPHOLIPASES

Substances	LD_{50} mice (intravenous) (mg/kg)	Enzymatic activity (μequiv. OH^-. min^{-1}. mg^{-1} phospholipase A)
Acidic phospholipase A	>10	67
Acidic phospholipase A + crotapotin	>10*	67**
Basic phospholipase A	0.54	194
Basic phospholipase A + crotapotin	0.05***	30**
Basic phospholipase A + acidic phospholipase A (0.5 mg/kg)	≈0.5	

* Injected together with crotapotin (8 mg/kg).
** Together with a 20-fold molar excess of crotapotin.
*** Injected together with crotapotin (0.5 mg/kg).

found to be about 70 μequiv. · min^{-1} · mg^{-1} protein in this system. The pH optima of all three phospholipases centered around pH 8.

The inhibitor crotapotin diminished the enzymatic activities of the basic phospholipases A, but did not influence the activity of the acidic enzyme (Table II).

Toxicity

The two basic *Crotalus* phospholipases could not be distinguished by their toxic actions as tested in mice, rats, rabbits and chicks. The LD_{50} of each enzyme was 0.54 mg/kg mice, intravenously (Table II). The animals died of respiratory paralysis. The acidic phospholipase A was fairly nontoxic (LD_{50} > 10 mg/kg mice, intravenous injection).

Crotapotin, increased the toxicity of both basic enzymes. However, 10 mg of the acidic phospholipase A per kg mice remained nontoxic when injected intravenously together with crotapotin (8 mg/kg).

Amino acid composition

The amino acid compositions of the three phospholipases are listed in Table III in terms of molar ratios. The differences in the composition of the two basic enzymes are minimal: Fraction V contains 13 aspartic and 12 glutamic acid residues in comparison to fraction VI with 11 and 10 residues, respectively.

Small differences were also found in the contents of lysine, histidine, arginine, serine, glycine, valine, and isoleucine. The amino acid composition of the acidic phospholipase A obtained from the atypical Miami crotoxin and from the typical Brazilian crotoxin was shown to be identical (Table III).

Absorbance spectra

The absorbance spectra of the two basic enzymes were identical. The molar absorbance coefficient of both proteins was 26 000 M^{-1} · cm^{-1} at 278.5 nm. The spectrum of the acidic phospholipase A was found to be similar. Its coefficient was 19 200 M^{-1} · cm^{-1} at 278.5 nm.

TABLE III

AMINO ACID COMPOSITION OF THE PHOSPHOLIPASE ISOENZYMES

Amino acid	Basic phospholipase A fraction VI*	Basic phospholipase A fraction V	Acidic phospholipase A Miami	Acidic phospholipase A Brazilian
Lys	11.36 (11)	10.16 (10)	12.15 (12)	11.84 (12)
His	2.26 (2)	1.09 (1)	1.37 (1)	0.92 (1)
Arg	11.44 (12)	9.59 (10)	6.14 (6)	6.00 (6)
Asp	10.91 (11)	13.22 (13)	20.93 (21)	21.04 (21)
Thr	7.71 (8)	7.85 (8)	5.46 (6)	5.82 (6)
Ser**	6.74 (7)	7.74 (8)	7.84 (8)	7.63 (8)
Glu	9.85 (10)	12.37 (12)	13.72 (14)	14.00 (14)
Pro	5.30 (5)	5.26 (5)	7.72 (8)	7.74 (8)
Gly	12.50 (13)	12.25 (12)	12.50 (13)	12.80 (13)
Ala	7.06 (7)	7.12 (7)	7.56 (8)	7.81 (8)
1/2 Cys	15.61 (16)	15.80 (16)	16.54 (16)	15.56 (16)
Val	2.09 (2)	2.80 (3)	7.77 (8)	8.18 (8)
Met	2.16 (2)	2.25 (2)	2.77 (3)	3.29 (3)
Ileu	4.85 (5)	3.79 (4)	3.20 (3)	2.85 (3)
Leu	6.87 (7)	6.89 (7)	4.93 (5)	4.72 (5)
Tyr	11.55 (12)	11.70 (12)	9.50 (9)	9.05 (9)
Phe	6.91 (7)	6.94 (7)	4.84 (5)	4.91 (5)
Trp***	(3)	(3)	(3)	(3)
Total	140	140	149	149
Formula weight	16294	16379	16699	16699

* Data of this isoenzyme have already been presented elsewhere [7].
** Extrapolated to zero time.
*** Determined spectrophotometrically [35].

Chemical fragmentation

Cyanogen bromide cleavage of the native phospholipases revealed two split products, which were isolated by gel filtration on Sephadex G-50. The short cyanogen bromide fragment of each enzyme contained homoserine. The large peptide cyanogen bromide fragment II/III was reduced, carboxyamidomethylated and then fractionated by gel filtration on Sephadex G-25. Two pure peptides, a long cyanogen bromide fragment II (containing homoserine) and a short cyanogen bromide fragment III (without homoserine) were obtained for each of the two basic phospholipases (Table IV).

Three pure peptides were obtained from the long cyanogen bromide fragment (cyanogen bromide fragment II/IV) of the acidic phospholipase A. The positions of cyanogen bromide fragment II (with 50 amino acid residues) and cyanogen bromide fragment III (76 residues) were not established, whereas cyanogen bromide fragment IV (16 residues without homoserine) was found to be the C-terminal fragment (Table IV).

Fingerprint maps of the tryptic peptides of cyanogen bromide fragment II

The cyanogen bromide fragment II-peptides of the basic phospholipases as well as the cyanogen bromide fragment II/IV-peptide of the acidic enzyme were maleylated and then subjected to long time tryptic digestion. As shown

TABLE IV

AMINO ACID COMPOSITION OF CYANOGEN BROMIDE FRAGMENTS FROM THE PHOSPHOLIPASE ISOENZYMES

CB, cyanogen bromide fragment.

Amino acid	Peptide and residue numbers									
	Basic phospholipase A (VI)			Basic phospholipase A (V)			Acidic phospholipase A			
	CB I	CB II	CB III	CB I	CB II	CB III	CB I	CB II	CB III	CB IV
Lys	1.1 (1)	9.9 (10)		1.0 (1)	9.1 (9)			4.1 (4)	6.7 (7)	1.0 (1)
His	1.0 (1)	1.2 (1)		0.2	0.8 (1)				0.9 (1)	
Arg		9.6 (10)	1.9 (2)		7.9 (8)	2.0 (2)		2.7 (3)	3.1 (3)	
Asp	1.0 (1)	9.1 (9)	1.0 (1)	1.1 (1)	10.5 (11)	1.0 (1)	1.3 (1)	7.1 (7)	9.2 (9)	4.0 (4)
Thr		7.1 (7)	1.1 (1)		6.6 (7)	0.9 (1)		1.8 (2)	3.2 (3)	0.9 (1)
Ser*		5.3 (5)	1.9 (2)	0.7 (1)	5.0 (5)	1.6 (2)	1.0 (1)	2.6 (3)	1.9 (2)	1.7 (2)
Glu	1.0 (1)	7.9 (8)	1.1 (1)	1.0 (1)	9.8 (10)	1.4 (1)	1.0 (1)	7.2 (7)	3.8 (4)	1.8 (2)
Pro		3.2 (3)	1.9 (2)		3.8 (3)	2.3 (2)		4.5 (5)	2.8 (3)	
Gly		12.5 (12)	1.2 (1)		10.6 (11)	0.6 (1)		2.1 (2)	10.4 (10)	1.2 (1)
Ala		6.9 (7)			6.6 (7)			2.7 (3)	4.8 (5)	
1/2Cys**		13.9 (14)	1.9 (2)		13.8 (14)	2.0 (2)		3.9 (4)	11.4 (11)	1.1 (1)
Val		1.8 (2)			2.9 (3)			2.1 (2)	3.7 (4)	1.8 (2)
Met***	+ (1)	+ (1)		+ (1)	+ (1)		+ (1)	+ (1)	+ (1)	
Ileu		4.6 (5)			3.8 (4)			1.6 (2)	0.7 (1)	0.3
Leu	1.9 (2)	5.1 (5)		1.9 (2)	5.0 (5)		1.7 (2)		2.8 (3)	
Tyr		11.3 (11)	0.7 (1)		10.6 (11)	1.0 (1)		2.9 (3)	4.2 (4)	1.8 (2)
Phe	1.0 (1)	4.9 (5)	1.1 (1)	1.0 (1)	4.9 (5)	1.0 (1)	1.0 (1)	1.9 (2)	1.7 (2)	
Trp†		+ (3)			+ (3)				+ (3)	
Total residues	8	118	14	8	118	14	7	50	76	16

* Extrapolated to zero time.

** Carboxamidomethylcysteine.

*** Homoserine and homoserine lactone.

† Positive color reaction on paper.

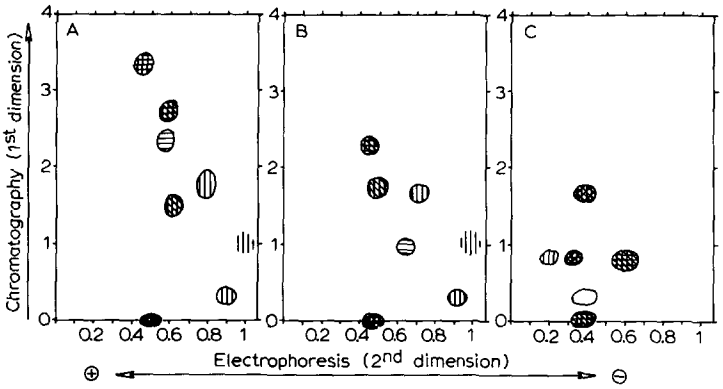

Fig. 6. Peptide maps of the tryptic hydrolysates of the maleylated and S-carboxamidomethylated cyanogen bromide fragments II, prepared from the basic phospholipases fraction VI (A), fraction V (B), and of the cyanogen bromide fragment II—IV prepared from the acidic phospholipase (C), respectively. The spots were detected by chlorination reaction for peptide bonds (○), by phenanthrene quinone reagent for arginine (▥), by nitrosonaphthol reagent for tyrosine (▤), by Ehrlich's reagent for tryptophane (▨), and by platinic iodide reagent for sulfur containing compounds (▩). Abscissa and ordinate indicate reference values relative to the arginine spot.

by Fig. 6, 8 positive spots in the fingerprint maps were obtained from the tryptic hydrolysate of cyanogen bromide fragment II of fraction VI, 7 spots were found for fraction V, and 6 spots for the large cyanogen bromide fragment of the acidic phospholipase A. The reference values R_F (relative to the arginine spot) indicate some similarities between the maps of the basic phospholipases. The patterns of arginine, tryptophan, tyrosine and sulfur positive spots supported the similarity of the basic enzymes.

Partial sequence analyses

The N-terminal amino acid of the basic phospholipase A fraction V was serine and the N-terminus of the fraction VI enzyme was histidine. The following amino acid sequences of both enzymes were identical (Table V), as shown

TABLE V

COMPARISON OF AMINO-TERMINAL SEQUENCES OF SEVERAL PHOSPHOLIPASES AND TWO NEUROTOXINS WITH WEAK PHOSPHOLIPASE A ACTIVITY

Crotalus d. terr. (fr. VI)	H_2N-His - Leu -Leu- Gln-Phe -Asn-Lys- Met - Lys -Leu-Phe-
Crotalus d. terr. (fr. V)	H_2N-Ser - Leu -Leu- Gln-Phe -Asn-Lys- Met -
Crotalus d. terr. (acidic)	H_2N-Ser - Leu -Leu- Asp-Phe -Glu - Met -
Bitis arietans [49]	H_2N-Ser - Leu -Phe- Glu-Phe -Gly-Gln- Met - Ile -Lys-Thr-
Bitis gabonica [17]	H_2N-Asp- Leu -Thr- Gln-Phe -Gly-Asn- Met - Ile -Asn-Lys-
Agkistrodon halys blomhoffii [18]	Glu-Phe -Glu-Thr- Leu - Ile -Met-Ser-
Naja melanoleuca [19]	H_2N-Asn- Leu -Tyr- Gln-Phe -Lys-Asn- Met - Ile -His -Cys-
Naja nigricollis [13]	H_2N-Asn- Leu -Tyr- Gln-Phe -Lys-Asn- Met - Ile -His -Cys-
Naja naja atra [12]	H_2N-Asn- Leu -Trp- Gln-Phe -Lys-Asn- Met - Ile -Gln-
Taipoxin [13]	H_2N-Asn- Leu -Val- Gln-Phe -Gly -Phe- Met - Ile -Glu -Cys-
Notexin [13]	H_2N-Asn- Leu -Val - Gln-Phe -Ser -Tyr- Leu - Ile -Gln-Cys-
Porcine pancreas [15]	H_2N-Ala - Leu -Trp- Gln-Phe -Arg-Ser - Met - Ile -Lys-Cys-
Apis mellifica [16]	H_2N-Ile - Ile -Tyr- Pro-Gly -Thr-Leu- Trp - Cys -Gly-His-

TABLE VI

COMPARISON OF C-TERMINAL AMINO ACID SEQUENCES OF REPTILIAN, MAMMALIAN AND BEE VENOM PHOSPHOLIPASES A

Crotalus d. terr. (fr. VI)	-Met- Phe -Tyr-Pro -Asp- Ser -Arg - Cys -Arg-Gly-Pro-Ser - Glu -Thr- CysOH
Crotalus d. terr. (fr. V)	-Met- Phe -Tyr-Pro -Asp- Ser -Arg - Cys -Arg-Gly-Pro-Ser - Glu -Thr- CysOH
Bitis gabonica	-Tyr- Phe -Gly -His -Ser - Ser -Lys - Cys -Thr-Gly- -Thr- Glu -Gln- CysOH
Aghistrodon halys bl.	-Pro- Phe -Ala -Lys-Asn- Cys-Gln - Cys -Glu-Ser -Pro- - Glu -Glu- CysOH
Naja melanoleuca	-Asp- Phe -Asn-Ala -Arg- Cys-Gln - OH
Porcine pancreas	-Asn- Leu -Asn-Thr-Lys- Lys-Tyr - Cys -OH
Apis mellifica	-Trp- Phe -Asp-Leu-Arg- Lys-Tyr - OH

by Dansyl-Edman degradation of the cyanogen bromide fragment I as well as by sequence analyses of the native phospholipases using the modified Dansyl-Edman method. The N-terminal sequence of the acidic phospholipase A showed more differences.

The C-terminal sequences of the two basic *Crotalus* phospholipases were found to be identical (Table VI).

Table V and VI demonstrate sequence homologies among several phospholipases, of which sequence data were available to us.

Discussion

Crotalus durissus terrificus venom of different sources contains high amounts of the crotoxin complex (65%, w/w). Crotoxin consists mainly of two basic, toxic phospholipases A and the acidic peptide crotapotin. In addition to these components, an acidic phospholipase A was isolated from the crotoxin complex. The isolation procedures for the *Crotalus* phospholipases are rather simple and give good yields of homogeneous enzymes in a minimal number of operations. Multiple forms of phospholipases A have been demonstrated to occur in other *Crotalid* venoms [50—52] and in *Elapid* venoms [53,54].

The molecular weights of the three phospholipases were found to be about 16 000, in good agreement with the values reported in the literature for other phospholipases A [55].

The two basic phospholipases A can be distinguished by their chromatographic behaviour on carboxymethyl cellulose, probably reflecting small differences in their amino acid compositions. The higher amount of acidic amino acid residues in fraction V does not influence the biological activities as compared to fraction VI.

The acidic *Crotalus* phospholipase A is rich in aspartic and glutamic acids. Another distinct difference is found in the high content of valine residues of the acidic enzyme. The overall picture of the amino acid compositions, however, indicates a clear similarity between the three phospholipases. In contrast to these structural similarities the acidic phospholipase A shows biochemical, immunological and pharmacological properties different from the basic enzymes.

An increasing number of phospholipase sequences has been published recently: porcine pancreas [15], bee venom [16], *Bitis gabonica* [17], *Agkistrodon halys blomhoffii* [18], and *Naja melanoleuca* [19]. As reported by

Samejima et al. [18], sequence homology of the phospholipase A from *Agkistrodon halys blomhoffii* with those of *Bitis gabonica* venom, *Naja melanoleuca* venom and porcine pancreas was 34, 20 and 22%, respectively. A synopsis of all NH_2- and C-terminal phospholipase sequence data available to us indicates characteristic features in the linear sequences of the reptilian and mammalian phospholipases A. Only the honey bee venom enzyme differs in many respects from these.

The basic *Crotalus* phospholipases are toxic enzymes. A presynaptic action is reported for the frog neuromuscular junction [56] and a block of the neuromuscular transmission as well as a myotoxic effect is found in mammalians [57].

Further structural analyses of the basic phospholipases and their acidic nontoxic homologue may elucidate the interrelationship between toxicity and enzymatic activity. Comparisons of toxic phospholipases from the *Crotalus terrificus* venom and from other sources [10—13] with the presynaptic neurotoxins having weak phospholipase A activity [13] should be helpful to understand the molecular conditions responsible for toxicity and enzymatic activity. Studies on mechanisms by which crotapotin potentiates the toxicity and inhibits the enzymatic activity of the basic *Crotalus* phospholipases A are another way to get further insight in the molecular biology of these toxic enzymes.

The sequence homologies of the toxic *Crotalus* phospholipases A with other phospholipases and neurotoxins support the hypothesis of Eaker [13] that the presynaptic neurotoxins (notexin, subunits of taipoxin, β-bungarotoxin, etc.) have evolved from a phospholipase A structure, preserved in venoms of reptilians and in digestive secrets of vertebrates. The toxic *Crotalus* phospholipase isoenzymes might be considered as the connecting links in the molecular evolution of presynaptic neurotoxins derived from the class of phospholipases A.

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