Venom from the Snake *Bothrops asper* Garman

PURIFICATION AND CHARACTERIZATION OF THREE PHOSPHOLIPASES A₂

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The water-soluble venom of *Bothrops asper* Garman (San Juan Evangelista, Veracruz, México) showed 15 polypeptide bands on polyacrylamide-gel electrophoresis. This material exhibited phospholipase, hyaluronidase, *N*-benzoyl-L-arginine ethyl hydrolase, *N*-benzoyl-L-tyrosine ethyl hydrolase and phosphodiesterase activity, but no alkaline phosphatase or acid phosphatase activity. Fractionation on Sephadex G-75 afforded seven protein fractions, which were apparently less toxic than the whole venom ($LD_{50} = 4.3 \mu\text{g/g}$ mouse wt.). Subsequent separation of the phospholipase-positive fraction (II) on DEAE-cellulose with potassium phosphate buffers (pH 7.55) gave several fractions, two being phospholipase-positive (II.6 and II.8). These fractions were further purified on DEAE-cellulose columns with potassium phosphate buffers (pH 8.6). Fraction II.8.4 was rechromatographed in the same DEAE-cellulose column, giving a pure protein designated phospholipase 1. The fraction II.6.3 was further separated by gel disc electrophoresis yielding two more pure proteins designated phospholipase 2 and phospholipase 3. Analysis of phospholipids hydrolysed by these enzymes have shown that all three phospholipases belong to type A₂. Amino acid analysis has shown that phospholipase A₂ (type 1) has 97 residues with a calculated mol.wt. of 10978 ± 11 . Phospholipase A₂ (type 2) has 96 residues with a mol.wt. of 10959 ± 11 . Phospholipase A₂ (type 3) has 266 residues with 16 half-cystine residues and a calculated mol.wt. of 29042 ± 31 . Automated Edman degradation showed the *N*-terminal sequence to be: Asx-Leu-Trp-Glx-Phe-Gly-Glx-Met-Met-Ser-Asx-Val-Met-Arg-Lys-Asx-Val-Val-Phe-Lys-Tyr-Leu- for phospholipase A₂ (type 2).

Phospholipase A₂ (EC 3.1.1.4) catalyses the selective hydrolysis of the 2-acyl groups in *sn*-3-phosphatidyl derivatives (de Haas & van Deenen, 1961). It is a heat-stable widely distributed enzyme playing a central role in lipid metabolism and having an important application in several fields of investigation (Dawson, 1973). The isolation and complete primary-structure determination of phospholipases A₂ have been accomplished by several laboratories from material extracted from pig (de Haas *et al.*, 1970) and horse (Evenberg *et al.*, 1977) pancreas, from bee venom (Shipolini *et al.*, 1971, 1974) and

especially from numerous snake venoms (Botes & Viljoen, 1974; Samejima *et al.*, 1974; Joubert, 1975a,b,c; Eaker, 1975; Halpert & Eaker, 1975; Heinrickson *et al.*, 1977). The present paper describes the isolation and partial characterization of three phospholipases A₂ from the venom of the Mexican snake *Bothrops asper* Garman. An abstract of this work was presented during the meeting of the Mexican Society for Zoology (Alagón *et al.*, 1978).

Materials and Methods

The venom of the snake *B. asper* was collected from adult snakes, captured in San Juan Evan-

Abbreviations used: Bz-Arg-OEt, *N*-benzoyl-L-arginine ethyl ester; Bz-Tyr-OEt, *N*-benzoyl-L-tyrosine ethyl ester; SDS, sodium dodecyl sulphate.

gelista (State of Veracruz, México). The snakes were maintained in a serpentarium at the Instituto Nacional de Higiene, S.S.A., Mexico City, where the crude venom was collected and immediately centrifuged at low speed (1000g), and the supernatant was freeze-dried and stored at -20°C . Hyaluronic acid was from Nutritional Biochemical Co. Bz-Arg-OEt and Bz-Tyr-OEt were from Sigma Chemical Co. Bis-(*p*-nitrophenyl) phosphate and 4-nitrophenyl phosphate were purchased from Aldrich Chemical Co. Sephadex G-75 and DE-32 DEAE-cellulose were from Pharmacia and Whatman Biochemicals respectively. Bio-Gel P-2 was from Bio-Rad Laboratories. Phospholipase assays were done by measuring the clearing of egg-yolk suspension in agarose gel (Habermann & Hardt, 1972) or by a titrimetric procedure with egg yolk as substrate (Shiloah *et al.*, 1973). One unit of phospholipase activity is defined as the amount of enzyme releasing $1\mu\text{mol}$ of acid per minute at pH 8 and 25°C in 3.0 ml of a 10% (w/v) solution of egg yolk in 0.1 M-NaCl. Hyaluronidase activity was measured by a turbidimetric method (Tolksdorf *et al.*, 1949) at 25°C . One unit of activity is defined as that amount of enzyme required to hydrolyse $1\mu\text{g}$ of hyaluronic acid per minute at 25°C in $200\mu\text{l}$ of a $200\mu\text{g}/\text{ml}$ solution of hyaluronic acid in pH 5.3 buffer. Esterase activity was measured spectrophotometrically with benzoyl esters (Bz-Arg-OEt and Bz-Tyr-OEt) as substrates. These assays were carried out at 25°C by adding the enzyme in solution to a cuvette containing 50 mM- CaCl_2 , 1 mM-Bz-Arg-OEt or 0.5 mM-Bz-Tyr-OEt in 40 mM-Tris/HCl buffer (pH 7.8) and measuring the absorbance at 255 nm at time intervals of 30 s for 3–5 min periods in 1.5 ml total volume. One unit of enzymic activity is defined as the amount of protein needed to hydrolyse $1\mu\text{mol}$ of substrate in 1 min in the above conditions.

Phosphodiesterase activity was measured by the method of Russell (1966). One unit of activity was defined as the amount of protein necessary to hydrolyse $1\mu\text{mol}$ of bis-(*p*-nitrophenyl) phosphate in 1 min. Alkaline phosphatase was tested by the technique described in the Worthington Enzyme Manual (1977) and acid phosphatase was measured by the same technique in 20 mM-ammonium acetate buffer (pH 4.7), instead of 1 M-Tris/HCl buffer (pH 8.0). In both assays 4-nitrophenyl phosphate was used as substrate.

Dialysis to remove excess salts after DEAE-cellulose chromatography was conducted against the proper buffer solution in Spectrapor type 3 dialysis tubing (molecular-weight 'cut-off' approx. 3500; Spectrum Medical Industries).

The mouse toxicity of various protein fractions was observed after intraperitoneal injection of different amounts of protein in 0.1–0.3 ml of NaCl or

ammonium acetate buffer into adult 18–20 g albino mice (local Mexican strain). The LD_{50} of the soluble venom was obtained graphically by plotting the percentage of deaths at 20 h versus the logarithm of the dose (ten mice at each dose and twelve different doses of increasing concentration). To define the toxicity of the various chromatographic components, three words were used. 'Lethal' means that the component at the dose injected was enough to kill the tested mouse within 20 h after injection. 'Toxic' means that the mouse shows any of the following symptoms: excitability, depression, anorexia or tissue damage (necrosis). 'Non-toxic' means normal behaviour similar to injection of 0.9% NaCl. Protein concentration, unless otherwise specified, was measured by spectrophotometry assuming that $1 A_{280}^{1\text{cm}}$ unit = $1\text{ mg}/\text{ml}$. Columns were run at room temperature (25°C).

Gel electrophoresis

Discontinuous electrophoresis in polyacrylamide gel (Bio-Rad Laboratories) was performed by the method of Jovin and his co-workers (1964) and the gels were stained with Coomassie Brilliant Blue G-250 (Serva Laboratories; Reisner *et al.*, 1975). SDS/polyacrylamide gels were run in cylindrical tubes containing 12% acrylamide, 0.6% methylene-bisacrylamide and 0.2% SDS (Swank & Munkres, 1971).

When required, phospholipase activity was measured in polyacrylamide gels. Gels were sliced longitudinally, one half stained for protein and the other was cut in 1.5 mm slices and measured for enzymic activity by the method of Habermann & Hardt (1972). The separation of phospholipase A_2 types 2 and 3, as described in the Results section, was obtained by gel electrophoresis by the technique of Jovin *et al.* (1964) in cylindrical gels (5 mm $\times$ 50 mm) made with 7.5% acrylamide in Tris/glycine buffer (pH 8.3). The amount of protein applied was $200\mu\text{g}$ in $200\mu\text{l}$ of sample solution containing Bromophenol Blue as tracking dye. Electrophoresis was carried out at room temperature (2 mA/tube) until the dye reached the bottom of the tube. Phospholipases were separately recovered from gels by cutting the portions with each of the two corresponding protein bands in small pieces, homogenizing with 0.5 M-NaCl in 20 mM-ammonium acetate buffer (pH 4.7) and centrifuging for 20 min. The rotor (Sorvall no. SS-34) was operated at 4°C and $27000g$ (r_{max} 10.8 cm). The supernatant was exhaustively dialysed against water and concentrated by freeze-drying. Before hydrolysis phospholipase A_2 types 2 and 3 were re-chromatographed once in a Bio-Gel P-2 column (0.9 cm $\times$ 40 cm) to remove traces of polyacrylamide and glycine that could interfere with the amino acid analysis.

Phospholipase specificity determination

Escherichia coli strain FT 17 (Taylor & Cronan, 1976) was used as a source of phospholipids (Haest *et al.*, 1969; Silbert, 1970; Cronan & Vagelos, 1972). For each experiment 50 mg of unfractionated phospholipids was dissolved in 2 ml of diethyl ether and added to 1 ml of 20 mM-Tris/HCl buffer (pH 7.5) containing 2.5 mM-CaCl₂, plus a sample of crude venom or purified phospholipase and vortex-mixed for 3 h at 22°C. The diethyl ether was evaporated off under N₂ and the phospholipids were extracted by the method of Bligh and Dyer as described by Christie (1973). The resulting chloroform phase was evaporated under N₂ and spotted on a silica gel G t.l.c. plate (Analtech), which was developed first in light petroleum (b.p. 40–60°C)/diethyl ether/acetic acid (7:3:2, by vol.) for 20 cm, then for 10 cm in chloroform/methanol/acetic acid (5:4:1, by vol.). The lysophospholipid spots were transesterified in 0.5 M-sodium methoxide in methanol and analysed by g.l.c. (Varian, series 2100) (Cronan & Gelmann, 1973).

Amino acid analysis and N-terminal sequence determination

Duplicate samples of protein were prepared for acid hydrolysis in 6 M-HCl with 0.5% phenol added for the protection of tyrosine in individually evacuated and sealed glass tubes. Amino acid analysis was carried out on samples hydrolysed at 110°C for 20, 40, 72 and 120 h for the determination of the amino acid composition, except for half-cystine and tryptophan residues. Half-cystine content of this protein was determined as cysteic acid by the method of Moore (1963). Tryptophan was determined after hydrolysis of the protein in 4.2 M-NaOH as reported by Hugli & Moore (1972) modified for 100 µl volumes. Thiodiglycol (4%) was used instead of soluble starch. The amino acid analysis was carried out on the Durrum D-500 amino acid analyser modified for high sensitivity with the mark II Data Analysis System.

Samples containing 23.8 nmol of phospholipase A₂ type 1 and 29.2 nmol of phospholipase A₂ type 2 were dissolved in anhydrous trifluoroacetic acid and applied independently to the spinning glass cup of a Beckman model 890 C automatic protein sequencer. Automatic Edman degradation, conversion and extraction of the fractions eluted after each cycle of operation was carried out as previously described (Possani *et al.*, 1977). G.l.c. of the phenylthiohydantoin amino acids was carried out both directly and after formation of silyl derivatives from the organic layer. Amino acid analysis was performed on 'back' hydrolysates of portions of both aqueous and organic layers prepared by the method of Smithies *et al.* (1971). Arginine and histidine were

further confirmed by spot test with phenanthron-quinone spray (Yamada & Itano, 1966) or the Pauly reagent.

Results

Characterization of the soluble venom

The venom from *B. asper* was solubilized at 4°C by mixing the freeze-dried venom with 20 mM-ammonium acetate buffer, pH 4.7, and then centrifuged. The rotor (Sorvall no. SS-34) was operated for 10 min at 4°C and 27000 g ($r_{\max.} = 10.8$ cm). The supernatant was termed soluble venom. The soluble venom showed the presence of 15 bands on polyacrylamide-gel electrophoresis (Fig. 1) and gave an LD₅₀ value of 4.3 µg/g mouse wt. The soluble venom contained the following enzymic activities (units, as defined in the Materials and Methods section, are normalized for 100 mg of protein $\pm$ s.d.): hyaluronidase, 3900 $\pm$ 490; phospholipase, 6300 $\pm$ 550; Bz-Arg-OEt hydrolase, 63 $\pm$ 0.2; Bz-Tyr-OEt hydrolase, 8.0 $\pm$ 0.2, phosphodiesterase, 2.5 $\pm$ 0.2. Three independent experiments were performed for each enzymic determination.

Fractionation of the soluble venom

The soluble venom was chromatographed on a column of Sephadex G-75 to yield seven fractions of

Fig. 1. Electrophoresis of soluble venom

Venom (left lane, 100 µg; right lane, 200 µg) was run on polyacrylamide cylindrical gels (5.5 cm; 2 mA) in discontinuous Tris/glycine buffer, pH 8.3 (Jovin *et al.*, 1964) and stained with Coomassie Brilliant Blue G-250. The proteins were run toward the anode. Initially 15 discrete bands were visible; six strong (6, 7, 8, 9, 13 and 15), four moderate (1, 10, 12 and 14) and five weak (2, 3, 4, 5 and 11).

different molecular sizes (Fig. 2). Fractions (I)–(IV) contained at least four enzymic activities and their distribution is shown in Table 1. Phospholipase and hyaluronidase are mainly concentrated in peaks (II) and (III) respectively, Bz-Tyr-OEt hydrolase activity is present in peaks (I) and (III) and Bz-Arg-OEt hydrolase activity is present in all four initial peaks of Sephadex G-75. A qualitative assay for toxicity has shown that fractions (I)–(IV) were lethal using 0.3–0.4 mg of protein in one or two mice weighing 20 g; fraction V was toxic but not lethal and frac-

tions (VI) and (VII) were non-toxic, at the same dose. Fraction (II), which accounted for 17.5% of the absorbance units at 280 nm and contained 88% of the total activity of phospholipase, was re-chromatographed on DEAE-cellulose, equilibrated and eluted with a linear salt gradient in potassium phosphate buffer (pH 7.55) (Fig. 3). Two components (II.6 and II.8) having phospholipase activity were obtained. Component (II.6) appeared to have two slightly separated phospholipase activities. The tubes corresponding to this fraction were pooled, dialysed

Fig. 2. Separation of *B. asper* venom components

The venom (200 mg dry wt.) was dissolved in 1.5 ml of buffer and centrifuged as described in the Materials and Methods section. The supernatant (213.8 mg by absorbance at 280 nm) was applied to a column (0.9 cm × 200 cm) of Sephadex G-75. The column was eluted with the same ammonium acetate buffer at a flow rate of 18 ml/h, and fractions of 30 drops (1.9 ml) were collected and pooled as indicated by the horizontal bars (I)–(VII), on the basis of absorbance at 280 nm and enzymic activities. Blue Dextran, ovalbumin (mol. wt. 45 000), horse heart cytochrome *c* (mol. wt. 12 400) and NaCl were chromatographed separately as molecular-weight markers.

Table 1. Distribution of the enzymic activities in different fractions of Sephadex G-75 chromatography. Results are expressed as percentage recoveries of the total activity present in all fractions.

Fractions . . .	Enzyme activities			
	I	II	III	IV
Phospholipase	7.36	87.94	3.22	1.48
Hyaluronidase	0.00	0.00	95.76	4.24
Bz-Arg-OEt hydrolase	22.56	40.06	33.78	3.60
Bz-Tyr-OEt hydrolase	66.92	8.85	24.23	0.00

and re-chromatographed on a column of DEAE-cellulose (Fig. 4). The column was equilibrated and developed, with a linear gradient of potassium phosphate buffer (pH 8.6), eluting a single major component (II.6.3). On gel disc electrophoresis two

bands were shown to be present in fraction (II.6.3), both having phospholipase activity determined directly on the gels as described in the Materials and Methods section. The faster moving protein was called phospholipase A₂ type 2 and the slower one

Fig. 3. Ion-exchange purification of phospholipases

Fraction (II) from Fig. 2 was adjusted to pH 7.55 by addition of 1.0 M-KOH. This sample (36 ml) containing 45.3 mg of protein was applied to a column (0.9 cm × 30 cm) of DEAE-cellulose. The column was eluted with a linear gradient containing 150 ml each of 15 mM-potassium phosphate (pH 7.55) and 0.2 M-KCl in the same phosphate buffer. The flow rate was 40 ml/h and 2 ml fractions were collected and pooled as shown by the horizontal bars. Components (II.6) and (II.8) were resolved on the basis of the absorbance at 280 nm and phospholipase activity. KCl concentration was experimentally determined in each fraction by conductivity measurement. Abbreviations: L, loading of the material; G, starting the gradient; W, washing with same ion concentration; S, washing with high salt concentration (2 M).

Fig. 4. Rechromatography of the fraction (II.6)

Fractions (II.6) (11.15 mg of protein) was dialysed against the starting buffer and applied to a column (0.65 cm × 42 cm) of DEAE-cellulose. The column was eluted with a linear gradient containing 200 ml each of 15 mM-potassium phosphate (pH 8.6) and 0.2 M-KCl in the same phosphate buffer. The flow rate was 30 ml/h and 2 ml fractions were collected and pooled as shown by the horizontal bar for recovery of component (II.6.3) on the basis of the absorbance at 280 nm and measurement of phospholipase activity. The abbreviations are defined in the legend to Fig. 3.

Table 2. *Summary of purification of phospholipase A₂ types 2 and 3 from B. asper venom*

Fraction	Source of fraction	Protein ($A_{280}^{1\text{cm}}$ units)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Ratio of specific activity to specific activity of soluble venom
Soluble venom	Freeze-dried venom	100.00	6300	63	100.00	1.00
II	Sephadex G-75	17.48	5541	317	87.94	5.03
II.6	DEAE-cellulose (pH 7.55)	2.40	1730	721	27.46	11.44
II.6.3	DEAE-cellulose (pH 8.60)	1.12	1176	1050	18.67	16.67
II.6.3 (phospholipase A ₂ type 2)	Gel electrophoresis	0.46	529	1149	8.40	18.24
II.6.3 (phospholipase A ₂ type 3)	Gel electrophoresis	0.15	112	748	1.78	11.87

Fig. 5. *Purification of phospholipase A₂ type 1*

(a) Fraction (II.8) from Fig. 2 (16.8 mg of protein) was dialysed and applied to a column (0.65 cm × 42 cm) of DEAE-cellulose. The column was developed with a linear gradient containing 200 ml each of 15 mM-potassium phosphate buffer (pH 8.6) and 0.2 M-KCl in the same phosphate buffer. The flow rate was 30 ml/h and 2 ml fractions were collected and pooled as shown by the horizontal bar for phospholipase A₂ type 2 (II.8.4) on the basis of the absorbance at 280 nm and phospholipase activity. (b) Component (II.8.4) was dialysed and re-chromatographed in the same conditions as above. Fractions were pooled as shown by the horizontal bar for phospholipase A₂ type 2 (II.8.4.2) on the basis of the absorbance at 230 nm and constant phospholipase specific activity in the peak. Abbreviations are defined in the legend to Fig. 3.

phospholipase A₂ type 3. Because these two enzymes were difficult to separate by gel filtration on Sephadex G-75 and on DEAE-cellulose ion-exchange chromatography, they were isolated after preparative gel electrophoresis, as also described in the Materials and Methods section. Analytical gels were used for separation, resulting in 55% recovery of enzymic activity. When subjected again to gel disc electrophoresis both phospholipases appeared as single bands (results not shown). Table 2 summarizes the isolation procedures and recoveries involved in the purification of phospholipase A₂ types 2 and 3. The phospholipase activity present in fraction (II.8) (Fig. 3) was further re-chromatographed on a DEAE-cellulose column equilibrated and eluted with a linear salt gradient in potassium phosphate buffer (pH 8.6). The sample was re-chromatographed on the same column (Fig. 5). The component (II.8.4.2) was homogeneous by several criteria (see below) and was called phospholipase A₂ type 1. Table 3 shows a summary of purification of P.1.

Characterization of the three purified phospholipases

We have numbered the purified phospholipases on the basis of their electrophoretic mobilities. The fastest moving protein was called phospholipase A₂ type

1, and the slowest one phospholipase A₂ type 3. Phospholipase A₂ types 1, 2 and 3 correspond probably to bands (15), (14) and (6) respectively in the gel electrophoresis pattern of Fig. 1.

The purity of the three phospholipases was ascertained by gel electrophoresis in the presence and absence of SDS. The apparent molecular weights as calculated from SDS/polyacrylamide gels were approx. 10000 for phospholipase A₂ types 1 and 2 and 27500 for phospholipase A₂ type 3. Phospholipase A₂ type 1 displayed a constant specific activity in fractions of the last chromatographic peak (Fig. 5b), which is a further indication of homogeneity. Table 4 shows the results of the experiment for determining the phospholipase A₂ type of specificity of the three phospholipases. The amino acid composition of the three enzymes was determined as described in the Materials and Methods section. Table 5 shows the calculated amino acid compositions. The *N*-terminal amino acid sequence for phospholipase A₂ types 1 and 2 was determined by automatic Edman degradation with only 25–30 nmol of protein available. For phospholipase A₂ type 1 we obtained the following *N*-terminal sequence: Asx-Leu-(Trp)Glx-Phe-Gly-Glx-Met-X-Ser-X-Val-X-X-Lys-Asx-Val(Val)Phe-. For phospholipase A₂ type 2 the corresponding amino acid sequence obtained was:

Table 3. Summary of purification of phospholipase A₂ type 1 from *B. asper* venom

Fraction	Source of fraction	Protein (A _{280 nm} units)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Ratio of specific activity to specific activity of soluble venom
Soluble venom	Freeze-dried venom	100.00	6300	63	100.00	1.00
II	Sephadex G-75	17.48	5541	317	87.94	5.03
II.8	DEAE-cellulose (pH 7.55)	3.12	1750	561	27.78	8.90
II.8.4	DEAE-cellulose (pH 8.60)	0.81	1038	1281	16.48	20.33
II.8.4.2 (phospholipase A ₂ type 1)	DEAE-cellulose (pH 8.60)	0.44	903	2052	14.33	32.57

Table 4. Fatty acid composition of the phospholipids and their lysophosphatidyl derivatives

Venoms of the rattlesnake *Crotalus adamanteus* (5 mg) and of the cobra *Naja naja kouthia* (5 mg) were used as controls. Phospholipase A₂ types 1, 2 and 3 were assayed with 2.8, 5.6 and 8.0 μg respectively.

	Fatty acid	Fatty acid methyl ester (% by wt.)				
		C ₁₄	C ₁₆	C _{16:1}	C ₁₈	C _{18:1}
Substrate phospholipids	...	3.0	40.1	37.4	1.4	18.0
Lysophosphatidyl derivatives						
<i>C. adamanteus</i>		1.4	86.3	Trace	0.0	12.3
Phospholipase A ₂ type 1		2.2	86.4	Trace	0.0	11.3
Phospholipase A ₂ type 2		2.2	86.8	Trace	0.0	11.0
Phospholipase A ₂ type 3		1.5	85.8	Trace	0.0	12.7
<i>N. naja kouthia</i>		1.0	83.7	Trace	0.0	14.5

Table 5. *Amino acid composition of phospholipases from B. asper venom*

The amino acid composition was calculated on the assumption that the number of Asp, Glu, Ala and Leu residues is respectively: 13, 9, 4 and 2 for phospholipase A₂ type 1; 14, 9, 4 and 2 for phospholipase A₂ type 2; 32, 30, 15 and 15 for phospholipase A₂ type 3. The number on the left on each column corresponds to the actual value (in nmoles of amino acid) used for determination of the nearest integer (odd number on the right), after average of the time course hydrolysis data. Ser and Thr values were extrapolated to zero time and the hydrophobic amino acids to infinite time hydrolysis. Abbreviations: P-1, phospholipase A₂ type 1; P-2, phospholipase A₂ type 2; P-3, phospholipase A₂ type 3.

Amino acid	Amino acid content					
	P-1		P-2		P-3	
	(nmol)	Nearest integer	(nmol)	Nearest integer	(nmol)	Nearest integer
Aspartic acid	12.90	13	13.91	14	32.19	32
Threonine	4.75	5	4.68	5	12.78	13
Serine	5.00	5	3.93	4	22.34	22
Glutamic acid	9.26	9	9.26	9	30.19	30
Proline	4.21	4	3.45	3	15.76	16
Glycine	9.88	10	9.29	9	24.73	25
Alanine	3.81	4	3.98	4	14.98	15
Half-cystine	8.69	9	9.14	9	16.32	16
Valine	6.29	6	6.24	6	16.42	17
Methionine	2.31	2	2.55	3	2.98	3
Isoleucine	3.09	3	2.93	3	13.60	14
Leucine	2.02	2	1.84	2	14.64	15
Tyrosine	6.80	7	7.60	8	6.98	7
Phenylalanine	3.56	4	3.75	4	6.31	6
Histidine	0.99	1	0.65	1	5.71	6
Lysine	6.88	7	7.28	7	14.31	14
Arginine	3.06	3	3.09	3	7.84	8
Tryptophan	3.14	3	2.10	2	6.79	7
Total		97		96		266
Calculated molecular weight		10978 ± 11		10959 ± 11		29042 ± 31

Asx-Leu-Trp-Glx-Phe-Gly-Glx-Met-Met-Ser-Asx-Val-Met-Arg-Lys-Asx-Val-Val-Phe-Lys-Tyr-Leu-

Discussion

The venom of *B. asper*, like most of the *Crotalidae* snake venoms, contains several hydrolytic enzymes, but has no detectable amounts of neurotoxins similar to the *Elapidae* snake venoms (Tu, 1977). The toxicity of *Elapidae* snake venoms (Chang & Lee, 1963) is at least one order of magnitude higher than the LD₅₀ value that we have found for *B. asper*. During purification each fraction obtained from *B. asper* venom is less toxic to mice than the total venom, indicating the presence of synergistic effects (see Fig. 2 and its toxicity in the Results section). The opposite situation occurs with the purification of neurotoxins from *Elapidae* snake venom where the pure toxins of β -type are more potent than the whole venom (Chang & Lee, 1963).

The presence of three phospholipases, four different types of esterases and at least one type of hyaluroni-

dase (see Fig. 2) does not account for all the positive protein bands found in gel disc electrophoresis from the total soluble venom of *B. asper* (Fig. 1). We have also determined the presence of a phosphodiesterase activity in this venom. Tests for acid or alkaline phosphatase activity were negative. The search for direct lytic factor in the venom was also negative, but we have found a positive indirect lytic factor, possibly associated with the phospholipase A₂ type of activity. When the venom was incubated with phosphatidylcholine (Sigma type V-E) for 10 min and then placed in contact with human erythrocytes a strong lytic effect is observed (Sosa *et al.*, 1979).

Peptide 'mapping' of samples obtained from fractions (VI) and (VII) of Fig. 2 have shown the presence of nine to ten different spots positive to ninhydrin (L. Possani & P. Fletcher, unpublished work). The biological significance of these different peptides is still unknown. We have no evidence for the presence of an inhibitor like that reported by Vidal &

Stoppani (1971) for the venom of several snakes of the genus *Bothrops* from South America. The recovery of the enzyme activity as shown in Tables 2 and 3 after the gel permeation on Sephadex G-75 does not indicate the presence of phospholipase inhibitors. In *B. asper* venom we also did not find a lag period for the phospholipase activity, but with the *B. jararaca* venom (a gift from the Instituto Butantan, São Paulo, Brazil), we have confirmed the results of Vidal & Stoppani (1971). In our hands this venom shows a lag period for the phospholipase activity, when measured titrimetrically (A. Alagón, R. Molinar, L. Possani, P. Fletcher, J. Cronan & J. Juliá, unpublished work).

The results shown in the present paper on the general characterization of the venom of the snake *B. asper* indicate that the venom is a complex mixture of different components in which the hydrolytic enzymes apparently predominate. The characterization of the three phospholipases, as described in the Results section, shows that the enzymes were homogeneous by several criteria. The specific activity in fractions corresponding to the last chromatographic peak (Fig. 5b) was constant. Gel disc electrophoresis of the three purified components have shown only one protein band and phospholipase activity was shown to be associated with the positive Coomassie Brilliant Blue band on the gels (results not shown). The recovery of the *N*-terminal amino acid (higher than 97%) was also consistent with the presence of only one protein component for phospholipase A₂ types 1 and 2 subjected to sequence determination studies. The results of the tests for the enzyme specificity as shown in Table 4 indicate that only a phospholipase A₂ type of specificity is associated with the enzymes. The phospholipids from *E. coli* strain FT 17 have more than 95% of unsaturated C_{16:1} fatty acid (palmitoleic acid) in position C-2 of the phospholipid, which makes these phospholipids very suitable material for determination of the type A₂ phospholipase activity. Separation of phospholipase A₂ types 2 and 3 was successfully achieved by disc electrophoresis of small amounts of protein (200 µg). This analytical method was used rather than a preparative one, due to the difficulty in separating the two enzymes by conventional chromatography (gel-permeation and ion-exchange columns). It is possible that phospholipase A₂ type 2 submitted to gel filtration under our experimental conditions behaves as a dimer, as was shown for other snake venoms (Tu, 1977). The final enzyme recovery from the different steps of purification was relatively low for phospholipase A₂ types 2 and 3 (Table 2), mainly because of the selection of special fractions for further purification (see Figs. 2–5). Some enzymic activity was left on side tubes. The recovery obtained in the purification of phospholipase A₂ type 1 was better than the pre-

vious one (Table 3). The starting material (soluble venom) is a mixture of several phospholipases and we do not know the contributions of the individual enzymes to the total phospholipase activity. This makes it difficult to report the purification factors for each phospholipase separately.

SDS/polyacrylamide gels have shown that phospholipase A₂ types 1 and 2 have apparent mol.wts. of 10000, and phospholipase A₂ type 3 has a mol.wt. of approx. 27500. Amino acid analysis of hydrolysates from the pure enzymes have revealed the presence of 97 residues for phospholipase A₂ type 1 and 96 residues for phospholipase A₂ type 2, both with a mol.wt. of 11000, and phospholipase A₂ type 3 has 266 amino acid residues with a mol.wt. of 29000 (Table 5).

On the basis of the amino acid composition (Table 5) phospholipase A₂ type 3 is not a polymeric form of either phospholipase A₂ type 1 or type 2. The three enzymes have a predominance of Glu and Asp residues over the basic amino acid residues, which explains the acidic behaviour of the phospholipases in DEAE-cellulose columns and in gel disc electrophoresis. Phospholipase A₂ types 1 and 2 have very similar amino acid composition, but phospholipase A₂ type 3 has a much higher content of the hydrophobic residues Ile and Leu when proportionally compared with phospholipase A₂ types 1 and 2. The total number of residues in phospholipase A₂ types 1 and 2 is lower than the phospholipases A₂ purified from pig pancreas and other snake venoms (Tu, 1977; Yang, 1978). As reviewed by Tu (1977) more than 40 different phospholipases A₂ have been purified from the venoms of 22 different species of *Hydrophiidae*, *Elapidae*, *Viperidae* and *Crotalidae* snakes. Phospholipase A₂ from the viper *Bitis gabonica* (Botes & Viljoen, 1974) has 118 residues with 12 half-cystine residues. Phospholipase DE-II from the elapid *Naja melanoleuca* (Joubert, 1975a,b,c) has 119 amino acids but 14 half-cystine residues, whereas phospholipase A₂ from the crotalid *Crotalus adamanteus* has 122 amino acid residues with 14 half-cystine residues (Heinrickson *et al.*, 1977). None of these enzymes are very similar in amino acid composition to *B. asper* phospholipase A₂ types 1, 2 and 3, except phospholipase P₄ from *Vipera berus*, which has 91 amino acids with 10 half-cystine residues and phospholipase P₂ from the same venom, which has 259 amino acids with 17 half-cystine residues (Delori, 1973).

A portion of the primary structure of phospholipase A₂ types 1 and 2, beginning at the *N*-terminal, was determined. Insufficient quantities of protein were available for even initial structural determinations of phospholipase A₂ type 3. Native protein was used instead of risking potential losses in cysteine-modification procedures that usually facilitate these

efforts. The *N*-terminal amino acid sequence for both phospholipase A₂ types 1 and 2, so far, seems to be identical (there are still some unknown residues, labelled X, in the Results section). If we compare these sequences with pig pancreas and other snake venom phospholipases A₂, a certain degree of homology is also apparent. The lack of half-cystine residues, so far, in the sequence of phospholipase A₂ types 1 and 2 is a serious problem for homology alignments of the primary structure with other known phospholipases (Yang, 1978). More data on the sequence determination of our phospholipases are required before further conclusions can be drawn.

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