Purification and Characterization of a Phospholipase A₂ from the Venom of the Coral Snake, *Micrurus fulvius microgalbineus* (Brown and Smith)

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A phospholipase A₂ was purified from the Mexican coral snake *Micrurus fulvius microgalbineus* (Brown and Smith). Gel filtration of the soluble crude venom on Sephadex G-50 resolved five fractions, of which fraction II had 98% of the total phospholipase activity. This fraction was rechromatographed on a CM-cellulose column that resolved eight fractions, four of which had an important phospholipase activity. The first fraction (II-1) was homogeneous by polyacrylamide-gel electrophoresis and displayed a phospholipase specific activity of 920 units/mg of protein. The apparent molecular weight as determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was approx. 14000. The amino acid analysis revealed the presence of 119 amino acid residues, with 12 half-cystines. The N-terminal sequence was shown to be Ser-Leu-Leu-Asx-Phe-Lys-Asx-Met-Ile-Glu-Ser-Thr ..., which is homologous with that of phospholipases from other snake venoms.

Phospholipase A₂ (EC 3.1.1.4) catalyses the selective hydrolysis of the 2-acyl groups in 3-*sn*-phosphatidyl derivatives (de Haas & van Deenen, 1961) playing a central role in lipid metabolism, and has been applied in important research in several fields of investigation. The purification and primary-structure determination of phospholipases A₂ have been accomplished by many groups using material extracted from pig (de Haas *et al.*, 1970) and horse (Evenberg *et al.*, 1977) pancreas, from bee venom (Shipolini *et al.*, 1974) and especially from numerous snake venoms (reviewed in Heinrikson *et al.*, 1977).

Phospholipase A₂ has been found in all snake venoms thus far examined (Tu, 1977) and varies from approx. 10000 to about 36000 in molecular weight. The primary sequences determined for several of these phospholipases have extensive structural homology with each other as well as with the same enzyme from mammalian pancreas. Although purified phospholipase A₂ from snake venom does not appear to exert either lethal or toxic effects in live animals, cells incubated in the presence of the venom enzyme undergo radical alterations to the plasmalemma and mitochondria. The morphology of rat liver cells remains intact in the presence of low concentrations of enzyme, but the cell begins to lose intracellular proteins and mitochondrial respiration becomes uncoupled (Gallai-Hatchard & Gray, 1968). Erythrocytes are very slowly haemo-

lysed by purified phospholipase A₂, except when phosphatidylcholine is added as well. This apparently implicates lysophosphatidylcholine as the lytic agent (Roy, 1945).

The apparent ubiquitous presence of phospholipase A₂ in snake venom has interesting implications. Although its pathological effects appear to be indirect from the evidence thus far obtained, its role in the overall toxicity of these venoms is certain. Since the protein is ubiquitous in virtually all snake venoms its primary structure provides valuable information about the evolutionary origin and mutational history of these venomous reptiles.

The present paper describes the isolation and partial characterization of phospholipase A₂ from the venom of the coral snake *Micrurus fulvius microgalbineus* (Brown and Smith), an elapid snake from Mexico (range, State of San Luis Potosi). An abstract of this work was presented during the meeting of the Mexican Society for Zoology (Possani *et al.*, 1978).

Materials and Methods

Only analytical-grade chemicals and solvents were used. The venom was obtained monthly from a single snake (73 cm in length). A 2 ml plastic spoon covered with polyester fabric was taken into the snake's mouth several times in the course of a single collection

and bitten. The venom (approx. 200 μ l) was recovered by washing the spoon and the fabric three times with water, followed by centrifugation. The rotor (Sorvall no. SS-34) was operated for 15 min at 4°C and 18 000g (r_{max} , 10.8 cm). The supernatant was stored immediately at -20°C. Gel-permeation chromatography was conducted on Sephadex G-50 (Pharmacia Fine Chemicals, Uppsala, Sweden). The Sephadex G-50 column (0.9 cm $\times$ 200 cm) was equilibrated and eluted with 20 mM-ammonium acetate buffer, pH 4.7. The phospholipase-positive fraction obtained after gel filtration was rechromatographed on CM-cellulose (CM-32 microgranular cation exchanger; Whatman, Clifton, NJ, U.S.A.). The CM-cellulose column (0.9 cm $\times$ 30 cm) was equilibrated with 20 mM-ammonium acetate buffer, pH 4.7, and the proteins were eluted in the same buffer with a linear gradient (total volume 400 ml) of NaCl from 0 to 0.5 M. Phospholipase activity was measured titrimetrically (Shiloah *et al.*, 1973). One unit of activity was defined as the amount of enzyme that liberated 1 μ mol of unesterified fatty acid/min, with egg-yolk emulsion as substrate. Hyaluronidase activity was measured turbidimetrically as reported by Tolkdorf *et al.* (1949), and proteinase activity was determined as previously described (Possani *et al.*, 1977). Toxicity was tested in albino mice (local strain) by intraperitoneal injections. Usually two to four mice were injected with each fraction and the approximately minimum lethal dose was determined. Specificity was kindly determined by Dr. John Cronan from Yale University, using techniques previously described (Christie, 1973). Polyacrylamide-gel electrophoresis was carried out with cylindrical and slab gels, as described previously (Possani *et al.*, 1977). Amino acid composition was obtained by the timed-hydrolysis method described by Moore (1972), by using duplicate samples hydrolysed under vacuum in a sealed tube with 6 M-HCl containing 0.5% phenol. Half-cystine was converted into cysteic acid before acid hydrolysis (Moore, 1963). Tryptophan was determined by alkaline hydrolysis by the technique of Hugli & Moore (1972), with small modifications: volumes were decreased proportionally and thioglycol (4%, v/v) was used instead of starch. The N-terminal sequence was obtained as previously described (Possani *et al.*, 1977), except that the phospholipase was not reduced and carboxymethylated before sequence determination. All protein values, unless otherwise stated, were determined by spectrophotometry at 280 nm by assuming that 1 $A_{280}^{1\text{cm}}$ unit = 1 mg/ml.

Results and Discussion

The amount of venom obtained in eight extractions gave a mean value of 20.1 (± 1.78 , s.d.) mg of soluble protein per extraction. The venom showed phos-

Fig. 1. Fractionation of soluble venom. Soluble venom (1.7 ml, containing $48.5 A_{280}^{1\text{cm}}$ units) was applied to a Sephadex G-50 column (0.9 cm $\times$ 200 cm) equilibrated and eluted with 20 mM-ammonium acetate buffer (pH 4.7). The column was run at a flow rate of 16 ml/h and fractions (1.8–1.9 ml) were collected. The horizontal bar indicates pooled fractions based on A_{280} and phospholipase activity. Roman numerals (I–V) indicate different pooled fractions. Recovery, toxicity and phospholipase activity data are given in Table 1. Blue Dextran (BD), ovalbumin (Oa), myoglobin (Mb) and bacitracin (Bac) were used as molecular-weight markers and their elution volumes are indicated by vertical arrows.

pholipase and hyaluronidase activity in 2.8 μ g and 1.4 mg samples respectively, with no detectable proteinase activity in a 56 μ g sample. The LD₅₀ value for the crude soluble venom was not determined, but quantities of 50 μ g killed a mouse of 25 g; within 2 h the mouse showed the following main symptoms: progressive paralysis, dyspnoea and respiratory failure. The necropsy showed hyperaemia of the parietal peritoneum, contracted intestine, abnormal pancreas and liver, with hyperaemia of the diaphragm.

Fig. 1 shows the gel-filtration profile of crude soluble venom containing $48.5 A_{280}^{1\text{cm}}$ units. Of the five fractions shown, only number II was lethal to mice (at 50 μ g of protein for a 25 g mouse) and was highly positive for phospholipase activity.

Fig. 2 shows eight fractions after ion-exchange chromatography of fraction II on a CM-cellulose column. Fractions II-1, II-2, II-4 and II-5 were positive for phospholipase activity. The component II-1 was homogeneous by polyacrylamide-gel electro-

Fig. 2. Ion-exchange purification of phospholipase 1

Fraction II from Fig. 1 containing 32.33 $A_{280}^{1\text{cm}}$ units (28 ml) was applied to a CM-cellulose column (0.9 cm $\times$ 30 cm) equilibrated with 20 mM-ammonium acetate buffer, pH 4.7, and eluted in the same buffer with a linear gradient of NaCl from 0 to 0.5 M (200 ml each) at a flow rate of 40 ml/h. Fractions (1.9 ml) were collected and pooled as shown by the horizontal bars to give fractions 1-8 based on the A_{280} . T denotes toxic fractions; L, G and W indicate position of loading the sample, starting gradient and washing with 1 M-NaCl solution in the same running buffer, respectively. Recovery, toxicity and phospholipase data are shown in Table 1.

Table 1. Recovery and toxicity of chromatographic components

For toxicity studies soluble venom or protein fractions were injected intraperitoneally into a 25 g mouse. Numerical values are approx. μg of protein required to kill a test mouse. 'Non-toxic' means normal behaviour similar to injection of 0.9% NaCl, with dose up to 200 μg of protein. The negative values for phospholipase activity were obtained with protein samples up to 15 μg /assay.

Column used	Protein component	Recovery ($A_{280}^{1\text{cm}}$ units)	Toxicity (μg of protein)	Phospholipase ($\mu\text{mol/min}$ per mg of protein)
Sephadex G-50 (Fig. 1)	Soluble venom	48.5	50	187
	Fraction I	4.68	Non-toxic	18
	Fraction II	34.51	50	228
	Fraction III	1.58	Non-toxic	46
	Fraction IV	1.33	Non-toxic	Negative
	Fraction V	2.72	Non-toxic	Negative
	Protein recovery	44.82 (92.4%)		
CM-cellulose (Fig. 2)	Fraction II loaded	32.33	50	228
	Unbound protein	0.27	Non-toxic	Negative
	Phospholipase 1	1.54	Non-toxic	920
	Fraction 2	1.91	Non-toxic	480
	Fraction 3	0.88	Non-toxic	147
	Fraction 4	2.10	50	397
	Fraction 5	9.45	160	227
	Fraction 6	8.68	160	3
	Fraction 7	3.26	50	18
	Fraction 8	0.66	Non-toxic	Negative
	Side tubes*	0.75	Not tested	Not tested
	Protein recovery	29.5 (91.2%)		

* Protein from tubes located in valleys between the fraction peaks.

phoresis in β -alanine/urea/acetate gels (Reisfeld *et al.*, 1962) and also on sodium dodecyl sulphate slab gels (Hubbard & Cohn, 1975) with an acrylamide gradient (10–20%), and was named phospholipase 1.

Fatty acid analysis of phospholipids treated with this enzyme has shown that phospholipase 1 from *M. fulvius microgalbineus* has a phospholipase A₂-type specificity, hydrolysing more than 99% of the ester bond in position 2 of phospholipids obtained from *Escherichia coli* strain FT 17 under our experimental conditions (Christie, 1973). The apparent molecular weight determined by electrophoresis in cylindrical gels containing sodium dodecyl sulphate was of the order of 14000. Table 1 summarizes the recovery and toxicity of components isolated by gel-permeation and ion-exchange chromatography.

Time-course hydrolysis in 6M-HCl (Moore, 1972), basic hydrolysis in 4.2M-NaOH (Hugli & Moore, 1972) and cysteine acid analysis (Moore, 1963) have shown that the amino acid composition of phospholipase 1 is: Asp 17, Thr 8, Ser 8, Glu 12, Pro 5, Gly 9, Ala 6, $\frac{1}{2}$ -Cys 12, Val 6, Met 1, Ile 2, Leu 7, Tyr 5, Phe 4, His 4, Lys 8, Arg 4, Trp 1, making a total of 119 residues with a calculated molecular weight of 13228. The same number of residues but with 14 half-cystines was found in the DE-II phospholipase A₂ from the cobra *Naja melanoleuca* (Joubert, 1975), based on sequence analysis, and phospholipase A₂ from the viper *Bitis gabonica* (Botes & Viljoen, 1974) has 118 amino acids (only 12 half-cystines) and the phospholipase A₂ from the rattlesnake *Crotalus adamanteus* (Heinrikson *et al.*, 1977) has 122 amino acid residues (14 half-cystines), also determined from sequence analysis. At least on the basis of molecular weight and specificity all these phospholipases are very similar. The N-terminal sequence of phospholipase 1 from *M. fulvius microgalbineus* was determined from only 24 nmol of starting material (as quantified by amino acid analysis) and was shown to be: Ser-Leu-Leu-Asx-Phe-Lys-Asx-Met-Ile-Glu-Ser-Thr . . .

Comparison of the N-terminal sequence of this phospholipase with the sequences of other snake phospholipases shows that it is more closely related to group I than to group II proposed by Heinrikson *et al.* (1977). This result would be expected, since *M. fulvius microgalbineus* is an elapid, like *Naja melanoleuca* and *Naja mossambica mossambica* (Mehashe *et al.*, 1976). The group II is represented by Crotalidae and Viperidae snakes having phospholipase sequences that differ more from that of *M. fulvius microgalbineus*. Nevertheless, the lack of half-cystine at position 11 would qualify this phospholipase 1 for group II, or it may represent the first example of a group-III type phospholipase (see Heinrikson *et al.*, 1977).

We hope that the remaining sequence will provide

either definitive proof of structural homology with group II, including exceptions, or further proof of the existence of a group-III type of phospholipase from *Micrurus fulvius microgalbineus*.

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