Isolation and properties of lysophospholipases from the venom of an Australian elapid snake, *Pseudechis australis*

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Two lysophospholipases were isolated from the venom of an Australian elapid snake (subfamily *Acanthophiinae*), *Pseudechis australis*, by sequential chromatography on CM-52 cellulose, Sephadex G-75 and DE-52 cellulose columns. They were very similar to each other. One of them, lysophospholipase I, was obtained as a homodimer, the monomer of which consisted of 123 amino acid residues with seven disulphide bridges. The amino acid composition and the *N*-terminal amino acid sequence of the enzyme were similar to those of phospholipase A₂. Ca²⁺ was required for its activity and the maximum activity was attained at 2 mM-CaCl₂ in the presence of 1 mM-EDTA. The optimum pH was 7.5. Lysophospholipase I hydrolysed lysophosphatidylcholine more rapidly than lysophosphatidylethanolamine. It did not hydrolyse, however, phosphatidylcholine, 1-palmitoylglycerol, tripalmitoylglycerol or *p*-nitrophenyl acetate. Modification of the enzyme with *p*-bromophenacyl bromide or 2-nitrophenylsulphenyl chloride suppressed the activity. A strong direct haemolytic activity was exhibited when the lysophospholipase was present together with phospholipase A₂.

Phospholipase A₂ (EC 3.1.1.4) hydrolyses specifically the fatty acid ester bond at the 2-position of 1,2-diacyl-phospholipid and has been found in various snake venoms.

Doery & Pearson (1964) found phospholipase B activity in the venom of Australian elapid snakes (subfamily *Acanthophiinae*) of the *Pseudechis* group, whereas Shiloah *et al.* (1973a,b) reported that the phospholipase A₂ from the venom of *Vipera palestinae* and *Naja naja* showed lysophospholipase activity at alkaline pH (pH > 10).

The present paper describes the isolation and properties of the lysophospholipases (EC 3.1.1.5) from the venom of an Australian elapid snake, *Pseudechis australis*. The enzyme hydrolyses specifically the *sn*-1-acyl ester bond of lysophospholipid.

Materials and methods

Snake venom

The snakes (*P. australis*) were collected at Townsville, Queensland, Australia. The venom was 'milked', diluted with an equal volume of 0.2 M-acetic acid and brought back to the laboratory.

Abbreviation used: SDS, sodium dodecyl sulphate.

Chemicals

1-Monopalmitoylglycerophosphocholine, 1-monoleoylglycerophosphocholine, 1-monopalmitoylglycerophosphoethanolamine, dipalmitoylglycerophosphocholine, 1-palmitoylglycerol and tripalmitoylglycerol were the products of Serdary Research Laboratories Inc. (Ontario, Canada). Egg-yolk phosphatidylcholine was purified from fresh egg-yolk by the cadmium-complex method (Pangborn, 1951), followed by silica-gel column chromatography. Egg-yolk lysophosphatidylcholine was obtained from egg-yolk phosphatidylcholine (3.50 g) by digestion with phospholipase A₂-I (2 mg; see below) of *Laticauda semifasciata* in a medium consisting of diethyl ether (170 ml), ethanol (10 ml), 0.1 M-Tris/HCl buffer (16 ml), pH 7.5, and 0.1 M-CaCl₂ (4 ml) for 4 h at room temperature. The reaction mixture was evaporated under reduced pressure and the components in the residue were separated by silica-gel column chromatography. The lysophosphatidylcholine fraction was evaporated under reduced pressure. Lysophosphatidylcholine was obtained as a white powder (yield 1.47 g) by the addition of diethyl ether to the residual oil. The molar ratio (with references for methods of analysis in parentheses) of phosphorus (Allen, 1940)/choline (Dittmer & Wells, 1969)/fatty

acid ester (Snyder & Stephens, 1959) in the purified lysophosphatidylcholine was 1:1.03:1.05.

Erabutoxin b and phospholipase A₂-I were obtained from *L. semifasciata* venom as described previously (Tamiya & Arai, 1966; Yoshida *et al.*, 1979). Other standard proteins for molecular-weight determination were from Sigma Chemical Co., St. Louis, MO, U.S.A.

CM-52 CM-cellulose and DE-52 DEAE-cellulose were from Whatman (Maidstone, Kent, U.K.).

Phenyl isothiocyanate, trifluoroacetic acid, dimethylallylamine, benzene and ethyl acetate (all Sequenal grade) were obtained from Wako Pure Chemical Industries, Osaka, Japan. Monoiodoacetic acid was recrystallized from chloroform/light petroleum (b.p. 30–70°C) (1:8, v/v).

Molecular-weight determination

Gel electrophoresis on 15% polyacrylamide gel in the presence of SDS was performed essentially by the method of Weber & Osborn (1969) with a slight modification. The pre-incubation of the protein samples with 1% SDS was carried out in boiling water for 1 h.

Molecular-weight determinations by gel filtration were carried out on a Sephadex G-75 column (1.5 cm × 75 cm) equilibrated with 0.05 M-ammonium acetate or 0.1 M-acetic acid on a Sephadex G-75 column (1.2 cm × 133 cm) equilibrated with 0.05 M-sodium/potassium phosphate buffer, pH 7.0, containing 6 M-guanidine hydrochloride. The standard proteins (molecular weights in parentheses) used were ovalbumin (45 000), α -chymotrypsin (25 000), myoglobin (17 000), phospholipase A₂-I (13 500) and erabutoxin b (6800).

Amino acid analysis

About 30 μ mol of protein samples were hydrolysed in sealed tubes *in vacuo* at 105°C with 6 M-HCl for 24 h or 72 h, or with 4 M-methanesulphonic acid containing 3-(2-aminoethyl)indole (0.2%, w/w; Pierce Chemical Co., Rockford, IL, U.S.A.) for 48 h. The hydrolysates were analysed with an automatic amino acid analyser (JLC-8AH; Jeol, Tokyo, Japan).

Manual Edman degradation

The N-terminal amino acid sequence of reduced and S-carboxymethylated lysophospholipase I was determined by the direct phenylthiohydantoin method (Iwanaga *et al.*, 1969). The ethyl acetate-soluble phenylthiohydantoin derivatives were identified by t.l.c. (Iwanaga *et al.*, 1969) and by high-pressure liquid chromatography (633A model; Hitachi, Tokyo, Japan) with a column of Lichrosorb RP-18 (0.4 cm × 25 cm; Sigma Chemical Co.) and, sometimes, by regeneration of amino acids

from phenylthiohydantoin as described by Mendez & Lai (1975).

Assay of lysophospholipase activity

Lysophospholipase activity was determined by the titrimetric method. Titration was carried out with 10 mM-NaOH by a pH-stat (HSM-10A; Toa Electronics, Tokyo, Japan) at pH 7.5 and 37°C. For each determination, 3.98 ml of the substrate solution containing 16 μ mol of egg-yolk lysophosphatidylcholine, 8 μ mol of CaCl₂ and 4 μ mol of EDTA was used and a suitable amount of the enzyme solution (20 μ l) was added (column eluates were used as such or after dilution with 0.14 M-NaCl). One unit of enzyme activity was defined as the amount of the enzyme that released 1 μ mol of fatty acid/min.

Assay of haemolytic activity

Adult-mouse blood was collected by suction from the tail vein into an equal volume of 0.3 M-sodium citrate buffer, pH 7.4, as an anti-coagulant. After centrifugation for 10 min at 10°C and 450 g and careful removal of the plasma and buffy layer by aspiration, the erythrocytes were resuspended in 20 times their volume of 50 mM-Tris/HCl buffer, pH 7.5, containing 0.14 M-NaCl. The suspension was centrifuged again for 10 min at 10°C and 450 g. The washing process was repeated twice more. The erythrocytes were finally resuspended in 100 vol. of the same buffer containing 0.14 M-NaCl and 10 mM-CaCl₂. Of the enzyme sample solution (column eluate, or after dilution with 0.14 M-NaCl) 50 μ l was added to 1 ml of the fresh erythrocyte suspension and incubated at 37°C for 10 min. The reaction was stopped by the addition of 3 ml of 0.14 M-NaCl containing 5 mM-EDTA and the mixture centrifuged for 10 min at 10°C and 750 g. The A₅₄₀ of the supernatant was measured. The total haemolysis was measured by the addition of water instead of NaCl/EDTA solution. One unit of haemolytic activity was defined as the amount of the sample that caused 50% haemolysis under these conditions.

Chemical modification of lysophospholipase I with p-bromophenacyl bromide

Lysophospholipase I (1.2 mg) was dissolved in 1 ml of 0.1 M-sodium cacodylate buffer, pH 6.0, containing 0.1 M-NaCl, and 50 μ l of acetone containing 500 nmol of p-bromophenacyl bromide was added (Volwerk *et al.*, 1974). The reaction mixture was left for 16 h at 30°C and applied to a column (1.2 cm × 28 cm) of Sephadex G-25 equilibrated with 0.1 M-acetic acid. The protein containing part of the eluate was pooled and freeze-dried.

Chemical modification of lysophospholipase I with 2-nitrophenylsulphenyl chloride

Lysophospholipase I (1.8 mg) was dissolved in

1 ml of 50% (v/v) acetic acid, and 0.1 ml of acetic acid containing 200 μ g of 2-nitrophenylsulphenyl chloride was added (Scoffone *et al.*, 1968). The reaction mixture was left for 1 h at room temperature, and applied to a column (1.2 cm $\times$ 28 cm) of Sephadex G-25 equilibrated with 10% (v/v) acetic acid. The protein-containing part of the eluate was pooled and freeze-dried.

Heat stability

Lysophospholipase I (20 μ g) in 0.1 ml of 25 mM-buffer (sodium citrate, pH 3.0–5.5; sodium/potassium phosphate, pH 4.5–7.5) was treated at 100°C for 10 min in a sealed tube, cooled in an ice-bath for 5 min and assayed for activity.

Results and discussion

Purification of lysophospholipases from the venom of *P. australis*

Freeze-dried *P. australis* venom (600 mg) was dissolved in 20 ml of 0.01 M-sodium/potassium phosphate buffer, pH 6.4, and applied to a column (1.5 cm $\times$ 8 cm) of CM-52 CM-cellulose equilibrated with the same buffer. The unabsorbed-protein-containing part of the eluate was treated in a Diaflo apparatus with a UM-2 filter (Amicon Co., Lexington, MA, U.S.A.) to convert the solvent into 0.1 M-acetic acid and freeze-dried. The residue was dissolved in 10 ml of 0.01 M-sodium/potassium phosphate buffer, pH 5.9, and applied to a CM-52 CM-cellulose column (2.2 cm $\times$ 27.5 cm) equilibrated with the same buffer. Most of the lysophospholipase activity was eluted again without retention. The unabsorbed-protein-containing part of the eluate (fraction I, Table 1) was freeze-dried (yield 35.1 mg). This material was dissolved in 8 ml of 0.05 M-ammonium acetate and gel-filtered through a column (2.6 cm $\times$ 110 cm) of Sephadex G-75 (Fig. 1). The

lysophospholipase-containing part of the eluate (S-II) was pooled, freeze-dried and freeze-dried again after the addition of 10 ml of water (yield 8.4 mg). The residue was dissolved in 4 ml of 0.02 M-Tris/HCl buffer, pH 7.5, and applied to a column (1.5 cm $\times$ 15 cm) of DE-52 DEAE-cellulose equilibrated with the same buffer (Fig. 2). Fractions DE-I, -II, -III and -IV with lysophospholipase activity were pooled separately and freeze-dried. Each residue was dissolved in 0.1 M-acetic acid (2 ml) and desalted by passing through a Sephadex G-25 column (1.2 cm $\times$ 30 cm) in 0.1 M-acetic acid and freeze-dried again.

The preparations from DE-II and DE-III fractions were monodisperse on polyacrylamide-disc-gel

Fig. 1. Gel filtration of fraction I on a Sephadex G-75. Freeze-dried fraction I (35.1 mg) was dissolved in 8 ml of 0.05 M-ammonium acetate and applied to a column (2.6 cm $\times$ 110 cm) of Sephadex G-75 equilibrated with the same solvent. The flow rate was 28 ml/h; 8.0 ml fractions were collected. The protein fractions (S-I–S-III), indicated by the bars, were collected separately. The shaded part (S-II) possessed the haemolytic activity. O, A_{280} ; ●, lysophospholipase activity.

Table 1. Purification of lysophospholipases from *P. australis* venom

Fraction	Total protein (mg)	Lysophospholipase recovery activity		Specific activity (units/mg)	Purification (-fold)	Haemolytic activity (haemolytic units)
		(units)	(%)			
Crude venom	600	709	100	1.18	1	21 000
CM-52, pH 6.4 (unabsorbed fraction)	145	658	93	4.54	3.8	18 000
CM-52, pH 5.9 (fraction I)	35.1	534	75	15.2	13	11 000
Sephadex G-75 (S-II)	8.40	470	66	56.0	47	630
DE-52						
DE-II (lysophospholipase I)	3.96	230	32	58.1	49	80
DE-III (lysophospholipase II)	1.74	108	15	62.1	53	N.D.*

* Not determined.

Fig. 2. DE-52 DEAE-cellulose column chromatography of S-II

Freeze-dried S-II (8.4 mg) was dissolved in 4 ml of 0.02 M-Tris/HCl buffer, pH 7.5, and applied to a column (1.5 cm $\times$ 15 cm) of DE-52 cellulose equilibrated with the same buffer. The elution was performed with the same buffer (60 ml), followed by a linear concentration gradient elution from 0 to 0.1 M-NaCl in the same buffer in a total volume of 600 ml, and then with 0.5 M-NaCl in the same buffer (arrow). The flow rate was 85 ml/h; 5.1 ml fractions were collected. The protein fractions (DE-I–DE-IV), indicated by the bars, were collected separately. — — —, [NaCl]. ●, Lysophospholipase activity; ○, A_{280} .

electrophoresis in 7.5% acrylamide gel at pH 8.9 and termed lysophospholipases 'I' and 'II' respectively.

By the above purification procedure, lysophospholipases I and II were purified 50-fold with overall yields of 32% and 15% respectively as summarized in Table 1. The haemolytic activity of the preparation (S-II) was decreased markedly by the gel-filtration step.

Molecular-weight determination

The molecular weight was determined by analytical gel filtration and by SDS/polyacrylamide-gel electrophoresis. At neutral and acidic pH, purified lysophospholipase I was eluted from the Sephadex G-75 column as a single symmetrical peak with a distribution coefficient (K_D) value of 0.32. Comparison of the elution volume with those of standard proteins gave a mol.wt. of 26 500 for lysophospholipase I. On the other hand, lysophospholipase I was eluted from the Sephadex G-75 column in 6 M-guanidine hydrochloride with a K_D value of 0.20, from which a mol.wt. of 13 900 was calculated. Lysophospholipase I migrated as a single band on SDS/polyacrylamide-gel electrophoresis, giving a mobility value corresponding to a mol.wt. of 13 600.

These results suggest that lysophospholipase I is a homodimer in neutral or acidic solution and dissociates into subunits in 6 M-guanidine hydrochloride or SDS solution.

The molecular weights of phospholipases A_2 from snake venoms are either 13 500 [for example, *Agkistrodon halys blomhoffii* A.I. and A.II (Kawauchi *et al.*, 1971), *Notechis scutatus scutatus* notexin (Karlsson *et al.*, 1972), *L. semifasciata* I, III and IV (Yoshida *et al.*, 1979)] or 28 000 [for example, *Crotalus adamanteus* α and β (Wells &

Hanahan, 1969), *Trimeresurus flavoviridis* PLA (Ishimaru *et al.*, 1980)] suggesting dimerization in some cases.

Amino acid composition

The amino acid composition computed for the molecular weight of the subunit (13 600) is presented in Table 2. The protein is characterized by high contents of aspartic acid, glycine, alanine, tyrosine and lysine. It also contains 14 half-cysteine residues. The whole composition is very similar to those of phospholipases A_2 .

Lysophospholipase II gave the same amino acid composition as lysophospholipase I.

N-terminal amino acid sequence

The N-terminal amino acid sequence of lysophospholipase I, determined by 12 cycles of Edman degradation on reduced and S-carboxymethylated derivative, is compared in Fig. 3 with those of phospholipases A_2 from snake venoms and porcine pancreas. The sequence homology is evident. The sequence of lysophospholipase I is probably the same as that of 'P.a.-11', a phospholipase A_2 obtained from the same venom (M. Terashima, S. Nishida, C. Takasaki & N. Tamiya, unpublished work).

Time course of lysophosphatidylcholine hydrolysis with lysophospholipase I

The time course of the reaction is shown in Fig. 4. After a lag period, the reaction proceeded at constant rate. The reaction velocity was calculated from the slope of the linear part. The addition of 0.2 mM-palmitate, which is the product of the

Table 2. Amino acid composition of lysophospholipase I

	Composition (residues/subunit)			Nearest integer
	(a)	(b)	(c)	
Lys	8.94	8.80	8.96	9
His	1.93	1.86	1.95	2
Arg	3.98	3.85	4.01	4
Asp	18.06	18.30	18.05	18
Thr	6.87	6.74	6.62	7
Ser	2.94	2.53	3.02	3
Glu	9.83	10.10	10.77	10
Pro	4.68	5.47	4.96	5
Gly	11.93	11.78	11.46	12
Ala	10.74	11.25	11.77	11
½Cys*	13.22	11.80	12.76	14
Val	2.76	3.28	2.37	3
Met	1.90	1.77	2.18	2
Ile	4.29	4.81	3.70	5
Leu	4.55	5.07	4.48	5
Tyr	7.73	7.44	7.79	8
Phe	4.19	4.05	3.89	4
Trp	—	—	0.72	1
Total				123

(a) Hydrolysed with 6 M-HCl for 24 h.

(b) Hydrolysed with 6 M-HCl for 72 h.

(c) Hydrolysed with 4 M-methanesulphonic acid for 48 h.

* Cystine tends to give a lower value owing to destruction during hydrolysis.

- (1) Asn-Leu-Ile-Gln-Phe-Gly-Asx-Met-Ile-Gln-Cys-Ala-⁵
- (2) Asn-Leu-Ile-Gln-Phe-Gly-Asn-Met-Ile-Gln-Cys-Ala-¹⁰
- (3) Asn-Leu-Val-Gln-Phe-Ser-Tyr-Leu-Ile-Gln-Cys-Ala-
- (4) Asn-Leu-Val-Gln-Phe-Ser-Asn-Val-Ile-Gln-Cys-Asn-
- (5) Asn-Leu-Tyr-Gln-Phe-Lys-Asn-Met-Ile-His-Cys-Thr-
- (6) Ala-Leu-Trp-Gln-Phe-Arg-Ser-Met-Ile-Lys-Cys-Ala-

Fig. 3. The N-terminal amino acid sequences of lysophospholipase I and phospholipases A₂

The sources for the sequence data are as follows: (1) *Pseudechis australis* lysophospholipase I, the present experiment; (2) *Pseudechis australis* P.a.-11 (M. Terashima, S. Nishida, C. Takasaki & N. Tamiya, unpublished work); (3) *Notechis scutatus* notexin (Halpert & Eaker, 1975); (4) *Laticauda semifasciata* phospholipase A₂-I (S. Nishida & N. Tamiya, unpublished work); (5) *Naja melanoleuca* DE-I (Joubert, 1975); (6) porcine pancreas phospholipase A₂ (Puyk *et al.*, 1977).

reaction, did not affect the reaction velocity, although it shortened the lag period. Caprylic acid (2 mM) did not affect the reaction.

Fig. 4. Time course of lysophosphatidylcholine hydrolysis with lysophospholipase I

The reaction was carried out with egg-yolk lysophosphatidylcholine as substrate at pH 7.5 and 37°C. *v*, reaction velocity; *t*, lag period.

Effect of pH or Ca²⁺ concentration on the lysophospholipase activity

By using egg-yolk lysophosphatidylcholine as substrate it was shown that the enzyme activity was maximal at pH 7.5. Ca²⁺ was required and the maximum activity was attained with 2 mM-CaCl₂ in the presence of 1 mM-EDTA. All the phospholipases A₂ of snake venoms and mammalian pancreas show the maximum activity at slightly alkaline pH and require the presence of Ca²⁺ for activity.

Substrate specificity

As shown in Table 3, lysophospholipase I hydrolysed 1-mono-oleoylglycerophosphocholine, as well as 1-monopalmitoylglycerophosphocholine, indicating that the enzyme did not distinguish saturated- and unsaturated-fatty acid moiety at the 1-position of lysophosphatidylcholine. Lysophosphatidylethanolamine was tested for its susceptibility to the enzyme action in the presence of a 2-fold molar excess of Triton X-100, which helped its solubilization. The enzyme hydrolysed lysophosphatidylethanolamine very slowly, indicating that it discriminated the amine part of the lysophospholipid.

Lysophospholipase I (40 μg, 2.4 units) was tested for phospholipase B activity at pH 7.5 against 4 mM egg-yolk phosphatidylcholine or 4 mM-di-palmitoylglycerophosphocholine in the absence or presence of a 2-fold molar excess of Triton X-100

Table 3. *Substrate specificity of lysophospholipase I*

Substrate	Apparent K_m (mM)	V_{max} (μ mol/min per mg)
Egg-yolk lysophosphatidylcholine	0.157	61.8
Lysophosphatidylcholine/Triton X-100 (1:2, molar ratio)	0.165	19.5
1-Monopalmitoylglycerophosphocholine	0.153	58.2
1-Mono-oleoylglycerophosphocholine	0.354	79.4
1-Monopalmitoylglycerophosphoethanolamine/Triton X-100 (1:2)	0.251	1.06

Fig. 5. Heat stability of lysophospholipase I. Lysophospholipase I in buffer solution was heated as described in the text. The control experiment was performed in the same manner without heating. O, Citrate buffer; ●, phosphate buffer.

Table 4. *Effect of various reagents on lysophospholipase activity*

The control experiment was performed in the same manner without the addition.

Addition	Concentration	Relative activity (%)
None	—	100
Sodium palmitate*	0.2 mM	97
HgCl ₂	2.0 mM	0
CuCl ₂	2.0 mM	0
FeSO ₄	2.0 mM	102
FeCl ₃	2.0 mM	96
Triton X-100	0.1%	41
	0.25%	21
PCMB†	(a)	103
PMSF†	(b)	94

* Sodium palmitate (0.1 ml; 8 mM) in 50% propanol (v/v) was added to the reaction mixture.

† The purified enzyme (140 μ g) was incubated previously: (a) with 0.2 ml of 10 mM-*p*-chloromercuribenzoate (PCMB) in 5 mM-phosphate buffer (pH 6.8)/5% ethanol at 25°C for 1 h; (b) with 0.4 ml of 2.5 mM-phenylmethanesulphonyl fluoride (PMSF) in 5% propanol/dilute KOH (pH 8.0) at 25°C for 1 h.

and in the presence of 2 mM-CaCl₂ and 1 mM-EDTA. No fatty acid was released within 20 min. It differs, therefore, from phospholipase B, which hydrolyses both lysophospholipid and phospholipid. Lysophospholipase I hydrolysed neither *p*-nitrophenyl acetate, 1-palmitoylglycerol nor tripalmitoylglycerol. It is concluded, therefore, that lysophospholipase I is a specific lysophospholipase without phospholipase A, phospholipase B, carboxyesterase or lipase activity.

The presence of phospholipases A₂ in the snake venoms has been well established. Doery & Pearson (1964) reported the presence of phospholipase B in the venoms of the *Pseudechis* group. Shiloah and his co-workers reported that the purified phospholipase A₂ of *V. palestinae* (Shiloah *et al.*, 1973a) and *N. naja* (Shiloah *et al.*, 1973b) had lysophospholipase activity at alkaline pH (relative activity to phospholipase A₂ was 0.2%). On the other hand, lysophospholipase I has its optimum pH at 7.5 and has a much higher specific activity (about 60 units/mg) than the enzymes studied by Shiloah and his co-workers.

Heat stability

Lysophospholipase I was heated at 100°C for 10 min in various buffer solutions and found to be stable in the pH range 5–6 (Fig. 5). Phospholipases A₂ are also heat-stable in an acidic medium (Condrea & de Vries, 1965).

Effects of some reagents on the enzyme activity

Lysophospholipase I was completely inhibited by the addition of 2 mM-HgCl₂ or 2 mM-CuCl₂ (Table 4). The enzyme was not affected with 2 mM-FeSO₄ and 2 mM-FeCl₃, which are the inhibitors for lysophospholipases of *Escherichia coli* (Doi & Nojima, 1975) and *Vibrio parahaemolyticus* (Misaki & Matsumoto, 1978). The addition of Triton X-100 was also inhibitory.

Lysophospholipase I (mol.wt. 25 000) and II (mol.wt. 60 000) of beef liver (de Jong *et al.*, 1974) are inactivated with *p*-chloromercuribenzoate, and lysophospholipases of *E. coli* [mol.wt. 39 500 (Doi & Nojima, 1975)] and *V. parahaemolyticus* [mol.wt. 89 000 (Misaki & Matsumoto, 1978)] are inactivated by di-isopropyl fluorophosphate. These four enzymes do not require Ca²⁺ for their activities. On

Table 5. Some properties of native and *p*-bromophenacylated lysophospholipase I

	Native	Modified
Number of histidine residues*/subunit	1.93	0.94
ϵ_{270}	22 000	42 000
PBP† residues/subunit‡	0	1.15
Lysophospholipase activity (units/mg)	58.1	0.35
Haemolytic activity (units/mg)	20	<0.5

* Measured by amino acid analysis.

† PBP, *p*-bromophenacyl.

‡ ϵ_{270} (PBP-conjugated) = 17 000 M·cm⁻¹ (Halpert *et al.*, 1976).

Table 6. Some properties of native and 2-nitrophenylsulphenylated lysophospholipase I

	Native	Modified
Number of tryptophan residues*/subunit	0.72	<0.1
ϵ_{365}	0	3820
Number of NPS† residues/subunit‡	0	0.96
Lysophospholipase activity		
K_m (mM)	0.157	0.170
V_{max} (μmol/min per mg)	61.8	5.13

* Measured by amino acid analysis.

† NPS, 2-nitrophenylsulphenyl residue.

‡ ϵ_{365} (NPS-conjugated) = 4000 M·cm⁻¹ (Scoffone *et al.*, 1968).

Fig. 6. Effect of phospholipase A₂ amount on haemolysis. Lysophospholipase I (5 μg) was mixed with various amounts of phospholipase A₂-I of *L. semifasciata* and the mixture tested for haemolytic activity by incubation with erythrocyte solution at 37°C for 30 min.

the other hand, lysophospholipase I of *P. australis* was not affected by the addition of *p*-chloromercuribenzoate or of phenylmethanesulphonyl fluoride, which is known to react with the active-site serine residue of serine-enzymes, similarly to diisopropyl fluorophosphate. Thus lysophospholipase I of *P. australis* differs from the other reported lysophospholipases.

Chemical modifications of lysophospholipase I

When lysophospholipase I was treated with a 6-fold molar excess of *p*-bromophenacyl bromide, one histidine residue/subunit was modified and the activity was destroyed. The weak haemolytic activity of the enzyme was also destroyed by the modification (Table 5).

All the phospholipases A₂ of snake venoms and mammalian pancreas lose their activity on chemical modification with *p*-bromophenacyl bromide, which is known to react with the histidine residue found at the corresponding positions of all the enzymes [for example, porcine pancreatic phospholipase A₂ (Volwerk *et al.*, 1974)]. Dijkstra *et al.* (1979) proposed an active-site model for beef pancreatic phospholipase A₂, in which His-48, Asp-99, Ca²⁺, water molecules and some other residues participate in the catalysis of phospholipid hydrolysis. Lysophospholipase I also requires Ca²⁺ for its activity and is inactivated by *p*-bromophenacyl bromide treatment,

suggesting a similar mechanism in its catalytic activity. All these results suggest that lysophospholipase I is very similar to phospholipases A₂ in its molecular structure and catalytic mechanism. It is different, however, from phospholipase A₂ in its substrate specificity.

The modification of the single tryptophan residue of lysophospholipase I with 2-nitrophenylsulphenyl chloride reduced the $V_{\max}$ value without affecting its K_m value (Table 6). The remaining activity may be due to the unmodified enzyme.

Co-operative haemolysis with lysophospholipase and phospholipase A₂

Lysophospholipase I (5 µg) was mixed with various amounts of phospholipase A₂-I of *L. semifasciata*, which had no haemolytic activity by itself. As shown in Fig. 6, a strong haemolytic activity was observed with the mixture. When either one of the component enzymes was modified with *p*-bromophenacyl bromide, the haemolytic activity was not observed (results not shown).

Snake venoms lyse red cells *in vitro* 'indirectly', i.e. in the presence of phosphatidylcholine, whereas only some of them are lytic to washed red cells 'directly', i.e. without any addition. In the cases of cobra venoms, direct lytic factor (termed 'DLF', cardiotoxin or cytotoxin) and phospholipase A₂ are the components that cause haemolysis (see, for example, Condrea, 1974).

Doery & Pearson (1961) reported another type of direct lytic component, termed 'haemolysin', in the venoms of Australian elapid snakes, especially of the *Pseudechis* group, but they failed to identify haemolysin as a single component.

Present results show that the purified lysophospholipase I with only a faint haemolytic activity (see Table 1) recovered the activity on the addition of purified phospholipase A₂-I of *L. semifasciata*. In addition, lysophospholipase fraction from the *P. australis* venom (S-II, Fig. 2) displayed strong haemolytic activity when mixed with the phospholipase A₂ fraction (S-III, Fig. 3) from the same venom (results not shown). The haemolysis caused with *P. australis* venom seems to be due to the synergetic action of lysophospholipase and phospholipase A₂.

Direct lytic factors of snake venoms of subfamily *Elapinae* are strongly basic low-molecular-weight proteins with high contents of hydrophobic amino acids (Lee, 1972). On the other hand, lysophospholipase I described in the present paper and phospholipase A₂-I of *L. semifasciata* are not basic proteins. The mechanism of haemolysis observed with *Pseudechis* venom must be different from that of cobra venoms.

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