Isolation, properties and amino acid sequences of a phospholipase A₂ and its homologue without activity from the venom of a sea snake, *Laticauda colubrina*, from the Solomon Islands

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A phospholipase A₂, *Laticauda colubrina* phospholipase A₂ II (LcPLA-II), and a phospholipase A₂ homologue, *Laticauda colubrina* phospholipase A₂ homologue I (LcPLH-I), were isolated from the venom of the yellow-lipped sea snake, *Laticauda colubrina*, from the Solomon Islands. LcPLA-II showed phospholipase A₂ activity towards egg-yolk phosphatidylcholine (24 µmol/min per mg at optimal conditions at 37 °C) and lethal potency (LD₅₀ 45 µg/kg body wt. intravenously in mice). Both of the activities were lost by treatment with *p*-bromophenacyl bromide. LcPLH-I showed neither phospholipase A₂ activity nor lethal potency at a dose of 4.5 mg/kg body wt. in mice. It was not modified by the treatment with *p*-bromophenacyl bromide. LcPLA-II and LcPLH-I bound Ca²⁺ at a 1:1 molar ratio with *K*_{Ca} values of 105 µM and 44 µM at pH 8.0 respectively. Elucidation of the amino acid sequences of these two proteins showed that each protein consisted of a single chain of 118 amino acid residues, including 14 half-cystine residues. The two sequences are different from each other at 22 residues and highly homologous to those from other sources. The essential histidine residue for the phospholipase A₂ activity at position 48 is replaced by an asparagine residue in the homologue LcPLH-I. Details of the separation of the peptides obtained by proteinase digestions of LcPLA-II and LcPLA-I and the determination of their amino acid sequences are given in Supplementary Publication SUP 50145 (14 pages), which has been deposited at the British Library Lending Division, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in Biochem. J. (1988) 249, 5.

INTRODUCTION

A number of phospholipases A₂ (EC 3.1.1.4) have been purified, and their amino acid sequences determined, from the venoms of various snakes, including those belonging to the three families Elapidae, Colubridae and Viperidae (see reviews by Verheij *et al.*, 1981). Furthermore, a non-neurotoxic non-enzymic phospholipase A₂ homologue, *Notechis* II-1, was isolated from an Australian elapid snake, *Notechis scutatus* (Halpert & Eaker, 1976), and its amino acid sequence determined (Lind & Eaker, 1980). From a member of the sea kraits, *Laticauda semifasciata*, three non-neurotoxic phospholipases A₂, LsPLA I, LsPLA III and LsPLA IV, were isolated (Yoshida *et al.*, 1979) and the amino acid sequences determined (Nishida *et al.*, 1982).

The yellow-lipped sea snake, *Laticauda colubrina*, (subfamily Laticaudinae, family Elapidae), is widely distributed over a region including the Bay of Bengal, Indo-Malaysia, New Guinea, southern Japan and South Pacific islands (Smith, 1926). The venom composition of *L. colubrina* varies according to the locality of the collection, and phospholipase A₂ (LcPLA-II, see below) was detected so far only in the venom of *L. colubrina* from the Solomon Islands (Kim & Tamiya, 1982).

The present paper describes the isolation, properties and the complete amino acid sequences of a

phospholipase A₂ and its homologue from the venom of *L. colubrina* from the Solomon Islands.

MATERIALS AND METHODS

Snake venom

The yellow-lipped sea snakes (*L. colubrina*) were collected in Lake Te-Nggano of Rennell Island, the Solomon Islands, in December 1980 (four specimens) and November 1981 (23 specimens). The venom was milked from the snakes, diluted with an equal volume of 0.2 M-acetic acid, brought back to the laboratory and freeze-dried.

Materials

Phospholipases A₂ LsPLA I, LsPLA III and LsPLA IV from *Laticauda semifasciata* venom were purified as described previously (Yoshida *et al.*, 1979). Radioactive ⁴⁵CaCl₂ (specified radioactivity 1.06 Ci/mmol) was obtained from Amersham International (Amersham, Bucks., U.K.). Trypsin (twice crystallized) and chymotrypsin (three-times crystallized) were purchased from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.). Lysyl-endopeptidase (EC 3.4.14.50) from *Achromobacter lyticus*, egg-yolk phosphatidylcholine and *p*-bromophenacyl bromide were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Abbreviations used: LcPLA-II, *Laticauda colubrina* phospholipase A₂ II; LcPLH-I, *Laticauda colubrina* phospholipase A₂ homologue I.

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M_r determination

M_r determination by gel filtration was carried out with the Pharmacia f.p.l.c. system equipped with a column (1 cm × 30 cm) of Superose 12 (Pharmacia Fine Chemicals, Uppsala, Sweden) as described previously (Takasaki & Tamiya, 1985).

Chemical modification of phospholipase A_2 and its homologue with *p*-bromophenacyl bromide

Each protein (1.2 mg for LcPLA-II, 0.15 mg for LcPLH-I) was dissolved in 1.0 ml of 0.1 M-Tris/HCl buffer, pH 8.0, containing 0.1 M-NaCl and 5 mM-EDTA. To this solution was added 0.1 ml of 0.1 M-*p*-bromophenacyl bromide solution in acetone. The reaction mixture was left for 20 h at 37 °C and applied to a column (1.2 cm × 30 cm) of Sephadex G-25 equilibrated with 0.1 M-acetic acid. The protein-containing fractions were pooled and freeze-dried.

Measurement of Ca^{2+} binding

Measurements of Ca^{2+} binding to protein were performed in accordance with a modification of the equilibrium gel-filtration technique described by Pieterse *et al.* (1974). A column (1.0 cm × 22 cm) of Sephadex G-50 (fine grade) was equilibrated with 50 mM-Tris/HCl buffer, pH 8.0, containing 0.1 M-NaCl and a known concentration of $^{45}CaCl_2$ ($4 \times 10^4 - 8 \times 10^4$ d.p.m./ml). A 0.2 ml portion of metal-ion-free protein (approx. 1 mg) in the Ca^{2+} -containing buffer solution was applied to the column and eluted with the same buffer. Radioactivity in the eluate was determined with a liquid-scintillation counter (model LSC-700; Aloka, Tokyo, Japan). The protein concentration of the eluate was calculated from the absorbance at 280 nm by using $A_{280}^{1\%}$ 11.0 and 8.9 for LcPLA-II and LcPLH-I respectively. The data were treated in accordance with the method of Fletcher *et al.* (1970) for the Scatchard (1949) model of ligand-protein interaction. For a single class of binding sites the following equation holds, where ν_{Ca} is the saturation degree, n the number of binding sites/mol, K_{Ca} the average dissociation constant of the protein- Ca^{2+} complex and $[Ca]$ the free Ca^{2+} concentration:

$$\nu_{Ca}/[Ca] = (n/K_{Ca}) - (\nu_{Ca}/K_{Ca})$$

Thus a plot of $\nu_{Ca}/[Ca]$ versus ν_{Ca} yields a straight line from which the value of n can be derived by extrapolation and the slope corresponds to $1/K_{Ca}$. For a single gel-filtration experiment as shown in Fig. 2, the saturation degree ν_{Ca} can be derived from the individual fractions or from the total peak according to:

$$\nu_{Ca} = [\text{protein}]_{\text{bound}}/[\text{protein}]_{\text{total}}$$

Amino acid analysis and Edman degradation

Amino acid analysis and the manual Edman degradation were carried out as described previously (Takasaki & Tamiya, 1982). Automated Edman degradation with a gas-phase sequencer (model 470A; Applied Biosystems, Foster, CA, U.S.A.) was carried out essentially as described by Hewick *et al.* (1981).

Oxidation of LcPLA-II and LcPLH-I with performic acid

LcPLA-II (75.1 mg) and LcPLH-I (28.0 mg) were separately oxidized with performic acid as described by Hirs (1956). After 7-fold dilution the reaction mixtures were freeze-dried.

RESULTS

Isolation of phospholipase A_2 and its homologue

The preliminary fractionation *L. colubrina* (Solomon Islands)-venom components by CM-cellulose column chromatography was described previously (Kim & Tamiya, 1982). Desalted fraction B (226 total A_{280} units) obtained from the freeze-dried venom (851 mg from 27 specimens) was applied to a column (2.2 cm × 23 cm) of CM-cellulose (Whatman CM-52) equilibrated with 0.01 M-sodium phosphate buffer, pH 5.9 (Supplementary Publication SUP 50145 Fig. 1). Fractions B-I and B-II, which were eluted at NaCl concentrations of 0.16 M and 0.18 M respectively, were pooled separately and freeze-dried. Each residue was dissolved in 0.1 M-acetic acid (15 ml) and desalted by passing through a Sephadex G-25 column (2.6 cm × 42 cm) in 0.1 M-acetic acid and freeze-dried again (yields: component B-I, 104.7 mg; component B-II, 80.5 mg). Each component was monodisperse on polyacrylamide-disc-gel electrophoresis in 7.5% acrylamide gel at pH 4.3.

Amino acid composition

The amino acid compositions of the proteins are given in Table 1. Each protein consisted of 118 amino acid residues. The true numbers of half-cystine residues and tyrosine residues in each protein were obtained from the sequence study.

Phospholipase A_2 activity

The preparation from fraction B-II showed in its enzymic reaction a lag period that disappeared on the addition of 1.4 mM-sodium oleate at a substrate concentration of 4 mM. The enzyme activity was maximal at pH 8.2. Ca^{2+} was required and the maximal activity was attained with 3 mM- $CaCl_2$ in the presence of 0.7 mM-EDTA. The specific activity of the preparation from fraction B-II measured by a titrimetric method (Yoshida *et al.*, 1979) was 24 $\mu\text{mol}/\text{min}$ per mg with egg-yolk phosphatidylcholine (4 mM) as a substrate at 37 °C in the presence of 8 mM-Triton X-100, 3 mM- $CaCl_2$, 0.7 mM-EDTA and 1.4 mM-sodium oleate at pH 8.2 (Table 2). On the other hand, the preparation from fraction B-I showed no phospholipase A_2 activity in the presence or in the absence of 1.4 mM-sodium oleate and in the presence of 3 mM- or 30 mM- $CaCl_2$. The components in fractions B-II and B-I are named *Laticauda colubrina* phospholipase A_2 II (LcPLA-II) and *Laticauda colubrina* phospholipase A_2 homologue I (LcPLH-I) respectively.

Lethal potency

The lethal potency (LD_{50}) of LcPLA-II by intravenous injection in mice (male, strain ddY, 19–21 g) was 45 $\mu\text{g}/\text{kg}$ body wt. LcPLH-I did not show lethal activity at a dose of 4.5 mg/kg body wt.

Modification of protein with *p*-bromophenacyl bromide

Treatment of LcPLA-II with *p*-bromophenacyl bromide resulted in loss of both enzymic activity and lethal potency (Table 2). Amino acid analysis showed that LcPLA-II lost the single histidine residue whereas no other amino acid residues were affected. On the other hand the single histidine residue of LcPLH-I was not modified under the same conditions.

Table 1. Amino acid compositions of LcPLA-II and LcPLH-I

The compositions of the proteins are given as the molecular proportions of amino acid residues determined.

Amino acid	Amino acid composition (mol/mol of protein)			
	LcPLA-II		LcPLH-I	
	Found	Nearest integer	Found	Nearest integer
Asp*	16.8	17	18.6	19
Thr	6.7	7	6.7	7
Ser	3.9	4	4.7	5
Glu*	7.3	7	7.1	7
Pro	5.2	5	7.5	7
Gly	10.6	11	11.9	12
Ala*	5.8	6	5.2	5
Cys†	12.2	14	12.4	14
Val	2.3	2	1.8	2
Met	1.1	1	1.2	1
Ile	5.5	6	2.7	3
Leu*	4.9	5	7.2	7
Tyr†	7.9	9	5.2	6
Phe	7.5	8	6.0	6
His	1.2	1	1.3	1
Lys	9.3	9	12.4	12
Arg	5.8	6	4.1	4
Trp‡	—	0	—	0
Total		118		118
Formula M_r		13465		13038
M_r (found)		12400		12800
ϵ_{280} ($M^{-1} \cdot cm^{-1}$)		14800		11600

* The amino acid contents of the proteins were calculated by taking the total molar contents of these amino acids as round numbers.

† These residue tend to give lower values owing to destruction during hydrolysis. The round numbers were confirmed by the sequence study.

‡ Each protein contains no tryptophan residues as judged from the value of ϵ_{280} .

Table 2. Some properties of native and *p*-bromophenacyl bromide-treated LcPLA-II and LcPLH-I

Abbreviation. N.D., not determined.

	LcPLA-II		LcPLH-I	
	Native	Treated	Native	Treated
No. of histidine residues (mol/mol)*	1.2	0.0	1.3	1.2
$\epsilon_{270}/\epsilon_{280}$	0.89	1.24	0.92	0.91
Phospholipase A ₂ activity (μ mol/min per mg)	24	< 2	< 2	N.D.
LD ₅₀ (μ g/kg body wt. in mice)	45	> 2100	> 4500	N.D.
K_{Ca} at pH 8.0 (μ M)	105	N.D.	44	N.D.
No. of Ca ²⁺ -binding sites (mol/mol)	0.96	N.D.	0.98	N.D.

* Measured by amino acid analysis.

Equilibrium gel filtration

Fig. 1 shows the typical elution pattern of 0.10 μ mol of LcPLH-I at pH 8.0 and an equilibrating Ca²⁺ concentration of 25 μ M. The saturation degrees can be calculated not only from the individual fractions and the total peak, but also from the trough. The peak and

trough areas yield a saturation degree of 0.35 and 0.36 respectively. Fig. 2 summarizes the data for LcPLA-II and LcPLH-I obtained with various equilibrating Ca²⁺ concentrations at pH 8.0. A straight line was obtained for each protein, indicating that there exists only a single class of binding sites for each protein. Extrapolation to the abscissa gives the values $n = 0.96$ and 0.98 for

Fig. 1. Typical elution profile for measurement of the binding of Ca^{2+} by LcPLH-I on the Sephadex G-50 column

The column (1.0 cm $\times$ 22 cm) of Sephadex G-50 was equilibrated with 25 μM - $^{45}\text{CaCl}_2$ (8.4×10^4 d.p.m./ml) in 50 mM-Tris/HCl buffer, pH 8.0, containing 0.1 M-NaCl; 1.4 mg of LcPLH-I was dissolved in 0.2 ml of elution buffer and developed along the column. The flow rate was 20 ml/h; 0.65 ml fractions were collected. Determinations of protein and Ca^{2+} concentration were performed as described in the Materials and methods section.

LcPLA-II and LcPLH-I respectively; thus it is concluded that the binding sites consists of just one class. From the slopes the K_{es} values were calculated to be 105 μM and 44 μM for LcPLA-II and LcPLH-I respectively.

Amino acid sequence of LcPLA-II

The separation of peptides obtained by proteinase digestions are given in Supplementary Publication 50145.

N-Terminal amino acid sequence. Manual Edman degradation on intact performic acid-oxidized LcPLA-II (120 nmol) was performed up to the 12th step (Fig. 3).

Tryptic peptides. Tryptic digestion of performic acid-oxidized LcPLA-II (44.8 mg) was carried out at 37 $^{\circ}\text{C}$ for 19 h in 5 ml of 0.1 M- NH_4HCO_3 , pH 8.0, at an enzyme/substrate ratio of 1:65 (w/w). The results of the manual Edman degradation on most of the peptides are given in Fig. 3.

Chymotryptic peptidase. Chymotryptic digestion of performic acid-oxidized LcPLA-II (25.3 mg) was carried out at 37 $^{\circ}\text{C}$ for 3 h in 2 ml of 0.1 M- NH_4HCO_3 , pH 8.0, at an enzyme/substrate ratio of 1:170 (w/w). The results of manual Edman degradation on some of the peptides are summarized in Fig. 3.

Amino acid sequence of LcPLH-I

N-Terminal amino acid sequence. The sequence analysis was performed on intact performic acid-oxidized

Fig. 2. Scatchard plot for binding data

L_{Ca} values were obtained from peak areas of the elution profiles as in Fig. 1. $\circ$, LcPLA-II; $\bullet$, LcPLH-I.

Fig. 3. Amino acid sequence of LcPLA-II

Underlines indicate amino acid residues detected by Edman degradation.

Fig. 4. Amino acid sequence of LcPLH-I

Underlines indicate amino acid residues detected by Edman degradation.

LcPLH-I (5 nmol) with a gas-phase sequencer for 50 steps with the repetitive yield of 94.5%. The data are shown in Supplementary Publication 50145 Table 3.

Lysyl-endopeptidase peptides. Lysyl-endopeptidase

digestion of performic acid-oxidized LcPLH-I (2.8 mg) was carried out at 37 °C for 5 h in 0.5 ml of 0.05 M-Tris/HCl buffer, pH 9.1, at an enzyme/substrate ratio of 1:140 (w/w). The results of manual Edman degradation on most of the peptides are given in Fig. 4.

Chymotryptic peptides. Performic acid-oxidized LcPLH-I (3.5 mg) was digested with chymotrypsin at 37 °C for 30 min to an enzyme/substrate ratio of 1:140 (w/w). The results of manual Edman degradation on some of the peptides are summarized in Fig. 4.

DISCUSSION

A neurotoxic phospholipase A₂, LcPLA-II, and non-enzymic, non-neurotoxic phospholipase A₂ homologue, LcPLH-I, were purified from the venom of the yellow-lipped sea snake, *L. colubrina*, from the Solomon Islands. Their recovery was 9.5% and 12.3% of the crude dried venom for LcPLA-II and LcPLH-I respectively.

The amino acid sequences of LcPLA-II and LcPLH-I are given in Figs. 3 and 4. Both protein molecules are composed of 118 amino acid residues including 14 half-cystine residues.

The proteinase digestions of LcPLA-II and LcPLH-I gave, in general, the expected peptides. The peptide alignments at positions 46–47, 57–58, 80–81, 86–87 and 109–110 in the sequence of LcPLA-II and those at position 57–58 and 94–95 in the sequence of LcPLH-I were determined by the amino acid compositions of corresponding overlapping peptides. The overlapping peptide was not obtained for the positions 67–68 of LcPLA-II. Further experiments to complete the sequence of this part could not be carried out because of scarcity of the sample material. The sequence of 67–68 was deduced, however, because the sum of the amino acid compositions of the parts of 1–67 and 68–118 agrees with the amino acid composition of the whole LcPLA-II, and the *N*-terminal amino acid sequence of the part of 1–67 agrees with that of whole LcPLA-II. The Tyr–Asn bond (67–68) of LcPLA-II was cleaved with trypsin as well as with staphylococcal proteinase and clostripain. A similar cleavage was reported on the Tyr–Ser bond (67–68) of *Pseudechis australis* phospholipase A₂ Pa-11 with trypsin, staphylococcal proteinase and carboxypeptidase Y (Nishida *et al.*, 1985). The tyrosyl bonds (67–68) in phospholipases A₂ may be in a conformation especially susceptible to proteinase digestion. There are two Lys–Lys bonds in the sequence of LcPLH-I. Amino acid

analysis showed that lysyl-endopeptidase cleaved these Lys–Lys bonds, but not the new *N*-terminal lysyl bonds.

LcPLA-II showed biphasic kinetics due to activation by the reaction product (oleate). A similar observation was reported on the *L. semifasciata* phospholipases A₂, LsPLA III and LsPLA IV (Yoshida *et al.*, 1979). The addition of oleate shortened the lag period without changing the highest velocity, similarly to the cases of LsPLA III and LsPLA IV. The length of lag period depended on the molar ratio of substrate (egg-yolk phosphatidylcholine) to oleate, and in the presence of 20 mol of oleate/100 mol the lag period disappeared (results not shown). The enzymic properties of LcPLA-II were very similar to those of LsPLA III and LsPLA IV, whereas LsPLA I showed normal kinetics and had approximately 30-fold higher specific activity (Table 3).

LsPLA I, which shows normal kinetics, can be converted into LsPLA III or LsPLA IV type with biphasic kinetics on the oxidation of Trp-64 (Yoshida *et al.*, 1979). LcPLA-II has Leu-64, similarly to LsPLA III or LsPLA IV. There are, however, phospholipases A₂ that show normal kinetics with leucine residues at position 64 (phospholipases A₂ Pa-11 and Pa-13 from *Pseudechis australis* venom; Nishida *et al.*, 1985). Therefore the 64th residue is not the only residue responsible for the biphasic kinetics.

On the other hand, only LcPLA-II, not LcPLH-I, showed the lethal activity. The three types of phospholipases A₂, highly lethal and less enzymic (LcPLA-II), less lethal and highly enzymic (LsPLA I) and non-toxic and less enzymic (LsPLA III and LsPLA IV), were obtained from venoms of sea snakes of the genus *Laticauda*.

Kondo *et al.* (1982) reported that Lys-58 and Lys-63 are essential for the neurotoxicity on the basis of amino acid sequence comparison of phospholipases A₂. LcPLA-II has Lys-58 but not Lys-63. The carbamoylation of Lys-58 in Pa-11, however, does not affect its lethal activity (C. Takasaki, unpublished work). The direct involvement of Lys-58 and Lys-63 in the lethal activity is therefore doubtful.

Each molecule of LcPLA-II and LcPLH-I has one Ca²⁺-binding site with *K*_{Ca} values of 105 μM and 44 μM at pH 8.0 respectively. Under the same conditions LsPLA I and *Pseudechis australis* phospholipase A₂ Pa-11 showed *K*_{Ca} values of 38 μM (*n* = 0.96) and 90 μM (*n* = 1.05)

Table 3. Properties of phospholipases A₂ from *Laticauda* venoms

	<i>L. colubrina</i>		<i>L. semifasciata</i>		
	LcPLA-II	LcPLH-I	LsPLA I	LsPLA III	LsPLA IV
Progress curve	Biphasic	–	Normal	Biphasic	Biphasic
Optimum pH	8.2	–	7.6†	7.8†	8.2†
Optimum Ca ²⁺ concn. (mM)*	2	–	20†	3†	2†
Highest velocity (μmol/min per mg)†	24	< 2	896	29	36
Toxicity (LD ₅₀ , μg/kg body wt.)	45	> 4500	9000	> 6000	> 6000

* Without EDTA.

† At the substrate (egg-yolk phosphatidylcholine) concentration of 4 mM.

‡ Taken from Yoshida *et al.* (1979).

	1	2	3	4	5
	0	0	0	0	0
(a) <i>Laticauda colubrina</i> LcPLA-II	NLIQFSELIQCANKGKRATYYMDYGCYCGKGGSGTPVDDLDRCCKTHDD				
(b) <i>Laticauda colubrina</i> LcPLH-I	NLIQFSQLIQCANCKRPTLHYMDYGCYCGPGGSGTPVDDLDRCCKTND				
(c) <i>Laticauda semifasciata</i> LsPLA I	NLVQFSNLIQCANKGSRASYHYADYGCYCGAGGSGTPVDELDRCCKIHDN				
(d) <i>Laticauda semifasciata</i> LsPLA III	NLVQFTYLIQCANSKGRASYHYADYGCYCGAGGSGTPVDELDRCCKIHDN				
(e) <i>Laticauda semifasciata</i> LsPLA IV	NLVQFSYLIQCANTGKRASYHYADYGCYCGAGGSGTPVDELDRCCKIHDN				
(f) <i>Enhydryna schistosa</i> Myotoxin	NLVQFSYVITCANHNRRSSLDYADYGCYCGAGGSGTPVDELDRCCKIHDD				
(g) <i>Pseudechis australis</i> Pa-11	NLIQFGNMIQCANKGSRPSLDYADYGCYCGWGGSGTPVDELDRCCQVHDN				
(h) <i>Notechis s. scutatus</i> Notechis II-1	NLVQFSNMIQCANKGSRPSLAYADYGCYCSAGGSGTPVDELDRCCKTHDD				
(i) <i>Notechis s. scutatus</i> Notexin	NLVQFSYLIQCANKGKRPTWHYMDYGCYCGAGGSGTPVDELDRCCKIHDD				
(j) <i>Naja m. mossambica</i> CM-I	NLYQFKNMIHCTVP-NRPWWHFADYGCYCGRGGGKTAVDDLDRCQVHDN				
(k) <i>Naja melanoleuca</i> DE-III	NLYQFKNMIHCTVP-NRSWWHFANYGCYCGRGGSGTPVDDLDRCCQIHDN				
(l) <i>Hemachatus haemachatus</i> DE-I	NLYQFKNMIKCTVP-SRSWWHFANYGCYCGRGGSGTPVDDLDRCCQTHDN				
(m) Pig pancreas	ALWQFRSMIKCAIPGSHPLMDFNNGYGCYCGLGSGTPVDELDRCCETHDN				
(n) Bovine pancreas	ALWQFNGMIKCKIPSEPLDFNNGYGCYCGLGSGTPVDDLDRCCQTHDN				

	6	7	8	9	1	1	1
	* 0*	0*	* 0 *	0	0	1	1
(a)	CYQAEKK-GC-F----	PFLTLYNFI-CFPGGPT-CDRGT	T-CQRFVCD	CDIQAACFARSPYNNKNYNINISKRCK			
(b)	CYQAEKK-GC-S----	PLSTNYNFD-CFPGGPQ-CGKGT	T-CQRFVCD	CDLKAALCFAKSPYNNKNFNIDTKRCK			
(c)	CYGEAEKM-GC-Y----	PKWTLYTYD-CSTEERN-CSTKT	G-CQGFVCA	CDLEAAKCFARSPYNNKNYNIDTSKRCK			
(d)	CYGEAEKM-GC-Y----	PKLTMYNYY-CGTQSP	T-CDDKTG-CQRYVCA	CDLEAAKCFARSPYNNKNYNIDTSKRCK			
(e)	CYQAEKM-GC-Y----	PKLTMYNYY-CGTQSP	T-CDNKTG-CQRYVCA	CDLEAAKCFARSPYNNKNYNIDTSKRCK			
(f)	CYGEAEKL-GC-Y----	PKMLMYDYY-CGSNGPY-CRNVKK	CNRKVCDC	LVAAAECFARNAYNNANYNIDTKRCK			
(g)	CYEQAGKK-GC-F----	PKLTLYSWK-CTGNVPT-CNSKPG	-CKSFVCA	CDAAAAKCFAPYKKNYNIDTKRCK			
(h)	CYARATKSYSC-T----	PYWTLYSWQ-CIEKTPT-CDSKT	G-CQRFVCD	CDATAAKCFAPYNNKNYNIDPKKRCQ			
(i)	CYDEAGKK-GC-F----	PKMSAYDYY-CGNGPY-CRNIKK	KCLRFVCD	CDVEAAFCFAPYNNANWNIDTKRCK			
(j)	CYGEAEKL-GC-W----	PYLTLYKYE-CSQGKLT	-CSGNNKCEAAVNC	CDLVAAACFAGAPYIDANYVNLKERCK			
(k)	CYGEAEKISGC-W----	PYIKTYTYDSC-QGTLT	SCGAANN-CAASVCD	CDRVAANCFARAPYIDKNYNIDFNARCQ			
(l)	CYSDAEKISGC-R----	PYFKTYSYD-CTKGKLT	-CKEKGNECAAFVCK	CDRLAAICFAGAHYNDNNYIDLARHCQ			
(m)	CYRDAKNLDSCKFLVDNPTYTESYSYS-CSNTEIT-CNSKNNACEAFICNCDRNAAICFSKAPYNKEHKNLDTKKYC-CYQAKKLDSCKVLVDNPTYTNYSYS-CSNNEIT-CSSENNAEAFICNCDRNAAICFSKVPYNKEHKNLD-KKNC-						

Fig. 5. Comparison of the amino acid sequences of various phospholipases A₂.

The phospholipases A₂ were obtained from: (a) and (b) *Laticauda colubrina* (present paper); (c) *L. semifasciata* (Nishida *et al.*, 1982; revised by C. Takasaki, H. Kuramochi, T. Shimazu & N. Tamiya, unpublished work); (d) and (e) *L. semifasciata* (Nishida *et al.*, 1982); (f) *Enhydryna schistosa* (Lind & Eaker, 1981); (g) *Pseudechis australis* (Nishida *et al.*, 1985); (h) *Notechis scutatus* (Lind & Eaker, 1980); (i) *N. s. scutatus* (Halpert & Eaker, 1975); (j) *Naja mossambica mossambica* (Joubert, 1977); (k) *N. melanoleuca* (Joubert, 1975b); (l) *Hemachatus haemachatus* (Joubert, 1975a); (m) pig pancreas (Puijck *et al.*, 1977); (n) bovine pancreas (Fleer *et al.*, 1978). The sequences were aligned with respect to the positions of the half-cystine residues.

respectively (C. Takasaki, unpublished work). Somewhat higher K_{Ca} values were calculated for notexin (140 μ M at pH 7.4; Halpert *et al.*, 1976), *Notechis* II-1 (600 μ M at pH 7.4; Halpert & Eaker, 1976) and pig phospholipase A₂ (250 μ M at pH 8.0; Pieterse *et al.*, 1974).

All the phospholipases A₂, including non-enzymic homologue *Notechis* II-1 from snake venoms and mammalian pancreas, possess a histidine residue at their active centre (His-48), which is specifically modified with *p*-bromophenacyl bromide (Volwerk *et al.*, 1974), and the modified enzymes lose both the enzymic and lethal activities. LcPLA-II modified at its single histidine residue (His-48) with *p*-bromophenacyl bromide also lost both of the activities. On the other hand, the single histidine residue in LcPLH-I (His-21) was not modified with *p*-bromophenacyl bromide (Table 2).

LcPLH-I has very similar properties with respect to M_r , amino acid composition and Ca²⁺-binding activity to those of phospholipase A₂ LcPLA-II, but it showed neither enzymic nor lethal activities. It contains all the invariant residues present among the phospholipases A₂ except for histidine-48 (Fig. 5). This is replaced by an asparagine residue in LcPLH-I, and the single histidine residue in the molecule is situated at position 21. This must be the reason that LcPLH-I has no enzymic activity and is not modified with *p*-bromophenacyl bromide. The

non-enzymic homologue LcPLH-I is different in this respect from *Notechis* II-1, a non-toxic and non-enzymic phospholipase A₂ homologue from the Australian elapid snake *Notechis scutatus scutatus* described by Lind & Eaker (1980). The latter has the invariant His-48 but has a serine residue instead of invariant Gly-30. It consistently accounts for 15–20% of the weight of the dried venom. The lethality of notexin, which is the most lethal component in *N. s. scutatus* venom, is about the same as the crude venom itself. Lind & Eaker (1980) considered that the high lethality of the crude venom must be accountable to some kind of synergism, perhaps involving the 'inactive' component *Notechis* II-1.

On the other hand, LcPLH-I consistently accounts for 13% of the weight of the dried venom. The main toxic components in *L. colubrina* venom are long-chain and short-chain post-synaptic neurotoxins named Lc a (Kim & Tamiya, 1982) and Lc c (Tamiya *et al.*, 1983) respectively, and the lethality in fraction B can be almost explained by the content of LcPLA-II; therefore it seems that LcPLH-I has no function on the lethality.

Heinrikson *et al.* (1977) classified phospholipases A₂ from mammalian pancreas and various snake venoms into two groups from their amino acid sequences. Group I is composed of the phospholipases A₂ from pancreas and elapid venoms, whereas phospholipases A₂ from

crotalid and viperid venoms belong to Group II. The amino acid sequences of LcPLA-II and LcPLH-I are compared with some phospholipase A₂ sequences belonging to Group I (Fig. 5).

The amino acid sequences of LcPLA-II and LcPLH-I differ from each other at 22 positions out of 118 amino acid residues. LcPLA-II is homologous with phospholipases A₂ from another snake of the genus, *Laticauda semifasciata* (73–77%), and to some extent with those from Hydrophiinae (64%) and Acanthopinae (64–68%), whereas it is less homologous with those of the other subfamily of the Elapidae, Elapinae (53–58%). It has 44–45% homology with the enzymes from mammalian pancreas and 34–42% homology with Group II enzymes.

We thank Dr. H. Heatwole, Dr. Y. Ishikawa and Mr. M. McCoy for their co-operation in the collection of snakes. We are grateful to Professor K. Ogura of Tohoku University, Sendai, Japan, for the use of the Pharmacia f.p.l.c. system. We thank Mr. H. Abe for the amino acid analysis. The collection trips were performed on Japan Society for the Promotion of Science Grant 041006 in 1980 and 1981.

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Received 2 September 1987/26 January 1988; accepted 27 April 1988