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Amino acid sequences of three phospholipases A I, III and IV from the venom of the sea snake *Laticauda semifasciata*

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Amino acid sequences of three phospholipases A, I, III and IV, from the venom of the sea snake *Laticauda semifasciata* were elucidated. Each protein consisted of a single chain of 118 amino acid residues, including 14 half-cystine residues. They showed high homology among themselves, and with the other snake-venom phospholipases A and with the enzymes from mammalian pancreas. Phospholipases A III and IV were especially similar to each other, with only four differences out of their 118 amino acid residues. Phospholipase A I contained one tryptophan residue at position 64, which was important for enzymic activity, whereas III and IV did not contain tryptophan residues and their corresponding positions were occupied by leucine residues. The substitution by leucine resulted in a decreased, but definite, phospholipase A activity. The substituted enzymes have a more potent neuromuscular blocking activity. Full experimental details and evidence for the amino acid sequences of the proteins have been deposited as Supplementary Publication SUP 50118 (39 pages) 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.* (1981) **193**, 5.

Phospholipases A have been isolated from several sources, including snake and bee venoms and mammalian pancreas. Four phospholipase A active components (I, II, III and IV) were detected in the venom of a sea snake, *Laticauda semifasciata*, on CM-cellulose column chromatography. Phospholipases A I, III and IV were isolated in a homogeneous state and their properties and amino acid compositions studied by Yoshida *et al.* (1979). Phospholipase A II was a minor component and could not be purified in a homogeneous state, and therefore it was not sequenced. The phospholipases A I, III and IV are classified into two groups by the differences in their enzymic properties. One group is phospholipase A I, which is the major form and has a higher specific activity. The other group includes phospholipases A III and IV, which are minor forms and have lower specific activities. The latter enzymes are activated by the reaction products, namely by unsaturated fatty acids and/or lysophosphatidylcholine. Although these phospholipases A are similar in amino acid compositions and in molecular weights, only phospholipase A I has a tryptophan residue. On modification with *N*-bromosuccinimide, phospholipase A I is converted into an enzyme very similar to III and IV in enzymic properties (Yoshida *et al.*, 1979). On the other hand, phospholipase A III is different from phospholipase A I in having a more potent neuromuscular blocking activity and in binding to the acetylcholine receptor site (Harvey & Tamiya, 1980). The elucidation of the amino acid sequences is thus of interest in relation to the structure–function relationship of these phospholipases A.

Primary structures have been reported for phospholipases A from mammalian pancreas, from honey-bee (*Apis mellifica*) venom and from the venoms of various snakes including those belonging to the three families Elapidae, Colubridae and Viperidae. According to the recent classification by Dowling & Duellman (1978), family Elapidae is classified into five subfamilies, namely Laticaudinae, Hydrophiinae, Acanthophiinae, Micrurinae and Elapinae. The amino acid sequences of phospholipases A from members of the subfamily Laticaudinae have not yet been elucidated.

The present paper describes the complete amino acid sequences of three phospholipases A, I, III and IV, from the venom of the sea snake *Laticauda semifasciata*.

Methods and results

Full experimental details and evidence for the amino acid sequences of the proteins have been deposited as Supplementary Publication SUP 50118.

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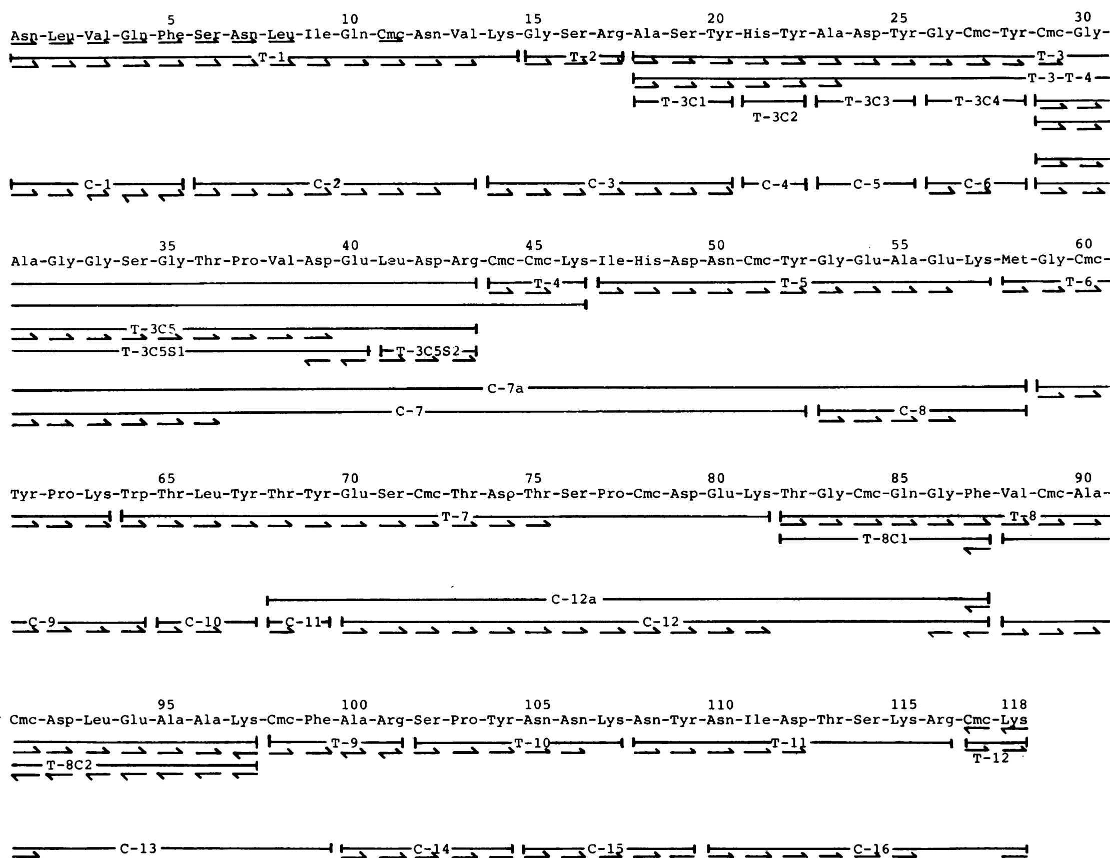

Fig. 1. *Amino acid sequence of L. semifasciata phospholipase A I*

The prefixes T, C and S stand for tryptic, chymotryptic and staphylococcal-proteinase peptides respectively. Symbols used are as follows: ⇀, residue detected by Edman degradation; ↽, residue released by carboxypeptidase Y; ⇁, residue released by leucine aminopeptidase. Abbreviation used: Cmc, *S*-carboxymethylcysteine.

Discussion

The amino acid sequences of *L. semifasciata* phospholipases A I, III and IV are given in Figs. 1, 2 and 3 and their amino acid compositions are given in Table 1. The three enzymes are composed of 118 amino acid residues. The *S*-carboxymethylcysteine (Cmc in sequences) contents of peptides determined by amino acid analysis tend to be lower than values found in sequence studies. However, sequence analysis of each peptide showed that the three enzymes contain 14 half-cystine residues each.

The digestion of phospholipase A I with trypsin and chymotrypsin gave in general the anticipated peptides. However, the -Lys–Arg- (115–116) bond was not split, and the -Arg–Cmc- (43–44) bond was rather slowly cleaved by tryptic digestion, probably owing to the acidic environment around the residues -Asp-Arg-Cmc-Cmc- (42–45). On chymotryptic digestion, the -Leu–Val- (2–3) and -Leu–Asp- (41–42) bonds were not split, and incomplete hydrolysis was observed at the -Tyr–Gly- (52–53), -Tyr–Glu- (69–70) and -Leu–Glu- (93–94) bonds.

The amino acid sequences of phospholipases A I, III and IV from *L. semifasciata* are shown in Fig. 4, together with some of the known phospholipase A sequences from snake venoms and from mammalian pancreas. The sequence of bee-venom phospholipase A (Shipolini *et al.*, 1974) is omitted. To obtain the maximum degree of homology, phospholipases A were aligned in order to locate half-cystine residues at the same positions as much as possible. The sequences of phospholipases A I, III and IV from *L. semifasciata* are highly homologous with the other snake-venom phospholipases A and with pancreatic phospholipases A. Only four substitutions were observed between phospholipases A III and IV (threonine/serine substitution at position 6, serine/threonine at position 14, glutamic acid/glut-

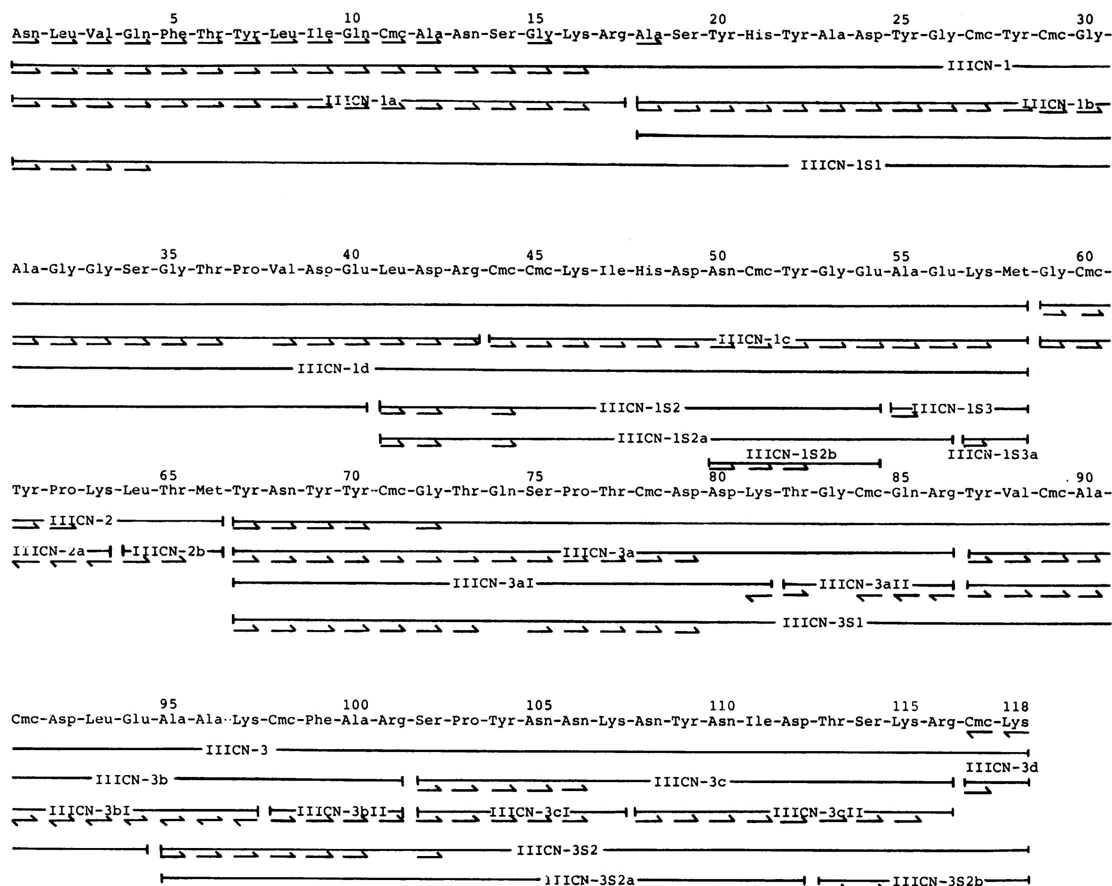

Fig. 2. *Amino acid sequence of L. semifasciata phospholipase A III*
The prefix CN stands for CNBr peptides. The other symbols and abbreviations used are the same as those in Fig. 1.

amine at position 54 and aspartic acid/asparagine at position 80). Each amino acid substitution can arise from a single base replacement in their genetic codons for the amino acids involved. There are more differences between phospholipases A I and III, including two deletions and 16 replacements, and between I and IV, including two deletions and 17 replacements. It was observed that phospholipases A I, III and IV shared 29 invariant residues (including ten half-cystine residues) with all other phospholipases A, 32 invariant residues with snake-venom enzymes and 41 invariant residues with enzymes from other members of the Elapidae.

No phospholipase A from the venom of subfamily Laticaudinae has previously been reported. The three phospholipases A from *L. semifasciata* described in the present paper show higher homology (up to 64%) with phospholipases A from other species of the family Elapidae than with those from other snake families. Phospholipase A I is less homologous with *Crotalus adamanteus* (rattlesnake) (43%; Colubridae, Heinrikson *et al.*, 1977) or with *Bitis gabonica* (Gaboon viper) (44%; Viperidae; Botes & Viljoen, 1974) enzyme. The three enzymes from *L. semifasciata* described in the present paper share 14 half-cystine residues with the enzymes from mammalian pancreas and Elapidae venoms, the only exception being the β_1-bungarotoxin A chain (Kondo *et al.*, 1978*a*). The enzymes from Colubridae and Viperidae venoms lack two and four half-cystine residues out of the above mentioned 14 residues respectively and have two additional half-cystine residues at positions 50 and 133. The homology between *L. semifasciata* phospholipase A I and notexin (Halpert & Eaker, 1975) is especially high at regions from Lys-14 to Lys-57 (81.9%) and from Phe-95 to Lys-125 (71.9%), whereas 19 residues between residues 65 and 88

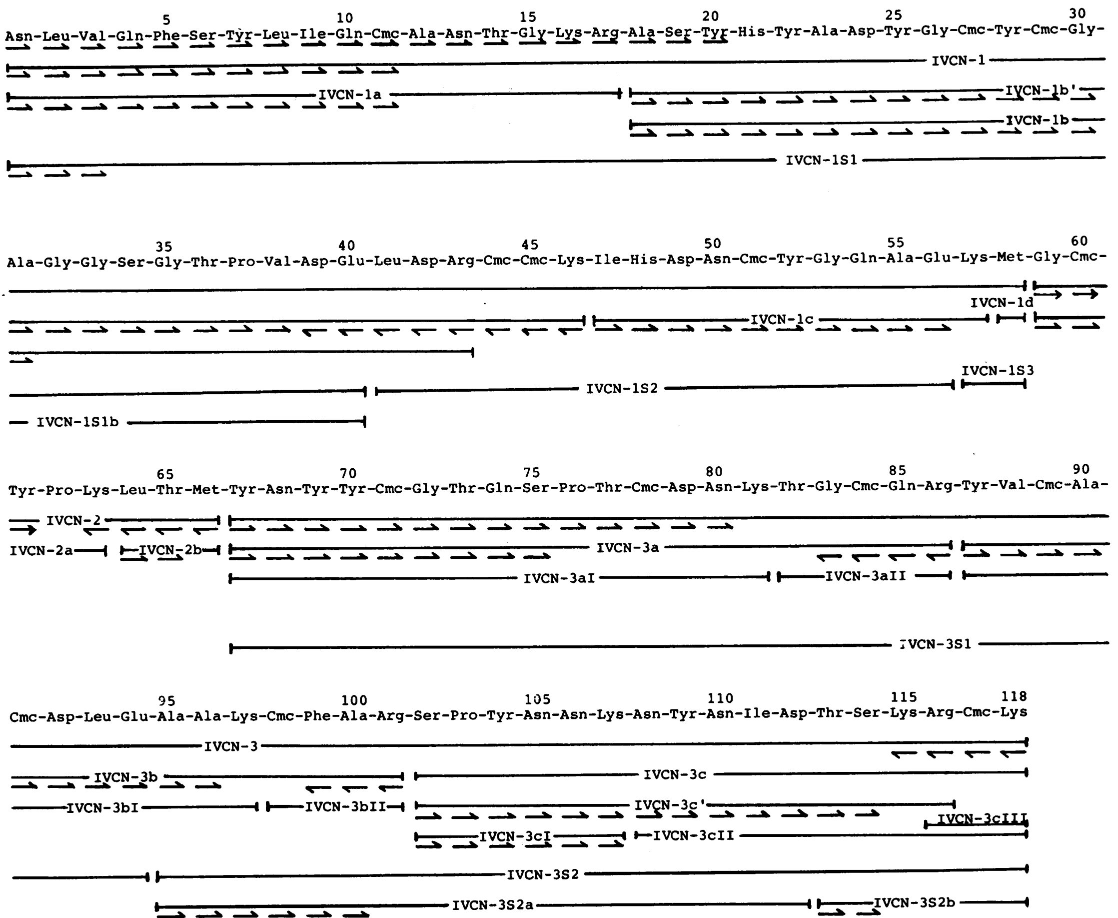

Fig. 3. *Amino acid sequence of L. semifasciata phospholipase A IV*
Prefixes, symbols and abbreviations used are the same as those in Fig. 1.

show only 31.6% homology in these proteins. *L. semifasciata* enzyme I has 55 residues (45.1%) identical with those of porcine pancreas phospholipase A (Puijk *et al.*, 1977).

Phospholipases A I, III and IV from *L. semifasciata* venom contain two histidine residues at the same positions (His-21 and His-48). Only one histidine residue (His-48) is invariant for all the sequences reported thus far, and the residue is concluded to be one of the active-site residues of the enzyme by the results of chemical modification with *p*-bromophenacyl bromide (Volwerk *et al.*, 1974; Halpert *et al.*, 1976; Kondo *et al.*, 1978*b*). The proposed active-site and calcium-binding residues in the enzymes are His-48, Asp-49, Tyr-52 and Asp-101, which are among 29 common residues for all the phospholipases A compared.

A tryptophan residue is present only in phospholipase A I and it occupies position 64; no tryptophan residues are present in enzymes III and IV from the same venom. Tryptophan-64 in phospholipase A I is replaced by leucine residues in enzymes III and IV. Yoshida *et al.* (1979) observed that phospholipases A III and IV were only one-thirtieth to one-fortieth as active as phospholipase A I and that III and IV showed in their reaction a lag time which disappeared on the addition of reaction products. It is noteworthy, however, that enzymes III and IV have a more potent neuromuscular blocking activity (Harvey & Tamiya, 1980). Modification of the tryptophan residue of enzyme I with *N*-bromosuccinimide converted the enzyme into a less-active and lag-time-showing form. The three-dimensional structures of porcine (Drenth *et al.*, 1976) and bovine (Dijkstra *et al.*, 1978) phospholipases A have been

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                                                    1         2         3         4         5
Elapidae                                            0         0         0         0         0
 (a) L. semifasciata I                     NLVQFSNLIQCNVKGSRASYHYADYGCYCGAGGSGTPVDELDRCCKIHDN
 (b) L. semifasciata III                   NLVQFTYLIQCANSGKRASYHYADYGCYCGAGGSGTPVDELDRCCKIHDN
 (c) L. semifasciata IV                    NLVQFSYLIQCANTGKRASYHYADYGCYCGAGGSGTPVDELDRCCKIHDN
 (d) N. s. scutatus, notexin               NLVQFSYLIQCANHGKRPTWHYMDYGCYCGAGGSGTPVDELDRCCKIHDD
 (e) N. s. scutatus, notechis II-5         NLVQFSYLIQCANHGRRPTRHYMDYGCYCGWGGSGTPVDELDRCCKIHDD
 (f) N. m. mossambica, CM-I                NLYQFKNMIHCTVPS-RPWWHFADYGCYCGRGGKGTAVDDLDRCCQVHDN
 (g) N. m. mossambica, CM-II               NLYQFKNMIHCTVPS-RPWWHFADYGCYCGRGGKGTAVDDLDRCCQVHDN
 (h) N. m. mossambica, CM-III              NLYQFKNMIHCTVPS-RPWWHFADYGCYCGRGGKGTPVDDLDRCCQVHDN
 (i) N. nigricollis, basic                 NLYQFKNMIHCTVPS-RPWWHFADYGCYCGRGGKGTPVDDLDRCCQVHDN
 (j) N. n. kaouthia, CM-II                 NLYQFKNMIQCTVPN-RSWWDFADYGCYCGRGGSGTPVDDLDRCCQVHDN
 (k) N. n. kaouthia, CM-III                NLYQFKNMIQCTVPS-RSWWNFADYGCYCGRGGSGTPVDDLDRCCQVHDN
 (l) H. haemachatus, DE-I                  NLYQFKNMIKCTVPS-RSWWHFANYGCYCGRGGSGTPVDDLDRCCQTHDN
 (m) N. melanoleuca, DE-I                  NLYQFKNMIHCTVPN-RPWWHFANYGCYCGRGGKGTPVDDLDRCCQIHDK
 (n) N. melanoleuca, DE-II                 NLYQFKNMIQCTVPN-RSWWHFANYGCYCGRGGSGTPVDDLDRCCQIHDN
 (o) N. melanoleuca, DE-III                NLYQFKNMIHCTVPN-RSWWHFANYGCYCGRGGSGTPVDDLDRCCQIHDN
 (p) B. multicinctus                       NLYQFKNMIVCAGT--RPWIGYVNYGCYCGAGGSGTPVDELDRCCYVHDN
 (q) B. multicinctus, β1-bungarotoxin      NLINFMEMIRYTIPCEKTWGEYADYGCYCGAGGSGRPIDALDRCCYVHDN
Colubridae
 (r) C. adamanteus, α                      SLVQFETLIM-KVAKRSGLLWYSAYGCYCGWGGHGRPQDATDRCCFVHDC
Viperidae
 (s) B. gabonica                           DLTQFGNMIN---KMGQSVFDYIYYGCYCGWGGKGKPIDATDRCCFVHDC
Mammalian pancreas
 (t) Porcine pancreas                      ALWQFRSMIKCAIPGSHPLMDFNNYGCYCGLGGSGTPVDELDRCCETHDN
 (u) Equine pancreas                       AVWQFRSMIQCTIPNSKPYLEFNDYGCYCGLGGSGTPVDELDACCQVHDN
 (v) Bovine pancreas                       ALWQFNGMIKCKIPSSEPLLDFNNYGCYCGLGGSGTPVDDLDRCCQTHDN

                                                          1         1         1         1
          6         7         8         9         0         1         2         3
          0         0         0         0         0         0         0         0
CYGEAEKM-GC-----YPKWTLYTYESCTDTSP--CDE-KTGCQGFVCACDLEAAKCFARSPYNNKNYNIDTSKR-CK
CYGEAEKM-GC-----YPKLTMYNYY-CGTQSPT-CDD-KTGCQRYVCACDLEAAKCFARSPYNNKNYNIDTSKR-CK
CYGQAEKM-GC-----YPKLTMYNYY-CGTQSPT-CDN-KTGCQRYVCACDLEAAKCFARSPYNNKNYNIDTSKR-CK
CYDEAGKK-GC-----FPKMSAYDYY-CGENGPY-CRNIKKKCLRFVCDCDVEAAFCFAKAPYNNANWNIDTKKR-CQ
CYSDAEKK-GC-----SPKMSAYDYY-CGENGPY-CRNIKKKCLRFVCDCDVEAAFCFAKAPYNNANWNIDTKKR-CQ
CYGEAEKL-GC-----WPYLTLYKYE-CSQGKLT-CSGGNNKCEAAVCNCDLVAANCFAGAPYIDANYNVNLKER-CQ
CYGEAEKL-GC-----WPYLTLYKYE-CSQGKLT-CSGGNNKCAAAVCNCDLVAANCFAGARYIDANYNINLKER-CQ
CYEKAGKM-GC-----WPYFTLYKYK-CSQGKLT-CSGGNSKCGAAVCNCDLVAANCFAGARYIDANYNINFKKR-CQ
CYEKAGKM-GC-----WPYLTLYKYK-CSQGKLT-CSGGNSKCGAAVCNCDLVAANCFAGARYIDANYNINFKKR-CQ
CYDEAEKISRC-----WPYFKTYSYE-CSQGTLT-CKNGNNACAAAVCDCDRLAAICFAGAPYNNNNYNIDLKAR-CQ
CYDEAEKISGC-----WPYFKTYSYE-CSQGTLT-CKGGNNACAAAVCDCDRLAAICFAGAPYNNNNYNINLKAR-CQ
CYSDAEKISGC-----RPYFKTYSYD-CTKGKLT-CKEGNNECAAFVCKCDRLAAICFAGAHYNDNNNYIDLARH-CQ
CYDEAEKISGC-----WPYIKTYTYESC-QGTLT-CKDGGK-CAASVCDCDRVAANCFARATYNDKNYNIDFNAR-CQ
CYGEAEKISGC-----WPYIKTYTYESC-QGTLTSCGANNK-CAASVCDCDRVAANCFARATYNDKNYNIDFNAR-CQ
CYGEAEKISGC-----WPYIKTYTYDSC-QGTLTSCGAANN-CAASVCDCDRVAANCFARAPYIDKNYNIDFNAR-CQ
CYGEAEKIPGC-----NPKTKTYSYT-CTKPNLT-CTDAAGTCARIVCDCDRTAAICFAAAPYNINNFNISSSTH-CQ
CYGDAEKKHKC-----NPKTSQYSYKL-TKRTII-CYGAAGTCGRIVCDCDRTAALCFGQSDYIEGHKNIDTARF-CQ

CYGKAT---NC-----NPKTVSYTYSE-ENGEIV-CGGDDP-CGTQICECDKAAAICFRDNIPSYDNKYWLFPPKDCRQEPEPC

CYGKMGTYDT---------KTSYNYEI-QNGGID-CDEDPQK--KELCECDRVAAICFANNRNTYNSNYFGHSSSKCTG-TEQC

CYRDAKNLDSCKFLVDNPYTESYSYS-CSNTEIT-CNSKNNACEAFICNCDRNAAICFSKAPYNKEHKNLDTK-KYC
CYTQAKELSSCRFLVDNPYTESYKFS-CSGTEVT-CSDKNNACEAFICNCDRNAAICFSKAPYNPENKNLDSKRKAC
CYKQAKKLDSCKVLVDNPYTNNYSYS-CSNNEIT-CSSENNACEAFICNCDRNAAICFSKVPYNKEHKNLD-K-KNC
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Fig. 4. *Comparison of the amino acid sequences of various phospholipases A*

The phospholipases A were obtained from: (a) *Laticauda semifasciata*, I (the present paper); (b) *Laticauda semifasciata*, III (the present paper); (c) *Laticauda semifasciata*, IV (the present paper); (d) *Notechis scutatus scutatus* (tiger snake), notexin (Halpert & Eaker, 1975); (e) *Notechis scutatus scutatus*, notechis II-5 (Halpert & Eaker, 1976); (f) *Naja mossambica mossambica*, CM-I (Joubert, 1977); (g) *Naja mossambica mossambica*, CM-II (Joubert, 1977); (h) *Naja mossambica mossambica*, CM-III (Joubert, 1977); (i) *Naja nigricollis* (spitting cobra), basic [Odidairo *et al.* (1977) cited in Joubert (1977)]; (j) *Naju naja kaouthia* (monocellate cobra), CM-II (Joubert & Taljaard, 1980); (k) *Naja naja kaouthia*, CM-III (Joubert & Taljaard, 1980); (l) *Hemachatus haemachatus* (ringhals), DE-I (Joubert, 1975*a*); (m) *Naja melanoleuca* (forest cobra), DE-I (Joubert, 1975*c*); (n) *Naja melanoleuca*, DE-II (Joubert, 1975*c*); (o) *Naja melanoleuca*, DE-III (Joubert, 1975*b*); (p) *Bungarus multicinctus* (many-banded krait) (Kondo *et al.*, 1981); (q) *Bungarus multicinctus*, β_1-bungarotoxin A chain (Kondo *et al.*, 1978*a*); (r) *Crotalus adamanteus* (rattlesnake), α (Heinrikson *et al.*, 1977); (s) *Bitis gabonica* (Gabon viper) (Botes & Viljoen, 1974); (t) porcine pancreas (Puijk *et al.*, 1977); (u) equine pancreas (Evenberg *et al.*, 1977); (v) bovine pancreas (Fleer *et al.*, 1978). The sequences were aligned with respect to the positions of the half-cystine residues. The one-letter notation for amino acid sequences is given in *Biochem. J.* (1969) **113**, 1–4.

Table 1. *Amino acid compositions of phospholipases A I, III and IV*
Corrections were made for the destruction of threonine and serine. Valine and isoleucine were estimated from the 72h-hydrolysis values. Tryptophan was determined by spectrophotometric measurement (Goodwin & Morton, 1946). The values in parentheses are those confirmed by the sequence study.

	Composition (mol of residue/mol of protein)					
	Phospholipase A I		Phospholipase A III		Phospholipase A IV	
Amino acid	Analysis	Sequence	Analysis	Sequence	Analysis	Sequence
Lys	8.8	(9)	9.2	(9)	9.3	(9)
His	2.0	(2)	1.9	(2)	1.7	(2)
Arg	4.2	(4)	4.8	(5)	4.7	(5)
Asx	16.0	(16)	15.6	(16)	16.3	(16)
Thr	6.9	(7)	7.0	(7)	6.9	(7)
Ser	7.6	(8)	5.7	(6)	5.7	(6)
Glx	9.3	(9)	8.8	(8)	7.6	(8)
Pro	4.2	(4)	4.3	(4)	5.1	(4)
Gly	10.1	(10)	10.1	(10)	10.2	(10)
Ala	8.1	(8)	9.4	(9)	8.5	(9)
½-Cys	12.4	(14)	13.0	(14)	12.5	(14)
Val	3.7	(4)	3.1	(3)	3.1	(3)
Met	1.1	(1)	1.9	(2)	2.0	(2)
Ile	3.1	(3)	2.8	(3)	2.7	(3)
Leu	5.1	(5)	5.1	(5)	4.7	(5)
Tyr	9.6	(10)	12.4	(13)	12.3	(13)
Phe	3.0	(3)	2.3	(2)	1.9	(2)
Trp	0.7	(1)	0	(0)	0	(0)
Total		118		118		118

elucidated by X-ray crystallography. The sequence homology of 45.1% between phospholipase A I and the porcine enzyme suggests a similarity in their tertiary structures. The residue tryptophan-64 seems to lie in a hydrophobic region just outside of the active-site crevice of the enzyme molecule. The residue probably plays an important role in substrate binding and affects the enzyme properties.

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