Journal of Chromatography, 530 (1990) 129–136
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5361

Note

Simple fractionation of phospholipase A_2 analogues from snake venom by high-performance liquid chromatography

CHIH-HUNG LO and SHYH-HORNG CHIOU*

Institute of Biochemical Sciences, National Taiwan University and Institute of Biological Chemistry, P.O. Box 23-106 Academia Sinica, Taipei 10098 (Taiwan)

(First received January 29th, 1990; revised manuscript received April 6th, 1990)

The venoms of the elapid snakes have been studied extensively both chemically and pharmacologically during the past four decades [1,2]. The components isolated from the crude venoms, in addition to some miscellaneous proteins/peptides and enzymes, generally fall into three major categories based on their structures and activities, *i.e.* (A) phospholipases A_2, (B) neurotoxins, and (C) cardiotoxins (or cytotoxins). All these biologically active proteins have been used widely as tools in the studies of various biological phenomena of molecular and cell biology.

Previous fractionation and purification of snake venom proteins involved multiple chromatographic steps, including both ion-exchange chromatography and gel permeation techniques, which seemed laborious and tedious. Although most venom toxins can be obtained by conventional open-column techniques, some new methods employing the recent advances in instrumentation for high-performance liquid chromatography (HPLC) are highly desirable to improve the speed of analysis and resolution of various isotoxins from the venom.

This paper describes a rapid and semipreparative HPLC method using cation exchange for the isolation and purification of phospholipase A_2 (PLA_2) from Thailand cobra (*Naja naja siamensis*), which can be used as the alternative enzyme source in the studies of inflammatory responses associated with PLA_2 of mammalian tissues.

EXPERIMENTAL

Materials and samples

The lyophilized venom powder was obtained from Biotoxins (St. Cloud, FL, U.S.A.). Cation-exchange resin, TSK CM-650 (S), ammonium acetate, sodium dodecyl-sulphate (SDS) and Coomassie blue were from E. Merck (Darmstadt, F.R.G.). The semipreparative HPLC SynChropak CM-300 column (250 × 10

0378-4347/90/$03.50

mm I.D.) and the reversed-pase C_{18} column (300 × 4.0 mm I.D., SynChropak RP-P, 6.5 μm) were from SynChrom (Lafayette, IN, U.S.A.). Molecular mass standards for SDS-PAGE were from Sigma (St. Louis, MO, U.S.A.). Constant-boiling 6 *M* HCl and 4 *M* methanesulphonic acid containing 0.2% 3-(2-aminoethyl)indole were from Pierce (Rockford, IL, U.S.A.). High-purity deinonized water was prepared with a Milli-Q purification system (Millipore, Bedford, MA, U.S.A.).

Apparatus and chromatography

Various toxins were first isolated by cation-exchange chromatography on an open column (15 × 2.5 cm I.D.) packed with TSK CM-650 (S). Dissolved venom powder in 0.05 *M* ammonium acetate (pH 5.7) starting buffer (20–50 mg/ml) was applied to a TSK CM-650 (total 5 ml) open column and then eluted in a linear gradient of 0.05–0.5 *M* ammonium acetate, followed by two stepwise elutions in 0.5 *M* and 1.0 *M* ammonium acetate (pH 5.9) buffers. For semipreparative separation of venom toxins, an ISCO biocompatible, buffer-impervious HPLC chromatograph (ISCO, Lincoln, NE, U.S.A.) was used, connected to a CM-300 column adapted with a 250-μl sample injection coil. A. 100–200 μl crude toxin extract was injected each time. The solvent and elution conditions for the HPLC system are described in the figure legend. Reversed-phase HPLC was also carried out with a Hitachi liquid chromatograph (Hitachi, Tokyo, Japan) with a Model L-6200 pump and a variable-wavelength UV monitor. This step was used to purify and desalt the toxin fractions isolated from the ion-exchange chromatographies.

Polyacrylamide gel electrophoresis and amino acid analysis

The purities of the isolated toxins were checked by SDS-polyacrylamide slab gel (5% stacking/14% resolving gel) as described before [3], with some modifications (5% crosslinking N,N′-methylenebisacrylamide in the gel solution). The amino acid compositions were determined with a Beckman 6300 amino acid analyser and a single-column system based on conventional ion-exchange chromatography. The special rapid procedure for the preparation of protein hydrolysates in 6 *M* HCl or 4 *M* methanesulphonic acid using microwave irradiation before amino acid analysis was essentially according to the previous reports [4,5].

N-Terminal sequence analysis

The N-terminal sequences of the peak fractions isolated from the HPLC column were carried out by automated Edman degradation with a pulsed-liquid phase protein sequencer (Model 477A, Applied Biosystems, Foster City, CA, U.S.A.). The samples, each containing *ca.* 1–5 nmol of protein, were dissolved in 100 μl of 0.1% trifluoroacetic acid (TFA), and 5 μl each were used for sequence determinations.

Activity assay

Pharmacological assays for neurotoxicity and PLA_2 activity were as described previously [6]. The synthetic substrate L-α-lecithin was used for phospholipase assay by the fatty-acid titration method [7].

RESULTS AND DISCUSSION

It is well known that various isotoxins or PLA_2 variants are present in the snake venom even from a single species. Investigation of the biochemical or genetic basis for the generation of multiple toxin isoforms in the same or closely related species remains a great challenge in venom research. It is imperative to isolate and characterize various isoforms of these toxins or enzymes in order to gain some insight into the mechanism underlying the process of sequence variation among these proteins. The present study was performed as part of the endeavour to isolate and characterize various PLA_2.

Fig. 1 shows the general elution pattern of the crude venom on the TSK CM-650 cation-exchange column. The poorly resolved peaks eluted in the first part of chromatogram represent the PLA_2 fractions with strong enzymic activity, and constitute *ca.* 20% of total venom proteins. The relative percentage yields for each toxin fraction and their respective enzymic or toxic properties were reported

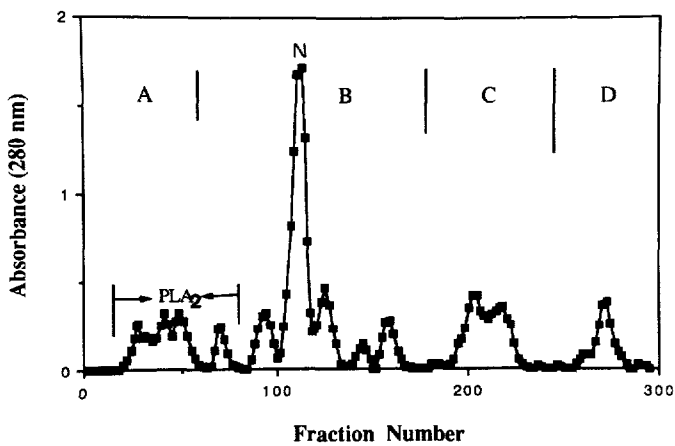

Fig. 1. Cation-exchange chromatography on the TSK CM-650(S) column of crude venom from *Naja naja siamensis*. About 200 mg of lyophilized crude venom dissolved in the starting buffer of 0.05 *M* ammonium acetate with 0.01% 2-mercaptoethanol (pH 5.7) was applied to the column equilibrated in the same buffer. Elution was carried out in four steps: (A) elution with starting buffer; (B) elution with a linear gradient of 0.05–0.5 *M* ammonium acetate in 0.01% 2-mercaptoethanol (pH 5.9); (C) 0.5 *M* ammonium acetate (pH 5.9); (D) 1.0 *M* ammonium acetate (pH 5.9). The column eluates (2.8 ml/tube per 3.2 min) were monitored for absorbance at 280 nm. The peak fractions were collected, lyophilized and used for chemical and pharmacological studies. Fraction N indicates the peak of a major long-chain neurotoxin, which constitutes *ca.* 35% of total crude venom. The region labelled as PLA_2 encompasses the fractions showing the highest phospholipase A_2 activity.

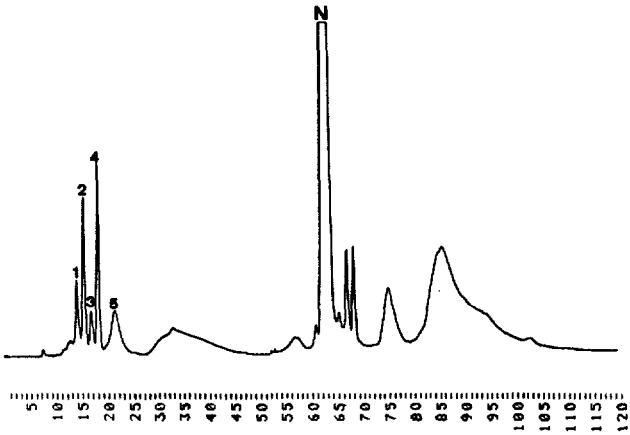

Fig. 2. Preparative cation-exchange chromatography of crude venom on the CM-300 column. About 3–5 mg of lyophilized crude venom dissolved in 100–200 μl of starting buffer of 0.05 *M* ammonium acetate with 0.01% 2-mercaptoethanol (pH 5.7) was applied to the column equilibrated in the same buffer. Elution was carried out in three steps: (A) 30 min elution with starting buffer; (B) a linear gradient of 0–100% of 1.0 *M* ammonium acetate (pH 5.7) for 60 min; (C) a final isocratic run of 1.0 *M* ammonium acetate for 30 min. The flow-rate was set at 1.0 ml/min for the complete cycle, and the fractions were detected with a variable-wavelength UV monitor set at 280 nm (a.u.f.s. 0.512). Fractions 1–5 were collected manually, dried under vacuum and used for SDS-PAGE (Fig. 3) and amino acid analysis (Tables I and II). Fraction N indicates the peak of the same major long-chain neurotoxin as in Fig. 1.

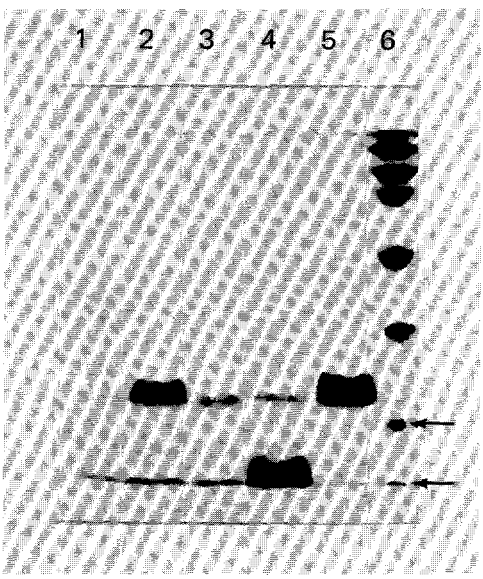

Fig. 3. Gel electrophoresis of the fractionated PLA_2 fractions under denaturing conditions (SDS-PAGE) in the presence of 5 m*M* dithiothreitol. Lane 6 contains standard proteins used as molecular mass markers (in kDa): transferrin (80), bovine serum albumin (66), ovalbumin (45), carbonic anhydrase (30), soybean trypsin inhibitor (20.1) and lysozyme (14). Lanes 1–5 correspond to the five numbered fractions indicated in Fig. 2. The gel was stained with Coomassie blue. The arrows point to the electrophoretic positions of lysozyme and bromophenol blue dye.

previously [6]. The PLA_2 fractions require a second chromatographic step before sequence analysis. We have found that these multiple PLA_2 components are very soluble in TFA and acetonitrile, and can be easily desalted and purified by reversed-phase (RP) HPLC without loss of activity (data not shown). At least three distinguishable PLA_2 with different retention times could be obtained by rechromatography of the pooled PLA_2 fractions on RP-HPLC. These observations prompted us to analyse the crude venom on an HPLC column packed with cation-exchange CM-300, a material similar to TSK CM-650 used in Fig. 1. It is noteworthy that by using cation-exchange HPLC the PLA_2 fractions could be separated into at least five well-defined fractions (Fig. 2).

The purities of each fraction were also checked with SDS-PAGE (Fig. 3). The patterns for most fractions are quite clear-cut regarding the homogeneity of each separated PLA_2, based on their molecular size. The fraction F-1 apparently contains several components with molecular masses greater than the typical 14 000–16 000 range for most elapid PLA_2 identified on SDS-PAGE. Therefore the

TABLE I

AMINO ACID COMPOSITIONS OF FRACTIONS WITH PHOSPOLIPASE A_2 ACTIVITY

Fractions 2, 3 and 5 correspond to the fractions labelled in Fig. 2. Data are expressed as the number of residues per molecule of protein using alanine as the reference to calculate the residues of other amino acids. Values represent the mean of duplicate determinations. The hydrolysis condition is microwave irradiation for 5 min using 6 *M* HCl of 4 *M* methanesulphonic acid containing 0.2% 3-(2-aminoethyl) indole.

Amino acid	F-2	F-3	F-5
1/2 Cys	12.8 (14)	13.1 (14)	13.4 (14)
Asx	22.2 (22)	21.4 (21)	21.3 (21)
Thr	4.7 (5)	4.3 (5)	4.6 (5)
Ser	4.9 (5)	4.2 (5)	5.3 (6)
Glx	7.6 (8)	7.8 (8)	7.2 (7)
Pro	3.8 (4)	3.8 (4)	3.7 (4)
Gly	8.8 (9)	8.9 (9)	9.3 (9)
Ala	11	11	11
Val	3.7 (4)	3.9 (4)	3.8 (4)
Met	0.8 (1)	0.6 (1)	0.9 (1)
Ile	4.3 (4)	4.4 (4)	4.5 (5)
Leu	4.9 (5)	4.7 (5)	4.7 (5)
Tyr	8.7 (9)	8.6 (9)	8.7 (9)
Phe	3.8 (4)	3.2 (3)	3.6 (4)
His	0.7 (1)	0.9 (1)	0.8 (1)
Lys	4.8 (5)	4.9 (5)	4.7 (5)
Arg	5.3 (5)	5.8 (6)	5.7 (6)
Trp	2.8 (3)	2.7 (3)	2.5 (3)
Total residues	(119)	(118)	(120)

characterization of PLA_2 is restricted to F-2, F-3 and F-5. Fraction 4 (F-4) was found to be contaminated with a toxin component of lower molecular mass.

The amino acid compositions of F-2, F-3 and F-5 with apparent electrophoretic homogeneity are shown in Table I. They were all shown to be similar to PLA_2 of *Naja naja kaouthia* [8], which is supposed to be a species closely related or identical to *Naja naja siamensis* [6]. It is of interest to note that the amino acid compositions are almost identical, except for some minor variation in the contents of aspartic acid, glutamic acid, isoleucine, phenylalanine and arginine. Since F-4 showed a major component of *ca.* 8 kDa instead of 15 kDa for PLA_2, its amino acid composition was determined and compared with that of the major long-chain neurotoxin denoted as Fr. N (Table II). The slight difference between these two fractions is caused by the contaminant PLA_2, as indicated in SDS-PAGE of Fig. 3. Notably this toxin possesses smaller amounts of aromatic amino acids, such as tyrosine and tryptophan, than PLA_2 (Table I).

The sequence changes among these PLA_2 isoforms (F-2, F-3 and F-5) must be small, probably indicating only limited variation of the amino acid residues in

TABLE II

AMINO ACID COMPOSITIONS OF FRACTIONS WITH NEUROTOXICITY

Fractions 4 and N correspond to the fractions labelled in Fig. 2. Data are expressed as the number of residues per molecule of protein using alanine as the reference to calculate the residues of other amino acids. Values represent the mean of duplicate determinations. The hydrolysis condition is microwave irradiation for 5 min using 6 *M* HCl of 4 *M* methanesulphonic acid containing 0.2% 3-(2-aminoethyl) indole.

Amino acid	Fr. N	F-4
1/2 Cys	9.5 (10)	9.8 (10)
Asx	8.6 (9)	9.6 (10)
Thr	8.5 (9)	7.6 (8)
Ser	2.5 (3)	2.7 (3)
Glx	0.8 (1)	2.2 (2)
Pro	5.6 (6)	4.7 (5)
Gly	3.9 (4)	4.9 (5)
Ala	3	3
Val	3.8 (4)	3.8 (4)
Met	0.2 (0)	0.8 (1)
Ile	4.5 (5)	4.7 (5)
Leu	0.9 (1)	1.7 (2)
Tyr	0.8 (1)	1.3 (1)
Phe	2.9 (3)	2.8 (3)
His	0.8 (1)	0.6 (1)
Lys	4.8 (5)	4.7 (5)
Arg	4.9 (5)	4.7 (5)
Trp	0.8 (1)	0.5 (1)
Total residues	(71)	(74)

their primary structures. The most common one is likely to be the deamidation of asparagine of glutamine to aspartic acid of glutamic acid, respectively. Indeed, N-terminal sequence analysis of these three PLA_2 by microsequencing indicated only one amino acid difference among the first 20 amino acid, with Asn being identified as the 20th residue in F-5 in contrast to Asp present in F-2 and F-3 (Table III). We have also sequenced neurotoxic F-4 and Fr. N by straightforward Edman degradation: the partial sequences are shown in Table III for comparison. Microsequencing of F-4 revealed two residues at each stap of Edman degradation, which is reflective of the two toxins in the sample as indicated by SDS-PAGE (Fig. 3).

With the advances in HPLC instrumentation and microsequencing, the complete sequences of these small toxins and PLA_2 could be carried out with the fractions obtained from single-step semipreparative HPLC on the automatic protein sequencer in a relatively short time. The upshot of this report is to establish venom separation and purification by HPLC as a convenient means of toxin isolation, with special regard to obtaining sufficient pure material for sequence

TABLE III

THE AMINO-TERMINAL SEQUENCES OF ISOLATED FRACTIONS FROM CM-300 HPLC

The residues with more than one amino acid denote those positions where more than one phenylthiohydantoin derivative was detected by automatic sequencing. Note that F-4 has a composite sequence consisting of F-5 and Fr. N in a molar ratio of 1:8, as estimated from the yield ratio of detected amino acids in each step by microsequencing.

Amino acid	F-3	F-5	F-4	Fr. N
1	Asn	Asn	Asn/Ile	Ile
2	Leu	Leu	Leu/Arg	Arg
3	Tyr	Tyr	Tyr/Cys	Cys
4	Gln	Gln	Gln/Phe	Phe
5	Phe	Phe	Phe/Ile	Ile
6	Lys	Lys	Lys/Thr	Thr
7	Asn	Asn	Asn/Pro	Pro
8	Met	Met	Met/Asp	Asp
9	Ile	Ile	Ile	Ile
10	Gln	Gln	Gln/Thr	Thr
11	Cys	Cys	Cys/Ser	Ser
12	Thr	Thr	Thr/Lys	Lys
13	Val	Val	Val/Asp	Asp
14	Pro	Pro	Pro/Cys	Cys
15	Asn	Asn	Asn/Pro	Pro
16	Arg	Arg	Arg/Asn	Asn
17	Ser	Ser	Ser/Gly	Gly
18	Trp	Trp	Trp/His	His
19	Trp	Trp	Trp/Val	Val
20	Asp	Asn	Asn/Cys	Cys

analysis. The preparative HPLC method has shortened the overall analysis time for protein separation to less than a few hours for most crude extracts of venom samples. Coupled with current microsequencing techniques, it facilitates the verification of published sequences and the determination of the new enzyme or toxin analogues from the same or closely related snake species.

ACKNOWLEDGEMENTS

This work was supported in part by Academia Sinica and the National Science Council, Taipei, Taiwan, Republic of China.

REFERENCES

1 C. Y. Lee, in B. Ceccarelli and F. Clementi (Editors), *Advances in Cytopharmacology*, Vol 3, Raven Press, New York, 1979. pp. 1–16.
2 E. Karlsson, in C. Y. Lee (Editor), *Snake Venoms, Handbook of Experimental Pharmacology*, Springer, Berlin, Vol. 52, 1979, pp. 159–212.
3 U. K. Laemmli, *Nature*, 227 (1970) 680.
4 S.-H. Chiou and K.-T. Wang, *J. Chromatogr.*, 448 (1988) 404.
5 S.-H. Chiou and K.-T.- Wang, *J. Chromatogr.*, 491 (1989) 424.
6 S.-H. Chiou and W.-W. Lin and W.-P. Chang, *Int. J. Peptide Protein Res.*, 34 (1989) 148.
7 M. A. Wells and D. J. Hanahan, *Methods Enzymol.*, 14 (1969) 178.
8 F. J. Joubert and N. Taljaard, *Eur. J. Biochem.*, 112 (1980) 493.