Purification and Characterization of Phospholipase A and A Short Peptide from Venom of Cobra (Naja naja naja) by High Performance Liquid Chromatography

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Summary: Phospholipase-A has been purified to homogeneity by combination of reverse-phase HPLC with gel permeation or ion-exchange HPLC. The amino acid composition and N-terminal sequence analysis has also been determined. A difference at position 10 has been observed from other species of Naja naja naja. The preparation is being employed for further structural characterization.

A short peptide (MW 3kDa) has also been purified from the PL-A containing fraction. The amino acid composition, N-terminal sequence, molecular mass by SDS-PAGE and mobility on PAGE has been determined. The peptide seems to represent a new type of small peptide in Cobra venom.

Introduction

Phospholipase-A (PL-A) is an extensively studied enzyme of snake venoms and has been purified by various methods including heat treatment, gel filtration, electrofocusing and multiple ion-exchange chromatography [1-5]. Recently separation of PL-A from rat spleen was achieved by reverse phase HPLC [6]. In present studies we have employed a rapid two step procedure for purification of PL-A using HPLC. The purification was achieved by either (a) reverse phase and gel permeation HPLC or (b) reverse phase and ion exchange HPLC. The procedure provides a better separation of protein fractions than conventional methods. A low molecular mass peptide which elutes alongwith PL-A has also been purified and characterized.

Materials and Methods:

The collection and fractionation of the venom from Naja naja naja on reverse-phase HPLC has been reported earlier [7]. The peak containing PL-A activity was further purified by gel permeation HPLC on a column of TSK 2000 (LKB, Bromma, Sweden), using 0.1% Trifloroacetic acid (TFA). The other method used in separation of the peak was on DEAE ion-exchange HPLC column TSK-DEAE SPW, (LKB, Bromma Sweden) using ammonium acetate buffer (20mM, pH6.5) with a linear gradient of 0.5M NaCl in the same buffer.

The assay for PL-A activity was carried out at room temperature according to De Haas et al. [8].

Molecular mass of the components were determined by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE, 15% gels) in vertical slabs [9].

Polyacrylamide gel electrophoresis (PAGE) was carried out in vertical slab gels at pH 8.9 using 10% gels [10].

Amino acid composition was determined on a Biotronik, amino acid analyzer LC6001, (Biotronik GmbH,

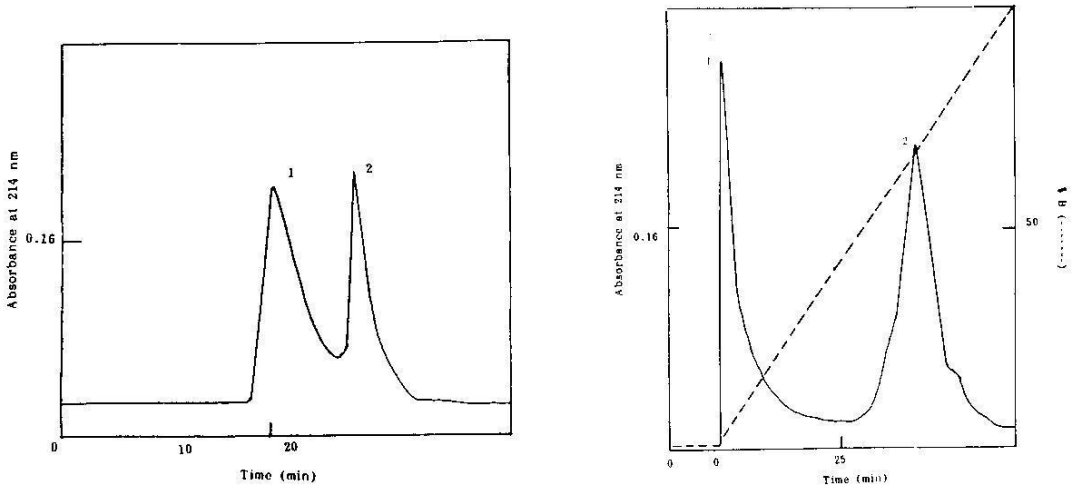


Fig-1: Elution profile of phospholipase-A activity containing peak from RP-HPLC by (A) gel permeation-HPLC on a column of TSK-2000 SW in 0.1% TFA (B) ion exchange-HPLC on a TSK-DEAE column in ammonium acetate buffer 20mM, pH 6.5, with a linear gradient of same buffer containing 0.5M NaCl. Peak 1 represents PL-A and peak 2 is the short peptide in both profiles. Absorbance was monitored at 214 nm.

Munich, West Germany). The samples were hydrolyzed with 5.7N HCl containing 0.5 % phenol under vacuum at 110°C for 20 h. [11].

N-terminal amino acid sequence was determined by Dimethylamino-azobenzene 4'-isothiocyanate/Phenyl-isothiocyanate (DABITC/PITC) double coupling micro sequencing method [12] and DABTH amino acids were identified on polyamide sheets (Chen Chin Trading Co. Taiwan) by two dimensional chromatography using water and acetic acid (2:1) in the first dimension and toulene, hexane and acetic acid (6:4:1) in the second dimension. The plates were developed by HCl vapours [12].

Results

The separation profile of the crude venom was similar to the one reported earlier [7]. The elution pattern of PL-A activity containing peak on a TSK-2000 column is shown in fig.(1A) and on TSK-DEAE column in fig.(1B). The separation profiles show that the peak containing PL-A also contain another protein component.

SDS-PAGE of PL-A active fraction from reverse-phase HPLC in both separation procedures showed the presence of a 14kDa protein component and a 3kDa protein (fig.2) as reported by us earlier [7]. The results of PAGE at pH8.9 showed two bands, confirming the presence of two proteins (fig.3). The separation on TSK-2000 or DEAE ion-exchange column completely resolved the two components (fig.2 and 3).

The PL-A activity was found in Peak 1 (figs.1A, 1B), while peak 2 (figs.1A, 1B) did not show any PL-A activity.

TABLE-I

AMINO ACIDS	PL-A	Short Peptide (3 kDa)
Asp	19.97 (20)	2.8 (3)
Thr	5.55 (6)	1.4 (1)
Ser	6.17 (6)	1.2 (1)
Glu	8.5 (8)	1.7 (2)
Pro	6.17 (6)	3.2 (3)
Gly	10.0 (10)	1.6 (2)
Ala	9.14 (9)	0.9 (1)
Cys	10.18 (10)	1.5 (2)
Val	6.22 (6)	1.0 (1)
Met	1.34 (1)	0.4 (0)
Ile	4.40 (4)	1.9 (2)
Leu	7.17 (7)	1.2 (1)
Tyr	8.82 (9)	1.2 (1)
Phe	2.86 (3)	0.7 (1)
His	1.41 (1)	0.7 (1)*
Trp	n.d.	- (1)*
Lys	7.5 (8)	2.2 (2)
Arg	6.17 (6)	- (0)
Total	120	25

* Tryptophan was determined by N-terminal sequence analysis.

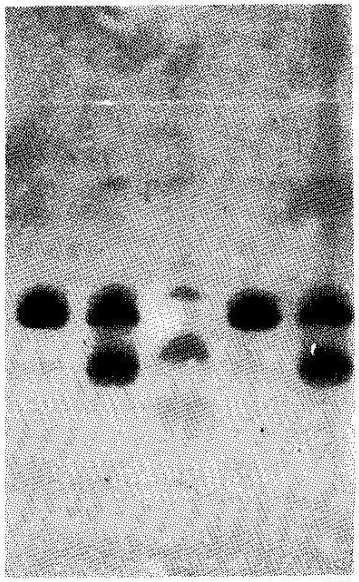
Amino acid composition of phospholipase A and 3kDa short peptide after acid hydrolysis. Values are given in molar ratios and those in parenthesis are closest integer and are calculated by considering a total of 120 residues for PL-A and 25 for short peptide. Values for cysteine are without modification and therefore lower as compared to other phospholipases. Tryptophan was not determined due to destruction during acid hydrolysis, value of tryptophan in the short peptide was determined by sequence analysis.

The amino acid compositions of peak 1 and peak 2 are given in table-I. The PL-A composition (peak 1) of Naja naja naja is compared with those of known phospholipases from other Naja species and shown in table-II.

N-terminal amino acid sequence of peak 1 and 2 are given in table-IIIA and IIIB respectively. The PL-A characterized from Naja naja naja shows similarity with other naja species (Table-IIIA) whereas the N-terminal amino acid sequence of peak 2 showed no homology with PL-A or toxins reported (Table-IIIB).

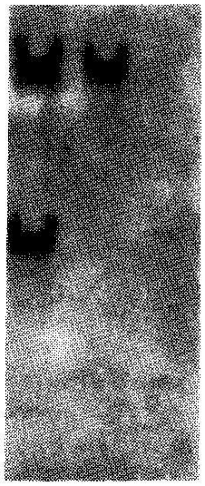
Discussion

The use of HPLC for separation of venom made it possible to obtain PL-A from venom of cobra in pure state by a simple two step procedure. It was observed during the separation of crude venom on reverse phase column of Vydac C-18, that all the PL-A activity eluted as a single peak [7]. The SDS-PAGE and PAGE showed the presence of two proteins (figs.2, 3). The separation of these two



1 RP-PL LM 1 RP-PL

Fig-2: SDS-PAGE of phospholipase and that of short 3 kDa peptide. M = low molecular mass standard (2.5 - 17 kDa). Numbers represent peaks from Fig 1A and 1B after gel permeation-HPLC and ion exchange-HPLC. RP-PL is the phospholipase containing peak after reverse phase HPLC.



PP-PL 1 2

Fig-3: PAGE of phospholipase-A containing peak of RP-HPLC and after purification on gel permeation-HPLC. Peak numbers as in Fig 1A.

proteins was achieved by gel permeation HPLC or ion-exchange HPLC as both the protein possess different charges and molecular mass. The activity was present in peak 1 (figs.1A, 1B) while peak 2 (figs.1A, 1B) did not show any PL-A activity.

The separation on gel permeation using TSK-2000 column was found to be relatively simpler than on DEAE column as it eliminates the desalting step.

The use of acetonitrile and 0.1 % TFA as eluent was selected in this system as it dissolves phospholipases and does not affect its activity, even at higher concentrations of acetonitrile [13]. Furthermore these solvents could be removed easily by lyophilization.

TABLE-II

AMINO ACIDS	Naja naja naja *	Naja naja atra [14]	Naja naja sputatrix[15] I	Naja naja sputatrix[15] II	Naja naja siamensis[16] I	Naja naja siamensis[16] II	Naja naja kaouthia[17] CMII	Naja naja kaouthia[17] CMIII
Asp	20	20	20	20	22	20	22	20
Thr	6	5	5	5	5	5	5	5
Ser	6	6	5	5	5	5	5	6
Glu	8	8	9	8	8	8	8	8
Pro	6	5	5	5	4	4	4	4
Gly	10	10	11	11	8	9	8	10
Ala	9	11	12	12	11	11	11	11
Cys	10	12	12	12	14	14	14	14
Val	6	4	4	4	4	4	4	4
Met	1	1	1	1	1	1	1	1
Ile	4	5	4	4	4	4	4	4
Leu	7	5	6	6	5	5	5	5
Tyr	9	9	10	9	9	9	9	9
Phe	3	4	4	4	4	4	4	4
His	1	1	1	1	1	1	1	1
Trp	n.d.	3	0	0	3	4	3	3
Lys	8	5	7	7	5	5	5	5
Arg	6	5	6	6	6	6	6	5
TOTAL	120	119	122	121	119	119	119	119

* As determined in present study.

Amino acid composition of phospholipases from different Naja species characterized so far

TABLE-III

A:

	1.				5					10
1.	Asn	Leu	Tyr	Gln	Phe	lys	Asn	Met	Ile	lys
2.	Asn	Leu	Tyr	Gln	Phe	Lys	Asn	Met	Ile	His
3.	Asn	Leu	Tyr	Gln	Phe	lys	Asn	Met	Ile	Gln

1. N.n. naja
2. N. melanoleuca [18]
3. N. n. Kaouthia [17]

B:

1	2	3	4	5	6	7	-
Phe	Val	Trp	Ala	Pro	Cys	Pro	-

Short peptide (3 kDa).

Table-IIIA:
N-Terminal homology alignment of PL-A from Naja naja naja with other Naja species.

Table-IIIB:
N-Terminal amino acid sequence of short peptide 3kDa eluted along with PL-A on RP-HPLC.

The PL-A obtained by both the methods gave single homogenous bands on SDS-PAGE (fig.2) and PAGE (fig.3), confirming the purity. The amino acid composition of Naja naja naja PL-A (Table-I) shows similarity to other phospholipases (Table-II). This similarity is further supported by N-terminal amino acid sequence (Table-IIIA). The N-terminal amino acid sequence of the 3 kDa component (Table-IIIB) showed no resemblance to any part of PL-A sequence.

The earlier report on PL-A from Naja naja naja also mentioned the presence of contaminating peptide and suggested it to be a related PL-A type or a processed fragment [5]. In the present study this peptide has been identified to be a low molecular mass peptide having a higher mobility towards cathode on PAGE, with different amino acid composition and N-terminal sequence from characterized venom proteins.

In the present study only one type of PL-A was isolated and characterized, no evidence for PL-A activity was found in other fractions of RP-HPLC, whereas other reports showed the presence of more than one PL-A form Naja naja sputatrix, Naja naja siamensis and Naja naja kaouthia except Naja naja atra which is reported to have one PL-A. The amino acid composition of PL-A from Naja naja naja and that from Naja naja atra are very similar. The complete amino acid sequence of PL-A from Naja naja naja is in progress and will be reported elsewhere.

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