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The amino acid sequence and properties of an edema-inducing Lys-49 phospholipase A_2 homolog from the venom of *Trimeresurus mucrosquamatus*

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Three phospholipase A_2 enzymes or homologs were purified from the venom of *Trimeresurus mucrosquamatus* (Taiwan habu). The most abundant one was found to be a phospholipase homolog without enzyme activity, and its complete amino acid sequence was determined using oligopeptide fragments derived from digestion by endopeptidases Glu-C, Asp-N, Lys-C and α-chymotrypsin, and by means of gas-phase sequencing. The sequence revealed that the protein belonged to the Lys-49 family of snake venom phospholipase A_2. This protein's function was characterized as edema-inducing. The Lys-49 protein has the potential to bind membrane phospholipid and Ca^{2+} ($K_d = 1.6 \cdot 10^{-4}$ M) as shown by ultraviolet difference spectra; however, the catalytic site appeared to be inactive and the edematous response was independent of the protein's hydrolytic activity. Mast cells and platelets were shown to be subject to activation by the Lys-49 protein.

Introduction

Snake venom is a rich source of both the structural and functional varieties of phospholipase A_2 (PLA_2, EC 3.1.1.4). For example, in the venom of king brown snake (*Pseudechis australis*), there are more than thirteen PLA_2's all differing in structures and enzyme capacities [1,2]. Besides a catalyst for the hydrolysis of phospholipids, PLA_2 from snake venoms functions in other ways, such as playing the roles of neurotoxin [3], myotoxin [4], carditoxin [5], anticoagulant [6] and an edema-inducing principle [7,9]. Based on their primary structures, PLA_2 were classified into two groups [8]. Group I comprises those from mammalian pancreatic juices and the venoms of snake families Elapidae and Hydrophidae while group II includes those from the venoms of Crotalidae and Viperidae.

Teng et al. have isolated the most basic and abundant phospholipase A_2 homolog, termed FXXII-2 [9] or $TMVPLA_2$ II [12,13], from the venom of *Trimeresurus mucrosquamatus* and shown it to be an edema-producing principle [9–13]. In our work here, we have further purified this component by means of HPLC, and elucidated its primary structure. It turns out to be a member of Lys-49 PLA_2 which was originally discovered in the venom of *Agkistrodon p. piscivorus* by Maraganore and Heinrikson [14] and more recently in the venom of *Trimeresurus flavoviridis* by Liu and Ohno et al. [15]. The abundant existence of the Lys-49 phospholipase A_2 homologs in some Crotalidae and Viperidae venoms has been puzzling because their enzyme activities are not easily detectable [20] and their biofunctions ambiguous [22]. This study may help to explain certain aspects of their structure and function.

Materials and Methods

Materials

Crude venom of *T. mucrosquamatus* was supplied by Dr. M. I. Liao of National Institute of Preventive medicine, Taipei, Taiwan. CM-Sephadex C-50 and Sephadex G-75 were obtained from Sigma. Chemcosorb ODS-H (C_{18}, 5 μ) was obtained from Chemcosorb Scientific, Osaka. The enzymes used in the fragmenta-

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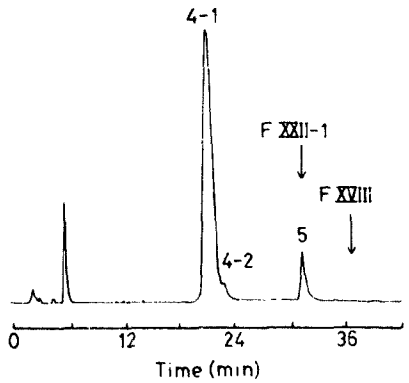

Fig. 1. Purification of TMV-K49 by reverse-phase HPLC. About 1 or 2 mg of FXXII-2 obtained from ion-exchanger and gel-filtration chromatographies were injected on an HPLC system with a Chemcosorb ODS-H (C_{18}) column (10×250 mm). The elution was effected with a linear gradient of 27–40% mobile phase (0.07% TFA in acetonitrile) over 1 h. Flow rate was 2 ml/min and the absorbance scale at 280 nm was 0.5. Arrows indicate the condition of PLA_2 eluted if FXXII-1 or XVIII [9] were applied.

tion experiments were lysyl endopeptidase (Wako, Japan), endoproteinase Glu-C (Boehringer), endoproteinase Asp-N (Boehringer) and α-chymotrypsin (Worthington). Other reagents were of the highest quality commercially available.

Isolation and purification of the protein

Fractions XVIII, XXII-1 and XXII-2 were obtained from CM-Sephadex C-50 and G-75 chromatography of *T. mucrosquamatus* venom, as previously described [9]. The lyophilized sample was dissolved in 0.07% trifluoroacetic acid (TFA) and further purified by reverse-phase HPLC (Fig. 1). The first emerging, major component, denoted as TMV-K49, was used in the present study. The purity was assessed by SDS-PAGE and N-terminal sequencing.

Enzyme digestions and separation of the peptides

After reduction and alkylation by iodoacetamide, the RCM protein (TMV-K49) was subjected to enzyme digestions as follows: (1) lysyl endopeptidase in 0.05 M Tris-HCl (pH 9.0) and 1 M guanidine HCl at 37°C for 3 h with substrate/enzyme ratio of 50/1 (w/w); (2) endoproteinase Asp-N in 0.05 M sodium phosphate buffer (pH 7.5) and 1 M guanidine HCl at 37°C for overnight with substrate/enzyme ratio of 250/1 (w/w); (3) endoproteinase Glu-C in 0.05 M ammonium bicarbonate (pH 8.0) and 1 M guanidine HCl at 37°C for 3 h with substrate/enzyme ratio of 40/1 (w/w); and (4) α-chymotrypsin in 0.05 M ammonium bicarbonate (pH adjusted to 6.5 by dilute acetic acid) at 25°C for 1 h with substrate/enzyme ratio of 110/1 (w/w).

Digestion was halted by addition of acetic acid and the aliquot was injected into a HPLC column. Separation of the peptides was effected by a linear gradient consisting of 0.07% TFA in water (solvent A) and 0.07% TFA in acetonitrile (solvent B). Amino acid compositions were determined by HPLC separation of DABSYL-amino acids, after pre-column dabsylation of the amino acids generated by a 30-min gas-phase acid hydrolysis [16,17].

Sequence determination

After HPLC purification, TMV-K49 and its oligopeptide fragments were subjected to automatic amino acid sequencing. Edman degradation was carried out in a pulsed-liquid type sequenator (model 477A, Applied Biosystems) accompanied with an on-line PTH amino acid analyzer. The program 'normal-1' was used.

Assay of phospholipase activity

The activity was measured using the pH-stat titration method [1,15]. Synthetic dipalmitoyl L-phosphatidylcholine (Sigma) was emulsified at a concentration of 2.5 mM in a substrate solution containing 2.5 mM sodium deoxycholate, 0.05 mM Na_2-EDTA, and 100 mM NaCl. After introducing 6–10 mM $CaCl_2$, the PLA_2 sample (≤ 50 μl) was added to 2.5 ml of the substrate and the reaction proceeded at 37°C, the amounts of fatty acid liberated being automatically titrated at pH 7.5 with 4 mM NaOH solution under purging N_2 gas on a pH-stat apparatus (RTS 822, Radiometer, Denmark). The rate of hydrolysis was calculated from the alkali consumption during the first 5 min and was expressed as mol of fatty acid liberated/min per mg protein.

Ultraviolet difference spectroscopy

Difference spectra were recorded on a double-beam spectrophotometer (Hitachi U3200) using 1-cm pathlength cells. Protein concentrations in both cells were 50 μM. Different amounts of $CaCl_2$ (18 mM) stock solutions were added to the sample cell and an equal vol. of buffer to the reference cell. Buffer solutions used in the titrations were 50 mM Tris-HCl and 0.1 M NaCl (pH 8.0). Analyses of the spectral data on the interaction between phospholipase A_2 and Ca^{2+} were carried out as previously described [18] for the Scatchard (1949) model of ligand-protein interaction. For a single class of binding sites the following equation holds,

$$(\Delta A/\Delta A_{max})/[Ca^{2+}] = (n/K_d) - (\Delta A/\Delta A_{max}/K_d)$$

where $(\Delta A/\Delta A_{max})$ is the saturation degree, n the number of binding sites, K_d the average dissociation constant of the enzyme-Ca^{2+} complexes and $[Ca^{2+}]$ the free calcium concentration. Thus a plot of $(\Delta A/\Delta A_{max})/[Ca^{2+}]$ vs. $\Delta A/\Delta A_{max}$ yields a straight line

from which the value of n can be derived by extrapolation and the slope corresponds to $-1/K_d$.

Effect on platelets and mast cells

Washed platelet suspension was obtained from rabbit blood anticoagulated with EDTA (6 mM) by several centrifugations and the washing procedure as described previously [24]. The final platelet pellets were suspended in Tyrode's solution of the following composition (mM): NaCl (136.8), KCl (2.8), $NaHCO_3$ (11.9), $MgCl_2$ (1.1), NaH_2PO_4 (0.33), $CaCl_2$ (1.0) and glucose (11.2). Platelet aggregation was measured by the turbidimetric method of O'Brien [25] at 37°C using a Chrono-Log Lumiaggregometer. The platelet suspension was stirred at 1200 rpm just 1 min before the addition of venom protein. The percentage of aggregation was calculated assuming the absorbance of platelet suspension as 0% aggregation and the absorbance of Tyrode's solution as 100% aggregation.

The isolation of mast cells from a rat's peritoneal cavity, together with the study of their release of histamine and β-glucuronidase, were as previously described [12,13].

Measurement of rat hind-paw edema and effect on nerve-muscle preparation

Wistar rats (180–220 g) were used. Hind-paw edema was induced by a single subplantar injection of 10 µl of irritant in 0.05 M phosphate buffer saline (PBS, pH 7.4) and an equal vol. of PBS into the right and left hind-paw, respectively. The vol. of both hind-paws of each rat were measured with a plethysmometer (Model 7150, Ugo Basile) at different times after the injection. Percent hind-paw swelling was calcuated as following: hind-paw edema (%) =

$$\left(\frac{\text{right paw vol.}}{\text{initial vol.}} - \frac{\text{left paw vol.}}{\text{initial vol.}} \right) \times 100$$

$$\text{right paw initial vol.} \qquad \text{left paw initial vol.}$$

The data were also analyzed to compare the area under the time course curve. Neurotoxicity and myotoxicity were measured electrophysiologically as previously described [5].

Results

Determination of amino acid sequence of TMV-K49

In previous papers by Teng and the co-workers, three PLA_2's were isolated from the venom of *T. mucrosquamatus* and designated respectively as FXVIII, FXXII-1 and FXXII-2 [9]. FXVIII and FXXII-2 were named TMVPLA$_2$ I and II in the subsequent study of their function [12,13]. By means of HPLC, we found that FXVIII (i.e., TMVPLA$_2$ I) and FXXII-1 were rather pure (showing a single peak), but FXXII-2 were the combination of 75% TMV-K49 and 20% FXXII-1. As shown in Fig. 1, HPLC effectively separated TMV-K49 (peak 4–1) from FXXII-1 (peak 5) and another minor PLA_2 (peak 4–2). Homogeneity of the purified protein is confirmed by the results of automatic N-terminal amino acid sequencing and the single band with a molecular mass of 14 kDa in the SDS-PAGE pattern under non-reducing and reducing conditions (not shown). Automatic amino acid sequencing of RCM protein established the N-terminal 47 amino acid se-

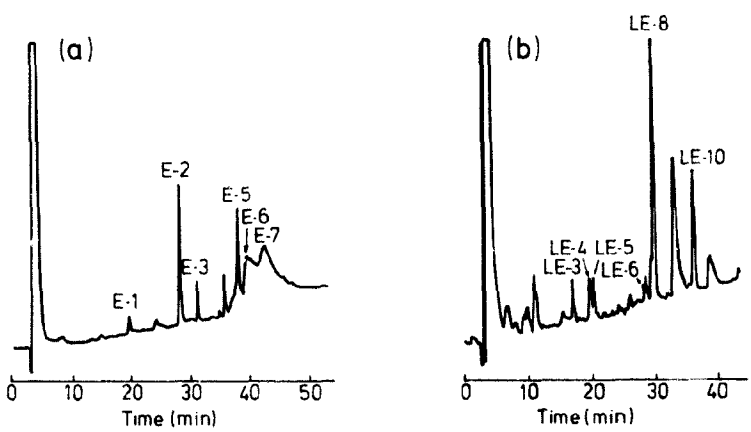

Fig. 2. Preparative HPLC peptide maps of (a) endoproteinase Glu-C digest and (b) lysyl endopeptidase digest of TMV-K49. (a) Aliquot containing 0.1 mg digest was applied to a Nucleosil C18 column (4.6×250 mm). Solvent A was 0.07% TFA in water. The gradient was linear from 10–50% solvent B (0.07% TFA in acetonitrile) over 80 min and monitored at 220 nm with a flow rate of 1 ml/min. Full absorbance was set at 0.06. (b) Aliquot containing 0.13 mg digest was chromatographed under the same condition mentioned above except that the elution was carried out by 5–40% solvent B for 50 min.

TABLE I

Amino acid compositions of peptides obtained from various enzyme digestions (see Figs. 2 and 3)

Values in parenthesis are those found in the sequence determination.

	E-1	E-2	E-3	E-5-a	E-5-b	LE-3	LE-4	LE-5	LE-6	LE-8-a	LE-8-b	LE-10	DN-3	Ch-4	Ch-5
Asx		2.5(2)	1.1(1)	1.9(2)			1.5(1)				1.8(2)	2.3(2)	1.4(2)		2.2(2)
Glx	1.0(1)	3.0(3)	1.0(1)	2.0(2)	2.0(2)	1.2(1)			0.8(1)	1.8(2)	0.8(1)	1.3(2)			
CMC		2.7(3)	1.4(2)	1.1(1)		0.5(1)	0.6(1)	1.0(1)	1.9(2)			0.6(1)	4.1(5)	1.9 2)	1.0(1)
Ser	1.2(1)								1.6(1)		1.8(2)				
Thr				1.7(2)						0.6(1)		1.0(1)	1.0(1)		0.9(1)
Gly		1.5(1)			1.5(1)	1.3(1)				1.0(1)			1.5(1)		1.1(1)
Ala		1.5(1)	1.6(2)	1.1(1)		1.4(1)			1.6(1)			1.9(2)			
Arg			1.0(1)	0.9(1)							1.0(1)	1.0(1)	1.1(1)		1.0(1)
Pro		2.5(2)		1.5(1)		1.0(1)	1.6(2)		1.5(1)				0.8(1)		1.0(1)
Val		1.4(2)	0.9(1)	1.5(2)								0.9(1)	1.5(2)	0.6(1)	1.1(1)
Met					1.6(1)					0.8(1)					
Ile	0.8(1)	0.9(1)	0.6(1)		1.5(1)	0.8(1)				0.6(1)		0.7(1)			
Leu	0.8(1)	1.0(1)	0.8(1)	2.0(2)	1.0(1)		0.8(1)	0.8(1)	0.8(1)			1.7(2)			
Phe				1.0(1)	1.0(1)			0.8(1)	0.9(1)	0.7(1)			1.0(1)		
Lys		2.4(3)	0.9(1)	3.7(4)	0.8(1)	1.0(1)	1.0(1)	1.0(1)	– (1)	1.0(1)	1.0(1)	1.0(1)	3.7(5)	1.0(1)	3.7(4)
His				0.9(1)								1.1(1)	1.4(1)	0.8(1)	
Tyr				0.6(1)							1.8(2)		0.7(1)	0.7(1)	1.1(1)
Trp											– (1)				
Sequence position	1–4	70–88	89–99	100–120	5–12	72–78	79–84	114–117	114–122	8–15	62–71	92–106	41–61	46–51	52–64

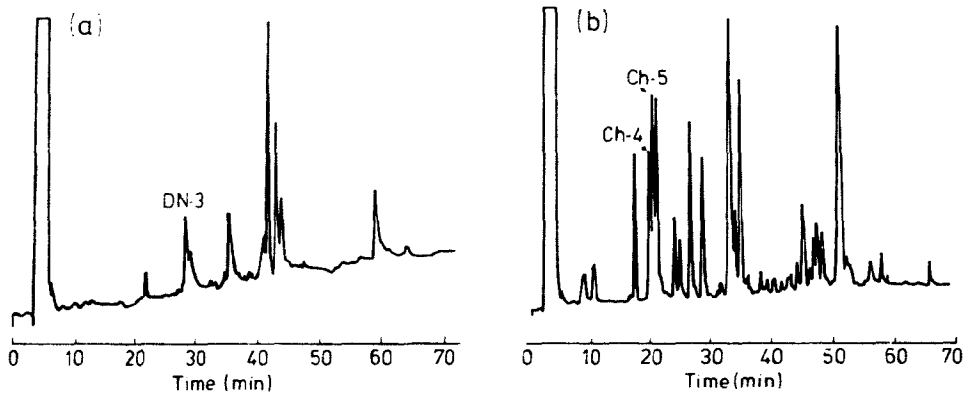

Fig. 3. Peptide maps of (a) endoproteinase Asp-N digest and (b) α-chymotrypsin digest of TMV-K49. (a) Aliquot containing 0.1 mg digest was chromatographed as mentioned in the legend of Fig. 2(a) except that the elution was 5–60% solvent B over 70 min and full absorbance was 0.1. (b) Aliquot containing 0.15 mg digest was chromatographed under the same condition of (a) except that the elution was 5–45% solvent B over 70 min and full absorbance was 0.2.

quences. A typical amino acid composition of RCM protein was obtained as follows: D11.9 (13) E10.4 (9) CMC13.4 (14) S3.8 (4) T4.5 (5) G10.0 (10) A5.6 (5) R5.2 (5) P5.8 (6) V7.9 (10) M1.8 (1) I3.2 (4) L7.0 (7) F3.0 (3) K13.1 (17) H2.1 (2) Y5.2 (6). Repeated experiments always showed the lower values for Lys and this can be attributed to incomplete hydrolysis of three Lys–Lys linkages in the molecule.

In Fig. 2a and b, the preparative HPLC of peptides from endoproteinase Glu-C and lysyl endopeptidase digests are displayed. Only those peptide peaks involved in the sequencing are numerated. Likewise, two other peptides maps arising from endoproteinase Asp-N and α-chymotrypsin digests are shown respectively in Fig. 3a and b. Amino acid compositions of the relevant peptides are shown in Table I, as well as the location of each peptide. Since the peptide E-5 was the mixture of two peptides at about equal molarity, as evidenced from the PTH amino acids, the values of E-5-a and E-5-b were apportioned from the gross compositions of E-5. Likewise LE-8-a and LE-8-b received appropriate values from that of LE-8. The two peptides, E-6 and E-7, were further purified by HPLC with less steep gradient to obtain the major peak, respectively, as E-6-3 and E-7-2 (not shown). Owing to the scarcity of material, peptides E-6-3 and E-7-2 were directly used for the sequencing. The former peptide produced the alignment of 31 amino acid residues beginning from Thr-13 to CMCys-43 while the latter, 15 amino acid residues from Thr-13 to Asn-27. Combining all these results of sequencing, the alignment of the whole sequence is shown in Fig. 4.

A primary structure comparison between the K49 homologs and some representative group II PLA_2's is shown in Fig. 5. The common feature of Lys-49 PLA_2's as opposed to the Asp-49 PLA_2's is evident in the replacement of Asp-49 by Lys-49 in the No. 1 to No. 4 PLA_2's. Homology index calculation revealed their similarity: 79% for No. 2, 72% for No. 3, 51% for No. 5 and 55% for No. 6 as compared with No. 1.

Spectroscopic titration of TMV-K49 with Ca^{2+}

To study the calcium affinity of TMV-K49, we have monitored the ultraviolet spectral change of the protein upon adding increasing amounts of Ca^{2+} (Fig. 6). The absorbance increased at 264 nm while two troughs were observed at 290 nm and 240 nm (which were also observed in the Ca^{2+} titration spectra of the K49 protein from *T. flavoviridis* [15]). These two troughs are likely due to the respective perturbation of histidine and aromatic amino acid residues [18]. The dissociation constant (K_d) of Ca^{2+}-TMV-K49 complex was calculated to be $1.6 \cdot 10^{-4}$ M by a Scatchard plot analysis (Fig. 6 insert), where ΔA is the difference absorbance at 290 nm and ΔA_{max} is that upon Ca^{2+} maturation. Similar K_d values were obtained when the increases of absorbances at 264 nm corresponding to increasing [Ca^{2+}] were analysed.

Hydrolytic activity of pure TMV-K49

It was reported that FXXII-2 had moderate enzymatic activity toward the mixed micelles of deoxycholate and dipalmitoyl phosphatidylcholine [9]. However, the specific activity of FXXII-2 decreased when further purified by G-75 gel-filtration or by HPLC (Fig. 1). Its original hydrolytic activity appeared to be due to coeluted FXXII-1 [9] PLA_2 (corresponding to peak 5, Fig. 1) plus another very minor contaminating enzyme (peak 4–2, Fig. 1). The purified TMV-K49 after HPLC (peak 4–1, Fig. 1) had almost no enzymatic activity (< 0.04 μmol/min per mg) (Table II). The absence of hydrolytic activity of TMV-K49 was not caused by

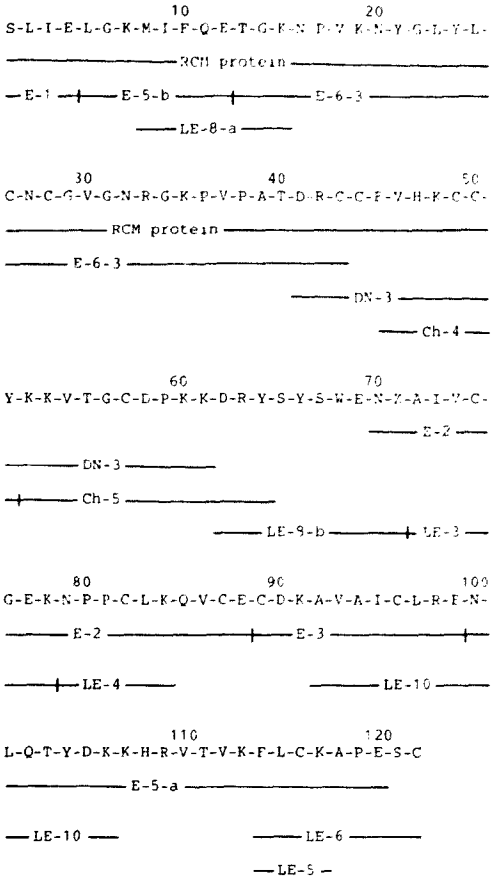

Fig. 4. Amino acid sequence of TMV-K49 deduced from peptide fragments. Abbreviations are: E, Glu-C or Staphylococcal V8 proteinase peptides; LE, lysyl endopeptidase peptides; DN, endoproteinase Asp-N peptides; Ch, chymotrypsin peptides.

inactivation during the HPLC since in the control experiment, where partially purified FXXII-2 was incubated with 0.07% TFA and 40% CH_3CN(v/v) at 25°C for 6 h, the enzyme activity remained almost the same. Therefore we may deduce that the enzyme activity of pure TMV-K49 is barely detectable.

Pharmacological activities of TMV-K49

Results of previous analysis with the FXXII-2 (i.e., TMVPLA$_2$ II) [10-13] suggest that this venom protein induces rat hind-paw edema in a dose-dependent manner, because it stimulates serotonin and histamine release from PMN-leukocytes and mast cells. The edema-inducing activity of HPLC-purified TMV-K49 was confirmed in the present study (Fig. 7). The purified TMV-K49 (at low dosage, 10 μg/ml) activated the platelet membrane by inducing shape change and causing reversible aggregation, and it also activated mast cells to release histamine and β-glucuronidase (Table II). However, upon testing, we found that TMV-K49 had very low myotoxic and neurotoxic activity at dosage as high as 100 μg/ml.

Discussion

Purification of the venom components from *T. mucrosquamatus* by various chromatographies including HPLC resolved at least three or four PLA_2 isoforms (Fig. 1). The present study has completed the amino acid sequence analysis of a new member of the Lys-49 family of PLA_2 (i.e., the K49 proteins) [14,21]. This and the previous reports [14,15] together suggest that this family of PLA_2's frequently occur as components of the Crotalidae snake venoms (especially those of *Agkistrodon*, *Bothrops* and *Trimeresurus*) and that Nature has preserved group II phospholipase A_2 homologs, devoid

TABLE II

Enzymatic and biological properties of phospholipases A_2 isolated from Trimeresurus mucrosquamatus venom

Purified protein	HPLC [a] elution, % B solvent	PLA_2 activity μmol/min per mg	Edema [b] (%)	Platelet [c] aggregation (%)	% release of mast cell [d]	
					Histamine	β-glucuronidase
TMV-K49	22	< 0.04 [e]	40 [e]	31.5 [e]	44.8	25.1
FXXII-1	32	123	34	73.2	27.5	14.8
TMVPLA$_2$ I	37	160	30	80.0	-	-

[a] Conditions used as in Fig. 1. TMV-K49 and FXXII-1 were obtained from HPLC of FXXII-2 and TMVPLA$_2$ I from FXVIII [9].
[b] Percent hind-paw swelling at 6 h after subplantar injection of venom protein (5 μg, paw).
[c] Percent aggregation of washed rabbit platelets at 6 min after the addition of the venom protein (10 μg/ml).
[d] At 15 min after the addition of the venom protein (10 μg/ml), mean values of three experiments are shown.
[e] Mean values of two experiments are presented.

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(K49)           **    *                    *   *                   *   *
                      10        20        30        40        50        60
1  TMV      SLIELGKMIFQETGKN-PVKNYGLYGCNCGVGNRGKPVDATDRCCFVHKCCYKKVTGCDPK

2  TFV      SLVWLWKMIFQETGKE-AAKNYGLYGCNCGVGRRGKPKDATDSCCYVHKCCYKKVTGCDPK

3  APP      SVLELGKMILQETGKN-AITSYGSYGCNCGWGHRGQPKDATDRCCFVHKCCYKKLTDCNHK

4  BAV      SLVELGKMILQETGKN-PLTSYGVYGCNCGVGSRHKPKDDTDRCCFVHKCCY/
 (D49)

5  TFV      GLWQFENMIIKVVKK-SGILSYSAYGCYCGWGGRGKPKDATDRCCFVHDCCYGKVTGCNPK

6  APP      NLFQFEKLIKKMTGK-SGMLWYSAYGCYCGWGGQGRPKDATDRCCFVHDCCYGKVTGCNPK


                          **                  *   *
              70        80        90        100       110       120
1   KDRYSYSWE-NKAIVCGEKNPPCLKQVCECDKAVAICLRENLGTYDK-KH-RVTVKFLCKA--PESC

2   MDSYSYSWK-NKAIVCGEKNPPCLKWVCECDKAVAICLRENLGTYNK-K-YTIYPKPFCKK--ADTC

3   TDRYSYSWK-NKAIICEEKN-PCLKQMCECDKAVAICLRENLDTYNK-K-YKAYFKLKCKK--PDTC

5   LGKYTYSWQGN--IVCGG-DDPCDKEVCECDRAAAICFRDNLDTYDRNK-YWRYPASNCQ-EDSEPC

6   MDIYTYSVE-NGNIVCGGTN-PCKKQICECDRAAAICFRDNLLTYD-SKTYWKYPKN-CTKEESEPC
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Fig. 5. Comparison between the Lys-49 and the Asp-49 phospholipase A_2 families from some Crotalidae venoms. Abbreviations of venoms are: TMV, *Trimeresurus mucrosquamatus* (the present study); TFV, *Trimeresurus flavoviridis* [15]; APP, *Agkistrodon piscivorus piscivorus* [20,21]; BAV, *Bothrops atrox* [21]. An asterisk denotes the special substitution in the Lys-49 proteins in contrast to the other PLA_2's.

of enzymatic activity [20], which share a high degree of structural homology in Old and New World snakes.

Being homologous to the group II venom PLA_2's, the K49 proteins have some structural features in common (residue numbering as in Fig. 5): (1) In their N-terminal α-helix, Glu-4, Leu-5 is in contrast to the highly conserved Gln-4, Phe-5 in other PLA_2's; (2) Asn-28, (Asn, Arg, His or Ser)-33 and Lys-49 in the

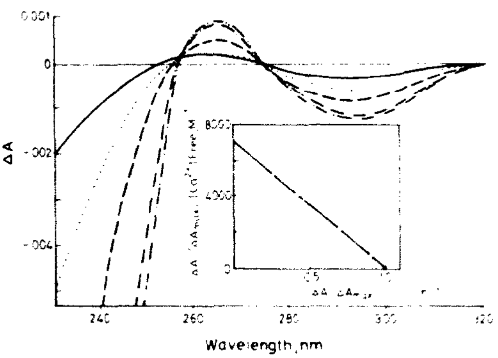

Fig. 6. Difference spectra of TMV-K49 induced by Ca^{2+} and the Scatchard plot (Insert). The sample cuvette contained purified TMV-K49 0.5 mg/ml in 50 mM Tris-0.1 M NaCl (pH 8.0) at 25°C in the presence of 0.05, 0.1, 0.3, 0.7 or 1.4 mM $[Ca^{2+}]$ (———, ·······, – – –, -·-·-·, -··-··-, respectively), while the reference cuvette contained the same concentration of the protein and buffer. Scatchard plots for obtaining the dissociation constant of the metal complex was based on the absorbances at 290 nm (Insert).

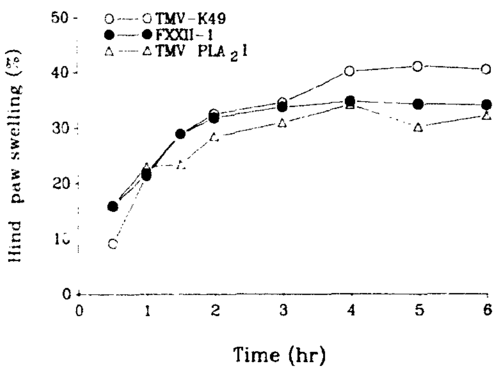

Fig. 7. Time-course of edema induced by three PLA_2 isoforms from *T. mucrosquamatus* venom. TMV-K49, FXXII-1 and TMVPLA$_2$ I used were purified by HPLC (Fig. 1). Hind paw edema was induced by a single subplantar injection of 5 μg protein in 10 μl buffer (see Materials and Methods).

K49 proteins substituted the calcium binding sites Tyr-28, Gly-33 and Asp-49 invariably found in other PLA_2's [20]; (3) Gln-11, Lys-53, Glu-80, Val-95, Leu-99 are conserved in the K49 proteins whereas in group II venom PLA_2's Lys-11, Gly-53, -80, Ala-95, Phe-99 are conserved (Fig. 5 and Ref. 20); (4) the K49 proteins are very basic (p*I* > 10), and contain many basic residues at region 110–122 (Fig. 5).

The K49 protein from *A. p. piscivorus* was shown to have the capacity to bind phospholipid [20,21] and in this study the activating effect of TMV-K49 on platelets and mast cells (Table II) suggests that it binds to the biomembranes. Another basic and membrane-binding venom protein, cardiotoxin, was also shown to cause edema in rat hind-paws [19]. The effective dosages for the edema induction by TMV-K49 and cardiotoxin [19] are 2–5 μg and 5–15 μg per paw, respectively. Thus the edema-forming activity of TMV-K49 is more potent than those of the cardiotoxin [19], cobra PLA_2 [13] and also those of the PLA_2 from the same venom, FXXII-1 and TMVPLA$_2$ I (Table II). The phospholipid-hydrolysing activities are not parallel to the edema inducing activities of these proteins. The *p*-bromophenacyl bromide-modified FXXII-2, whose enzyme catalytic activities were completely lost, still induced paw edema and the effect was greatly suppressed by heparin [13], which further supports the contention that the basic charges, rather than the enzymatic hydrolysis, are responsible for the edema-inducing property of TMV-K49. Furthermore, the PLA_2 inhibitor, aristolochic acid [23], did not interfere with the effect of TMV-K49 (not shown), but this edematous response was totally reversed in the rats pretreated with aspirin in combination with anti-serotonin (methysergide) and anti-histamine (diphenhydramine) [13].

It was shown previously that the K49 protein from *A. p. piscivorus* venom had very low intraventricular lethal potency, low anticoagulant and hemolytic activities and that it also had little effect on cardiac and neuromuscular tissues (70 and 35 μg/ml of the nearly-purified K49 are required to be effective, respectively) [22]. Our results confirmed the low activity of TMV-K49 on the nerve-muscle preparation. Moreover, this and the previous studies [11–13] indicate that one of the major functions of the K49 family is direct binding to PMN leukocytes, platelets and mast cells to cause release of serotonin, histamine and other inflammatory mediators, thus causing vasodilation and edema. It was found that various proteins (PLA_2's, esterases and proteinases) in *T. mucrosquamatus* venom had edema-inducing capabilities [9]. Thus TMV-K49 may be part of the synergistic system which contributes to the overall edematous response caused by the venom. Although the *Trimeresurus* K49 proteins bind Ca^{2+} (Fig. 6 and Ref. 15), the role of the prosthetic Ca^{2+} remain to be investigated. The lack of enzyme activity of TMV-K49 may be due to unfavorable conformation at the active site caused by substitution of residue 49 and possibly other substitutions [20].

Recently, the crystallographic structure of the K49 PLA_2-homolog from *Agkistrodon* was solved [26]. The 3-D map showed an intact phospholipid binding site and a conserved catalytic center (His-48, Tyr-73 and Asp-99) except Lys-49 displacing Asp-49 in the calcium-binding site. However, the structure-function relationship of the K49 protein remains to be studied before its dynamic role toward specific cell types (e.g., mast cell, PMN) can be understood at the molecular level.

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