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Electrospray ionization mass spectrometry as a critical tool for revealing new properties of snake venom phospholipase A_2

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Results from high-performance liquid chromatography/nano-electrospray ionization tandem mass spectrometry (HPLC/nESI-MS/MS) coupled to two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (2D SDS-PAGE) indicated that the monomer and dimer of phospholipase A_2 (PLA_2) coexisted in crude Chinese *Agkistrodon blomhoffii Ussurensis* snake venom (ABUSV). Then, an acidic PLA_2 with the accurate molecular mass of 13979.6 Da was purified from ABUSV (mo-ABUSV-aPLA_2). MS/MS-derived peptides from ABUSV-aPLA_2 were compared with other homologous snake venom PLA_2s, which in turn showed that ABUSV-aPLA_2 is a novel snake venom PLA_2. Meanwhile, the ABUSV-aPLA_2 dimer (di-ABUSV-aPLA_2) was also obtained. MS/MS analysis identified the same peptides from di-ABUSV-aPLA_2 as from mo-ABUSV-aPLA_2, which indicates that di-ABUSV-aPLA_2 is a homodimer. One Ca^{2+} ion is contained per ABUSV-aPLA_2. The Ca^{2+} ion is critical for both the hydrolytic activity and the structure of ABUSV-aPLA_2. Pro-Q Emerald and Pro-Q Diamond specific glycoprotein and phosphoprotein staining combined with MS/MS analysis indicated that the ABUSV-aPLA_2 is both a glycoprotein and a phosphoprotein, which to our knowledge is the first such report for a snake venom PLA_2 and thus provides new threads for the study of the functions and structures of snake venom PLA_2s. One phosphorylation site and the size of the glycan chain are determined by using HPLC/nESI-MS/MS and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS. The delicate utilization of ESI-MS can exert tremendous impact on protein sciences. Copyright © 2009 John Wiley & Sons, Ltd.

Phospholipase A_2 (PLA_2, EC 3.1.1.4) belongs to the superfamily of proteins which hydrolyzes the *sn*2 linkage in phosphoglycerides to release arachidonic acid and lysophospholipids.[1] PLA_2s are among the most abundant enzymes found in venoms. Snake venoms containing secreted PLA_2s belong to groups I (elapid snakes) or II (viperid and crotalid snakes) species. Snake venom PLA_2s possess important toxic/pharmacological effects including myonecrosis, neurotoxicity, cardiotoxicity, hemolytic, hemorrhagic, hypotensive, anticoagulant, platelet aggregation inhibition, and edema-inducing activities in addition to their primary catalytic activity.[2,3] Diverse isoforms of the PLA_2 families that vary in their biophysical and biochemical properties have been identified and well characterized.[1–4] However, new techniques should be delicately designed to exploit the biological and structural characteristics of proteins including the SV PLA_2s.

A high-performance liquid chromatography (HPLC) method coupled to tandem mass spectrometry (MS/MS) has been established as a critical and powerful analytical tool for protein analysis, nucleic acid analysis, molecular biology and structural characterization of macromolecules as well as in discovery of biomarkers for a variety of diseases.[5–15] The present study reports the application of HPLC/nESI-MS/MS (nESI: nano-electrospray ionization) in revealing new structural characteristics of an acid PLA_2 isolated from Chinese *Agkistrodon blomhoffii Ussurensis* snake venom (ABUSV). First, dimerization of ABUSV-aPLA_2 is confirmed by the HPLC/nESI-MS/MS method coupled to two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (2D SDS-PAGE) in the crude sample of ABUSV. Secondly, the monomer and dimer of ABUSV-aPLA_2 were obtained through purification by ion-exchange and gel-filtration chromatography. Thirdly, protein identification results from MS/MS analysis indicated that the ABUSV-aPLA_2 is a novel snake venom PLA_2 and its dimer is

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a homodimer. Furthermore, the Ca^{2+} ion was found to be critical for the hydrolytic activity and the structure of ABUSV-$aPLA_2$. Finally, results from Pro-Q Emerald and Pro-Q Diamond staining coupled to MS/MS analysis indicated that the ABUSV-$aPLA_2$ was both a glycoprotein and a phosphoprotein. HPLC/nESI-MS/MS analysis is the key tool for revealing new molecular properties of ABUSV-$aPLA_2$.

EXPERIMENTAL

Crude venom and reagents

Lyophilized ABUSV was obtained from Jilin Panshi snake plant. Acrylamide, ammonia bicarbonate, ammonium persulfate, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS), Cy2, DEAE Sephadex A-50 (A-50), dithiothreitol (DTT), Deep Purple stain, *O,O′*-bis(2-aminoethyl)ethyleneglycol-*N,N,N′,N′*-tetraacetic acid (EGTA), iodoacetamide (IAM), immobilized pH gradient (IPG) buffer, pH 4–7 IPG (24 cm), *N,N′*-methylenebisacrylamide, protein assay kit, Sephadex G-75 (G-75) resin, sodium dodecyl sulfate (SDS), TEMED, thiourea and urea were obtained from GE Healthcare. Formic acid (FA) and trifluoroacetic acid (TFA) were from Sigma. Pro-Q Diamond and Pro-Q Emerald staining kits were from Molecular Probes (USA). HPLC purity water and acetronitrile (ACN) were from Brodick Johnson Inc. Sequencing-grade trypsin was from Promega. All other chemicals were analytical grade from commercial sources.

Two-dimensional SDS-PAGE of crude ABUSV

ABUSV (500 μg) dissolved in Tris-HCl buffer (0.05 M, pH 8.0) was labeled using Cy2 dye using the DIGE system from GE Healthcare. Labeling reactions were carried out on ice in the dark for 30 min before being quenched with 10 mM lysine. The sample was then diluted with an equal volume of rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2% IPG buffer, 20 mM DTT). A 24 cm pH 4–7 NL IPG strip was subjected to electrophoresis using an Ettan IPGphor II system. DTT (1%) and IAM (4.5%) were added into the solutions of the first and second equilibration steps, respectively. The IPG strip was placed on top of a 15% polyacrylamide gel precast with a low-fluorescence glass plate. SDS-PAGE was carried out at 20°C at 0.2 W/gel for 1 h, 0.4 W/gel for 1 h, and 1.8 W/gel for 15 h. After electrophoresis, the gel was subjected to Deep Purple staining (GE Healthcare). Gel images were acquired using excitation/emission wavelengths of 488/610 nm. The protein spots were robotically excised in a 96-well plate.[12]

Protein identification by HPLC/nESI-MS/MS and sequence comparison

In-gel tryptic digestion[8,9] was performed prior to protein identification by HPLC/ESI-MS/MS on a nanoflow multidimensional liquid chromatography system (Ettan MDLC) coupled to an LTQ mass spectrometer (Thermo).[12] Two C18 trap columns (5 × 0.3 mm, Agilent) and two nanoscale C18 analytical columns (150 × 0.075 mm, Dionex) were used for MDLC.[16] Mobile phase A consisted of water with 0.1% FA while mobile phase B was 84% ACN with 0.1% FA. Separation was achieved at a flow rate of 260 nL/min by applying a linear gradient of 0–60% B over 50 min. Mass spectra were collected using data-dependent acquisition of one MS scan (400 to 2000 *m/z*) followed by MS/MS scans of the five most abundant ions. MS/MS spectra were interpreted using TurboSequest Bioworks searching against the NCBInr database.[17] Peptide cross-correlation scores (Xcorr) of 1.9, 2.5, and 3.75 were set for charge states 1, 2, and 3, respectively. Protein probability was set below 10^{-3}. Sequence similarity between ABUSV-$aPLA_2$ and other snake venom PLA_2 sequences was accessed by ClustalW and the multi-sequence alignment was conducted by CLUSTALX2.[18,19]

Purification of ABUSV-$aPLA_2$

Crude ABUSV was first applied to an A-50 column (1.6 × 48 cm). The column was washed with buffer (0.05 M Tris-HCl, pH 7.6) and proteins were eluted with a linear 0–0.6 M NaCl gradient. The ABUSV-$aPLA_2$ was then purified through a G-75 column (1.6 × 96 cm) using a buffer of 0.05 M Tris-HCl, pH 7.5, 0.15 M NaCl. The active PLA_2 fractions were further purified through a Mono Q column (1.0 × 5.0 cm). The protein was eluted with a linear 0–0.8 M NaCl in 0.05 M Tris-HCl (pH 7.5). The final purification step was performed on a second G-100 column (1.6 × 96 cm). All the isolation processes were carried out at 4°C.

Enzyme activity and protein concentration determination of ABUSV-$aPLA_2$

The hydrolytic activity of ABUSV-$aPLA_2$ was determined using egg yolk as substrate.[20,21] Protein concentration was determined by the Lowry method.[22]

SDS-PAGE, MALDI- and ESI-MS

SDS-PAGE was performed using a 15% separation gel.[8,21] Sinapinic acid was used as the matrix for matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS analysis. Mass spectra were obtained with the accumulation of 280 single shots.[5,8,9] The molecular mass and homogeneity of ABUSV-$aPLA_2$ were also analyzed by electrospray ionization mass spectrometry (ESI-MS) on a Finnigan LCQ quadrupole ion-trap mass spectrometer coupled to a C4 reversed-phase column (1 × 15 mm; Vydac, Hesperia, CA, USA).

Ca^{2+} measurement and the influences of the Ca^{2+} ion on the hydrolytic activity and intrinsic fluorescence of ABUSV-$aPLA_2$

Ca^{2+} measurement was performed by inductively coupled plasma-atomic emission spectrometry (ICP-AES) analysis.[8,9,12] Apo-ABUSV-$aPLA_2$s were obtained by dialyzing ABUSV-$aPLA_2$ extensively against EGTA buffer (0.05 M EGTA, 0.05 M Tris-HCl, pH 7.2). Three readings were obtained for each sample to enable statistical averaging. The relative hydrolytic activities of native and apo-ABUSV-$aPLA_2$ were measured. Fluorescence spectroscopy of native and apo-ABUSV-$aPLA_2$ was carried out with a RF-5000 fluorescence spectrometer (Shimadzu, Japan).[8,9] Protein concentration was 2 μM. Both the excitation and emission slits were set at 5 nm.

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Glycosylation and phosphorylation of ABUSV-aPLA$_2$

SDS-PAGE gels were obtained for the mixture of mo-ABUSV-aPLA$_2$ and di-ABUSV-aPLA$_2$ at the ratio of 2:1 (mass ratio). After fixing the gel overnight by two incubations in 50% (v/v) methanol and 10% (v/v) acetic acid, gels were washed twice in water and incubated in the dark with either Pro-Q Emerald 488 glycoprotein stain or Pro-Q Diamond phosphoprotein stain (Molecular Probes),[23,24] and were scanned and analyzed with DecyDer software.

The tryptic peptides of ABUSV-aPLA$_2$ were enriched by using TiO_2 in combination with HPLC/nESI-MS/MS analysis. The tryptic digest was dried down by SpeedVac, re-dissolved in 1% TFA/50% ACN and mixed with TiO_2 suspended in the same solvent. After vortexing for 10 min the supernatant was discarded, and the TiO_2 was washed three to five times with the same solvent. Phosphopeptides were eluted with 1% NH_4OH.[25,26]

The size of the glycan chain of ABUSV-aPLA$_2$ was analyzed by using PNGase proteolysis in combination with MALDI-TOF MS analysis. mo-ABUSV-aPLA$_2$ (100 μg) was added into to an Eppendorf tube and adjusted to 18 μL final volume with deionized water. Then, 5 μL 5× PNGase F reaction buffer 7.5 and 1.5 μL of PNGase F denaturation solution were added and the final complex solution was heated at 100°C for 5 min. Then 1.3 μL of Triton X-100 and 1.0 μL of PNGase F were added to the reaction. Proteolysis time lasted for 3 h at 37°C.[27,28]

RESULTS AND DISCUSSION

Measurements and identifications of PLA$_2$ monomers and dimers in crude ABUSV by 2D SDS-PAGE and HPLC/nESI-MS/MS

A proteomic approach, the combination of 2D SDS-PAGE and HPLC/nESI-MS/MS,[12] was established to analyze the protein components in the crude ABUSV. The sample was separated first by isoelectric focusing (IEF) on a 24 cm IPG strip followed by the second dimensional separation on a 12.5% gel. After electrophoresis, the gel with a silanized spacer low-fluorescence glass plate was subsequently subjected to Deep Purple staining and used for spot picking. Deep Purple stain is compatible with protein identification by mass spectrometry. Proteins are statistically more likely to be identified from Deep-Purple-stained gel with higher quality.[9,12]

More than 300 protein spots were visualized from the crude ABUSV by Deep Purple staining (Fig. 1). The same peptides were detected from seven protein spots numbered in Fig. 1 by ESI-MS/MS analysis (Table 1), which results in the same PLA$_2$. Normally, the snake venom PLA$_2$s have molecular weights around 13.8–14.4 kDa.[1–4,12,29–32] Thus, the protein spots (1950, 2015, 2017 and 2169) with molecular weights between 13.7 and 14.5 kDa are considered to be the monomers of ABUSV-aPLA$_2$. Meanwhile, the molecular weights of three protein spots, 1200, 1236 and 1511 between 27.6 and 29 kDa (Fig. 1), that are twice the molecular weights

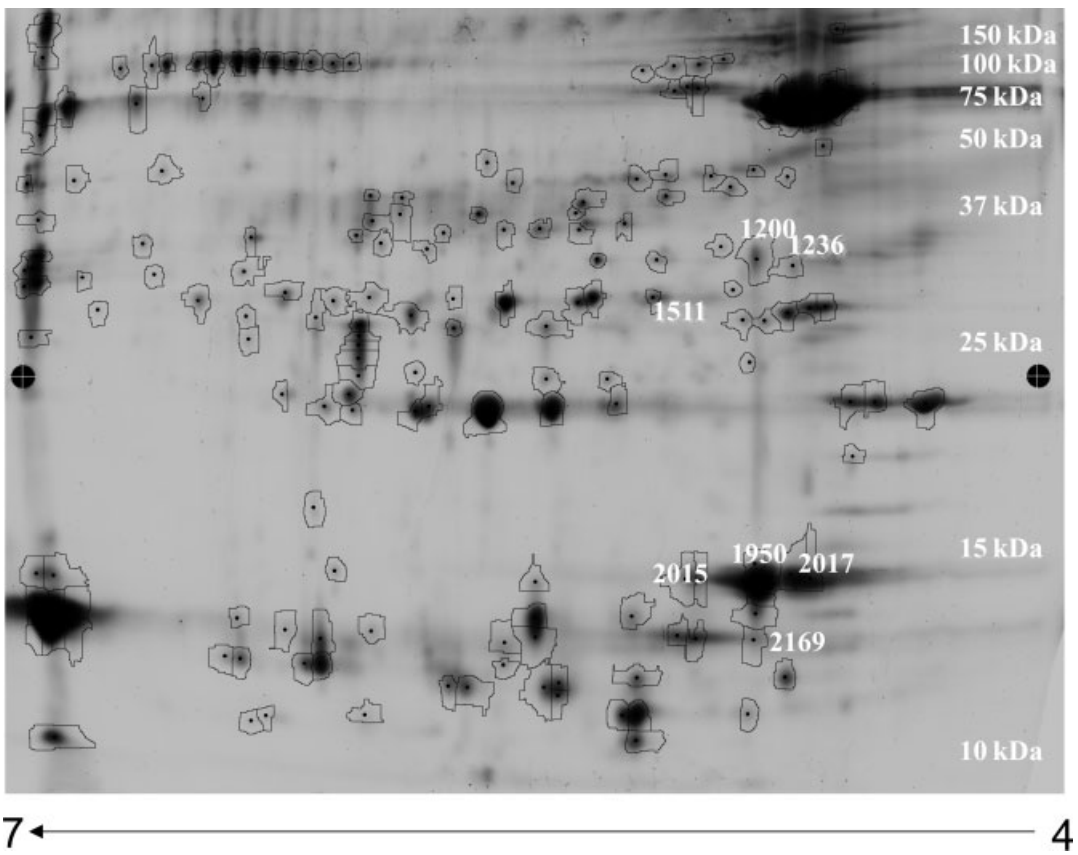

Figure 1. Deep Purple staining image of 2D SDS-PAGE of crude ABUSV. A 12.5% SDS-PAGE gel was applied for the second dimension. The protein spots identified as snake venom PLA$_2$ are marked with numbers.

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Table 1. Protein identification results for PLA_2s in crude ABUSV

Protein spot	MS/MS-derived sequence	MH^+	Z	P (pep)$^{\Delta}$
1200	DNLNTYNDKK[a,c,d,e]	1224.59	2	4.71E-03
	M[#]DVYSFSEENGDIVC[*]GGDDPC[*]KK[a,b,c,d,e]	2510.04	2	1.08E-10
	SLIQFETLIMK[a,b,f,g]	1322.74	2	1.54E-05
	WDDYTYSWK[h,i,j]	1263.53	1	1.86E-03
1236	M[#]DVYSFSEENGDIVC[*]GGDDPC[*]KK[a,b,c,d,e]	2638.14	3	1.19E-07
	SLIQFETLIMK[a,b,f,g]	1338.73	2	1.31E-03
	WDDYTYSWK[h,i,j]	1263.53	1	3.53E-04
1511	NC[*]PQEESEPC[*][a,b,c,d,h]	1249.51	1	8.42E-03
	AAAIC[*]FRDNLNTYNDKK[a,c,d,e,h]	2014.01	3	1.21E-09
	KYWAFGAK[a,b,c,d,e,h]	970.51	2	1.24E-02
	SLIQFETLIMK[a,b,f,g]	1322.74	2	2.33E-05
	KSGM[#]FWYSNYGC[*]YC[*]GWGGQGRPQDATDR[a,b]	3320.43	3	4.55E-06
	M[#]DVYSFSEENGDIVC[*]GGDDPC[*]K[a,b,c,d,e]	2510.04	3	1.67E-14
	WDDYTYSWK[h,i,j]	1263.53	1	3.2E-04
1950	AAAIC[*]FRDNLNTYNDKK[a,c,d,e,h]	2014.01	3	1.21E-09
	KYWAFGAK[a,b,c,d,e,h]	970.51	2	1.24E-02
	MDVYSFSEENGDIVC[*]GGDDPC[*]KK[a,b,c,d,e]	2622.14	2	2.50E-10
	MDVYSFSEENGDIVC[*]GGDDPC[*]K[a,b,c,d,e]	2494.05	2	5.84E-12
	SLIQFETLIMK[a,b,f,g]	1322.74	2	2.33E-05
2015	KYWAFGAK[a,b,c,d,e,h]	970.51	2	1.24E-02
	KSGM[#]FWYSNYGC[*]YC[*]GWGGQGRPQDATDR[a,b]	3320.43	3	4.55E-06
	MDVYSFSEENGDIVC[*]GGDDPC[*]KK[a,b,c,d,e]	2622.14	2	2.50E-10
	MDVYSFSEENGDIVC[*]GGDDPC[*]K[a,b,c,d,e]	2494.05	2	5.84E-12
	SLIQFETLIMK[a,b,f,g]	1322.74	2	2.33E-05
	WDDYTYSWK[h,i,j]	1263.53	1	1.22E-04
2017	AAAIC[*]FRDNLNTYNDKK[a,c,d,e,h]	2014.01	3	1.21E-09
	KYWAFGAK[a,b,c,d,e,h]	970.51	2	1.24E-02
	MDVYSFSEENGDIVC[*]GGDDPC[*]KK[a,b,c,d,e]	2622.14	2	2.50E-10
	WDDYTYSWK[h,i,j]	1263.53	1	1.22E-04
	MDVYSFSEENGDIVC[*]GGDDPC[*]K[a,b,c,d,e]	2494.05	2	5.84E-12
2169	AAAIC[*]FRDNLNTYNDKK[a,c,d,e,h]	2014.01	3	1.21E-09
	KYWAFGAK[a,b,c,d,e,h]	970.51	2	1.24E-02
	MDVYSFSEENGDIVC[*]GGDDPC[*]KK[a,b,c,d,e]	2622.14	2	2.50E-10
	MDVYSFSEENGDIVC[*]GGDDPC[*]K[a,b,c,d,e]	2494.05	2	5.84E-12

$^{\Delta}$ Protein probability.
[a] PLA_2, acidic (phosphatidylcholine 2-acylhydrolase, *Gloydius ussuriensis*, **GI: 47117139**; Protein entry number: **Q7LZQ4**).
[b] PLA_2, (acidic phosphatidylcholine 2-acylhydrolase, *Gloydius halys*, **GI:129399**; Protein entry number: **P14418**).
[c] PLA_2 (phosphatidylcholine 2-acylhydrolase, *Gloydius halys* (halys viper), GI: **27151651**; Protein entry number: **O42191**).
[d] Acidic PLA_2 (*Gloydius halys*, **GI:28948590**; Protein entry number: **1M8S_A**).
[e] PLA_2 (*Gloydius halys*, **GI:2460031**; Protein entry number: **AAB71847**).
[f] Phospholipase A_2, acidic precursor (*Deinagkistrodon acutus*, **GI:90265326**; Protein entry number: **CAJ85790.1**).
[g] Acidic phospholipase A_2 (*Agkistrodon Acutus*, **GI:18158787**; Protein entry number: **1IJL_B**.).
[h] Phospholipase A_2 (*Gloydius halys*, **GI:2460029**; Protein entry number: **AAB71846.1**).
[i] Phospholipase A_2 (*Agkistrodon halys Pallas*, **GI:6980609**; Protein entry number: **1B4W**).
[j] Phospholipase A_2 (*Gloydius halys*, **GI:27151647**; Protein entry number**: O42817**).
[#] Oxidation of the residue of Met.
[*] Cysteine residue modification by IAM.

of monomeric PLA_2s, should be the dimers of ABUSV-aPLA_2. Our proteomic results clearly indicate that the monomers and dimers of PLA_2 coexist in crude ABUSV. Moreover, the di-ABUSV-aPLA_2 is stable enough to bear the rough conditions for SDS-PAGE. Mo-ABUSV-aPLA_2 and di-ABUSV-aPLA_2 were purified from ABUSV and characterized by SDS-PAGE coupled to HPLC/nESI-MS/MS analysis testifying the authenticity, see next section. Interestingly, the same protein was identified from various spots with different molecular weights, which suggests the ABUSV-aPLA_2 may be glycosylated. Furthermore, the same protein was identified from several spots with different PIs, suggesting that ABUSV-aPLA_2 might be phosphorylated. Pro-Q Emerald and Pro-Q Diamond staining experiments (see below) also confirmed the glycosylation and phosphorylation of ABUSV-aPLA_2.

Purification of ABUSV-aPLA_2s

Mo-ABUSV-aPLA_2 and di-ABUSV-aPLA_2 were obtained through a four-step column purification process as described in the Experimental section. Finally, the mo-ABUSV-aPLA_2 and di-ABUSV-aPLA_2 were purified to the extent of 22-fold with 3.0% recovery of protein and 66% recovery of enzymatic activity for mo-ABUSV-aPLA_2 and 35-fold with 0.3% recovery of protein and 10.5% recovery of enzymatic activity for di-ABUSV-aPLA_2, respectively (Table 2). A single band was observed for both the mo-ABUSV-aPLA_2 and the di-ABUSV-aPLA_2 on the SDS-PAGE gel (Fig. 2).

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Table 2. Purification results for ABUSV-aPLA$_2$

Purification Steps		Mass (mg)	Protein yield (%)	Hydrolysis activity (U/min.mg)[a]	Purification factor[b]
Crude venom		1000	100	16	1
A-50		240	24	38	2.4
G-75		80	8	148	9.2
	moABUSV-aPLA$_2$	45	4.5	300	18.8
2nd G-75	diABUSV- aPLA$_2$	12	1.2	384	24
S-100	moABUSV-aPLA$_2$	30	3.0	400	22
	diABUSV- aPLA$_2$	3	0.3	560	35

[a] One activity unit is defined as the amount of enzyme that hydrolyzes substrate to give an absorbance change of 1 absorbance unit per minute.
[b] The specific activity of the enzyme fraction from each step divided by that of crude snake venom.

Molecular mass, homogeneity and dimerization analyses and protein identification

Figure 3(a) shows the MALDI TOF mass spectrum of mo-ABUSV-aPLA$_2$. The singly charged molecular ion $[MH]^+$, doubly charged ion $[MH_2]^{2+}$, dimer ion $[M_2H]^+$, trimer ion $[M_3H]^+$ and tetramer ion $[M_4H]^+$ of mo-ABUSV-aPLA$_2$ are obtained. The MALDI TOF mass spectrum of di-ABUSV-aPLA$_2$ is similar to that of the mo-ABUSV-aPLA$_2$. The mass spectrum is dominated by the mass ion signal peak (*m*/*z* 13980.8) instead of its molecular ion $[MH]^+$ (*m*/*z* 27958.9); the mass spectrum is not shown here as it is the same as the spectrum of mo-ABUSV-aPLA$_2$. The multiply charged mass ion signals, $[MH7]^{7+}$, $[MH8]^{8+}$, $[MH9]^{9+}$, $[MH10]^{10+}$, $[MH11]^{11+}$, $[MH12]^{12+}$, $[MH13]^{13+}$, $[MH14]^{14+}$, $[MH15]^{15+}$ and $[MH16]^{16+}$, were observed in the ESI mass spectrum of ABUSV-aPLA$_2$ (Fig. 3(b)). The ESI-MS spectrum of ABUSV-aPLA$_2$ was deconvoluted to provide a more accurate molecular mass of 13985.6. No ion peaks of impurities are observable indicating the monomer and dimer of ABUSV-aPLA$_2$ are homogeneous. The mass spectrum of di-ABUSV-aPLA$_2$ is the same as that of mo-ABUSV-aPLA$_2$, which potentially indicates that each subunit of di-ABUSV-aPLA$_2$ can be charged with one proton ($[(M/2+H)^+ + (M/2+H)^+]^{2+}$) to form an artifact $[MH]^+$ ion peak as the mo-ABUSV-aPLA$_2$. The subunit might be released from di-ABUSV-aPLA$_2$ during the sample preparations for mass spectrometry.

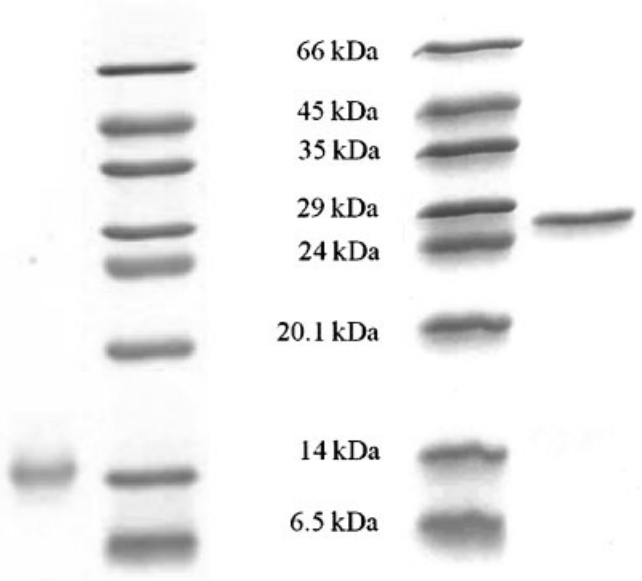

Figure 2. SDS-PAGE (12.5%) for ABUSV-aPLA$_2$s. Left lane: mo-ABUSV-aPLA$_2$; right lane: di-ABUSV-aPLA$_2$.

However, the possibility that disulfide bridges might be involved in ABUSV-aPLA$_2$ cannot be precluded.

Eight peptides derived from the MS/MS spectra, NCPQEESEPC, AAAICFRDNLNTYN DKK, DNLNTYNDK-KYWAFGAK, KSGMFWYSNYGCYCGWGGQGRPQDATDR, MD VYSFSEENGDIVCGGDDPCK, SLIQFETLIMK, WDDY-TYSWK and REICECDR, were identified by matching the sequences of ten snake venom PLA$_2$s from *Gloydius usuriensis*, *Gloydius halys*, *Gloydius halys* (halys viper), *Deinagkistrodon acutus*, and *Agkistodon halys Pallas* [33–37] (Table 3). Figure 3(c) shows a representative tandem mass spectrum of AAAICFRDNLNTYNDKK from ABUSV-aPLA$_2$. The y and b series of fragment ions as well as three internal ions, DIVCGGDDPCK-28, SFSEENGDIVCG and DVYSFSEENGDIVCGGD, were detected and matched for this peptide (the results are shown in Fig. 3(c)).

Sequence comparison between ABUSV-aPLA$_2$ and other snake venom PLA$_2$s

Sequence alignment (Fig. 4) results indicate that the sequence KSGMFWYSNYGCYCGWG GQGRPQDATDR is found in two PLA$_2$s from *Gloydius ussuriensis* and *Gloydius halys*. The sequence REICECDR is also found in only two PLA$_2$s from *Deinagkistrodon acutus* and *Agkistrodon acutus*. Six out of the eight MS/MS-derived peptides of ABUSV-aPLA$_2$ are matched in the PLA$_2$ (GI: 47117139/Q7LZQ4) isolated from *Glyodius ussurensis* venom (Table 3, Fig. 4), which also suggests that the ABUSV-aPLA$_2$ is evolutionarily closest related with the latter. WDDYTSWK is matched in the sequences of PLA$_2$s obtained from the venoms of *Gloydius halys* and *Agkistrodon halys Pallas*, which is not matched in the sequences of other snake venom PLA$_2$s. The sequences of NCPQEESEPC, AAAICFRDNL NTYNDKK, DNLNTYNDK-KYWAFGAK, MDVYSFSEENGDIVCGGDDPCK and SLIQF ETLIMK are only detected in certain snake venom PLA$_2$s (Table 3, Fig. 4). Since no single known PLA$_2$ matches all the peptides we detected by HPLC/nESI-MS/MS, ABUSV-aPLA$_2$ therefore can be regarded as a new snake venom PLA$_2$.

MS/MS spectra of ABUSV-aPLA$_2$ enable the identification of eight peptides representing 92.9% of the sequence (deduced from the identified peptides and the molecular mass of the protein, 12989.4/13979.6*100%), which shows only about 990 Da difference. Since our subsequent experiments indicate that the ABUSV-aPLA$_2$ has phosphorylation and glycosylation modifications, meanwhile, the ESI-MS/MS analysis has difficulties in providing unknown sequences of novel proteins. Therefore, by using the HPLC/-nESI-MS/

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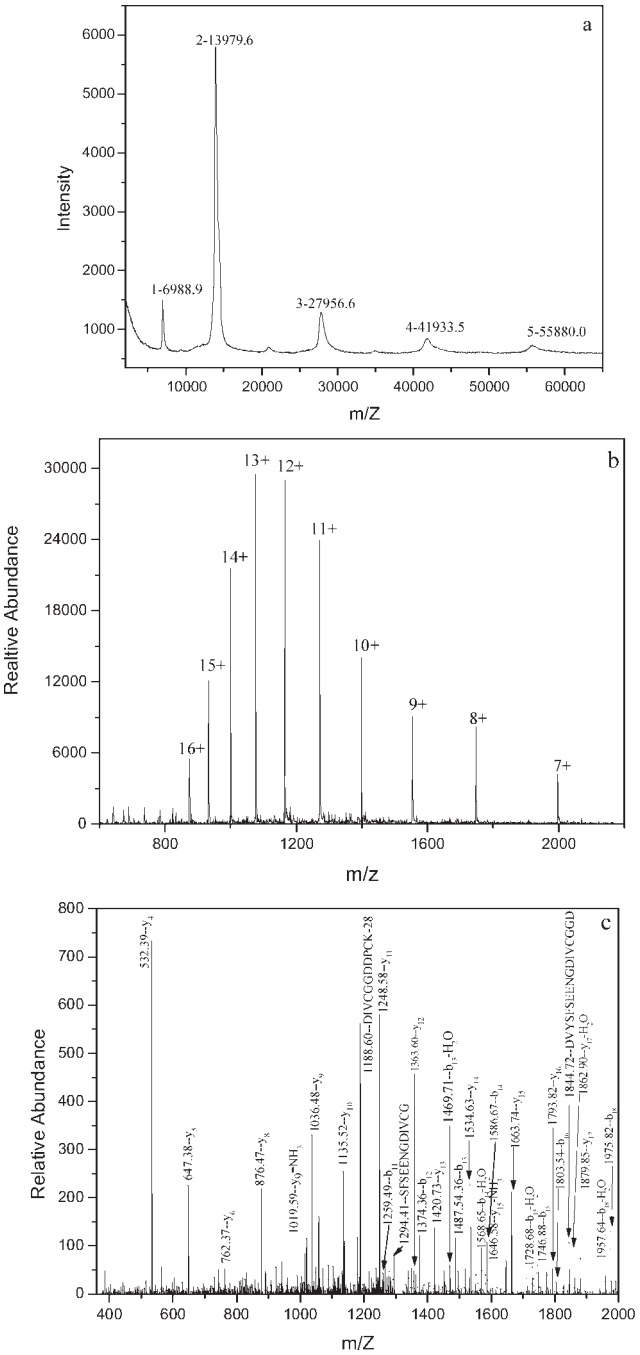

Figure 3. (a) MALDI-TOF mass spectrum of ABUSV-aPLA$_2$. The protein concentration was 2.8 μM and the spectrum was obtained from the accumulation of 1000 single shots. (b) Mo-ABUSV-aPLA$_2$ ESI mass spectrum. Multiply charged signals, +7, +8, +9, +10, +11, +12, +13, +14, +15, +16 were observed. (c) Representative tandem mass spectrum of AAAICF RDNLNTYNDKK from ABUSV-aPLA$_2$; b and y series of mass ions are marked in the plot.

MS analysis, we still have difficulties in obtaining the complete sequence of ABUSV-aPLA$_2$. Molecular cloning and DNA sequencing analysis will be the most reliable and convenient way to obtain its complete sequence. The current article mainly focuses on the feasibility of the applications of different techniques for revealing novel molecular properties for snake venom PLA$_2$s.

The effect of the Ca^{2+} ion on the hydrolytic activity and structure of ABUSV-aPLA$_2$

Previous molecular mass measurements of ABUSV-aPLA$_2$s by MALDI-TOF and ESI-MS ensure the precise measurement of metal ions present. The ICP-AES analysis shows that ABUSV-aPLA$_2$s are Ca^{2+}-containing proteins with a calcium stoichiometry of 1:1 [Ca^{2+}]/[mo-ABU SV-aPLA$_2$] (1.08 ± 0.08 from triplicate measurements) or [Ca^{2+}]/[di-ABUSV-aPLA$_2$] (1.12 ± 0.10). To investigate the functions of Ca^{2+} in ABUSV-aPLA$_2$, apo-mo-ABUSV-aPLA$_2$ was obtained ([Ca^{2+}]/[apo-mo-ABUSV-aPLA$_2$] = 0.06:1, as determined by ICP-AES). The removal of the Ca^{2+} ion leaves only a residual hydrolytic activity of ~10% (Fig. 5(a)), and leads to a 2.4 nm red shift for the maximum emission wavelength from 349.6 to 352.0 nm, and induces a 20% decrease for the maximal emission intensity of the intrinsic fluorescence of mo-ABUSV-aPLA$_2$ (Fig. 5(b)). These results clearly indicate that the Ca^{2+} ion is involved in maintaining both the hydrolytic activity and the structural integrity[8,9] of ABUSV-aPLA$_2$.

Ca^{2+} coordination residues are commonly located at the N-terminus of snake venom PLA$_2$s featured with the conserved sequence of Y-X2(4)-Y-X-G-X-G-X7-D-X9-D.[29-31,33–37] In this region, the oxygen atoms from the conserved residues coordinate Ca^{2+} in a pentagonal fashion. ABUSV-aPLA$_2$ has the sequence YGCYCGWGGQGRPQ-DATD (Table 3), which forms its calcium-binding hydrophobic consensus sequence. The PLA$_2$ catalytic site essential residues, H(49) and Y(53), are closely located to this region.[29,30] Consequently, the calcium-binding hydrophobic channel supports the access of hydrophobic phospholipids to the catalytic site of PLA$_2$ by enhancing its catalytic activity.[29–31] Apparently, the presence of the Ca^{2+} ion increases the hydrophobicity of the calcium-binding channel, enhances the substrate binding ability and improves the enzymatic hydrolysis activity of ABUSV-aPLA$_2$.

Glycosylation and phosphorylation analysis of ABUSV-aPLA$_2$

Pro-Q Emerald and Pro-Q Diamond are specific glycoprotein and phosphoprotein stains widely used especially in high-throughput glycoproteomics and phosphoproteomics.[23,24,38–42] Mo-ABUSV-aPLA$_2$ and di-ABUSV-aPLA$_2$ were mixed at the mass ratio of 2:1 (4 μg:2 μg) and run on a 12.5% SDS-PAGE gel. After electrophoresis, the gels were fixed and visualized by Pro-Q Emerald 488 glycoprotein staining and Pro-Q Diamond phosphoprotein staining (the results are shown in Figs. 6(a) and 6(b), respectively). Gel bands 1 and 2 represent the bands for mo-ABUSV-aPLA$_2$ and di-ABUSV-aPLA$_2$ (Fig. 6(a)). Gel bands a and b in Fig. 6(b) are the gel bands of mo-ABUSV-aPLA$_2$ and di-ABUSV-aPLA$_2$, respectively. The four protein bands were sliced from the gel, digested by trypsin and analyzed by HPLC/ESI-MS/MS, which gave almost the same results as those reported in Table 3. However, the number of peptides identified by HPLC/nESI-MS/MS for di-ABUSV-aPLA$_2$ are smaller than that of mo-ABUSV-aPLA$_2$, which indicates that the dimerization of ABUSV-aPLA$_2$ changes the conformational environment for certain peptides by making it more difficult to access.

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Table 3. Protein identification results for ABUSV-aPLA$_2$ by ESI-MS/MS

MS/MS-Derived Sequence	MH$^+$ (monoisotopic)	z	Peptide Probability
			2.78E-12[Δ]
NC*PQEESEPC*[a,b,c,d,h]	1249.51	1	5.30E-02
AAAIC*FRDNLNTYNDKK[a,c,d,e,h]	2014.01	3	4.47E-06
DNLNTYNDKKYWAFGAK[a,c,d,e]	2047.99	3	2.36E-05
KSGMFWYSNYGC*YC*GWGGQGRPQDATDR[a,b]	3304.44	3	2.63E-11
MDVYSFSEENGDIVC*GGDDPC*K[a,b,c,d,e]	2494.05	2	2.78E-12
SLIQFETLIMK[a,b,f,g]	1322.74	2	6.86E-05
WDDYTYSWK[h,i,j]	1263.53	1	1.22E-04
REICECDR[f,g]	1137.56	2	1.29E-02

[Δ] Protein probability.
[a] PLA$_2$, acidic (phosphatidylcholine 2-acylhydrolase, *Gloydius ussuriensis*, **GI: 47117139**; Protein entry number: **Q7LZQ4**).
[b] PLA$_2$, (acidic phosphatidylcholine 2-acylhydrolase, *Gloydius halys*, **GI:129399**; Protein entry number: **P14418**).
[c] PLA$_2$ (phosphatidylcholine 2-acylhydrolase, *Gloydius halys* (halys viper), **GI: 27151651**; Protein entry number: **O42191**).
[d] Acidic PLA$_2$ (*Gloydius halys*, **GI:28948590**; Protein entry number: **1M8S_A**).
[e] PLA$_2$ (*Gloydius halys*, **GI:2460031**; Protein entry number: **AAB71847**).
[f] Phospholipase A$_2$, acidic precursor (*Deinagkistrodon acutus*, **GI:90265326**; Protein entry number: **CAJ85790.1**).
[g] Acidic Phospholipase A$_2$ (*Agkistrodon Acutus*, **GI:18158787**; Protein entry number: **1IJL_B**).
[h] Phospholipase A$_2$ (*Gloydius halys*, **GI:2460029**; Protein entry number: **AAB71846.1**).
[i] Phospholipase A$_2$ (*Agkistrodon halys Pallas*, **GI:6980609**; Protein entry number: **1B4W**).
[j] Phospholipase A$_2$ (*Gloydius halys*, **GI:27151647**; Protein entry number: **O42817**).

PLA$_2$s from bee venoms have been reported to be glycosylated,[32,43] but, to the best of our knowledge, the present paper is the first study reporting that snake venom PLA$_2$s when isolated are glycoproteins. Although snake venom PLA$_2$s were reported to have various roles in inhibition of protein phosphorylation,[44–46] they have not been reported to be phosphoproteins themselves. Three glycyrrhizin (GL)-binding PLA$_2$s (gbPLA$_2$) from the *Habu* snake venom and a gbPLA$_2$ purified from the synovial fluids of patients with rheumatoid arthritis were reported to be phosphorylated by casein kinase II (CK-II).[47,48] However, no report has been made indicating that snake venom PLA$_2$s are phosphoproteins when isolated. Here, our results clearly indicate that the present ABUSV-aPLA$_2$ is a phosphoprotein.

Usually, the phosphorylation level of proteins is pretty low, so prior to phosphopeptide analysis by HPLC/nESI-MS/MS, we used the TiO_2 enrichment method[24,25] to enrich the phosphorylated peptides produced by tryptic digestion. One phosphorylated peptide with the sequence of WDDYTYSWK was captured and the phosphorylated site is identified as the residue of T (results shown in Fig. 7(a)). In order to analyze the size of the glycan chain of ABUSV-aPLA$_2$, PNGase was utilized to remove the small glycan chain from ABUSV-aPLA$_2$. MALDI-TOF MS analysis results indicated that the molecular size of the glycan chain was approximately 581 Da (Fig. 7(b)). The above result clearly indicated that the glycan chain contains a GLcNAc. Considering the molecular mass of 581 Da (the glycan chain) against the molecular mass of ABUSV-aPLA$_2$, the unmatched protein mass of the ABUSV-aPLA$_2$ is only about 409 Da (990–581). The above results also implicate that only about four amino acid residues might be undetectable by using the HPLC/nESI-MS/MS approach due to the fact that tryptic peptides might be too small to be caught by the HPLC separation.

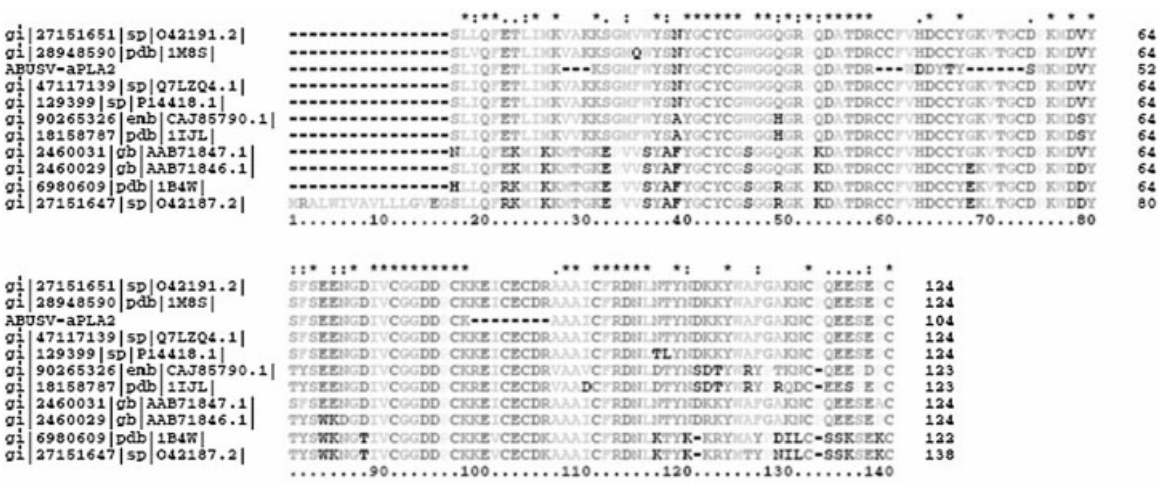

Figure 4. Sequence comparison between ABUSV-aPLA$_2$ and other ten snake venom PLA$_2$s sharing the homologous peptides with ABUSV-aPLA$_2$.

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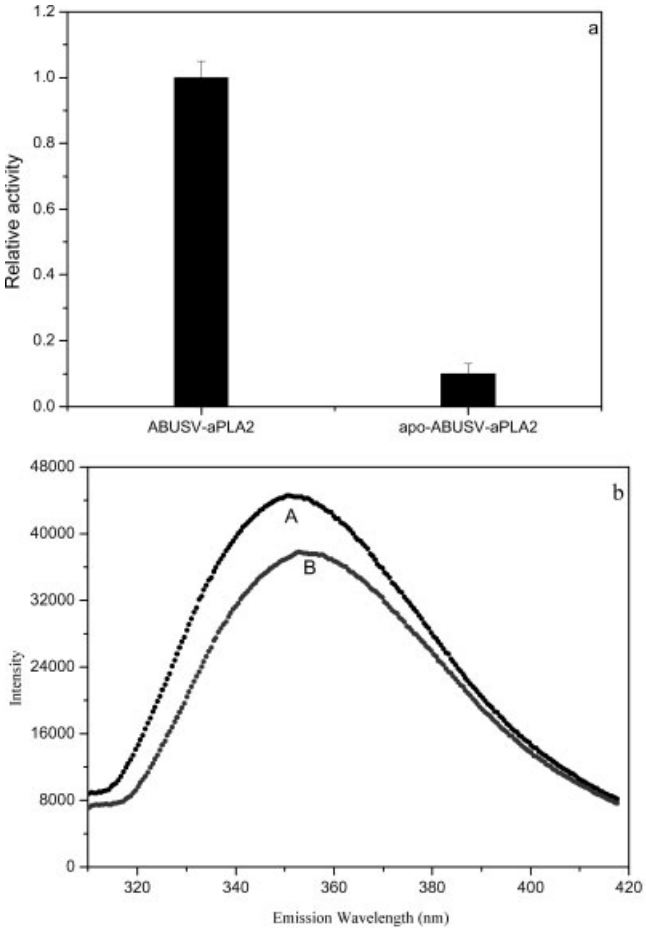

Figure 5. The effect of the Ca^{2+} ion on (a) the hydrolytic activity of ABUSV-aPLA$_2$ and (b) the intrinsic fluorescence of ABUSV-aPLA$_2$. Line A is for native ABUSV-aPLA$_2$ and line B is for apo-ABUSV-aPLA$_2$.

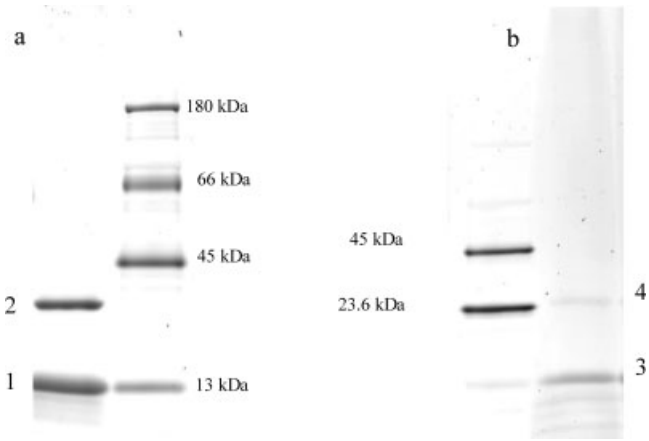

Figure 6. Glycosylation and phosphorylation analysis of ABUSV-aPLA$_2$. (a) Pro-Q Emerald staining image. Left lane: 1, mo-ABUSV-aPLA$_2$; 2, di-ABUSV-aPLA$_2$; right lane: gel bands for standard glycoproteins. (b) Pro-Q Diamond staining image. Left lane: gel bands for standard phosphoproteins; right lane: 3, mo-ABUSV-aPLA$_2$; 4, di-ABUSV-aPLA$_2$.

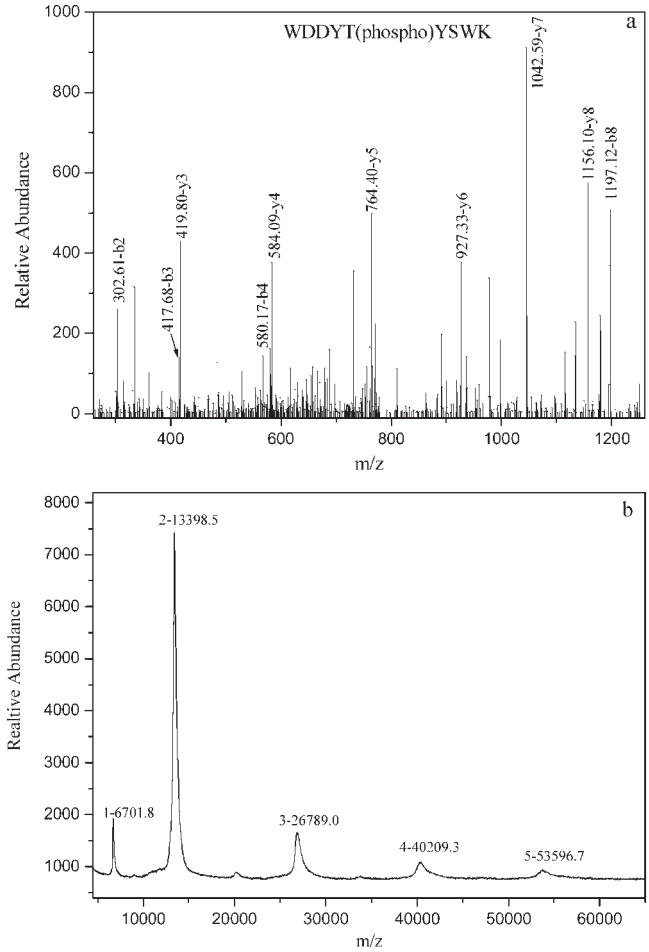

Figure 7. (a) Phosphorylation site analysis of ABUSV-aPLA$_2$ by ESI-MS/MS. The peptide with sequence WDDYTYSWK was identified phosphorylated at the T residue. (B) PNGase proteolysis in combination with MADT-TOF MS determines the size of the glycan chain of ABUSV-aPLA$_2$. Deglycosylated mo-ABUSV-aPLA$_2$ has a molecular mass of 13398.5 that has a 581 Da difference from that of the mo-ABUSV-aPLA$_2$.

ABUSV-aPLA$_2$ is both a glycoprotein and a phosphoprotein, which also implies that other snake venom PLA$_2$s are likely to be glycoproteins and phosphoproteins. Thus, the results from this paper provide new threads into studies on the structure and function of snake venom PLA$_2$s.

CONCLUSIONS

In the present study, HPLC/nESI-MS/MS analysis has been proved to be a critical tool for revealing new molecular characteristics of protein molecules. Undeniably, this study was initially guided by the initial protein identification results of the 2D SDS-PAGE spots of crude ABUSV from HPLC/nESI-MS/MS analysis. In addition, the completeness of the current study was totally driven by the initial data from HPLC/nESI-MS/MS analysis.

A two-dimensional electrophoresis technique coupled to the Deep Purple staining method was carried out to investigate the proteome of ABUSV. Protein identification of the gel spots resulted in good HPLC/nESI-MS/MS data

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with high quality and indicated clearly that a PLA_2 monomer and a PLA_2 dimer coexisted in crude ABUSV. Then, homogenous mo-ABUSV-aPLA_2 and di-ABUSV-aPLA_2 were successfully purified from the crude venom by testifying its authenticity. The MS/MS-derived sequence of ABUSV-aPLA_2 was compared with the amino acid sequences of other homologous snake venom PLA_2s, which indicated that the ABUSV-aPLA_2 was a novel snake venom PLA_2. The accurate Ca^{2+} content of ABUSV-aPLA_2 was acquired by utilizing MALDI-TOF MS coupled to ICP-AES analysis. The Ca^{2+} ion was found to be critical for both the hydrolytic activity and the structure of ABUSV-aPLA_2. Finally, with the aid of protein identification by ESI-MS/MS, Pro-Q Emerald and Pro-Q Diamond glycoprotein and phosphoprotein staining analysis indicated clearly that the ABUSV-aPLA_2 was both a glycoprotein and a phosphoprotein. To our knowledge, this is the first report of a snake venom PLA_2. In summary, HPLC/nESI-MS/MS analysis is the key tool for revealing new protein characteristics of ABUSV-aPLA_2 and potentially providing new threads for the functional study of snake venom PLA_2s. Our results indicate clearly that the dedicated experimental design in HPLC/ESI-MS/MS may exert a tremendous impact on protein science.

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