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Cloning, characterization and phylogenetic analyses of members of three major venom families from a single specimen of *Walterinnesia aegyptia*☆,☆☆

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ABSTRACT

Walterinnesia aegyptia is a monotypic elapid snake inhabiting in Africa and Mideast. Although its envenoming is known to cause rapid deaths and paralysis, structural data of its venom proteins are rather limited. Using gel filtration and reverse-phase HPLC, phospholipases A_2 (PLAs), three-fingered toxins (3FTxs), and Kunitz-type protease inhibitors (KIns) were purified from the venom of a single specimen of this species caught in northern Egypt. In addition, specific primers were designed and PCR was carried out to amplify the cDNAs encoding members of the three venom families, respectively, using total cDNA prepared from its venom glands. Complete amino acid sequences of two acidic PLAs, three short chain 3FTxs, and four KIns of this venom species were thus deduced after their cDNAs were cloned and sequenced. They are all novel sequences and match the mass data of purified proteins. For members of each toxin family, protein sequences were aligned and subjected to molecular phylogenetic analyses. The results indicated that the PLAs and a Kunitz inhibitor of *W. aegyptia* are most similar to those of king cobra venom, and its 3FTxs belongs to either Type I α-neurotoxins or weak toxins of orphan-II subtype. It is remarkable that both king cobra and *W. aegyptia* cause rapid deaths of the victims, and a close evolutionary relationship between them is speculated.

1. Introduction

Walterinnesia aegyptia (black desert cobra) is a monotypic species distributed over the desert areas of Egypt, Israel, Syria, Iraq, Iran, Saudi Arabia, and Turkey. Like other elapid venoms, its envenoming causes muscular paralysis and respiratory failure in mice and human victims (Ismail et al., 1998; Lee et al., 1976; Lee and Tsai, 1972; Samejima et al., 1997). Notably, injection of lethal doses of its venom on rats or mice caused rapid deaths. Previously, two neurotoxic three-finger toxins (3FTxs) of *W. aegyptia* venom from Israel have been purified and sequenced, but cobra cardiotoxin-like proteins were not detected (Lee et al., 1976; Samejima et al., 1997). Being the non-enzymatic markers of elapid venom, the 3FTxs form a superfamily that has a stable three-β sheets scaffold. They

Abbreviations: PLA, phospholipase A_2; 3FTx, three-finger toxin; KIn, Kunitz-type protease inhibitor; dPPC, dipalmitoyl glycerophosphocholine; HPLC, high performance liquid chromatography; Wa, *Walterinnesia aegyptia*.

☆ The sequence data were deposited in the GenBank database with the following accession numbers: EU196553–EU196554 for Wa-PLII and Wa-PLI, EU196555–EU196557 for Wa-III, Wa-IV and Wa-V, EU196558–EU196561 for KIn-I, III, II, and IV.

☆☆ *Ethical statement*: The experiments carried out in the present report did not involve live animals.

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display a broad array of known or undefined functions, e.g. antagonism of various subtypes of nicotinic acetylcholine receptors, cytotoxicity or cardiotoxicity, inhibition of L-type calcium channel, and acetylocholinesterase (Kini, 2002; Tsetlin and Hucho, 2004).

Another common non-enzymatic component of elapid venom is Kunitz protease inhibitor (KIn), which has never been studied in the *W. aegyptia* venom. In addition, the venom of *W. aegyptia* was found to contain various enzymes including phospholipases A_2 (PLAs) (Simon and Bdolah, 1980), L-amino acid oxidase, and proteolytic enzymes (Gitter and de Vries, 1968). In fact, two acidic PLAs of *W. aegyptia* venom have been purified but their sequences were not reported (Simon and Bdolah, 1980). The 13–14 kDa PLAs hydrolyze phospholipids and release fatty acids and lysophospholipids in a Ca^{2+}-dependent manner (Danse et al., 1997).

The aims of this study are to investigate the proteome and the transcriptome of venom components of *W. aegyptia* and to understand the biosystematics of this elapid species. Here, three low molecular weight and common venom families (i.e. PLA, 3FTx, and KIn) would be focused. We have purified and characterized the venom proteins of a single specimen of *W. aegyptia* from Egypt. In the meantime, we used PCR and facile cDNA cloning to study the transcriptome of each of the three venom families. The cDNA-deduced sequence data were matched to those of the purified proteins. These sequences were then subjected to phylogenetic analyses to expand our knowledge about the elapid systematics. Structural features of the *W. aegyptia* proteins would also be discussed for a better understanding of the structure–function relationships.

2. Materials and methods

2.1. Materials

Crude venom was milked from a single specimen of *W. aegyptia* (northeastern Egypt). Two days later its venom glands were dissected after sacrificing the snake. The tissue was preserved for several weeks in the RNAlater solution (Ambion, USA) before total RNA was extracted. Modification and restriction enzymes and the pGEM-T vectors were purchased from Promega Corp. (Madison, USA). Synthetic dipalmitoyl phosphatidylcholine was from Avanti-Biochemical (Alabama, USA). Triton X-100 and sodium deoxycholate were from Sigma Chemical Co. (USA). Other chemicals used were of reagent grade.

2.2. Purification and characterization of venom proteins

Lyophilized venom was dissolved in a small volume of 100 mM CH_3COONH_4 (pH 6.24), followed by centrifugation. The supernatant was applied to a Superdex-G75 gel filtration column and eluted with the same buffer on a FPLC system. Fractions containing PLAs were pooled and lyophilized before further purification by reverse-phase HPLC. After being injected into a Vydac column of C18 silica gel (4.6 × 250 mm), the sample was eluted by a gradient of buffer A and B, made of 0.07% (v/v) trifluoroacetic acid and acetonitrile, respectively. Proteins collected were dried in a vacuum-centrifuge device (Labconco, USA). We also assumed that the averaged extinction coefficients at 280 nm for fractions A and B were approximately twice those for fractions C and D of the gel filtration profile. The relative content (%, w/w) of each protein in the crude venom was estimated, based on the relative absorbance at 280 nm of the area under the four fractions collected in gel filtration (Fig. 1) as well as the relative absorbance at 230 nm of each HPLC peaks (Fig. 2). The molecular weight of each purified protein was analyzed by QSTAR XL Nano-ESI Mass Spectrometer System (Applied Biosystems) in the protein-core facility of our institute.

2.3. Assays of PLAs

Micelles of 3 mM L-dipalmitoyl phosphatidylcholin ($diC_{16}PC$) with 3 mM sodium deoxycholate or 6 mM Triton X-100, and 100 mM NaCl were prepared in a glass-Teflon tissue homogenizer, and 2.5 ml of the solution was transferred to the thermostated reaction cup of a pH-stat apparatus (Radiometer, Copenhagen, Denmark). With constant stirring, a small aliquot of $CaCl_2$ was added to a final concentration of 10 mM just before the addition of enzyme. With pH set to 7.40, the release of acid during substrate hydrolysis was followed by titration with 8 mM NaOH at 37 °C. The initial hydrolysis rate was corrected for the non-enzymatic rate in each experiment.

2.4. Cloning and sequencing of venom toxins

The RNA from *W. aegyptia* venom glands was extracted using mRNA extraction kit. Their complementary DNAs

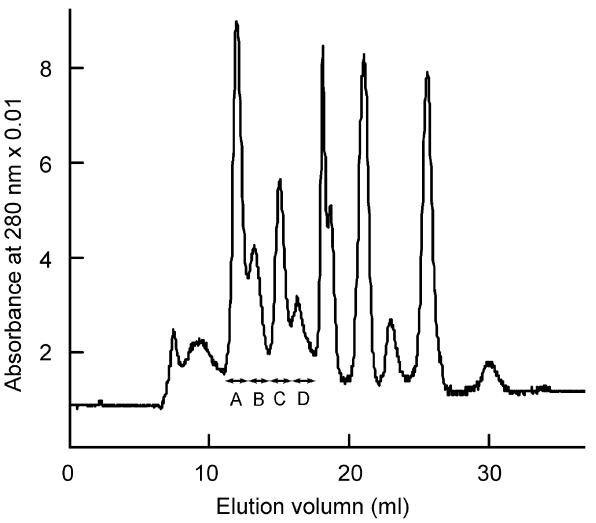

Fig. 1. Separation of *W. aegyptia* venom proteins by gel filtration. The venom powder (1.5 mg) was dissolved in 100 μl water and loaded onto a Superdex G75 (HR10/30) column pre-equilibrated with 0.1 N ammonium acetate buffer (pH 7.1). Elution of venom proteins was carried out on a FPLC system at a flow rate of 1 ml/min and monitored at 280 nm. Fractions were collected separately and lyophilized.

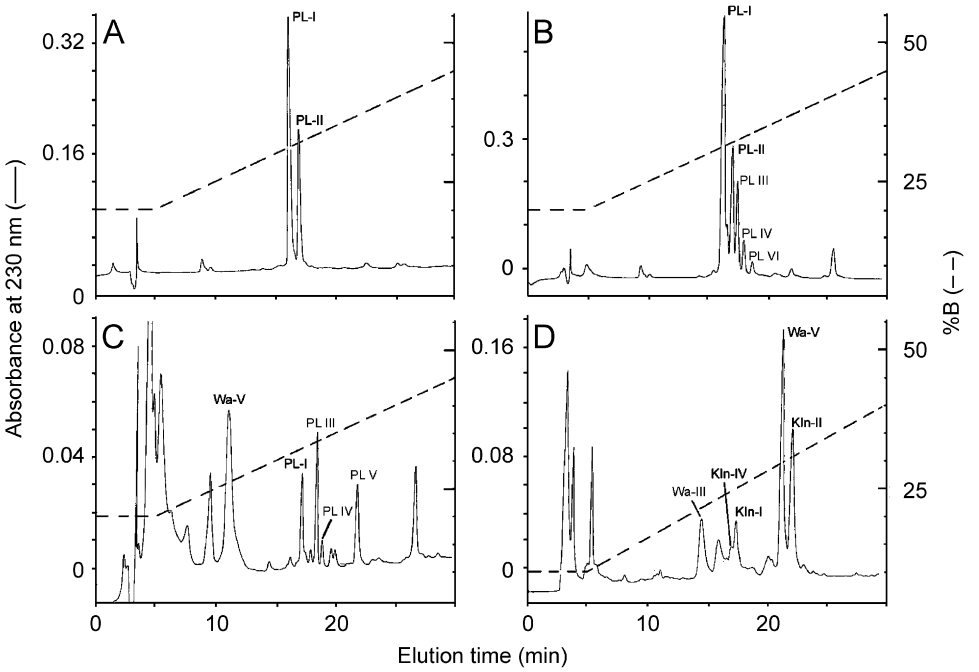

Fig. 2. Purification of venom proteins by RP-HPLC. Proteins obtained from fractions A–D in the gel filtration (Fig. 1) were re-dissolved and injected into a Vydac RP-C18 column separately. The elution procedure started with 20% buffer B for 5 min followed by a gradual increase of buffer B for 25 min, at a flow rate of 1 ml/min to establish a concentration gradient (dashed lines). Eluted polypeptides were monitored at 230 nm. Annotations of the proteins are as shown in Table 1.

(cDNAs) were prepared using cDNA synthesis kit according to the manufacturer's instructions (Stratagene, USA). It is known that snake venoms undergo positive Darwinian evolution (Ohno et al., 1998), which features high conservation at the 5′ and 3′ untranslated regions (UTR) but rapid evolution of the exon regions. Using primers designed from conserved UTR sequences, we were able to amplify specific cDNAs by PCR, and clone various venom-specific cDNAs. PCR primers were synthesized based on the conserved UTR and/or signal peptide regions of the cDNA sequences previously published for elapid venom PLAs (Tsai et al., 2002), 3FTxs (Chang et al., 1999) and KIns (Chang et al., 2001). To amplify the 3FTx cDNAs, Primer 1 used was 5′-ATGAAAACTCTGCTGCTGWCCTTG-3′ and Primer 2 was 5′-CTCAAGGAAWTTAGSCACTCRKAGAG-3′. To amplify the PLA cDNAs, Primers 3 and 4 used were 5′-GCAGTTTGTGTCTCC CTCTTAGGA-3′ and 5′-CACAGTCCTT-GAGCTGAAGCTTCTC-3′, respectively. To amplify the KIn cDNAs, Primers 5 and 6 used were 5′-CCAGACGGCTTCAT-CATG-3′ and 5′-AAAAGGAATRATCCAGG-3′, respectively. Primers 1, 3, and 5 were in the sense orientation of the 5′-end sequences, whereas primer 2, 4, and 6 were in the antisense orientation of the 3′-end UTR.

PCR (Mullis and Faloona, 1987) was conducted using the cDNAs derived from the venom glands as templates and SuperTaq DNA polymerase (HT Biotech, UK) was used. Conditions of each of the 35 cycles were set to 92 °C for 1.0 min (denaturation), 52 °C for 1.0 min (annealing), and 72 °C for 1.0 min (extension). After amplification, the DNA fragments of expected size were resolved by 1% agarose gel electrophoresis and harvested. After treating with polynucleotide kinase, they were inserted into the pGEM-T vector (Promega Biotech), and then used to transform *Escherichia coli* strain JM109 (Maniatis et al., 1989). The plasmid DNA was extracted from white transformants and its restriction pattern was examined by agarose gel electrophoresis. Each cloned cDNA was sequenced by the DNA-Sequencing-System (model 373A, PE-Applied Biosystems, USA). The sequences were confirmed at least twice and translated into amino acid sequences.

2.5. Phylogenetic trees

The amino acid sequences were aligned using the CLASTAL W (Thompson et al., 1994). Cladograms were constructed based on the aligned sequences by a neighbor-joining algorithm using the PHYLIP program (Felsenstein, 1992), degree of confidence for the internal lineage was determined by bootstrap methods (Felsenstein, 1985).

3. Results and discussion

3.1. Venom protein purification and characterization

The *W. aegyptia* venom collected from an individual snake was in low quantity. It was first separated by gel filtration. As shown in Fig. 1, four fractions (A–D) contained most of the venom proteins were harvested before 20 ml of the elution volume and lyophilized. The later peaks were also analyzed but gave poor yields of proteins. By HPLC, PLAs were further purified from fractions A and B, while the 3FTxs and KIns (6–7 kDa) were purified from fractions C and D (Fig. 2). Molecular

Table 1
Characterization and enzymatic activities of purified venom PLAs of *W. aegyptia*

PLA	Content % (w/w)	Mass (Da)	Specific activity (μmol/mg/min)	
			DPPC plus deoxycholate	DPPC plus Triton X-100
PL-I	13.0	13,342	793 ± 27	591 ± 3.3
PL-II	6.0	13,343	54.1 ± 3.6	15.0 ± 0.1
PL-III	1.0	13,455	n.d.	n.d.
PL-IV	1.5	13,562	n.d.	n.d.
PL-V	0.2	14,206	n.d.	n.d.

Table 2
Characterization of purified 3FTx and KIn of *W. aegyptia* venom

Protein	Content % (w/w)	Mass (Da)	N-terminal sequence 1–16
(3FTx)			
Wa-III	2.0	6852	FVCHNQQSSQPPTTTN
Wa-V	2.4	7400	LTCLICPKKYCNQVHT
(KIn)			
KIn-I	0.2	6347	RPGLCELPAETGPCKA
KIn-II	0.8	6394	RPRLCELPAESGLCNA
KIn-IV	0.4	6779	LGGPKYCHLPADPGPC

mass of each purified protein was determined by ESI-Mass spectrometry (Tables 1 and 2). The esterase activities of Wa PL-I and PL-II were also assayed by pH stat method (Table 1). There are another three PLA-like components, which could be purified at very low yields. Some of the purified proteins were subjected to automatic sequencing by protein sequencer (Tables 1 and 2).

We also used azocasein to assay the protease activities in *W. aegyptia* venom. The activities were greatly reduced to control level when 5 mM EDTA was added to inhibit the proteases (data not shown). Thus, *W. aegyptia* venom probably contains metalloprotease.

3.2. Cloning and sequencing

The primers used in our PCR to amplify cDNA of the three families worked well and many of the venom cDNAs were successfully cloned. After sequencing 50 isolated cDNA clones for each of the venom family, distinct sequences for two PLAs, three 3FTxs, and four KIns were obtained. Only those clones that have been confirmed at least twice were included in the results (Table 2). Notably, the 27-residue conserved signal peptides of the PLAs are highly similar to those of the other venom group I PLAs, the 21-residue conserved signal peptides of the 3FTxs are also identical to those of the 3FTxs from other venom species (Chang, 2007). The 24-residue signal peptides of the four Wa-KIns are identical except that of KIn-IV, which contains a V21I substitution. These signal peptides are also very similar to those of the other venom KIns (Zupunski et al., 2003).

The N-terminal sequences and the molecular weights of most of the venom proteins (Tables 1 and 2) could match those predicted from their cDNA sequences (Table 3). However, two cDNA clones appeared to have low expression level and their encoded proteins (Wa-IV and KIn-III) could not be purified. In addition, several minor PLAs were purified and identified (Table 1) from fractions B and C (Fig. 2), but none of the cDNAs we cloned could match their masses determined by ESI-MS.

Table 3
Data for members of three venom families of *W. aegyptia* deduced from their cDNA sequences

Encoded protein	Calculated mass	Predicted pI	Signal peptide sequence
(PLA)			1–27
PL-I	13,342	4.6	MYPAHLLVLL AVCVSLLGAA NIPPQPL
PL-II	13,343	4.6	MYPAHLLVLL AVCVSLLGAA NIPPQPL
(3FTx)			1–21
Wa-III	6853	>8.9	MKTLLLTLVV VTIVCLDLGYT
Wa-IV	6782	>8.7	MKTLLLTLVV VTIVCLDLGYT
Wa-V	7400	>8.8	MKTLLLTLVV VTIVCLDLGYT
(KIn)			1–24
KIn-I	6347	8.6	MSSGGLLLLL GLLTLWAELT PVSG
KIn-II	6394	8.3	MSSGCLLLLL GLLTLWAELT PVSG
KIn-III	6378	8.6	MSSGCLLLLL GLLTLWAELT PVSG
KIn-IV	6780	>9.2	MSSGCLLLLL GLLTLWAELT PISG

Isoelectric points (pI) and masses were calculated from the predicted protein sequences.

3.3. Venom PLA isoforms

Prior to our study, two non-lethal acidic PLAs from *W. aegyptia* venom have been reported (Simon and Bdolah, 1980). Here we have purified and cloned two acidic PLA variants and designated them as Wa PL-I and PL-II. Similar to the other group IA venom PLAs, both PLA precursors have 14-conserved Cys residues in the mature protein of 120 residues. A BLASTP search (Altschul et al., 1997) identified a PLA from king cobra venom (Xu et al., 2003) as the most similar enzyme to Wa PL-I and PL-II. Complete amino acid sequences of Wa PL-I and PL-II and other homologous PLAs were aligned and compared in Fig. 3.

Both Wa PL-I and PL-II contain active site residues H48, D49, Y52, and D99, Ca^{2+}-binding loop at positions 25–33, and a substrate binding hydrophobic channel comprised of L2, F5, I9, W19, Y66, A102, and F106 (White et al., 1990). However, the N-terminal residues H1, G3, N6 are not frequently present in the elapid venom PLAs, although H1 and N6 have been found in myotoxic and neurotoxic PLAs of pitvipers' venom (Tsai et al., 2004; Chen et al., 2004), and N6 is also present in bovine pancreatic PLA. Previous mutagenesis study on pancreatic PLA showed that W3A point mutation resulted in decreased catalysis (Liu et al., 1995). Thus small amino acids at position 3, such as G3, might not be favorable for the enzymatic activity. Wa PL-I and II differ by only four substitutions (T70R, S101L,

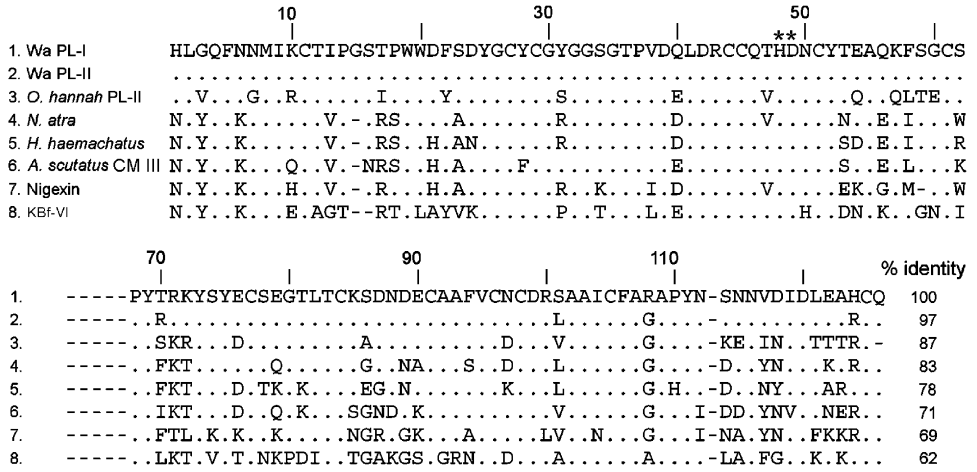

Fig. 3. Alignment of amino acid sequences of Wa-PLAs and related venom PLAs. Their GenBank or SwissProt accession numbers are shown in legend of Fig. 5. Single-letter codes of amino acids are used, gaps are marked with hyphens, the active sites are marked with asterisks. A commonly used numbering system is adopted (Tsai et al., 2002).

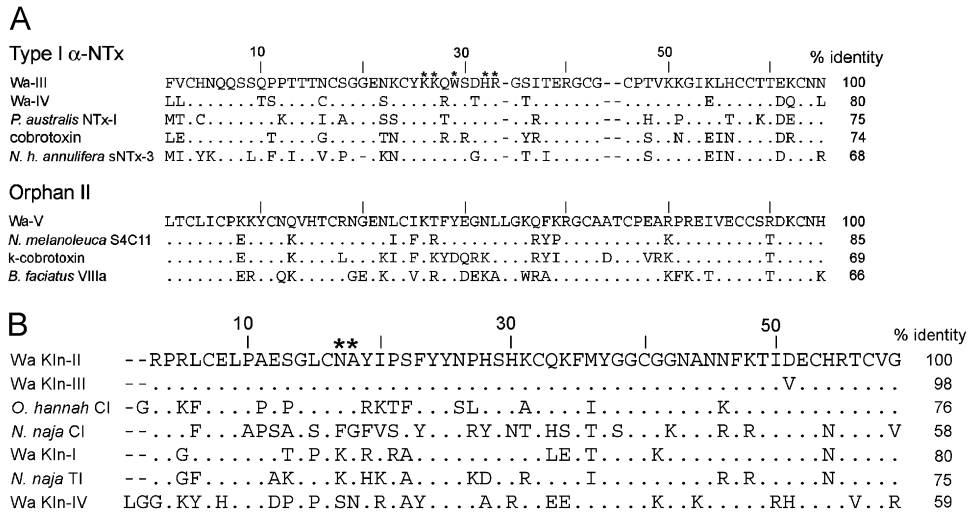

Fig. 4. Multiple alignment of amino acid sequences of 3FTxs and Kunitz inhibitors. (A) Wa-III, Wa-IV, Wa-V and related 3FTxs. (B) Wa KIns and related elapid venom KIns. Their GenBank or SwissProt accession numbers are shown in legends of Figs. 6 and 7, respectively. Single-letter codes of amino acids are used and gaps are marked with hyphens. Important sites were marked with asterisks.

R108G, and H124R), and their predicted pI values of 4.6 are almost identical. However Wa PL-I showed 15-folds higher hydrolytic activity than Wa PL-II in the *in vitro* assay (Table 1), and its content was much higher than that of Wa PL-II (Fig. 2). The reduced enzymatic activity of Wa PL-II could possibly be attributed to the T70R substitution because the 70th residue of other active elapid PLAs are usually neutral, while substitutions L101, G108, and R124 are rather common in other PLAs (Danse et al.,1997).

3.4. Three-fingered toxins

We now cloned three novel 3FTxs from the venom of *W. aegyptia*, and designated the proteins as Wa-III, Wa-IV, and Wa-V (Table 3) since the sequences of Wa-III and Wa-IV were very similar to W-III and W-IV, respectively (Samejima et al., 1997). The sequences of Wa-III, Wa-IV, and Wa-V were aligned in Fig. 4A. Wa-III and Wa-IV are highly basic polypeptides of 62 amino acids, probably containing four conserved disulfide bonds, while Wa-V contains 65 residues with a fifth disulfide bridge in loop I (Tsetlin and Hucho, 2004). The deduced Wa-III sequence differs from the previously reported W-III sequence by two substitutions, namely, S36 and T38 instead of K36 and I38; and the predicted Wa-IV sequence differs from the W-IV sequence by a P30S substitution (Samejima et al., 1997). A special Cys residue is present at position 16 of Wa-IV, which seems to have no significant effect on its toxicity.

Among the 3FTxs sequenced, Wa-III and Wa-IV are about 80% similar, and they both have conserved residues

K27, K28, W29, H32, and R33 in loop II (Fig. 4A). It has been known that a W29 and several positively charged residues (e.g. R33) on loop II are important for the postsynaptic neurotoxicities of these α-neurotoxins (Pillet et al., 1993; Tremeau et al., 1995; Antil et al., 1999; Antil-Delbeke et al., 2000). However, sequence of Wa-V is only 35% similar to that of Wa-III or Wa-IV, but 85% identical to that of a cobra weak toxin, Nm S4C11 (Carlsson, 1975).

Previously, three postsynaptically acting neurotoxins (W-III, W-IV, and W-V) were purified from the pooled venom of Isreal *W. aegyptia* and their quantities were in the order of W-IV > W-V > W-III (Lee et al., 1976). For mice, the medium lethal doses (LD_{50}) of W-III and W-IV previously determined were 0.95 and 0.14 μg/g, respectively (Samejima et al., 1997). Here, both Wa-III and Wa-V were purified from a single *W. aegyptia* venom (Fig. 2) and the content of Wa-V is about 5-fold higher than that of Wa-III (Table 2), but Wa-IV was hardly detectable (Fig. 2C and D). Apparently, differential expression of the 3FTxs variants may cause individual or geographic variations in the toxin proportions and affect the snakebite symptoms.

3.5. Kunitz-type inhibitors

We have cloned and sequenced four Kunitz inhibitors from *W. aegyptia* venom, and they are designated as KIn-I–IV. Except for KIn-III, all of them could be purified from the venom. KIn-I, KIn-II, and KIn-III all contain 57 amino acid residues while KIn-IV contains 59 residues with two extra residues at the N-terminus, similar to some other venom KIns (Fig. 4B). It was shown by *in vitro* mutagenesis study that removal of the N-terminal three residues do not affect the affinity of a cobra venom KIn for the protease (Cheng et al., 2005). The KIn-II and KIn-III sequences are almost identical except that the D51 in KIn-II is replaced by V51 in KIn-III, however, their sequences are ⩽80% identical to those of KIn-I and KIn-IV.

Specificities of KIn towards the target protease is usually determined by the main protease contact site (residue 17 in Fig. 4B, short-handed as P1) (Schechter et al., 1967) and its adjacent region (Millers et al., 2006). The P1 site of KIn-I is K17, that of KIn-II and KIn-III is N17 and that of KIn-IV is S17. KIns with K17 or R17 have been classified as trypsin inhibitors, and those with a hydrophobic or aromatic P1-site have been classified as chymotrypsin inhibitors (Laskowski and Kato, 1980). Presumably, Wa KIn-I might inhibit trypsin, while KIn-II and III might inhibit chymotrypsin if both pancreatic enzymes were used in the *in vitro* assay. However, their true protease targets *in vivo* remain to be identified.

In addition, the P1′ sites of Wa KIn-I–III are all A18. A18 at P1′ position of the inhibitors are highly conserved and is important for their binding affinity to the target protease (Zhou et al., 2004), as shown by the mutational studies of bovine pancreatic trypsin inhibitors (BPTI) (Grzesiak et al., 2000). In contrast, dendrotoxins and other ion-channel binding KIn-like toxins have a charged amino acid at position 18 (Apostoluk and Otlewski, 1998). Remarkably, S17N18 (P1 and P1′ sites) is present in KIn-IV, suggesting its possible role as another type of toxin rather than a protease inhibitor. Although KIns from snake venom have been shown to play a pro-coagulation role (Filippovich et al., 2002; Flight et al., 2005), functions and targets of these KIns are interesting subjects to be further investigated.

3.6. Molecular phylogeny of the three venom families

Venom proteins are known to undergo accelerated evolutions. Gene duplication followed by variation is an efficient route for venom to evolve into new toxins (Ohno et al., 1998). The efficiency of duplication/variation is suggested by the emergence of several large venom families whose structural scaffolds and/or active sites are conserved or related. The more closely the snakes are related, the more similar the sequences of their genes and proteins. If a sufficient number of related sequences are available, the origin of the venom gene can be determined, or at least their evolutionary history can be inferred.

While viperid and crotalid venoms express only group II PLAs, group I PLAs have evolved in elapid and hydrophiid venoms (Davidson and Dennis, 1990). To find out the evolutionary relationship between *W. aegyptia* and other species, a phylogenetic tree was built based on the amino sequences of the representative group I venom PLAs, using a group-IIB Lys49-PLA (AAR14171) as the outgroup (Fig. 5). The tree topology unveiled the presence of five or six major clusters, including African and Asian cobras, king cobra, kraits, and two or three groups of hydrophiid/Australian elapids. The topology is similar to that of the species tree which was based on mtDNA sequences (Slowinski et al., 1997). Remarkably, the Wa-PLAs are closely associated with a PLA of *Ophiophagus hannah* venom (Xu et al., 2003) with a high bootstrap score (Fig. 5).

Elapid venom 3FTxs are known to exhibit a great diversity of biological properties (Fry et al., 2003; Karlsson, 1979; Menez, 1998). A phylogenetic tree was also constructed based on 40 amino acid sequences of the short chain 3FTxs (59–64 residues), including the *W. aegyptia* 3FTxs but excluding those of cardiotoxins, while a long chain neurotoxin, α-bungarotoxin, was assigned as an outgroup (Fig. 6). This tree reveals that Wa-III and Wa-IV form a unique cluster and are loosely related to the Type I α-neurotoxins of other species, which have been known to antagonize muscle nicotinic acetylcholine receptors. Another abundant 3FTx, Wa-V with five disulfide bridges, is closely related to NmS4C11 of *Naja mossanbica* (Carlsson, 1975) as well as Bf-VIIIa and Bc-wtx from kraits (Fig. 6). They appeared to be weak or poorly characterized toxins, belonging to the orphan subtype-II of a previous 3FTx classification (Fry et al., 2003).

The data set for this tree also includes several newly published sequences of the short chain 3FTxs of Chinese king cobra venom (Li et al., 2006; Rajagopalan et al., 2007). As shown in Fig. 6, king cobra venom expresses at least three kinds of weak 3FTxs, namely orphan type XVIII, orphan type X, and muscarinic toxin. The latter two subtypes are close to those found in venom of African *Hemachatus hemachatus* (Rinkhals), *Aspidelaps scutatus* (shield-nose cobra), and *Dendroaspis angusticeps* (black

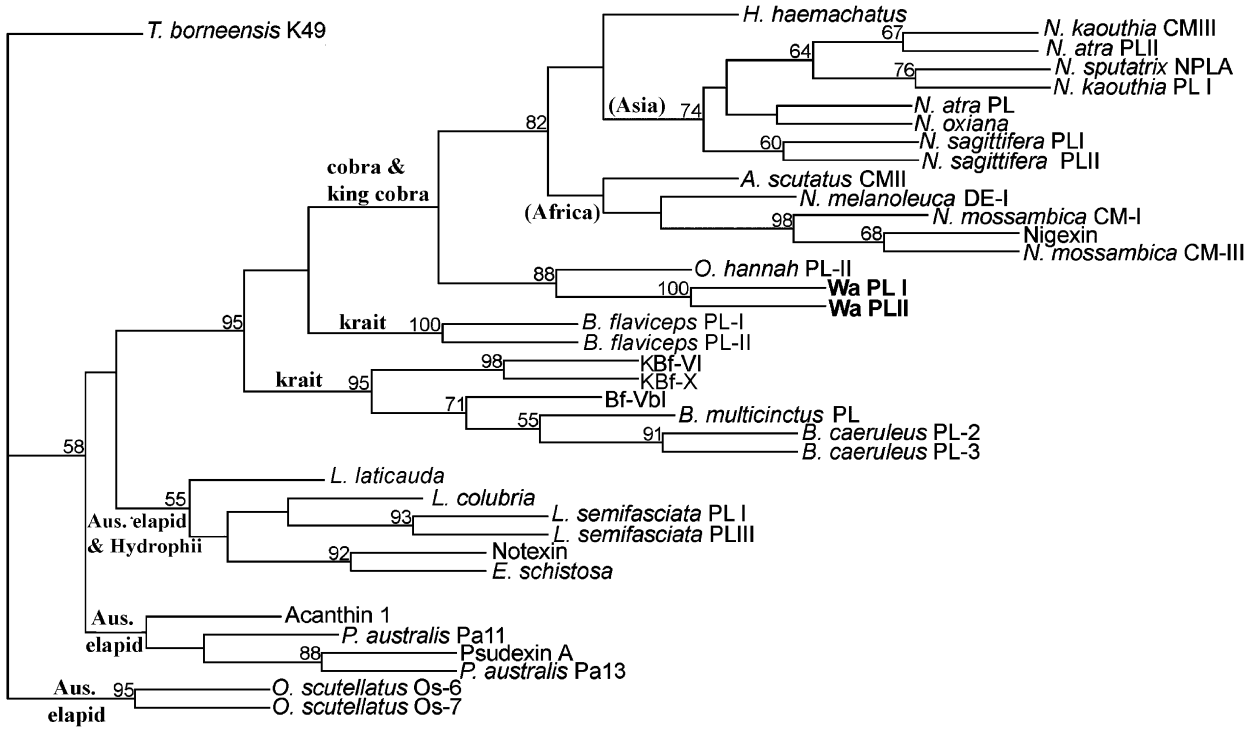

Fig. 5. Phylogenetic analysis of elapid venom PLAs. *Trimeresurus borneensis* venom Lys49-PLA (AAR14171) is assigned as an outgroup. Bootstrap values, if >50, are shown at each node. Species, abbreviations, and accession numbers are: *Acanthophis antarcticus* Acanthin I, P81236; *Aspidelaps scutatus* CMII, P07037; *Bungarus caeruleus* PL-2 and 3 AAR19228–AAR19229; *Bungarus flaviceps* PL-I and II, Ab112359–Ab112360; *Bungarus faciatus* KBf-VI DQ508411, KBf -X DQ508414, Bf-Vb-1 (Liu and Lo, 1994); *Bungarus multicinctus* PL 0702209A; *Enhydrina schistosa* P00610; *Haemachatus haemachatus* P00595; *Laticauda colubria* P10116; *Laticauda laticaudata* CAA68449; *Laticauda semifasciata* PLI BAB72247, PLIII P00612; *Naja atra* PL, CAA45372; PL-II, Q91133; *Naja Kaouthia* PLI, BAA36403, CMIII, P00597; *Naja melanoleuca* DE-I, P00599; *Naja mossambica* CM-I, P00602, CM-III, P00604; *Naja oxiana*, P25498; *Naja pallida* Nigexin, P14556; *Naja sagittifera* PLI, AAR16428, PLII, AAR00254; *Naja sputatrix* NPLA, Q92084; *Notechis scutatus* Notexin, P00608; *Ophiophagus hannah* PL-II, AF302907; *Oxyuranus scutellatus* Os-6 and 7, AAY47070–AAY47071; *Pseudechis australis* Pa11 P04056, Pa13 P04057; *Pseudechis porphyriacus* pseudexin A, P20258.

mamba). The functions of most of these short chain 3FTxs are not well defined.

KIns have usually been isolated from the venoms of elapids and true vipers but not from those of pitvipers (Zupunski et al., 2003). The molecular phylogenetic tree of the elapid venom KIns was constructed using a neurotoxin from mamba venom, Dtx-E, as an outgroup (Fig. 7). The tree topology revealed the clustering of elapid venom KIns under five groups, i.e. Australian Elapidae and sea snakes, kraits, Asian cobras, African cobras, and king cobras. This is apparently in accord with the phylogeographic relationships of these species. The data set used to build this tree does not include the B-chains of β-bungarotoxins, dendrotoxins, and viperid venom KIns. If included, they would be looped out from the majority of elapid KIn.

Interestingly, Wa KIn-II and III are linked to a chymotrypsin-inhibitor of king cobra venom and Wa KIn-I and IV are not associated with KIns of African cobras or other elapids. Thus, the cladograms of both venom PLAs and KIns (Figs. 5 and 7) suggest a close relationship between *W. aegyptia* and king cobra, and these molecular phylogeny trees might give new insights for the elapid biosystematics. In a recent species tree of elapids constructed based on sequences of mitochondrial DNA or other nuclear genes (Castoe et al., 2007), *W. aegyptia* was close or linked to *A. scutatus* rather than to king cobra, but the bootstrap value was too low to assure the relationship.

In conclusion, we solved the cDNA and the protein sequences of a total of seven *W. aegyptia* venom toxins (Figs. 3 and 4) and characterized the toxins. While its 3FTxs are either typical neurotoxins (e.g. Wa-III) or weak toxins (Wa-V), its PLAs and KIns are found to be similar or closely related to those of king cobra venom (Figs. 5 and 7). Morphological variations in the genus *Walterinnesia* across its distribution were recently examined, and the eastern populations (Turkey, Saudi Arabia, Iran) were found to differ consistently from those further west (Egypt, Israel, Jordan) in having lower scale row counts around the neck (21–23, vs. usually 25–27 in western populations), and in having a banded juvenile pattern (Nilson and Rastegar-Pouyani, 2007). Interestingly, king cobra has banded pattern for all juveniles and some of the adults, but lower scale row counts (15–19) than those of *Walterinnesia*. Taken the results together, king cobra (an Asian elapid) is likely to descent from African elapids in recent Natural history. Wa-PLAs are acidic enzymes and may inhibit platelets' function or affect the cardiovascular system (Al-Shammary et al., 1998), like king cobra venom PLAs (Huang et al., 1997a, b; Xu et al., 2003). Whether the

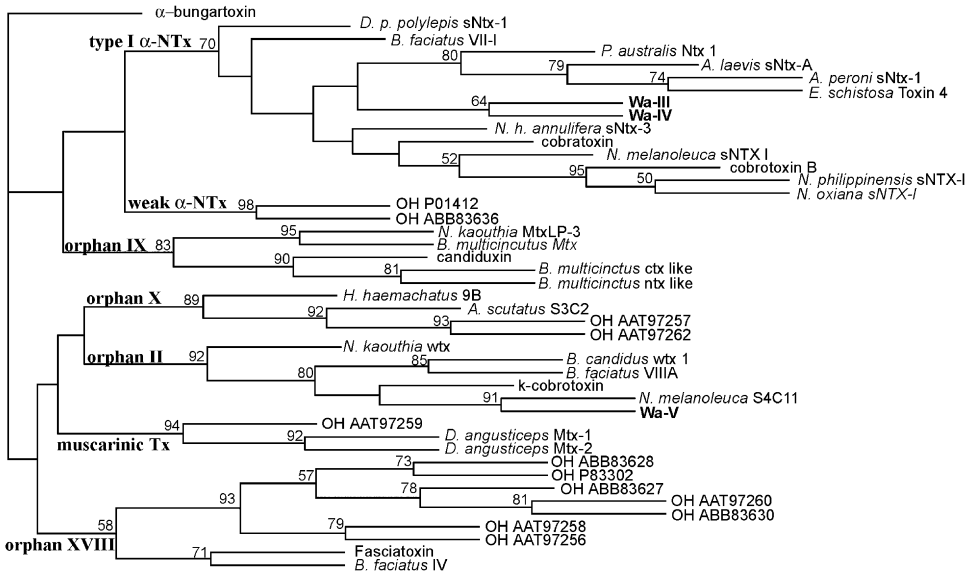

Fig. 6. Phylogenetic analysis of short chain 3FTxs. α-Bungarotoxin is assigned as the outgroup. Bootstrap values, if >50, are shown at each node. Species, abbreviations, and accession numbers are: *Acalyptophis peroni* sNtx-1, AAV33393; *Aipysurus laevis* sNtx-A, P32879; *Aspidelaps scutatus* AsS3C2, P19003; *B. candidus* wtx 1, AAL30059, candiduxin 1, AAL30057; *B. faciatus* fasciatoxin, P14534, VII-1, P10808, VIIIa, A2CKF6, IV (Liu et al., 1991); *B. multicinctus* Ctxl, CAA71820, Bm X-2, Q9YGI0, Mtx, Q9W727; *Dendroaspis angusticeps* Mtx-1 and Mtx-2, P81030 and P18328; *Dendroaspis p. polylepis* sNtx-1, P01416; *Enhydrina schistosa* Toxin 4, P68415; *H. haemachatus* 9B P24778; *N. atra* κ-cobrotoxin CAA76846, cobrotoxin, P60770, cobrotoxin-b, P80958; *N. haje annulifera* Nha sNtx-3 P01420; *N. kaouthia* mtxlp-3, P82464, wtx P82935; *N. melanoleuc*a sNTX-I P01424, S4C11 P01400; *N. oxiana* sNTX-I P01427; *N. philippinensis* sNTX-I P60773; *Pseudechis australis* Ntx-1 P25497.

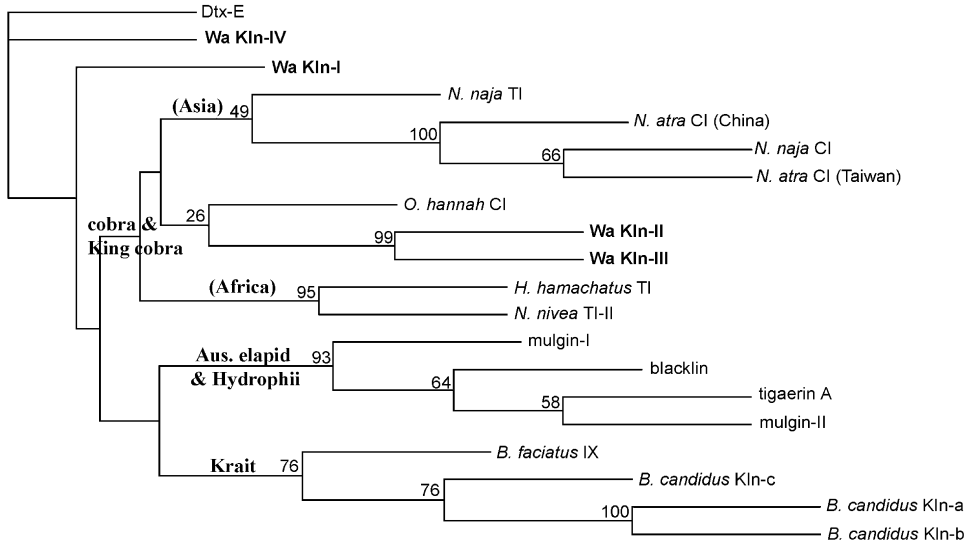

Fig. 7. Phylogenetic analysis of the venom KIns. *D. p. polylepis* Dtx-E (P00984) is assigned as the outgroup. Bootstrap values, if >50, are shown at each node. Species, abbreviations, and accession numbers are: *B. candidus* KIn-a–c, AAL30068–AAL30070; *B. fasciatus* IX P25660; *H. haemachatus* TI P00985; Chinese *N. atra* CI (Zhou et al., 2004), and Taiwanese *N. atra* CI, CAE51866; *N. naja* CI CAE51866, TI P20229; *N. nivea* TI-II P00986; *N. scutatus*, tigerin AAT45409; *O. hannah* CI P82966; *P. australis* mulgin I and II AAT45400–AAT45401; *P. porphyriacus* blacklin AAT45410.

W. aegyptia venom metalloprotease is similar to the P-III found in king cobra venom (Guo et al., 2007) remains to be investigated. Synergistic actions between these venom components possibly contribute to the rapid death reported for envenoming by either species. Further studies on the functions and properties of these venom components are underway.

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