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Complementary DNA sequencing and identification of mRNAs from venomous gland of *Agkistrodon piscivorus leucostoma*

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Abstract

To advance our knowledge on the snake venom composition and transcripts expressed in venom gland at the molecular level, we constructed a cDNA library from venom gland of *Agkistrodon piscivorus leucostoma* for the generation of expressed sequence tags (ESTs) database. From the randomly sequenced 2,112 independent clones, we have obtained ESTs for 1,309 (62%) cDNAs which showed significant deduced amino acid sequence similarity (scores > 80) to previously characterized proteins in NCBI database. Ribosomal proteins make up 47 clones (2%) and the remaining 756 (36%) cDNAs represent either unknown identity or show BLASTX sequence identity scores of < 80 with known GenBank accessions. The most highly expressed gene encoding phospholipase A₂ (PLA₂) accounting for 35% of *A. p. leucostoma* venom gland cDNAs was identified and further confirmed by crude venom applied to SDS-PAGE electrophoresis and protein sequencing. A total of 180 representative genes were obtained from the sequence assemblies and deposited to EST database. Clones showing sequence identity to *disintegrins*, *thrombin-like enzymes*, *hemorrhagic toxins*, *fibrinogen clotting inhibitors* and *plasminogen activators* were also identified in our EST database. These data can be used to develop a research program that will help us identify genes encoding proteins that are of medical importance or proteins involved in the mechanisms of the toxin venom.

Keywords

Agkistrodon piscivorus leucostoma; Venoms; cDNA library; ESTs; Phospholipase A₂

1. Introduction

Snake venoms are exquisitely complex mixture of many different proteins, enzymes, carbohydrates, lipids and biologically active peptides (Bieber, 1979; Perez and Sanchez, 1990). They tend to belong to relatively small stable protein families (Tsetlin, 1999). Different venomous snake taxa employ different combination of mechanisms and no single species employs them all (Aird, 2002). There are differences between venoms of juvenile and adults, and even among different geographic regions (Chippaux et al., 1991; Daltry et al., 1996). These differences may be due to different evolutionary pressures, like different ancestry, prey and environments. The primary function of snake venom is to immobilize,

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paralyze, kill and digest prey organisms (Thomas and Pough, 1979; Karlsson, 1979). In spite of the harmful effects of snake venom, it has an important place in scientific discovery and medicine. For example, some venom components, including disintegrins (Niewiarowski et al., 1989; Huang et al., 1991), phospholipase A₂ (Holland et al., 1990; Tu, 1991), metalloproteinase (Baravoma et al., 1989; Selistre and Ownby, 1995) and other components have an important place in the treatment of thrombosis, arthritis, cancer, strokes, heart attacks and many other diseases.

The generation of large numbers of partial cDNA sequences, or expressed sequence tags (ESTs), has provided a method with which to sample a large number of genes from an organism (Rounsley et al., 1996). The EST analysis is an effective method for collecting expressed genes, gene expression profiling (Adams et al., 1991), and development of resources useful for functional genomics studies (Kashima et al., 2004). To our knowledge, Adams (1991) constructed the first human brain EST database, and then a large number of EST databases were developed from plant to animal kingdom. Several groups have been accumulating snake venom ESTs. Junqueira-de-Azevedo and Ho (2002) obtained 210 distinct genes from the *viperidae* snake *Bothrops insularis*. Francischetti et al. (2004) randomly sequenced 600 of independent clones from venomous gland cDNA library of the snake *Bitis gabonica* (Gaboon viper). Wagstaff and Harrison (2006) assembled a toxin transcriptome based upon 1000 expressed sequence tags (ESTs) from pooled venom glands of 10 individual *Echis ocellatus*. Zhang et al. (2006) generated 8696 expressed sequence tags from a non-normalized venomous gland cDNA library of *Deinagkistrodon acutus*, Pahari et al. (2007) constructed a venomous gland cDNA library of rattlesnake, *Sistrurus catenatus edwardsii* and generated 576 ESTs. With the increasing number of ESTs present in public databases (National Center for Biotechnology Information (NCBI), SWISS-PROT, EMBL, etc.), it may be possible to identify important and useful genes by comparing EST database obtained from the sequencing of randomly isolated clones from any given cDNA library. EST data from the venom glands of medically important snakes enables a rapid examination of venom composition and relative expression levels of the individual venom components (Wagstaff and Harrison, 2006). Particularly, the cDNA clones containing full-length coding sequences (CDSs) are very beneficial for analyses using protein products translated from clones, such as the preparation of antibodies against venom antigens. Furthermore, the recombinant proteins generated from insect cell expression system can be used for structural and functional study of therapeutically relevant bio-molecules.

The western cottonmouth, *Agkistrodon piscivorus leucostoma*, is a venomous pit viper subspecies, and geographical distributed from southern Illinois south to Alabama, west to Oklahoma and Central Texas in the USA. As an attempt to examine the transcripts expressed in the venom gland of *A. p. leucostoma* and to determine if ESTs would be useful for advancing our knowledge on the complex mixture of snake venom, we constructed a plasmid cDNA library to identify genes and their putative proteins. A total of 180 representative ESTs was obtained from randomly sequenced 2,112 independent clones. One of the highly expressed genes encoding phospholipase A₂ was identified and further characterized. This database offers information which help us to elucidate the structure-function relationships of snake venom genes and as a foundation for enhancing genomic studies in the future.

2. Materials and methods

2.1 Snake venom gland and cDNA library construction

Glands were dissected from an euthanized adult snake (*A. p. leucostoma*) and immediately frozen in liquid nitrogen. Approximately 20 µg of gland was used to extract total RNAs by using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). The integrity of total RNA was

checked by discerning the 28S and 18S bands of ribosomal RNA in formaldehyde denaturing 1% agarose gel. A directional cDNA library using 0.5 µg of total RNA was constructed by using Creator SMART cDNA Library Construction Kit (Clontech Laboratories, Inc., Mountain View, CA, USA) according to the manufacturer's instructions with minor modifications. First, total RNA (0.5 µg) from venom gland was reversed transcribed to cDNA using PowerScript Reverse Transcriptase (Clontech) and the CDSIII/3' PCR primer (Clontech) for 1h at 42 °C. Second, double stranded cDNA (ds cDNA) synthesis was performed on a iCycler Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) by LD PCR reaction containing 80 µL of deionized H₂O, 10 µL of 10 x Advantage 2 PCR buffer, and 2 µL of first-strand cDNA, 50 x dNTP Mix, 5' PCR primer, CDS III/3' PCR primer and 50 x advantage 2 polymerase Mix. The final volume will be 100 µL. PCR conditions included an initial denaturation step at 95 °C for 1 min followed by 18 cycles for 95°C for 15s, 68°C for 6 min. Lastly, the digested ds cDNA was fractionated using CHROMA SPIN-400 column (Clontech). Samples of 3 µL of each fraction were electrophoresed on a 1.1% agarose/EtBr gel to determine the peak fractions by visualizing the intensity of the bands under UV. The fractions were pooled as large-, medium- and small-sized cDNA, concentrated and separately ligated into pDNR-LIB vector (Clontech) for overnight at 16°C. The resulting ligation reactions were transformed into One shot Electrocomp GeneHogs *E.coli* (Invitrogen Corporation, Carlsbad, CA, USA) separately. The three libraries (large-, medium- and small-sized) were mixed. The final resulting plasmid library had over 1 million independent clones having inserts with an approximate average size of 700 bp by checking over 200 clones.

2.2 Plasmid preparation and DNA sequencing

Individual colonies were randomly picked from a Luria-Bertani (LB)/Chloroamphenical plate and transferred to 96-well plate containing 1.4 ml of LB and 50 µg mM⁻¹ chloroamphenical. Plates were incubated overnight with shaking of 225 rpm at temperature of 37 °C. Plasmid DNAs were isolated by using Montage Plasmid Miniprep Kit (Millipore Corporation, Billerica MA, U.S.A), according to the manufacture's instruction manual. Extracted plasmid DNAs were sent to Purdue Genomics Core Facility for automated sequencing from the 5' end of each cDNA by using BigDye3.1 on Applied Biosystems 9700 thermal cycler.

2.3 Sequence Analysis and EST database construction

Sequence chromatogram files from each sequence reaction were processed manually and using the Lasergene 7.1 software (DANSTAR, Inc., Madison, WI) to remove low quality regions, contaminating vector sequences and poly A+ tracts. Each edited EST sequence (average size 612bp) was searched against GenBank BLASTN and BLASTX algorithms (Altschul et al., 1997) via the web site (<http://www.ncbi.nlm.nih.gov/BLAST/>) to obtain the best match sequence identities. Scores of 80 or *E*-value of 4.00E-14 (a sequence > 150 nucleotides) was used as a criterion for indicating potentially significant homology (Newman et al., 1994). Sequence identities (Scores< 80) were considered to be unknown. The electronic version of sequences with hyperlinks to web-based databases, each contig in DNASTAR files and the best match to the GenBank of each sequence are available on request (ying.jia@tamuk.edu). The 1,309 ESTs were obtained from our cDNA library. A total number of 180 representative ESTs from each cDNA assembly were submitted to NCBI EST database, where they were given GenBank accession numbers (EV854764–EV854943) and can be accessed EST database (<http://www.ncbi.nlm.nih.gov/>).

2.4 Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE)

Thirty micrograms of crude venom from *A. p. leucostoma* was heated in NuPAGE LDS sample buffer (Invitrogen) containing SDS without reducing agents for 10 min and

subjected to a NU-PAGE 4–12% Bis-Tris gel (1 mm thick) using NuPAGE MES SDS Running buffer (Invitrogen). Gel was stained in 0.2% Coomassie Blue. For N-terminal sequencing of proteins, samples of 30 µg was electrophoresed and transferred to polyvinylidene difluoride (PVDF) membrane using 10 mM CAPS, pH 11.00, 10% methanol as transfer buffer on a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (BIO-RAD). The membrane was stained in 0.02% Coomassie Blue without acetic acid for 5 min, thoroughly washed in distilled water, and dried at room temperature. Stained bands were subjected to Edman degradation using a Procise sequencer (Iowa State University).

3 Results and Discussion

3.1 Blast search and sequence analysis

To improve our understanding of the composition of *A. p. leucostoma* venom and its transcriptional activity at the molecular level, we elected to construct a plasmid cDNA library to develop an EST database. To ensure the high molecular weight transcripts were included, the synthesized double strand cDNA and size-selection of cDNAs were electrophoresed on a 1.1% agarose/EtBr gel, resulting in a ds cDNA of 0.1–4 kb smear appearing on the gel. The fractions were pooled as large-, medium- and small-sized cDNA, and separately ligated into vector. The resulting ligation reactions were transformed into *E. coli* separately. The three libraries (large-, medium- and small-sized) were mixed and generated a final cDNA library with a titer of 1.3×10^8 . The 2,112 clones from cDNA library were randomly selected and sequenced from 5' end. A total of 1,932 sequences with an average insert size 612bp (ranging from 250–1,229bp) were obtained after removing low quality, sequences of less than 150 bp and contaminating vector sequences. Almost all sequences possess poly (A⁺) tail except for those having more than 700 bp nucleotides and the sequencing was not reached to 3' end. To determine the number of matches in NR nucleotide NCBI databases, a BLAST N search (Altschul et al., 1997) was carried out against NCBI database using 1,932 sequences, and BLAST scores of 80 or *E*-value of $4.00\text{E-}14$ (a sequence >150 nucleotides) was used as a criterion for indicating potentially significant homology (Newman et al., 1994). BLASTN search had 1,661 (86%) database hits and the remaining 271 (14%) had no hits in GenBank database. To determine the number of matches in all protein, a BLASTX search (Altschul et al., 1997) was performed against protein database of NCBI. A total number of 1,309 (68%) ESTs were obtained through BLASTX search, which had significant similarities in GenBank, and the remaining 623 (32%) sequences either gave no BLASTX identity matches (26%) or had scores of less than 80 (6%). The significant homologies of sequences were given more by BLASTN search than by BLASTX partly due to most venom proteins are relatively small proteins with long 5' and 3' end untranslated regions (UTRs) (Junqueira-de-Azevedo and Ho, 2002), which make significant database hits in GenBank at the nucleotide level. Not surprisingly, one of the most highly expressed genes in snake venom coding phospholipase A₂ accounting for 35% (684 cDNAs) of *A. p. leucostoma* venom gland cDNAs was identified and further characterized. The highly expressed house-keeping genes, especially genes related to the transcription and translation machinery such as ribosomal protein (2.4%), translation initiation factors, and elongation and release factors were also identified. Hypothetical proteins consist of 40 sequences (2.0%) in our EST database. Fig. 1 shows the relative proportion of the number of individual cDNA over the total number of 1,932 randomly sequenced clones.

We are interested in identifying genes encoding proteins which can be used in biomedical applications. For example, some components in snake venom shows very promising anti-cancer effects and may prevent the growth of cancerous tumors. The rich source of serine proteases and metalloproteases induces a variety of toxic effects such as haemorrhage, platelet aggregation and blood coagulation (Markland, 1998). Homology searches of our

ESTs in GenBank database reveal a number of genes that were likely to play an important role in human diseases. These genes included several with homology to metalloproteinases and disintegrin related genes, genes with similarity to thrombin-like proteins, serine proteases, hemorrhagic toxins, fibrinogen clotting inhibitors and plasminogen activator related genes. Our EST database was constructed to store sequences and similarity data of snake venom transcript assemblies and to make this information available to users for obtaining sequence data and names for clones of interest.

Sequence analysis revealed that 1,932 sequences were clustered into 465 putative gene categories, including 329(17%) singletons and the other 1,603(83%) sequences assembled into 136 contigs, with each contig having 2–370 overlapping sequences. We estimated that at least 512 unique sequences can represent the transcriptome of this viper venom gland. This 512 unique sequences includes polymorphic variants (few nucleotide substitutions), sequences with scores less than 80, ribosomal proteins and hypothetical proteins. The electronic version of these 512 sequences with hyperlinks to web-based databases, and the best match to the GenBank of each sequence are available on request (ying.jia@tamuk.edu). Our objective was not to separate all polymorphic variants into unique gene but to select one of the representative sequences from each assembly. Only the genes that have been cloned repetitively and their cDNA nucleotide sequences confirmed by absolutely confident chromatogram were reported here. As a result, we have obtained a total number of 180 representative ESTs from the sequence assemblies based on having the longest sequence, most highly represented among the sequences and having significant homolog score >80. The complete sequences of the inserts of these 180 representatives ESTs are being determined. Table 1 shows the putative protein identity match and GenBank accession numbers for each of the 180 representative ESTs that showed protein identity matches other than ribosomal proteins or hypothetical proteins. These 180 ESTs listed in Table 1 have been submitted to NCBI EST database with accession number EV854764–EV854943 and can be accessed in public dbEST (<http://www.ncbi.nlm.nih.gov/>).

3.2. Highly Expressed Gene Encoding Phospholipase A₂ (PLA₂)

Phospholipase A₂ (PLA₂), one of the most extensively studied small, globular enzymes found ubiquitously in nature, is a diverse class of enzymes based on function, localization, regulation, mechanism, structure, and role of divalent metal ions. The primary function of phospholipases A₂ (PLA₂s) is to cleave phospholipids at the A₂ position, and more than 150 PLA₂s have been identified in several snake venoms (Gutierrez and Rucavado, 2000; Valentin and Lambeau, 2000). In snake venoms the PLA₂s have been described as an essential role in toxicity including envenomation, neurotoxicity, myotoxicity, anticoagulant, proinflammatory effects and digestion (Rosenberg, 1991; Tu, 1991; Bejhini et al., 2008; Gutierrez et al., 2008). Some of the envenomation symptoms involve not only the hemostatic system, with an anticoagulant and an antiplatelet profile (Fuly et al., 1997; Lizano et al., 2003), but also inflammatory and myotoxic effects (Thimmegowda et al., 2007; Narendra et al., 2007). The snake presynaptic neurotoxins can also present PLA₂ activity, which leads to the release of acetylcholine followed by impairment of synaptic functions. These neurotoxins are spread through several families of Colubroidea superfamily (Hodgson and Wickramaratna, 2002; Rossetto et al., 2004; Pungercar and Krizaj, 2007). Kashima et al. (2004) generated 549 expressed sequence tags (ESTs) from venom gland of *Bothrops jararacussu*, of which 36% was phospholipase, the largest proportion among the ESTs. Due to the fact that no effort was made to eliminate redundant sequences, the highly expressed gene presented in our database was also PLA₂, which consists of 694 cDNAs including 684 having significant homology in GenBank and 10 with scores of less than 80. Of the 694 PLA₂ cDNAs, 419 cDNAs were full-length, usually containing 130–140 amino acids beginning with MRT and ending with PC amino acids. These 684 cDNAs, being

composed of 35% of *A. p. leucostoma* venom gland, was assembled into 6 contigs with each contig having 2–370 cDNAs. We chose one of representative PLA₂ from each contigs. Contig 8 (representative clone, 16D02) contains 370 PLA₂s corresponding to D-49 type PLA₂, Contig 17(13C11) is composed of 169 PLA₂s belonging to K-49 type, and Contig 9 (02A06) is comprised 64 PLA₂s corresponding to D-49 type but structure of 02A06 is much different from 16D02. Contig 25(01D06), 46 (06H09) and 10 (08D05) contain 3, 2 and 6 PLA₂s, respectively. 01D06, a hybrid D-49 type of PLA₂s, consists of a C-terminal sequence highly similar to that of 16D02, whereas the N-terminal is almost identical to 02A06. Hybrid types of PLA₂s were also found in *Callosellasma rhodostoma* (Tsai et al., 2000) and *Agkistrodon halys* (Pan et al., 1999). Contig 46 and 10 are neither D-49 type nor K-49 type, and each was missing 33 and 30 amino acids. There is no registered full-length PLA₂s isolated from *A. p. leucostoma* in GenBank to date. Therefore, Fig. 2 shows the comparison of these 6 PLA₂s from each contig with amino acid sequence of CvvPLA₂s isolated from *Crotalus viridis viridis* (Tsai et al., 2003). Clone 02A06, 16D02, 01D06 and 13C11 are composed of 137, 139, 139 and 137 amino acids, respectively. Clone 08D05 with 109 amino acids and 06H09 with 106 amino acids are truncated inside the sequences. By comparison with amino acid sequence of CvvPLA, the identities of each clone 02A06, 16D02, 01D06, 08D05, 13C11 and 06H09 were 70%, 68%, 71%, 56% and 53%, and 37%, respectively. The nucleotide sequences of these proteins at coding regions are much more divergent than the sequences at 5′- and 3′-untranslated regions (UTRs) (data not shown), and this finding is consistent with the previous studies (John et al., 1994; Nakashima et al., 1995). It is important to note that these 6 representative sequences had no nucleotide substitution, and derived from absolutely confident comparison of chromatograms. The majority of the other full-length phospholipases had at least 1 nucleotide substitution. The causes of the generating changes of nucleotide in PLA₂s were either due to PCR involved in synthesis of double strand cDNA during cDNA library construction, or changes derived by duplication of a common ancestral gene. Nucleotide substitutions were also found in phospholipases by Junqueira-de-Azevedo and Ho (2002) and in serine proteases (Deshimaru et al., 1996).

To further confirm that the PLA₂ is the highest percentage component in *A. p. leucostoma* venom, approximately 30 µg of crude venom was applied to SDS-PAGE, stained by Coomassie Blue (Fig. 3). The gel shows four strong bands and several weak bands. Among the clearly visible bands, band 2 was the brightest band. To identify these proteins, these bands were transferred to PVDF membrane and subjected to Edman degradation. N-terminal amino acid sequences were successfully obtained for these bands. To find match to known proteins, these sequences were blasted against the NR GenBank database. The sequence QFETLIMKIA for band 1 and NLFQFEKLIKMM for the brightest band 2 were identical to phospholipase A₂ (P20249) from *Gloydius blomhoffi* (Tomoo et al. 1992) and phospholipase A₂ (AAB26655) from *Agkistrodon piscivorus* (Welches et al. 1993), respectively. This result is consistent with our cDNA library containing the largest amount of cDNA (35%) coding for phospholipase A₂. In addition, both Band 3 and band 4 share a similar sequence: VIGGDEKNINEH and VIGGDEWNINEH, respectively. These sequences are similar to the N-terminal sequence (VIGGDECNINEH) of serine protease (thrombin-like enzyme) from *Trimeresurus stejnegeri* (Zhang et al, 1998). Band 5 sequence ADGRNPLEECFR is identical to N-terminal of L-amino acid oxidase precursor (LAAO) from *Gloydius blomhoffi* (Takatsuka et al, 2001). Overall, both band 1 and the brightest band 2 corresponding to phospholipase A₂ make up the highest constituent of this snake venom, and this data strongly supports our cDNA library in which the largest proportion of cDNAs was phospholipase A₂.

This EST database shows the immediate effect that can be obtained from the development of an EST database. Not only does our snake EST database allow us to initiate an EST based research programs to study the components of snake venom, but it also provides a genetic

resource for other research programs interested in gene expression in snake venom. Clearly, the development of an EST database for snake venom provides a wealth of resources for monitoring the components in snake venom at the molecular level. Future research projects associated with the development of snake venom EST database will enhance our knowledge of the biology of snake venom and will provide valuable new information to enhance integrated biochemistry for studying such an interesting animal.

In conclusion, combined with clone availability, this EST database can offer a researcher a convenient path to full sequences of many venom gland genes, given an appropriate query. This database also offers information regarding nucleic acid sequences, and cDNA clones will help users to explore genes that play important roles in the treatment of human diseases and medical applications.

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Abbreviations

cDNA	complementary DNA
EST	expressed sequence tag
UTR	untranslated region
PLA₂	phospholipase A ₂

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Fig. 1.

The composition of *A. piscivorus leucostoma* venom gland cDNAs encoding for putative proteins. The number of sequences for each putative transcripts annotated by function terms based on significant BlastX matches (score > 80) against NCBI GenBank. Unknown includes sequences either gave no BLASTX identity matches or had scores of less than 80. Ribosomal proteins (2.4%) and hypothetical protein (2.0%) were not included. Eight thrombin-like enzymes were included in serine. Others include putative proteins with less than 5 members (e.g. vascular apoptosis-inducing protein (5), Parvalbumin (5), fibrinogen clotting inhibitor (3), stejnobin (5), plasminogen activator (4), hemorrhagic toxin (4), S-adenosylmethionine decarboxylase (1), etc. The numbers on top of bar represent each clone numbers in 1932 random sequences).

CvvPLA	MRTLWIVAVL	LLGVEGSLVQ	FETLIMKIAG	RSGLLWYSAY	GCYCGWGGHG	LPQDATDRCC
02A06	M...	D.M.	K	...MF.....	Q.	R.....
16D02	M...	N.F.	..K..K.MT.	K..M.....	Q.	R.K.....
01D06	M...	D.M.	K	...MF.....	Q.	R.K.....
08D05	M...	D.M.	K	...MF.....	Q.	R.K.....
13C11	L.	.V.....VLE	LGKM.LQET.	KNAITS.GS.	..N....HR.	Q.K.....
06H09	L.	V.....VLE	LGKM.LQET.	KNAITS.GS.	..N....HR.	Q.K.....-
CvvPLA	FVHDCCYGKA	TDCNPKTVS	YSVKNGEII	CEDDDPCKRQ	VCECDRVA	AVCFRDNIPSYN
02A06	...D.....V	.G.D..LD..	E..DVV	.GGN....KE	I.....A..I	KVT.D
16D02	...D.....V	.G.D..LD..	E..DVV	.GGN....KE	I.....A..I	KVT.D
01D06	...D.....V	.G....MDI.	E..N.V	.GGTN...K.	I.....A..I	LKT.D
08D05	-----	---D..LD..	E..DVV	.GGN....KE	I.....A..I	KVT.D
13C11	...K...K.L	H..DR.	S..W..KA..	..EKN..LKE	M....KAV.I	.L.E.LDT..
06H09	-----	-----	-----	..EKN..LKE	M....KAV.I	.L.E.LDT..
			% Identity		Predicted Mass	
CvvPLA	NN-YKRFP	AE	NCREEP	EPC	100	15540
02A06	.K-.W.P.QN	CKE.-S...			70	15685
16D02	SKT.WKY.KK	..K..S...			68	11840
01D06	SKT.WKY.KK	..K..S...			71	15833
08D05	.K-.W...PQ	..K..S...			56	12582
13C11	K.-.K.YFKL	K.KK-.DT.			53	15713
06H09	KK-.K.YFKL	K.KK-.DT.			37	11934

Fig. 2.

Alignment of deduced amino acid sequences and molecular masses of PLA₂s from snake venom gland. Alignment was constructed by Lasergene 7.1 software and manually. The boxes represent the calcium binding site. The sources of sequences were as follow: CvvPLA (Acc. No. AAO93140) as a control isolated from *Crotalus viridis viridis*. Clone 02A06 (Acc. No. EV854772), 16D02 (Acc. No. EV854875), 01D06 (Acc. No. EV854874), 13C11(Acc. No. EV854871), 06H09 (Acc. No. EV854870), and 08D05(Acc. No. EV854873) were isolated from venom gland of snake (*Agkistrodon piscivorus leucostoma*)

Fig. 3. SDS-PAGE of snake (*Agkistrodon piscivorus leucostoma*) total venom under denaturing but non-reducing condition (NuPAGE LDS sample buffer). Sample of 30 ul total venom was subjected to a 4–12% NuPAGE Novex Bis-Tris mini gel with MES running buffer. Pre-stained standard is shown on the left. Sequence for each band and its identification are on the right.

Table 1

A representative list of putative protein identity matches for expressed sequence tags (ESTs) obtained from randomly sequenced clones from *A. p. leucostoma* venom gland. GenBank accession numbers are shown for each submitted EST

Accession No.	HIT ID	E-value (score)	Putative identification
EV854764	NP_036228	3.00E-74 (280)	Adaptor protein,
EV854765	BAC579722.00	2.00E-68 (261)	Alpha globin
EV854766	XP_001506169.1	1.00E-118 (429)	Annexin A1
EV854767	NP_001007941	8.00E-67 (257)	Adenine phosphoribosyltransferase
EV854768	XP_001368124	7.00E-81 (303)	Arginine-rich early stage tumors
EV854769	NP_001091001.1	1.00E-35 (152)	ATPase,
EV854770	CAA62600.1	7.00E-27 (122)	Atrolysin e
EV854771	NP_990681	9.00E-83 (310)	B-cell translocation gene
EV854772	AAL48289.1	2.00E-15 (86.7)	Beta-2-microglobulin protein
EV854773	Q8AY75	1.00E-83 (312)	Calglandulin
EV854774	Q91053	6.00E-82 (307)	Calobin precursor
EV854775	XP_418914	6.00E-50 (199)	CDK5 regulatory associated protein
EV854776	CAJ83813	2.00E-17 (92.8)	CHK1 checkpoint homolog
EV854777	EAW95563.1	4.00E-55 (218)	Chromodomain protein
EV854778	AAL99919	3.00E-96 (353)	CLL-associated antigen KW-12
EV854779	XP_235705	8.00E-92 (339)	Coatomer subunit zeta-1
EV854780	NP_001029211.1	1.00E-17 (91.7)	Collagen, type I, alpha 1
EV854781	NP_001025946	2.00E-54 (215)	COP9 complex subunit 7a
EV854782	XP_419151	2.00E-61 (237)	Corneal epithelial Rho-associated-kinase
EV854783	AAF76899	1.00E-112 (408)	Creatine kinase
EV854784	ABB42830.1	4.00E-34 (147)	Crotastatin
EV854785	AAQ11364	5.00E-209 (99.8)	Crotocetin-1
EV854786	AAQ11364	7.00E-62 (240)	Crotocetin-1
EV854787	AAQ11364	7.00E-62 (240)	Crotocetin-1
EV854788	AAM22788.1	7.00E-70 (266)	C-type lectin
EV854789	AAM22788	1.00E-71 (272)	C-type lectin
EV854790	AAM22788	2.00E-69 (265)	C-type lectin
EV854791	XP_425393.2	5.00E-60 (232)	CUTL1 protein
EV854792	YP_313674	1.00E-45 (184)	Cytochrome c oxidase
EV854793	YP_313671	4.00E-93 (343)	Cytochrome c oxidase subunit II
EV854794	XP_429181	5.00E-15 (83.2)	Cytochrome c oxidase subunit VIIc
EV854795	NP_001007474.1	1.00E-50 (203)	Defender against cell death 1
EV854796	Q805F5	2.00E-62 (242)	Disintegrin piscivostatin alpha precursor
EV854797	Q805F5	2.00E-62 (242)	Disintegrin piscivostatin alpha precursor
EV854798	XP_001507891	1.00E-100 (367)	Elongation factor 1 alpha-2
EV854799	XP_001493068.1	6.00E-59 (229)	Elongation factor SIII p15 subunit
EV854800	NP_001075254	8.00E-50 (198)	Eukaryotic translation elongation factor
EV854801	AAM93483.1	5.00E-22 (106)	Eukaryotic translation elongation factor 1

Accession No.	HIT ID	E-value (score)	Putative identification
EV854802	XP_534847.2	2.00E-33 (144)	Family with sequence similarity 60
EV854803	NP_001006535	3.00E-94 (348)	Far upstream element binding protein
EV854804	Q7SXA5	3.00E-51 (204)	Ferritin light chain
EV854805	P07229	5.00E-71 (271)	Ferritin, higher subunit
EV854806	AAD18055	1.00E-48 (196)	Fibrinogen clotting inhibitor A chain
EV854807	XP_425040.1	1.00E127 (457)	Fructose 1,6-bisphosphatase
EV854808	XP_001367289.1	3.00E-31 (137)	G2/mitotic-specific cyclin-B1
EV854809	AAN77283	8.00E-72 (273)	Gag-Pro-Pol protein
EV854810	Q9PSN0	1.00E-53 (212)	Galactose-specific lectin (PAL)
EV854811	Q9PSN0	3.00E-82 (308)	Galactose-specific lectin (PAL)
EV854812	NP_001025786	3.00E-74 (280)	Gallus gallus clathrin interactor 1
EV854813	XP_001368349	1.00E-107 (391)	Gallus gallus SEC63 homolog
EV854814	XP_424406	3.00E-27 (123)	Gekko japonicus GekBS201P mRNA
EV854815	NP_990696.1	1.00E-80 (301)	Glutathione S-transferase
EV854816	AAG17178	7.00E-63 (244)	Halyxin A-chain precursor
EV854817	AAG28522	1.00E-69 (266)	Halyxin B-chain precursor
EV854818	NP_990398.1	3.00E-46 (187)	Heat shock 10kDa protein
EV854819	NP_989620	1.00E-103 (378)	Heat shock protein 90kDa
EV854820	AAA03326.1	5.00E-38 (160)	Hemorrhagic toxin a
EV854821	NP_990427.1	1.00E-129 (465)	High density lipoprotein binding protein
EV854822	XP_001513003	4.00E-51 (202)	Histone H2a(A)-613
EV854823	AAB23201	4.00E-54 (214)	Ht-e, hemorrhagic toxin e
EV854824	XP_001231746	6.00E-94 (346)	Human P5 isoform
EV854825	ABI33937	1.00E-93 (345)	Hyaluronidase
EV854826	AAH73769	3.00E-48 (195)	Ig kappa chain precursor
EV854827	ABG72683	2.00E-32 (141)	Immunoglobulin A
EV854828	AAL37211	7.00E-57 (224)	Immunoglobulin lambda light chain
EV854829	AAA39434	2.00E-62 (242)	Immunoglobulin lambda-chain
EV854830	AAF68173.1	5.00E-46 (186)	Insulin-like growth factor 2 receptor
EV854831	XP_001373175.1	2.00E-62 (241)	Insulinoma protein (rig)
EV854832	XP_001366516.1	2.00E-89 (332)	Interleukin enhancer binding factor 3
EV854833	XP_511489	1.00E-51 (207)	Isolog of yeast sui1 and rice gos2
EV854834	XP_001115408.1	8.00E-51 (202)	Keratinocyte associated protein 2
EV854835	XP_419719	1.00E-61 (239)	Keratinocytes associated protein
EV854836	NP_001079355	1.00E-50 (203)	Kielin [Xenopus laevis]
EV854837	Q6STF1	1.00E-130 (468)	L-amino-acid oxidase precursor (LAAO)
EV854838	XP_416590.1	4.00E-69 (265)	Leucine zipper nuclear factor 1
EV854839	XP_001233279	2.00E-68 (261)	LOC398473 protein
EV854840	XP_001489661	3.00E-34 (146)	Macrophage migration inhibitory factor
EV854841	EDL11708.1	3.00E-52 (206)	MCG19578, isoform CRA_a
EV854842	XP_001362614.1	2.00E-56 (221)	Membrane protein palmitoylated 4
EV854843	AAP78951.1	1.00E-121 (438)	Metalloprotease

Accession No.	HIT ID	E-value (score)	Putative identification
EV854844	AAC59703.1	1.00E-110 (401)	Metalloproteinase
EV854845	ABD34834.1	2.00E-31 (139)	Metalloproteinase and disintegrin jarastatin
EV854846	AAC59704	2.00E-45 (186)	Metalloproteinase precursor
EV854847	XP_001363068.1	5.00E-27 (123)	MGC52838 protein
EV854848	XP_416800.2	1.00E-114 (414)	MGC82793 protein isoform 2
EV854849	ABB22797.1	2.00E-53 (213)	MHC class II-associated invariant chain
EV854850	AAU25921.1	8.00E-91 (335)	Myosin light chain
EV854851	NP_990808.1	2.00E-37 (157)	Myosin regulatory light chain 2A
EV854852	XP_415166	5.00E-81 (303)	Myosin, heavy polypeptide
EV854853	XP_419862	2.00E-54 (213)	N-acetylglucosamine-phosphate mutase
EV854854	AAF77622	2.00E-17 (90.9)	NADH dehydrogenase
EV854855	XP_001111436	8.00E-33 (142)	NADH dehydrogenase
EV854856	NP_001072252	9.00E-86 (319)	NADH dehydrogenase
EV854857	YP_313668	5.00E-95 (350)	NADH dehydrogenase
EV854858	NP_001017047.1	1.00E-20 (102)	NADH dehydrogenase
EV854859	AAF77622	1.00E-75 (284)	NADH dehydrogenase subunit 4
EV854860	XP_532446.1	6.00E-47 (189)	NADH-ubiquinone oxidoreductase
EV854861	XP_001511377.1	5.00E-55 (216)	Nonclathrin coat protein gamma2-COP
EV854862	XP_422088	1.00E-21 (104)	Nucleolar phosphoprotein
EV854863	XP_849017	2.00E-27 (125)	Nucleolar protein family A
EV854864	XP_001500165.1	1.00E-66 (256)	Nucleotidase
EV854865	ABO25859.1	7.00E-58 (227)	Oryctolagus cuniculus lectin
EV854866	AAV33972.1	1.00E-509 (201)	Oxyuranus scutellatus scutellatus PDI
EV854867	P02615	1.00E-50 (202)	Parvalbumin beta
EV854868	NP_001006253	2.00E-63 (245)	PEST-containing nuclear protein
EV854869	BAA06333.1	1.00E-102 (376)	Phosphodiesterase I
EV854870	P49121	7.00E-34 (146)	Phospholipase A2
EV854871	P49121	3.00E-60 (234)	Phospholipase A2 (Myotoxin)
EV854872	A53872	3.00E-74 (281)	Phospholipase A2 monomers
EV854873	A53872	3.00E-48 (194)	Phospholipase A2 monomers
EV854874	P51972	2.00E-69 (265)	Phospholipase A2 (APP-D-49)
EV854875	P51972	7.00E-75 (283)	Phospholipase A2 (APP-D-49)
EV854876	XP_414907.2	1.00E-113 (411)	PI-3-kinase-related kinase SMG-1
EV854877	Q7ZTA0	5.00E-86 (321)	Piscivirin precursor
EV854878	Q7ZTA0	1.00E-133 (476)	Piscivirin precursor
EV854879	Q7ZTA0	1.00E-122 (440)	Piscivirin precursor
EV854880	AAD01624.1	8.00E-94 (347)	Plasminogen activator precursor
EV854881	XP_001364529	6.00E-33 (142)	Plexin A1,
EV854882	XP_001362540.1	1.00E-17 (92)	Polymerase (RNA) II polypeptide G-like
EV854883	XP_001369563.1	9.00E-77 (290)	Proteasome activator PA28 beta
EV854884	AAO85513.1	1.00E-25 (118)	Protein C activator
EV854885	AAO85513.1	1.00E-101 (373)	Protein C activator

Accession No.	HIT ID	E-value (score)	Putative identification
EV854886	EDM02045.1	2.00E-25117	Protein kinase
EV854887	XP_414584.2	1.00E-38 (163)	Protocadherin Fat 2
EV854888	ABG82012.1	1.00E-117 (422)	Putative ubiquitin B
EV854889	XP_541939	1.00E-48 (196)	Pyroglutamyl-peptidase I
EV854890	XP_001231251	1.00E-44 (181)	Rattus norvegicus growth factor
EV854891	EDL87016.1	6.00E-96 (353)	RCG50513, isoform CRA_b
EV854892	EDM10822	2.00E-17 (90.1)	RCG53232, isoform CRA_c
EV854893	BAC82595.1	2.00E-15 (84.7)	Reverse transcriptase
EV854894	P84617	6.00E-32 (140)	Rhealcalcin-1 (RCA-1)
EV854895	AAH03896.2	8.00E-95 (349)	Rpl17 protein
EV854896	AAH94640	1.00E-80 (301)	Rpl17 protein [Mus musculus]
EV854897	XP_416616	2.00E-52 (207)	Rpl24 protein
EV854898	XP_417405	2.00E-33 (144)	Rps21-prov protein
EV854899	NP_990497	7.00E-34 (145)	S100 calcium binding protein A11
EV854900	NP_001012587	2.00E-63 (246)	S-adenosylmethionine decarboxylase 1
EV854901	NP_001019905.1	3.00E-47 (192)	Selenoprotein S
EV854902	ABB76279.1	1.00E-109 (397)	Serine protease
EV854903	AAN52346.1	4.00E-95 (351)	Serine protease
EV854904	ABA42115.1	1.00E-108 (394)	Serine proteinase
EV854905	Q9PTU8	8.00E-83 (310)	Serine proteinase A precursor
EV854906	EDL22601.1	5.00E-95 (350)	Serine/threonine kinase 38
EV854907	Q9DG84	1.00E-87 (326)	Serpentokallikrein-2 precursor
EV854908	XP_414251.2	3.00E-48 (194)	Signal peptidase
EV854909	NP_001006561	3.00E-89 (330)	Signal sequence receptor
EV854910	XP_001231983.1	1.00E-20 (102)	Small EDRK-rich factor 1
EV854911	XP_001504327	4.00E-14 (82)	Solute carrier family
EV854912	NP_001008471.1	4.00E-62 (241)	Solute carrier family 39
EV854913	NP_001025974	4.00E-33 (143)	Spermine synthase
EV854914	AAQ15166	8.00E-78 (293)	Stejaggregin-A alpha chain
EV854915	AAQ15166.1	2.00E-69 (265)	Stejaggregin-A alpha chain
EV854916	AAQ15167	1.00E-58 (229)	Stejaggregin-A beta chain
EV854917	AAQ15169.1	6.00E-59 (230)	Stejaggregin-A beta chain-3
EV854918	AAN52347.1	1.00E-127 (459)	Stejnegeri stejnobin
EV854919	NP_001006302.1	3.00E-67 (258)	Stromal cell derived factor 4
EV854920	XP_001519428.1	8.00E-25 (116)	SUN2 [Ornithorhynchus anatinus]
EV854921	XP_001369869	5.00E-33 (144)	Superfast myosin heavy chain
EV854922	XP_421618	3.00E-24 (113)	Synaptopodin 2-like
EV854923	AAP20638	8.00E-45 (182)	Thrombin-like enzyme defibrase
EV854924	AAL54898	3.00E-40 (167)	Transport protein
EV854925	AAW69916	4.00E-45 (184)	Trimecetin beta-chain precursor
EV854926	AAW69916.1	2.00E-45 (185)	Trimecetin beta-chain precursor
EV854927	NP_990748.1	1.00E-79 (299)	Troponin I, skeletal, fast

Accession No.	HIT ID	E-value (score)	Putative identification
EV854928	NP_990729.1	6.00E-45 (183)	Tumor protein, translationally-controlled
EV854929	AAC95470.1	2.00E-30 (136)	Type 2 iodothyronine deiodinase
EV854930	XP_001135183	2.00E-68 (260)	Ubiquitin residue ribosomal protein
EV854931	XP_509962.1	2.00E-74 (280)	Ubiquitin-conjugating enzyme
EV854932	XP_001370741.1	4.00E-56 (221)	hypothalamus protein
EV854933	AAR19276	2.00E-84 (315)	Venom gland cyclophilin
EV854934	Q91516	8.00E-47 (188)	Venom plasminogen activator
EV854935	ABK97625	7.00E-38 (159)	Venom thrombin-like enzyme
EV854936	ABK97627	6.00E-20 (120)	Venom thrombin-like enzyme
EV854937	BAA88337.1	1.00E-31 (138)	Vipera ammodytes Bov-B LINE
EV854938	BAA88337	2.00E-38 (161)	Vipera ammodytes Bov-B LINE
EV854939	NP_001026621	2.00E-14 (81.3)	WD repeat domain 70
EV854940	XP_001377165.1	6.00E-81 (304)	ZFAND1 protein
EV854941	XP_545867.2	1.00E-103 (379)	Zinc finger protein 29 isoform
EV854942	NP_006340	2.00E-46 (189)	Zinc finger, HIT domain containing
EV854943	Q805F4	1.00E-139 (498)	Zinc metalloproteinase piscivostatin