

# Molecular Diversity in Venom from the Australian Brown Snake, *Pseudonaja textilis*\*<sup>§</sup>

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**Venom from the Australian elapid *Pseudonaja textilis* (Common or Eastern Brown snake), is the second most toxic snake venom known and is the most common cause of death from snake bite in Australia. This venom is known to contain a prothrombin activator complex, serine proteinase inhibitors, various phospholipase A<sub>2</sub>s, and pre- and postsynaptic neurotoxins. In this study, we performed a proteomic identification of the venom using two-dimensional gel electrophoresis, mass spectrometry, and *de novo* peptide sequencing. We identified most of the venom proteins including proteins previously not known to be present in the venom. In addition, we used immunoblotting and post-translational modification-specific enzyme stains and antibodies that reveal the complexity and regional diversity of the venom. Modifications observed include phosphorylation,  $\gamma$ -carboxylation, and glycosylation. Glycoproteins were further characterized by enzymatic deglycosylation and by lectin binding specificity. The venom contains an abundance of glycoproteins with N-linked sugars that include glucose/mannose, N-acetylgalactosamine, N-acetylglucosamine, and sialic acids. Additionally there are multiple isoforms of mammalian coagulation factors that comprise a significant proportion of the venom. Indeed two of the identified proteins, a procoagulant and a plasmin inhibitor, are currently in development as human therapeutic agents. *Molecular & Cellular Proteomics* 5:379–389, 2006.**

Snakes from the Australian elapid genus *Pseudonaja* are fast moving and highly venomous and are responsible for most deaths by snake bite in Australia. Of the eight classified species of *Pseudonaja*, *Pseudonaja textilis*, the Common or Eastern Brown snake, has the most lethal venom, surpassed only by *Oxyuranus microlepidotus*, the Inland Taipan. The venom is strongly procoagulant and has little hemolytic or myolytic activity (1). Several researchers have examined the venom from *P. textilis* and have isolated numerous toxins.

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These include postsynaptic  $\alpha$ -neurotoxins (2–4), the presynaptic neurotoxin textilotoxin (5–7), phospholipase A<sub>2</sub>s (PLA<sub>2</sub>s)<sup>1</sup> (6, 8); serine protease inhibitors (9, 10), and a prothrombin activator (11, 12). Fry (13) has written a comprehensive review on the properties of venom components from Australian elapids.

Barnett *et al.* (2) isolated a postsynaptic  $\alpha$ -neurotoxin from the venom and named it pseudonajatoxin A. This protein is 117 amino acids in length, which is considerably larger than other snake neurotoxins, and like other  $\alpha$ -neurotoxins acts by binding to acetylcholine receptors. Pseudonajatoxin B by contrast (3) contains only 71 amino acids and has considerable homology with other postsynaptic long  $\alpha$ -neurotoxins. Six other  $\alpha$ -neurotoxins have been cloned and expressed from venom gland RNA (4). These have fewer amino acids (57 or 58 residues) and possess lower neurotoxic activities than the short-chain  $\alpha$ -neurotoxins found in other snakes. Textilotoxin is the most potent neurotoxin yet isolated from the venom of a land snake. It represents 3% by weight of the crude venom and 70% of its total lethality (1). It is comprised of five PLA<sub>2</sub> subunits, two of which are identical, and acts by blocking the release of acetylcholine following the arrival of an action potential at a nerve terminal (14). Armugam *et al.* (8) isolated four other PLA<sub>2</sub>s from the venom and found them to be group 1B PLA<sub>2</sub>s. In that study, analysis of DNA from the venom gland showed that only two genes and two cDNAs were responsible for the four PLA<sub>2</sub> proteins produced. This is similar to the short-chain  $\alpha$ -neurotoxins (4) where alternative splicing of the *P. textilis* short neurotoxin 1 gene (*PT sntx1*) gives rise to two mRNAs and two neurotoxins. Masci *et al.* (9) identified and characterized serine protease inhibitors from the venom, named them textilins, and showed that they significantly reduce bleeding in an animal model. More recently, six isoforms of textilinin have been identified in *P. textilis* venom gland-derived cDNA (10). A prothrombin-activating complex was identified by Masci *et al.* (11) and was found to comprise a large proportion of the venom. This complex was subsequently named pseutarin C and found to

<sup>1</sup> The abbreviations used are: PLA<sub>2</sub>, phospholipase A<sub>2</sub>; 2D, two-dimensional; Gla,  $\gamma$ -carboxyglutamate; mAb, monoclonal antibody; PNGase F, peptidyl-N-glycosidase F; ConA, concanavalin A; WGA, wheat germ agglutinin; RCA<sub>120</sub>, *Ricinus communis* agglutinin 120; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; GRP78, glucose-regulated protein 78; HSP, heat shock protein.

be a group C prothrombin activator consisting of a Factor Xa-like enzymatic subunit and a non-enzymatic Factor Va-like subunit (12). Injection of purified pseutarin C results in massive disseminated intravascular coagulation within the body of the snake's prey, ultimately resulting in death (11). In addition to the identification of a number of toxins, it has been reported that the venom composition from a single specimen can vary substantially from season to season (15).

In this study, we performed the first comprehensive proteomic analysis of the venom composition using two-dimensional (2D) PAGE, mass spectrometry, and immunoblotting to identify the toxins. We also used post-translational modification-specific enzyme stains and antibodies that reveal the complexity and regional diversity of the venom. Post-translational modifications examined include phosphorylation, acetylation,  $\gamma$ -carboxylation, and glycosylation. Glycoproteins were further characterized by chemical and enzymatic deglycosylation and by lectin binding specificity.

#### EXPERIMENTAL PROCEDURES

**Materials: Snake Venoms and Chemicals**—Venom samples were obtained from Venom Supplies Pty. Ltd. (Tanunda, South Australia). Venom samples were collected over the course of a year and pooled from over 40 individual snakes to reduce seasonal and individual variation. Sample collection was performed for both Queensland and South Australian specimens to enable comparison of geographic variation. Venoms were lyophilized and reconstituted at 10 mg/ml in PBS, 50% glycerol. Water was prepared using a Milli-Q system (Millipore, Bedford, MA). FITC-labeled lectins were from Vector Laboratories (Burlingame, CA). Cy dyes were from Amersham Biosciences. Pro-Q Diamond and Pro-Q Emerald were from Molecular Probes Inc. (Eugene, OR). Neuraminidase and peptidyl-N-glycosidase F (PNGase F) were from New England Biolabs (Beverly, MA). O-Glycosidase was from Roche Applied Science. Antisera to various venom components were raised in rabbits or sheep in University of Queensland animal house facilities with appropriate ethical clearances. Acetylated lysine monoclonal antibody (mAb) was from Cell Signaling Technology (Beverly, MA). mAb against  $\gamma$ -carboxyglutamate (Gla) residues was from American Diagnostica Inc. (Greenwich, CT). All other chemicals were from Sigma unless stated otherwise.

**2D PAGE**—Venom samples (500  $\mu$ g) were diluted to 400  $\mu$ l with UTC buffer (7 M urea, 2 M thiourea, 4% CHAPS with a trace of bromphenol blue), 50 mM DTT, and 1% ampholytes (Biolytes 3–10, Bio-Rad). Samples were mixed and centrifuged for 5 min at 13,000  $\times$  g to pellet insoluble material, and the supernatant was loaded onto IEF strips (Bio-Rad ReadyStrip, pH 3–10, 24 cm) for 16-h passive rehydration. Proteins were focused on a Bio-Rad IEF cell. The IEF running conditions were: 250 V for 15 min, ramp to 8000 V for 3 h, hold at 8000 V for 90,000 V-h. After IEF, IPG strips were equilibrated for 15 min in an equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 2% SDS, 30% glycerol, 2% DTT) followed by 15 min in an equilibration buffer that had DTT replaced with 2.5% iodoacetamide. IPG strips were briefly rinsed in SDS-PAGE running buffer and embedded on top of 12% SDS-PAGE gels (Bio-Rad Protean Plus, 25  $\times$  20 cm) and covered with 0.5% agarose. Gels were run at 200 V constant voltage until the bromphenol blue dye front reached the bottom of the gel. Silver staining was performed as described by Shevchenko *et al.* (16). Colloidal Coomassie staining was performed as described by Neuhoff *et al.* (17). For immunoblotting; amino-terminal sequencing; phosphoprotein, glycoprotein, and lectin binding studies 11-cm IEF strips (Bio-Rad ReadyStrip, pH 3–10, 11 cm) were used with 50- $\mu$ g

venom samples with IEF conditions scaled down to 40,000 V-h total and samples run on 12% SDS-PAGE gels (Bio-Rad Criterion format gels).

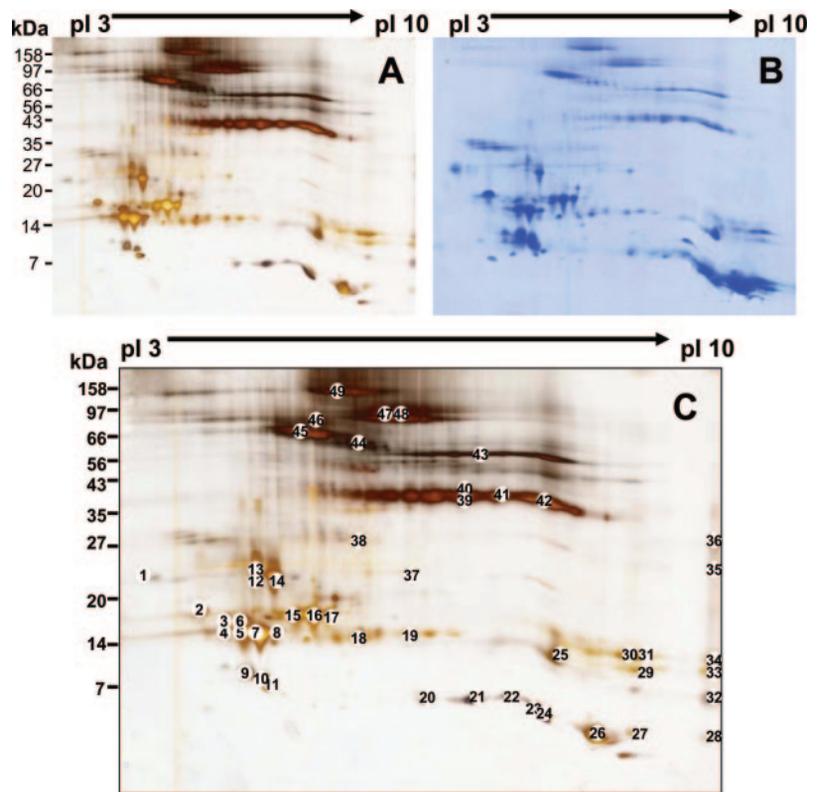
**Cy Dye Labeling**—Pooled *P. textilis* venom samples from Queensland or South Australia were precipitated in 8:1 acetone:methanol for 16 h at  $-20^{\circ}\text{C}$ . Samples were centrifuged for 30 min at 16,000  $\times$  g to pellet protein, the supernatants were removed and discarded, and the protein pellets were allowed to air dry for 10 min. Proteins were resuspended in 20  $\mu$ l of 20 mM Tris in UTC buffer to bring the pH to  $\sim$ 9. Cy3 and Cy5 monofunctional reactive Cy dyes (Amersham Biosciences) were used to label proteins according to the manufacturer's instructions. Labeling reactions were quenched by the addition of 2  $\mu$ l of 10 mM lysine. Reaction mixtures were combined and loaded onto 11-cm pH 3–10 IEF strips as described under "2D PAGE" above. After electrophoresis, gels were scanned on a Typhoon laser scanner (Amersham Biosciences) for both Cy3 and Cy5, and the resultant images were overlaid and analyzed using PDQuest software (Bio-Rad).

**Identification of Venom Proteins Using MALDI-TOF and TOF-TOF Mass Spectrometry and de Novo Peptide Sequencing**—Protein spots from silver-stained 2D PAGE were excised, washed in water, and destained as described by Gharahdaghi *et al.* (18). Trypsin was added, and proteins were allowed to digest overnight at 37  $^{\circ}\text{C}$  prior to extraction. Extracted peptides were dried, resuspended in 50% ACN, 0.1% TFA, mixed 1:1 with matrix ( $\alpha$ -cyano-4-hydroxycinnamic acid), and spotted on a MALDI plate. Samples were analyzed using a Voyager-DE STR mass spectrophotometer (Applied Biosystems) in positive reflector mode with an accelerating voltage of 20,000 V. One hundred and fifty laser shots per spectrum were acquired in the mass range 600–4000 Da. The 50 most intense peptide masses were searched against the Chordata taxonomic subset of the National Center for Biotechnology Information (NCBI) protein database using the Mascot search engine. MS peptide tolerance was 100 ppm, the search allowed for carbamidomethylated cysteine, and no other post-translational modifications were taken into account.

For TOF-TOF analysis, peptides were analyzed using a 4700 Proteomics Analyzer (Applied Biosystems) operated in positive ion reflectron mode. MS data were acquired using 2000 shots of a neodymium:yttrium-aluminum-garnet laser at 355 nm with a 200-Hz repetition rate and fixed intensity. The top 50 most intense peptides detected for each spot in the MS mode were automatically selected for MS/MS analysis using 3000 laser shots at a fixed intensity, which was  $\sim$ 20% greater than that used for MS. MS/MS data were calibrated against the MS/MS fragments of the  $m/z = 1296.685$  Angiotensin I peptide in the standards. MALDI-TOF/TOF MS/MS data were automatically analyzed using the GPS Explorer suite of software (Applied Biosystems). For each spot a combined MS and MS/MS analysis was performed in-house using a Mascot search engine and the Chordata taxonomic subset of Celera Discovery System database. MS peptide tolerance was 100 ppm, and MS/MS tolerance was 0.3 Da. The search allowed for carbamidomethylated cysteine and oxidized methionine. For the purposes of protein identification, no other post-translational modifications were taken into account.

For *de novo* peptide sequencing, MALDI-TOF/TOF MS/MS data were opened in Data Explorer (Applied Biosystems, Version 4.2) and deisotoped, and raw text peak lists were exported. The peak lists were analyzed using the automatic *de novo* function of PEAKS Studio software Version 2.4 (19) (Bioinformatics Solutions Inc., Ontario, Canada). Contiguous stretches of seven or more amino acids with 100% confidence call using the default parameters of the softwares were collected and matched to the NCBI non-redundant protein database using the protein BLAST algorithm ([www.ncbi.nlm.nih.gov/BLAST/](http://www.ncbi.nlm.nih.gov/BLAST/)).

**Amino-terminal Sequence Determination**—Venom proteins were electroblotted from 2D PAGE onto PVDF membrane (Pall Scientific,



**FIG. 1. 2D PAGE of *P. textilis* (Queensland) venom.** Duplicate samples of 500  $\mu\text{g}$  of venom proteins were subjected to isoelectric focusing on 24-cm pH 3–10 IEF strips and subsequently resolved by 12% SDS-PAGE. The silver-stained gel (A) shows stronger staining intensity of high molecular size proteins compared with the Coomassie-stained equivalent gel (B). Forty-nine protein spots were excised (C) for identification by mass spectrometry, *de novo* peptide sequencing, and  $\text{NH}_2$ -terminal sequencing (Supplemental Table 1).

Melbourne, Australia) in Towbin buffer at 15 V for 16 h. The membrane was briefly stained with Coomassie Blue, destained, washed extensively with several changes of water for 2 h, and dried. Selected spots were excised from the membrane with a scalpel blade, and the  $\text{NH}_2$ -terminal sequences were analyzed by Edman degradation using an Applied Biosystems Model 492 sequencer (Applied Biosystems). The observed sequences were matched to the NCBI non-redundant protein database using the protein BLAST algorithm ([www.ncbi.nlm.nih.gov/BLAST/](http://www.ncbi.nlm.nih.gov/BLAST/)).

**Immunoblotting**—Proteins from 2D PAGE were electrotransferred to nitrocellulose membranes (Pall Scientific, Brisbane, Australia), and membranes were blocked by immersion in 5% skim milk in PBS, 0.05% Tween 20 (SM/PBS/T). Membranes were probed with rabbit or sheep polyclonal antisera raised against specific venom proteins or venom protein complexes (26). The antisera were diluted 1:2000 in SM/PBS/T, and membranes were incubated overnight with gentle agitation. After washing in PBS/T for  $3 \times 10$  min, membranes were incubated for 1 h in secondary antisera (goat anti-rabbit Ig/horseradish peroxidase or rabbit anti-sheep Ig/horseradish peroxidase, Chemicon, Melbourne, Australia) diluted 1:5000 in PBS/T. After  $5 \times 10$ -min washes in PBS/T, blots were developed using ECL reagent (Western Lightning, Perkin Elmer Life Sciences) and visualized with a chemiluminescence detector (Fujilmager 3000, Fuji).

**Lectin Binding Specificity**—Glycosylation profiles of 2D PAGE-separated proteins transferred to nitrocellulose membrane were examined using a panel of eight lectins with FITC labels (Fluorescein Lectin Kit I, Vector Laboratories) as described by Nawarak *et al.* (20).

**Phosphoprotein and Glycoprotein Staining**—Detection of phosphoproteins and glycoproteins in 2D PAGE was performed using the ProQ Diamond and ProQ Emerald fluorescent reagents, respectively (Molecular Probes Inc.) according to the manufacturer's instructions. ProQ Emerald is a fluorescent-based form of the periodic acid-Schiff stain for carbohydrates that has a broad range of specificity for glycol-containing molecules.

**Enzymatic Deglycosylation**—Venom samples were precipitated in methanol:acetone (8:1) as above and air-dried prior to exposure to neuraminidase (New England Biolabs), O-glycosidase (New England Biolabs), or PNGase F (New England Biolabs) in buffers and conditions recommended by the manufacturer. Reactions were allowed to proceed for 1 h at 37  $^{\circ}\text{C}$ .

## RESULTS

**Separation and Comparative Analysis of *P. textilis* Venom Proteins**—Venom proteins from *P. textilis* (Queensland specimen pool) were initially analyzed using 2D PAGE. Sample processing and isoelectric focusing conditions were chosen to give the greatest number of resolved protein spots per gel. Over the pH range 3–10, 219 protein spots were detected using PDQuest software (Bio-Rad) from the silver-stained gel (Fig. 1A), whereas 192 spots were identified on the corresponding Coomassie Blue-stained gel (Fig. 1B). The proteins ranged in size from 5 kDa to greater than 200 kDa. Numerous horizontal trains of spots were evident especially in the molecular mass range greater than 30 kDa. These are suggestive of post-translational modifications of individual proteins. The abundance of spots in the molecular mass range 10–15 kDa with low pI corresponds to that expected of  $\text{PLA}_2$ s, which have already been shown to be present in *P. textilis* venom (6, 8). The overall pattern of staining was similar when colloidal Coomassie Blue staining was used; however, the relative intensity of some protein spots was different compared with detection by silver staining. Both methods of staining are compatible with further analysis by mass spectrometry and

are likely between them to facilitate characterization of most proteins in the venom.

**Protein Identification by Mass Spectrometry**—To identify venom proteins, gel spots were excised from 2D gels and digested with trypsin, and peptides were analyzed by MALDI-TOF MS and MALDI-TOF/TOF MS/MS. Forty-nine spots were selected from the 2D gels across a range of molecular masses. The distribution of these is indicated in Fig. 1C, and a complete list of the peptides and proteins identified appears in Supplemental Table 1. A prominent protein represented as a train of ~10 spots at 40 kDa (Fig. 1C, spots 39–42 and adjacent spots) was identified as the Factor Xa-like heavy chain. Another protein also present in the prothrombin activator complex of *P. textilis*, Factor Va-like protein, was identified at its expected molecular mass of ~160 kDa also as a train of spots (Fig. 1C, spot 49 and adjacent spots). There were several other Factor Va-like polypeptides corresponding to the A1 domain (heavy chain, 100 kDa; Fig. 1C, spots 47 and 48), A3 domain (light chain, 65 kDa; Fig. 1C, spot 45), and A2 domain (52 kDa; Fig. 1C, spots 43 and 44). These were identified by the presence of tryptic peptides that mapped specifically to the respective domains (Supplemental Table 1). The discrepancy between expected and observed molecular size is thought to be due to potential *N*-glycosylation sites in the A2 and A3 domains (12). Another protein identified in the high molecular mass region of the gel was a single protein spot identified as glucose-regulated protein 78 (GRP78; Fig. 1C, spot 46), a member of the heat shock protein 70 (HSP70) family. The presence of GRP78 in snake venom has not been reported previously.

A pseudetoxin-like protein was identified by *de novo* peptide sequencing as a protein spot of 25 kDa and pI ~10 (Fig. 1C, spot 35). Pseudetoxin and its homolog pseudecin are members of the cysteine-rich secreted proteins that were previously identified in two related Australian snakes, *Pseudechis australis* and *Pseudechis porphyriacus*, respectively (21). More recently, a cDNA clone corresponding to this protein was identified from the venom gland of *P. textilis* in a separate study (22). Additional members of this family have also been identified in several other Australian snake species using cDNA cloning (22). Two *de novo* sequenced peptides were used to identify protein spot 35 in the present study as the pseudetoxin-like protein from *P. textilis*. We also identified what appeared to be several members of the PLA<sub>2</sub> family at ~15 kDa and over a pI range of 4–6. Due to the limited amount of sequence information in the NCBI database, it was not possible to identify all PLA<sub>2</sub>s as individual isoforms. However, the differences in pI and size between the isoforms and previously reported data on the presence of multiple PLA<sub>2</sub> isoforms in *P. textilis* venom (8) supports the presence of sequence variants. The four subunits of the textilotoxin complex were identified in multiple protein spots (Fig. 1C, spots 1, 3, 6, 12, 13, 14, 29, 30, 31, 33, and 36). These molecules are members of the PLA<sub>2</sub> family and were also

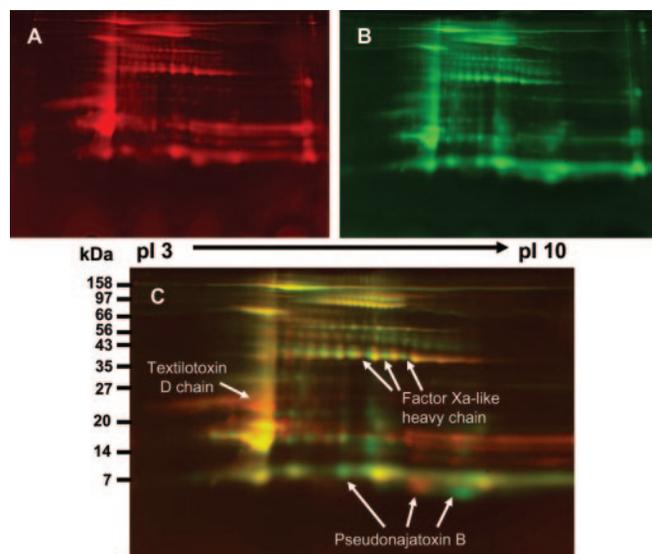


FIG. 2. **Regional diversity of *P. textilis* venom.** Pooled venoms from Queensland specimens (A) were labeled with Cy 3 dye, whereas those from South Australia (B) were labeled with Cy 5 dye. Both Cy3 and Cy5 samples were combined and subjected to 2D gel electrophoresis. The gel was scanned at wavelengths specific for each Cy dye, and the images were false colored and superimposed (C) to reveal protein spots unique to each region. Proteins common to both regions are colored yellow. Red spots are unique to Queensland; green spots are unique to South Australia. Arrows indicate proteins that appear at noticeably different levels.

identified using specific antisera in a corresponding immunoblot (Fig. 4E). Somewhat surprisingly, two isoforms of textilinin were identified migrating at an approximate molecular size of 10 kDa (Fig. 1C, spots 9 and 10). Although multiple textilinin cDNAs have been reported for *P. textilis*, the size of predicted proteins based on these cDNAs is 6–7 kDa, which is considerably lower than that observed here. As expected, several neurotoxins were identified at their anticipated pI and molecular size (Fig. 1C, spots 20, 22, 24, 25, 26, 27, 28, 33, and 34).

**Geographic Variation in Venom Composition**—Previous reports have shown that the toxicity of *P. textilis* venom can vary in a geographic manner (1, 23). To examine for geographic variation in venom composition we compared venom proteins in *P. textilis* from two geographically separated regions, Queensland and South Australia. Proteins from the two samples were differentially labeled with Cy3 and Cy5 dyes to allow comparison within a single gel. Similar numbers of proteins were observed in both samples with greater intensity of labeling of proteins in the lower molecular mass region compared with the silver or Coomassie Blue stain (Fig. 2, A and B). Overlay of the two images revealed that there was concordance between the labeled proteins in greater than 95% of cases (Fig. 2C). There were notable differences in proteins in the molecular mass range of 5–10 kDa. It has been reported that short and long neurotoxins and textilins are known to occur in this size range (3, 4, 9). By reference to Fig. 1C, we propose the most notable differences to be an increase in

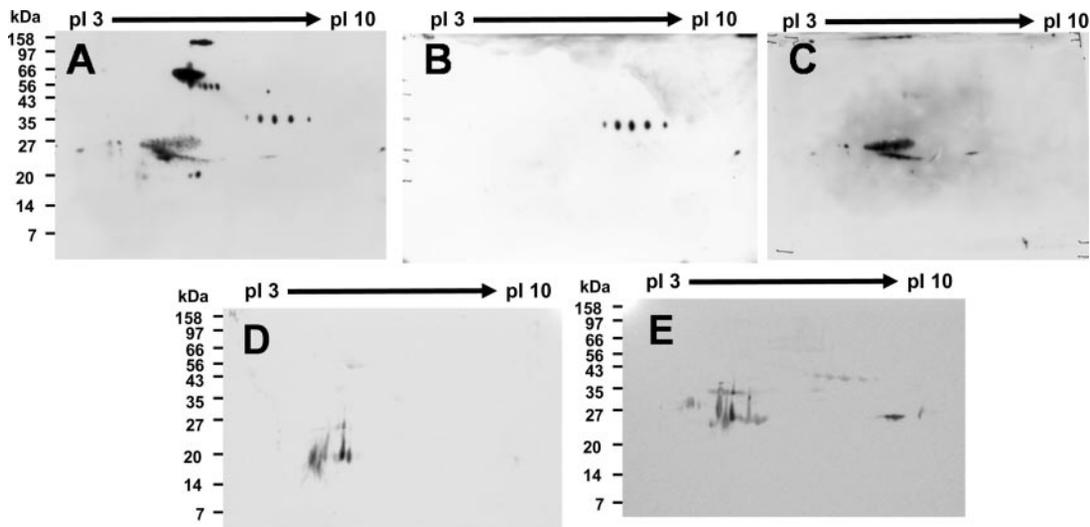


FIG. 3. **Immunoblots of 2D separated venom proteins.** Venom proteins were separated on 2D gels, and proteins were transferred to nitrocellulose membrane for immunoblotting with antisera to prothrombinase complex (A), heavy chain of the Factor Xa-like protease (B), Gla residues (C), textilins (D), and textilotoxin (E).

abundance of the phospholipase  $A_2$ , textilotoxin D chain (Fig. 2C, arrow), and differences in the abundance of forms of the long neurotoxin pseudonajatoxin B. Small differences were also observed for the Factor Xa-like heavy chain suggesting subtle differences in post-translational modifications of this train of spots. Our findings suggest that overall there is great similarity between the venoms from the two regions and that the differences are unlikely to account for significant changes in overall toxicity.

**Protein Identification by 2D Immunoblotting**—As an additional approach to identifying proteins, antisera specific to various components of the venom were used to identify the composition and location of the individual proteins (Fig. 3). The prothrombin activator complex in *P. textilis* is composed of a Factor Va-like molecule and a Factor Xa-like molecule. Three different antibodies were used to examine the prothrombin activator complex in the venom. These include a rabbit polyclonal antisera raised against the purified complex (Fig. 3A), a sheep polyclonal antisera raised against a recombinant form of the Factor Xa-like heavy chain (Fig. 3B), and a mouse monoclonal antibody specific for Gla residues (American Diagnostica Inc.).

Antisera against the prothrombin activator complex (Fig. 3A) detected three isoforms of the Factor Va-like molecule. This corresponded well with the data in Fig. 1C where these isoforms were identified by mass spectrometry, although the antisera failed to detect isoforms corresponding to the A1 domain (Fig. 1C, spots 47 and 48) and the A2 domain (Fig. 1C, spot 43). The Factor Xa-like molecule was also identified as a series of discrete spots as observed above using silver staining (Fig. 1C, spots 39–42). Antisera raised against a recombinant form of the heavy chain of Factor Xa-like molecule detected a train of protein spots identical to that seen with antisera against prothrombin activator complex (Fig. 3B). Al-

though the heavy chain was readily detectable in 2D gels using silver and Coomassie Blue staining, the Factor Xa-like light chain was not evident in these gels. However, with the prothrombin activator antisera, a complex staining pattern was also evident in a region corresponding to the light chain of Factor Xa-like molecule. Because it has been reported that the light chains of Factor Xa from both mammalian species and snakes are post-translationally modified at their  $NH_2$  terminus by  $\gamma$ -carboxylation of glutamic acid residues (11), we used a monoclonal antibody that detects this modification. The pattern observed with this antibody corresponded to a region that was also observed with the prothrombin activator complex antiserum (Fig. 3C). This region also corresponds with the expected molecular size and pI of the Factor Xa-like light chain.

A series of textilinin spots were detected using rabbit antisera raised against purified textilinin 1. This pattern was consistent with the presence of multiple isoforms of textilinin identified in *P. textilis* venom gland using cDNA cloning (10). These spots corresponded very well to those identified by mass spectrometry (see Fig. 1C). Multiple spots were detected for textilotoxin using rabbit antisera raised against the purified complex at both acidic and basic pI values. This was consistent with mass spectrometric identification of textilotoxin, which is composed of four related  $PLA_2$  molecules, textilotoxins A–D (7). Textilotoxins A and B were identified by mass spectrometry at high pI (pH 9–10). Antisera used here detected spots in this region (Fig. 3E). The multiple forms detected at a lower pI (pH 3–5) corresponded well to textilotoxins C and D (7), also identified by mass spectrometry. It is of interest that textilotoxins B and D were identified from several distinct spots.

**Post-translational Modifications**—One of the intriguing observations of the 2D gel separations of the *P. textilis* venom

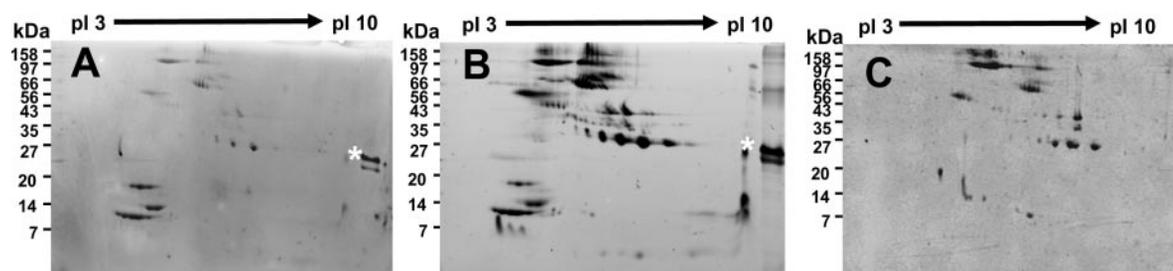


FIG. 4. **Post-translational modification stains.** Venom proteins were separated on 2D gels, and proteins were transferred to nitrocellulose membrane for staining with fluorescent stains specific for phosphoproteins (A), total protein (B), and glycoproteins (C). Asterisks in A and B indicate casein run as a phosphoprotein positive control lane on the edge of the second dimension gel.

proteins was that there were multiple horizontal trains of spots in different regions of the gels. These were most apparent in the higher molecular mass region where both Factor Va-like and Factor Xa-like components were identified (Fig. 1C, spots 39–42). These suggested the presence of multiple isoforms differentiated either by amino acid composition, post-translational modifications, or a combination of both. One of the most common post-translational modifications of proteins is phosphorylation. We assayed for this modification using a fluorescent stain, Pro-Q Diamond (Molecular Probes Inc.), reported to be specific for phosphoproteins (24). The results in Fig. 4A revealed the presence of several apparently phosphorylated proteins. When the same gel was subsequently stained with a total protein stain (Deep Purple, Amersham Biosciences), the phosphorylated spots correlated well with some of the proteins detected (Fig. 4B). Several of the high molecular mass phosphorylated proteins correlated well with Factor Va-like and Factor Xa-like isoforms on the basis of size and isoelectric point. A set of spots in the 15–20-kDa region with pI ~4–5 were consistent with phosphorylated PLA<sub>2</sub>s. Glycoproteins were also detected in a separate gel (Fig. 4C) using a specific stain (Pro-Q Emerald, Molecular Probes Inc.). This revealed evidence of multiple proteins modified across the range of different sizes and pI values.

Treatment of crude venom with neuraminidase and separation by single dimension SDS-PAGE revealed a shift in molecular size for most proteins above 20 kDa (Fig. 5, lanes 1 and 2) suggesting an abundance of sialic acids. We also used the glycosidases PNGase F and O-glycosidase to distinguish between N-glycosidic and O-glycosidic linked sugars (Fig. 5, lanes 6 and 3, respectively). PNGase F treatment resulted in a number of band shifts above 20 kDa in the treated (Fig. 5, lane 6) compared with untreated (lane 7) samples. On the other hand, no change was evident after treatment with O-glycosidase alone (lane 3). Because it has been reported that O-glycosidase cleavage is often more efficient after pretreatment with a sialidase, we used this combination of enzymes (lane 4). The pattern of proteins observed was identical to that observed with sialidase alone, providing additional support for the lack of O-linked sugars in venom proteins. The combination of the three enzymes (lane 5) supports the presence of sialic acid residues and N-linked sugars. The use of acid

hydrolysis to remove sugars from the venom proteins resulted in the appearance of protein smearing in both one-dimensional and 2D gels (data not shown). This may have been due to cleavage of peptide bonds as well as glycosidic bonds.

To relate the presence of glycosylation to the appearance of trains of spots on 2D gels, we used Cy dye methodology. We treated samples with and without neuraminidase and with and without PNGase F and subjected them to 2D PAGE (Fig. 5, B and C, respectively). Both results showed a reduction in molecular size for several trains of spots after deglycosylation with very little effect on the pI of the proteins. The reduction in molecular size after deglycosylation was particularly evident above 20 kDa where these trains were most apparent. Although not as clear, the lower molecular mass region showed direct overlay (yellow) between sets of spots. We also examined blots for the presence of acetylated lysine residues using a mAb specific for this modification. Acetylation of lysine is an important reversible modification controlling protein activity (25). We saw no evidence of lysine acetylation on 2D blots (data not shown).

**Lectin Binding Specificity**—Fluorescently labeled (FITC) lectins were used to detect glycoproteins on nitrocellulose membranes after 2D PAGE of venom proteins from *P. textilis*. Because it was possible that peripheral sugars may be lost during the preparation and separation of samples for 2D PAGE and subsequent electrotransfer to nitrocellulose, we initially spotted different dilutions of crude venom onto nitrocellulose membrane and probed it with a selection of eight lectins chosen to identify a wide range of specific sugar groups. Under these conditions, four of eight different lectins used showed no binding to venom proteins (results not shown). These four non-binding lectins were soybean agglutinin (specific for N-acetylgalactosamine and galactose), *Ulex europaeus* I lectin (specific for fucose), elderberry bark lectin (specific for  $\alpha$ 2–3-linked sialic acid), and peanut agglutinin (specific for galactose). It should also be noted that lectins can have additional structural requirements for binding ([www.vectorlabs.com/infopage.asp?dpID=24&locID=146](http://www.vectorlabs.com/infopage.asp?dpID=24&locID=146)). The four lectins that showed binding by dot blot analysis were subsequently used to probe 2D separated venom proteins (Fig. 6, A–D). Incubation with ConA, which is specific for glucose and mannose, revealed the presence of multiple binding proteins

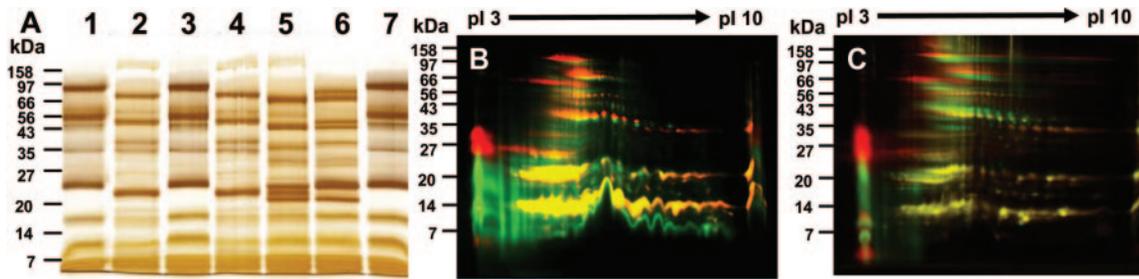


FIG. 5. **Enzymatic deglycosylation.** Venom samples were treated with various glycosidic enzymes and resolved by SDS-PAGE to examine for proteins with altered mobility. *A*, lane 1, untreated; lane 2, neuraminidase-treated; lane 3, *O*-glycosidase-treated; lane 4, neuraminidase- and *O*-glycosidase-treated; lane 5, neuraminidase-, *O*-glycosidase-, and PNGase F-treated; lane 6, PNGase F-treated; lane 7, untreated. *B*, venom samples were treated without and with neuraminidase and subsequently labeled with Cy3 and Cy5 dyes, respectively. The samples were combined and subjected to 2D PAGE, and the gel was scanned at wavelengths specific for the two Cy dyes. The images were false colored and superimposed to reveal proteins affected by deglycosylation. (Red, Cy3, without neuraminidase treatment; green, Cy5, with neuraminidase treatment. Overlap of red and green spots appear as yellow.) *C*, venom samples were treated as in *B* except neuraminidase was replaced with PNGase F.

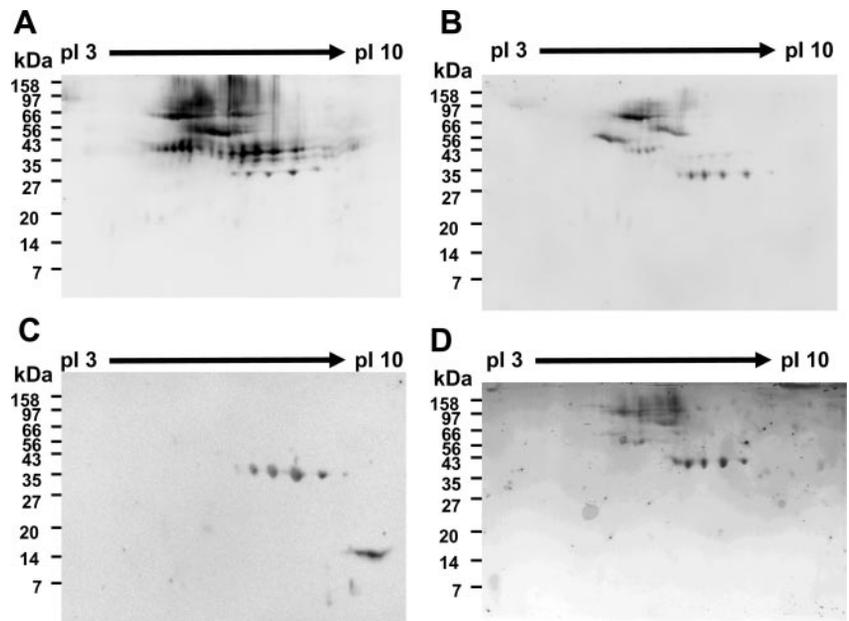


FIG. 6. **Lectin binding.** Venom proteins were separated on 2D gels, and proteins were transferred to nitrocellulose membrane to examine binding of various lectins. Lectin binding specificities are shown in parentheses: *A*, ConA (mannose, glucose); *B*, WGA (sialic acid, GlcNAc); *C*, *D. biflorus* agglutinin (GalNAc); *D*, RCA<sub>120</sub> (GalNAc, Gal).

above 30 kDa in size (Fig. 6A). Comparison of the pattern of ConA binding with 2D silver-stained gels showed a strong resemblance indicating that Factor Va-like and Factor Xa-like proteins were being modified by glycosylation. Use of wheat germ agglutinin (WGA), which detects GlcNAc and sialic acids, revealed a simpler and more defined pattern of staining. The lower molecular mass train of spots (pI 7–9) appeared to correspond to the Factor Xa-like heavy chain (Fig. 6B). It was notable that the train of spots immediately above the Factor Xa-like heavy chain, revealed by ConA staining, was undetectable with the WGA lectin. A second lectin known to have specificity for  $\alpha$ 2–3-linked sialic acid, *Sambucus nigra* agglutinin, failed to stain any proteins (results not shown). *Dolichos biflorus* agglutinin, which detects GalNAc, bound to the train of spots corresponding to the Factor Xa-like heavy chain (Fig. 6C). The only other lectin that detected protein binding was

*Ricinus communis* agglutinin (RCA<sub>120</sub>), which detects GalNAc and galactose. Again the Factor Xa-like protease heavy chain was detected as well as discrete fragments corresponding to Factor Va-like protein (Fig. 6D).

#### DISCUSSION

The venoms of Australian elapid snakes represent a rich source of highly active molecules that affect a variety of homeostatic mechanisms, including the coagulation cascade, neuromuscular signaling, and the cardiovascular system (13). A greater understanding of the mechanism of action of these molecules has potential for the development of human therapeutic agents. To understand how these proteins function, it is necessary to fractionate and identify the individual factors involved. In this study we carried out a proteomic profiling of the venom proteins from *P. textilis* and showed that although

~200 of the protein spots could be identified by 2D PAGE and MS, as few as 20 individual proteins are represented due to isoform variation. The number of proteins detected here was comparable to that observed in bovine milk, another secreted fluid where >95% of spots on 2D PAGE are represented by as few as six proteins again with extensive isoform variation (27).

Series of horizontal trains of spots on 2D PAGE often represent glycoforms of specific proteins. Glycoforms can vary in site occupation and diversity of glycan structures, both of which contribute to the separation of protein isoforms in 2D PAGE (28). These were especially apparent in the present study in regions of the gels where the Factor Va-like and the Factor Xa-like molecules were identified. The identity of these proteins was confirmed by selecting a series of individual spots across the trains and analyzing them by MALDI-TOF MS, MALDI-TOF/TOF MS/MS, and NH<sub>2</sub>-terminal sequence analysis. Multiple trains of spots have also been observed in venoms from the Asian snakes *Naja naja atra* and *Agkistrodon halys* (29) and for several snakes of the Elapidae and Viperidae families (20). Li *et al.* (29) also used 2D PAGE and observed trains of multiple spots that were subsequently shown to contain protein glycoforms using tandem MS. It is notable that in the case of the *A. halys* venomous proteins, the spots were distributed over a narrow range of isoelectric points of 4–6, whereas in the case of *Naja*, most proteins migrated to basic regions (pH > 7). In the present study of *P. textilis*, the venom proteins were distributed over the entire pH range of 3–10. Furthermore the molecular mass range for the *A. halys* proteins extended from <20 kDa to a maximum of ~60 kDa compared with a range observed in the present study from 5 to 160 kDa. The difference in the higher molecular mass region can be largely accounted for by the presence of Factor Va-like proteins in *P. textilis* that are not present in *A. halys*. This is consistent with the different mechanisms used by the two snakes to interfere with the mammalian coagulation process. The most abundant protein complex in *P. textilis* venom is the prothrombin activator complex, which is composed of Factor Va-like and Factor Xa-like proteins that activate prothrombin to initiate coagulation (11). On the other hand *A. halys* has largely anticoagulant activity. The major factors responsible for this anticoagulant activity are a 65-kDa inhibitor of platelet aggregation (30) and an L-amino acid oxidase that impairs intrinsic clotting by inhibiting Factor IX (31).

Although the prothrombinase complex is abundant in *P. textilis*, it has been reported that variation in the composition of the venom from a single snake undergoes seasonal variation (15). In that study, coagulant activity dropped to a minimum during the summer months; however, they did not determine the levels of the individual proteins in the prothrombinase complex. By using venom pooled from over 40 individual snakes, collected over the course of a year, we minimized the variation inherent in individual samples. The multiple spots observed for Factor Va-like and Factor Xa-like proteins as well as for several other proteins separated on the

2D gels could be due to the presence of post-translational modifications, proteolysis, protein splicing, or a combination of these. Furthermore several cDNAs coding for the different forms could be another explanation. The only evidence of proteolysis observed was fragments of the Factor Va-like protein (Fig. 1C, spots 21, 23, and 32) corresponding to the NH<sub>2</sub>-terminal region of the light chain. In the case of Factor Va-like and Factor Xa-like proteins it is unlikely that multiple cDNA isoforms exist because we have isolated full-length cDNAs for these genes and found only two isoforms for Factor Xa-like and a single species for Factor Va-like (32, 33). In the present study, the use of mass spectrometry failed to distinguish between these isoforms due to limited tryptic peptide sequence coverage. The Factor Xa-like protein is in development as a single agent procoagulant (33) ([www.qrxpharma.com/Q8010.htm](http://www.qrxpharma.com/Q8010.htm)) useful in situations for control of bleeding and tissue sealing following surgery. It has also been shown to induce blood clotting both *in vitro* and *in vivo* in a calcium-independent manner.

The presence of different post-translational modifications, largely in the higher molecular mass region, was evident after treatment with both sialidase and PNGase F using SDS-PAGE in a single dimension. Bands corresponding in size to both Factor Va and Factor Xa-like proteins were clearly shifted in their migration after treatment by either enzyme (see Fig. 5A). This was confirmed using 2D gel electrophoresis and Cy dye labeling (Fig. 5, B and C). When venom proteins were treated with PNGase F and subsequently labeled with Cy dyes, minimal overlap between the trains of spots that correspond to Factor Va-like protein was seen (Fig. 5C). This treatment did not reduce the number of individual species but simply led to a shift in their overall migration indicative of a loss of acidic sugar groups. The continuing presence of multiple spots under these conditions supports the presence of other post-translational modifications on this molecule. We provided evidence here that Factor Va-like protein is also modified by N-glycosylation and to a lesser extent by phosphorylation. It seems likely that the trains of spots observed for *P. textilis* Factor Xa-like proteins represent similar post-translational modifications. The multiple species observed for other proteins is unlikely to be explained by such modifications. For example in the case of PLA<sub>2</sub>s, it is more likely to be due to multiple isoforms for which there is evidence in *P. textilis* (7, 8). Furthermore in the Cy dye overlay experiments there seemed to be a high degree of concordance between treated and untreated samples for the PLA<sub>2</sub>s.

One of the characteristics of the light chain of the Factor Xa-like protease is a series of Gla residues in the NH<sub>2</sub>-terminal region of the molecule. This modification is conserved across different species (34, 32). The Gla residues chelate calcium ions and induce a conformational transition in the Gla domain that results in phospholipid membrane binding and stimulation of the procoagulant activity of the prothrombinase complex (35). As well as mammalian coagulation factors, Gla

domains have been found in the toxins of predatory cone snails of the genus *Conus* (36, 37). Recently precursors of Gla-containing conotoxins have been found to contain a carboxyl-terminal recognition site that directs  $\gamma$ -carboxylation (38), thus providing evidence that  $\gamma$ -carboxylation occurs as a post-translational rather than cotranslational event. The use of antisera against the prothrombinase complex detected a diffuse region of staining that corresponded in size to the light chain of Factor Xa-like protein. This was supported by the observation of coincident reactivity using a Gla-specific antibody (Fig. 3, A and C). The Factor Xa-like heavy chain was readily detectable on 2D gels by protein staining and also identified with antisera against this protein and the whole complex (Fig. 3, A and B). However, protein staining alone failed to detect the presence of the light chain possibly due to the presence of the charged Gla residues. The Factor Va- and Factor Xa-like proteins represented only a small proportion of the total number of proteins separated on the 2D gels in this study yet comprised a significant proportion by mass. This was evident in the prothrombinase complex Western blot (Fig. 3A).

Another protein identified in the higher molecular mass region was GRP78, a member of the HSP70 family of chaperones and protein folding catalysts (39). GRP78 may play a role in facilitating the assembly of multimeric protein complexes known to exist in the venom. GRP78 has not been identified previously in snake venom; however, protein sequences for the related proteins HSP70 (NCBI accession number AAY33973.1) and HSP90 (NCBI accession number AAY67995.1) have been found in the Australian taipan, *Oxyuranus scutellatus*. However, these share only 50 and 21% sequence identity, respectively, with the GRP78 sequence identified by tandem mass spectrometry in the present study.

The majority of venom proteins were observed in the <5–25-kDa region. Mass spectrometry identified a number of PLA<sub>2</sub>s across the entire pI range at a molecular size of 12–25 kDa. These enzymes catalyze the hydrolysis of insoluble phospholipid substrates at the *sn*-2 position of the glycerol backbone giving rise to fatty acids and lysophospholipids. In addition, PLA<sub>2</sub>s from Australian elapids inhibit platelet aggregation, they have procoagulant and myotoxic activities, and they are presynaptic neurotoxins (for a review, see Ref. 40). Accelerated evolution within the snake PLA<sub>2</sub> family has generated multiple isoforms that have resulted in diverse biological activities (41). Armugam *et al.* (8) have reported four new PLA<sub>2</sub>s in the venom of *P. textilis* bringing the total number of isoforms reported for this snake to eight. RT-PCR analysis of *P. textilis* venom gland RNA has also identified additional PLA<sub>2</sub> isoforms (23). Of 49 protein spots from 2D PAGE analyzed by mass spectrometry and NH<sub>2</sub>-terminal sequencing, 21 contained peptides from PLA<sub>2</sub>s. Although it was not possible to identify them as individual isoforms due to incomplete peptide sequence coverage (Fig. 1 and Supplemental Table

1), MALDI-TOF/TOF MS/MS analysis of tryptic peptides identified individual spots corresponding to known PLA<sub>2</sub>s, including the four PLA<sub>2</sub> subunits of the presynaptic neurotoxin textilotoxin.

We also identified textilins and  $\alpha$ -neurotoxins among the low molecular mass proteins and found  $\alpha$ -neurotoxins among two PLA<sub>2</sub> spots. Textilins have been shown to inhibit plasmin, a key enzyme in the fibrinolytic pathway involved in dissolving blood clots (9). Textilins share ~45% protein sequence similarity with aprotinin (Trasylo<sup>®</sup>, Bayer) the current “protein-based” antifibrinolytic agent on the market. Aprotinin is derived from bovine lung and is otherwise known as bovine pancreatic trypsin inhibitor. As the name suggests the inhibitor is able to inhibit trypsin and is also shown to inhibit a broad range of trypsin-like proteases, many of which are involved in the control of fibrinolysis and hemostasis. We have previously isolated textilins from the venom by conventional chromatography and by cDNA cloning (10, 11). The 2D PAGE separation described here distributed the textilins into two distinct spots of ~9 kDa. However, use of immunoblotting detected what appeared to be four separate species in the corresponding region (Fig. 4D). This is not surprising because six separate cDNAs coding for textilins have been described in *P. textilis* (10) with four of them having pI values (pI 4–5) corresponding with those observed here. The observed molecular size of 9 kDa corresponds to the precursor protein predicted from the full-length cDNA, which contains an open reading frame of 61 amino acids and a propeptide of 22 amino acids (10). On the other hand the mature textilin proteins would be expected to migrate at ~6–7 kDa in size once the propeptide had been removed. This discrepancy might be explained by failure to completely reduce the three disulphide linkages that occur in these proteins, post-translational modifications, inaccurate sizing in this molecular mass range, or abnormal migration of the protein. Seven spots were identified as neurotoxins based on NH<sub>2</sub>-terminal sequencing and MALDI-TOF/TOF MS/MS analysis. These correspond to both long-chain and short-chain  $\alpha$ -neurotoxins previously identified in *P. textilis* (3, 4). Five spots were identified as the long-chain neurotoxin pseudonajatoxin B, which is consistent with reports of at least four isoforms in the venom of *P. textilis* (42). These protein spots were observed at a range of molecular size from 6 to 15 kDa, whereas the mature proteins are expected to be 8 kDa in size. Long-chain  $\alpha$ -neurotoxins typically have five disulphide bridges that if not fully denatured may account for altered electrophoretic migration. Short-chain neurotoxins contain four disulphide bonds and share a similar three-finger-loop structure with long-chain neurotoxins (43). The two short-chain neurotoxins identified correspond to  $\alpha$ -neurotoxin 6, one of six isoforms previously identified by cDNA cloning (4). Interestingly both were identified in 2D gel spots that also contained a PLA<sub>2</sub>, the A-chain of textilotoxin. A complex containing an  $\alpha$ -neurotoxin, a PLA<sub>2</sub>, and a serine protease inhibitor has been identified in

the related Australian elapid *O. scutellatus* (44). However, purification of the major serine protease inhibitor present in *P. textilis* venom, textilinin, has found no evidence of a similar complex.<sup>2</sup> The two  $\alpha$ -neurotoxin 6 spots were identified at a pI of >10 and at molecular sizes of 12 and 14 kDa, which are significantly greater than their predicted size of 8.5 kDa.

Our lectin binding studies showed the venom to be rich in ConA-binding (mannose- and glucose-specific) glycoproteins in the higher molecular size region. This is consistent with a study by Nawarak *et al.* (20) who used ConA-agarose to isolate such glycoproteins from *N. naja* venom. The WGA lectin (sialic acid- and GlcNAc-specific) and RCA<sub>120</sub> lectin (galactose- and GalNAc-specific) showed similar binding of both the Factor Va-like and Factor Xa-like molecules. It seems unlikely that these glycosylations are required for prothrombinase activity as neither of the mammalian homologs are glycosylated. The large amount of glycosylation present, as confirmed by the glycosidase experiments (Fig. 5, A, B, and C), may be required for enzyme stability rather than function. It is interesting to note that the mammalian coagulation factor homologs are found in the activated form in snake venoms (12), which is not the case in mammalian circulation. These procoagulant proteins are useful in treating various thrombotic and hemostatic conditions (45).

Examples of clinically useful venom proteins from other snakes include contortrostatin from the Southern Copperhead viper that is being tested as an anticancer agent and Ancrod from American pit viper that is being tested as an anticoagulant for stroke victims. Another drug, Integrilin, is a small molecule based on the RGD peptide motif found in a platelet aggregation inhibitor from rattlesnake venom and has been approved by the United States Food and Drug Administration to treat patients with severe chest pain, minor heart attacks, and other cardiovascular conditions.

The results described here represent the first approach to identifying all of the venom proteins in *P. textilis*. Representatives of all the major classes were identified after 2D gel electrophoresis using a combination of immunoblotting, MALDI-TOF MS and MALDI-TOF/TOF MS/MS analysis, and NH<sub>2</sub>-terminal sequencing. A common characteristic of these 2D gel separations of venom proteins was the detection of multiple trains of spots corresponding to a single protein. This was shown to be the case by immunoblotting and by mass spectrometry. We also demonstrated that these were at least partially generated by post-translational modifications and by amino acid variation. Two of the identified proteins, the procoagulant Factor Xa-like protein and the plasmin inhibitor textilinin, are currently in development as human therapeutic agents ([www.qrxpharma.com/pipeline.htm](http://www.qrxpharma.com/pipeline.htm)) and are the subject of international patent filings. Both of these proteins were shown to have multiple isoforms expressed in the venom. The challenge ahead is to identify all of the other proteins in the

venom of *P. textilis* and to extend these studies to a variety of other elapid snake venoms.

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