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# Analysis of Lectin-Bound Glycoproteins in Snake Venom from the Elapidae and Viperidae Families

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This paper describes an efficient method of studying the glycoproteins found in snake venom. The glycosylation profiles of the Elapidae and Viperidae snake families were analyzed using FITC-labeled lectin glycoconjugates. The Con A-agarose affinity enrichment technique was used to fractionate glycoproteins from the *N. naja kaouthia* venom. The results revealed a large number of Con A binding glycoproteins, most of which have moderate to high molecular weights. To identify the proteins, the isolated glycoprotein fractions were subjected to two-dimensional electrophoresis and MALDI-TOF MS. Protein sequences were compared with published protein databases to determine for their biological functions.

**Keywords:** snake venom • proteomics • lectin • glycoprotein

## Introduction

Snake venoms contain active enzymes for killing or immobilizing prey and for supporting the digestion of untriturated, swallowed food. To date, more than 150 proteases have been isolated with partial or full characterizations.[1] Many of these enzymes are reported to have post-translational modifications such as glycosylation.[2] Identified snake venom glycoproteins include cobra venom factor,[3−8] prothrombin activator,[9,10] prothrombin-converting enzyme,[11] thrombin-like enzyme,[12] factor V activator,[13] fibrinogen clotting enzyme,[14] metalloproteinase,[15,16] C-type lectin,[17,18] lectin,[19] disintegrin,[20] L-amino acid oxidase,[21] protease inhibitor,[22] nerve growth factor,[23,24] kininogenase,[25] kininogenin,[26] NAD glycohydrolase,[27,28] hemorrhagic factor,[29] arginine ester hydrolases,[30] hyaluronidase,[31] and acetylcholinesterase.[32] All of these enzymes contribute to the toxicity of snake venom.

Several venom enzymes, particularly from the Viperidae and Elapidae families, contain proteins that interact with components of the human hemostatic system. These proteins are used clinically as anticoagulants and also in pre-clinical research to examine their therapeutic potential.[33] Many of these enzymes convert target proteins to their activated forms, which consequently affect the coagulation and fibrinolytic pathways. For example, a coagulant factor from Russell's viper venom (RVV-V) alters Factor V (FV) to the activated form (FVa), by cleaving a single peptide bond.[34] A further example is found in the *Tr. stejnegeri* venom plasminogen activator (TSV-PA), a serine proteinase glycoprotein, which also uses enzymatic digestion to activate the fibrinolysis system. Anticoagulant enzymes from *Agkistrodon contortrix contortrix* venom digest zymogen protein C, resulting in anticoagulant effects.[35]

Snake venoms also contain neurotoxins, the majority of which act on the peripheral nervous system.[36] Different toxins have different mechanisms of action on specific targets. Snake toxins are of specific interest in designing drugs as they possess pharmacological properties.[37] Interestingly, many snake venom constituents are reported to be glycoproteins. Glycosylation is required to support their biological activities.

Although two-dimensional electrophoresis (2-DE) is a high-resolution technique for resolving protein mixtures, characterization of proteins with post-translational modifications require additional methods for sample preparation, identification, isolation, and enrichment:[38,39] these methods are key to obtaining good 2-DE results. Sample fractionation techniques such as sequential extraction methods, sample precipitation and chromatographic methods have been used. Lectins such as Concanavalin A (Con A) have been widely used in glycoconjugate research.[40−42] In addition to the advantages of reducing the complexity of samples before IEF, the specificity of different lectins for different sugar moieties may indicate the important features of carbohydrate chains on glycoproteins.

In this study, we used lectins to detect and to fractionate snake venoms for glycoproteins. The lectin-binding fractions were separated by 2-DE and selected glycoproteins were identified by mass spectrometry. Seven FITC labeled-lectins, with different saccharide specificities were used to study the lectin-binding affinity in snake venoms of the Elapidae and Viperidae families. Agarose bound-Con A and 2-DE were used to fractionate glycoproteins of *N. naja kaouthia* venom. This is a simple process to enrich post-translational modified proteins and to rapidly fractionate the glycoproteins from complex mixtures. The process provides a partial characteriza-

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tion of the glycoprotein composition in snake venoms and leads to a primary understanding of the overall protein modifications of snake venom constituents and their relationship to protein functions.

Our goal is to establish a fast and efficient analysis method for the study of snake venom glycoproteins. The results of the lectin-binding experiments indicated that all of the snake venoms studied have binding affinity to at least one kind of lectin. Many snake venoms have binding affinity to lectins with specificities for mannose, glucose, sialic acid, GalNAc, and GlcNAc. Con A affinity purification of *N. naja kaouthia* venom revealed many glycoproteins in the venom. The results suggest that glycoproteins are important constituents of snake venoms. This information will be useful in the future when studying the role of carbohydrates and glycoproteins in snake venom.

## Materials and Methods

**Reagents and Apparatus.** IPGphor, immobiline dry strips, carrier ampholytes, ammonium persulfate and TEMED were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). 2-DE was performed in a Bio-Rad Multi-Cell Casting chamber and a Protean II xi Cell. Urea and CHAPS were obtained from J. T. Baker, Iodoacetamide (IAA) and glutaraldehyde from Fluka and Sodium acetate, formaldehyde, acetic acid, and citric acid from Merck. DTE was obtained from AppliChem, Silver nitrate from Mallinckrodt, Sypro Ruby from Molecular Probe, Methanol from BDH, Tris(hydroxymethyl)-aminomethan from Merck (Germany) and Bio-Dot SF was from Bio-Rad. The FITC-labeled lectins used in the experiments were Ulex Europaeus Agglutinin (UEL1), Maackia Amurensis Lectin I (MAL1), Penut Agglutinin (PNA), Elderberry Bark Lectin (EBL), Erythrina Cristagalli Lectin (ECL), Wheat Germ Agglutinin (WGA), and Concanavalin A (Con A). All lectins, both the FITC-labeled and the agarose bound-Con A were from VECTOR Laboratories (Burlingame, USA). Deionized water used for all buffers was prepared with a tandem Milli-Q system (Millipore).

**Snake Venoms.** Ten samples of venom from eight snake species were investigated for lectin specificity. Snake venoms of the *N. naja kaouthia, O. hannah, B. fasciatus,* and *V. russellii siamensis* species were obtained from the Queen Saovabha Memorial Institute, Thai Red Cross Society, Bangkok, Thailand. Snake venoms from *N. naja atra, D. acutus, B. multicinctus, V. russelli formosensis, Tr. mucrosquamatus,* and *Tr. stejnegeri,* were kindly provided by Mr. Ming-Yi Liau from the Department of Biotechnology, Fooyin Institute of Technology, Kaohsiung, Taiwan. All venoms were lyophilized prior to use.

**Lectin-Binding Specificity in Snake Venom.** The 10 lyophilized snake venoms were dissolved in a PBS buffer at a concentration of 50 $\mu$g/mL. Using Bio-dot Blot (Bio-Rad), the 10 venoms were blotted (10 $\mu$g per spot) onto a single PVDF membrane to test for lectin-binding properties. After blotting, the membrane surfaces were blocked with 2% polyvinylidone in PBS for 30 min at room temperature in a shaker. The membranes were then washed twice with 50 mM Tris-HCl containing 150 mM NaCl pH 7.5 (buffer A) for 5 min each. Seven membranes were prepared and tested individually, with seven different lectins. Each blotted membrane was incubated in a specific lectin solution (20 $\mu$g/mL) at room temperature for 1 h in a shaker. Each membrane was rinsed twice with buffer A, air-dried, and the fluorescent images were digitized using a CCD camera.

**Agarose-Con A affinity purification of *N. naja kaouthia* Venom.** *N. naja kaouthia* venom was subjected to a single round of lectin-affinity purification using Sepharose-bound Con A gel (protein 6.0 mg/mL of settled gel, 1:1 v/v slurry). *N. naja kaouthia* venom was dissolved in buffer A at 10 mg/mL and 1 mL of the solution was added to a 0.1 mL slurry of Sepharose-Con A gel. The mixture was incubated at room-temperature overnight with continuous shaking. Buffer A (2 mL) was used five times to wash out unbound proteins; this was followed by washing twice with $ddH_2O$ (2 mL). The bound glycoproteins were extracted from the Sepharose-Con A gel with a lysis buffer (7 M urea, 4% CHAPS, 4 mM Tris base, 2 M Thiourea, 2% IPG buffer, 65 mM DTE, 0.4 mL) for 2-DE analysis. The negative control was a lysis buffer extract of Sepharose-Con A gel. In an additional control experiment, the bound glycoproteins were eluted from Sepharose-Con A gel with 1 mL of 0.2 M $\alpha$-methyl mannose. The eluted glycoproteins mixture was lyophilized and dissolved in a lysis buffer for 2-DE analysis.

**Deglycosylation by Acid Hydrolysis.** The Con A binding proteins of *N. naja kaouthia*, which were eluted using a free mannose solution, were subjected to a deglycosylation experiment by acid hydrolysis. One ml of 1 M HCl was added to a clean, glass screw-capped vial containing 3 mg of *N. naja kaouthia* venom. The mixture was incubated at 55 °C for 1 h. After reacting, the sample was neutralized then dialyzed against $ddH_2O$ before being lyophilized. The dried proteins were dissolved in a lysis buffer and subjected to 2-DE analysis.

**Two-Dimensional Electrophoresis of Snake Venoms. (a) Wide Range pH 2-DE.** For pH 3−10 separations, 0.50 mg each of the lectin-binding fraction, the nonbinding fraction (Con A-agarose eluent), the control and *N. naja kaouthia* venom were separately solubilized in a lysis buffer. Each sample was sonicated and centrifuged. Each sample was applied to an IPG strip (Amersham Pharmacia Biotech, 18 cm, 3−10 NL) by an IPG strip rehydration application method. The IPG strip was rehydrated for 12 h on an IPGphor machine (Amersham Pharmacia Biotech). An electric current was applied at 30 V during the rehydration time. The iso-electric focusing (IEF) running conditions were: 100 V for 1 h, 250 V for 1 h, 500 V for 1 h, 1000 V for 1 h and 6000 V for up to 30 kVh. For the preparative polyacrylamide gel, 1 mg samples were used and the final application of current was 6000 V for up to 45 kVh. Sypro Ruby was used for gel staining.

**(b) Narrow Range pH 2-DE.** For pH 6−11 separations, 0.10 mg of total *N. naja kaouthia* venom was solubilized in a lysis buffer modified with the addition of 10% 2-propanol and 5% glycerol to improve protein separation. Before the sample application, an IPG strip (18 cm, 6−11 L) was allowed to rehydrate with the same lysis buffer for 10 h. After this, the sample was applied by the cup loading method. The loading cup was placed on the acidic end of the IPG gel. Samples were allowed to penetrate into the IPG strip by applying current at 30 V for 3 h subsequently focusing by using current at 100 V for 1 h, 250 V for 1 h, 500 V for 1 h, 1000 V for 1 h, and 6000 for up to 15 kVh.

**(c) SDS-PAGE.** After IEF, both wide and narrow range IPG strips were equilibrated for 15 min in an equilibration solution (50 mM Tris-HCl pH 8.8, 6 M urea, 2% SDS, 30% glycerol, 2% DTE and a trace of bromophenol blue), followed by a further 15 min in the equilibration solution which had DTE replaced with 2.5% iodoacetamide. IPG gel strips were embedded on top of the 2-D gels and covered with 0.5% agarose. 2-D SDS-PAGE was carried out on 10−20% acrylamide gradient gel (18 × 18 cm) at 45 mA/gel until the bromophenol blue dye front reached the bottom of the gel.

**(d) Gel Staining. Silver Staining.** Analytical gels were silver stained as previously described.[43] Analytical gels were first fixed with 40% ethanol and 10% acetic acid solution for 1 h then placed in 5% ethanol and 5% acetic acid solution for 2 h. The gels were washed in $ddH_2O$ for 5 min and incubated in 1% glutaraldehyde and 0.5 M sodium acetate for 30 min. The gels were washed with $ddH_2O$ and incubated in 0.05% 2,7-naphthalene-disulfonic acid solution for 20 min, then this step was repeated. After this, the gels were washed with $ddH_2O$ and stained with freshly made ammonical silver nitrate solution for 45 min. The gels were washed and developed in 0.005% citric acid and 0.05% formaldehyde solution. Staining was halted with 5% Tris and 2% acetic acid.

**Sypro Ruby Staining.** Analytical and preparative gels were fixed with 10% methanol and 7% acetic acid for 30 min. The gels were washed with $ddH_2O$ and then stained with 500 mL of Sypro Ruby dye solution for 3 h. After staining, the gels were washed with $ddH_2O$ and the images were scanned.

**(e) Image Analysis.** Silver-stained gels were digitized using a Hewlett-Packard Scan Jet 4100 C. Sypro Ruby-stained gels were digitized by a Typhoon 9200 image scanner (Amersham Pharmacia Biotech). 2-DE gel images were analyzed using ImageMaster® image software (Amersham Pharmacia Biotech).

**(f) Amino-Terminal Sequence Determination.** 2-DE gel of snake venom was electroblotted onto a PVDF membrane using a semi-dry blotting apparatus (Amersham Pharmacia Biotech, Sweden). Electroblotting was carried out with a 10 mM CAPS buffer pH 11.0 in 10% methanol at 400 mA for 3 h. The PVDF membrane was stained with 1% amido black and destained in 50% methanol until the image developed. The membrane was washed with $ddH_2O$ and air-dried. Selected spots were excised from the dried PVDF membrane and the $NH_2$-terminal sequences were analyzed by Edman degradation using an Applied Biosystems Model 492 sequencer (Applied Biosystems, Weiterstadt, Germany). The $NH_2$-terminal sequence comparisons were carried out using FASTA, BLAST, and TFASTA software from protein databases at the ExPASy website, http://tw.expasy.org/tool/#similarity.

**(g) Mass Spectrometric Analysis. Protein Digestion.** Protein bands or spots were manually excised from the prepared polyacrylamide gels and transferred to 500 $\mu$L siliconized eppendorf. Each gel piece was reduced with 200 $\mu$L 65 mM DTE in 100 mM ammonium bicarbonate pH 8.5 for 1 h at 37 °C and subsequently alkylated with 200 $\mu$L 55 mM IAA in 100 mM ammonium bicarbonate buffer pH 8.5 for 1 h in the dark at room temperature. The gel piece was then washed twice with 200 $\mu$L of 50% acetonitrile/25 mM ammonium bicarbonate buffer pH 8.0 for 15 min, washed once with 200 $\mu$L of 100% acetonitrile and vacuum-dried in a Speed Vac concentrator. Each dried gel piece was soaked in 10 $\mu$L of 25 mM ammonium bicarbonate containing 0.1 $\mu$g trypsin (Promega, sequencing grade) and then crushed with siliconized blue stick and incubated at 37 °C for at least 16 h. The crushed gels were subsequently extracted twice with 50 $\mu$L of 50% acetonitrile/ 5% trifluoroacetic acid, and the extract dried using the Speed-Vac concentrator. The peptides or pellets were resuspended in 20 $\mu$L of 0.1% trifluoroacetic acid and the suspension purified using Zip-Tips (Millipore, Bedford, MA) according to the manufacturer's instructions. The desalted peptides retained in Zip-Tip were eluted out using 6 $\mu$L 75% acetonitrile /0.1% formic acid into a new 500-$\mu$L siliconized eppendorf.

**MALDI-TOF MS and LC MS.** Peptide mass spectra were acquired using a Q-Tof mass spectrometer (Micromass, UK) operating in a delayed extraction reflector mode. Each sample (1 $\mu$L in 75% acetonitrile/ 0.1% formic acid) was mixed with 1 $\mu$L matrix solution (5−10 mg α-cyano-4-hydroxy cinnamic acid in 60% ACN/1% TFA) and spotted on the sample plate. External calibration used 1 pmol/$\mu$L glufibrinopeptide B (GFB). Mass spectrometry was performed using an accelerating voltage of 20 kV. Selected peptides in the mass range of 1000−3500 Da were used for the matched databases.

For LC MS/MS, mass spectra were acquired on a Q-Tof Ultima API, hybrid quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer equipped with a Z-spray nanoelectrospray ion source (all from Micromass, UK). The nanoLC sprayer and Z-spray source parameters were optimized while infusing 50 fmol/$\mu$L GFP. Calibration was performed using the product ions generated from fragmentation of the doubly charged molecular ion of GFP at $m/z = 785.8$. Argon was used as the collision gas ($\sim 4.0 \times 10^{-5}$ mbar).

**Databases Search.** Peptide mass fingerprint data from MALDI-TOF were used to match with protein candidates in the NCBI and SWISS-PROT protein databases using the Protein Prospector MS-FIT (http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm) and MASCOT (http://www.matrixscience.com) software programs. The search parameters allowed for oxidation of methionine, carbamidomethylation of cysteine, one missed cleavage site, and a peptide mass tolerance of 50 ppm. Due to limitations in the snake venom proteins databases, the taxonomy of proteins could not be specified. However, the type of proteins that may be contained in snake venom and the score of the protein candidates under other search parameters were considered. Protein identification was confirmed at least once using spots from different gels. Peptide fragment-ion data obtained from ESI-Q-TOF were used to search for protein candidates in protein sequence databases used for MALDI-TOF MS via the MASCOT (http://www.matrixscience.com) software program. The search parameters were as follows: carbamidomethylation of cysteine, oxidation of methionine, one miss cleavage site, peptide mass tolerance of 50 ppm, MS/MS tolerance of ± 0.25 Da, and peptide charges of +2 and +3.

## Results

**Lectin-Binding Glycoproteins.** FITC-labeled lectins were used to detect glycoproteins in 10 different venoms: 5 from the Elapidae family (*B. multicinctus*, *B. fasciatus*, *N. naja atra*, *N. naja kaouthia*, and *O. hannah*) and 5 from the Viperidae family (*V. russelli formosensis*, *V. russelli siamensis*, *Tr. mucrosquamatus*, *Tr. stejnegeri*, and *D. acutus*). Seven lectins were used to bind venom glycoproteins (UEL1, MAL1, PNA, EBL, ECL, WGA, and Con A). Five lectins (UEL1, PNA, ECL, WGA, and Con A) have binding specificity for different sugar moieties, while MAL1, EBL, and WGA have binding specificity to *N*-acetyl neuraminic acid (sialic acid) (Table 1).

The results of the lectin-binding affinities of snake venoms from Table 1 and Figure 1 show that each venom has different lectin-binding properties. These observations imply that the glycoproteins of each snake venom contain different sugar moieties. Only three snake venoms, *B. fasciatus*, *O. hannah*, and *Tr. stejnegeri*, were found to have fucose residues. All venom has proteins that bind to EBL lectin, which specifically binds terminal 2,6-sialic acid. In addition, proteins from nearly all of the snake venoms from both families, which bind to MAL1 and WGA, also have specificity to sialic acid. Proteins from several snake venoms, *B. multicinctus*, *B. fasciatus*, *N. naja atra*, and *O. hannah*, from the Elapidae family, and *Tr. stejnegeri*

**Table 1.** Lectin Affinity of Snake Venoms in the Elapidae and Viperidae Families

| | | Elapidae family | | | | | Viperidae family | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *B. multi-cinctus* | *B. fasciatus* | *N. naja atra* | *N. naja kaouthia* | *O. hannah* | *V. russellii formosensis* | *V. russellii siamensis* | *Tr. mucro-squamatus* | *Tr. stej-negeri* | *D. acutus* |
| lectin | carbohydrate specificity | | | | | | | | | | |
| UEL1 | fucose | | √ | | | √ | | | | √ | |
| MAL1 | sialic acid | √ | √ | √ | | √ | √ | √ | √ | √ | √ |
| PNA | Gal-GalNAc | √ | √ | √ | | √ | | | | √ | √ |
| EBL | 2,6 sialic acid | √ | √ | √ | √ | √ | √ | √ | √ | √ | √ |
| ECL | Gal 1,4 GlcNAc | | √ | √ | | √ | | | | | √ |
| WGA | GlcNAc, sialic acid | √ | √ | √ | | √ | √ | √ | √ | √ | √ |
| Con A | mannose, glucose | √ | √ | √ | √ | √ | √ | √ | √ | √ | √ |

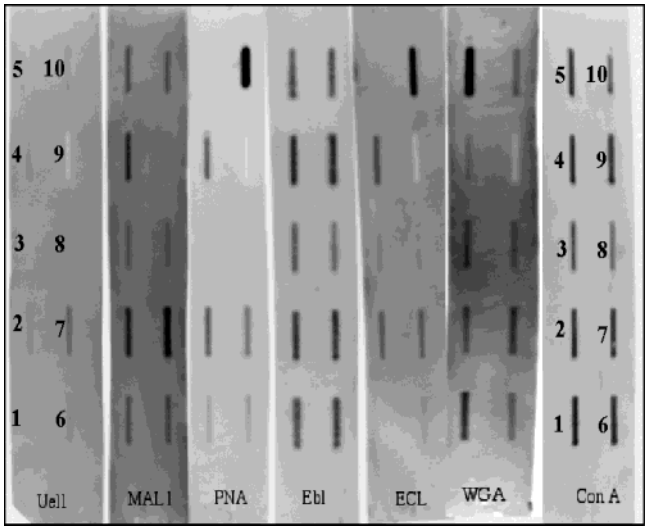


**Figure 1.** Dot blot analysis of 10 snake venoms using FITC-labeled lectins. Lectins used are UEL1, MAL1, PNA, EBL, ECL, WGA, and Con A. Each PVDF membrane was blotted with 10 $\mu$g each of 10 kinds of snake venom. The order of snake venoms are as follows: 1, *B. multicinctus*; 2, *D. acutus*; 3, *V. russelli formosensis*; 4, *N. naja atra*; 5, *Tr. mucrosquamatus*; 6, *Tr. stejnegeri*; 7, *B. fasciatus*; 8, *V. russelli siamensis*; 9, *N. naja kaouthia*; 10, *O. hannah* venom.

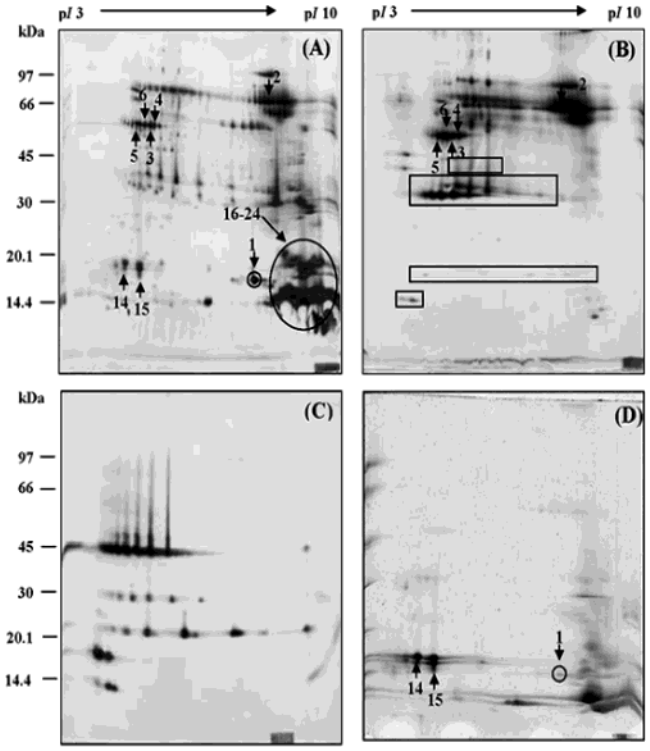


**Figure 2.** 2-DE gel images of *N. naja kaouthia* venom: (A) the whole venom; (B) Venom from Con A-agarose binding fraction; (C) control, Con A-agarose extracted with lysis buffer; and (D) venom from Con A-agarose nonbinding fraction. All samples were dissolved in the same lysis buffer and use the same conditions for 2-DE. Squared spots are Con A-agarose proteins. Numbers are in order of identified proteins.

and *D. acutus* from the Viperidae family, bind to PNA, which has specificity for terminal Gal-GalNAc groups. High-intensity fluorescence occurred in *O. hannah* venom (dot spot No. 10). This venom has both neurotoxic and heamotoxic properties, which differs from other venoms in this family. King cobra, *B multicinctus, B. fasciatus,* and *D. acutus* venoms have proteins that bind to ECL lectin, with binding specificity to GlcNAc. All snake venoms in both families have proteins which bind to Con A, specifically binding to mannose and glucose groups. The intensity of fluorescence signals (Figure 1) are high compared to the signals obtained from others lectins.

**Con A-Sepharose Affinity Purification of *N. naja kaouthia* Venom and Proteomics.** Both Con A-binding and nonbinding fractions were subjected to 2-DE analysis. Proteinspots were stained using the silver stain (Figure 2), whereas Sypro Ruby stained gels were used for protein identification by mass spectrometry.

The 2-DE images revealed that Con A-binding glycoproteins, eluted from agarose gel by using a lysis buffer, have moderate to high molecular weight and separated in the upper part of the 2-DE gel (Figure 2B). The protein pattern (Figure 2B) is similar to that for *N. naja kaouthia* total proteins at the upper part of the 2-DE gel (Figure 2A). The spots proteins have more intensity. The unbound proteins from Con A-affinity contain low molecular weight proteins (Figure 2D). The Con A-agarose extracted with a lysis buffer in the control experiment showed about twenty spots (Figure 2C).

In an additional experiment, the Con A-binding glycoproteins were eluted using free mannose. The 2-DE image shows that the Con A-binding proteins are also present on the upper part of the gel with a moderate to high molecular weight (Figure 3A). There are more spot proteins than are presented on the 2-DE gel of which were eluted by lysis buffer, and the protein pattern seems different. The Con A-unbinding fraction exhibited the same pattern as obtained in the process of elution by lysis buffer in Figure 2D (Figure 3B). In our experiment, we did not elute Con A-binding fraction with glucose. Proteins that do not appear when eluted with free mannose may have glucose on the reducing end of their carbohydrate chains. In

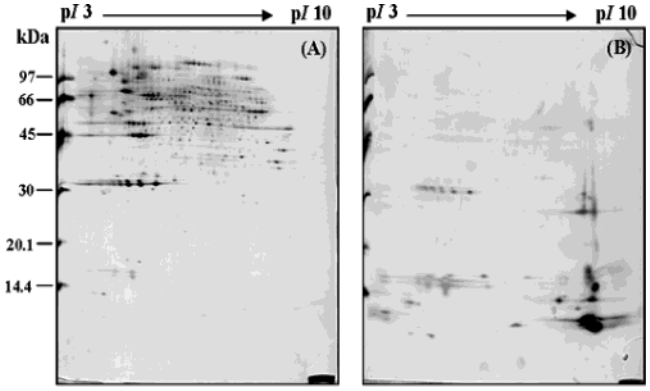


**Figure 3.** 2-DE images of *N. naja kaouthia* venom: (A) is Con A-agarose binding fraction extracted by using 0.2 M α-methyl mannose, (B) is the nonbinding fraction. The proteins were dissolved in lysis buffer and used 2-DE conditions the same as in Figure 2.

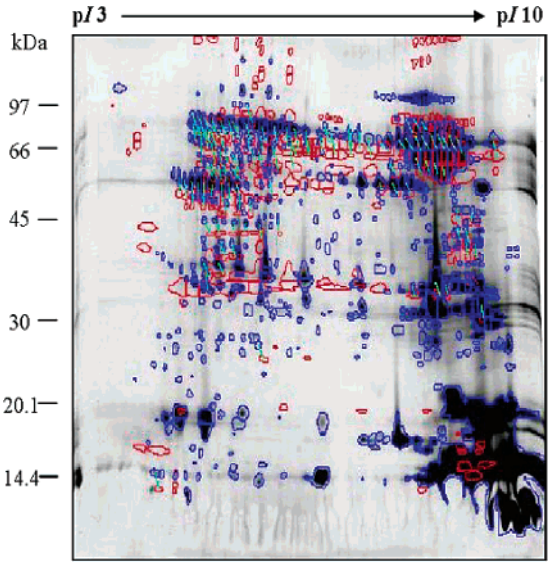


**Figure 4.** Superimposed 2-DE gel images of *N. naja kaouthia* whole venom and venom from Con A-agarose binding glycoprotein fraction using ImageMaster image software. The total number of detected spots on the 2-DE gel of whole proteins (blue) are 503 spots (not including low MW proteins not separated on the lower basic area). Total number of detected spots for venom from Con A-agarose binding fraction are 282 spots (red) matched spots are indicated by connected green lines.

the other case, it may be due to a different interaction of the proteins with Con A. The experiment confirms, however, that Con A-binding glycoproteins have moderate to high molecular weight and Con A-unbinding proteins have a lower molecular weight.

In the deglycosylation experiment, *N. naja kaouthia*'s Con A binding proteins were eluted using a free mannose solution. The proteins were then neutralized, separated from salt, then dried and analyzed by 2-DE. It was found that the pattern of proteins was different (result not shown).

Using ImageMaster software, we compared the 2-DE gels for the total venom proteins and the Con A-binding proteins obtained by elution with a lysis buffer (Figure 4). There are 503 spots (blue) for the *N. naja kaouthia* venom and 282 spots isolated for the Con-A binding fraction (red). Twenty-six spots were detected in the negative control (Figure 2C). The control spots are likely due to the Con A glycoprotein or its fragments resulting from part of the sample preparation. By comparing images, about half of the *N. naja kaouthia* total proteins were found to be Con A-binding glycoproteins. There were only 107 matched spots (green line, 21% of venom proteins) between total venom proteins and the Con A-binding glycoproteins.

**Protein Identification by Mass Spectrometry.** In the gel digestion and mass spectrometry of total venom proteins and nonbinding fraction, thirteen proteins were identified from different areas in 2-DE gel (Table 2). These spots were chosen for identification because they exhibited high intensity in the 2-DE gels of *N. naja kaouthia* venom. Of interest, spot No. 1 is a *NAJA* nerve growth factor. This is a glycoprotein also found in many species of snakes, in addition to the Elapidae family.[23,24]

Other basic, low molecular weight spots of whole venom proteins, in the nonbinding fraction on the 2-DE gel pH 3−10, were also identified. Low molecular weight proteins, with basic properties, have important roles for the bioactivity and properties of the venom. Due to incomplete separation of the protein spots on the 2-DE gels, they were identified using ESI−MS/MS (Table 3, spots 16−24). Some of the proteins in this area separated within a narrow pH range (pH 6−11) were also identified by MALDI-TOF (Table 2, spots 7−13 on Figure 5). Spots 14 and 15 underwent N-terminal analysis to confirm MALDI-TOF results. The protein spots are very prominent on the 2-DE gel of the total venom proteins. The N-termini of the two proteins is NLYQFKNMIQ, which identifies them as $PLA_2$ isoforms. These proteins had not been previously reported as glycoproteins, and our findings support these results, because they appeared in the nonbinding fractions (Figure 2D).

Protein identifications were also carried out on spots in the upper quadrant of the 2-DE gels that were of a high molecular weight and had a basic p*I* (Figure 2A and 2B). We found that spot 2 was consistent with the sequence for Cobra venom factor (CVF). CVF is a 184 519 Da glycoprotein consisting of three disulfide-linked chains of three polypeptides.[44] The spot was identified as the α-chain with a molecular mass of about 68 500 Da. We confirmed the MALDI-TOF result by identification with ESI−MS/MS. (Table 4, with full amino acid sequence shown in Figure 6). Spots 3 and 4 (Table 2) were also identified as CVF. These two spots may be the *β*-chain. The other 2 glycoprotein spots (Table 2, spots 5,6) were identified as serum albumin.

## Discussion

**Lectin-Binding Glycoproteins.** The results of the lectin-binding affinities of snake venoms are shown in Table 1 and Figure 1. The glycoproteins from three snake venoms, *B. fasciatus*, *O. hannah*, and *Tr. stejnegeri*, bind to fucose. From the results obtained in our experiment, this sugar occurs in few snake venoms. The biological properties of the three venoms, in which fucose was present, were also different.

All of the venom glycoproteins have an affinity for sialic acid binding lectins. This may indicate significant amounts and a significant role for sialic acid in snake venoms proteins.[45,46] Sialylation of glycoproteins confers a negative charge and increases resistance to enzymatic degradation.[47] High levels of sialylation are reasonable for snake venom proteins, which contain a mixture of hydrolytic enzymes. The sugar moieties may help many protein constituents resist enzymatic digestion and provide stability for proteins in the venom gland. Nega-

**Table 2.** Protein Identification in *N. naja kaouthia* Venom by MALDI-TOF Mass Spectrometry

| protein name | app. mass/p*I* | theo. mass/p*I* | no. peptides matched | matched sequences | sequence coverage (%) | accession no. | spot no. |
|---|---|---|---|---|---|---|---|
| nerve growth\| factor | 17000/6.6 | 13023/5.8 | 4 | (K)ALTMEGNQASWR (F)<br>(K)ALTMEGNQASWR(F)<br>(K)GNTVTVMENVNLDNKVYK (E)<br>(K)TTATDIKGNTVTVMENVNLDNK (V) | 31.0 | PO1140 | 1 |
| cobra venom factor | 67000/6.8 | 184519/5.99 | 13 | (K)YVLPSFEVR(L)<br>(K)YFTYLILNK(G)<br>(R)KYVLPSFEVR(L)<br>(K)GIYTPGSPVLYR(V)<br>(K)QLDIFVHDFPR(K)<br>(R)QNQYVVVQVTGPQVR(L)<br>(K)LILNIPLNAQSLPITVR(T)<br>(R)TDTEEQILVEAHGDSTPK(Q)<br>(K)FFYIDGNENFHVSITAR(Y)<br>(R)ASSSWLTAYVVKVFAMAAK(M)<br>(R)DGQNLVTMNLHITPDLIPSFR(F)<br>(R)RDGQNLVTMNLHITPDLIPSFR(F)<br>(R)LQPSEKFFYIDGNENFHVSITAR(Y)2PO4 | 7.0 | 2118405 | 2 |
| cobra venom factor | 50000/5.2 | 184519/5.99 | 8 | DLNLDITIELPDREVPIR<br>YRINYENALLAR<br>INYENALLAR<br>VAVIIYLNKVSHSEDECLHFK<br>HFEVGFIQPGSVK<br>FYHPDKGTGLLNK<br>CAGETCSSLNHQERIDVPLQIEK<br>IDVPLQIEKACETNVDYVYK | 6.0 | gi\|2118405 | 3 |
| cobra venom factor | 50100/5.3 | 184519/5.99 | 14 | DLNLDITIELPDREVPIR<br>YRINYENALLAR<br>VAVIIYLNKVSHSEDECLHFK<br>ILKHFEVGFIQPGSVK<br>HFEVGFIQPGSVK<br>FYHPDKGTGLLNK<br>CAGETCSSLNHQER<br>CAGETCSSLNHQERIDVPLQIEK | 10.0 | I51018 | 4 |
| cobra venom factor | 50100/5.3 | 184519/5.99 | 14 | CAGETCSSLNHQERIDVPLQIEK<br>IDVPLQIEKACETNVDYVYK<br>CQEALNLKVNDDYLIWGSR<br>VNDDYLIWGSR<br>NTWIERWPHEDECQEEEFQK<br>LCDDFAQFSYTLTEFGCPT | 10.0 | I51018 | 4 |
| serum albumin | 50100/5.1 | 68915/5.8 | 9 | AFFGHYLYEVAR<br>RHPYFYAPELLYYAQK<br>HPYFYAPELLYYAQK<br>ADLAKYMCEHQETISSHLK<br>RHPDYSVVLLLR<br>HPDYSVVLLLR<br>VLDEFQPLVDEPK<br>KVPQVSTPTLVEISR<br>QTALVELVKHKPHATNDQLK | 17.0 | P49065 | 5 |
| serum albumin | 50100/5.1 | 69411/5.9 | 11 | FNDVGEEHFIGLVLITFSQYLQK<br>SLHDIFGDKICALPSLR<br>AFHDDEKAFFGHYLYEVAR<br>AFFGHYLYEVAR<br>RHPYFYAPELLYYAQK<br>HPYFYAPELLYYAQK<br>HPYFYAPELLYYAQKYK<br>QNCELYEQLGDYNFQNALLVR<br>RPCFSALGPDETYVPK<br>EFNAETFTFHADICTLPETER<br>EFNAETFTFHADICTLPETERK | 22.0 | ALBU_RABIT | 6 |
| phospholipase A2 isozyme | 13600 /8.8 | 13200/8.6 | 4 | (K)DFVCACDAAAAKCFAK(A)<br>(K)IHDDCYIEAGKDGCYPK(L)<br>(R)AAWHYLDYGCYCGPGGR(G)<br>(K)IHDDCYIEAGKDGCYPK(L) | 42.0 | P20257 | 7 |
| phospholipase A2 | 13540/9.6 | 15739/5.2 | 5 | (R)CCFVHDCCYGKLTACSPK(L)<br>(K)LDMYTYSQKNEDIVCGGDDPCK(K)<br>(K)EPLTTYLFYACYCGWGGRGEPK(D)<br>(K)EATGKEPLTTYLFYACYCGWGGR(G)<br>(K)EATGKEPLTTYLFYACYCGWGGR(G) | 48.0 | Q9I968 | 8 |
| phospholipase A2 | 13540/10.2 | 15424/5.05 | 3 | TGIFGIMSYIYYGCYCGWGGK<br>CCFVHDCCYGRVNGCDPK<br>MGTYSYSFQNGDIVCGGDDPCLR | 44.0 | Q910A1 | 9 |

**Table 2.** (continued)

| protein name | app. mass/p*I* | theo. mass/p*I* | no. peptides matched | matched sequences | sequence coverage (%) | accession no. | spot no. |
|---|---|---|---|---|---|---|---|
| neurotoxin α | 13,540/9.6 | 6578/8.72 | 4 | MICHNQQSSQPPTIKTCSEGQCYK<br>MICHNQQSSQPPTIKTCSEGQCYK<br>ICHNQQSSQPPTIKTCSEGQCYK<br>GTISERGCGCPTVKPGIHISCCASDK | 83.0 | P80548 | 10 |
| similar to long chain α-neurotoxins | 20010/8.8 | 9559/7.42 | 4 | ECYLNPHDTQTCPSGQEK<br>GKVLEFGCAATCPSVNTGTEIK<br>VLEFGCAATCPSVNTGTEIK<br>VLEFGCAATCPSVNTGTEIKCCSADK | 52.0 | gi\|3551478 | 11 |
| cytochrome *c* | 32450/9.1 | 26653/9.39 | 5 | PISMCGTGFQK<br>FISCSSMDPYSSAQINGIR<br>FISCSSMDPYSSAQINGIRR<br>HPGCGSCIVMQLDNFDK<br>HPGCGSCIVMQLDNFDKYSK | 21.0 | gi\|29741831 | 12 |
| similar to immunoglobulin heavy chain | 13540/9.2 | 13773/8.0 | 4 | (R) LSCAASGFPFDDYAMHWVR (Q)<br>(K)NALYLQMNSLRAEDTALYYCVK (T)<br>(R)LSCAASGFPFDDYAMHWVRQAPGK (G)<br>(R)LSCAASGFPFDDYAMHWVRQAPGK(G) | 55.0 | 414567 | 13 |

**Table 3.** Protein Identification in *N. naja kaouthia* Venom by ESI−MS/MS

| name of protein | accession no. | matched sequences | protein no. |
|---|---|---|---|
| cobrotoxin b (CBT b) (short neurotoxin) | gi\|28380028 | LECHNQQSSQTPTTK<br>TCSGETNCYKK<br>VKPGVNLNCCR | 16 |
| cardiotoxin 1c [*Naja atra*] | gi\|1688221 | MFMMSDLTIPVK<br>MFMMSDLTIPVKR<br>MFMMSDLTIPVKR<br>RGCIDVCPK<br>GCIDVCPK<br>YVCCNTDRCN | 17 |
| cardiotoxin 7 precursor [*Naja sputatrix*] | gi\|3176835 | MFMMSNKTVPVK<br>RGCIDVCPK<br>GCIDVCPK<br>YVCCNTDRCN | 18 |
| short neurotoxin 1 −monocled cobra [*Naja kaouthia*] | gi\|7428586 | LECHNQQSSQAPTTK<br>TCSGETNCYKK<br>VKPGVNLNCCR | 19 |
| neurotoxin 6 −Indian cobra | gi\|85982 | GCADTCPVRKPR | 20 |
| neurotoxin-like protein [*Naja naja*] | gi\|5102575 | VYVDCCAR | 21 |
| toxin C [*Naja naja*] | gi\|223302 | IRCFITPDITSK<br>IRCFITPDITSK<br>DCPNGHVCYTK<br>DCPNGHVCYTK<br>TWCDAFCSIR<br>TWCDAFCSIRGK | 22 |
| toxin VII2 [*Naja haje annulifera*] | gi\|229547 | MYMVATPMLPVKR<br>MYMVATPMLPVKR<br>RGCIDVCPK<br>GCIDVCPK | 23 |
| weak toxin CM-9a | gi\|14195693 | LTCLNCPEMFCGK<br>GCADTCPVGYPK | 24 |

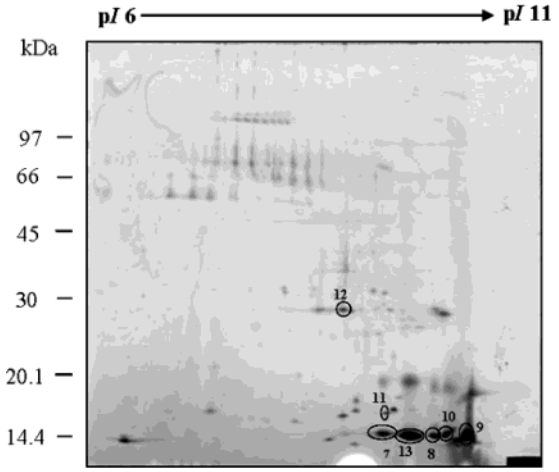


**Figure 5.** 2-DE gel image of *N. naja kaouthia* venom. The whole venom was solubilized in modified lysis buffer and separated using IEF with IPG strip pH 6−11 (linear) using cup loading as the sample application method. The narrow range pH of the IPG strip gives more separation of proteins in this area and allows for more identification.

tively charged sialic acid groups on proteins are implicated in the modulation of cell adhesion in some organ systems.[46]

Venoms of the Elapidae family bind to PNA lectin, which has specificity for terminal Gal-GalNAc groups. Our results show that this may be one of the characteristics of snake venoms from this family, but the intensity of fluorescence exhibited by each venom is different.

Some venoms have proteins which bind to Gal-1,4-GlcNAc. As is the case with fucose, this sugar type is found in only a few snake venoms. Even within in the same *Bungarus genus*, the fluorescence from the blotted dots of each snake venom exhibited different intensities.

All snake venoms have proteins that bind to Con A, which specifically binds mannose and glucose groups. Many glycoproteins with affinity to Con A are reported to be N-linked glycoproteins. This may suggest that N-linked glycoproteins, which are more common than mannose type O-linked glycoproteins, may be present in many snake venoms. N-linked glycoproteins have been found to be essential for many proteins where the binding property is important for their bioactivities.[48−50]

It must be stressed that the seven lectins tested in our investigation do not cover all of the carbohydrate moieties found in glycoproteins. Furthermore, no conclusive evidence is demonstrated that the carbohydrates found in snake venoms contain only the seven moieties tested. From the results presented here, we can conclude that there are heterogeneous glycoforms present in snake venom glycoproteins.

The synthesis of the polypeptide chain of a glycoprotein is under genetic control and the carbohydrate chains are attached and processed by a series of enzyme reactions without the rigid detection of a nucleic acid template.[51] For this reason, a single polypeptide which is glycosylated generally emerges from the

**Table 4.** Protein Identification of Cobra Venom Factor by ESI−MS/MS

| name of protein | accession no. | total score | match sequences | ion score |
|---|---|---|---|---|
| cobra venom factor | gi\|2118405 | 870 | TDTEEQILVEAHGDSTPK | 58 |
| | | | QLDIFVHDFPR | 63 |
| | | | VDMNPAGGMLVTPTIEIPAKEVSTDSR | 50 |
| | | | GIYTPGSPVLYR | 46 |
| | | | YVLPSFEVR | 63 |
| | | | YLYGEEVEGVAFVLFGVK | 82 |
| | | | SIPDSLTR | 16 |
| | | | SIPDSLTRIPIIDGDGK | 14 |
| | | | IPIIDGDGK | 43 |
| | | | IPIIDGDGKATLK | 45 |
| | | | LILNIPLNAQSLPITVR | 94 |
| | | | TNHGDLPR | 59 |
| | | | YFTYLILNK | 42 |
| | | | RDGQNLVTMNLHITPDLIPSFR | 48 |
| | | | IKLEGDPGAR | 47 |
| | | | LEGDPGAR | 13 |
| | | | VGLVAVDK | 37 |
| | | | AVPFVIVPLEQGLHDVEIK | 50 |

biosynthetic pathway as a mixture of glycoforms.[51] In addition, it is well established that these glycoform populations are both species and cell specific.[52,53] The differences in the glycoforms of glycoproteins from related species of snake venoms may indicate diversity at the genetic level.

The total proteins of one type of venom can have binding affinity to many kinds of lectins. The fluorescence intensity obtained from all kinds of FITC-labeled lectin to venom of *V. russelli formosensis* and *V. russelli siamensis* look very similar (dot spots Nos. 3 and 8), whereas the binding affinity to venom composition is different in related species such as in *Bungarus* (dot spots Nos. 1 and 7) and *Naja sp*. (dot spots No 4 and 9). This indicates some differences in the protein composition of the venoms. It may indicate a difference in the protein post-translational modification process if the protein composition appears to be the same. This may result from differences in the protein amino acid sequence as a result of mutation. Mutation is a process that constantly causes diversity in the genes of animals of related species, and causes different protein homologies. The differences in protein sequence homology can cause a different composition of carbohydrates in the same proteins.

Large portions of snake venom contain glycoproteins with the same or different carbohydrate moieties. Different sugar moieties may be responsible for similar or different functions due to the specific characteristics of each molecule and its environment. In the case of snake venom, the glycosylation process may play an important role in helping proteins create the correct folding topology, a consequent of which is the possession of the biological activity necessary to access their targets. Snake venoms are presented as a pooled mixture of concentrated proteins with different toxic properties, many of which contain hydrolytic enzymes. These kinds of co- or post-translational modifications help to keep each of their individual properties and resist being degraded by the action of other toxic components.

It can be said that glycosylation determines a protein's affinities and interactions. This is supported by the results of our experiment in which all of the snake venoms studied had protein compositions with an affinity for sialic acid binding lectins which are often reported to have a role in protein−protein interaction. In addition to the biological activity effect of the glycoprotein toxins themselves, the role of carbohydrates in the viscosity of snake venoms is important for the rapid and efficient dissemination of the venom from the envenomation site. Further detailed studies are necessary to elucidate the role of sugars for specific glycoproteins.

**Con A-Sepharose Affinity Purification of *N. naja kaouthia* Venom and Proteomics.** In the preceding experiments, we have demonstrated that all 10 snake venoms have Con A-binding components. Therefore, we investigated the protein composition of the Con A-binding fraction of *N. naja kaouthia* venom. *N. naja kaouthia* is one of the most dangerous snakes in Thailand and poses a danger to public health in many tropical and sub-tropical countries.[54] Con A is a lectin widely used for carbohydrate studies: for example, Con A has been used to monitor the enzymatic reaction and lectin specificity for sugar moieties in a sugar microarray format.[55−57]

We found that Con A-binding glycoproteins have a moderate to high molecular weight. Glycosylation increases proteins' molecular weight and affects bioactivity. The higher molecular mass results in a bulkier molecular size. This may provide a further advantage in protecting the proteins from other toxic components in venom.

**Protein Identification by Mass Spectrometry and Protein Functions.** *NAJA* nerve growth factor was identified in the Con A nonbinding fraction, the protein not found to bind with Con A-agarose. The carbohydrate structure of this protein is different and does not contain mannose or glucose in its structure.

In the process of identifying basic and low molecular weight proteins, we obtained results similar to other studies, where no protein of this type was reported to be a glycoprotein. Many low molecular weight protein toxins with basic properties have been reported to have many disulfide bonds in their structure, which may help stabilize the conformation of the proteins and help in other bioactivity thus canceling the need for further post-translational modifications such as glycosylation.

Three of the protein spots were identified as CVF, including the α- and $\beta$-chains of the protein. The α- and $\beta$-chains of the CVF were glycosylated with 2 and 1 carbohydrate chains, respectively. This protein is a nontoxic complement activating glycoprotein found in cobra venom. It is a functional analogue of mammalian complement component C3b. Spot 2 appeared along a train of spots in the 2-DE. We speculate that the neighboring spots may be different glycoforms of the same protein. The train of spots may be caused by different p*I* in the glycoproteins and/or variation of sialylation for different glycoforms.[58]

Serum albumin spots were identified on a train of spots (spots 5−6). The protein found in the serum of *N. naja kaouthia*

1 MERMALYLVA ALLIGFPGSS HGALYTLITP AVLR**TDTEEQ ILVEAHGDST**

51 **PKQLDIFVHD FPR**KQKTLFQ TR**VDMNPAGG MLVTPTIEIP AKEVSTDSR**Q

101 NQYVVVQVTG PQVRLEKVVL LSYQSSFLFI QTDK**GIYTPG SPVLYR**VFSM

151 DHNTSKMNKT VIVEFQTPEG ILVSSNSVDL NFFWPYNLPD LVSLGTWRIV

201 AKYEHSPENY TAYFDVRK**YV LPSFEVR**LQP SEKFFYIDGN ENFHVSITAR

251 **YLYGEEVEGV AFVLFGVK**ID DAKK**SIPDSL TRIPIIDGDG KATLK**RDTFR

301 SRFPNLNELV GHTLYASVTV MTESGSDMVV TEQSGIHIVA SPYQIHFTKT

351 PKYFKPGMPY ELTVYVTNPD GSPAAHVPVV SEAFHSMGTT LSDGTAK**LIL**

401 **NIPLNAQSLP ITVRTNHGDL PR**ERQATKSM TAIAYQTQGG SGNYLHVAIT

451 STEIKPGDNL PVNFNVKGNA NSLKQIK**YFT YLILNK**GKIF KVGRQPR**RDG**

501 **QNLVTMNLHI TPDLIPSFR**F VAYYQVGNNE IVADSVWVDV KDTCMGTLVV

551 KGDNLIQMPG AAMK**IKLEGD PGARVGLVAV DK**AVYVLNDK YKISQAKIWD

601 TIEKSDFGCT AGSGQNNLGV FEDAGLALTT STNLNTKQRS AAKCPQPANR

651 RRRSSVLLLD SNASKAAEFQ DQDLRKCCED VMHENPMGYT CEKRAKYIQE

701 GDACKAAFLE CCRYIKGVRD ENQRESELFL ARDDNEDGFI ADSDIISRSD

751 FPKSWLWLTK DLTEEPNSQG ISSKTMSFYL RDSITTWVVL AVSFTPTKGI

801 CVAEPYEIRV MKVFFIDLQM PYSVVKNEQV EIRAILHNYV NEDIYVRVEL

851 LYNPAFCSAS TKGQRYRQQF PIKALSSR**AV PFVIVPLEQG LHDVEIK**ASV

901 QEALWSDGVR KKLKVVPEGV QKSIVTIVKL DPRAKGVGGT QLEVIKARKL

951 DDRVPDTEIE TKIIIQGDPV AQIIENSIDG SKLNHLIITP SGCGEQNMIR

1001 MAAPVIATYY LDTTEQWETL GINRRTEAVN QIVTGYAQQM VYKKADHSYA

1051 AFTNRASSSW LTAYVVKVFA MAAKMVAGIS HEIICGGVRW LILNRQQPDG

1101 AFKENAPVLS GTMQGGIQGA EEEVYLTAFI LVALLESKTI CNDYVNSLDS

1151 SIKKATNYLL KKYEKLQRPY TTALTAYALA AADQLNDDRV LMAASTGRDH

1201 WEEYNAHTHN IEGTSYALLA LLKMKKFDQT GPIVRWLTDQ NFYGETYGQT

1251 QATVMAFQAL AEYEIQMPTH KDLNLDITIE LPDREVPIRY RINYENALLA

1301 RTVETKLNQD ITVTASGDGK ATMTILTFYN AQLQEKANVC NKFHLNVSVE

1351 NIHLNAMGAK GALMLKICTR YLGEVDSTMT IIDISMLTGF LPDAEDLTRL

1401 SKGVDRYISR YEVDNNMAQK VAVIIYLNKV SHSEDECLHF KILKHFEVGF

1451 IQPGSVKVYS YYNLDEKCTK FYHPDKGTGL LNKICIGNVC RCAGETCSSL

1501 NHQERIDVPL QIEKACETNV DYVYKTKLLR IEEQDGNDIY VMDVLEVIKQ

1551 GTDENPRAKT HQYISQRKCQ EALNLKVNDD YLIWGSRSDL LPTKDKISYI

1601 ITKNTWIERW PHEDECQEEE FQKLCDDFAQ FSYTLTEFGC PT

**Figure 6.** Amino acid sequence of Cobra venom factor and matched sequences (bold) obtained by ESI−MS/MS identification.

has a role in sequestering the lethal endogenous toxin in the serum of the snake.[59] Interestingly, serum albumin is not glycosylated. This protein has an affinity for other proteins in biological fluids[60,61] and can be found in a wide range of animals including mammals, amphibians, and reptiles. There are many types of proteins belonging to the family of related serum albumins, [62] which aids in the transport of many ligands. In addition to blood plasma, serum albumins are also found in tissues and secretions throughout the body.[63] It is possible that serum albumin binds to the glycoprotein components in snake venom. This may lead to coexistence of serum albumin in the Con A-affinity purified fractions. The lectin-affinity was subsequently broken by protein dissociation in 2-DE, resulting in serum albumin being separately identified.

The results of our experiment indicate that large portions of snake venom proteins are post-translationally modified by glycosylation. Carbohydrate modification is the primary cause of microheterogeneity in proteins (glycoforms).[64] Glycosylation is known to alter the physical properties of proteins such as folding, trafficking, packing, stabilization, protease protection, quaternary structure, and organization of water structure or function as specific recognition epitopes.[64,65] Many proteins from snake venoms have been purified and characterized as glycoproteins. There are, however, few reports on the effects of carbohydrates on snake venom proteins.

Using lectins to assess and separate the carbohydrate moieties, we sequentially performed a glycoproteomics analysis of the snake venom, *N. naja kaouthia*. This technique is useful for the study of the snake venom proteome. The analysis *N. naja kaouthia* venom revealed large amounts of glycoproteins. Dot blot analysis using FITC-labeled lectins found that snake venom glycoproteins have an affinity for sialic acid binding lectins. This may indicate the target binding role of sialic acid for the toxin glycoproteins. We have shown that N-linked oligosaccharides may make up the majority of *N. naja kaouthia* venom glycoproteins, due to their specificity for Con A and GlcNAc, which primarily occur in N-linked type glycoproteins.

**Abbreviations.** *B. fasciatus*, *Bungarus fasciatus*; *B. multicinctus*, *Bungarus multicinctus*; *D. acutus*, *Deinagkistrodon acutus*; *N. naja atra*, *Naja naja atra*; *N. naja kaouthia*, *Naja naja kaouthia*; *O. hannah*, *Ophiophagus hannah*; *V. russelli formensis*, *Vipera russelli formosensis*; *V. russelli siamensis*, *Vipera russellii siamensis*; *Tr. mucrosquamatus*, *Trimeresurus mucrosquamatus*; *Tr. stejnegeri*, *Trimeresurus stejnegeri*; FITC, fluorescein isothiocyanate.

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