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STRUCTURAL FEATURES OF CARBOHYDRATE MOIETIES IN SNAKE VENOM GLYCOPROTEINS

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The structures of the carbohydrate moieties of glycoproteins in snake venoms are largely unknown. In the present study, we have analyzed venoms of several species of snakes as well as plasma and tissue glycoproteins from one species of cobra (*Naja naja kaouthia*) by lectin affinity staining of Western blots. The data demonstrate that glycoproteins in cobra venom invariably contain terminal α-galactosyl residues with negligible proportions of sialic acids. Interestingly, however, terminal α-galactosyl residues are present in significantly lower proportions in cobra tissues such as brain, liver, lung, kidney, spleen, muscle, and totally absent in cobra plasma glycoproteins. In sharp contrast to cobras, venom glycoproteins of other snakes do not contain terminal α-galactosyl residues but do contain terminal 2,3- and/or 2,6-linked sialic acids as well as β-galactosyl residues. Cobra venom also contains high molecular weight heavily glycosylated proteins bearing poly-*N*-acetyllactosaminyl oligosaccharides, the majority of which appear to be linked to the protein core via *O*-glycosidic bonds.

Snake venoms contain several biologically active proteins including complement activating factor, blood coagulation enzymes, thrombin-like enzymes, nerve growth factors and platelet aggregation factors, most of which are glycosylated (1-3). Some of these glycoproteins have been proven to be useful in clinical applications and in studying physiological processes (4-7). The occurrence of carbohydrate moieties and their qualitative sugar compositions have been reported for several venom glycoproteins (1-3). However, the structures and functional role of the oligosaccharides have not been investigated. For some glycoproteins, the reported sugar compositions are even questionable (8,9). The carbohydrate contents of snake venom proteins ranges from 5 to 90% (1-3).

Recently a detailed study on the structures of the oligosaccharides in cobra venom factor, the complement activating glycoprotein in cobra venom (*Naja naja kaouthia*), has been performed (10, Gowda *et al* unpublished results). It was found that cobra venom factor contains novel oligosaccharide structures with α-galactosylated Lewis X antigenic epitope, Galα1-3Galβ1-4(Fucα1-3)GlcNAcβ1. This study represents

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the first detailed investigation regarding the nature of oligosaccharides in snake venom glycoproteins. Preliminary studies in our laboratory indicated that other glycoproteins in the venom of *Naja naja kaouthia* also contain oligosaccharides bearing the above carbohydrate epitope.

Our long term goal is to determine the structures of novel oligosaccharides in biologically active snake venom glycoproteins and to understand the functional roles of saccharide residues in determining their biological activities. Here, we report the results of initial characterization of the carbohydrate moieties in various snake venom glycoproteins.

MATERIALS AND METHODS

Lyophilized snake venoms were obtained from Miami Serpentarium Laboratories, Punta Gorda, FL and from Ventoxin Laboratories Inc., Frederick, MD. *Griffonia simplicifolia* agglutinin I from E-Y Laboratories, San Mateo, CA and was radiolabeled with $Na^{125}I$ (Amersham) according to Lee and Griffith (11) to give ~ 7.5 x 10^5 cpm/μg protein. Digoxigenin-labeled *Maackia amurensis* agglutinin, *Sambucus nigra* agglutinin and *Datura stramonium* agglutinin were part of the glycan differentiation kit from Boehringer Mannheim, Indianapolis, IN. Polyvinylidene difluoride membranes were from Millipore, Bedford, MA.

Cobra plasma and tissues were collected by sacrificing a *Naja naja kaouthia* snake. The snake was anesthetized by intramuscular injection of ketamine hydrochloride (Avco, Ft. Dodge, Iowa). The cardiac cavity was entered surgically and exsanguinated by aortal puncture. Blood was collected with a 50 ml syringe containing approximately 5 ml of 0.2 M EDTA, pH 7.4 and immediately centrifuged at 2000 x g at 4°C for 30 min. The erythrocyte pellet was removed, the EDTA-plasma collected and stored at -90°C. Organs such as brain, liver, lung, kidney, spleen were removed by dissection, immediately frozen in liquid nitrogen and stored at -90°C.

About 0.5 to 1 g of each tissue was ground with a pestle and extracted with 0.5 to 1 ml of 20 mM Tris.HCl, 0.2 M NaCl, pH 8.0 containing 1 mM phenylmethylsulfonyl chloride, 5 mM N-ethylmaleimide and 0.5% SDS. The extracts were heated in a boiling water bath for 2 min and centrifuged at 14,000 x g for 30 min.

SDS-Polyacrylamide Gel Electrophoresis. Snake venoms (150 to 200 μg dry weight) and tissue proteins (30 to 40 μg protein) were electrophoresed on 10% (w/v) or 12% (w/v) SDS-polyacrylamide gels in the presence of β-mercaptoethanol according to Laemmli (12). The gels were stained with Coomassie Blue or periodate-Schiff (13).

Lectin Affinity Staining. Snake venoms (150-200 μg dry weight) were electrophoresed on 10% (w/v) SDS-polyacrylamide gels under reducing conditions and electro-transferred onto polyvinylidene difluoride membranes. The blots were stained with either Amido Black or lectins. For staining with *Griffonia simplicifolia agglutinin* I, the membranes were blocked with 1% bovine serum albumin and then treated with [^{125}I]*Griffonia simplicifolia* agglutinin I (10^7 cpm) in 10 ml of 10 mM sodium phosphate, 0.15 M NaCl, 1 mM $CaCl_2$, pH 7.4 containing 1% bovine serum albumin. Staining with digoxigenin-labeled lectins was performed using the glycan differentiation kit from Boehringer Mannheim according to the manufacturer's protocol.

Carbohydrate Composition. Glycoprotein samples (50 to 100 μg) were hydrolyzed with 2.5 M trifluoroacetic acid at 100°C for 5 h and lyophilized. The released

sugars were analyzed on a CarboPac PA1 column by high-performance anion-exchange chromatography coupled to pulsed amperometric detection. The sugars were eluted with 18 mM sodium hydroxide isocratically.

RESULTS AND DISCUSSION

Coomassie Blue staining of venoms from various species of snakes separated on SDS-polyacrylamide gels showed that snake venoms are indeed complex mixtures of proteins and peptides (Figure 1A). Staining with the periodate-Schiff demonstrated that several venom proteins in each species of snake are glycosylated (Figure 1B). Some proteins appear to contain a rather high proportion of carbohydrate as they poorly stained with Coomassie Blue and stained intensely with periodate-Schiff (compare lanes 1 to 3 and 5 in Figures 1A and IB).

Practically no information is available on the structures of oligosaccharides in snake venom glycoproteins. Therefore, we initially studied the glycosylation pattern in venom glycoproteins of some representative species of snakes. The total venom components were analyzed on SDS-polyacrylamide gels, the protein bands were transferred onto polyvinylidene difluoride membranes and affinity stained with a series of lectins. *Griffonia simplicifolia* agglutinin I binds with high affinity the oligosaccharides containing terminal α-galactosyl residues. Almost all the glycoproteins in venoms of different species and subspecies of cobras, *Naja naja kaouthia, Naja naja naja, Naja naja atra, Naja nivea, Naja nigricollis crawshayi, Naja melanoleuca*, that we studied stained strongly with ^{125}I-labeled *Griffonia simplicifolia* agglutinin I (Figure 2). The staining was completely abolished on treatment with coffee bean α-galactosidase (Figure 2). In contrast, none of the glycoproteins in venoms of *Agkistrodon piscivorus piscivorus* and *Bitis arietans arietans* (Figure 2) and several other species of snakes such as *Crotalus adamanteus, Crotalus atra, Agkistrodon piscivorus conanti, Bungurus fasciatus, Trimeresurus okinavensis,* and *Vipera russelli russelli* (data not shown) stained with *Griffonia simplicifolia* agglutinin I. These data suggest that terminal α-galactosyl residues are expressed in cobra venom glycoproteins in a species specific manner.

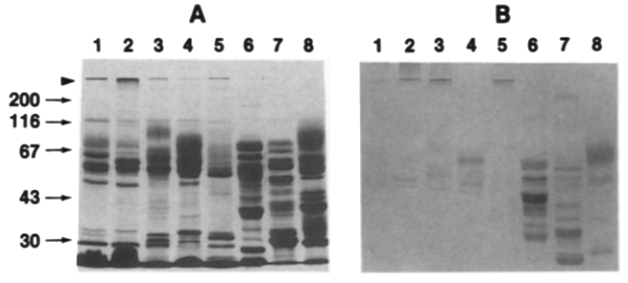

Fig. 1. SDS-polyacrylamide gel electrophoresis of snake venoms. Snake venoms were analyzed on 10 % SDS-polyacrylamide gels under reducing condition and stained with Coomassie Blue (A) and with periodate-Schiff (B). Lanes: 1, *Naja Naja kaouthia*; 2, *Naja naja atra*; 3, *Naja nivea*; 4, *Naja melanoleuca*; 5, *Naja nigricollis crawshayi*; 6, *Bitis arietans arietans*; 7, *Agkistrodon piscivorus piscivorus;* 8, *Crotalus adamanteus.*

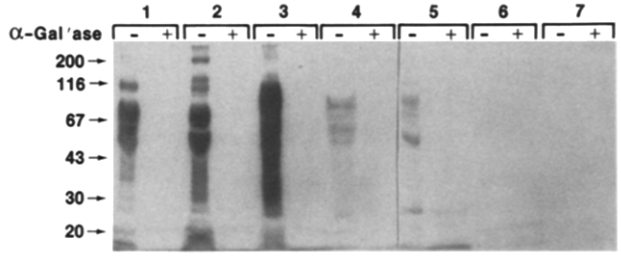

Fig. 2. Lectin affinity staining of snake venom glycoproteins before (-) and after (+) treatment with α-galactosidase. Snake venoms components were separated on SDS-polyacrylamide gels under reducing condition and the protein bands on the gels were transferred to polyvinylidene difluoride membranes. The blots were affinity stained with ^{125}I-labeled *Griffonia simplicifolia* agglutinin I. Lanes: 1, *Naja Naja kaouthia*; 2, *Naja naja atra*; 3, *Naja nivea*; 4, *Naja nigricollis crawshayi*; 5, *Naja melanoleuca*; 6, *Bitis arietans arietans* ; 7, *Agkistrodon piscivorus piscivorus.*

Although glycoproteins containing terminal α-galactosyl residues have been reported to occur in a number of animals in a varieties of tissues (14-16), they are not expressed in humans, apes and Old World monkeys (17). In fact, humans contain circulating antibodies (~1% of total IgG) directed against Galα1,3Galβ1 epitope (18) and the α-galactosyltransferase gene is evolutionarily suppressed (19, 20). Among reptiles, cobra venom glycoproteins represent the first documentation regarding the occurrence of oligosaccharides containing terminal α-galactosyl residues. It remains to be determined why terminal α-galactosyl residues are expressed specifically in cobra venom glycoproteins but not in other snake venom glycoproteins.

To investigate whether the expression of terminal α-galactosyl residues in cobras is tissue specific, glycoproteins of plasma, erythrocytes and tissues such as muscle, heart, brain, kidney, spleen, liver, and lung from one of the species of cobras, *Naja naja kaouthia,* were examined. SDS-polyacrylamide gel electrophoresis and staining of the gels with Coomassie Blue and periodate-Schiff showed that cobra plasma and tissue extracts contain a complex mixture of proteins, several of which are glycosylated (Figures 3A and 3B). A 68 kDa glycoprotein in cobra plasma appears to be rather

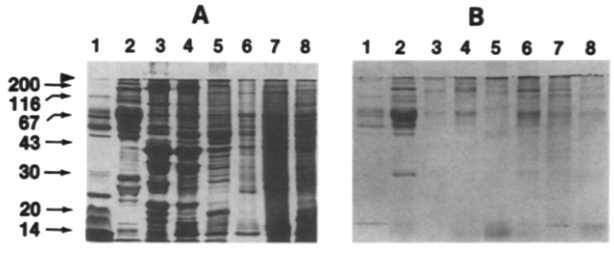

Fig. 3. SDS-polyacrylamide gel electrophoresis of cobra plasma and tissue proteins. *Naja naja kaouthia* venom, plasma and tissue proteins were separated on 12% polyacrylamide gels under reducing condition and stained with Coomassie Blue (A) and periodate-Schiff (B). Lanes: 1, venom; 2, plasma; 3, muscle; 4, heart ; 5, brain; 6, kidney; 7, spleen; 8, liver.

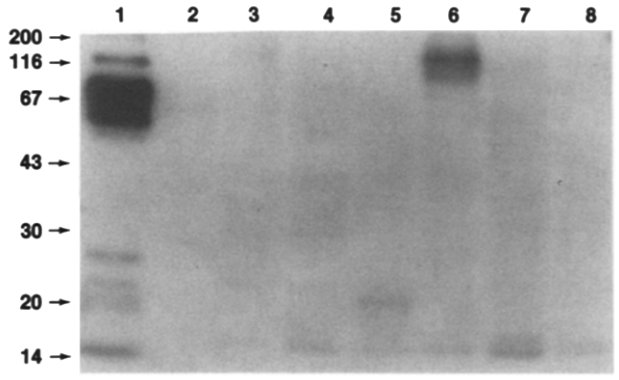

Fig. 4. Affinity staining of Cobra plasma and tissue glycoproteins with *Griffonia simplicifolia* agglutinin I. The venom, plasma and tissue proteins from *Naja naja kaouthia* were separated on 12% SDS-polyacrylamide gels under reducing condition and the protein bands on the gels were transferred to polyvinylidene difluoride membranes. The blots were affinity stained with ^{125}I-labeled *Griffonia simplicifolia* agglutinin I. Samples in lanes are as described in figure 3.

heavily glycosylated as it was stained intensely with periodate-Schiff (Figure 3B, lane 2). The protein bands on polyacrylamide gels were transferred to polyvinylidene difluoride membranes and affinity stained with ^{125}I-labeled *Griffonia simplicifolia* agglutinin I. The results indicate that terminal α-galactosyl residues are totally absent in cobra plasma and present in significantly lower proportion in glycoproteins of various tissues that were studied (Figure 4). However, staining of these glycoproteins with digoxigenin-labeled *Maackia amurensis* and *Sambucus nigra* agglutinins suggest that they contain terminal 2,3- and 2,6-linked sialic acids (data not shown).

To determine the nature of terminal sugar residues in venom glycoproteins of snakes other than cobras, the venom glycoproteins from representative species of snakes were blotted onto polyvinylidene difluoride membranes and stained with digoxigenin-labeled *Maackia amurensis*, *Sambucus nigra* and *Datura stramonium* agglutinins. The data for venoms of four snake species are shown in Figure 5. The

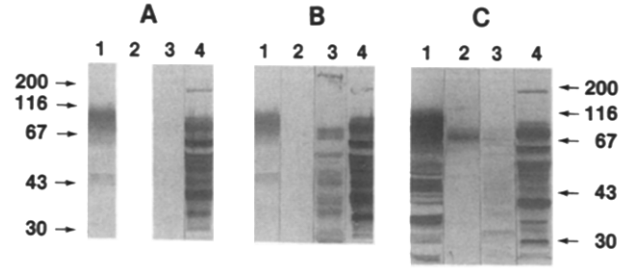

Fig. 5. Lectin affinity staining of snake venom glycoproteins. Snake venoms were analyzed on 10% SDS-polyacrylamide gels under reducing condition, blotted onto polyvinylidene difluoride membranes and stained with ^{125}I-labeled *Maackia amurensis* agglutinin (A), *Sambucus nigra* agglutinin (B) and *Datura stramonium* agglutinin (C). Lanes: 1, *Crotalus adamanteus*; 2, *Naja naja kaouthia*; 3, *Agkistrodon piscivorus piscivorus*; 4, *Bitis arietans arietans*.

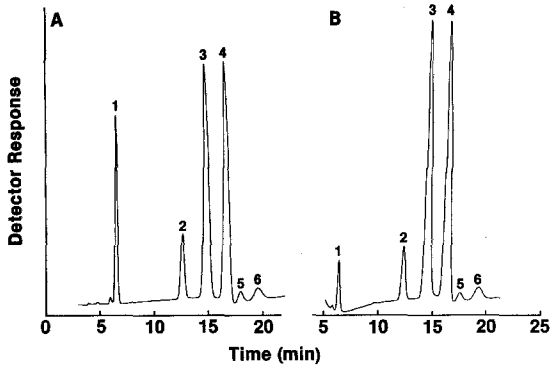

Fig.6. Carbohydrate compositional analysis of cobra venom glycoproteins containing poly-*N*-acetyllactosaminyl oligosaccharides. The heavily glycosylated proteins of cobra venom which eluted at the excluded volume on Bio-Gel A-0.5m column were hydrolyzed with 2.5 M trifluoroacetic acid and the hydrolyzates were analyzed on a CarboPac PA1 column by high-performance anion-exchange chromatography coupled to pulsed amperometric detector. A, from *Naja naja kaouthia* venom and B, from *Naja naja naja* venom. Peaks: 1, fucose; 2, galactosamine; 3, glucosamine; 4, galactose; 5, glucose; 6, mannose.

results demonstrate that the glycoproteins from the venoms of snakes contain terminal sialic acid and/or β-galactosyl residues. For example, the glycoproteins of *Agkistrodon piscivorus piscivorus* and *Agkistrodon piscivorus conanti* contain exclusively 2,3-linked sialic acid. The glycoproteins in *Bitis arietans arietans, Crotalus adamanteus, Crotalus atra, Bungarus fasciatus* contain both 2,3- and 2,6-linked sialic acids as well as β-galactosyl residues.

Venoms from three types of cobras, *Naja naja naja, Naja naja kaouthia* and *Naja nivea,* were fractionated on a Bio-Gel A-0.5m column. In each venom, a high molecular weight glycoprotein fraction, containing >50% carbohydrate by weight, was eluted at the excluded volume. Carbohydrate compositional analysis indicated that these heavily glycosylated proteins contain a high proportion of galactose and *N*-acetylglucosamine in approximately equimolar ratio, and relatively lower proportion of fucose, mannose and *N*-acetylgalactosamine (Figure 6). Preliminary studies suggest that these glycoproteins contain, in addition to *N*-linked saccharides, extended *O*-linked poly-*N*-acetyllactosaminyl chains which are variably substituted with fucose depending on the cobra species (data not shown). Methylation analysis demonstrated that the poly-*N*-acetyllactosaminyl chains obtained from the venom glycoproteins of *Naja naja kaouthia* are extensively substituted with fucose by 1,3-glycosidic linkage to *N*-acetylglucosamine residues.

The biological function of poly-*N*-acetyllactosaminyl oligosaccharides in glycoproteins of various animal cells and tissues is not understood at present. Poly-*N*-acetyllactosaminyl chains are usually found in membrane bound proteins (21-26) and in extracellular matrix-associated proteins of tumor cells such as laminin from embryonic fibronectin (27), EHS murine tumor (28) and thyroglobulin from transformed thyroid cells

(29). The occurrence of such chains in secreted glycoproteins of cobra venom gland is unusual.

In summary, the data demonstrate that several proteins in snake venom proteins are glycosylated in a species specific manner and appear to contain oligosaccharides with novel and diverse structures. The detailed structures of these carbohydrate moieties and their roles in determining the biological activities of various pharmacologically active snake venom glycoproteins remain to be investigated.

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