

Structure of carbohydrate chain of a thrombin-like protease from the venom of *Agkistrodon halys breviceaudus stejneger* snake*

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Abstract

The structure of the carbohydrate chain of kangshuanmei, a thrombin-like serine protease isolated from *Agkistrodon halys breviceaudus stejneger* snake venom, was determined. The carbohydrate content of the kangshuanmei was 18%. The sugar composition was analyzed by the acid hydrolysis followed by aminobenzoic ethyl ester labeling. Galactose, N-acetylglucosamin, mannose, and fucose were detected, indicating that the binding carbohydrate chain is asparagine-linked type oligosaccharides. N-Acetylneuraminic acid located at non-reduced terminal of the carbohydrate chain was identified by neuraminidase digestion. The carbohydrate chain moiety was separated from kangshuanmei by hydrazinolysis treatment followed by aminobenzoic octyl ester (ABOE) labeling. The isolated ABOE-modified carbohydrate chain was compared to the asparagine-linked type standard oligosaccharides. The carbohydrate chains were consisted of sialylated bi(39.4%)-, tri(50.4%)- and tetra(10.2%)-antennary lactosamins complex containing fucose. The structure of the conjugated carbohydrate chain of kangshuanmei was significantly different from that of thrombin, which has a bisected antennary structure of oligosaccharide.

Key words: snake venom, thrombin-like protease, carbohydrate

Introduction

We isolated a thrombin-like serine protease, kangshuanmei from the venom of *Agkistrodon halys breviceaudus stejneger* snake¹⁾. The protease converts fibrinogen to fibrin, while the protease also has a characteristic distinct from thrombin. The protease was found to be a single chain glycoprotein

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with a molecular weight 32 kDa. N-Terminal amino acid sequence of this protease revealed homology to the other thrombin-like enzyme derived from Viperlidae and Crotalidae snakes. The conjugated carbohydrate chain structure of kangshuanmei remains to be unknown. We attempted to determine the structure of the carbohydrate chain in this report.

Materials and Methods

Chemicals

Aminobenzoic ethyl ester (ABEE) and aminobenzoic octyl ester (ABOE) was purchased from Honen, Japan. N-Acetylneuraminic acid (NeuNAc) and N-glycolylneuraminic acid (NeuGc) were obtained from Sigma, USA. Standard monosaccharides, galactose (Gal), N-acetylgalactosamin (GalNAc), N-acetylglucosamin (GlcNAc), mannose (Man), N-acetylmannosamin (ManNAc) and fucose (Fuc) were purchased from Honen, Japan. All other chemicals were analytical grade commercially available.

Purification of kangshuanmei

Kangshuanmei was purified from the venom of *Agkistrodon halys brevicaudus stejneger* snake according to the method of Zhang¹⁾.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

To estimate the molecular weight of kangshuanmei and glycosidase treated kangshuanmei, SDS-PAGE was carried out under the reducing condition according to Laemmli²⁾. Gradient concentration of gel (4-20%) was used. Molecular weight standard markers (phosphorylase b: 97.4 kDa, bovine serum albumin: 66.2 kDa, aldolase: 39.2 kDa, triose phosphate isomerase: 26.6 kDa, trypsin inhibitor: 21.5 kDa, lysozyme: 14.4 kDa), Roche Diagnostics, Germany were used for calibration. After running the electrophoresis, the gel staining was carried out using a Coomassie brilliant blue and the gel was photographed.

Determination of molecular mass

The molecular mass of kangshuanmei and glycosidase-treated kangshuanmei was determined by using a matrix assisted laser desorption ionization/time of flight type mass spectrometry (MALDI-TOF/MS), model Voyager Elite, PerSeptive Inc, USA. Calibration of the mass was performed with myoglobin ($[M+H]^+$: 16,951.7) and bovine serum albumin ($[M+H]^+$: 66,428.4). A saturated solution (10 mg/ml) of sinapinic acid in 2:1 purified water/acetonitrile containing 0.1% trifluoroacetic acid (TFA) was prepared for matrix solution. The lyophilized sample was dissolved with 54 μ l of 50% acetonitrile. The sample solution (0.5 μ l) and the matrix solution (0.5 μ l) were mixed and the sample was fixed on a sample target plate.

Acid hydrolysis of oligosaccharide of kangshuanmei

The kangshuanmei, 5 μg placed in tapered glass tube (45 \times 9.5 mm id) fitted with a screw cap for sealing, was evaporated to dryness under reduced pressure with a centrifugal concentrator, CC-100, TOMY, Japan and a linked vacuum pump. N-Acetylneuraminic acid aldolase (5 munits/ μl) was dissolved in 20 mM sodium phosphate buffer at pH 7.3. Sialidase (1 munits/ μl) was dissolved in 10 mM sodium phosphate buffer at pH 7.3. In order to release the sialic acid residues, 5 μl of N-acetylneuraminic acid aldolase solution, 2 μl of sialidase solution, and 3 μl of 83 mM sodium acetate buffer at pH 5.0 were added the dried kangshuanmei, and the reaction mixture was incubated at 45 $^{\circ}\text{C}$ for 1 hr. Ten microliters of 8 M TFA solution was added to the reaction mixture that had been previously treated with, and the reaction mixture was incubated at 100 $^{\circ}\text{C}$ for 3 hr. After being cooled to room temperature, the reaction mixture was evaporated to dryness under reduced pressure. The residue was dissolved in 40 μl of 2-propanol and again evaporated to remove residual TFA.

Acetylation of monosaccharides

The mixture of monosaccharides was subjected to N-acetylation reaction. The residue was treated with 40 μl of pyridine/methanol (5/95, v/v) and 10 μl of acetic anhydride at 25 $^{\circ}\text{C}$ for 30 min. The reaction mixture was evaporated to dryness.

ABEE labeling of monosaccharides

The monosaccharides released from kangshuanmei were converted at their reducing end with ABEE. Ten microliters of purified water and 40 μl of the ABEE reagent were added to the reaction residues. The mixture was vortexed and the incubated at 80 $^{\circ}\text{C}$ for 60 min. After being cooled to room temperature, 200 μl of the purified water and equal volume of distilled chloroform were added to the reaction mixture. After vigorous vortexing, the mixture was centrifuged, and the aqueous phase was subjected to the reverse-phase high-performance liquid chromatography (RPLC) analysis. The ABEE-modification of the internal standard monosaccharides was simultaneously carried out and applied to RPLC.

Analytical RPLC procedure

The sample for RPLC was filtrated using a 0.45 μm filter and then applied to a RPLC system, Waters 600E, Waters, USA. The ABEE-modified monosaccharides were analyzed according to the HPLC conditions of Yasuno³⁾ and Honda⁴⁾ with a slight modifications. An octadodesyl silan column (75 mm \times 4.6 mm id), Honenpak C18 column, Honen, Japan was used. The ABEE-modified monosaccharides were injected into the column at a flow rate of 1.0 ml/min at 30 $^{\circ}\text{C}$ of the oven temperature and detected by fluorescence monitoring, excitation at 305 nm and emission at 360 nm, with a Scanning Fluorescence Detector, model 474, Waters USA. Solvent A was 0.2 M potassium borate buffer at pH

8.9 containing 7% acetonitrile. Solvent B was 0.02% TFA solution containing 50% acetonitrile. Solvent A was used for 50 min for analytical separation, and solvent B was then passed through the column for 5 min for washing. In order to analyze the ABOE-saccharides, the following solvents A and B were used. Solvent A was 0.1 M ammonium acetate buffer at pH 4.0/acetonitrile (75/25), and solvent B was the same buffer/acetonitrile (55/45). The following gradient was used: a 10 min isocratic hold at 10% solvent B followed by a linear gradient to 80% solvent B over 40 min. The column oven temperature was at 45°C. The flow rate and the fluorescence monitoring were kept under the same condition of the ABEE-sugar analysis. Chromatograms were processed by Millennium 32 chromatography Manager, Waters, USA.

Sialic acid analysis

The identification of sialic acids at non-reduced terminal was carried out similarly in an above-mentioned manner of the ABEE-sugar analysis. The acid hydrolysis and the N-acetylation were omitted.

ABOE labeling of oligosaccharides

The carbohydrate chain moiety from kangshuanmei was released by Hydraclub® model 206, Honen, Japan. The released oligosaccharides was placed in test tube (13×100 mm, id) with a screw cap, and lyophilized. The dry residues were dissolved by 10 μ l of purified water, and 40 μ l of ABOE labeling reagent was added to the solution. The reaction mixture was incubated at 80°C for 30 min. After being cooled to room temperature, 1 ml of purified water and equal volume of distilled chloroform were added to the reaction mixture. After vigorous vortexing, the reaction solution was centrifuged and the water phase was obtained. The triple extractions with 1 ml of water were carried out, the water phases were combined and concentrated. The concentrated extract was applied to RPLC analysis.

Results and Discussion

Sugar content of kangshuanmei

Molecular weight estimation of kangshuanmei and glycosidase-treated kangshuanmei was performed by SDS-PAGE. The result was shown in Fig 1. Accurate difference of the molecular mass between intact- and treated-kangshuanmei was measured by MALDI/TOF mass spectrometer. The molecular mass of the intact kangshuanmei and the deglycosylated derivative was 32,278 and 26,476, respectively. The molecular mass difference both the proteases was 5,802. These results indicate that the sugar content of kangshuanmei was 18.0%.

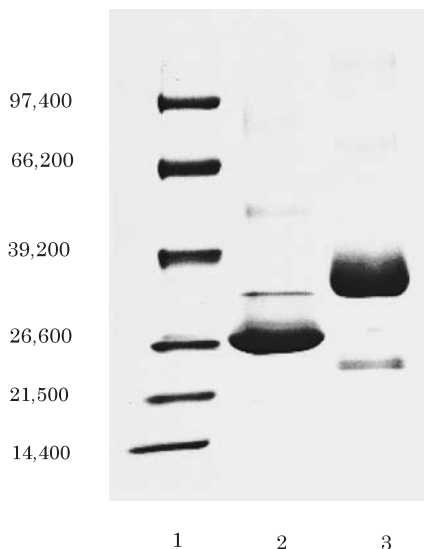


Fig 1 SDS-PAGE of kangshuanmei

Lane 1: molecular weight standard markers. The molecular weight markers used were phosphorylase b: 97.4 kDa, bovine serum albumin: 66.2 kDa, aldolase: 39.2 kDa, triose phosphate isomerase: 26.6 kDa, trypsin inhibitor: 21.5 kDa, lysozyme: 14.4 kDa. Lane 2: glycosidase-treated kangshuanmei. Lane 3: isolated kangshuanmei.

Table 1 Sugar composition of kangshuanmei

carbohydrate	Gal	Man	ManNAc	GlcNAc	Fuc	GalNAc
<i>p</i> moles	222.8	209	227.8	549.6	101.4	n.d.
molar ratio	3.2	3	3.3	7.8	1.4	—

Gal, D-galactose; Man, D-mannose; GlcNAc, N-acetyl-D-glucosamine; GalNAc, N-acetyl-D-galactosamine; ManNAc, N-acetyl-D-mannosamine; Fuc, L-fucose. n.d. indicates not detected.

Sugar composition of kangshuanmei

Carbohydrate moiety of kangshuanmei was hydrolyzed by TFA. The resulting monosaccharides were modified with ABEE and were applied to RPLC analysis. The result of the sugar composition is shown in Table 1. Gal, Man and GlcNAc were detected, indicating that the carbohydrate chain is asparagine binding type oligosaccharide. ManNAc was occurred coincidentally in the reaction, suggesting that sialic acids such as NeuAc or NeuGc were conjugated at non-reducing terminal of the carbohydrate chain. Fuc was also detected, indicating that the carbohydrate chain structure of kangshuanmei distinct from that of thrombin which did not contain Fuc. GalNAc was not detected, showing that O-linked sugar chain was not contained.

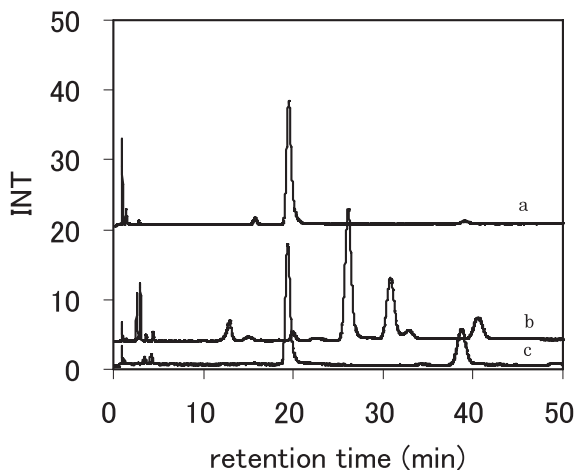


Fig 2 RPLC profile of the ABEE-modified sialic acids
 Chromatograms of a, b and c represent the sialic acid released from kangshuanmei,
 N-glycolylneuraminic acid and N-acetylneuraminic acid, respectively.

Analysis of sialic acid

Analysis of sialic acid was also performed. The release of sialic acids was carried out similarly with sialic acid aldolase, and the free sialic acid residue was released. The released sialic acids were modified by ABEE reagent, and were subjected to the RPLC analysis. The profile of RPLC was shown in Fig 2. The result indicates that N-acetylneuraminic acid is located at the non-reducing terminal of the carbohydrate chain of kangshuanmei.

Carbohydrate chain structure of kangshuanmei

The result of the sugar composition analysis indicates that the conjugating carbohydrate chain was asparagine binding type oligosaccharide. The carbohydrate chain moiety was isolated with hydrazinolysis followed by ABOE labeling, and was subjected to the RPLC analysis. The profile of the RPLC is shown in Fig 3. The carbohydrate chain was consistent with bi(39.4%)-, tri(50.4%)- and tetra(10.2%)-antennary lactosamins complex containing Fuc (Fig 4).

Tanaka⁵⁾ reported that the structure of the carbohydrate chain of batroxobin isolated from the venom of *Agkistrodon atrox moojeni* snake. The carbohydrate chain contained a novel linkage, NeuAc α 2 \rightarrow 3GalNAc. In the sugar composition analysis of kangshuanmei, the GalNAc residue was not contained. The Fuc residue was detected, indicating that the structure of carbohydrate chain is different from that of thrombin, which have not involved Fuc residue. Furthermore the branched chain structure of carbohydrate was different from that of thrombin. The carbohydrate chain structure of thrombin is typical bi-antennary oligosaccharides⁶⁾. The structure of kangshuanmei was a complex of bi-, tri-, tetra-antennary oligosaccharides. NeuAc residues were located at the non-reduced terminal of the branched chain of kangshuanmei. From these findings, the glycosylated sites of kangshuanmei and

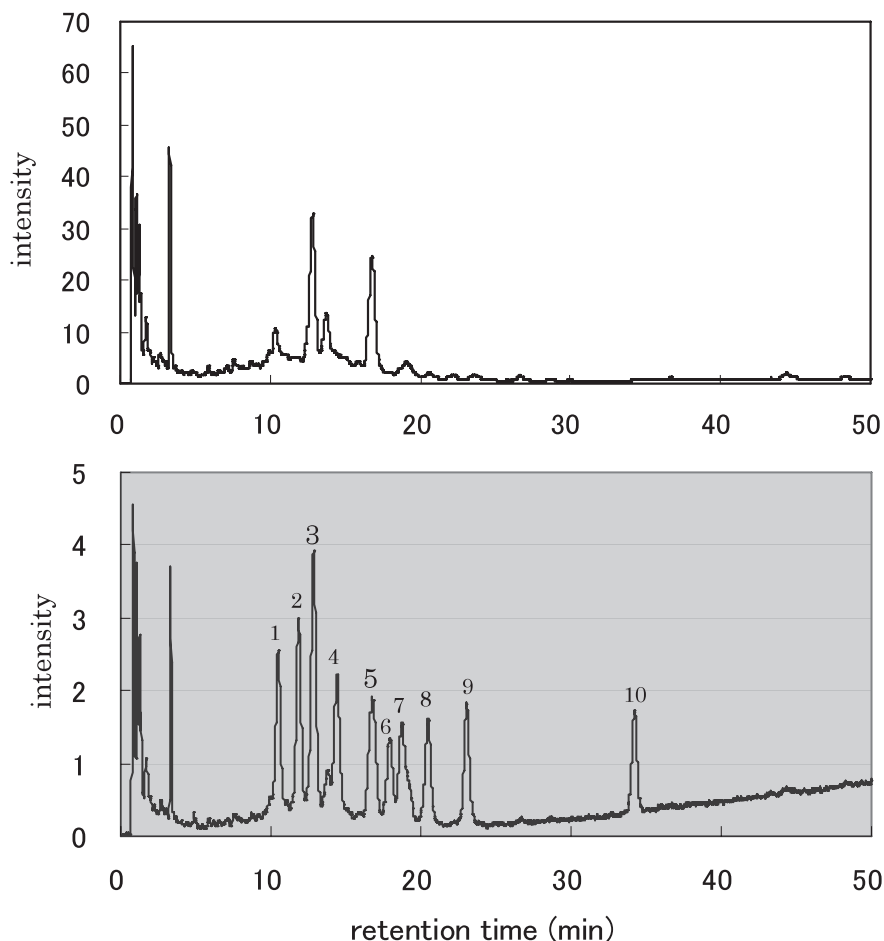
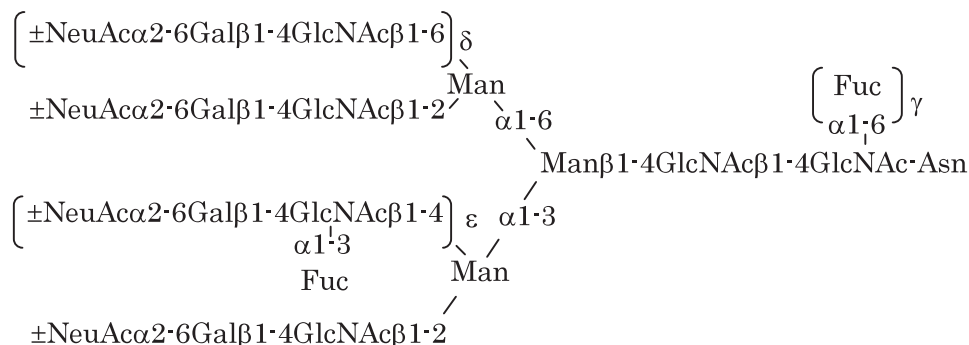


Fig 3 RPLC profile of ABOE-modified oligosaccharides

Upper panel indicates oligosaccharide of kangshuanmei. Lower panel indicates standard markers of oligosaccharide (peak 1, tetra-antennary; peak 3, tri-antennary; peak 5, bi-antennary).

its marginal residues may be exposed to the lateral surface of the protease molecule. Tanaka et al have described that the removal of oligosaccharide of batroxobin reduced its clotting activity considerably. Nikai et al have isolated a coagulant enzyme, bilineobin, from *Agkistrodon bilineatus* venom⁷. They have described that glycosidase F-treated bilineobin released fibrinopeptide A more than the native enzyme. Our preliminary study have demonstrated that deglycosylation of kangshuanmei induced the reduced release of fibrinopeptide B and B β 1-42⁸. These reports suggest that the carbohydrate chain moiety has an important implication for the enzymatic property, and the linkaged site of the carbohydrate chain may be near the catalytic center. In fact, the glycosylated site of thrombin is at Asn53 that located near the hydrophobic pocket of its active site cleft⁹. We recently analyzed the amino acid sequence of kangshuanmei¹⁰. The glycosylated sites are at Asn81, 99, 148 and 229. Three



bisected type	content (%)	γ	δ	ϵ
bi- antennary	39.4	+	—	—
tri- a.	50.4	—	—	+
tetra- a.	10.2	—	+	+

Fig 4 The structure of the carbohydrate chain of kangshuanmei

sites of them are located near the residues that constructed the catalytic center. The precise role of the carbohydrate chain of kangshuanmei remains unknown, however, the carbohydrate chain must be involved in characteristics of the protease activity.

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