The Carbohydrate Moiety of Human Urinary Kallikrein

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Highly purified human urinary kallikrein (HUK) [EC 3.4.21.35] was subjected to hydrazinolysis in order to release the carbohydrate moieties. The released oligosaccharides were N-acetylated with acetic anhydride and reduced with sodium borohydride (NaB^{3}H$_4$). The radioactive oligosaccharides were applied to a column of Hitachi Custom Resin #2630. One neutral (N) and five acidic (AI-AV) peaks were observed, indicating that the HUK was heterogeneous as regards the charge of the oligosaccharide moieties. Sialic acid residues of tritium-labelled AI and AII oligosaccharides were separately removed by neuraminidase treatment, and the asialo oligosaccharides of AI and AII thus obtained were separated by successive chromatographies on columns of Bio-Gel P-4, concanavalin A-Sepharose 4B and Ricinus communis agglutinin-Sepharose 4B, and their structures were investigated by a combination of endo- and exo-glycosidase digestions. Tritium labelled asialo-oligosaccharides AI and AII were each applied to a column of Bio-Gel P-4, and several peaks (AI-1, AI-2 and AI-3 from AI, and AII-1, AII-2 and AII-3 from AII) were obtained. The main asialo-oligosaccharides, AI-2 and AII-2, were each digested with a mixture of β-galactosidase and β-N-acetylhexosaminidase, and yielded small oligosaccharides having the structures αMan$_2$-βMan-βGlcNAc-GlcNAc and αMan$_2$-βMan-βGlcNAc-(Fuc-)GlcNac, respectively. These results indicate that a major portion of the HUK oligosaccharide has a common core structure, and that heterogeneity arises from 1) variation of the charge of the oligosaccharides, 2) variation of the molecular weights of the oligosaccharides.

Keywords——human urinary kallikrein; glycoprotein; carbohydrate moiety; sialic acid; glycosidase digestion; concanavalin A-Sepharose 4B; Ricinus communis agglutinin-Sepharose 4B

From the viewpoint of the physiological function of the carbohydrate moieties of glycoproteins, there is currently much interest in glycoprotein hormones[1,2] and glycoprotein enzymes.[3-5] Recentry, new techniques in enzyme chemistry have resulted in the separation of glundular kallikreins, such as human urinary,[6,7] hog pancreatic,[8] rat submandibular[9,10] kallikreins, into heterogeneous and further into micro-heterogeneous forms. In our previous papers,[11-14] the carbohydrate contents in heterogeneous components of hog pancreatic kallikrein were analyzed and a possible role of the sialic acid residues was discussed. Differences of carbohydrate compositions within these multiple components were also observed and discussed. However, the carbohydrate structures and the functional role of the carbohydrate moieties of human urinary kallikrein (HUK) have never been clarified. In this paper, a preliminary characterization of the primary structures of the carbohydrate moieties of HUK is described.

Materials and Methods

Purification of HUK——HUK was purified according to our previously described method[7] (with minor modifications) from crude urinary protein fractions kindly supplied by Dr. E. Sako of Green Cross Corp., Osaka, Japan.

The purified enzyme was analyzed by electrophoresis. Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate[15] was performed in 10%(w/v) gel at pH 7.0, for 6 h at 2 mA/tube. The

gels were stained for protein with Coomassie Brilliant Blue R-250 or for sugar with periodate-Schiff reagent.[16]

Isolation of Oligosaccharides from HUK——The oligosaccharides were released from the purified HUK by hydrazinolysis according to the method of Fukuda et al.[17] Dried purified HUK (10 mg) was heated at 105°C for 4 h in a sealed, evacuated tube with 2 ml of freshly distilled anhydrous hydrazine containing 20 mg of dried hydrazine sulfate. After evaporation of the hydrazine *in vacuo*, the residue was dried over conc. H_2SO_4 in a vacuum desiccator and then dissolved in a small amount of 4.5 M sodium acetate solution. The oligosaccharides were N-acetylated by the method of Spiro.[18] N-Acetylated oligosaccharides were then isolated by subjecting the reaction mixture to gel chromatography on a column (1.5 × 100 cm) of Sephadex G-25 at room temperature.

Radioactive Labelling of Oligosaccharides——Tritium labelling of oligosaccharides with NaB^3H_4 was performed according to Mizuochi et al.[19] About 400 μg of oligosaccharides was reduced with 10 μmol (2.5 mCi) of NaB^3H_4 (New England Nuclear, Boston, Mass) in 100 μl of 0.01 M NaOH at 25°C for 2 h. Unlabelled $NaBH_4$ (5 mg) was then added and the reaction was allowed to continue at 25°C for another 1 h. The reaction was stopped by acidifying the reaction mixture by passing it through Dowex 50W × 8 (H+ form). The remaining borate was removed by repeated evaporation with methanol. Radioactive contaminants in the NaB^3H_4 reagent were removed by descending paper chromatography for 16 h on Whatman No. 1 filter paper with ethyl acetate/acetic acid/formic acid/water (18: 3: 1: 4, by volume) as a solvent.[20] The area of the radioactive sample was detected with a radiochromatogram scanner (Packard model 7220). The radioactive material remaining at the origin was eluted with water.

Fractionation of Oligosaccharides——NaB^3H_4-reduced oligosaccharides were subjected to ion-exchange chromatography on a Hitachi Custom Resin #2630 column (4.6 × 250 mm) equilibrated with water at a flow rate of 0.5 ml/min using a high-pressure liquid chromatograph (Jasco Tri-rotor; Japan Spectroscopic Co., Tokyo, Japan). The column was washed with water and after unbound oligosaccharides had been eluted, acidic oligosaccharides were eluted with a concave gradient of NaCl (0—250 mM). The oligosaccharides were further fractionated by gel-permeation chromatography on Bio-Gel P-4 and affinity chromatography on concanavalin A- and *Ricinus communis* agglutinin-Sepharose 4B.

Gel-permeation Chromatography——Gel-permeation chromatography was carried out according to the method of Irimura et al.[21] Bio-Gel P-4 (−400 mesh) column chromatography was performed on a high-pressure liquid chromatograph (0.8 × 100 cm) column at a flow rate of 0.3 ml/min. During the produced, the column was maintained at 55°C by means of a water jacket. A mixture of oligomers of N-acetylglucosamine obtained by the method of Rupley[22] and the unit B-type oligosaccharides of porcine thyroglobulin[23] were used as standards. Sugars were detected with a UV spectrophotometer (Uvidec 100-II; Jasco, Tokyo, Japan) and a liquid scintillation spectrometer (LSC-700; Aloka Corp., Tokyo, Japan).

Affinity Chromatography with Immobilized Lectins——Concanavalin A and *Ricinus communis* agglutinin were purified according to Agrawal and Goldstein[24] and Tomita et al.,[25] respectively. These lectins were coupled to carboxyl-Sepharose 4B prepared by the method of Matsumoto et al.[26] For concanavalin A-Sepharose 4B column chromatography, the column was eluted first with 0.01 M sodium acetate buffer (pH 6.0) containing 0.15 M NaCl, 1 mM $CaCl_2$ and 1 mM $MnCl_2$ after application of the samples. The absorbed oligosaccharides were eluted with the same buffer containing 10 mM or 100 mM methyl-α-mannoside. For *Ricinus communis* agglutinin-Sepharose 4B column chromatography, elution was performed first with 0.1 M sodium phosphate buffer (pH 7.3) containing 0.15 M NaCl and then with the same buffer containing 0.1 M lactose.

Treatment of Glycosidases——$β$-Galactosidase and $β$-N-acetylhexosaminidase were purified from jackbean meal by the method of Li and Li.[27] Neuraminidase from *Arthrobacter ureafaciences* and endo-$β$-N-acetylglucosaminidase D from *Diplococcus pneumoniae* were purchased from Nakarai Chemicals, Ltd. (Kyoto Japan) and Seikagaku Kogyo Co. (Tokyo, Japan), respectively.

NaB^3H_4-reduced oligosaccharides (about 4—5 × 10³ dpm) were digested at 37°C with glycosidases (0.1—0.5 units) in 0.1 ml of the appropriate buffer under a toluene layer for 24—48 h followed by heating at 100°C for 3 min to stop the reaction. Then the reaction mixture was passed through small columns of Dowex 50W × 8 (H+ form) and Bio-Rad AG3 (OH− form) and analyzed by gel-permeation chromatography on a Bio-Gel P-4 column. For digestion with neuraminidase, 10 mM sodium phosphate buffer (pH 6.8) was used. $β$-Galactosidase plus $β$-N-acetylhexosaminidase digestion was carried out in 50 mM sodium acetate buffer (pH 4.0). Endo-$β$-N-acetylglucosaminidase D digestion was done in 0.1 M sodium citrate/0.1 M sodium phosphate buffer (pH 6.0).

Results and Discussion

Purification of HUK

The purified kallikrein, isolated from human urine, was homogeneous as judged from the results of the last chromatography, *i.e.* Sephacryl S-200 gel chromatography. It gave a single band on sodium dodecyl sulfate polyacrylamide-gel electrophoresis when stained for
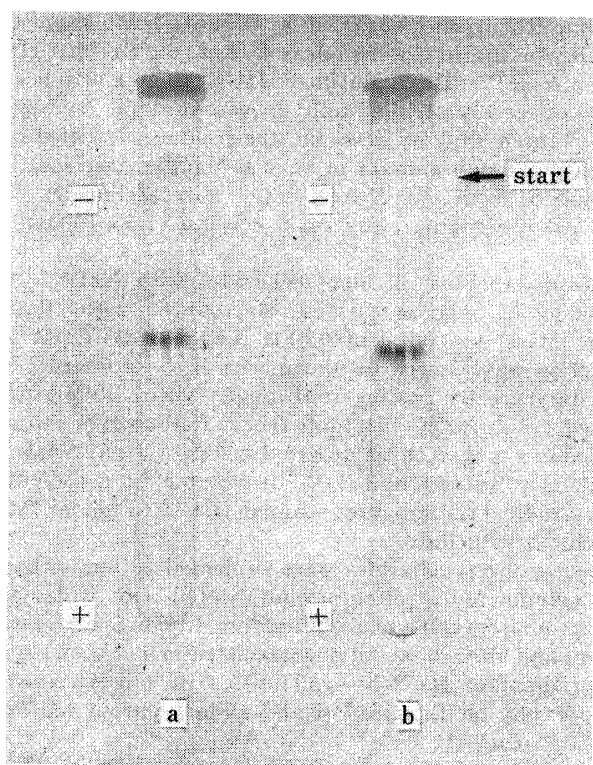
Fig. 1. Electrophoretograms of HUK on Sodium
Dodecyl Sulfate Polyacrylamide-Gel

(a) and (b): HUK (10 μg and 150 μg, respectively).
After electrophoresis, gels a and b were stained with
0.1 % (w/v) Coomassie Brilliant Blue R-250 and
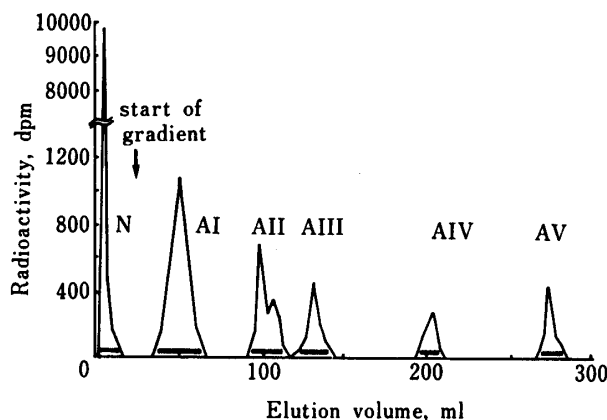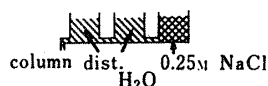periodate-Schiff reagent, respectively.

Fig. 2. High-Pressure Liquid Chromatography
of ³H-Labelled Oligosaccharides from HUK on
a Hitachi Custom Resin #2630 Column

Experimental details are given in the text. The radioactiv-
ity was counted with a liquid scintillation spectrometer.
Fractions were combined as indicated by bars.

Column: Hitachi Custom Resin #2630 φ4.6×250 mm.
Flow rate: 0.5 ml/min.
Pressure: 10 kg/cm².
Elution method (Each Chamber: 120 ml)

column dist. 0.25M NaCl
H₂O

protein with Coomassie Brilliant Blue
R-250 and for sugar with periodate-Schiff
reagent (Fig. 1, a and b, respectively).

Fractionation and Characterization of Oligosaccharides of HUK

Oligosaccharides were liberated from
HUK by hydrazinolysis followed by N-
acetylation with acetic anhydride and
reduction with NaB^3H_4 (for details, see
"Materials and Methods"). When triti-
um-labelled oligosaccharides were subject-
ed to high-pressure liquid chromatography
on Hitachi Custom Resin # 2630, six major
oligosaccharide fractions were detected
(Fig. 2). These peaks, designated N, AI,
AII, AIII, AIV and AV, were pooled as
indicated in Fig. 2. About 40% of oligo-
saccharides (fraction N) was recovered
in the pass-through fraction, and these
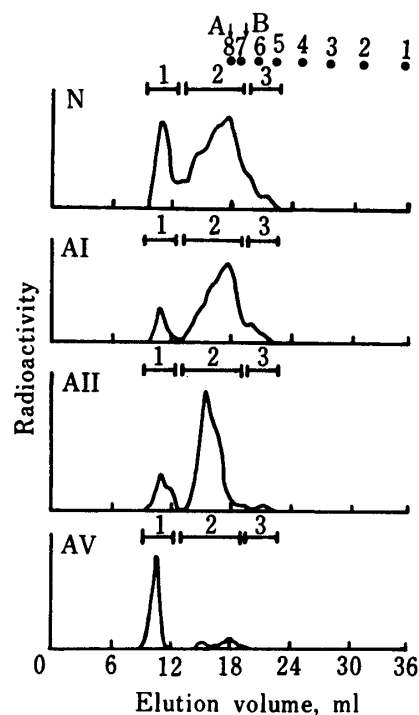were presumed to be neutral oligosac-

Fig. 3. Gel-Permeation Chromatography
of N and Asialo-AI, -AII and -AV on
a Bio-Gel P-4 Column

Experimental details are given in the text. Dots
(1 to 8) indicate the elution positions of authentic
N-acetylglucosamine oligomers and the numbers
indicate the number of N-acetylglucosamine units.
A and B indicate the elution positions of authentic
oligosaccharide-alditol standards prepared from
porcine thyroglobulin: A, Gal-GlcNAc-(Gal-
GlcNAc-)Man-(Gal-GlcNAc-Man-)Man-GlcNAc-
(Fuc-)GlcNAcol; B,Gal-GlcNAc-Man-(Gal-Glc-
NAc-Man-)Man-GlcNAc-(Fuc-)GlcNAcol.

charides, *i.e.* high mannose-type oligosaccharide, and/or complex-type oligosaccharide free of sialic acid. On the other hand, acidic fractions AI, AII, AIII, AIV and AV, which were eluted with a concave concentration gradient of NaCl, were found to be acidic oligosaccharides containing sialic acid, because these oligosaccharides lost their acidity after treatment with neuraminidase.

It has been suggested that the acidities of micro-heterogeneous forms of hog pancreatic kallikreins A and B depend on the amount of sialic acid.[11-14] As in the case of hog pancreatic kallikrein, HUK migrated as a rather broad band on polyacrylamide-gel electrophoresis and was separated into several heterogeneous forms on isoelectric focusing.[7] The possibility exists that the electrophoretic behavior of HUK depends on the heterogeneity of the carbohydrate moieties of HUK. Each fraction was pooled as indicated in Fig. 2 and analyzed by gel-permeation chromatography on a Bio-Gel P-4 column (Fig. 3). Each oligosaccharide was detected by radioactivity measurement.

The acidic fractions AI, AII and AV each appeared to be heterogeneous, since gel-permeation chromatography of each fraction on a column of Bio-Gel P-4 after removal of sialic acids gave three or more partially overlapping peaks (Fig. 3). This result suggested that the difference in the elution positions of the peaks on a Bio-Gel P-4 column was due to differences in the molecular weights of these oligosaccharides.

The fractions AI-2 and AII-2 (Fig. 3) were further characterized by affinity chromatography with immobilized lectins. As shown in Fig. 4a, fraction AI-2 was separated into two fractions in approximately equal amounts on a concanavalin A-Sepharose 4B column.
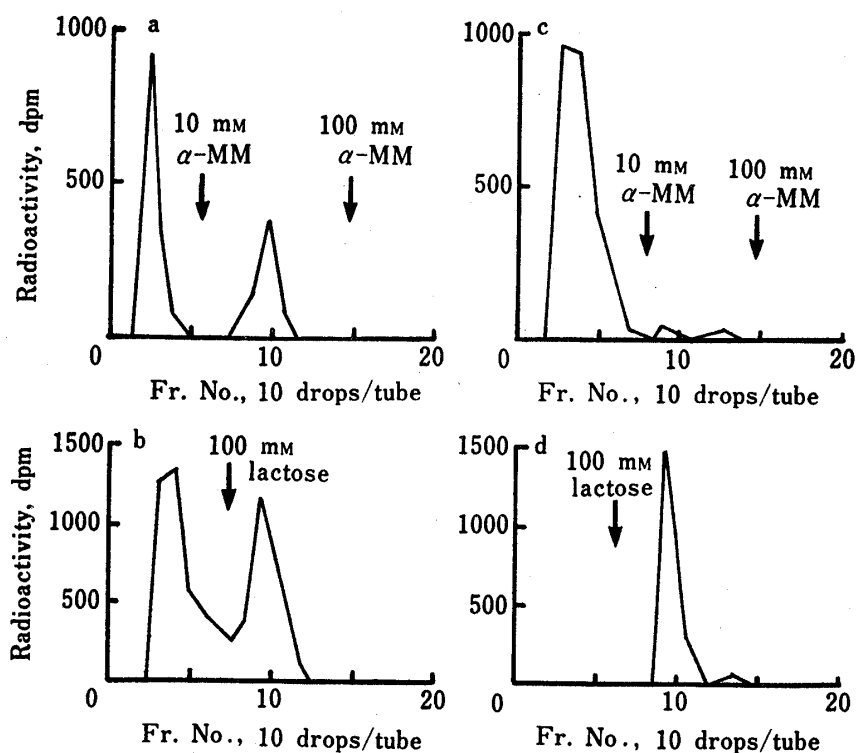

Fig. 4. Behavior of ³H-Labelled Oligosaccharides AI-2 (a and b) and AII-2 (c and d) on Columns of Lectin-Sepharose 4B

Oligosaccharides (about 4—5 × 10³ dpm) were applied to the columns (1.2 × 8 cm), incubated at room temperature for 30 min and then eluted with the appropriate buffer. (a) and (c): a concanavalin A-Sepharose 4B column was equilibrated with 0.01 M sodium acetate buffer (pH 6.0) containing 0.15 M NaCl, and absorbed oligosaccharides were eluted with the same buffer containing 10 mM and 100 mM methyl-α-mannoside (α-MM). (b) and (d): a *Ricinus communis* agglutinin-Sepharose 4B column was eluted first with 0.1 M sodium phosphate buffer (pH 7.3) containing 0.15 M NaCl and then with the same buffer containing 0.1 M lactose.

One groups should be the so-called triantennary sugar chains (unbound oligosaccharides), and the other the so-called biantennary sugar chains (bound oligosaccharides) because they could be separated by concanavalin A-Sepharose 4B chromatography as reported by Ogata et al.[28] and Krusius et al.[29] Triantennary complex-type sugar chains have been found in several glycoproteins; for example, carbohydrate unit B of porcine thyroglobulin,[23] calf fetuin,[30,31] α_1-proteinase inhibitor,[32,33] and oligosaccharide A-2 of human ceruloplasmin.[34] When AII-2 oligosaccharides were applied to a concanavalin A-Sepharose 4B column, all oligosaccharides passed through the column (Fig. 4c). Therefore, fraction AII-2 may consist of triantennary sugar chains. AI-2 and AII-2 were further characterized by means of affinity chromatography using Ricinus communis agglutinin-Sepharose 4B which was shown to have affinity for the Galβ1-4GlcNAc sugar sequence.[35] When ^{3}H-labelled asialo-AI-2 was applied to a column of Ricinus agglutinin-Sepharose 4B, two peaks were observed in approximately equal amounts (Fig. 4b), while fraction AII-2 was completely bound to Ricinus agglutinin-Sepharose 4B and was eluted by 100 mM lactose (Fig. 4d). These results indicate that AII-2 oligosaccharides may have β-galactose residues at the nonreducing termini. It is possible that the unbound oligosaccharides may also have much more terminal or core fucose and N-acetylglucosamine residues than the bound oligosaccharides, as reported by Tsuji et al.[36]

Treatment of AI-2 and AII-2 Oligosaccharides with Glycosidases

In order to determine the sequence of the core oligosaccharides obtained from asialo-AI-2 and -AII-2, the ^{3}H-labelled oligosaccharides were subjected to sequential glycosidase digestion and the reaction mixtures were analyzed by gel-permeation chromatography on a Bio-Gel P-4 column. The results are shown in Fig. 5. Most of AI-2 and AII-2 could be digested with a mixture of β-galactosidase and β-N-acetylhexosaminidase to give small oligosaccharides (Fig. 5b and e). This suggests that AI-2 and AII-2 oligosaccharides have a common structure containing galactose and N-acetylglucosamine residues. These β-galactosidase- and β-N-acetylhexosaminidase-digested AI-2 oligosaccharides were eluted in a major peak at the same elution position as αMan$_2$-βMan-βGlcNAc-GlcNAcol (Fig. 5b), while the elution position of
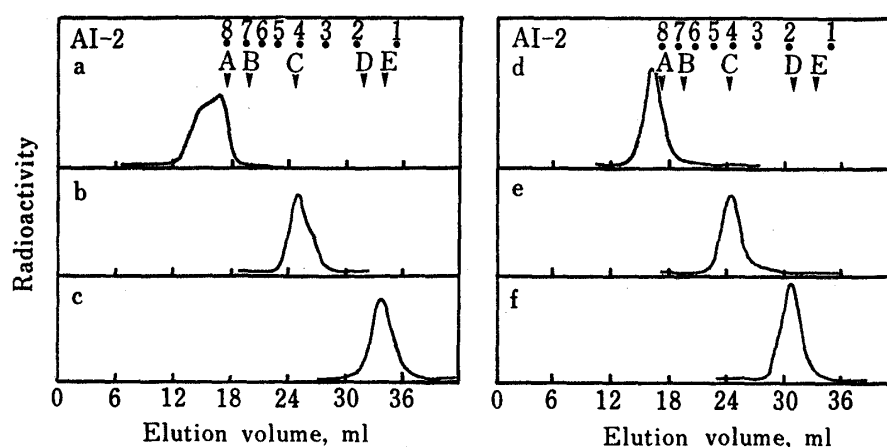

Fig. 5. Elution Profiles of ^{3}H-Labelled Oligosaccharides AI-2 and AII-2 after Glycosidase Digestion

Experimental details are given in the text. Sequential glycosidase treatments of AI-2 and AII-2 were performed separately and the product at each step was analyzed by gel-permeation chromatography on a Bio-Gel P-4 column. (a) and (d): original oligosaccharides AI-2 and AII-2; (b) and(e): products of β-galactosidase and β-N-acetylhexosaminidase treatments of a and d, respectively; (c) and (f): products of endo-β-N-acetylglucosaminidase D treatment of b and e, respectively. A to E indicate the elution positions of authentic standard oligosaccharide-alditols prepared from porcine thyroglobulin unit B: A, Gal-GlcNAc-(Gal-GlcNAc)-Man-(Gal-GlcNAc-Man-)Man-GlcNac-(Fuc-)GlcN-Acol; B, GlcNAc-Man-(GlcNAc-Man-)Man-GlcNAc-(Fuc-)GlcNAcol; C, Man-(Man-)Man-Glc-NAc-(Fuc-)GlcNAcol; D, Fuc-GlcNAcol; E, GlcNAcol. Dots (1—8) indicate the elutionpositions of N-acetylglucosamie noligomers and the numbers indicate the number of N-acetylglucosamine units.

similarly digested AII-2 was found to be the same as that of standard αMan$_2$-βMan-(αFuc-)-GlcNAcol (Fig. 5e). When asialo-AI-2 had previously been digested with a mixture of β-galactosidase and β-N-acetylhexosaminidase and then further with endo-β-N-acetylglucosaminidase D from *Diplococcus pneumoniae*, one major peak was detected at an elution position identical to that of N-acetylglucosaminitol (Fig. 5c). When the fragment of the core oligosaccharide of asialo-AII-2 digested with endo-β-N-acetylglucosaminidase was analyzed by gel-permeation chromatography, it was found to be the same as that of standard α-fucosyl-N-acetylglucosaminitol (Fig. 5f). Hence, asialo-AII-2 seemed to contain a fucosyl residue in the core portion.

From the results described above, the sugar sequence of the asialo fraction of AI-2 is proposed to be (βGal-βGlcNAc-)$_n$-(αMan-)$_2$-βMan-βGlcNAc-GlcNAc and that of the asialo fraction of AII-2 is proposed to be (βGal-βGlcNAc-)$_n$-(αMan-)$_2$-βMan-βGlcNAc-(X-)-GlcNAc, as illustrated in Fig. 6.

AI-2
[SA-](-βGal-βGlcNAc-)$_n$-αMan$_2$-βMan-βGlcNAc-GlcNAc
AII-2   X
                                                                         |
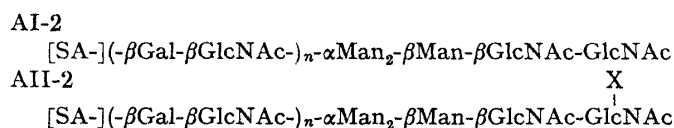[SA-](-βGal-βGlcNAc-)$_n$-αMan$_2$-βMan-βGlcNAc-GlcNAc

Fig. 6.   Proposed Structures of Oligosaccharides of AI-2 and AII-2
SA, sialic acid; n, the number of repeating βgal-βGlcNAc disaccharides or branches.

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