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OCCURRENCE OF Galβ(1→3)GalNAc-Ser/Thr IN THE LINKAGE REGION OF POLYGALACTOSAMINE CONTAINING FUNGAL GLYCOPROTEIN FROM *Cordyceps ophioglossoides*

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The nitrous acid deamination liberated the N-acetyl galactosamine containing glycoproteins from galactosminoglycan (CO-N) which has been isolated from *Cordyceps ophioglossoides* culture. Mild alkaline borohydride degradation of the purified glycoprotein released oligosaccharide alditols. The smallest oligosaccharide alditol was characterized to be Galβ(1→3)-GalNAcol. This result indicated that polygalactosamine part attached to protein part *via* Galβ(1→3)GalNAc-Ser/Thr as the linkage region. To our knowledge, this is the first report of Gal-GalNAc-Ser/Thr linkage from fungal glycoprotein.

C. ophioglossoides is known to synthesize water and alkaline insoluble galactosaminoglycan(CO-N) that is composed predominantly of α(1→4)-linked D-galactosamine units which are N-acetylated to only a small extent(1). CO-N contained small proportion of glucose, galactose and mannose and 3.6% of protein, and the previous reports suggested that the polygalactosamine in CO-N might be linked to protein(1). Fungal mannoprotein is composed of carbohydrate chains attached to the protein core *via* asparagine residues and by base labile O-glycosidic linkages(2). Although it has been reported that several mannooligosaccharides attached to serine or threonine

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Abbreviation: Galβ(1→3)GalNAcol, galactosylβ(1→3)-N-acetyl galactosaminitol.

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residues in the fungal glycoprotein(2,3) no other linkage region has been reported.

Here we describe the linkage region between polygalactosamine and protein part of CO-N from C. ophioglossoides that is composed of Galβ(1→3)GalNAc-Ser/Thr which is a common linkage region occurred in the animal glycoprotein.

MATERIALS AND METHODS

Materials: CO-N was prepared from C. ophioglossoides culture as described previously(1). Concanavalin A(Con A)-Sepharose, DEAE-Sephadex A-25 and Sephadex G-50(fine) were purchased from Pharmacia. Bio-Gel P-2(200-400mesh) and Dowex 1x8 was obtained from Bio-Rad, and SPECTRA/POR6(M.W. cut off;1,000) cellulose tubing from SPECTRUM Medical Industries Inc.(U.S.A.).
General Methods: Colorimetric methods were used for monitoring elution profiles during the chromatography, phenol-H_2SO_4 method(4) for the total hexose and both modified indole-HCl(5) and Elson-Morgan(6) methods for total hexosamines. The contents of amino acids were measured by using the amino acid analyser Hitachi 835 after hydrolysis (6M HCl, 110°, 20h).
Preparation of Galβ(1→3)GalNAcol: The O-glycosidically linked carbohydrate chains of fetuin were prepared according to the the procedure described by Spiro et al.(7). The oligosaccharides were desialylated by mild acid hydrolysis and sialic acids were removed by passing through a column of Dowex 1x8.
Preparation of glycoproteins from CO-N: The polygalactosamine part of CO-N was decomposed according to the deamination procedure described by Yamauchi et el.(8). CO-N(5g) was dissolved in 20% acetic acid(500ml), and 5% aqueous sodium nitrite(500ml) was added to this solution. The reaction mixture was stirred at room temperature for 18h, and then dialysed using SPECTRA/POR6 cellulose tubing against tapping water for 2 days and against distilled water for 3 days at 4°. The non-dialysable portion was lyophilised and the deamination resistant products(360mg) were obtained. For removal of galactomannan in CO-N, the deamination resistant products were dissolved in 50mM sodium phosphate buffer, pH 7.2, containing 0.5M sodium chloride and applied to a column(2.5x 20cm) of Con A-Sepharose equilibrated with buffer. Non-absorbed fractions were collected and dialysed using SPECTRA/POR6 cellulose tubing. The inner portion was collected and lyophilised. This fraction was dissolved in distilled water and applied to a column(2.6x36cm) of DEAE-Sephadex A-25(Cl^-). The glycoprotein fractions were eluted with a linear sodium chloride gradient from 0 to 1.0M. The glycoproteins were then dialysed using SPECTRA/POR6 cellulose tubing and further purified on a column(2.6x98cm) of Sephadex G-50(fine).
Preparation and analyses of oligosaccharides from glycoproteins: The purified glycoproteins were dissolved in 0.1M sodium hydroxide containing 1M sodium borohydride and incubated at 37°. After 48h, the incubataion mixture was neutralised with acetic acid and applied to a column of Sephadex G-50. The fractions which were positive on indole-HCl reaction, were combined and lyophilised. These oligosaccharide alditols

released by alkaline borohydride degradation were applied to a column(2.0x97cm) of Bio-Gel P-2(200-400mesh).
Methylation analyses of oligosaccharide alditols: Oligosaccharide alditols were methylated by Hakomori's method(9), respectively. The products were extracted with chloroform and the permethylated oligosaccharide alditols were subjected to gas-liquid chromatography(g.l.c.). G.l.c. was performed with a Shimadzu GC-R1A gas chromatograph equipped with flame-ionisation detector and a glass column(0.26x 210cm) of 2% silicone OV-17 on Uniport HP at 170-300°(8°/min) and with nitrogen as carrier gas at 60ml/min. G.l.c.-m.s. was performed with a JEOL DX-303 mass spectrometer equipped with a glass column(0.26x100cm) of 2% silicone OV-17 on Uniport HP at 160-280°(8°/min) with helium as carrier gas at 25ml/min and operated at an ionisation voltage 70eV, ionisation current 300 μA with an ion source at 160°.

RESULTS AND DISCUSSION

The nitrous acid deamination completely cleaved galactosaminyl residues in CO-N and its small deaminated fragments were removed by dialysis. The deamination resistant products of which a molecular weight of >1,000 corresponded to 7.2% of CO-N, and composed of N-acetyl galactosamine, mannose, glucose, and galactose and proteins. The products were separated into Con A unbound and bound fractions by Con A-Sepharose. Con A unbound fraction consisted of 1.9% of N-acetyl galactosamine, 64.4% of hexose(mainly glucose), 14.9% of protein whereas Con A bound fraction consisted of galactose and mannose. This result indicated that galactomannan in the products was recovered as the Con A bound fraction. This Con A unabsorbed fraction was further purified by DEAE-Sephadex A-25 chromatography(Fig.1). Unbound fraction(A) consisted of glucose and a small amount of N-acetyl galactosamine. Of the absorbed fraction, B-I consisted of galactose, mannose and protein whereas B- II consisted of 50.6% of protein, 11.6% of N-acetyl galactosamine and 37% of hexose. B- II was further purified by gel filtration on Sephadex G-50 and N-acetyl galactosamine containing glycoprotein was eluted as a wide band (data not

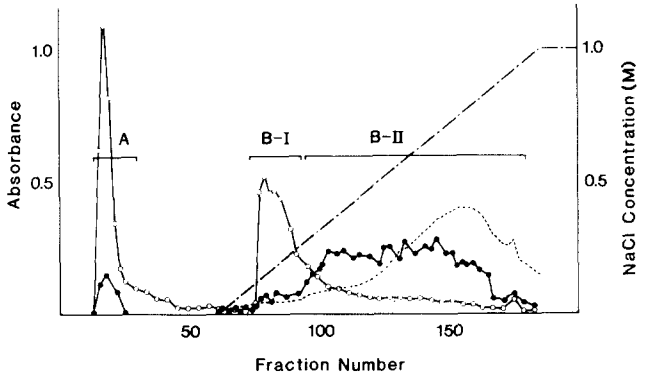

Fig. 1. DEAE-Sephadex chromatography of Con A unbound fraction. (—●—, N-acetyl hexosamine at 492nm; —○—, hexose at 490nm; ·····, at 280nm).

shown). Molecular weight heterogeneity might be arised by different chain length of peptides and carbohydrate moieties.

Mild alkaline borohydride treatment of the glycoprotein released the oligosaccharide alditols and remarkably decreased serine and threonine residues. This treatment also resulted in an increase of alanine, and the appearance of α-amino butylic acid after hydrolysis with 6M HCl(Table I). These results showed that the carbohydrate chains attached to peptides O-glycosidically in the glycoprotein. The oligo-

Table I. Changes of amino acid compositions in the glycoprotein after alkaline borohydride degradation

Amino acid*	Reaction time (hours)			
	0	5	24	48
Aspartic acid	3.9	3.9	4.1	4.1
Glutamic acid	7.4	7.0	7.4	7.2
Leucine	1	1	1	1
Glycine	2.5	2.9	3.0	2.9
Serine	3.3	1.3	1.2	1.0
Threonine	2.2	1.5	1.2	1.0
Alanine	1.6	2.2	2.5	2.6
α-amino butylic acid	0	0	0.1	0.2

*These values are represented as molar ratio to leucine. The other amino acids were essentially unchanged.

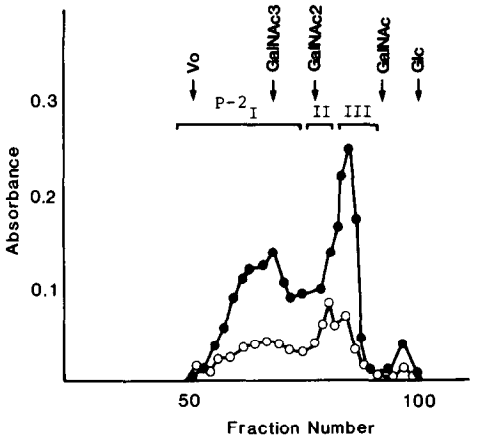

Fig. 2. Gel filtration of oligosaccharide alditols on Bio-Gel P-2. The contents of N-acetyl hexosamine (—●—) was measured by the Elson-Morgan method and hexose (—○—) by phenol-sulfuric acid method. The arrows indicate the elution positions of void volume, α(1→4)GalNAc oligosaccharides, and glucose.

saccharide alditols were purified by gel filtration of Sephadex G-50 and N-acetyl galactosamine containing oligosaccharide alditols were further separated into three fractions (P-$2_{I,II}$ and $_{III}$) by Bio-Gel P-2 gel filtration (Fig.2). Oligosaccharide fractions were methylated and analysed by g.l.c.-m.s. Only methylated P-2_{II} fraction gave peaks on g.l.c. (Fig.3). The mass spectrum of major peak (B) in permethylated P-2_{II} showed a characteristic fragmentation pattern to permethylated hexosyl(1→3)-N-acetyl hexosaminitol and its retention time was agreed with permethylated Galβ(1→3)GalNAcol obtained from fetuin. Both fragmentation patterns in mass spectra were essentially identical. The minor peak (A) was identified as N-unmethylated same hexosyl-N-acetyl hexosaminitol. These results showed that P-2_{II} fraction contained Galβ(1→3)GalNAcol. P-2_{I} fraction was suggested to be highly polymerised oligosaccharide alditols because no peaks was observed by g.l.c. The present results indicated that partially N-acetylated polygalactosamine bound to protein O-glycosidically via Galβ(1→3)GalNAc as the linkage region of CO-N.

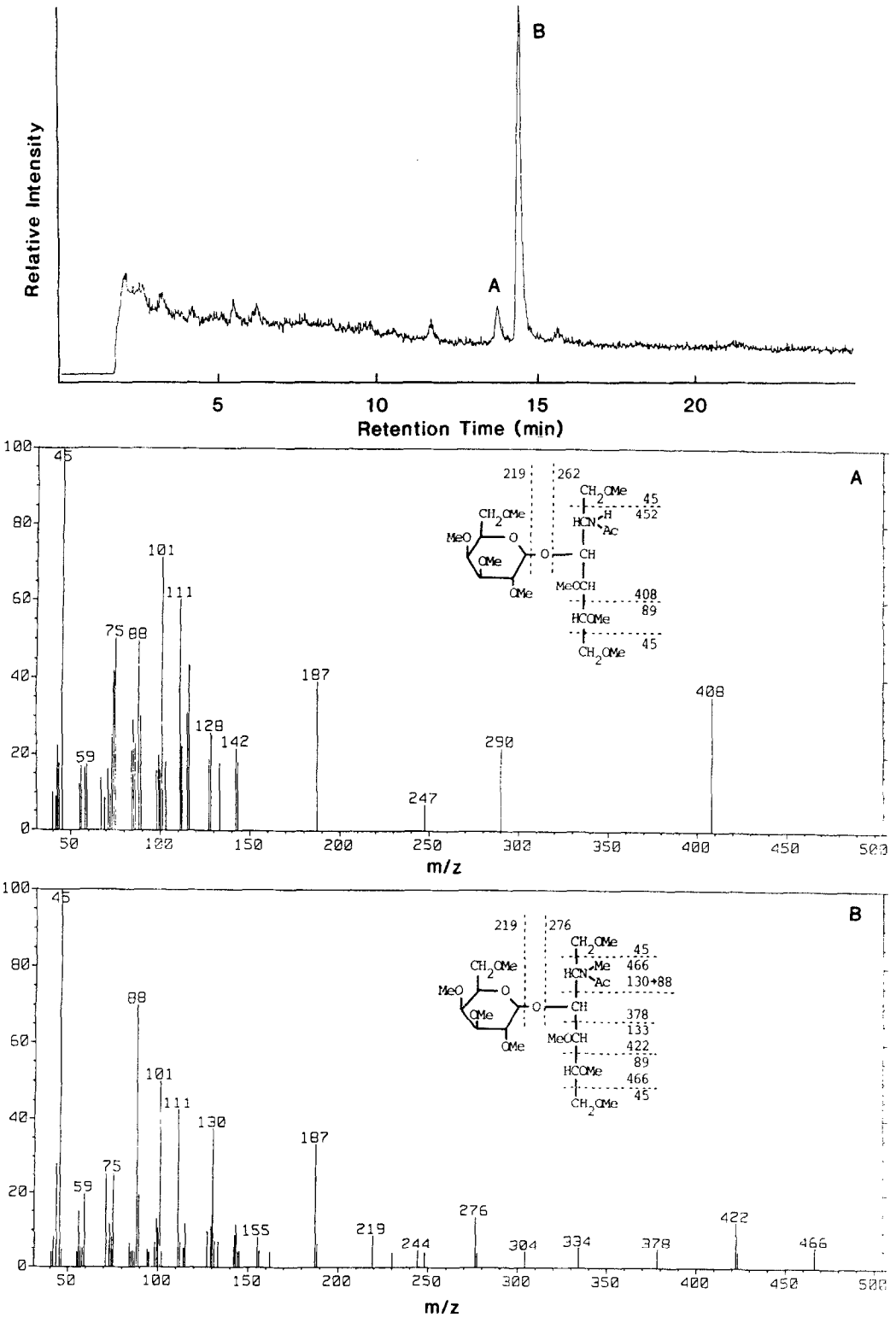

Fig. 3. Gas-liquid chromatogram, mass spectra and fragmentation schemes of permethylated P-2$_{II}$ fraction. The chromatogram and mass spectra were obtained under the condition described in MATERIALS AND METHODS.

Many of O-glycosidically linked carbohydrate chains have been isolatd from the glycoproteins of various sources(10). Gal-GalNAc-Ser/Thr linkage has usually been observed as a common O-glycosidic linkage region of glycoproteins in animal cells, but not in the kingdom of Protista. This is first report showing the existence of Gal-GalNAc-Ser/Thr linkage in fungi.

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