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Novel *N*-linked oligo-mannose type oligosaccharides containing an α-D-galactofuranosyl linkage found in α-D-galactosidase from *Aspergillus niger*

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Structures of oligosaccharides from *Aspergillus niger* α-D-galactosidase [EC 3.2.1.22] were studied. Purified α-D-galactosidase was treated with *N*-glycosidase F, and six kinds of oligosaccharides were isolated by gel chromatography and anion-exchange chromatography. The structures of the oligosaccharides were determined by ^{1}H-NMR and compositional analysis to be $Man_5GlcNAc_2$, $Man_6GlcNAc_2$, $Man_9GlcNAc_2$, $GlcMan_9GlcNAc_2$, $GalMan_4GlcNAc_2$ and $GalMan_5GlcNAc_2$. From mild acid hydrolysis, methylation analysis and ROESY spectral analysis, it was ascertained that the galactosyl residue in two oligosaccharides was in the furanose form and was bound to mannose at the nonreducing end with an α1-2 linkage ($Gal\textit{f}Man_4GlcNAc_2$ and $Gal\textit{f}Man_5GlcNAc_2$).

Keywords: α-D-galactosidase, *N*-linked oligosaccharide, α-D-galactofuranosyl linkage, *Aspergillus niger*

Many kinds of fungi secrete glycosidases in culture broth. Most of these enzymes have been found to be glycoproteins, and the biosynthetic processes and the roles of the sugar chains have been studied extensively. From studies on the invertase from *Saccharomyces cerevisiae*, the relation between secretion and glycosylation has been investigated [1, 2], and, furthermore, the function of the sugar chain has been reported by Reddy *et al.* [3].

Aspergillus niger also secretes various kinds of glycosidases in the culture broth [4]. Some of these enzymes have been reported to be glycoproteins, and the composition of the sugar chains has been studied [5–8]. However, the primary structures of the sugar chains in the glycoenzymes from this mold have not been clarified.

An enzyme product, Transglucosidase Amano, from *A. niger* contains a large amount of α-D-glucosidase. In addition, other glycosidase activities (α- and β-D-galactosidase, β-D-mannosidase, β-D-glucosidase, α-L-fucosidase, *N*-acetyl-β-D-glucosaminidase, and *N*-acetyl-α-D-galactosaminidase) can also be detected.

In the present study, we isolated the α-D-galactosidase (EC 3.2.1.22) from Transglucosidase Amano and investigated the structure of the oligosaccharides of the enzyme.

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Materials and methods

Materials

An enzyme product, Transglucosidase Amano, from *A. niger* was obtained from Amano Pharmaceutical Co., Ltd. and used as a source of α-D-galactosidase. *p*-Nitrophenyl glycosides of various monosaccharides were obtained commercially.

Purification of α-D-galactosidase

A solution of Transglucosidase Amano (2 ml, approximately 150 mg protein) was loaded on a column of Sephacryl S-200 (Pharmacia, 2.6 cm × 90 cm), which had been equilibrated with 20 mM sodium acetate buffer, pH 5.0, containing 0.2 M NaCl, and eluted with the same buffer at a flow rate of 0.2 ml min^{-1}. The fractions containing α-D-galactosidase activity were collected and rechromatographed over the same column. The purified α-D-galactosidase fractions were collected, then concentrated in a pressure cell with a YM-30 membrane (Amicon).

Each glycosidase activity was assayed by using the corresponding *p*-nitrophenyl L- or D-glycopyranoside. A reaction mixture consisting of 0.2 ml 50 mM sodium acetate buffer, pH 5.0, 0.1 ml 5 mM *p*-nitrophenyl L- or D-glycopyranoside, and 0.1 ml enzyme solution, was incubated for 5 min at 37 °C. The reaction was stopped by

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adding 0.4 ml 0.2 M Na_2CO_3 solution and the released *p*-nitrophenol was measured spectrophotometrically.

SDS-PAGE was performed with 4–20% linear gradient acrylamide slab gels [9]. Proteins were electrophoretically transferred from the slab gel to nitrocellulose membranes [10], and stained with eight different peroxidase-conjugated lectins, Con A (concanavalin A), WGA (wheat germ agglutinin), PNA (peanut agglutinin), UEA-I (*Ulex europaeus* agglutinin-I), RCA120 (*Ricinus communis* agglutinin-120), PHA-E4 (phytohemagglutinin-E4), DBA (*Dolichos biflorus* lectin), LCA (lentil agglutinin) and colloidal gold total protein detection kit (Bio-Rad). The protein–lectin–peroxidase complex was visualized with 4-chloro-1-naphthol.

Separation of sugar chains

The solution of the purified α-D-galactosidase (30 mg per 5 ml) was heated at 100 °C for 10 min. The denatured enzyme was hydrolyzed with Pronase (50 μg) for 2 h at 40 °C, followed by lyophilization. The product was dissolved in 800 μl 50 mM potassium phosphate buffer (pH 7.4) containing 20 mM EDTA, 0.1% SDS, 1% 2-mercaptoethanol, and 1% Triton X-100. The mixture was heated for 10 min at 100 °C, and then incubated with *N*-glycosidase F (2 units) for 24 h at 35 °C. The reaction mixture was fractionated on a Bio-Gel P-4 column (Bio-Rad, 2.6 cm × 90 cm) which had been equilibrated with water. The fractions which contained carbohydrate (measured by the phenol–sulfuric acid method [11]) were combined and concentrated. The carbohydrate solution was injected onto HPLC equipped with a CarboPac PA-1 column (Dionex, 9 mm × 250 mm). The sugar chains were eluted with a linear gradient of 0–200 mM sodium acetate in 50 mM NaOH at a flow rate of 3.0 ml min^{-1} and the eluent was monitored with the pulsed amperometric detector (Dionex). The eluent containing oligosaccharide was collected and desalted by a micro acylyzer S1 (Asahikasei Co., Ltd.).

^{1}H-NMR spectroscopy

The isolated sugar chains were dissolved in 0.5 ml deuterium oxide (99.98% $^{2}H_2O$) containing 0.001% acetone after deuterium exchange of labile hydrogen atom. ^{1}H-NMR spectra were recorded on an XL-400 or a Unity-400 spectrometer (Varian). Chemical shifts (δ) were expressed in ppm relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured by reference to internal acetone in $^{2}H_2O$ (2.22 ppm).

Compositional analysis

An oligosaccharide sample (10 μg) was dissolved in 500 μl 2 M TFA containing 0.1 N HCl and incubated for 6 h at 100 °C. The solution was neutralized with 1 N NaOH, followed by lyophilization. The product was injected onto HPLC equipped with a CarboPac PA-1 column (4 mm × 250 mm) and eluted with 15 mM NaOH. The eluted oligosaccharides were monitored with a pulsed amperometric detector. Each peak was identified by comparing the retention time with that of a reference monosaccharide standard.

Mild acid hydrolysis

An oligosaccharide sample (100 μg) was dissolved in 1.0 ml trifluoroacetic acid (TFA) solution, pH 2.2, and incubated at 100 °C. At appropriate time intervals, 300 μl of the reaction solution were withdrawn and immediately lyophilized. The lyophilized sample was dissolved in 0.5 ml of $^{2}H_2O$ and analyzed by ^{1}H-NMR.

Methylation analysis

An oligosaccharide sample (100 μg) was methylated with methylsulfinylcarbanion and methyl iodide in dimethyl sulfoxide [12]. The methylated glycan was recovered from the chloroform layer after partition between water and chloroform, and hydrolyzed in 4 M TFA (100 μl) at 100 °C for 4 h. After repeated coevaporation with toluene, the hydrolyzate was reduced with $NaBH_4$ (10 mg ml^{-1} in 50% methanol) at 25 °C for 2 h. Small amounts of acetic acid were added to destroy the remaining $NaBH_4$, then boric acid was removed by repeated coevaporation with ethanol. After acetylation with acetic anhydride (100 μl, 120 °C, 2 h) the sample was dried under reduced pressure. The dried sample was dissolved in 50 μl dichloromethane, and then analyzed on a gas–liquid chromatograph equipped with an OV-17 column (GL Science Co. Ltd., 3 mm × 2 m). The sample was eluted with nitrogen gas at 170–230 °C (1 °C min^{-1}) and monitored by FID or Hitachi M-2000 mass spectrometer.

Results

Purification of α-D-galactosidase

The purification of α-D-galactosidase from *Aspergillus niger* culture broth has been reported by Bahl and Agrawal [13] and Christakopoulos *et al.* [14]. In the present experiment, α-D-galactosidase was purified in one step by gel chromatography on a column of Sephacryl S-200. Only α-D-galactosidase was eluted after all the other enzymes were eluted. Some specific interaction between α-D-galactosidase and matrix components of Sephacryl S-200 was assumed. The isolated α-D-galactosidase showed a single protein band (60 kDa) on SDS-PAGE as demonstrated in Fig. 1. However, the preparation was contaminated with a small amount of *N*-acetyl-α-D-galactosaminidase activity and *N*-acetyl-α-D-glucosaminidase activity (3.5% and 0.3%, respectively, of α-D-galactosidase activity). For the structural analysis of oligosaccharides attached to the α-D-galactosidase, the enzyme preparation was used without further purification.

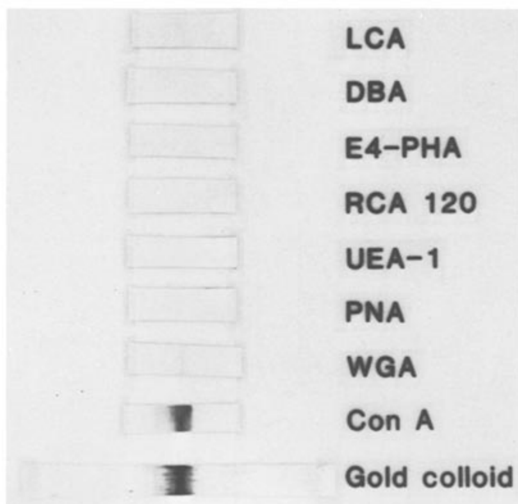

Figure 1. Lectin-staining of purified α-D-galactosidase. The protein bands which were electrophoretically transferred to nitrocellulose were stained with a colloidal gold total protein detection kit and eight peroxidase-conjugated lectins (Con A, WGA, PNA, UEA-I, RCA120, PHA-E4, DBA, LCA). The protein–lectin–peroxidase complex was visualized with 4-chloro-1-naphthol.

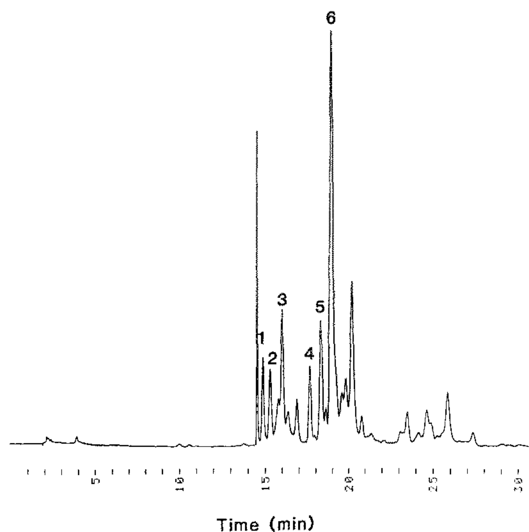

Figure 2. Anion-exchange chromatography of the oligosaccharides from the α-D-galactosidase. The oligosaccharide mixture released by *N*-glycosidase F was injected onto HPLC equipped with a CarboPac PA-1 column (9 mm × 250 mm) and eluted with a linear gradient of 0–200 mM sodium acetate in 50 mM NaOH at a flow rate of 3.0 ml min^{-1}. The eluted oligosaccharides were monitored by a pulsed amperometric detector. 1, OS-1; 2, OS-2; 3, OS-3, 4, OS-4; 5, OS-5; 6, OS-6.

Isolation of oligosaccharides from α-D-galactosidase

The total carbohydrate content of the α-D-galactosidase from *A. niger* was shown to be approximately 15% by the phenol–sulfuric acid method. Affinity staining of Western blots with lectins showed that Con A bound well with the enzyme, whereas the other lectins bound scarcely at all (see Fig. 1). Therefore, it was predicted that the major portion of the oligosaccharides present were of the oligo-mannose type.

The purified α-D-galactosidase was first hydrolyzed with Pronase, then treated with *N*-glycosidase F. The released sugar chains were separated by Bio-Gel P-4 column chromatography. The fractions containing carbohydrates were fractionated by use of anion exchange column chromatography. As demonstrated in Fig. 2, the chromatogram of the sugar fractions from Bio-Gel P-4 afforded more than 15 peaks, of which six peaks were isolated to determine the structure. The isolated fractions were numbered from OS-1 to OS-6 as shown in Fig. 2.

Structural analysis of oligosaccharides from α-D-galactosidase

^{1}H-NMR spectra of all the isolated oligosaccharides were measured and the chemical shifts of H-1 and H-2 of each monosaccharide component were compared with the reported data [15, 16]. The chemical shift profiles of OS-1–OS-4 coincided with the reported values and the structure of each oligosaccharide was deduced as demonstrated in Fig. 3.

Figure 4 shows the ^{1}H-NMR spectrum of OS-6. Although

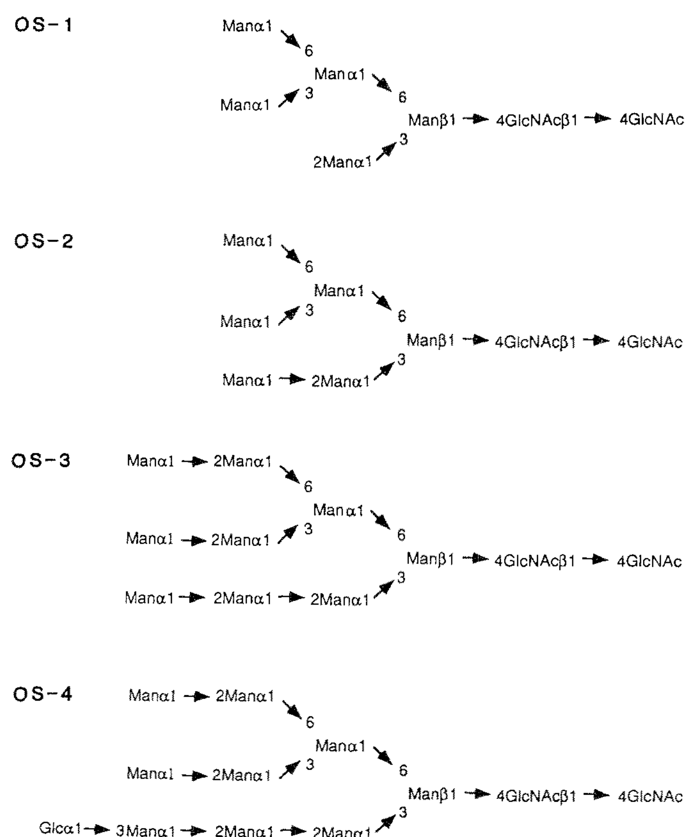

Figure 3. Structures of OS-1–OS-4. The structures were identified by comparison of H-1 and H-2 chemical shifts of OS-1–OS-4 with data previously reported [15, 16].

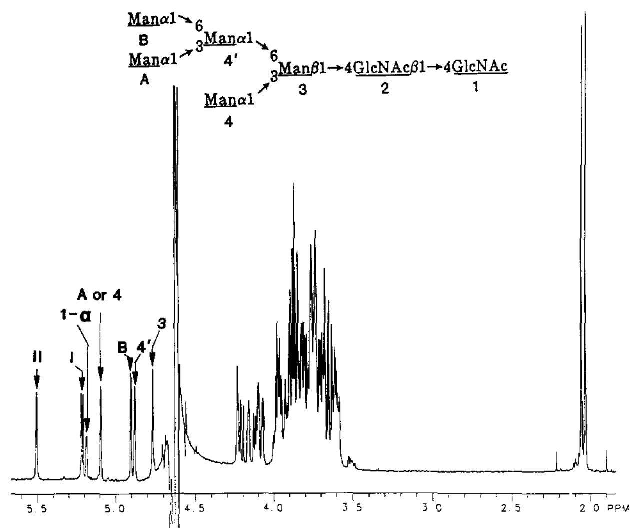

Figure 4. ^{1}H-NMR spectrum of OS-6. The fraction containing OS-6 was desalted by a micro acylyzer S1. After deuterium exchange of labile hydrogen atoms, OS-6 was dissolved in 0.5 ml deuterium oxide (99.98% ^2H_2O) containing 0.001% acetone. The ^{1}H-NMR spectrum was recorded on a Varian XL-400 spectrometer at 40 °C. Chemical shifts are expressed in ppm with reference to internal acetone (2.22 ppm). The signals 3, 4, 4′, A and B were assigned to H-1 of Man(3), Man(4), Man(4′), Man(A) and Man(B), respectively. The signal 1-α was assigned to the H-1 of the α-anomer of GlcNAc(1).

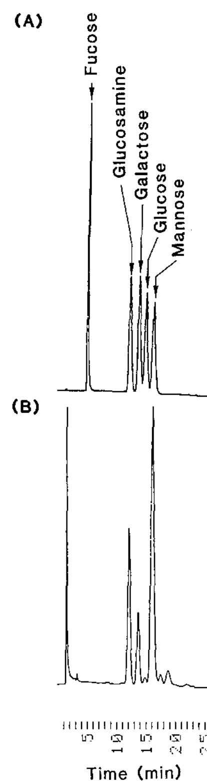

Figure 5. Compositional analysis of OS-6. OS-6 (10 μg) was dissolved in 500 μl of 2 M TFA containing 0.1 N HCl, and the solution was incubated for 6 h at 100 °C. After neutralization with 1 N NaOH, the solution was lyophilized. The product was injected onto HPLC equipped with a CarboPac PA-1 column (4 mm × 250 mm) and eluted with 15 mM NaOH at a flow rate of 1.0 ml min^{-1}. A pulsed amperometric detector was used for the detection of monosaccharides (B). The peaks due to *N*-acetylglycosamine, galactose, and mannose were identified by comparison of the retention times with those of the reference monosaccharide standard (A). The ratios of galactose, mannose, and N-acetylglucosamine were 0.9, 5.1, and 2.0, respectively.

this oligosaccharide was also assumed to be of the oligo-mannose type, its ^{1}H-NMR spectrum indicated unusual signals at 5.21 ppm (peak I, $J_{1,2} = 4.7$ Hz) and at 5.51 ppm (peak II). These unusual chemical shift values were not found in the reported data of Vliegenthart *et al.* [15].

The compositional analysis of OS-6 showed that the oligosaccharide consisted of one D-galactose, five D-mannose, and two *N*-acetyl-D-glucosamine residues, as demonstrated in Fig. 5. Therefore, the unusual signals, peak I in Fig. 4, were suggested to be caused by the linkage of a D-galactose residue to a mannose residue at the nonreducing end of the oligo-mannose type oligosaccharide. However, the coupling constant ($J_{1,2} = 4.7$ Hz) was different from those of methyl α- and β-D-galactopyranoside ($J_{1,2} = 3.0$ Hz and 7.9 Hz, respectively) and was rather close to that of methyl α-D-galactofuranoside ($J_{1,2} = 3.7$ Hz). A hexofuranosyl linkage is known to be more acid labile than a hexopyranosyl linkage. In order to examine whether the D-galactosyl residue was in the pyranose or furanose form, OS-6 was hydrolyzed under mild acidic conditions [17]. The ^{1}H-NMR spectrum of OS-6 after 4h of mild acid hydrolysis showed a new signal at 5.25 ppm ($J_{1,2} = 3.6$ Hz) which corresponded to the H-1 signal of free α-D-galactose together with a signal at 5.09 ppm which coincided with an H-1 signal of Man(A) or Man(4) in $Man_5GlcNAc_2$ (see Fig. 6). Thus, only the D-galactosyl linkage was hydrolyzed and the $Man_5GlcNAc_2$ structure remained without degradation by the present hydrolysis condition. From this result, the D-galactosyl residue was concluded to be a furanose structure and bound to Man(A) or Man(4) of $Man_5GlcNAc_2$. Moreover, the methylation analysis of OS-6 showed the presence of 1,4-di-*O*-acetyl-2,3,5,6-tetra-*O*-methylhexitol as shown in Fig. 7. Thus the presence of a D-galactofuranosyl linkage was supported.

From the above experiment, peak II was assigned to H-1 of either Man(A) or Man(4). In order to clarify the branching position of the D-galactofuranosyl residue (D-Gal*f*), a ROESY spectrum was measured. The presence of cross peaks between peak II ($\delta = 5.51$ ppm) and H-2, H-3, and H-4 of Man(4′) in the ROESY spectrum in Fig. 8 shows that the component sugar of peak II is attached to Man (4′) at the 2-, 3-, or 4-position. Therefore, peak II was determined to be H-1 of Man(A). In oligo-mannose type oligosaccharides, 2-*O*- substitution by mannose gives rise to a large downfield shift (0.311 ppm) of the H-1 signal of

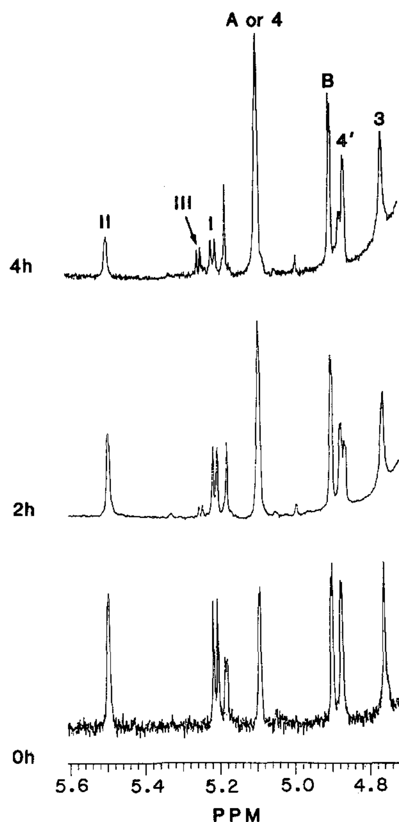

Figure 6. Mild acid hydrolysis of OS-6. OS-6 (100 μg) was dissolved in 1.0 ml of TFA solution, pH 2.2, and incubated at 100 °C. At appropriate time intervals (0, 2 h, 4 h), 300 μl of the reaction mixture were withdrawn and lyophilized immediately. The product was dissolved in 0.5 ml of ^2H_2O and analyzed by ^{1}H-NMR spectroscopy. The signals 3, 4, 4′, A and B correspond to the H-1 signals of Man(3), Man(4), Man(4′), Man(A) and Man(B) in Fig. 4.

Man(A) [15]. In this instance, the large downfield shift (0.42 ppm) of the H-1 of Man(A) may be due to 2-*O*-substitution by Gal*f*. The presence of 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylhexitol in the methylation analysis supported a structure where D-Gal*f* is bound to the 2-position of Man(A) (see Fig. 7). In Fig. 8, the cross peaks for (4′)H1-(4′)H2 and (4)H1-(4)H2 were too weak to be observed, while (A)H1-(A)H2 and (B)H1-(B)H2 showed clear cross peaks. Some conformational difference in each mannosyl unit might have caused the different appearances of the cross peaks.

From the coupling constant value ($J_{1,2}$ = 4.7 Hz), D-Gal*f* was concluded to have an α-configuration, considering that $J_{1,2}$ of methyl α-D-galactofuranoside is 3.7 Hz and that of methyl β-D-galactofuranoside is 2.0 Hz. Therefore, the structure of OS-6 could be as shown in Fig. 9.

The ^{1}H-NMR spectrum of OS-5 in Fig. 9 is quite similar to that of OS-6, except for the signal due to Man(4) (5.09 ppm). Consequently, the structure of OS-5 is assumed to be OS-6 without the Man(4) residue, as demonstrated also in Fig. 9.

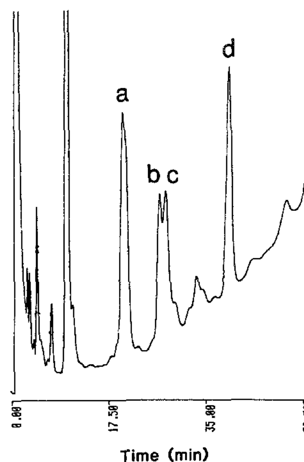

Figure 7. Methylation analysis of OS-6. The partially methylated alditol acetates from OS-6 were separated by GC equipped with an OV-17 column (3 mm × 2 m). The sample was eluted with nitrogen gas at 170–230 °C (1 °C per min) and monitored by FID or a Hitachi M-2000 mass spectrometer. (a) 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol; (b) 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylhexitol; (c) 1,4-di-*O*-acetyl-2,3,5,6-tetra-*O*-methylhexitol; (d) 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methylhexitol.

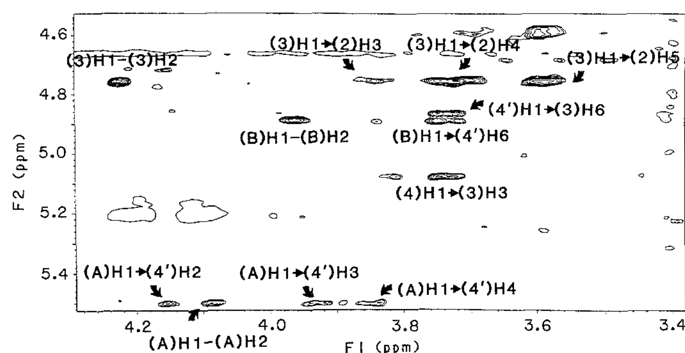

Figure 8. Anomeric region of the 400 MHz 2D rotating-frame NOE spectrum (ROESY) of OS-6. The spectrum was recorded at 33 °C with a mixing time of 150 ms. The cross peak annotations refer to the corresponding residues in the structure (Fig. 4). The inter-residue NOE connectivities are indicated by arrows (→).

Discussion

The present study established the structure of the oligosaccharides from *A. niger* α-D-galactosidase. The oligosaccharides were purified by gel permeation chromatography and anion-exchange chromatography. In the anion-exchange chromatography (see Fig. 2), approximately 15 peaks of oligosaccharides were observed. However, only six of them were isolated, because the other fractions were contaminated with two or more oligosaccharides. OS-6 was the largest of the peaks and OS1–OS5 were also major oligosaccharides. Therefore, the oligosaccharides studied in the present investigation could be estimated as representative sugars in α-D-galactosidase from *A. niger*.

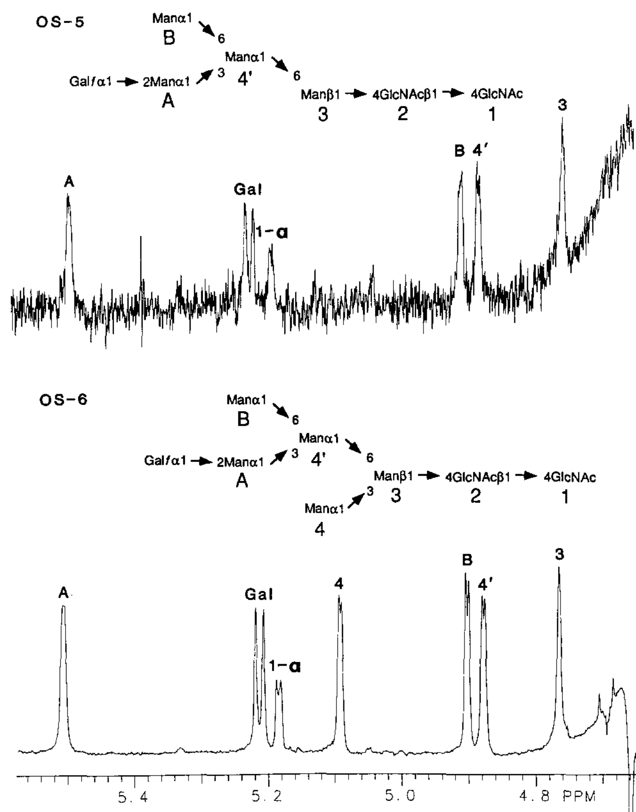

Figure 9. ^{1}H-NMR spectra and structures of OS-5 and OS-6. ^{1}H-NMR spectra show the H-1 region of each sugar residue in the oligo-mannose type oligosaccharides.

The faster eluted oligosaccharides, OS-1–OS-4, were oligo-mannose type oligosaccharides of known structure. In contrast, the slower eluted oligosaccharides, OS-5 and OS-6, were also oligo-mannose type oligosaccharides but contained α(1-2)-linked D-Gal*f*. Mild acid treatment, which is known to hydrolyze hexofuranosidic bonds but not hexopyranosidic bonds, has been used to prove the presence of D-Gal*f* in the sugar chains [17, 18]. We followed the time course of hydrolysis of the oligo-mannose type oligosaccharide containing Gal*f* (Gal*f*$Man_5GlcNAc_2$) by ^{1}H-NMR and confirmed the appearance of signals due to D-galactose and an oligo-mannose type oligosaccharide ($Man_5GlcNAc_2$).

Mendelzon and Parodi [19] first reported that D-Gal*f* was linked to mannose at the nonreducing end of oligo-mannose type oligosaccharide in *Crithidia fasciculata* and *Crithidia harmosa*. The Gal*f* was characterized as being the β-anomer from its sensitivity to β-galactofuranosidase. Also, in the trypanosome *Leptomonas samueli*, it was found that D-Gal*f* was linked to mannose at the nonreducing end of an oligo-mannose type oligosaccharide [18]. From the thin layer chromatography of permethylated oligosaccharides, it was assumed that D-Gal*f* was attaching to mannose by a (1-2) linkage, but the anomer type of D-Gal*f* has not been clarified. This Gal*f*-containing oligosaccharide from the protozoan was quite similar to that from *A. niger* in the present study.

Although D-galactopyranosyl residues have been found widely in the oligosaccharides from mammalian cells, D-Gal*f* has been found only in the sugar chains from various kinds of microorganism. In addition to oligo-mannose type oligosaccharides, D-Gal*f* has been found in *O*-linked carbohydrate chains in the cellulosome of *Clostridium thermocellum* [20] or the galactomannan from *Aspergillus*, *Pencillium*, and *Dermatophytes*.

In a study of the galactomannan from the zoopathogenic fungus *Aspergillus fumigatus*, Debeaupuis *et al.* [21] found that monoclonal antibodies directed to *A. fumigatus* galactomannan were specific for the galactofuranoside chain. Furthermore, they showed that galactofuranosyl residues were detected in exocellular antigenic materials from many species of the *A. fumigatus* group. The Gal*f* in the galactomannan of *A. fumigatus* was the β-anomer, and different from the oligo-mannose type oligosaccharide of *A. niger* α-galactosidase analyzed in the present study.

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