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Novel structures of N-linked high-mannose type oligosaccharides containing α-D-galactofuranosyl linkages in *Aspergillus niger* α-D-glucosidase

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ABSTRACT

Seven oligosaccharides were isolated from α-D-glucosidase (EC 3.2.1.20) from *Aspergillus niger*, and the structures of these oligosaccharides were studied by ^{1}H NMR spectroscopy. After treatment of the α-D-glucosidase with *N*-glycosidase F, seven major oligosaccharide peaks were detected by Dionex anion-exchange HPLC. The structures corresponding to the three peaks OS-1, OS-2, and OS-4 were determined to be $Man_8GlcNAc_2$, $Man_9GlcNAc_2$, and $GlcMan_9GlcNAc_2$, respectively, from ^{1}H NMR spectra of the isolated fractions. Each of the four oligosaccharides OS-5, OS-6, OS-7-1, and OS-7-2 contained an α-D-galactofuranosyl residue (Gal*f*) linked to Man (A) via an α-(1 → 2)-linkage. OS-7 was found to consist of two oligosaccharides. The structures of these four oligosaccharides were determined to be Gal*f*$Man_5GlcNAc_2$, Gal*f*$Man_6GlcNAc_2$, Gal*f*$Man_7GlcNAc_2$, and Gal*f*$Man_8GlcNAc_2$ by ^{1}H NMR spectroscopy and compositional analysis. The Gal*f* structure of Gal*f*$Man_5GlcNAc_2$ was found to be identical to that of an oligosaccharide previously isolated from the α-D-galactosidase of the same strain[1]. The structure of OS-3 remains undetermined.

INTRODUCTION

Aspergillus niger secretes various kinds of enzymes[2] such as α- and β-D-glucosidase, α- and β-D-galactosidase, α-L-fucosidase, *N*-acetyl-β-D-glucosaminindase, and *N*-acetyl-β-D-galactosaminidase. While most of these enzymes have been found to be glycoproteins[3–6], the primary structures of the sugar chains have yet to be completely elucidated.

Previously we determined the structures of the sugar chains of an α-D-galactosidase (EC 3.2.1.22) purified from a commercial enzyme preparation of *A. niger*[1]. The sugar chains were released enzymatically from the α-D-galactosidase

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and were isolated by Dionex anion-exchange HPLC. All of these oligosaccharides were high-mannose type structures and two contained galactofuranosyl residues bound via α-(1 → 2)-linkages to the mannosyl residues of the nonreducing ends.

The α-D-glucosidase (EC 3.2.1.20) is a main component of *A. niger* culture broth. The enzymatic properties[7] and complete amino acid sequence[8] have been reported by Kimura et al. The purified enzyme is a glycoprotein of molecular weight 12.5×10^4. The α-D-glucosidase contains fifteen Asn-X-Ser/Thr sequences, and the carbohydrate content is 25.5% of the molecular weight.

In the present study, we isolated the oligosaccharides from *A. niger* α-D-glucosidase and determined the structure of seven oligosaccharides. Comparison of the sugar chain structures between an α-D-glucosidase and an α-D-galactosidase from the same origin is expected to provide important information concerning the secretion mechanism of glycoproteins in *A. niger*.

EXPERIMENTAL

Purification of α-D-glucosidase.—*A. niger* α-D-glucosidase was purified and crystallized from a commercial product of Transglucosidase Amano™ (Amano Pharmaceutical Co., Ltd.) according to the method of Kita et al.[7].

Affinity staining of Western blots with lectins.—SDS-PAGE was performed with a 4–20% linear gradient polyacrylamide slab gel[9]. Protein bands on the slab gel were electrophoretically transferred to a nitrocellulose membrane[10]. The membrane was stained with the biotin-blot protein detection kit (Bio-Rad), G.P. Sensor (Honen), and five kinds of biotin-conjugated-lectins [*Aleuria aurantia* lectin (AAL), concanavalin A (Con A), *Datura stramonium* agglutinin (DSA), *Maackia amurens* leukoagglutinin (MAL), *Sambucus sieboldiana* agglutinin (SSA)]. The protein–lectin–biotin complex was treated with avidin-HRP and visualized with 4-chloro-1-naphthol.

Separation of sugar chains.—The purified α-D-glucosidase (20 mg) was dissolved in 5 mL of 10 mM potassium phosphate buffer (pH 7.2) and heated for 5 min at 100°C. The denatured enzyme was hydrolyzed with 25 μg of Pronase (Sigma) for 2 h at 40°C, followed by lyophilization. The lyophilizate was dissolved in 5.0 mL of 100 mM potassium phosphate buffer (pH 7.4) containing 20 mM EDTA, 0.1% SDS, 1% 2-mercaptoethanol, and 0.2% Triton X-100. The mixture was heated for 10 min at 100°C, and then incubated with *N*-glycosidase F (8 units) for 48 h at 35°C. The mixture was passed over Amberlite MB-1 (1 g) and Bio-beads SM-2 (1 g), and then fractionated over a Bio-gel P-4 column (Bio-Rad, 2.6 × 950 mm) which had been equilibrated with water. The fractions containing carbohydrate were measured by the phenol–H_2SO_4 method[11], and were concentrated to 400 μL. The carbohydrate solution was injected onto an HPLC equipped with a preparative CarboPac PA-1 column (Dionex). The oligosaccharides were eluted with a linear gradient of 0–200 mM sodium acetate in 100 mM NaOH at a flow rate of 3.0 mL/min and the eluent was monitored with a pulsed amperometric

detector (Dionex). The eluent containing each oligosaccharide was collected and desalted by a Micro Acilyzer S1 (Asahikasei Co., Ltd.).

^1H NMR spectroscopy.—The isolated sugar chains were dissolved in D_2O (0.7 mL, 99.98% D_2O) containing 0.001% acetone after deuterium exchange of the labile hydrogen atoms. ^1H NMR spectra were recorded on a Unity-500 NMR spectrometer (Varian). Chemical shifts (δ) were expressed in ppm relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually calibrated by reference to internal acetone in D_2O (2.217 ppm).

Compositional analysis.—Oligosaccharide samples were dissolved in 400 μL of 2.5 M CF_3CO_2H and incubated for 5 h at 100°C. The cooled CF_3CO_2H solutions were evaporated with a centrifugal concentrator at 50°C until the samples were dry. Methanol (100 μL) was added to the dry residues, and after thorough mixing, the sample solutions were evaporated with a centrifugal concentrator. The dry samples were injected onto an HPLC equipped with a CarboPac PA-1 column (4 × 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.

RESULTS AND DISCUSSION

Affinity staining of Western blots with lectins.—After the SDS-PAGE of α-D-glucosidase, the proteins were electrophoretically transferred from the slab gel to a nitrocellulose membrane. For visualization of the protein and sugar chains of glycoprotein, the nitrocellulose membrane was stained using a biotin-blot protein detection kit (Fig. 1, lane 1) and G.P. sensor (Fig. 1, lane 2), respectively. The G.P. sensor is also a commercial kit for the visualization of sugar chains of glycoproteins. The protein band in lane 1 corresponding to *A. niger* α-D-glucosidase coincided with the band in lane 2, which indicated the existence of glycoprotein. This result shows that the α-D-glucosidase from *A. niger* is a glycoprotein.

Affinity staining with lectins showed that the oligosaccharide of α-D-glucosidase bound well with Con A (Fig. 1, lane 4) but only slightly with DSA (Fig. 1, lane 5). The other lectins (AAL, MAL, and SSA) bound weakly or not at all (Fig. 1, lanes 3, 6, and 7). Consequently, it was assumed that the major portion of the present oligosaccharides was of the high-mannose type.

Isolation of oligosaccharides from A. niger *α-D-glucosidase.*—The α-D-glucosidase was digested with Pronase, and then treated with *N*-glycosidase F. The protease digestion was performed to facilitate the release of oligosaccharides in the *N*-glycosidase F treatment and to prevent gelation of the enzyme. The released oligosaccharides were separated from the peptides and amino acids by Bio-Gel P-4 column chromatography. The fractions containing carbohydrates were analyzed by HPLC using a CarboPac PA-1 column. As shown in Fig. 2, seven major peaks were detected and named OS-1–OS-7. The oligosaccharides corresponding to these peaks were isolated by use of a preparative scale column of CarboPac PA-1.

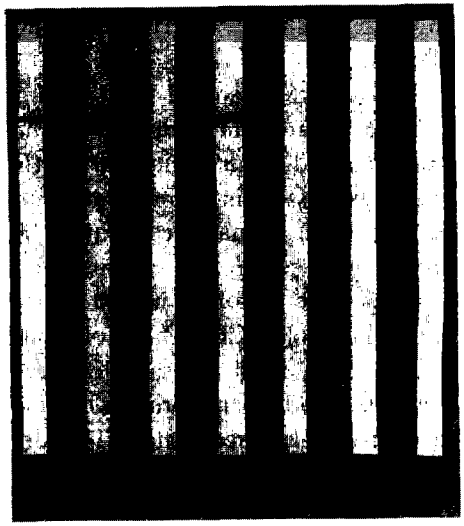


Fig. 1. Affinity staining of Western blots of α-D-glucosidase with lectins. SDS-PAGE was performed with a 4–20% linear gradient gel, and proteins on the slab gel were transferred to a nitrocellulose membrane. The membrane was stained with a biotin-blot protein kit (lane 1), G.P. Sensor (lane 2), and biotin-conjugated-lectin (lane 3-7). Lane-3, AAL; lane 4, Con A; lane 5, DSA; lane-6, MAL; lane-7, SSA.

Structure analyses of oligosaccharides isolated from α-D-glucosidase.—The H-1 proton chemical shifts of each monosaccharide component in the ^{1}H NMR spectra of OS-1–OS-7 have been summarized in Table I. The symbols of GlcNAc(1),

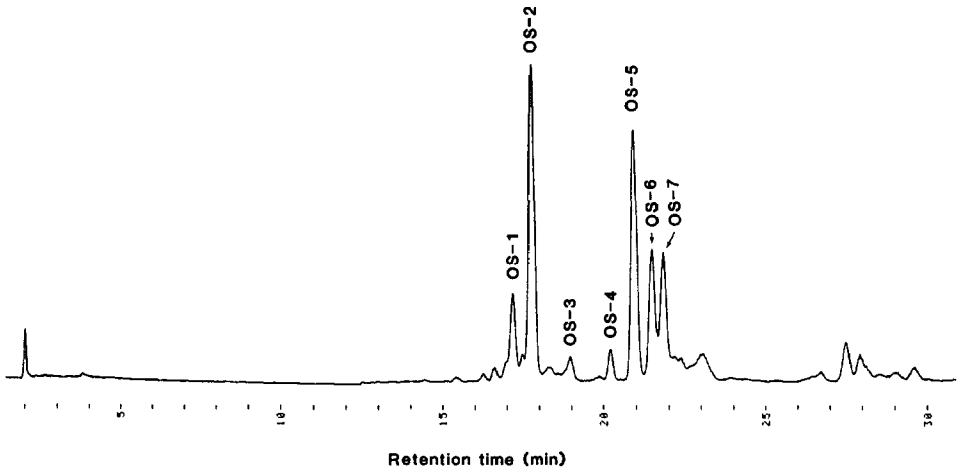


Fig. 2. Anion-exchange chromatography of the oligosaccharides from α-D-glucosidase. The oligosaccharide mixture released by *N*-glycosidase F was separated by HPLC using a CarboPac PA-1 column.

TABLE I

^{1}H NMR chemical shifts of H-1 of the constituent monosaccharides of OS-1–OS-7-2

Monosaccharide	Chemical shifts							
	OS-1	OS-2	OS-3	OS-4	OS-5	OS-6	OS-7-1	OS-7-2
GlcNAc(1-α)	5.188	5.187		5.188	5.186	5.185	5.186	5.186
Man(3)	4.757 (4.78)	4.756 (4.77)	4.757	4.757 (4.76)	4.764	4.753	4.753	4.753
Man(4)	5.318 (5.336)	5.315 (5.333)	5.316	5.316 (5.33)	5.099	5.326	5.315	5.315
Man(4′)	4.866 (4.869)	4.864 (4.868)	4.865	4.866 (4.87)	4.878	4.876	4.878	4.878
Man(A)	5.097 (5.09)	5.372 (5.401)	5.375	5.376 (5.40)	5.489	5.489	5.491	5.491
Man(B)	5.118 (5.145)	5.116 (5.141)	5.116	5.117 (5.14)	4.905	4.904	4.905	5.119
Man(C)	5.284 (5.304)	5.283 (5.308)	5.286	5.285 (5.31)		5.053	5.282	5.282
Man(D_1)	5.043 (5.044)	5.046 (5.047)	5.047	5.045 (5.03)			5.045	5.045
Man(D_2)		5.060 (5.059)	5.061	5.061 (5.05)				
Man(D_3)	5.043 (5.044)	5.040 (5.040)	5.042	5.041 (5.03)				5.045
Glc				5.249 (5.24)				
Gal*f*					5.213	5.212	5.211	5.217

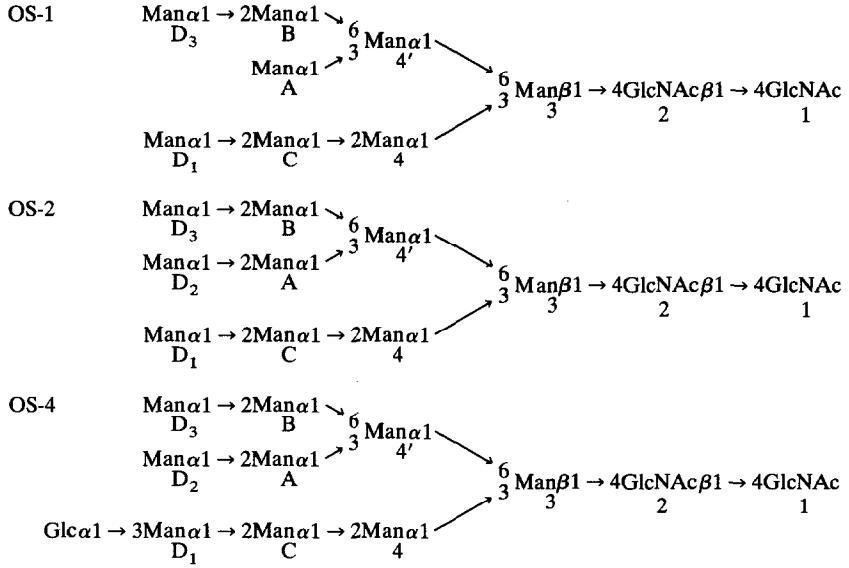
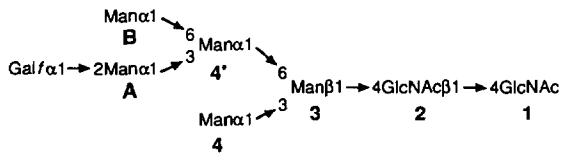


Fig. 3. Structures of OS-1, OS-2, and OS-4.



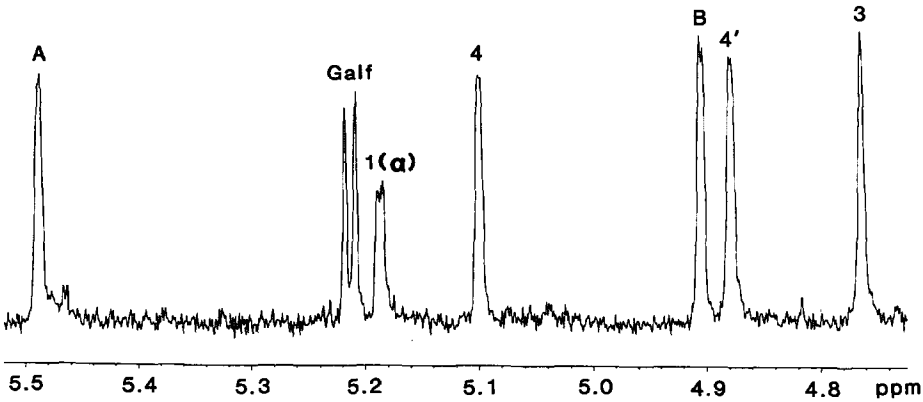


Fig. 4. The ^{1}H NMR spectrum and structure of OS-5. The signals of 3, 4, 4″, A, B, and Gal*f* were assigned to H-1 of Man(3), Man(4), Man(4′), Man(A), Man(B), and Gal*f*, respectively. The signal 1(α) was assigned to H-1 of the α anomer of GlcNAc(1).

TABLE II

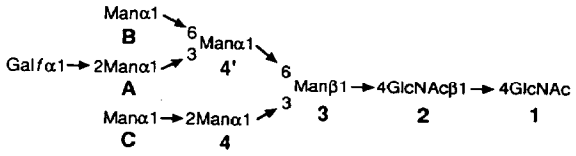
Compositional analyses of the oligosaccharides containing Gal*f*

	GlcNAc [a]	Gal	Glc	Man
OS-5	2.0	1.0	0	5.1
OS-6	2.0	1.0	0	6.4
OS-7	2.0	1.0	0	7.5

[a] The molar ratio of GlcNAc was taken as 2.0.

Man(3), Man(4), Man(4′), Man(A), Man(B), Man(C), Man(D_1), Man(D_2), and Man(D_3) in high-mannose type oligosaccharides correspond to those used by Vliegenthart et al.[12]. The chemical shift profiles of OS-1, OS-2, and OS-4 coincided well with the reported values[12,13]. Consequently, the structures of the oligosaccharides were determined as shown in Fig. 3. The chemical shifts of the H-1 protons in OS-3 were in agreement with those of OS-2 except for GlcNAc(1-α), which was missing in the spectrum of OS-3. In spite of this observation, the methyl proton signal corresponding to the *N*-acetyl group of GlcNAc(1-α) was present. Therefore, the structure of OS-3 at present remains undetermined.

The ^{1}H NMR spectrum of OS-5 in Fig. 4 was identical to that reported by us previously for the high-mannose type oligosaccharide containing an α-D-galactofuranosyl residue[1]. The signals at 5.213 and 5.489 ppm were characteristic of the Gal*f* containing oligosaccharides, and were assigned to H-1 of Gal*f* and Man(A),



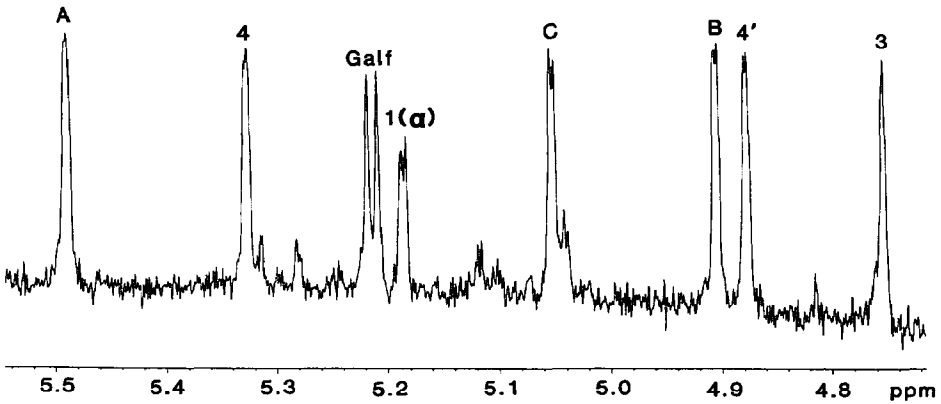


Fig. 5. The ^{1}H NMR spectrum and structure of OS-6. Signal C was assigned to H-1 of Man(C). The other symbols are the same as those in Fig. 4.

respectively. The coupling constant ($^3J_{H\text{-}1,H\text{-}2}$ 4.7 Hz) in Gal*f* indicates an α-linkage, as $^3J_{H\text{-}1,H\text{-}2}$ of methyl α-D-galactofuranoside was 3.7 Hz and that of the β anomer was 2.0 Hz[1]. The large downfield shift (0.392 ppm) of Man(A) is due to 2-*O*-substitution by Gal*f*[1]. Therefore, Gal*f* was determined to be attached to Man(A) via an α-(1 → 2)-linkage. The compositional analysis of OS-5 indicated that GlcNAc, Gal, and Man were in the molar ratio 2:1:5.1 (Table II), which supported the structure of OS-5 as Gal*f*$Man_5GlcNAc_2$.

In the spectrum of OS-6 in Fig. 5, new signals at 5.053 ppm and 5.326 ppm appeared in addition to those found in the spectrum of OS-5 in Fig. 4. These signals were assigned to H-1 of Man(C) and Man(4), respectively. The downfield shift (from 5.099 to 5.326 ppm) of H-1 of Man(4) was assumed to be caused by 2-*O*-substitution by Man(C)[12]. The compositional analysis of OS-6 for GlcNAc,

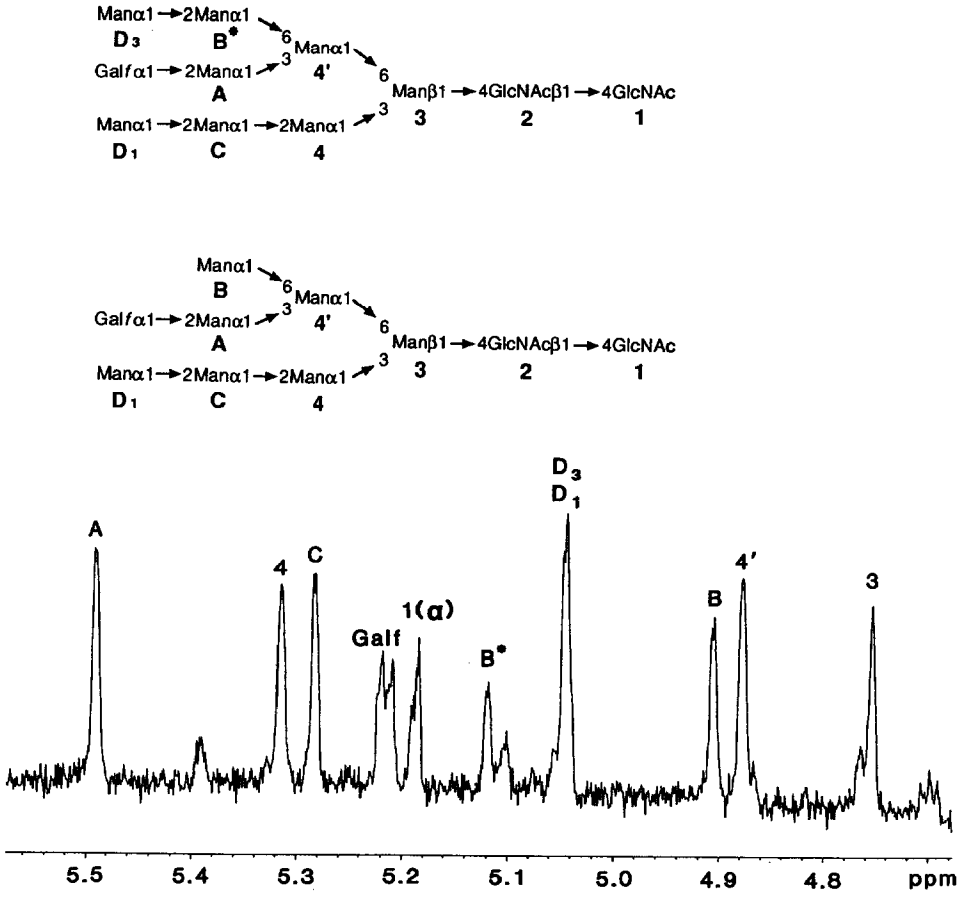


Fig. 6. The ^1H NMR spectrum of the OS-7 fraction and structures of two oligosaccharides. Signals D_1 and D_3 were assigned to H-1 of Man(D_1) and Man(D_3), respectively. The other symbols are the same as those in Fig. 4.

Gal, and Man gave the ratio 2:1:6.4 (Table II). Therefore, the structure of OS-6 was determined to be Gal*f*$Man_6GlcNAc_2$.

In the spectrum of OS-7, a new signal was observed at 5.282 ppm, which was assigned to the H-1 of Man(C) as shown in Fig. 6. The downfield shift of the H-1 of Man(C) was assumed to be caused by 2-*O*-substitution with Man(D_1). The H-1 signal of Man(D_1) was observed at 5.045 ppm[12]. In the spectrum of OS-7, however, an unexpected signal was observed at 5.119 ppm. This signal was assigned to H-1 of Man(B) possessing Man(D_3) with an α-(1 → 2)-linkage[12]. Therefore, it was concluded that OS-7 consisted of a mixture of Gal*f*$Man_7GlcNAc_2$ and Gal*f*$Man_8GlcNAc_2$. This conclusion is supported by a doubled Gal*f* signal at 5.211 ppm and 5.217 ppm. The H-1 signal of Man(D_3) at 5.045 ppm is assumed to overlap with the H-1 signal of Man(D_1), as the intensity of the peak at 5.045 ppm is 1.5-fold greater than those of the other peaks. Slight contamination by OS-1 and OS-2 account for the weak signals at 5.390 and 5.103 ppm. The compositional analysis of OS-7 (GlcNAc:Gal:Man = 2:1:7.5, Table II) also supported the conclusion that OS-7 consists of a mixture of Gal*f*$Man_7GlcNAc_2$ (OS-7-1) and Gal*f*$Man_8GlcNAc_2$ (OS-7-2) as demonstrated in Fig. 6.

CONCLUSIONS

High-mannose type oligosacharides containing Gal*f* were first reported in *Crithidia fasciculata* and *Crithidia harmosa* by Mendelzon and Parodi[14]. We subsequently reported the structure of two N-linked oligosaccharides of *A. niger* α-D-galactosidase to be of the high-mannose type containing α-(1 → 2)-linked Gal*f* residues[1]. The structure of the sugar chains from *A. niger* α-D-galactosidase were analogous to those of *Crithidia species*. The structure of OS-5 in the present study was also the same as the Gal*f* containing oligosaccharide in α-D-galactosidase. Although three other oligosaccharides. (OS-6, OS-7-1, and OS-7-2) were found only in α-D-glucosidase, Gal*f* was linked to Man(A) via an α-(1 → 2)-linkage in all cases. It may be proposed that the high-mannose type oligosaccharides of both enzymes are synthesized through the same glycosylation pathway if the Gal*f* is added in the Golgi apparatus of *A. niger* to Man(A) in high-mannose type sugar chains by a particular galactofuranosyl transferase.

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