Novel β-D-Galactofuranose-containing High-mannose Type Oligosaccharides in Ascorbate Oxidase from Acremonium sp. HI-25

Masaya Ohta, Shinichi Eji, Hiroyuki Iwamoto,* Junzo Hirose,* Keitaro Hiromi,* Homare Itoh,** Takashi Shin,** Sawao Murao,** and Fumito Matsuura*

Department of Biotechnology, and *Department of Food Science and Technology, Faculty of Engineering, Fukuoka University, Fukuoka, Hiroshima 729-02, Japan
**Department of Applied Microbial Technology, The Kumamoto Institute of Technology, Ikeda 4-22-1, Kumamoto 860, Japan

Received January 16, 1996

Ascorbate oxidase from the fungus Acremonium sp. HI-25 is a copper-containing glycoprotein that catalyzes the oxidation of ascorbic acid to dehydroascorbic acid. Monosaccharide composition analysis showed that the enzyme contains exclusively N-linked oligosaccharide chains. Following liberation by hydrazinolysis/re-N-acetylation, and fractionation by HPLC on anion exchange, Amide-80 and/or octadecyl silica columns after derivatization with p-aminobenzoic ethyl ester, the structures of the twelve major neutral oligosaccharides were identified by FAB-MS, 400 MHz 1H-NMR, methylation analysis, mild acid hydrolysis, and/or sequential exoglycosidase digestions. Acremonium sp. ascorbate oxidase was found to consist of high-mannose type oligosaccharides (76.3%) having 4 to 9 mannose residues and a series of novel D-galactofuranose-containing high-mannose type oligosaccharides (18.6%) with the following structure.

Galβ1
\[± Manz1 \rightarrow 2Manz1 \rightarrow ^{\frac{1}{2}} 6\text{Manz1} \rightarrow ^{\frac{1}{2}} 6\text{Manβ1} \rightarrow 4\text{GlcNAcβ1} \rightarrow 4\text{GlcNAc} \]

Key words: ascorbate oxidase; Acremonium; N-linked oligosaccharide; galactofuranose-containing oligosaccharide

Ascorbate oxidase [EC 1.10.3.3] (AOD), which catalyzes the oxidation of ascorbic acid to dehydroascorbic acid, is an enzyme widely found in higher plants, such as squash and cucumber.\(^{21}\) Ascorbate oxidase is used as an analytical reagent for clinical and food analyses of ascorbate. However, since ascorbate oxidase from cucumber, which is most commonly used as an analytical reagent, is unstable, a more stable ascorbate oxidase is required.

Efforts to find microorganisms producing ascorbate oxidase have been made.\(^{3-5}\) Although the occurrence of ascorbate oxidase in microorganisms has been reported,\(^{6-10}\) in Myrothecium verrucaria,\(^{11}\) and Aerobacter aerogenes,\(^{12}\) the enzyme has not been purified or characterized. Recently, Murao et al.\(^{13}\) succeeded in isolating a strain, identified as Acremonium sp. HI-25, producing an ascorbate oxidase, and purified it with no laccase activity. The ascorbate oxidase from Acremonium sp. is a monomeric glycoprotein with a molecular mass of 80 kDa containing four copper atoms per molecule.\(^{14}\) While the ascorbate oxidase from cucumber and squash are dimeric glycoproteins with molecular masses of 130-140 kDa and containing eight copper atoms per molecule.\(^{7,8}\) Hirose et al.\(^{15}\) reported that the coordination structures of types 1 and 2 copper atoms in ascorbate oxidase from Acremonium sp. are very similar to those in the cucumber enzyme. Furthermore, they have shown that the apparent $K_m$ and $k_{cat}$ for ascorbic acid of the ascorbate oxidase from Acremonium sp. are closely similar to those of the monomeric unit of the ascorbate oxidase isolated from cucumber.

The primary structures of the ascorbate oxidases from cucumber and squash have been identified by cDNA cloning.\(^{16,17}\) The amino acid sequences deduced from the nucleotide sequences of the cDNAs for the squash and cucumber enzymes had 80% identity, with four and three possible N-glycosylation sites, respectively. D’Andrea et al.\(^{18}\) analyzed the primary structures of the N-linked sugar chains of ascorbate oxidase of squash to be Manz1→6(Xylβ1→2)(Manz1→3)Manβ1→4GlcNAcβ1→4GlcNAc. However, no report is available on the primary structure of Acremonium sp. ascorbate oxidase. Glycosylation is known to be required for stability, catalytic activity, secretion, and so on. Thus, identification of the sugar chain structures as well as the amino acid sequences of microorganism ascorbate oxidases may be valuable.

In this paper, we report the structures of N-linked oligosaccharides of the ascorbate oxidase from Acremonium sp. and the presence of novel galactofuranose-containing high-mannose type oligosaccharides.

* To whom correspondence should be addressed.

Abbreviations: AOD, ascorbate oxidase; ABEE, p-aminobenzoic acid ethyl ester; Man, mannose; GlcNAc, N-acetylglucosamine; Hex, hexose; HexNAc, N-acetylhexosamine; Galf, D-galactofuranose; 2D-COSY, two-dimensional chemical-shift correlated spectroscopy.
Materials and Methods

Ascorbate oxidase. AOD was isolated from culture broth of *Acremonium spinosum* sp. HI-25 by the method of Murao et al.¹⁰

Chemicals and enzymes. t-(-)-2-Octanol and t-galactose were purchased from Wako Pure Chemical Industries Ltd., Japan. t-Galactose was purchased from Sigma Chemical Co. (St. Louis, USA). Sphingomyelin from bovine brain and apo-endo-β-N-acylglucosaminidase H was obtained from Seikagaku Corporation, Japan. Jack bean meal α-mannosidase, β-galactosidase, and β-N-acetylgalactosaminidase were prepared by modifications of the published methods.¹¹³ S. agarilus *satoii* α-mannosidase I was purified by the method of Amano and Kobata.¹¹ Methyl-galactoside of the furanose form was prepared by heating t-galactose with 0.015% methanolic HCl at 80°C for 6 h. Unless specified, all other chemicals and chromatographic solvents were purchased from Wako Pure Chemical Industries.

Standard oligosaccharides. The following standard oligosaccharides tagged with p-methoxybenzoyl ethyl ester (ABEE) were prepared as described previously.¹⁰⁻¹³ Manβ1→4GlcNAcβ1→4GlcNAc-ABEE (Man, GlcNAc-ABEE), Manz1→6Manβ1→4GlcNAcβ1→4GlcNAc-ABEE (Man, GlcNAc,ABEE), Manz1→6Manz1→3Manβ1→4GlcNAcβ1→4GlcNAc-ABEE (Man, GlcNAc,ABEE), Manz1→6Manz3→1Manβ1→4GlcNAcβ1→4GlcNAc-ABEE (Man, GlcNAc,ABEE), Manz1→6Manz3→1Manβ1→4GlcNAcβ1→4GlcNAc-ABEE (Man, GlcNAc,ABEE), Manz1→6Manz3→1Manβ1→4GlcNAcβ1→4GlcNAc-ABEE (Man, GlcNAc,ABEE), and Manz1→6Manz3→1Manβ1→4GlcNAcβ1→4GlcNAc-ABEE (Man, GlcNAc,ABEE). These were obtained with recombinant human β-galactosidase A (M. Ohta et al., manuscript submitted).

The following high-mannose type oligosaccharides having a single N-acetylgalactosamine residue at their reducing end were isolated from urine of patients with z-mannosidosis,¹⁰⁹ and their structures were analyzed by ¹H-NMR. Manz1→6Manz3→1Manβ1→4GlcNAc-ABEE (Man, GlcNAc,ABEE), Manz1→6Manz3→1Manβ1→4GlcNAc-ABEE (Man, GlcNAc,ABEE), Manz1→6Manz3→1Manβ1→4GlcNAc-ABEE (Man, GlcNAc,ABEE), Manz1→6Manz3→1Manβ1→4GlcNAc-ABEE (Man, GlcNAc,ABEE), Manz1→6Manz3→1Manβ1→4GlcNAc-ABEE (Man, GlcNAc,ABEE), Manz1→6Manz3→1Manβ1→4GlcNAc-ABEE (Man, GlcNAc,ABEE), Manz1→6Manz3→1Manβ1→4GlcNAc-ABEE (Man, GlcNAc,ABEE), Manz1→6Manz3→1Manβ1→4GlcNAc-ABEE (Man, GlcNAc,ABEE), Manz1→6Manz3→1Manβ1→4GlcNAc-ABEE (Man, GlcNAc,ABEE), Manz1→6Manz3→1Manβ1→4GlcNAc-ABEE (Man, GlcNAc,ABEE), Manz1→6Manz3→1Manβ1→4GlcNAc-ABEE (Man, GlcNAc,ABEE), Manz1→6Manz3→1Manβ1→4GlcNAc-ABEE (Man, GlcNAc,ABEE), Manz1→6Manz3→1Manβ1→4GlcNAc-ABEE (Man, GlcNAc,ABEE), Manz1→6Manz3→1Manβ1→4GlcNAc-ABEE (Man, GlcNAc,ABEE), Manz1→6Manz3→1Manβ1→4GlcNAc-ABEE (Man, GlcNAc,ABEE), Manz1→6Manz3→1Manβ1→4GlcNAc-ABEE (Man, GlcNAc,ABEE), Manz1→6Manz3→1Manβ1→4GlcNAc-ABEE (Man, GlcNAc,ABEE), Manz1→6Manz3→1Manβ1→4GlcNAc-ABEE (Man, GlcNAc,ABEE), Manz1→6Manz3→1Manβ1→4GlcNAc-ABEE (Man, GlcNAc,ABEE), Manz1→6Manz3→1Manβ1→4GlcNAc-ABEE (Man, GlcNAc,ABEE), and Manz1→6Manz3→1Manβ1→4GlcNAc-ABEE (Man, GlcNAc,ABEE). These were obtained with recombinant human β-galactosidase A (M. Ohta et al., manuscript submitted).

Liberation of N-linked oligosaccharides and preparation of ABE derivatives. N-Linked oligosaccharides were liberated from 50 mg of AOD by hydrazinolysis/re-N-acetylation as described.¹⁷ The liberated oligosaccharides were tagged with ABE by reductive amination, and then purified by chromatography first on a PRE-SEP C18 cartridge (Tessek, U.S.A.), followed by on a Bio-Gel P-4 column (200–400 mesh, 1.0 × 45 cm), as described previously.¹⁴,¹⁵,¹⁷

HPLC of ABE-oligosaccharides. HPLC was done with a Shimadzu LC-6A HPLC system. Anion-exchange HPLC was done on a TSKgel DEAE-SPW column (i.d. 1.6 × 7.5 cm, Tosoh Co., Japan) as described previously.¹⁸ Fractonation of neutral ABE-oligosaccharides was done either on a TSKgel Aminex-50 column (i.d. 0.8 × 7.5 cm, Toso Co.) or on a Wako gel SC-18 column (i.d. 0.4 × 25 cm, Wako Pure Chemical Industries) under the reported conditions.¹⁴ Two-dimensional mapping analysis (2D-HPLC) of the individual ABE-oligosaccharides was done as described previously.¹⁵,¹⁹

Monosaccharide analysis. Monosaccharide compositions were analyzed by gas liquid chromatography (GLC) of trimethylsilyl methyl glycosides obtained by methanalysis of native AOD in 1.5 M methanolic HCl.²⁰ The absolute configurations of oligosaccharides were identified by GLC of trimethylsilyl t-2-octyl glycosides according to the method of Takano et al.²¹ GLC of the trimethylsilyl methyl glycosides and trimethylsilyl t-2-octyl glycosides was done on a DB-5 fused silica capillary column (0.32 mm × 25 m, J&W Scientific, USA) in a Hewlett-Packard 5840A gas chromatograph. The column temperature was programmed from 50°C to 170°C at 20°C min, and then to 250°C at 3°C min.

Methylolation analysis. ABE-oligosaccharides were permethylated by the method of Ciucanu and Kerek.²² Each methylated sample was hydrolyzed, reduced, and acetylated by the method of Liang et al.,²³ and the resulting partially methylated alditol acetates were analyzed by gas chromatography/mass spectrometry (GC/MS HP-5, 0.25 mm × 100 m, Shimadzu Corp., Japan) as previously described.¹⁴ Authentic 1,4-di-O-acetyl-2,3,5,6-tetra-O-methylgalactitol was prepared from methyl-galactofuranoside in a similar manner to that described above.

Digestion with enzymes. Digestion of ABE-oligosaccharides with jack bean α-mannosidase, jack bean β-galactosidase, jack bean β-N-acetylhexosaminidase, and *A. arietii* α-mannosidase I, respectively, was done as described.¹⁴ Digestion with endo-β-N-acylglucosaminidase H was done as described by Mizuochi et al.²⁴

Partial acid hydrolysis. Mild acid hydrolysis of ABE-oligosaccharides, to hydrolyze galactofuranoside linkages but not hexopyranosidic linkages, was done in 0.01 N HCl (100°C, 30 min). Under these conditions, about 60% of the galactofuranoside (Gal/F) residues were selectively hydrolyzed without degradation of the ester linkages in ABE. Treatment with 0.02 N HCl at 100°C for 150 min was also done to hydrolyze galactofuranosidic linkages in free N-linked oligosaccharides, which were prepared from AOD by hydrazinolysis.¹⁶ The reaction mixture after mild acid hydrolysis was evaporated to dryness under reduced pressure and then the residue was repeatedly evaporated with water. The residue was then tagged with ABE and analyzed by 2D-HPLC.

Analysis of the isomeric structures of high-mannose type oligosaccharides. We recently found that separation of the isomeric structures of high-mannose type oligosaccharides with the same number of mannose residues can be done by 2D-HPLC analysis of ABE derivatives of oligosaccharides with a single GlcNAc residue at their reducing end (unpublished results). Thus, in this study, ABE-derivatives of high-mannose type oligosaccharides having a chonosaic structure at their reducing terminus were first digested with endo-β-N-acetylglucosaminidase H. The resulting counterparts having a single GlcNAc residue at the reducing terminus were analyzed by 2D-HPLC after ABE derivatization.

400 MHz ¹H-NMR spectrometry. ABE-oligosaccharides were repeatedly exchanged in H₂O (99.96 atom% D₂O), with intermediate lyophilization. The ¹H-NMR spectra were recorded with a Bruker AM 400 spectrometer operating at 400 MHz, with a probe temperature of 40°C. Chemical shifts (δ) are expressed in ppm relative to internal acetone in H₂O (δ = 2.21 ppm).

Fast-atom bombardment mass spectrometry. Positive ion fast-atom bombardment mass spectra (FAB-MS) were obtained with a JEOL JMS-HX 100 mass spectrometer as described elsewhere.¹⁴

Results

Monosaccharide analysis

Monosaccharide analysis of AOD showed that the enzyme contained mannose, galactose, and N-acetylglucosamine in a molar ratio of 7.2:3.4:1.0, suggesting that the enzyme consists of high-mannose type and unique galactose-containing high-mannose type oligosaccharides. On the basis of the carbohydrate content (10% (w/w)) and the molecular mass of the enzyme, it was estimated that AOD had four sugar chains per molecule.

Pattern of N-linked oligosaccharides in AOD

Oligosaccharides were liberated from AOD by hydrazinolysis/re-N-acetylation and then labeled with ABE.
 Upon anion-exchange HPLC on a DEAE-5PW column, the ABE-Oligosaccharides were separated into neutral (fraction N) and acidic fractions (fraction Al) in the molar ratio of 88.5 : 11.5 (Fig. 1). The elution position of Al corresponded to monophosphoryl high-mannose type oligosaccharides. However, no further structural analysis of this fraction was done due to the limited amounts of the sample. In this study, structural analysis of the oligosaccharides in fraction N was done. Upon size fractionation HPLC on an Amide-80 column, fraction N gave rise to twelve peaks, denoted as Na-Nl, as shown in Fig. 2. The relative proportions of the fractions were: Na, 13.1%; Nb, 2.4%; Ne, 4.9%; Nd, 2.1%; Ne, 29.5%; Nf, 10.3%; Ng, 16.0%; Nh, 2.5%; Ni, 7.0%; Nj, 1.4%; Nk, 7.1%; and Nl, 3.6%. Monosaccharide analysis of each fraction showed that Na, Ne, Ng, Nl, and Nk consisted of only mannose and N-acetylglucosamine, and fractions Ne, Nf, Nh, and Nj of galactose, mannose, and N-acetylglucosamine, suggesting that the former group was of the high-mannose type and the latter of a galactose-containing high-mannose type.

**Structures of high-mannose type oligosaccharides**

Upon two-dimensional mapping analysis, fractions Na, Ne, Ng, Nk, and Nl were eluted at the positions of standard Man_{3}GlcNAc_{2}-ABEE, Man_{4}GlcNAc_{2}-ABEE, Man_{5}GlcNAc_{2}-ABEE, Man_{6}GlcNAc_{2}-ABEE, and Man_{7}GlcNAc_{2}-ABEE, respectively (Fig. 3). These oligosaccharides were further characterized by sequential exoglycosidase digestion coupled with 2D-HPLC analysis. When fractions Ng, Nl, Nk, and Nl were digested with A. saitoi x-mannosidase I, they gave a single peak, with the same mobility as Ne, that comigrated with standard Man_{4}GlcNAc_{2}-ABEE (Fig. 3). Subsequent treatment of the product and Ne with jack bean x-mannosidase produced Man_{5}GlcNAc_{2}-ABEE. ABE-Oligosaccharide Na was stable as to A. saitoi x-mannosidase I digestion, but converted to Man_{5}GlcNAc_{2}-ABEE on jack bean x-mannosidase digestion. On the basis of these results together with their behavior on 2D-HPLC analysis, these oligosaccharides were established to be typical high-mannose type oligosaccharides with four to nine mannose residues, as shown in Table I.

Further characterization of the predominant oligosaccharides, Na, Ne, and Ng, was done by 400 MHz 1H-NMR analysis. The chemical shift values of structural reporter-group protons (Table I) were compared with those previously reported. Each fraction was found to contain...
<table>
<thead>
<tr>
<th>Residue</th>
<th>Ne</th>
<th>Ng</th>
<th>NF</th>
<th>Peak X</th>
</tr>
</thead>
<tbody>
<tr>
<td>ManαGlcNAc-2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>4.625</td>
</tr>
<tr>
<td>Man-3</td>
<td>4.760</td>
<td>4.765</td>
<td>4.756</td>
<td>4.762</td>
</tr>
<tr>
<td>Man-4</td>
<td>5.109</td>
<td>5.105</td>
<td>5.341</td>
<td>5.106</td>
</tr>
<tr>
<td>Man-4</td>
<td>4.897</td>
<td>4.870</td>
<td>4.871</td>
<td>5.035</td>
</tr>
<tr>
<td>Man-4</td>
<td>5.109</td>
<td>5.096</td>
<td>5.096</td>
<td>5.150</td>
</tr>
<tr>
<td>Man-4</td>
<td>4.908</td>
<td>4.908</td>
<td>4.908</td>
<td>4.919</td>
</tr>
<tr>
<td>Man-C</td>
<td>5.055</td>
<td>5.055</td>
<td>5.055</td>
<td>5.055</td>
</tr>
<tr>
<td>ManαGlcNAc-3</td>
<td>4.333</td>
<td>4.332</td>
<td>4.331</td>
<td>4.332</td>
</tr>
<tr>
<td>Man-3</td>
<td>4.215</td>
<td>4.224</td>
<td>4.215</td>
<td>4.225</td>
</tr>
<tr>
<td>Man-4</td>
<td>4.074</td>
<td>4.075</td>
<td>4.116</td>
<td>4.075</td>
</tr>
<tr>
<td>Man-4</td>
<td>4.127</td>
<td>4.140</td>
<td>4.140</td>
<td>4.137</td>
</tr>
<tr>
<td>Man-4</td>
<td>4.074</td>
<td>4.064</td>
<td>4.067</td>
<td>4.065</td>
</tr>
<tr>
<td>Man-4</td>
<td>3.988</td>
<td>3.988</td>
<td>3.988</td>
<td>3.988</td>
</tr>
<tr>
<td>Man-C</td>
<td>4.067</td>
<td>4.067</td>
<td>4.067</td>
<td>4.067</td>
</tr>
<tr>
<td>ManαGlcNAc-4</td>
<td>1.919</td>
<td>1.918</td>
<td>1.917</td>
<td>1.917</td>
</tr>
<tr>
<td>ManαGlcNAc-2</td>
<td>2.056</td>
<td>2.054</td>
<td>2.052</td>
<td>2.052</td>
</tr>
</tbody>
</table>

* See Fig. 2.

"The reference compound obtained from bovine β-mannosidosis kidney."

"Value could not be determined.

tain a single component based on the relative intensities of anomeric proton signals (data not shown). The assignments given in Table 1 are based on comparison with appropriate oligosaccharides.14,26,27 The structural reporter-group signals of oligosaccharide Ne completely matched those of the corresponding residues in Manz1-6-(Manz1-3)Manz1-6-Manz1-3)Manβ1-4GlcNAc-β1-4GlcNAc-ABEE. In the spectrum of Na, the H-1 signal for Man-4 showed a significant downfield shift and H-2 for Man-4 an upfield shift as compared to those for the corresponding residues in Ne, indicating the deletion of a Manz1-6-Man-B residue from Ne. Thus, oligosaccharide Na was identified as Manz1-3z1-6-Manz1-3)Manβ1-4GlcNAc-β1-4GlcNAc-ABEE (Table II). As to oligosaccharide Ng, the H-1 and H-2 signals for Man-4 underwent downfield shifts characteristic of the introduction of a Man-C into the z(1→2)-linkage at the C-2 position of oligosaccharide Ne. Furthermore, Man-C is characterized by its H-1 (δ = 5.055 ppm) and H-2 (δ = 4.067 ppm) signals. Thus, the structure of Ng was deduced to be as shown in Table II.

Since the amounts of Ni, Nk, and Ni were too low for 1H-NMR, their structures were further analyzed by the 2D-HPLC technique after their conversion to ABEE-oligosaccharides with a single GlcNAc residue at their reducing end. The endo-β-N-acetylgalactosaminidase H digest of fraction Ni gave two peaks corresponding to Manz1-2-Manz1-6-Manz1-3)Manz1-6-Manz1-2-Manz1-3)Manβ1-4GlcNAc-ABEE (Manz1-4-Manz1-1-ABEE) and Manz1-6-Manz1-3)Manz1-6-Manz1-2-Manz1-3)Manβ1-4GlcNAc-ABEE (Manz1-4-Manz1-3-ABEE) obtained from x-mannosidosis urine (data not shown) in a molar ratio of approximately 2:1 (Table II). The endo-β-N-acetylgalactosaminidase H digests of Nk and Ni each gave a single peak corresponding to Manz1-2-Manz1-6-Manz1-3)Manz1-6-Manz1-2-Manz1-3)Manβ1-4GlcNAc-ABEE and Manz1-6-Manz1-2-Manz1-3)Manβ1-4GlcNAc-ABEE, respectively. These results established the structures of fractions Nk and Ni to be as shown in Table II.

Structures of galactose-containing high-mannose type oligosaccharides

Positive ion FAB-MS of intact Ne, Nf, Nh, and Nj, which contained galactose in addition to mannose and N-acetylgalactosamine, had [M + Na]+ ions at m/z 1406, corresponding to Hex3HexNAc3-ABEE, m/z 1568, Hex3HexNAc2-ABEE, m/z 1730, Hex3HexNAc2-ABEE, and m/z 1892, Hex3HexNAc-ABEE, respectively. When Ne, Nh, and Nj were digested with jack bean z-mannosidase, they released 2, 3, 4, and 5 mannose residues, respectively, and gave an ABEE-oligosaccharide with the same mobility (oligosaccharide X; Fig. 4). These results suggested that the galactose-containing oligosaccharides had a common core structure. Positive ion FAB-MS and monosaccharide analysis indicated that oligosaccharide X had the composition, Gal-Man3GlcNAc-GlcNAc-ABEE (data not shown). Methylation analysis showed 2,3,5,6-tetra-O-methylgalactitol, 3,4,6-tri-O-methylmannitol, 2,3,4-tri-O-methylmannitol, and 3,6-di-O-methyl-2-N-methylacetalaminodideoxyglucitol in a molar ratio of 1:1:1:1:1, suggesting that the galactose residue was of the furanose form and linked to the C-2 position of the nonreducing terminal mannose residue of Manz1-6-Manβ1-4GlcNAc-ABEE. To examine the presence of a hexofuranosyl linkage, which is more acid-labile than a hexopyranosyl linkage, oligosaccharide X was hydrolyzed with mild acid and then the hydrolyzate was analyzed by HPLC. Since mild acid hydrolysis under the reported conditions (0.02 N HCl, 100 C,
Fig. 4. Two-Dimensional Mapping Analysis of the Monogalactosylated High-mannose Type ABEE-oligosaccharides and Their Products Obtained on α-Mannosidase Digestion and Mild Acid Hydrolysis.

Each ABEE-oligosaccharide was analyzed by HPLC on TSKgel Amide-80 and Wako soril SC18-200 columns, under the conditions given in Fig. 3. Arrows (→) and (←) indicate jack bean α-mannosidase digestion and mild acid hydrolysis (0.01 N HCl, 100 °C, 30 min), respectively, a, b, c, d, e, and f indicate the elution positions of authentic ABEE-oligosaccharides ManαGlNAc2-ABEE, Manβ-GlNAc2-ABEE, Manβα-GlNAc2-ABEE, Manα-GlNAc2-ABEE, Manβ-GlNAc2-ABEE, and Manα-GlNAc2-ABEE, respectively.

150 min)21 cleaved the ester linkage present in ABEE as well as the galactofuranose linkages, hydrolysis under milder conditions (0.01 N HCl, 100 °C, 30 min) was done for ABEE-oligosaccharide X. As shown in Fig. 4, approximately 60% of X was converted to an ABEE-oligosaccharide co-migrating with authentic Manx1→6Manβ1→4GlcNAcβ1→4GlcNAc-ABEE.

In a separate experiment, free oligosaccharides prepared from AOD by hydrazinolysis were hydrolyzed with mild acid under the reported conditions (0.02 N HCl, 100 °C, 150 min), and the hydrazylate was derivatized with ABEE before HPLC analysis. Peaks NC, NF, NH, and NJ in Fig. 2 were completely converted to Na, Ne, Ng, and Ni, respectively. It was, therefore, concluded that galactose was in the furanose form.

To identify the absolute configuration, free galactose obtained on mild acid hydrolysis was analyzed by GLC as the trimethylsilyl 1,2-octyl glycoside. It gave a single peak co-eluting with standard trimethylsilyl 1,2-octyl-d-galactoside, indicating the configuration of the galactose to be D.

To identify the anomeric configuration of the galactofuranose residue, oligosaccharide X was analyzed by 1H-NMR (Fig. 5). Relevant H-1 and H-2 chemical shifts for this oligosaccharide are listed in Table I together with the reference compound. The spectrum contains three anomeric proton signals of equal intensity, pointing to a single component. The H-1 signal for GlcNAc-1 was not recognizable due to the overlapping with H2O. The H-1 signal at δ=4.751 ppm and the H-2 signal at δ=4.073 ppm are indicative of the presence of β-linked Man (Man-3) substituted at its C-6 position (Table I). The signals at δ=5.045 ppm (J1,2=1.8 Hz) and δ=5.105 ppm (J1,2=2.0 Hz) can therefore be ascribed to H-1 of either Man-4' or Gal/f.

2D-COSY NMR analysis of oligosaccharide X showed a cross-peak between the signals at δ=5.045 ppm and δ=4.062 ppm. It had been reported that C-2 substitution of the nonreducing terminal mannose residue (Man-A) of Manαβ-GlNAc2 with α-galactofuranose or β-mannose gave rise to a large downfield shift (Δδ=0.42 ppm) of the H-1 for a mannose residue.26-29 However, no such large shift was observed on the C-2 substitution of the non-reducing terminal mannose residue (Man-4') of Manαβ-GlNAc2 by a galactofuranose residue (Table I), suggesting the anomeric form of Gal/f to be β. Substitution at C-2 of Man-4 or 4' by a β-GlNAc residue in complex type oligosaccharides causes only small increases in the shifts for their H-1 atoms (Δδ=0.011-0.015 ppm), but significant increases for their H-2 atoms (Δδ=0.12-0.14 ppm).30 In this instance, the signals at δ=5.045 ppm and δ=4.062 ppm are tentatively assigned to H-1 and H-2 for Man-4' substituted at C-2 by β-Gal/f, respectively, as compared to those for the corresponding residues in Manαβ-GlNAc2. The signal at δ=5.105 ppm can, therefore, be assigned to H-1 for the Gal/f residue. The coupling constant value of H-1 for the Gal/f residue (J1,2=2.0 Hz) is close to that of methyl β-galactofuranoside (J1,2=2.0 Hz), but different from those of methyl α-galactofuranoside (J1,2=3.7 Hz) and galactofuranose residues linked to Man-A in various high-mannose type oligosaccharides (J1,2=4.7 Hz).28,29 Taking these observations into account, the anomeric configuration of galactofuranose is proposed to be β. However, because the
galactofuranose residue may occur in several possible ring conformations, this assignment is tentative in the absence of an authentic standard. On the basis of these results, the common core oligosaccharide present in all galactose-containing oligosaccharides was characterized as Gal/β1→2Manz1→6Manβ1→4GlcNAcβ1→4GlcNAc-ABEE. The most prominent oligosaccharide, NF (Gal/Mann GlcNAc), was resistant to A. staito z-mannosidase I digestion. Upon mild acid hydrolysis, this oligosaccharide produced an ABE-oligosaccharide comigrating with authentic Man, GlcNAc-ABEE (Fig. 4), with the release of galactofuranose. The degalactosylated oligosaccharide released four mannose residues on digestion with jack bean z-mannosidase to produce Man, GlcNAc-ABEE, confirming that the degalactosylated oligosaccharide was a high-mannose type oligosaccharide with five mannose residues. Methylation analysis of NF showed the presence of 3 mol of 2,3,4,6-tetra-O-methylglactosyl, 1 mol of 2,3,5,6-tetra-O-methylgalactosyl, 1 mol of 2,4-di-O-methylmannitol, 1 mol of 4-monooacetamido-2-deoxyglucitol. From these results together with the results for oligosaccharide X, the structure of NF was assigned to be Manz1→(Manz1→3Gal/β1→2)Manz1→6(Manz1→3)Manβ1→4GlcNAcβ1→4GlcNAc-ABEE. H-1 NMR of NF is shown in Fig. 5. Six anomeric proton signals of equal intensity were observed. The three anomeric proton signals at δ = 4.762 ppm, δ = 5.106 ppm, and δ = 4.919 ppm can be assigned to H-1 of Man-3, Man-4, and Man-B, respectively, as compared to those of authentic Man, GlcNAc-ABEE. The signal at δ = 5.035 ppm is assigned to H-1 of Man-4 as compared to that of Gal/Mann, GlcNAc-ABEE. 2D-COSY NMR showed a cross-peak between the signals at δ = 5.035 ppm and δ = 4.137 ppm, as in the case of oligosaccharide X. The downfield shift (∆δ = 0.075 ppm) of the H-2 signal for Man-4 in comparison to that of Gal/β1→2Manz1→6Manβ1→4GlcNAcβ1→4GlcNAc-ABEE is indicative of substitution of this residue at O-3 and O-6 by Man-A and Man-B, respectively.26 The absence of a β(1→2)-linked β-galactofuranose residue is indicated by the broad broad line width at δ = 5.120 ppm with the coupling constant, J, 1,2 = 1.88 Hz. Another unusual signal at δ = 5.150 ppm, thus, can be assigned to H-1 of Man-A. Combining these results, it can be concluded that the structure of fraction NF is as shown in Table II.

The oligosaccharide in peak Ne was also resistant to A. staito z-mannosidase I. When Ne was hydrolyzed with mild acid, the ABE-oligosaccharide showed a peak shift corresponding to the release of one galactose residue to yield an ABE-oligosaccharide comigrating with authentic Man, GlcNAc-ABEE (Fig. 4). From these results, the structure of Ne was proposed to be as in Table II.

The ABE-oligosaccharide in peak Nh was converted to an ABE-oligosaccharide with the same mobility as Gal/Mann, GlcNAc-ABEE (oligosaccharide NF) on digestion with A. staito z-mannosidase I (Fig. 6). That the product was actually Gal/Mann, GlcNAc-ABEE was demonstrated by similar experiments to those for structural analysis of NF described above. To locate the z(1→2)-linked mannose residue, degalactosylated Nh obtained on mild acid hydrolysis was digested with endo-β-1-N-acetylgalactosaminidase H, followed by 2D-HPLC analysis. The endo-β-

![Fig. 6. Amide-80 HPLC Analysis of A. staito z-Mannosidase I Digests of the ABE-oligosaccharides in Peaks Ne and Nh.](image)

The digest of each fraction was subjected to HPLC on a TSKgel Amide-80 column under the conditions given in Fig. 2. When the batched peak material was subjected to z-mannosidase digestion, a peak corresponding to Gal/β1→2Manz1→6Manβ1→4GlcNAcβ1→4GlcNAc-ABEE appeared (shown by a dotted line). Arrows 1 and 2 indicate the same as in Fig. 2. Open arrows (3) indicate the elution positions of untreated ABE-oligosaccharides.

N-acetylgalactosaminidase H digest gave a peak at the same position as authentic Manz1→6(Manz1→3)Manβ1→6-(Manz1→2Manz1→3)Manβ1→4GlcNAcβ1→4GlcNAc-ABEE (Man, GlcNAc-ABEE-H-ABEE) (data not shown). These results were consistent with the proposed structure shown in Table II.

When the ABE-oligosaccharide in peak Nj was digested with A. staito z-mannosidase I, 50% of the fraction was converted to Gal/Mann, GlcNAc-ABEE with the release of two mannose residues, the other 50% remaining unchanged (Fig. 6). The endo-β-N-acetylgalactosaminidase H digest of degalactosylated Nj gave two peaks corresponding to Man, GlcNAc-1-ABEE and Man, GlcNAc-1-III-ABEE in a molar ratio of 2:1. These results suggested that this fraction contained the two isomers shown in Table II and a small amount of uncharacterized oligosaccharides.

**Discussion**

This study established the structures of N-linked oligosaccharides derived from AOD from a fungal strain, *Acromonium* sp. HI-25. AOD was found to only have N-linked oligosaccharide chains. Oligosaccharides were liberated from the enzyme by hydrazinolysis and then tagged with ABE. Among the total ABE-derivatized oligosaccharides from AOD, approximately 90% were neutral. The neutral oligosaccharides were resolved into twelve subfractions on normal-phase HPLC, the structures of which were identified by FAB-MS, 2D-HPLC analysis, 1H-NMR, methylation analysis, mild acid hydrolysis, and exoglycosidase digestion. In Table II, the structures are compiled together with their relative amounts. The results obtained in this study show that AOD from *Acromonium* sp. HI-25 contains two types of oligosaccharides, a series of high-mannose type oligosaccharides, Man, GlcNAc, (76.3%), and a series of β-galactofuranose-containing high-mannose type oligosaccharides, Gal/Mann, GlcNAc, (18.6%). Galactofuranose was linked to Man-4 in various high-mannose type oligosaccharides via a β(1→2)-linkage.
Table II. Proposed Structures of Neutral N-Linked Oligosaccharides of *Acremonium* sp. Ascorbate Oxidase

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Structure</th>
<th>Relative amount (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Na</strong></td>
<td>Galβ1/β1</td>
<td>13.1</td>
</tr>
<tr>
<td></td>
<td>Manβ1→4GlcNAcβ1→4GlcNAc</td>
<td></td>
</tr>
<tr>
<td><strong>Ne</strong></td>
<td>Galβ1/β1</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td>Manβ1→4GlcNAcβ1→4GlcNAc</td>
<td></td>
</tr>
<tr>
<td><strong>Nc</strong></td>
<td>Galβ1/β1</td>
<td>29.5</td>
</tr>
<tr>
<td></td>
<td>Manβ1→4GlcNAcβ1→4GlcNAc</td>
<td></td>
</tr>
<tr>
<td><strong>Nf</strong></td>
<td>Galβ1/β1</td>
<td>10.3</td>
</tr>
<tr>
<td></td>
<td>Manβ1→4GlcNAcβ1→4GlcNAc</td>
<td></td>
</tr>
<tr>
<td><strong>Ng</strong></td>
<td>Galβ1/β1</td>
<td>16.0</td>
</tr>
<tr>
<td></td>
<td>Manβ1→4GlcNAcβ1→4GlcNAc</td>
<td></td>
</tr>
<tr>
<td><strong>Nh</strong></td>
<td>Galβ1/β1</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>Manβ1→4GlcNAcβ1→4GlcNAc</td>
<td></td>
</tr>
<tr>
<td><strong>Ni</strong></td>
<td>Galβ1/β1</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>Manβ1→4GlcNAcβ1→4GlcNAc</td>
<td></td>
</tr>
<tr>
<td><strong>Nj</strong></td>
<td>Galβ1/β1</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>Manβ1→4GlcNAcβ1→4GlcNAc</td>
<td></td>
</tr>
<tr>
<td><strong>Nk</strong></td>
<td>Galβ1/β1</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>Manβ1→4GlcNAcβ1→4GlcNAc</td>
<td></td>
</tr>
<tr>
<td><strong>Nl</strong></td>
<td>Galβ1/β1</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>Manβ1→4GlcNAcβ1→4GlcNAc</td>
<td></td>
</tr>
</tbody>
</table>

*See Fig. 2.*
The presence of D-galactose of the furanose form is highly unusual. D-Galactose found in mammalian glycoproteins is in the pyranose form and present in complex type oligosaccharides with the sequence of Gal-GlcNAc-Man. In contrast, D-galactose residues in AOD are of the furanose form and present in high-mannose type oligosaccharides.

The presence of galactofuranose in N-linked oligosaccharides was first reported in protozoa, *Crithidia fasciculata* and *Crithidia homarsa*, and trypanosomatid flagellates. In these protozoa, D-galactofuranose was found to be linked to the nonreducing ends of high-mannose type oligosaccharides with the composition of Gal-Man, GlcNAc. The galactofuranose was of the β-anomeric form from its sensitivity to β-galactofuranosidase from *Penicillium charlesi*. However, the position of its linkage to a mannose residue has not been identified. Morais et al. subsequently reported that galactofuranose-containing high-mannose type oligosaccharides with compositions of Galα-Man,6GlcNAc2 and Galα-Man,8GlcNAc2 occurred in glycoproteins of the parasitic protozoan *Leptomonas suameli*. In this case, the galactose residue was found to be attached to the C-2 position of a mannose at the nonreducing end. The anomeric configuration has not been identified. Groisman and de Lederkremer reported that galactofuranose-containing oligosaccharides were also present in the fungus *Ascoschlorella furfuraceus*. On methylation analysis and 13C-NMR, it was found that the galactofuranose residues in *A. furfuraceus* were of the β-anomeric form and linked to position 2 of the mannose residue in the oligosaccharides sensitive to endo-β-N-acetylglucosaminidase. Recently, galactofuranose-containing high-mannose type oligosaccharides with similar structures to those of protozoan oligosaccharides were reported in an α-galactosidase from the fungus *Aspergillus niger*. In *A. niger*, the galactofuranose residues were found to be linked to nonreducing mannose (Man-A) of Man9GlcNAc2 via an α-(1→2)-linkage. Interestingly, the galactofuranose residues in *Acremonium* sp. AOD oligosaccharides were not linked to external mannose but internal mannose residue (Man-4), in contrast to all other reported cases. Furthermore, the anomeric configuration of the galactofuranose in *Acremonium* sp. AOD oligosaccharides differed from that of *A. niger* enzyme oligosaccharides.

It should be noted that the galactofuranose-containing oligosaccharides from *Leptomonas suameli* were sensitive to endo-β-N-acetylglucosaminidase but those from *Acremonium* sp. AOD reported in this study were resistant to it. Maley et al. reported that endo-β-N-acetylglucosaminidase required for hydrolysis the minimum structural unit, Man α1→3Man α1→6Man β1→4GlcNAcβ1→4GlcNAc. In addition, Yamashita reported that the presence of an α-(1→3)-linked mannosic residue (Man-A) to an α-(1→6)-linked mannosidic residue (Man-4) was essential for a substrate of the enzyme. Interestingly, the structures of the galactofuranose-containing oligosaccharides from *Acremonium* AOD met these requirements, they were not hydrolyzed under these conditions (0.1 units of the enzyme, 37 °C, 16 h). These results indicated that the attachment of Gal to the internal mannose residue (Man-4) via β-(1→2)-linkage inhibited the action of endo-β-N-acetylglucosaminidase.

The oligosaccharide structures of the fungus, *Acremonium* sp., AOD differ markedly from those of plant AOD. The biosynthesis and biological functions of galactofuranose-containing high-mannose type oligosaccharides in *Acremonium* sp. remain unknown.

References