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Structures of Oligosaccharides on β-Galactosidase from *Aspergillus oryzae*[1]

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The carbohydrate portions of β-galactosidase from *Aspergillus oryzae* were found to be composed of two types of sugar chains. They were released equally well with endo-β-*N*-acetylglucosaminidase H, but were distinct in their chain length. The long sugar chains (fraction I), corresponding to 4% of the total carbohydrate chains, were composed of galactomannan-type oligosaccharides, which consisted of mannose, galactose, glucose, and glucosamine in the molar ratios of 30.0, 16.4, 1.4, and 2.1 per mol of aspartic acid, respectively. The short sugar chains (fraction II), corresponding to 96% of the total carbohydrate chains, consisted of mannose, galactose, glucose, and glucosamine in the molar ratios of 9.4, 0.6, 0.3, and 1.7 per mol of aspartic acid, respectively. Both types of sugar chains were fractionated into neutral and acidic subfractions. The neutral subfraction of fraction I (I-N), corresponding to 1% of the total carbohydrate chains, was very heterogeneous in length and was resistant to digestion with α-mannosidase and β-galactosidase. The neutral subfraction of fraction II (II-N), corresponding to 91% of the total carbohydrate, was composed of a mixture of oligosaccharides with oligomannoside chains ($Man_nGlcNAcol$). The major components were similar to high mannose-type oligosaccharides of mammalian origin in their composition and size ($n=5$–9). However, digestion of II-N with α1,2-mannosidase produced considerable amounts of $Man_6GlcNAcol$, an unusual product in the case of high mannose-type oligosaccharides of mammalian origin, in addition to the common one, $Man_5GlcNAcol$. The following structure is proposed for $Man_6GlcNAcol$:

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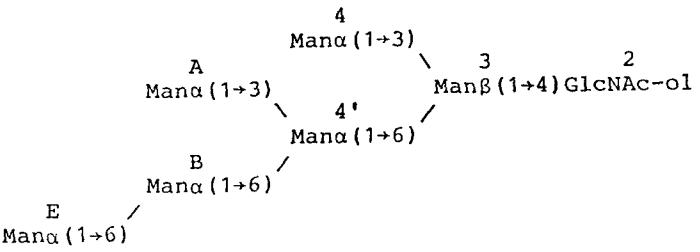


The acidic subfraction of fraction I, corresponding to 1.3% of the total carbohydrate chains, contained phosphate in mono- and diester linkages, whereas the acidic subfraction of fraction II, corresponding to 3% of the total carbohydrate chains, contained phosphate only in monoester linkages.

It has been shown that the recognition and uptake of lysosomal enzymes by various cells require the occurrence of recognition markers on lysosomal enzymes. These markers are believed to be oligomannoside-type oligosaccharides or their phosphorylated derivatives (*1–3*). These findings have improved the prospects of developing an enzyme replacement therapy for lysosomal storage diseases.

We have previously isolated β-galactosidase from *Aspergillus oryzae*. The enzyme is capable of degrading many galactose-containing glycoconjugates at a low pH, like endogenous lysosomal β-galactosidases, and can be prepared on a large scale (*4*, *5*). This prompted us to examine the possibility of using this enzyme for the enzyme replacement therapy of β-galactosidosis such as GM_1-gangliosidosis and Krabbe disease. For this, however, it is necessary to identify the structures of the carbohydrate moiety which serve as recognition markers for cellular uptake.

Therefore, we have undertaken to determine the carbohydrate structures of β-galactosidase from *A. oryzae* using enzymatic analysis with exoglycosidases and 500-MHz ^{1}H-NMR spectroscopy. The results are presented in this paper.

EXPERIMENTAL PROCEDURES

Materials—Bio-Gel P-2 (minus 400 mesh), Bio-Gel P-4 (minus 400 mesh), AG 1×2 (100–200 mesh) and AG 50W×4 (100–200 mesh) were purchased from Bio-Rad Laboratories, Richmond. Sephadex G-25 (fine) and Sephadex G-50 (fine) were from Pharmacia, Uppsala. NaB^3H_4 (469 mCi/mmol) was obtained from the Radiochemical Centre, Amersham. $Man_9GlcNAcol$ was prepared from α-mannosidase of porcine kidney as described previously (*6*). $Man_7GlcNAcol$, $Man_6GlcNAcol$, and $Man_5GlcNAcol$ were prepared from cathepsin D of porcine spleen as described previously (*7*). Manβ1→4GlcNAcol was obtained from $Man_5GlcNAcol$ by exhaustive digestion with jack bean α-mannosidase.

β-Galactosidase from *A. oryzae* strain RT102 (*4*, *5*) was kindly obtained from Tokyo Tanabe Co., Tokyo. Pronase P was purchased from Kaken Kagaku Co., Tokyo. Endo-β-*N*-acetylglucosaminidase H (endo-H) from *Streptomyces griseus* and jack bean β-*N*-acetylhexosaminidase were from Seikagaku Kogyo Co., Tokyo. Alkaline phosphatase from *Escherichia coli* was from Sigma, St. Louis. α1,2-Specific mannosidase from *Aspergillus saitoi* (*8*, *9*) was kindly provided by Dr. Eiji Ichishima, Tokyo Noko University, Tokyo. Jack bean α-mannosidase was prepared according to Li and Li (*10*) and β-mannosidase from a snail, *Achatina fulica*, according to Sugahara and Yamashina (*11*).

Methods—Preparation of glycopeptides: Preparation of glycopeptides from *A. oryzae* β-galactosidase was performed essentially as described previously (*7*).

Gel permeation chromatography: The Bio-Gel P-2 column (1.8×190 cm) and Bio-Gel P-4 column (1.6×180 cm) were developed with water. These column chromatographies were performed at 55°C using columns equipped with water jackets. Calibration of the column was carried out with glucose

oligomers and oligosaccharides of known structures, *e.g.* $Man_{5-9}GlcNAcol$ and $Man\beta1\rightarrow4GlcNAcol$ (*12*).

Paper chromatography: Descending paper chromatography was performed with Toyo No. 51A paper using the following solvents: I, ethylacetate–pyridine–acetic acid–H_2O (5 : 5 : 1 : 3, v/v); II, nitromethane–ethanol–acetic acid–sat. boric acid (8 : 1 : 1 : 1, v/v).

Enzymatic digestions: Digestions with Pronase P, endo-H, jack bean α-mannosidase, *A. saitoi* α-mannosidase, snail β-mannosidase, and *E. coli* alkaline phosphatase were carried out as described previously (*7*). *A. oryzae* β-galactosidase digestion was performed with 2.6 units of the enzyme in 50 μl of 0.2 M citric acid–0.4 M Na_2HPO_4 buffer, pH 4.5, for 48 h at 37°C under a toluene atmosphere.

Analytical methods: Hexose was determined the orcinol-H_2SO_4 method of Hewitt (*13*). Sialic acid was determined by the resorcinol method of Jourdian *et al.* (*14*). Hexosamine was determined with a Hitachi amino acid analyzer, model 835, after hydrolysis of the samples in 6 M HCl at 100°C for 16 h. Neutral sugars were separated and estimated by mass-fragmentography after conversion into alditol acetates as described previously (*15*). Radioactivity was measured with a Beckman liquid scintillation spectrophotometer, model LS7500, using a toluene-Triton X-100 scintillation mixture (*16*). Peptides were determined by the fluorescamine method of Nakai *et al.* (*17*).

500 MHz ^{1}H-NMR spectroscopy: The conditions for 500-MHz ^{1}H-NMR spectroscopy were as described in a previous paper (*7*).

RESULTS

Carbohydrate Composition of β-Galactosidase copeptides—Purified β-galactosidase from *A. oryzae* was found to contain hexose (8.6%) and *N*-acetylglucosamine (1.25%), but no sialic acid. The β-galactosidase (8.0 mg of protein) was exhaustively digested with Pronase P under conditions where the activities of phosphatase and phosphodiesterase detectable in the protease were completely inhibited (*7*). The digest was fractionated on a Sephadex G-50 column (1.8×119 cm) into three hexose fractions (Fig. 1). Fractions I and II contained the glycopeptides which

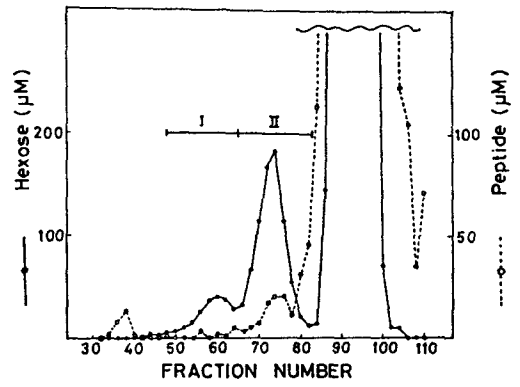


Fig. 1. Sephadex G-50 column chromatography of the pronase digest of β-galactosidase. The digest was applied to a Sephadex G-50 (fine) column (1.8×119 cm) which was then developed with 0.05 M pyridine-acetic acid buffer, pH 5.0. Fractions of 3.4 ml were collected and aliquots thereof were used for monitoring hexose (●) and peptide (○). The results are expressed as mannose and leucine equivalents, respectively. Fractions I and II were pooled as indicated by bars.

originated from β-galactosidase, and the third was identified as glucose 6-phosphate, which was added to the reaction mixture prior to the Pronase digestion in order to inhibit phosphatase(s) and phosphodiesterase(s) contaminating the protease (*7*).

Fraction I contained 15% of the total hexose and 4% of the total glucosamine of β-galactosidase, while fraction II contained 85 and 96%, respectively. The carbohydrate compositions of the two fractions are shown in Table I. The high contents of mannose and galactose in fraction I suggest that galactomannan-type oligosaccharides are dominant in this fraction. On the other hand, fraction II contained mannose as the major hexose, suggesting that the fraction consists predominantly of oligomannoside-type oligosaccharides.

Isolation of Oligosaccharides—Fractions I and II were digested with endo-H. Following isolation by gel filtration on a Sephadex G-25 column, each oligosaccharide was reduced with NaB^3H_4 as described previously (*7*). The resulting reduced oligosaccharides were fractionated on an AG 1×2 (Cl^- form) column (0.9×22 cm) after *N*-acetylation of NH_2-termini of the contaminating peptides. Elution was carried out in a stepwise manner with 0.01 M HCl, followed by 1 M NaCl. As shown in Fig. 2A, the oligosaccharides obtained from frac-

TABLE I. Carbohydrate and amino acid compositions of glycopeptides obtained from β-galactosidase. Analyses were carried out as described under "EXPERIMENTAL PROCEDURES." No corrections were made for loss during hydrolysis.

	Molar ratio[a]	
	Fraction I	Fraction II
Amino acid		
Aspartic acid	1.00	1.00
Threonine	0.24	0.17
Serine	0.35	0.13
Glutamic acid	0.07	0.14
Glycine	0.78	0.27
Alanine	0.09	0.09
Sugar		
Mannose	30.0	9.42
Galactose	16.4	0.59
Glucose	1.4	0.30
Glucosamine	2.1	1.69

[a] Values are molar ratios to aspartic acid.

tion I were separated into three fractions, *i.e.* I-N, I-A-1, and I-A-2, which accounted for 25, 35, and 40%, respectively, of the total ^{3}H-radioactivity. In a similar manner, the oligosaccharides from fraction II were separated into three fractions, *i.e.* II-N, II-A-1, and II-A-2, which accounted for 44, 39, and 17%, respectively, of the total ^{3}H-radioactivity, (Fig. 2B). However, when we estimated the number of oligosaccharide chains on the basis of ^{3}H-radioactivity recovered as GlcNAcol on paper chromatography after hydrolysis of each fraction followed by re-*N*-acetylation, it was shown that fraction I (long chains) and fraction II (short chains) accounted for 4 and 96% of the total oligosaccharide chains, respectively. The same calculation indicated that fractions I-N, I-A, II-N, and II-A accounted for 1, 1.3, 91, and 3% of the total chains, respectively. The amount of carbohydrate chains recovered in fraction II-A was much less than that deduced from the total radioactivity recovered in the fraction, suggesting that the large majority of the radioactivity recovered in II-A can be ascribed to peptide moieties.

Characterization of II-N—II-N, comprising 95% of the total hexose of fraction II, was further

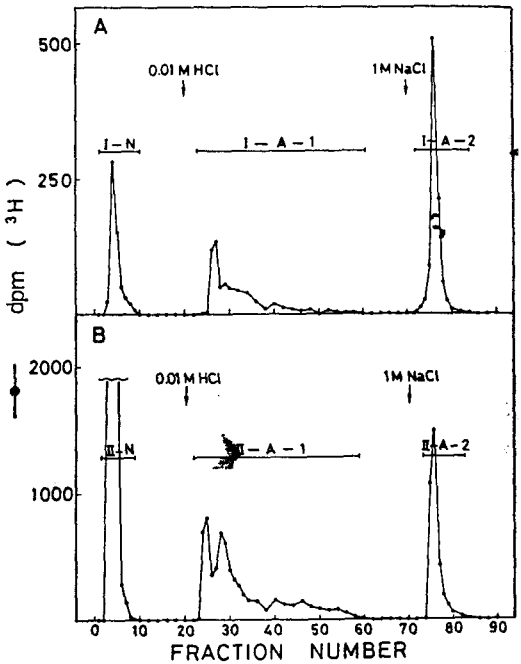


Fig. 2. AG 1×2 chromatography of the endo-H digests. The endo-H digests, after reduction with NaB^3H_4, were applied to an AG 1×2 (Cl^- form) column (0.9×22 cm). Elution was carried out in a stepwise manner with 0.01 M HCl and 1.0 M NaCl as indicated by arrows. Fractions of 2.0 ml were collected and aliquots thereof were used for monitoring radioactivity. A, endo-H digest of fraction I; B, endo-H digest of fraction II.

fractionated by paper chromatography on Toyo No. 51A paper with solvent I. As shown in Fig. 3A, II-N was separated into one major and three minor components, *i.e.* II-N-1, and II-N-2, II-N-3, and II-N-4, respectively. The major component, II-N-1, was re-chromatographed using the same solvent system for a longer time to produce three subfractions, *i.e.* II-N-1-1, II-N-1-2, and II-N-1-3 (Fig. 3B). On these paper chromatographies II-N-1-2, II-N-2, II-N-3, and II-N-4 migrated to positions corresponding to those of Man_9-GlcNAcol, Man_7GlcNAcol, Man_6GlcNAcol, and Man_5GlcNAcol, respectively.

These purified fractions were subjected to sequential exoglycosidase digestions, and the reaction products at each step were analyed by gel filtration on a Bio-Gel P-2 column. Upon digestion with α1,2-mannosidase, 91% of II-N-1-2,

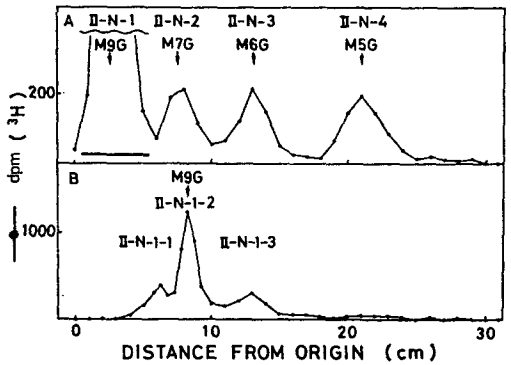


Fig. 3. Paper chromatography of II-N. II-N was subjected to paper chromatography, in which the paper was developed for 15 days with solvent I (A). The fractions indicated by bars in A were combined (II-N-1) and re-chromatographed for 44 days with the same solvent (B). The paper was cut into 0.5 cm segments and each was extracted with 0.05 M pyridine-acetic acid buffer, pH 5.0. Aliquots of each extract were used for monitoring radioactivity. The arrows denote positions of migration of standard oligosaccharides (M9G, $Man_9GlcNAcol$; M7G, $Man_7GlcNAcol$; M6G, $Man_6GlcNAcol$; M5G, $Man_5GlcNAcol$).

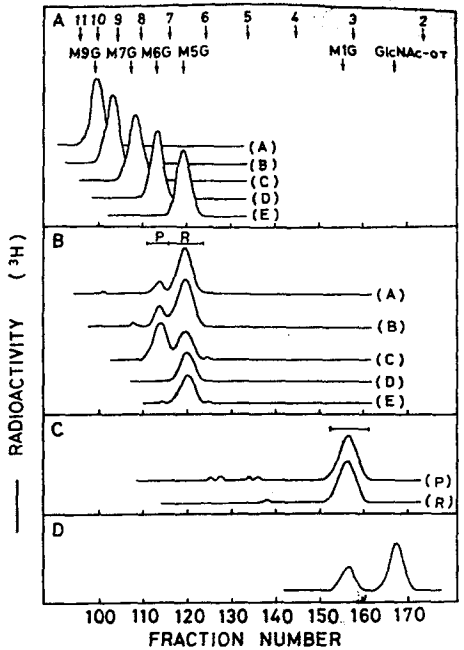


Fig. 4. Gel permeation chromatography of the oligosaccharides of II-N and their exoglycosidase digests. The radioactive oligosaccharides were applied to a Bio-Gel P-2 (minus 400 mesh) column (1.8×190 cm). Fractions of 1.9 ml were collected and aliquots thereof were used for monitoring radioactivity. A, II-N-1-2 (A), II-N-1-3 (B), II-N-2 (C), II-N-3 (D), and II-N-4 (E); B, α1,2-mannosidase digests of (A), (B), (C), (D), and (E) in panel A; C, jack bean α-mannosidase digests of Ps and Rs in Panel B; D, β-mannosidase digest of the mixture of (P) and (R) in Panel C. The arrows denote the elution positions of glucose oligomers, with numbers indicating the numbers of glucose units, and authentic oligosaccharides (M9G, $Man_9GlcNAcol$; M7G, $Man_7GlcNAcol$; M6G, $Man_6GlcNAcol$; M5G, $Man_5GlcNAcol$; M1G, ManGlcNAcol).

84% of II-N-1-3, 34% of II-N-2, and all of II-N-3 and II-N-4 were converted into a peak R that was eluted at the position corresponding to that of $Man_5GlcNAcol$; the remainder of each of the first three fractions appeared in a peak P that was eluted at the position corresponding to that of $Man_6GlcNAcol$ (Fig. 4B). All the α1,2-mannosidase digests, fractions P and R, were, in turn, converted to ManGlcNAcol on treatment with jack bean α-mannosidase, and then to GlcNAcol with β-mannosidase (Figs. 4, C and D). Nearly complete digestion with β-mannosidase was accomplished on repeated treatments (data not shown). Based on these lines of evidence and taking the specificity of endo-H (*18*) into account, II-N-1-2, II-N-1-3, II-N-2, and II-N-3 and II-N-4 could be deduced to be mixtures of $(Man\alpha1\rightarrow2)_n\alpha Man_4\beta ManGlcNAcol$ and $(Man\alpha1\rightarrow2)_{n-1}\alpha Man_5\beta ManGlcNAcol$ ($n=4$, 3, and 2 for II-N-1-2, II-N-1-3, and II-N-2, respectively) and II-N-3 and II-N-4 to be $(Man\alpha1\rightarrow2)_n\alpha Man_4\beta ManGlcNAcol$ ($n=1$ and 0 for II-N-3 and II-N-4, respectively).

The digestion of II-N-1-1 with mannosidases gave more complex results. As shown in Fig. 5A, this fraction was eluted on gel filtration as a single peak near the position of 11.5 glucose units. Treatment of this oligosaccharide with α1,2-mannosidase gave two peaks, *i.e.* A (83%) and B (17%), at the positions of 9.5 and 7.5 glucose units, respectively (Fig. 5B). Upon digestion with jack bean α-mannosidase, fraction B was degraded to ManGlcNAcol (Fig. 5D), while fraction A produced two peaks, C and D (Fig. 5C). These two components could no longer be degraded by treatment with relevant glycosidases (*A. oryzae* β-galactosidase, jack bean β-*N*-acetylhexosaminidase, yeast α-glucosidase, and almond β-gluco-

sidase). Neutral sugar analyses showed that fraction C contained on average 3.2 residues of mannose and 3.3 residues of glucose per oligosaccharide molecule, and fraction D contained 3.4 and 2.1, respectively. These results suggested that II-N-1-1 was a mixture of $(Man\alpha1\rightarrow2)_4$ or ${}_5\alpha Man_5\beta ManGlcNAcol$ and similar oligosaccharides containing glucose residues. The results of these studies on II-N oligosaccharides are summarized in Table II.

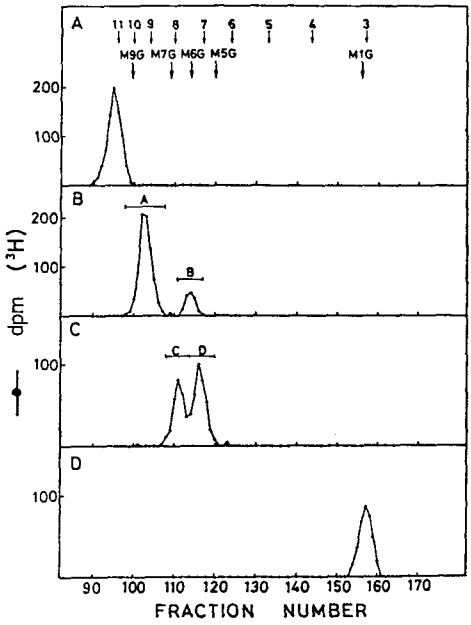
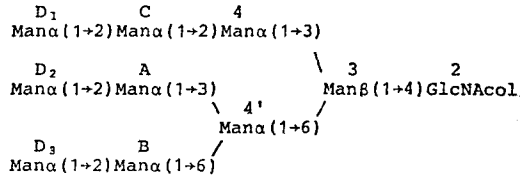


Fig. 5. Gel permeation chromatography of II-N-1-1 and its glycosidase digests. Analytical conditions for radioactive sugars are the same as in Fig. 4. A, II-N-1-1; B, α1,2-mannosidase digest of II-N-1-1; C, jack bean α-mannosidase digest of peak A in Panel B; D, jack bean α-mannosidase digest of peak B in Panel B.

Further characterizations of II-N-1-2, II-N-1-3, II-N-2, II-N-3, and II-N-4 were carried out with the use of 500-MHz ^{1}H-NMR spectroscopy. The chemical shifts of the structural reporter groups of these compounds are compiled in Table III. The ^{1}H-NMR spectrum of II-N-1-2 confirmed it to be a $Man_9GlcNAcol$ compound. Comparison of the NMR data for II-N-1-2 and a known $Man_9GlcNAcol$(N-1) (*6*) indicated that the structures were identical. The structure of II-N-1-2 is:



No isomeric $Man_9GlcNAcol$ compounds could be detected by ^{1}H-NMR spectroscopy.

Fraction II-N-1-3 is a mixture of $Man_8GlcNAcol$ compounds. In the ^{1}H-NMR spectrum of II-N-1-3 the major signals were attributed to the main compound. The signal at δ 5.304 ppm points to Man-C substituted with Man-D_1; the signal at δ 5.142 ppm indicates Man-B substituted with Man-D_3 and the signal at δ 5.132 ppm is charac-

TABLE II. Structures of major oligosaccharides (II-N) of β-galactosidase.

Fraction	%[a]	Structure	%[b]
II-N-1-1	(15)	$(Man\alpha1\rightarrow2)_4$ or ${}_5\alpha Man_5\beta ManGlcNAc_2$	(17)
		glucose containing oligosaccharides	(83)
II-N-1-2	(45)	$(Man\alpha1\rightarrow2)_3\alpha Man_5\beta ManGlcNAc_2$	(9)
		$(Man\alpha1\rightarrow2)_4\alpha Man_4\beta ManGlcNAc_2$	(91)
II-N-1-3	(20)	$(Man\alpha1\rightarrow2)_2\alpha Man_5\beta ManGlcNAc_2$	(16)
		$(Man\alpha1\rightarrow2)_3\alpha Man_4\beta ManGlcNAc_2$	(84)
II-N-2	(7)	$Man\alpha1\rightarrow2\alpha Man_5\beta ManGlcNAc_2$	(66)
		$(Man\alpha1\rightarrow2)_2\alpha Man_4\beta ManGlcNAc_2$	(34)
II-N-3	(8)	$Man\alpha1\rightarrow2\alpha Man_4\beta ManGlcNAc_2$	(100)
II-N-4	(5)	$\alpha Man_4\beta ManGlcNAc_2$	(100)

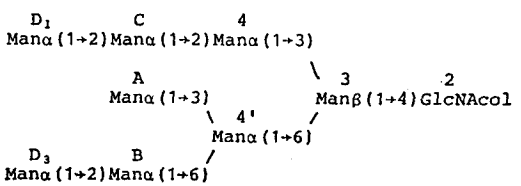
[a] Percentages of the total amount of fraction II-N. [b] Percentages of the total amount of each subfraction of II-N.

TABLE III. ^{1}H-Chemical shifts of structural reporter-group protons of the constituent monosaccharides for the major neutral oligosaccharide alditols derived from *A. oryzae* β-galactosidase.

Reporter group	Residue	Chemical shift[a] in[b] II-N-1-2	II-N-1-3	II-N-2	II-N-3	II-N-4
		D_1-C-4 \ D_2-A \ 3-2-ol, 4′ / D_3-B	D_1-C-4 \ A \ 3-2-ol, 4′ / D_3-B	C-4 \ A \ 3-2-ol, 4′ / B / E	C-4 \ A \ 3-2-ol, 4′ / B	4 \ A \ 3-2-ol, 4′ / B
H-1	4	5.332	5.333	5.340	5.343	5.103
	4′	4.873	4.881	4.881	4.879	4.882
	A	5.393	5.132	5.138	5.137	5.136
	B	5.142	5.142	4.905	4.906	4.908
	C	5.304	5.304	5.053	5.054	—
	D_1	5.045	5.040	—	—	—
	D_2	5.045	—	—	—	—
	D_3	5.039	5.040	—	—	—
	E	—	—	4.928	—	—
NAc	2-ol	2.059	2.059	2.059	2.058	2.059

[a] Chemical shifts are given in ppm downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS), but were actually measured relatively to internal acetone (δ2.225 ppm), in D_2O at 27°C. [b] For complete structures of the compounds, see the text.

teristic of Man-A in a terminal position. Therefore the structure of the main component of II-N-1-3 is:



Furthermore, in the spectrum of II-N-1-3 low intensity signals were present at δ 5.397, δ 4.905, and δ 5.056 ppm, which were attributed to the H-1 protons of Man-A substituted with Man-D_2, Man-D_3 terminal and Man-C terminal, respectively. In addition, a low intensity signal was present at δ 4.928 ppm. Such a chemical shift is in the range of an α(1→6) linked Man residue in a terminal position. For example, the H-1 signals of Man-4′ and Man-B in a terminal position appear at 4.915 and 4.907 ppm, respectively. Therefore, the signal at δ 4.928 ppm must be attributed to an α(1→6) linked Man residue that is not known to occur in oligomannoside-type chains derived from plants or animal tissues but which might well be present in fungus-derived oligosaccharides. In analogy with oligosaccharides from yeast (*19*) this unusual Man residue (denoted Man-E) may be linked to Man-B (see also the structure proposed for II-N-2).

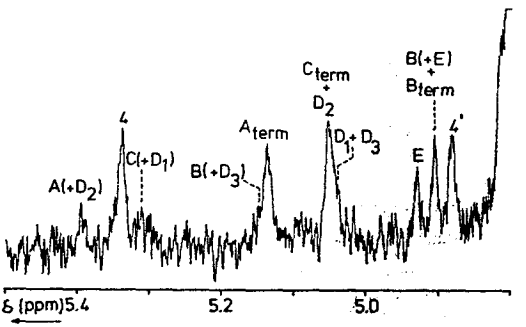
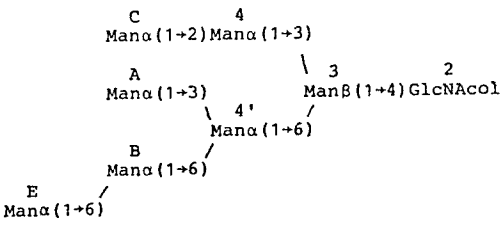


Fig. 6. The 500-MHz ^{1}H-NMR spectrum (D_2O, 27°C) of II-N-2. The numbers and letters in the spectrum refer to the corresponding residues in the structure as shown in Table III and the text.

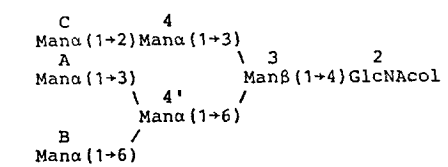
The ^{1}H-NMR spectrum of II-N-2 is shown in Fig. 6. It indicates the presence of an oligomannoside-type chain showing heterogeneity with

respect to the outer α-linked Man residues. All compounds of II-N-2 share the Man_6 moiety consisting of Man-3, -4, -4′, -A, -B, and -C. Furthermore, the spectrum contains a main signal at δ 4.928, attributed to the major component, and, as in II-N-1-3, this was interpreted as belonging to Man-E. Therefore, the following structure is proposed for the major compound of II-N-2:

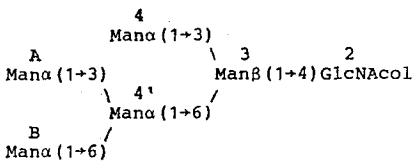


In the minor components of II-N-2, the Man_6 moiety is extended with Man-D_1, -D_2, or -D_3 instead of E. The structures of II-N-3 and II-N-4 could readily be identified by comparison of their NMR data with those of N-2 and N-3 from α-mannosidase (*6*). The structures are:

II-N-3



and II-N-4



Characterization of II-A-1 and II-A-2—In order to determine whether the net negative charges of II-A-1 and II-A-2 were due to phosphate groups or not, each fraction was digested with alkaline phosphatase with or without prior hydrolysis in mild acid (10 mM HCl, 100°C, 30 min) as described previously (*7*). It was found that II-A-1 contained small amounts of oligosaccharides with phosphate residues in monoester linkages (3% of the total carbohydrate of the enzyme) and II-A-2 contained no residue with phosphate. Most of the ^{3}H-radioactivities found in II-A-1 and II-A-2 may be attributable to contaminating peptides, as reported previously (*6, 7*). In fact, upon hydrolysis followed by re-*N*-acetylation, only small portions of ^{3}H-radioactivity of II-A-1 and none of II-A-2 could be identified, on paper chromatography with solvent II, as GlcNAcol that should have originated from the reducing termini of the oligosaccharides.

Characterization of I-N, I-A-1, and I-A-2—As with mannan-type oligosaccharides of yeast invertase (*20, 21*), fraction I-N, corresponding to 1% of the total carbohydrate, was separated into various subfractions on a Bio-Gel P-4 column, and the elution profiles did not change significantly on digestion with a mixture of *A. oryzae* β-galactosidase and jack bean α-mannosidase (data not shown).

To identify phosphate groups, I-A-1 and I-A-2 were treated with alkaline phosphatase with or without prior mild acid hydrolysis under the same conditions as used for II-A-1 and II-A-2. The results indicated that 73% of ^{3}H-radioactivity of I-A-1 and 6% of that of I-A-2 were due to oligosaccharides with phosphate groups in monoester linkages. They accounted for 1.1% of the total carbohydrate of the enzyme. Two and eight percent of ^{3}H-radioactivity of I-A-1 and I-A-2, respectively, belonged to oligosaccharides with diester linkages, which accounted for 0.2% of the total carbohydrate. The remainder of I-A-1 and I-A-2 radioactivities could not be identified, and nor could those of II-A-1 and II-A-2.

DISCUSSION

Assuming that all the *N*-acetylglucosamine residues (1.25%) are attributable to chitobiose units attached to the peptide portion, one molecule of this enzyme would possess four *N*-linked oligosaccharides units.

The sugar chains of this enzyme consisted of two types of units. One (fraction I) was suggested to be of a galactomannan type and comprised 15% of the total hexose. The other (fraction II) was of an oligomannoside type and comprised 85% of the total hexose. Fraction II-N comprised 95% of hexose of fraction II, indicating that more than 80% of the total hexose is accounted for by II-N. As shown in Table II, II-N can be classified into six subfractions based on size. These sub-

fractions were further divided into two groups based on another criterion. The structures of one group can be formulated as $(\alpha 1 \rightarrow 2\text{Man})_n\alpha\text{Man}_4\beta\text{ManGlcNAc}_2$ ($n=0$–4), and 500-MHz ^{1}H-NMR studies revealed that structures of this group are identical to those of oligomannoside-type oligosaccharides of lysosomal enzymes of mammalian origin (*6*, *7*, *22–24*). Approximately two-thirds of II-N belonged to this group. The sharing of common sugar chains raises the possibility that β-galactosidase from *A. oryzae* may be used for enzyme replacement therapy of β-galactosidosis, a lysosomal enzyme deficiency disease. The results of *in vitro* experiments with cultured fibroblasts from GM_1-gangliosidosis are in fact suggestive of this possibility (*25*). The structures of the other group can be formulated as $(\alpha 1 \rightarrow 2\text{Man})_n\alpha\text{Man}_5\beta\text{ManGlcNAc}_2$ ($n=0$–4 or 5). The 500-MHz ^{1}H-NMR spectroscopic studies suggested that the core portion of the latter ($\alpha\text{Man}_5\beta\text{ManGlcNAc}_2$) would be formed by attaching an additional α1,6-linked mannose residue to the nonreducing α1,6-linked mannose residue of the core portion of the former ($\alpha\text{Man}_4\beta\text{ManGlcNAc}_2$), analogously to oligosaccharides of yeast invertase (*20*).

Although significant amounts of ^{3}H-radioactivity were recovered in the acidic fractions of oligosaccharides (I-A-1, I-A-2, II-A-1, and II-A-2), the total amounts of sugar chains containing phosphate groups were calculated to comprise only approximately 4% of the total oligosaccharides. This value is less than those of lysosomal enzymes from *Dictyostelium discoideum* (*26*) and yeast carboxypeptidase Y (*21*), but nearly equal to those of lysosomal enzymes isolated from mammalian tissues (*6*, *7*).

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