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The Carbohydrate Structures of β-Galactosidase from Human Liver

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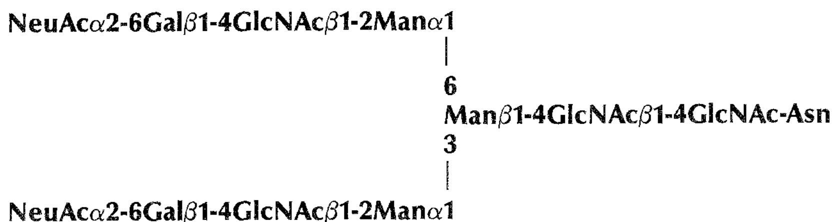
Acid β-galactosidase (EC 3.2.1.23) was obtained from human liver in a pure monomeric state (M_r63 000). The carbohydrate content of the enzyme was established to be 9% by weight; mannose, *N*-acetylglucosamine, galactose and *N*-acetylneuraminic acid were found to be the constituent monosaccharides. The carbohydrate structures of the enzyme were studied at the glycopeptide level by employing 500 MHz ^{1}H-NMR spectroscopy, carbohydrate composition analysis and methylation analysis involving GLC-MS. Based upon the intensities of relevant signals in the ^{1}H-NMR spectrum, approximately 60% of the chains were found to be of the *N*-acetyllactosamine type, having the structure



The rest appeared to be of the oligomannoside type ($Man_{5\text{-}6}GlcNAc_2Asn$). The carbohydrate composition and methylation analysis results sustained these findings, although the calculation of the distribution based upon these techniques indicated a somewhat lower percentage of *N*-acetyllactosamine type chains. There are approx-

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imately three oligosaccharide chains per molecule. These findings offer an explanation for the abnormal distribution of β-galactosidase in tissues and cultured fibroblasts of patients with I-cell disease.

β-Galactosidase is a lysosomal glycoprotein which, like most other soluble acid hydrolases, is widely distributed in mammalian tissues. In patients with I-cell disease, however, decreased tissue levels have been reported for β-galactosidase compared to other acid hydrolases such as β-*N*-acetylhexosaminidase (EC 3.2.1.30). Moreover, β-galactosidase is completely absent from cultured I-cell fibroblasts, whereas most other hydrolases exhibit only decreased activities [1]. Since lysosomal enzyme localization is determined by recognition processes involving their carbohydrate moiety [2], the aforementioned abnormalities in the distribution of β-galactosidase prompted us to elucidate the structure of the carbohydrate chains of this enzyme, in order to enable comparison with other lysosomal enzymes. We chose human liver for its isolation, since this organ is a particularly rich source of β-galactosidase. Moreover, the carbohydrate structures of other enzymes (like β-*N*-acetylhexosaminidase [3]) from this tissue have been described. A preliminary account of the present investigation has been published [4].

Materials and Methods

Materials

Human liver was frozen as soon as possible after autopsy and stored at -40°C. The following materials were obtained from the suppliers indicated: Sepharose and derivatives (Pharmacia, Uppsala, Sweden); pronase (Calbiochem, San Diego, CA, USA); glycosidase substrates (Koch-Light Ltd., Haverhill, UK); *p*-aminophenyl-β-D-thio-galactopyranoside, D-galactono-1,4-lactone, α-methylmannopyranoside, *N*-acetylglucosamine (Sigma, St Louis, MO, USA). All other reagents were of the highest grade available.

Isolation and Purification of β-Galactosidase

The isolation and purification of β-galactosidase were performed, following the method described by Van Diggelen *et al.* [5]. All operations were carried out at 4°C, unless otherwise mentioned. Human liver was homogenized in water (25%, w/v), using a Polytron homogenizer. The homogenate (3328 ml) was centrifuged for 1 h at 100 000 × *g*. The supernatant (2640 ml) was dialyzed against a buffer solution containing 50 mM sodium phosphate (pH 7.4), 0.5 M NaCl and 0.02% NaN_3, being the elution buffer of the Con A-Sepharose chromatography step. The enzyme solution was applied to a 100 ml column of Con A-Sepharose (5.1 × 5.0 cm i.d.), equilibrated in the aforementioned buffer solution containing 1.0 mM $CaCl_2$, 1.0 mM $MnCl_2$ and 1.0 mM $MgCl_2$, at a flow rate of 125 ml/h. After loading of the sample the column was washed with 300 ml of the starting buffer at 20°C. The fractions containing enzymic activity were pooled and dialyzed against a solution containing 10 mM sodium acetate, 0.1 M NaCl, 0.02% (w/v)

NaN_3, pH 5.0, being the starting buffer of the subsequent affinity chromatography step. The affinity column consisted of *p*-aminophenyl-β-D-thiogalactopyranoside, coupled to CH-Sepharose (according to Pharmacia's instructions). The sample (200 ml) was applied to this column (17 ml bed volume, contained in a plastic syringe), at a flow rate of 30 ml/h. The column was successively washed with 1) 50 ml of the buffer to which Triton X-100 (0.25% w/v) had been added; 2) 50 ml of the starting buffer containing 1.0 M NaCl; 3) 50 ml of the buffer containing 0.5 M NaCl. The enzyme was then eluted with 150 ml of buffer containing 100 mM D-galactono-1,4-lactone. Finally the fractions containing β-galactosidase activity were dialyzed against distilled water. The purity of the enzyme preparation was checked by polyacrylamide gel electrophoresis in sodium dodecylsulphate, performed as described [6].

Determination of the Molecular Mass

A sample of the purified β-galactosidase (0.14 U), dissolved in 10 ml elution buffer, was applied to a Sepharose 6B column (100 × 2.5 cm i.d.), equilibrated in 50 mM Tris/0.1 M KCl, adjusted to pH 7.4 with concentrated HCl. An analogous run was performed with a sample of the 100 000 × *g* supernatant. The column was calibrated with standard proteins [7].

Enzyme Assay

β-Galactosidase was determined as follows: 50 μl of the enzyme solution was incubated with 250 μl of a 1.0 mM solution of 4-methylumbelliferyl β-D-galactopyranoside in McIlvaine buffer (0.2 M Na_2HPO_4, adjusted to pH 4.4 with 0.1 M citric acid) for an appropriate time. The reaction was stopped by the addition of 2.2 ml of a buffer containing 0.25 M Na_2CO_3 adjusted to pH 10.5 with glycine. The liberated 4-methylumbelliferone was measured as described [8]. One unit of enzyme hydrolyzes 1 μmol of substrate per minute.

Determination of the Carbohydrate and Amino Acid Composition

The carbohydrate and amino acid composition of the purified β-galactosidase preparation were determined as described earlier [6].

Preparation of Glycopeptides

The purified β-galactosidase preparation (170 U) was dissolved in 1.5 ml 0.01 M $CaCl_2$/0.02% (w/v) NaN_3, adjusted to pH 8.0 with 1.0 M NaOH. Pronase digestion and purification of the resulting glycopeptides was as described earlier [9].

Methylation Analysis

The glycopeptides were permethylated according to the method of Hakomori [10], as modified by Finne *et al.* [11]. Subsequently they were subjected to methanolysis and acetylation. The products were identified and quantified by GLS-MS analysis [12] with a Hewlett-Packard 5993 instrument, equipped with a cross-linked methyl silicone capillary column (15 m × 0.2 mm i.d.). The pressure was 50 kPa.

Table 1. Purification of human liver β-galactosidase.

Purification step	Protein (mg)	Activity (U)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Liver homogenate	116480	1065	9.1×10^{-3}	1.0	100
Centrifugation (100,000 x *g*)	41580	449	10.8×10^{-3}	1.2	42
Con A-Sepharose	1000	220	2.2×10^{-1}	24.1	21
p-aminophenyl-β-D-thiogalacto-pyranoside-CH-Sepharose	5.2	174	33.6	3676	16

The above homogenate was prepared from 832 g of frozen liver. For experimental details, see the Materials and Methods section.

^{1}H-NMR Spectroscopy

Deuterium-exchanged glycopeptides were obtained by five-fold lyophilization of their ^2H_2O solutions, finally using 99.96 atom % deuterated water (Aldrich, Milwaukee, WI, USA). 500 MHz ^{1}H-NMR spectra were recorded on a Bruker WM-500 instrument (SON hf-NMR facility, Department of Biophysical Chemistry, University of Nijmegen, The Netherlands) under conditions described [13]. The applied probe temperature was 27°C. Chemical shifts (δ) are expressed in p.p.m. downfield from internal sodium 4,4-dimethyl-4-silapentanesulfonate (DSS), but were actually measured by reference to internal acetone (δ 2.225 p.p.m.) with an accuracy of 0.002 p.p.m.

Results

Enzyme Purification

The various steps in the purification of β-galactosidase from human liver were monitored by enzyme activity assays. The results are given in Table 1. Polyacrylamide-gel electrophoresis of reduced, sodium dodecylsulphate-treated samples showed three major protein bands, corresponding to M_r 63 000, 31 000 and 20 000. A minor band at M_r 45 000 was faintly visible on the original gel (Fig. 1).

Sepharose-6B gel filtration chromatography revealed that the final enzyme preparation consisted of a single molecular mass species of M_r 63 000 (elution volume similar to that of bovine serum albumin). The 100 000 $\times$ *g* supernatant was chromatographed under the same conditions; in addition to the M_r 63 000 peak it contained a peak of β-galactosidase activity having an M_r value of about 800 000 (Fig. 2).

The final β-galactosidase preparation did not contain detectable amounts of six other glycosidases (β-hexosaminidase, β-glucuronidase, β-glucosidase, α-mannosidase, α-galactosidase, α-fucosidase) as determined according to the method of Lisman and Overdijk [8].

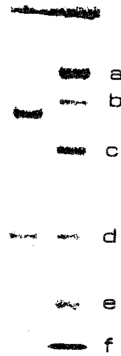


Figure 1. Polyacrylamide gel electrophoresis of human liver β-galactosidase. A sample of the purified enzyme (15 μg) was reduced, treated with sodium dodecylsulphate, and run in a 13% slab gel [6]. Molecular mass markers (2 μg each) are: a, phosphorylase B (92 500); b, bovine serum albumin (66 200); c, ovalbumin (45 000); d, carbonic anhydrase (31 000); e, soybean trypsin inhibitor (21 500); f, lysozyme (14 400). Gels were stained with Coomassie Brilliant Blue R250 and destained in methanol/acetic acid/water, 4/1/5, by vol. A minor band with M_r 45 000 was faintly visible on the original gel.

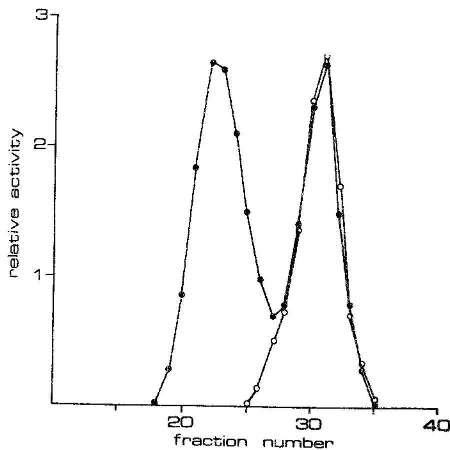


Figure 2. Sepharose 6B gel filtration chromatography of human liver β-galactosidase. A sample of the purified enzyme and a sample of the 100 000 × *g* supernatant were run separately on a column of Sepharose 6B (100 cm × 2.5 cm). Elution buffer: 50 mM Tris/0.1 M KCl/0.02% (w/v) NaN_3, adjusted to pH 7.4 with concentrated HCl. Sample volume was 10 ml. Flow rate 30 ml/h. Fraction volume 10.7 ml. ○, purified β-galactosidase; ●, β-galactosidase activity in the 100 000 × *g* supernatant.

Table 2. Amino acid composition of human liver β-galactosidase.

Compound	mol/100 mol
Aspartic acid	10.5
threonine	6.4
serine	8.6
glutamic acid	8.6
proline	6.0
glycine	8.4
alanine	6.2
cysteine	n.d.
valine	6.0
methionine	0.3
isoleucine	3.7
leucine	9.9
tyrosine	5.3
phenylalanine	5.8
histidine	2.3
lysine	6.1
arginine	5.7

n.d. = not detectable

Table 3. Carbohydrate composition of human liver β-galactosidase. Total carbohydrate 9% (w/w)

Monosaccharide[a]	μg/mg protein	mol/mol enzyme[b]
Man	37.4	13.1
Gal	8.4	3.0
GlcNAc	26.8	7.7
NeuAc	12.5	2.5

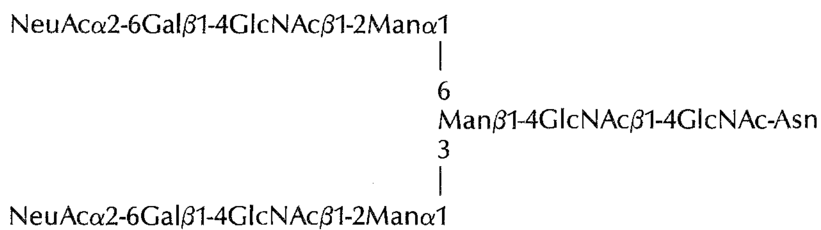
[a] In addition, traces of glucose have been observed.
[b] Calculated on the basis of an M_r value of 63 000.

The amino acid composition of the purified enzyme is given in Table 2. The data are in reasonable agreement with those obtained for human liver β-galactosidase by Frost *et al.* [14]. The carbohydrate composition of the enzyme preparation as determined by GLC is shown in Table 3. In addition to mannose and *N*-acetylglucosamine, substantial amounts of galactose and *N*-acetylneuraminic acid were found, suggesting the presence of *N*-acetyllactosamine-type chains.

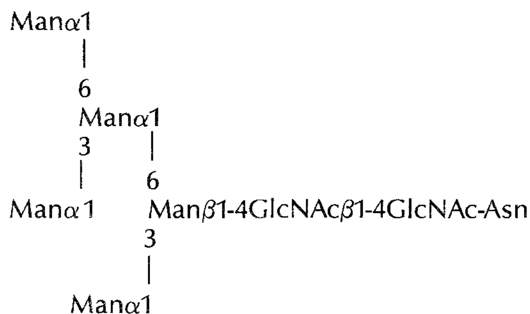
Structural Analysis of the Carbohydrate Chains

The molar carbohydrate composition of the glycopeptides obtained after pronase digestion of the purified β-galactosidase is included in Table 3. The glycopeptides were further analyzed by 500 MHz ^{1}H-NMR spectroscopy. The chemical shifts of pertinent structural-reporter groups have been listed in Table 4. The NMR-spectrum revealed that all carbohydrates present are *N*-glycosidically-linked to asparagine forming part of a short peptide chain. This is inferred from the occurrence of the H-1 and the *N*-acetyl resonances of GlcNAc-**1** at δ 5.047 p.p.m. and 2.01 p.p.m., respectively. The multiplicity of the latter signal points to heterogeneity of the peptide moiety [13].

As anticipated from the results of the sugar analysis, the glycopeptide preparation appeared to contain a mixture of *N*-acetyllactosamine-type and oligomannoside-type structures. Part of the ^{1}H-NMR spectral data obtained match those of the well-characterized bisialyl di-antennary glycopeptide



The rest is strongly reminiscent of the previously described ^{1}H-NMR features of oligomannoside-type glycopeptides, like



In Table 4, the chemical-shift data of the different components of the glycopeptide preparation from β-galactosidase are compared to the corresponding characteristics of the above reference glycopeptides [13]. The signals attributable to the *N*-acetyllactosamine-type compounds indicate that there is virtually no heterogeneity in

Table 4. Pertinent ^{1}H-chemical shifts of structural-reporter groups of the monosaccharides present in the glycopeptide preparation obtained from human liver β-galactosidase, together with those of similar reference glycopeptides.

Reporter group	Residue[a]	Chemical shift[b] in N-6-5-4 / N'-6'-5'-4' > 3-2-1-Asn: β-galactosidase	human serotransferrin[c]	Reporter group	Residue[a]	Chemical shift in 4 / A, B > 4' > 3-2-1-Asn: β-galactosidase	Gaucher disease urine[c]
H-1	GlcNAc-1	5.047	5.038	H-1	GlcNAc-1	5.047	5.071
	GlcNAc-2	4.617	4.616		GlcNAc-2	4.610	4.606
	Man-4	5.131	5.133		Man-4	5.095	5.099
	Man-4'	4.947	4.949		Man-4'	4.870	4.872
	GlcNAc-5	4.603	4.603		Man-A	5-090	5.093
	GlcNAc-5'	4.603	4.603		Man-B	4.908	4.908
	Gal-6	4.443	4.442				
	Gal-6'	4.447	4.447				
H-2	Man-3	4.254	4.254	H-2	Man-3	4.251	4.251
	Man-4	4.197	4.195		Man-4	4,068	4.077
	Man-4'	4.116	4.116		Man-4'	4.145	4.141
					Man-A	4-068	4.066
					Man-B	3.980	3.985
H-3ax	NeuAc	1.719	1.716				
	NeuAc'	1.719	1.716				
H-3eq	NeuAc	2.670	2.666				
	NeuAc'	2.670	2.672				
NAc	GlcNAc-1	2.006[d]	2.002	NAc	GlcNAc-1	2.010[d]	2.012
	GlcNAc-2	2.081	2.081		GlcNAc-2	2.061	2.060
	GlcNAc-5	2.069	2.067				
	GlcNAc-5'	2.065	2.063				
	NeuAc	2.030	2.029				
	NeuAc'	2.030	2.028				

[a] For complete structures and coding of monosaccharides, see text.
[b] Data were acquired at 500 MHz for neutral solutions in $^{2}H_{2}O$ at 27°C. N = NeuAc.
[c] Data taken from Vliegenthart *et al.*[13](compound **33** and **62,** respectively).
[d] Multiplicity of this signal indicates heterogeneity of the peptide portion.

this type of carbohydrate chain, neither in degree of branching nor in the presence and the type of linkage of the sialic acid residues. Both branches of the di-antennary compounds are α(2-6)-sialylated. In the carbohydrate portion of the oligomannoside-type compounds, however, some heterogeneity is observed with respect to the presence of an additional mannose residue α(1-2)-linked to the upper Manα1-3 unit. In addition to the signals compiled for $Man_5GlcNAc_2Asn$ in Table 4, a low-intensity signal is observed at $\delta \sim 5.35$ p.p.m.; this is related to a Man-**4** residue substituted by Man-**C** [9, 13, 15]. No chemical shift perturbations or extra spin couplings that could possibly indicate the presence of Mannose-6-phosphate residues were observed.

Table 5. Relative amounts of methylated, acetylated glycosides present in β-galactosidase from human liver.

Derivative	Molar ratio
2,3,4,6-tetra-Me-Gal	<0.1
2,3,4-tri-Me-6-Ac-Gal	13.1
2,4,6-tri-Me-3-Ac-Gal	2.0[a]
3,4,6-tri-Me-2-Ac-Gal	0.5
2,3,4,6-tetra-Me-Man	7.2
2,3,4-tri-Me-6-Ac-Man	6.0
2,4,6-tri-Me-3-Ac-Man	4.4
3,4,6-tri-Me-2-Ac-Man	14.1
2,4-di-Me-3,6-di-Ac-Man	16.6
3,6-di-Me-4-Ac-GlcNAc(Me)	22.2
3,4,6-tri-Me-GlcNAc(Me)	<0.1
4,7,8,9-tetra-Me-NeuAc(Me)	2.9

[a] Although the retention time and mass spectrum of this peak unquestionably belong to this compound, the molar ratio is too high due to a relatively high background of undefined character.

From the ratio of the intensity of the nearly coinciding H-1 signals of Gal-**6**/**6'** (at δ ~ 4.445 p.p.m.) to that of the combined H-1 signals of Man-**4** and Man-**A** in $Man_5GlcNAc_2Asn$ (at δ ~ 5.09 p.p.m.), it can be concluded that the glycopeptide mixture obtained from β-galactosidase consists of the above di-antennary *N*-acetyllactosamine-type structures and the $Man_5GlcNAc_2Asn$ compounds in a ratio 3:2. The relative amount of $Man_6GlcNAc_2Asn$ in the mixture is close to the detection limit of the applied NMR technique, that is, approximately 5%.

Subsequently, the glycopeptides were subjected to methylation analysis. From a quantitative point of view, methylation analysis yielded relatively low amounts of amino sugar (*N*-acetylglucosamine and *N*-acetylneuraminic acid) derivatives. This is ascribed to the use of a splitless injection mode [16]. Qualitatively, the results listed in Table 5 are in agreement with the structures proposed on the basis of ^{1}H-NMR spectroscopy and sugar analysis, with one exception. The presence of 2,4,6-tri-Me,3-mono-Ac-Gal is indicative of the existence of α(2-3)-sialylated galactose residues. However, the molar ratio of this derivative could not be estimated exactly, due to the high background that contaminated this peak. Since this type of linkage was not found in ^{1}H-NMR spectroscopy, it can be estimated that less than 10% of the *N*-acetyllactosamine-type branches are α(2-3)-sialylated on the basis of the detection limit of the latter technique. The finding of low quantities of 2,3,4-tri-Me,6-mono-Ac-Man and 2,4,6-tri-Me,3-mono-Ac-Man indicates a higher degree of heterogeneity of the oligomannside-type structures than could be deduced from ^{1}H-NMR spectroscopy for the same reason as above. Methylation of α-methylmannoside (as a control), yielded exclusively the permethylated sugar, thus making the possibility of partial undermethylation improbable.

Discussion

β-Galactosidase was obtained from human liver in a 3676-fold purified state. The final preparation had a specific enzyme activity of 33.6 U/mg. Gel filtration of the resulting preparation showed a single enzyme component, M_r 63 000, generally referred to as the monomeric form A_1 of β-galactosidase [17, 18]. No contaminating activities of six other hydrolases were detected. SDS-polyacrylamide-gel electrophoresis showed three major bands and a minor one, in agreement with the results of others [14, 19].

By combination of sugar analysis by GLC, methylation analysis by GLC-MS and ^{1}H-NMR spectroscopy at 500 MHz, human liver β-galactosidase was shown to contain comparable amounts of *N*-acetyllactosamine-type and oligomannoside-type carbohydrate chains. This substantiates the results of the sugar composition analysis of this enzyme, reported by Frost *et al.* [14]. Thus β-galactosidase is the first soluble lysosomal hydrolase to be reported to have such a high content of *N*-acetyllactosamine-type carbohydrates. Other lysosomal hydrolases obtained from the same tissue, like β-*N*-acetylhexosaminidase [3], contain almost exclusively oligomannoside-type ($Man_{5\text{-}9}GlcNAc_2$ Asn) side chains.

It is tempting to correlate these findings to the enhanced secretion of β-galactosidase observed in I-cell disease [1, 20]. The human liver enzyme does not contain any side chains that may act as optimal substrates for *N*-acetylglucosaminylphosphotransferase. So far, only relatively large ($Man_{7\text{-}9}GlcNAc_2Asn$) oligomannosidetype carbohydrate chains have been described as possible carriers of the mannose-6-phosphate recognition marker [21]. Apart from the apparent heterogeneity in the oligomannoside-type chains in the enzyme preparation studied here that is not detectable with 500 MHz ^{1}H-NMR spectroscopy, the majority of these chains has a low number of mannose residues. It is improbable that this is a result of the action of lysosomal α-mannosidase. In the case of cathepsin D, Nakao *et al.* found that lysosomal α-mannosidase was unable to hydrolyze these type of chains [22]. These authors also showed that phosphorylated oligosaccharides were more heterogeneous and contained a higher number of mannose residues than the non-phosphorylated neutral oligomannoside-type chains. Therefore, it seems logical that, once β-galactosidase has been secreted from the (liver) cell, no endocytosis or recycling of the enzyme can occur.

It is still unclear how lysosomal enzymes in tissues of patients with I-cell disease, bearing non-phosphorylated oligomannoside-type chains, become entrapped in the lysosomes [23, 24]. If the mannose-6-phosphate receptor would recognize non-phosphorylated oligomannoside-type chains but with a lower affinity, the relatively low percentage of this chain type present on liver β-galactosidase would explain the high secretion compared with that of most other lysosomal enzymes. In this respect it is of interest to note that Fischer *et al.* [25] found a higher inhibiting effect of mannose on the binding of β-glucuronidase to a fibroblast preparation than of mannose-1-phosphate and glucose-6-phosphate.

From the carbohydrate content of the liver β-galactosidase (~ 9%), its monomeric nature, its M_r (63 000), the M_r values of the two types of carbohydrate structures (M_r 2250 and M_r 1250) and their relative abundance (3:2), it can be estimated that the mean number of carbohydrate chains per molecule of enzyme is approximately three. It remains to be established whether each β-galactosidase molecule contains both types of

side chains, or that the enzyme preparations studied were composed of a mixture of molecules, each bearing exclusively one type of chain.

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