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GLYCOENZYMES: AN UNUSUAL TYPE OF GLYCOPROTEIN STRUCTURE FOR A GLUCOAMYLASE*

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ABSTRACT

Glucoamylase, (1→4)(1→6)-α-D-glucan glucohydrolase (EC 3.2.1.3), hydrolyzes starch and glycogen completely to D-glucose and is used industrially in the manufacture of D-glucose from starch. The enzyme is elaborated by many types of fungi and occurs in two isoenzymic forms (glucoamylase I and glucoamylase II) in extracts from certain fungi. The isoenzymes from *Aspergillus niger* are glycoenzymes containing D-mannose, D-glucose, and D-galactose as integral structural components. New data from experiments on reductive alkaline β-elimination and from methylation analyses show that the carbohydrate chains of glucoamylase I are linked *O*-glycosidically from D-mannose residues to L-serine or L-threonine residues of the protein moiety. In this enzyme, the carbohydrate residues are present as 20 single D-mannose residues, 11 disaccharides components having the structure 2-*O*-D-mannopyranosyl-D-mannose, 8 trisaccharides, and 5 tetrasaccharides composed of various combinations of D-mannose, D-glucose, and D-galactose residues joined by (1→3) and (1→6) glycosidic linkages. Such an array of carbohydrate chains in a glycoprotein is unusual, and may account for some of the unique properties exhibited by glucoamylase.

INTRODUCTION

Much progress has been made in the elucidation of the molecular architecture of glycoproteins from mammalian and yeast sources, especially with regard to the structure of the carbohydrate portion of these polymers[1,2]. For the most part, such glycoproteins contain heterosaccharide chains attached to the protein by *O*-glycosidic linkages between 2-acetamido-2-deoxy-D-galactose and the hydroxyl groups of L-serine and L-threonine, or by N–glycosyl linkages between 2-acetamido-2-deoxy-D-glucose and the amide group of L-asparagine. In contrast, fungal glycoenzymes are glycoproteins having a different type of molecular structure in which many short

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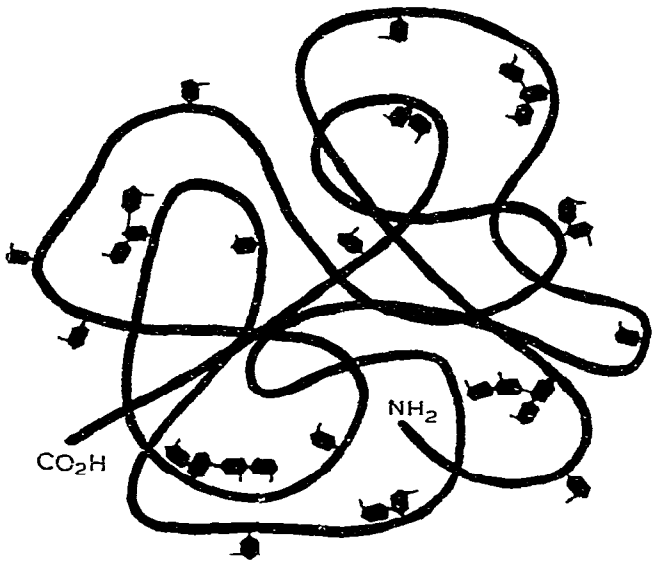


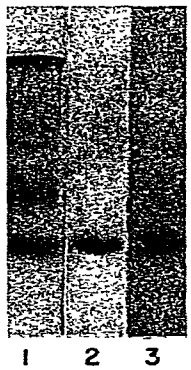
Fig. 1. Diagrammatic representation of the molecular structure of glucoamylase; solid line denotes the polypeptide chain and hexagons represent hexose residues.

carbohydrate-chains are attached by *O*-glycosidic linkages from D-mannose residues to L-serine or L-threonine of the protein. Two examples of such glycoenzymes are glucoamylase from *Aspergillus niger*[3] and mycodextranase from *Penicillium mellinii*[4]. Both enzymes occur in isoenzymic forms and are composed of 85% protein and 15% covalently linked carbohydrates consisting primarily of D-mannose and, to a lesser extent, of D-glucose and D-galactose.

New, quantitative data have been obtained on the number and structures of the carbohydrate chains in glucoamylase I and on the manner of attachment of the chains to the protein moiety of the enzyme. This isoenzyme of glucoamylase possesses the higher electrophoretic mobility and is produced[3] in larger amounts by *Aspergillus niger* than glucoamylase II. Methylation analysis and reactions of reductive alkaline β-elimination have been employed to obtain the new data. The results of these experiments show that glucoamylase I possesses 44 carbohydrate-chains per molecule of enzyme and that approximately half of the chains are single D-mannosyl residues; the remainder are disaccharides of D-mannose and branched trisaccharides and tetrasaccharides composed of D-mannose, D-glucose, and D-galactose. On the basis of the new results, a representation of the molecular architecture of glucoamylase I is depicted in Fig. 1. Such an arrangement of carbohydrate chains may well be responsible for some of the unique properties that are exhibited by the glucoamylase, such as its prolonged stability[5] and non-precipitability by electrolytes[6].

RESULTS AND DISCUSSION

A molecular architecture suggested for glucoamylase I on the basis of the new



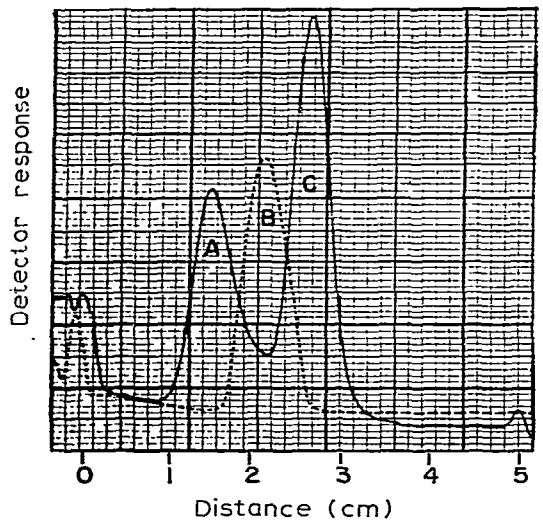


Fig. 2. Poly(acrylamide)-gel electrophoresis of glucoamylase; 1, nonpurified glucoamylase stained with Coomassie Blue G-250; 2, purified glucoamylase stained with Coomassie Blue G-250; and 3, purified glucoamylase stained with periodate–Schiff reagent.

Fig. 3. Density-gradient centrifugation patterns for 2-mg samples of bovine serum albumin (A), glucoamylase (B), and D-glucose oxidase (C).

results is shown diagrammatically in Fig. 1. In this diagram, the glucoamylase molecule is shown as a single polypeptide chain (solid line) having many carbohydrate side-chains of single residues (hexagons) or oligosaccharides of the di-, tri-, or tetrasaccharide type (segments of hexagons). The suggestion that the enzyme consists of a single polypeptide chain is based on *N*-terminal amino acid analysis[3]. The evidence for the number and types of carbohydrate chains in glucoamylase is considered later. In Fig. 1, not all of the carbohydrate chains of the enzyme are shown, and the distribution of the carbohydrate moieties along the polypeptide chain has not yet been determined.

The glycoprotein nature of glucoamylase has been verified by new results from gel electrophoresis and density-gradient centrifugation experiments. Photographs of poly(acrylamide) gels of the purified enzyme stained for proteins and for glycoproteins are reproduced in Fig. 2. It may be seen that the purified glucoamylase reacts both with the protein[7] and the glycoprotein reagents[8], yielding intense bands at the same position on the gels. There appears to be a trace of a slow-moving component that stained with the glycoprotein reagents, but this component was present in amounts less than 1 % of the major glycoprotein component. The non-purified sample yields an electrophoresis pattern showing multiprotein components.

Centrifugation patterns on a sucrose density-gradient were obtained by a published procedure[9] and the patterns for glucoamylase, bovine serum albumin, and glucose oxidase are shown in Fig. 3. The centrifugation data for the three proteins were obtained under identical conditions, and a composite figure of the patterns has

TABLE I

NUMBER OF AMINO ACID RESIDUES PER MOLE OF GLUCOAMYLASE BEFORE AND AFTER REDUCTIVE ALKALINE β-ELIMINATION

Residue	*Before*	*After*	*Difference*
Threonine	104	76	−28
Serine	119	102	−17
Aspartic	83	86	+3
Glutamic	56	57	+1
Arginine	23	22	−1
Glycine	59	72	+13
Alanine	78	101	+23
2-Aminobutanoic	0	10	+10

been prepared. It may be noted in Fig. 3 that glucoamylase sediments as a homogeneous substance of uniform molecular size at a rate intermediate between bovine serum albumin (mol.wt. 67,000) and D-glucose oxidase (mol.wt. 154,000). The molecular weight of glucoamylase calculated from these data and from the appropriate formula[10] is 102,000. This value is in agreement with an earlier value of 97,000 published from this laboratory[11], but not with the value of 62,000 recently published by others[12]. In another type of density-gradient centrifugation experiment, glucoamylase was

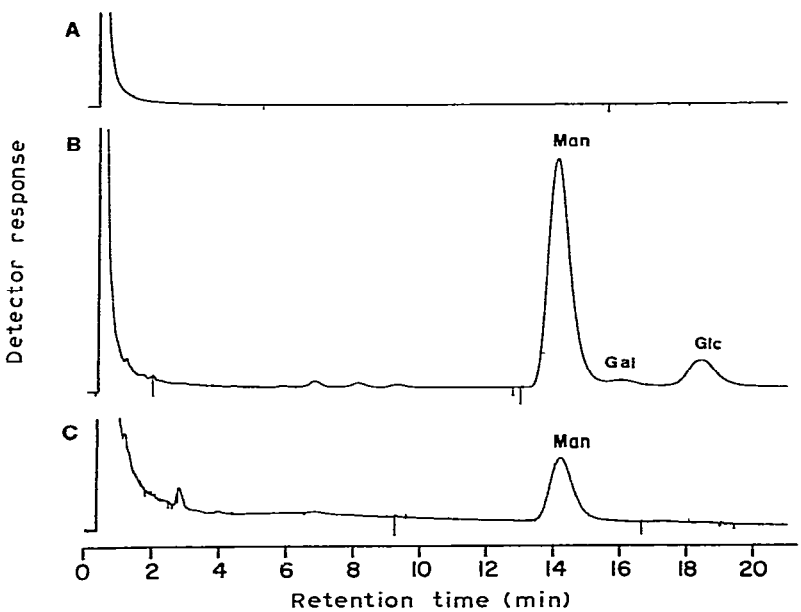


Fig. 4. G.l.c. patterns of hexitol acetates on a column of 3% SP-2340 at 230°: A, glucoamylase blank; B, glucoamylase after successive acid hydrolysis, reduction, and acetylation; C, glucoamylase after reductive alkaline β-elimination and acetylation; Man = mannitol hexaacetate, Gal = galactitol hexaacetate, and Glc = glucitol hexaacetate.

centrifuged in a glycerol density-gradient column. The finished column was fractionated into 0.2-mL samples and analyzed for carbohydrate by the orcinol method[13] and for protein by absorbance at 280 nm. The results of this experiment showed that the protein and the carbohydrate components sedimented at the same rate. Such electrophoretic and sedimentation behavior would be expected for a glycoprotein.

Paper chromatography was used earlier[3] to identify D-mannose, D-glucose, and D-galactose as the carbohydrate components in acid hydrolyzates of glucoamylase. The identity of these monosaccharides has been verified by g.l.c. analysis of the hexitol acetates prepared from the carbohydrates in the acid hydrolyzate of the enzyme. The g.l.c. pattern for the derivatives is shown in Frame B of Fig. 4. A similar analysis was performed on an enzyme sample that had not been hydrolyzed, and the g.l.c. pattern is shown in Frame A of Fig. 4. Frame C of Fig. 4 shows a g.l.c. pattern for the products present in a mixture following reductive alkaline β-elimination of the carbohydrate residues from the enzyme and acetylation of the products. Quantitative values for the monosaccharide composition of the enzyme were obtained by integration of the peaks in Frame B of Fig. 4. From these values, the ratio of D-mannose : D-glucose : D-galactose in glucoamylase was calculated to be 36 : 6 : 1. These values are in good agreement with the values of 35 : 8 : 1 calculated from the data of colorimetric analyses[3].

The number of carbohydrate chains attached to the polypeptide of glucoamylase was determined first by reductive alkaline β-elimination and analyses for amino acids before and after the elimination reaction. Some of the data from this experiment are recorded in Table I. It may be seen in Table I that the number of L-threonine and L-serine residues decreased and the number of L-alanine, L-2-amino-butanoic acid, and glycine residues increased in the enzyme sample subjected to β-elimination, in comparison with the values for the native enzyme. The loss of L-serine and L-threonine residues was 45 and the gain in L-alanine, 2-amino-L-butanoic acid, and glycine residues was 46. These values are in good agreement, and show that there are 45 or 46 points of attachment of carbohydrate chains. In the β-elimination reaction, some of the L-threonine residues were degraded to glycine and L-alanine, and, as a result, the loss and gain in amino acids of a single set does not balance. As already pointed out, the loss and gain for all of the amino acids that changed does indeed balance.

The remainder of the mixture from the reductive alkaline β-elimination experiment was evaporated under vacuum, acetylated at room temperature, and analyzed for alditol acetates by g.l.c. The g.l.c. pattern for this analysis is shown in Frame C of Fig. 4. It may be seen in Fig. 4 that only one major hexaacetate was produced. This derivative was identified as mannitol hexaacetate from g.l.c. retention-times and from mass-spectral data. In order for mannitol hexaacetate to be produced in the foregoing series of reactions, D-mannose must be present as single groups attached to the polypeptide chain of glucoamylase. A quantitative value for calculating the number of single D-mannose groups per molecule of enzyme was obtained from the data in Fig. 4. From this value and the total D-mannose content of the enzyme, it was calculated that one third of the D-mannose residues of the native enzyme occur

as single groups. The remaining D-mannosyl residues, as well as the D-glucose and D-galactose residues, were present as oligosaccharide chains. These compounds were released as reduced oligosaccharides in the elimination reaction and would be acetylated in subsequent reactions. However, these acetylated products were not detectable (Frame C, Fig. 4) under the conditions of the g.l.c. analysis.

The reduced oligosaccharides were detected in the non-acetylated mixture by paper-chromatographic methods as described in the Experimental section. Compounds that reacted with silver nitrate reagent were present at R_F values of 0.40, 0.25, 0.19, and 0.14. Alditols react more slowly with silver nitrate than do reducing sugars, but can be detected with this reagent. The R_F values of mannitol and mannobiitol were 0.41 and 0.24, respectively, under the same chromatographic conditions. All of the compounds in the mixture were isolated by preparative paper-chromatography. That having R_F 0.40 was identified as mannitol by acetylation and g.l.c. analysis. The compound having R_F 0.25 was identified by methylation, g.l.c. analysis, and mass spectrometry as 2-*O*-D-mannopyranosyl-D-mannitol. The R_F values of the compounds indicated that those having R_F 0.19 are trisaccharides and those having R_F 0.14 are tetrasaccharides. The samples were subjected to methylation and to g.l.c.–m.s. analysis. The preparation from R_F 0.19 yielded 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol (mannitol and glucitol), 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol, and 3,6-di-*O*-acetyl-1,2,4,5-tetra-*O*-methylmannitol. It was concluded from these and other data that the preparation consists of two types of trisaccharide. The preparation from R_F 0.14 yielded 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol (mannitol),

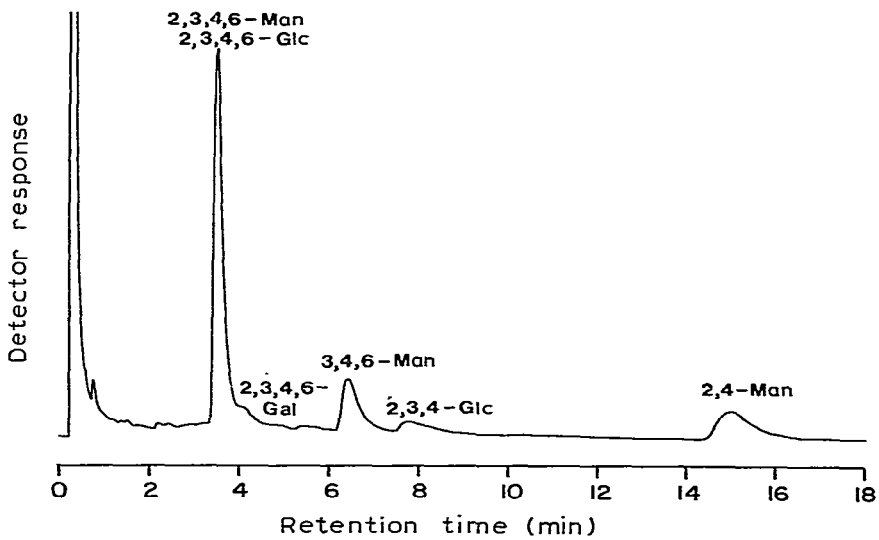


Fig. 5. G.l.c. pattern of the methylated alditol acetates obtained from fully methylated glucoamylase: 2,3,4,6-Man = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylmannitol; 2,3,4,6-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol; 2,3,4,6-Gal = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol; 3,4,6-Man = 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylmannitol; 2,3,4-Glc = 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylglucitol; and 2,4-Man = 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methylmannitol.

TABLE II

PARTIALLY METHYLATED ALDITOL ACETATES FROM THE METHYLATED GLUCOAMYLASE

Derivative	*Retention time*[a]	*Moles*
1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methylmannitol	0.99	49
1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol	1.00	6[b]
1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol	1.16	2
1,2,5-Tri-*O*-acetyl-3,4,6-tri-*O*-methylmannitol	1.82	11
1,5,6-Tri-*O*-acetyl-2,3,4-tri-*O*-methylglucitol	2.19	5
1,3,5,6-Tetra-*O*-acetyl-2,4-di-*O*-methylmannitol	4.22	13

[a]On OV-225 at 190° and relative to the retention time for 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol. [b]Determined by a difference method outlined in the text.

1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylglucitol, and 3,6-di-*O*-acetyl-1,2,4,5-tetra-*O*-methylmannitol; it appears to consist of a tetrasaccharide. The methylation results establish that the reduced oligosaccharides from the glucoamylase contain (1→3) and (1→6) glycosidic linkages.

The g.l.c. pattern for the methylated hexitol acetates[14,15] obtained from the fully methylated glucoamylase is shown in Fig. 5. The identity of the compounds corresponding to the various peaks was deduced from retention times and the mass-spectral data on the individual peaks of the pattern[16]. Integration of the areas under the peaks in Fig. 5 and appropriate calculations yielded the values in Table II for the methylated alditol acetates from the enzyme. The values for 1,5-di-*O*-acetyl-3,4,5,6-tetra-*O*-methylmannitol and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol were determined by a difference method utilizing the data in Figs. 4 and 5, because these derivatives are not separable by g.l.c.

On the basis of the yields and types of derivatives listed in Table II, it was calculated that the glucoamylase contains 20 single D-mannose residues, 11 disaccharide components, 8 trisaccharide components, and 5 tetrasaccharide components. The methylation data for the native enzyme and for the reduced oligosaccharides obtained from the enzyme by reductive alkaline β-elimination show that the disaccharide is a 2-*O*-D-mannopyranosyl-D-mannopyranose. On the basis of the methylation data and R_F values in paper chromatography, possible structures for the oligosaccharides are D-mannopyranosyl-(1→6)-[D-glucopyranosyl-(1→3)]-D-mannopyranose, D-mannopyranosyl-(1→6)-[D-galactopyranosyl-(1→3)]-D-mannopyranose, and D-mannopyranosyl-(1→6)-[D-mannopyranosyl-(1→6)-D-glucopyranosyl-(1→3)]-D-mannopyranose. Other structures consistent with these data are possible, and these have not been ruled out.

The total number of carbohydrate chains in glucoamylase as calculated from the methylation data is 44. This value is in good agreement with that (45) determined from the loss and gain of amino acids on reductive alkaline β-elimination reactions. Two lines of evidence thus substantiate the unique molecular architecture for glucoamylase shown in Fig. 1.

The biosynthetic pathway for glycoproteins having the type of structure shown in Fig. 1 is most probably different from that for mammalian glycoproteins. In the latter pathway[1,17], large, oligosaccharide side-chains are assembled separately as lipid–oligosaccharide intermediates, and the oligosaccharides are then transferred intact to the appropriate amino acid residues of the protein. Subsequently, a processing step occurs in which the excess of carbohydrate residues is removed, leaving a residual core that appears common to a wide variety of mammalian glycoproteins[18,19]. Finally, new carbohydrate residues were added to the core oligosaccharides, presumably from appropriate sugar nucleotides, to yield the completed glycoprotein. In the biosynthesis of glucoamylase, it is more likely that the single residues are transferred directly from a nucleotide sugar to the amino acids of the proteins. The glycosyl transferases responsible for the transfers are highly specific for substrate and acceptor molecules. Evidence for the direct transfer of single residues from guanosine 5′-(α-D-mannopyranosyl diphosphate) to a modified glucoamylase has been presented[20].

The molecular architecture of the type depicted in Fig. 1 may well impart some distinctive properties to glycoenzymes. Such properties may be their high stability[5], ability to pass through membranes[19,21], and non-precipitability by electrolytes[6]. Of special interest would be the elucidation of the role of carbohydrates in the transport of glycoproteins through membranes and of the role of the carbohydrates in the folding of the polypeptide chains of glycoproteins into specific conformational structures[5,19]. Glucoamylase may prove to be a suitable model system for investigating such biological processes.

EXPERIMENTAL

Preparation and purity of glucoamylase. — Glucoamylase was isolated from a commercial enzyme-preparation marketed under the trade name of Diazyme (Miles Laboratories). Diazyme is prepared from a strain of *Aspergillus niger* and is used in the commercial production of D-glucose and syrups from starch[22]. The organism produces two isoenzymic forms of glucoamylase[3]. The separation and purification of the two forms was achieved by chromatography on DEAE-cellulose and elution with buffers of decreasing pH, as described earlier[23]. One isoenzyme of glucoamylase is eluted at pH 6 and the other at pH 4.5. The experiments described in this report were performed with the isoenzyme that was eluted at pH 4.5. This isoenzyme possesses the higher electrophoretic mobility and, in accord with the recommendation of the Enzyme Commission[24], has been designated glucoamylase I.

Gel electrophoresis of glucoamylase I was performed on 50-μg samples in 7% poly(acrylamide) gels as outlined in a published procedure[7]. Gels were stained with 0.2% Coomassie Blue G-250 in 10% trichloroacetic acid to reveal protein components[7] and with periodate and Schiff reagents to reveal glycoprotein components[8]. A photograph of the gels of the purified and non-purified enzyme samples is reproduced in Fig. 2.

Ultracentrifugation of a 2-mg sample of glucoamylase I and of samples of serum bovine albumin and D-glucose oxidase was performed in 5–25% sucrose gradient for 6 h at 65,000 r.p.m. in a Beckman L65 centrifuge and SW-65 rotor. After completion of the centrifugation, the gradients were scanned for u.v.-absorbing components at 280 nm and fractionated into 0.2-mL fractions by use of an Isco fractionator[9]. Fig. 3 shows the u.v. scan for the three samples. The solid line in Fig. 3 represents the gradient solution containing bovine serume albumin and glucose oxidase, and the dotted line represents the gradient solution containing glucoamylase. The samples were centrifuged at the same time but in two different tubes. The composite figure was prepared from the data to facilitate comparison of the sedimentation rates. Centrifugation of glucoamylase was also performed by the foregoing method in a glycerol column (5–25%). Fractions from the glycerol column were analyzed for protein by absorbance at 280 nm and for carbohydrate by the orcinol method[13].

The specific activity of the purified glucoamylase remained constant on repeated chromatography on DEAE-cellulose. The assays for glucoamylase activity were performed as described in an earlier publication[3]. In the purification procedure, 5 g of Diazyme was generally used for the chromatography and ~0.2 g of purified glucoamylase I was obtained. The specific activity[3] of purified enzyme was 2300 units per mg of protein.

Carbohydrate analysis. — A sample (4 mg) of the purified glucoamylase was hydrolyzed in 0.1 mL of M hydrochloric acid in a boiling-water bath for 1 h and the carbohydrates in the hydrolyzate were identified by paper chromatography[3]. The individual compounds were isolated from the hydrolyzates by preparative paper-chromatography and determined quantitatively by a colorimetric method[25]. From these data, the ratio of D-mannose:D-glucose:D-galactose in the enzyme was calculated to be 35:8:1.

A second sample (4 mg) of the glucoamylase was hydrolyzed as before and the product was dried thoroughly in a vacuum desiccator. The carbohydrates in the residue were reduced with sodium borohydride (10 mg) and then acetylated with acetic anhydride (0.5 mL) and dry pyridine (0.5 mL) for 18 h at room temperature. The reagents were removed by evaporation with a stream of nitrogen, and the products dissolved in chloroform. Suitable samples of the solution were used for g.l.c. analysis on a column of 3% SP-2340 at 230° in a Varian Aerograph chromatograph. A solution of reference hexaacetates of mannitol, galactitol, and glucitol was also analyzed by the g.l.c. method. Mass-spectral data were obtained for all peaks with a DuPont 21-490 mass spectrometer. The unhydrolyzed glucoamylase (4 mg) was subjected to the foregoing series of reactions and to the g.l.c. analysis. Typical g.l.c. patterns obtained for these samples are shown in Fig. 4, Frames A and B. The areas under the peaks of Frame B were integrated to afford quantitative values for the monosaccharides. From these values, the ratio of 36:6:1 for D-mannose, D-glucose, and D-galactose was calculated. This ratio is in good agreement with that calculated from the data of the colorimetric analysis.

Reductive alkaline β-elimination. — The purified glucoamylase (30 mg) was subjected to reductive alkaline β-elimination reactions[26,27]. In this experiment, the enzyme was dissolved in 0.1M sodium hydroxide (7 mL) containing 0.3M sodium borohydride, and the mixture was maintained for 288 h at 4°. The excess of borohydride was then decomposed by acidification with M acetic acid, and the borate removed by evaporation of methanol several times from the product. The residue was dissolved in water (0.5 mL).

In order to check whether monosaccharides were released from the enzyme by the β-elimination reaction, a sample (0.1 mL) of the mixture was evaporated and the residue acetylated with acetic anhydride in pyridine. The reagents were evaporated off and the acetylated products dissolved in chloroform and subjected to g.l.c. analysis on a column of 3% of SP-2340 at 230°. The g.l.c. pattern for the mixture is reproduced in Frame C of Fig. 4.

Samples of the original mixture and of standards of mannitol, glucitol, galactitol, and mannobiitol were subjected to paper chromatography in 6:4:3 (v/v) 1-butyl alcohol–pyridine–water. The finished chromatogram was stained with a silver nitrate reagent[28] that oxidizes alditols slowly and reveals the position of such compounds on the paper chromatogram. Compounds that reacted with this reagent were detectable at R_F 0.40, 0.25, 0.19, and 0.14. The R_F values of reference alditols were: mannitol, 0.41; glucitol, 0.36; galactitol, 0.34; and mannobiitol, 0.24. The compounds in 0.3 mL of the mixture were isolated by preparative paper-chromatography. The compound having R_F 0.40 migrated similarly to mannitol; acetylation followed by g.l.c.–m.s. yielded the same data as reference mannitol hexaacetate. The other compounds were subjected to methylation analysis as described in a later section.

Amino acid analysis. — The remaining sample from the β-elimination experiment was used for amino acid analysis. This sample was first evaporated in an ampule and the residue dissolved in 0.5 mL of 6M hydrochloric acid. The ampule was sealed and heated for 24 h at 110°. A comparable amount of the native glucoamylase was hydrolyzed similarly. Both samples were analyzed for amino acids in a Beckman amino acid analyzer. From these values and the molecular weight of the glucoamylase, the number of residues of amino acids per molecule of native and modified glucoamylase were calculated. Factors of 0.87 and 0.75 were employed in calculating the threonine and serine values to correct for the decomposition of these residues during hydrolysis. These factors have been established for the 24-h hydrolysis in a series of control experiments[29]. The values for the amino acids that differed significantly in the two preparations, and for a few that did not differ, are recorded in Table I. Values for amino acids not listed in the Table did not differ appreciably in the two samples.

Methylation analysis. — The purified glucoamylase (5 mg) was methylated by the Hakomori method[30] and analyzed by g.l.c.–m.s. by the procedure of Lindberg[16]. The details of the procedures employed in our laboratory have been published recently[15]. The methylated alditol acetates obtained from the methylated enzyme were separated on a column of 3% of OV-225 at 190°. A typical g.l.c. pattern is repro-

duced in Fig. 5. The individual derivatives were identified from the retention times recorded in Table II and from literature values[16]. The derivatives were also identified by *m/e* values of their fragments on mass spectrometry[14]. Integration of the peak areas of Fig. 5 yields data from which the moles of the partially methylated alditol acetate per mole of enzyme were calculated. These values are recorded in Table II.

A 2-mg sample of 2-*O*-D-mannopyranosyl-D-mannose (provided by C. E. Ballou, Department of Biochemistry, University of California, Berkeley, CA) was dissolved in water (0.5 mL) and reduced with 5 mg of sodium borohydride. After 18 h, the excess of borohydride was decomposed with Dowex-50 (H^+) resin, and the pH of the solution fell to 4.5. The borate was removed by evaporation of methanol several times from the product. The reduced disaccharide and the reduced oligosaccharides from the β-elimination mixture were subjected to methylation, g.l.c. analysis, and mass spectrometry. The retention times for the derivatives from the methylated glucoamylase are recorded in Table II. The retention times for the methylated alditol acetates from the reduced disaccharide at R_F 0.25 were 0.42 for 2-*O*-acetyl-1,3,4,5,6-penta-*O*-methylmannitol and 1.00 for 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylmannitol; for the derivatives from the reduced trisaccharides at R_F 0.19, the retention times were 0.74 for 3,6-di-*O*-acetyl-1,2,4,5-tetra-*O*-methylmannitol, 1.00 for 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol (mannitol and glucitol), and 1.15 for 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol; for the derivatives from the reduced tetrasaccharide at R_F 0.14, retention times were 0.75 for 3,6-di-*O*-acetyl-1,2,4,5-tetra-*O*-methylmannitol, 1.00 for 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol (mannitol), and 2.10 for 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylglucitol. The retention times for the derivatives from reference compounds were in good agreement with the values for the derivatives from the reduced oligosaccharides and with those from the native glucoamylase. A standard derivative of 3,6-di-*O*-acetyl-1,2,4,5-tetra-*O*-methylmannitol was not available, and its retention time was estimated from data for other tetra-*O*-methylmannitol diacetates[16]. The estimated value agrees with the value for the fast-moving derivatives from the reduced tri- and tetra-saccharides.

The *m/e* values from the mass-spectral data for the derivatives from the glucoamylase, the reduced oligosaccharides, and the reference compounds were as follows: mannitol hexaacetate, 115 (100), 127 (40), 128 (40), 139 (60), 145 (50), 157 (40), 170 (30), 187 (60), 217 (40), and 259 (30); 2-*O*-acetyl-1,3,4,5,6-penta-*O*-methylhexitol, 45 (100), 89 (30), 101 (70), 145 (50), 161 (40), and 205 (20); 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol, 45 (80), 117 (100), 161 (80), and 205 (30); 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylhexitol, 45 (70), 161 (100), and 189 (60); 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylhexitol, 117 (100), 161 (30), 189 (20), and 233 (10); and 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methylhexitol, 45 (30), 117 (60), and 189 (100). The first figure of each set denotes the *m/e* value and the figure in parentheses the relative abundance, on the basis of 100 for the most abundant fragment.

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