# Structural Characterization of the Two Forms of Glucoamylase from Aspergillus niger[1]

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## ABSTRACT

Glucoamylases I [α-D-(1→4)-glucan glucohydrolase, E.C. 3.2.1.3] and II from *Aspergillus niger* were isolated in high purity by chromatography on DEAE-cellulose ion-exchange columns. Glucoamylase I contained 13% total carbohydrate and had a molecular weight of 74,900. Glucoamylase II contained 18% total carbohydrate and had a molecular weight of 54,300. The carbohydrate moieties in both glycoproteins were *O*-glycosidically linked through mannose to the hydroxyl groups of serine and threonine in the polypeptide chain. Treating the enzymes with alkaline borohydride eliminated a portion of the carbohydrate. The carbohydrate fragment was eluted from a column of Sephadex G-15 at a volume corresponding to that of a trisaccharide. This fragment was unstable in mild acid, decomposing to mannitol and a disaccharide. Evidence indicates that both enzymes contain the same carbohydrate moieties but differ in amino acid composition.

Glucoamylases [α-D-(1→4)-glucan glucohydrolases, E.C. 3.2.1.3] are produced by fungal, bacterial, and mammalian sources. These enzymes convert starch almost quantitatively into D-glucose, and hence are widely used in commercial production of D-glucose and in manufacture of corn syrups of high D-glucose content. They also have been used for quantitative analyses of starches (1,2). The glucoamylases from fungal sources have been more thoroughly studied than those from other sources.

The glucoamylase from *Aspergillus niger* has been isolated and purified by chromatography on DEAE-cellulose ion-exchange columns (3,4,5). The enzyme consists of two forms, designated glucoamylases I and II. Both have been partially

[1]Contribution No. 769, Department of Grain Science and Industry, Kansas Agricultural Experiment Station, Manhattan. Portions of this work were taken from the dissertation submitted by L. A. Aira to the Graduate Faculty of Kansas State University in partial fulfillment of requirements for the Ph.D. degree (1971) and from the thesis submitted by R. L. Horner to the Graduate Faculty of the University of Nebraska in partial fulfillment of requirements for the M.S. degree (1968). Portions of this work were presented at the 56th Annual Meeting, Dallas, October 1971. Address correspondence to D. R. Lineback.

characterized (3,5,6,7). Both have been reported to be glycoproteins containing D-mannose, D-glucose, and D-galactose (3,4). The carbohydrate moieties appear to be *O*-glycosidically linked through mannose to hydroxyl groups of serine and threonine in the polypeptide chain of the two enzymes (8,9). Biosynthetic studies indicate involvement of a nucleotide-hexose pathway to incorporate carbohydrate residues into glucoamylases I and II (10,11).

A more detailed characterization of the glycoprotein structure of glucoamylases I and II is presented here. The two forms differ in molecular weight (mol. wt.) but have the same absolute amount of carbohydrate (approximately 60 hexose residues per mol. wt.), indicating that they differ in amino acid composition but not in the carbohydrate portion of the glycoprotein.

## MATERIALS AND METHODS

### Materials

Takamine Diazyme (powder form) was provided by Miles Chemical Co., Elkhart, Ind. Ovalbumin, bovine serum albumin, alcohol dehydrogenase, D-amino acid oxidase, and soybean lipoxidase were obtained from Mann Research Laboratories, New York, N.Y. Methanol (reagent grade) was dried by the magnesium procedure. All other chemicals used were reagent or analytical grade, unless otherwise specified.

### Chromatographic Procedures

Ion-exchange columns of DEAE-cellulose (reagent grade, 0.86 meq. per g., Brown Company, Berlin, N.H.) were prepared and operated as previously described (5).

Sephadex G-15 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) was hydrated by stirring in 0.1M acetic acid. A thick slurry of the gel was poured into a column and allowed to settle to the desired height. Chromatography was performed at room temperature with 0.1M acetic acid as eluant at a flow rate of 0.7 ml. per min. Flow rates were maintained with a Mariotte Flask and fractions (3-ml.) were collected automatically at timed intervals. A column (1.5 × 140 cm.) of Sephadex G-15 was calibrated by chromatography of a partially hydrolyzed dextran, prepared by partial hydrolysis of a dextran (purified crude dextran, grade 2-P, Pharmachem Corporation, Bethlehem, Pa.) by the method of Taylor and Whelan (12). Elution volumes for the homologous series of isomaltooligosaccharides were determined by measuring the carbohydrate content of the effluent fractions by the phenol-sulfuric acid procedure (13,14).

Columns of Bio-Gel P-100 (Bio-Rad Laboratories, Richmond, Calif.) were prepared and operated in the same manner, after the gel was swollen in 0.1M sodium chloride (NaCl). Proteins were eluted with 0.1M NaCl at a flow rate of 0.5 ml. per min. Fractions (3-ml.) were collected automatically. A column (3 × 60 cm.) of Bio-Gel P-100 was calibrated as previously described (15). Elution volumes for purified glucoamylases I and II were determined on this column. Protein components were located in effluent fractions by automatically monitoring the effluent at 280 nm.

Gas-liquid chromatography (GLC) was performed with an F & M Model 5751B gas chromatograph (Hewlett Packard, Avondale, Pa.) equipped with flame-ionization detectors. A stainless-steel column (12 ft. × 0.125 in.) packed with

a stationary phase of 3% SE-52 on Anakrom SD (90/100 mesh, Analabs, Inc., North Haven, Conn.) was used to separate the trimethylsilyl ethers of methyl glycosides. The column was operated at 145°C. with nitrogen as the carrier gas (22 ml. per min.). A stainless-steel column (9 ft. × 0.125 in.) packed with the same stationary phase was used to separate the trimethylsilyl ethers of free hexoses. Nitrogen was used as carrier gas at a flow rate of 25 ml. per min. The chromatograph was programmed to maintain the temperature at 120°C. for 3 min. after the sample was injected, and then to increase at 6° per min. to 185°C., where the temperature was maintained until the components had emerged from the column. Trimethylsilyl ether derivatives of α-D-glucopyranose, β-D-galactopyranose, and β-D-mannopyranose were not completely resolved under these conditions. The amount of each of these isomers was determined from the corresponding anomeric peak, which was resolved, and the equilibrium composition of the anomers. The latter was determined from aqueous solutions of the carbohydrates, which were equilibrated at room temperature for 24 hr., freeze-dried, and trimethylsilyl ether derivatives prepared. The derivatives were separated by GLC and the areas under the peaks corresponding to each anomer were compared. *myo*-Inositol was added after hydrolyses as an internal standard for the analysis of hexoses. D-Xylose was added prior to methanolysis as an internal standard for the analysis of methyl glycosides.

Paper chromatography was performed on Whatman No. 1 chromatography paper in a descending system with an ethyl acetate:pyridine:water (8:2:1 v./v./v.) solvent (16) for about 30 hr. at room temperature. Carbohydrates were located on the dried chromatograms by staining with silver nitrate dip reagent (17).

#### Enzyme Assays

Glucoamylase activity was determined as previously described (5). One unit of glucoamylase activity was defined as the amount of enzyme required to liberate 1 μmole of glucose per min. under the defined reaction conditions. Specific activity was defined as units per mg. of protein, as determined by the Lowry method (18), with bovine serum albumin as a standard.

#### Isolation and Purification of Glucoamylases I and II

Glucoamylases I and II were separated from other constituents of the Diazyme by chromatography on an ion-exchange column of DEAE-cellulose by the gradient-elution procedure previously described (5). The two protein peaks containing glucoamylase activity were collected individually, dialyzed against running tap water, and adjusted to both the desired ionic strength and pH with a concentrated (0.2M) buffer solution. The solutions were further purified by chromatography on a column of DEAE-cellulose by the conditions previously described (5).

The protein concentration of the purified solutions was determined by the Lowry method (18), with bovine serum albumin as a standard. The purified enzyme solutions were dialyzed against running water to remove buffer salts before freeze-drying.

#### Sedimentation-Velocity and Equilibrium Measurements

Sedimentation-velocity measurements were made on solutions of purified

glucoamylases I and II, with the conditions previously described (5). Detailed boundary analyses were made of the sedimentation-velocity experiments (5). Experiments on sedimentation equilibrium were conducted as previously reported (15). Weight-average mol. wts. at the meniscus and bottom of the cell were calculated for each photograph, as described by Schachman (19).

#### Determination of the Partial Specific Volume

The partial specific volume of purified glucoamylase I was determined in the manner previously reported (15).

#### Amino Acid Analysis

Purified, freeze-dried glucoamylase I and II was dried under reduced pressure over phosphorus pentoxide-potassium hydroxide ($P_2O_5$-KOH) for 2 to 3 days at room temperature. Samples (about 10 mg.) were hydrolyzed in sealed tubes in constant boiling (6M) hydrochloric acid (HCl) for 24, 48, or 72 hr. The acid was removed under reduced pressure, the residue was dissolved in water, and the solution was again evaporated under reduced pressure. Amino acid analyses were performed on the residue by the method of Spackman et al. (20) with a Spinco Model 120C amino acid analyzer. For routine analyses, 24-hr. hydrolysis was used.

Amino acid analysis of the reaction products obtained from treating glucoamylases I and II with alkaline borohydride was accomplished as follows. After the desired reaction time, the reaction was terminated and processed as described later. The residue was dried under reduced pressure over $P_2O_5$-KOH for 2 to 3 days at room temperature. The dried residue was hydrolyzed in a sealed tube in constant-boiling HCl for 24 hr. The hydrolysate was processed and analyzed as previously described.

#### Carbohydrate Analyses

Total carbohydrate contents of glucoamylases I and II were determined by the phenol-sulfuric acid procedure (14). The desired weight of freeze-dried glucoamylase I or II was suspended in the desired volume of 1M acetic acid and allowed to stand overnight at room temperature with occasional gentle shaking. Dissolution of the enzyme was complete under those conditions; carbohydrate analyses showed an unacceptable variation without this procedure. D-Mannose (0 to 150 $\gamma$) was used to prepare a standard curve. Solutions were read against a reagent blank. Individual carbohydrate components were determined in each enzyme by GLC following methanolysis of the enzyme. Methanolysis of the freeze-dried enzymes and hydrolysis of carbohydrate fractions were accomplished as previously described for the hydrolysis and methanolysis of glucoamylase A from *A. phoenicis* (15).

#### Treatment of Glucoamylases I and II with Alkaline Borohydride

Freeze-dried glucoamylase I or II (150 mg.) was suspended in 15 ml. of 0.1M sodium hydroxide (NaOH)-0.3M sodium borohydride ($NaBH_4$) and maintained at 45°C. for 24 hr. The reaction was terminated by adding glacial acetic acid slowly to pH 5. The mixture was freeze-dried and the residue was evaporated several times from methanol to remove excess borate as the volatile methyl borate. The residue was dissolved in water and passed through a column (1 X 10-cm.) of Amberlite

IR-120 ($H^+$) resin. The column was eluted with several column volumes of water and the effluent was freeze-dried. In cases where amino acid analysis was to be performed on the reaction product, use of the column of ion-exchange resin was omitted.

An alternative procedure used the conditions reported by Carlson (21). Freeze-dried glucoamylase I or II (150 mg.) was suspended in 60 ml. of 0.05M NaOH-1.0M $NaBH_4$ and was maintained at 45°C. for 16 hr. The reaction was terminated and processed as described.

#### Isolation of Carbohydrate Fragment

The freeze-dried reaction product from treating the enzymes (150 mg.) with alkaline borohydride was suspended in 0.1M acetic acid (2 to 4 ml.), stirred vigorously with a vortex mixer, and centrifuged to remove insoluble material. The supernatant solution was charged to a column (1.5 X 140 cm.) of Sephadex G-15, which was eluted with 0.1M acetic acid at a flow rate of 0.7 ml. per min. Fractions (3-ml.) were collected automatically. Carbohydrate components were located in effluent fractions by the phenol-sulfuric acid procedure (14). Protein and peptide (amino acid) components were located in the fractions by the Lowry (18) and Rosen ninhydrin (22) assays, respectively. The fractions containing the two carbohydrate components were collected individually and freeze-dried. The component that contained carbohydrate, but no peaks for protein or peptides (amino acids), was dissolved in water and rechromatographed on the calibrated column of Sephadex G-15. Carbohydrate components were located in the effluent fractions as previously described, and the fractions containing the single carbohydrate component were collected and freeze-dried.

The freeze-dried carbohydrate component from the Sephadex G-15 column was dissolved in a small amount of water and streaked onto a sheet (20 X 57-cm.) of Whatman No. 1 chromatographic paper. The chromatogram was developed as described. Strips (1-cm.) were removed from each edge of the dried chromatogram and stained with silver nitrate dip reagent (17) to locate the carbohydrate components. The areas of the chromatogram corresponding to the mono-, di-, and trisaccharides located on the strips were cut out and eluted with water by a descending system. The solutions were freeze-dried. Analysis of the component sugars in each fraction was accomplished by GLC of the trimethylsilyl ether derivatives, after first hydrolyzing the di- and trisaccharide fractions with 3 ml. of 1M HCl, as previously described for the hydrolysis of glucoamylase from *A. phoenicis* (15).

A portion of the trisaccharide fraction was dissolved in 0.1M acetic acid and allowed to stand at room temperature for 60 hr. The solution was dried under reduced pressure over $P_2O_5$-KOH at room temperature. The residue was dissolved in a small amount of water and chromatographed on Whatman No. 1 chromatographic paper as described.

A portion of the alkaline borohydride reaction product from each enzyme, which had not been chromatographed on Sephadex G-15, was dissolved in water and submitted to the same procedure.

#### Isolation of α-Mannosidase

The α-D-mannosidase from jack-bean meal (Sigma Chemical Co., St. Louis, Mo.) was isolated as described by Snaith and Levvy (23).

**Treatment of Glucoamylases I and II and Isolated Carbohydrate Fragments with α-Mannosidase**

Freeze-dried glucoamylase I or II (50 to 100 mg.) was dissolved in 4 ml. of 0.125M acetate buffer, pH 5.0, and 1.0 ml. of α-mannosidase (45 units activity) in 0.05M acetate buffer was added. The solution was incubated at 37°C. for 72 hr. Aliquots were removed at 6, 12, 18, 24, 36, 48, 60, and 72 hr., and the enzyme was inactivated by boiling for about 3 min. The solutions were dried under reduced pressure over $P_2O_5$-KOH at room temperature and trimethylsilyl ether derivatives were prepared by adding 0.5 ml. of Tri-Sil reagent (Pierce Chemical Co., Rockford, Ill.). Presence of mannose liberated by the enzyme was determined by GLC.

Aliquots of the di- and trisaccharide fractions of the carbohydrate fragments eliminated from glucoamylases I and II by treatment with alkaline borohydride were incubated with 1.0 ml. of α-mannosidase (45 units activity) at 37°C. as described. Aliquots were removed at 2, 4, 6, 8, 10, and 12 hr. and processed as described.

## RESULTS AND DISCUSSION

**Isolation and Purification of the Enzymes**

Chromatography of the crude enzyme on columns of DEAE-cellulose separated three major protein components, two of which showed glucoamylase activity (5). Further purification of the two glucoamylases by chromatography on DEAE-cellulose yielded solutions of glucoamylases I and II with the same specific activity reported (5) for highly purified enzymes.

The high degree of purity of the purified enzymes was confirmed by ultracentrifugal studies. Both enzymes sedimented as single symmetrical boundaries when subjected to ultracentrifugal analysis (about 200,000 X g). A detailed boundary analysis of the ultracentrifugal experiments indicated a high degree of homogeneity (5).

**Properties of Glucoamylases I and II**

Molecular weights were determined for glucoamylases I and II by the Archibald approach-to-equilibrium procedure as described by Schachman (19). Molecular weights calculated at the liquid meniscus and bottom of the cell were 74,900 ±

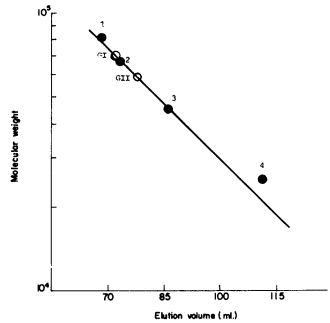


**Fig. 1. Calibration of the column of Bio-Gel P-100 (3 X 60 cm.): 1) Alcohol dehydrogenase, 2) bovine serum albumin, 3) ovalbumin, and 4) chymotrypsinogen A. Elution volumes for glucoamylase I (GI) and glucoamylase II (GII) are also indicated. The column was eluted with 0.1M NaCl at a flow rate of 0.5 ml. per min.**

1,550 for glucoamylase I and 54,300 ±6,690 for glucoamylase II. Those are averages determined at the meniscus and bottom of the cell in two different approach-to-equilibrium experiments using enzymes from different purifications. The partial specific volume of glucoamylase I was calculated as 0.72 ±0.02 ml. per g. by the method of Schachman (19). Glucoamylase II was assumed to have the same partial specific volume. Molecular weights for each enzyme were also estimated by gel filtration on a calibrated column of Bio-Gel P-100 (Fig. 1). A value of 70,000 was obtained for glucoamylase I and 59,000 for glucoamylase II. Both agree well with those obtained by the approach-to-equilibrium procedure. The value of 74,900 for the mol. wt. of glucoamylase I is considerably lower than the 97,000 (7) and 110,000 (9) reported by Pazur and co-workers. Their 97,000 was calculated from the measured sedimentation coefficient and diffusion coefficient and an estimated partial specific volume (0.74 ml. per g.) of the enzyme. Their 110,000 was determined from density-gradient-centrifugation data.

The amino acid compositions of glucoamylases I and II, based on the mol. wts. determined by the Archibald procedure, are shown in Table I. All values were obtained by standard procedures on acid hydrolysates of the enzymes. The enzymes were not subjected to oxidation by peroxyformic acid before analysis, so the half-cystine value is regarded as approximate. Tryptophane was not analyzed. Averaged values are given for each amino acid, except that values for serine and threonine were obtained by extrapolation to zero time because they were partially destroyed during hydrolysis. The value for ammonia was also obtained by extrapolation to zero time. Data from 72-hr. hydrolyses were used for valine and isoleucine because they were released more slowly. The amino acid analyses

TABLE I. AMINO ACID COMPOSITION OF GLUCOAMYLASES I AND II FROM *A. NIGER*[a]

| Amino Acid | Calculated Residues (nearest integer) | |
|---|---|---|
| | Glucoamylase I | Glucoamylase II |
| Lysine | 12 | 9 |
| Histidine | 3 | 3 |
| Ammonia[b] | 63 | 50 |
| Arginine | 18 | 11 |
| Aspartic acid | 75 | 57 |
| Threonine[b] | 79 | 53 |
| Serine[b] | 84 | 62 |
| Glutamic acid | 46 | 31 |
| Proline | 22 | 16 |
| Glycine | 47 | 36 |
| Alanine | 65 | 50 |
| Cystine/2 | 9 | 9 |
| Valine[c] | 45 | 32 |
| Methionine | 2 | 2 |
| Isoleucine[c] | 23 | 16 |
| Leucine | 44 | 33 |
| Tyrosine | 26 | 17 |
| Phenylalanine | 21 | 13 |

[a]All values are the average of 24-, 48-, and 72-hr. hydrolyses in 6M HCl, except where indicated.

[b]Extrapolated to zero time of hydrolysis.

[c]Values from 72-hr. hydrolysis.

indicate that glucoamylase I contains 121 residues of acidic amino acids and 96 residues of basic amino acids, including ammonia—a difference of 25. Glucoamylase II contains 87 residues of acidic amino acids and 73 residues of basic amino acids, including ammonia—a difference of 14. The difference of 11 acidic amino acid residues between the two enzymes could account for the more-rapid migration of glucoamylase I toward the positive pole during electrophoresis and also its lower isoelectric point (5).

Analyses of freeze-dried glucoamylases I and II by the phenol-sulfuric acid method (13,14) indicated that they contained 13 and 18% of total carbohydrate (relative to a standard of D-mannose), respectively. Close agreement with those two values was obtained over several different enzyme preparations from different purifications, as long as the dissolution procedure described was followed. Deviating from the procedure produced inconsistent variations in carbohydrate content, owing to incomplete solubilization of the enzyme. Our values for total carbohydrate content correspond to approximately 60 hexose residues per molecule of each enzyme. Glucoamylases I and II originally were reported to contain 10 and 18% total carbohydrate (24), respectively. Later, glucoamylase I was reported to contain 15% carbohydrate (10). GLC analysis of the *O*-trimethylsilyl ether derivatives of an acid hydrolysate of each gave products having the same relative retention times as the corresponding derivatives of mannose, glucose, and galactose. This agreed with observations of Pazur et al. that mannose was the preponderant carbohydrate component, with lesser amounts of glucose and galactose (24). No amino sugars were detected by paper or gas-liquid chromatography of the acid hydrolysates. Aliquots from samples of glucoamylases I and II, hydrolyzed with 0.1N sulfuric acid, assayed by the thiobarbituric acid assay (25), indicated no sialic acid in either enzyme. The *O*-trimethylsilyl ether derivatives of methanolysates of glucoamylases I and II were quantitatively analyzed by GLC (Table II). The values reported by Pazur et al. (24) are also shown in Table II. The discrepancy between total carbohydrate as determined by the phenol-sulfuric acid procedure and by methanolysis likely can be attributed: a) To the sugars being destroyed under the conditions of methanolysis, b) to incomplete release of the hexoses from the glycoproteins, or c) to protein contributing to color formation in the phenol-sulfuric acid assay.

TABLE II. CARBOHYDRATE COMPOSITION OF GLUCOAMYLASES I AND II FROM A. NIGER

| Procedure | % Mannose | % Glucose | % Galactose | % Total[a] |
|---|---|---|---|---|
| Glucoamylase I | | | | |
| Methanolysis | 6.8 | 2.1 | 0.3 | 10.7 |
| Pazur et al. (24) | 8 | 2 | 0.2 | |
| Glucoamylase II | | | | |
| Methanolysis | 10.6 | 4.4 | 0.7 | 18.2 |
| Pazur et al. (24) | 15 | 3 | 0.2 | |

[a]Total carbohydrate content as determined by the phenol-sulfuric acid assay.

**Nature of the Carbohydrate-Polypeptide Linkage**

Treatment of glycoproteins with alkaline borohydride affects β-elimination of carbohydrate moieties *O*-glycosidically linked to the hydroxyl group of serine or threonine residues, with concomitant reduction of the reducing end of the eliminated carbohydrate to the corresponding alditol (26-29). Glucoamylases I and II were treated with alkaline borohydride, using two different sets of reaction conditions. The first used 0.1M NaOH-0.3M $NaBH_4$ at 45°C. for 16 hr., the reagent concentrations reported by Lee and Lang (30). It has been reported that similar concentrations of alkaline borohydride, used for 5 to 7 days at room temperature, can destroy carbohydrate before it is reduced by the borohydride (21,31,32). The second set of reaction conditions used 0.05M NaOH-1.0M $NaBH_4$ at 45°C. for 16 hr., which has been reported to result in maximal release of the carbohydrate moieties from pig submaxillary mucin with minimal destruction of carbohydrates (21). The reaction mixtures obtained from the two different sets of conditions were submitted to gel filtration on columns of Sephadex G-15. In all cases two components that contained carbohydrate were eluted. One coincided with a major protein component and eluted at the void volume of the column. It was believed to be glycoprotein from which the carbohydrate had not been eliminated. The second carbohydrate component, retained by the column, did not coincide with any protein or peptide component. It represented 60% of the total carbohydrate in the initial enzyme when 0.1M NaOH-0.3M $NaBH_4$ was used, whereas 0.05M NaOH-1.0M $NaBH_4$ released 40% of the carbohydrate in the initial enzyme. So the latter conditions are milder and release less carbohydrate. Those values are considered minimal because they are based on the results of phenol-sulfuric acid assays. The terminal alditol units produce much less color in this assay than do corresponding hexoses because the alditols are not converted to the requisite furfural derivative under the conditions of the assay. Hence they are underestimated. When the carbohydrate component from each of the two elimination conditions was isolated, hydrolyzed, and the products identified by paper or gas-liquid chromatography, the same carbohydrates were present in the same relative proportions. This indicates that no carbohydrate was destroyed before reduction, using the conditions that eliminated more carbohydrate moieties from the enzymes.

When the glycoprotein component eluting at the void volume of the column was isolated and again treated with alkaline borohydride, using a longer period of reaction (several days), only a small proportion of the carbohydrate was eliminated. It is known that the amino and carboxyl functions adjacent to the *O*-glycosidically substituted serine or threonine residues must be substituted for β-elimination to occur (33). When these functions are free, the reaction ceases or is greatly retarded. This may account for the behavior we observed. Because some of the carbohydrate is eliminated slowly upon further treatment with alkaline borohydride, the component eluting at the void volume is believed to represent glycoprotein from which the carbohydrate has not been eliminated because fragmentation of the peptide chain has resulted in *O*-glycosidically-substituted fragments with free amino or carboxyl groups adjacent to the substituted serine and threonine residues. The unsaturated amino acid residues formed during the β-elimination are known to be easily hydrolyzed by alkali (33). This is considered more plausible than is the presence of a different type of linkage between the carbohydrate moieties and the peptide chain. However, the latter cannot yet be entirely ruled out.

Amino acid analyses of the reaction product from the treatment of glucoamylase I with alkaline borohydride revealed the loss of 20 residues of threonine and 14 residues of serine, with a concurrent increase of 19 residues of alanine and 5 moles of α-aminobutyric acid. Under analogous conditions, glucoamylase II revealed a loss of 14 residues of threonine and 11 residues of serine with a concomitant increase of 16 residues of alanine and 3 residues of α-aminobutyric acid. The decrease in the proportions of serine and threonine observed in glucoamylases I and II after treatment with alkaline borohydride, with the simultaneous increase in the proportion of alanine and the appearance of α-aminobutyric acid, indicates that serine and threonine are involved in glycosidic linkages through their hydroxyl groups. These observations confirm those reported in a preliminary communication (8). The alanine and α-aminobutyric acid observed are formed, presumably, under the reducing conditions, from the unsaturated amino acid residues produced by β-elimination of the glycosidically-linked carbohydrate substituents from serine and threonine, respectively. Our results are similar to those reported by Pazur et al. (9) in which the carbohydrate moieties of glucoamylase I were reported to be linked by *O*-glycosidic bonds to approximately 45 serine and threonine residues "presumably on the surface of the enzyme molecule." Their conclusion was based on the loss of 28 residues of threonine and 17 residues of serine (based on a mol. wt. of 110,000) from glucoamylase I on treatment with alkaline borohydride "for 4- or 6-day periods at 0° at reaction conditions which effect a reductive removal of a high percentage of the carbohydrate residues from the protein." The loss of serine and threonine and the gain in alanine and α-aminobutyric acid do not coincide, as dehydrothreonine (2-aminocrotonic acid) is not completely reduced to α-aminobutyric acid under the conditions used (29).

**Structure of Carbohydrate Moieties**

The carbohydrate fragment eliminated from the enzymes by treating with alkaline borohydride was isolated by chromatography on a column of Sephadex G-15. The fractions containing the single carbohydrate component retarded by the column were combined, freeze-dried, and chromatographed on a column of Sephadex G-15 which had been calibrated for molecular size by chromatography or a partially hydrolyzed dextran. The carbohydrate fragment from both enzymes eluted at a volume corresponding to a trisaccharide (Fig. 2). There was no evidence for a component eluting at a volume corresponding to a disaccharide except in two cases when a large amount of enzyme (about 1 g.) had been treated with alkaline borohydride. Then a small shoulder appeared at a volume corresponding to a disaccharide (shown in Fig. 2). This might be due to decomposition of the trisaccharide, as discussed later, or possibly to the presence of disaccharide units, although no direct evidence was obtained for the latter possibility. A monosaccharide would not have been detected by the phenol-sulfuric acid assay, as it would be an alditol. However, when fractions corresponding to the volume in which a monosaccharide would have been eluted were collected, freeze-dried, and analyzed chromatographically, no evidence for a monosaccharide component was obtained. This indicates that the carbohydrate moieties are present in both enzymes as trisaccharides.

When the carbohydrate fragment was subjected to paper chromatography, components migrating with the relative mobilities of mono-, di-, and trisaccharides

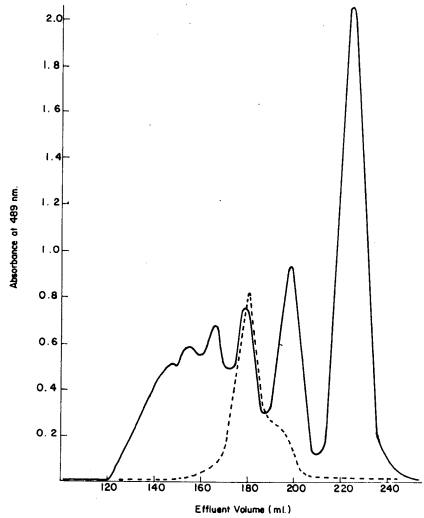


**Fig. 2. Chromatography of partially hydrolyzed dextran (solid line) and the carbohydrate fragment obtained from glucoamylase I following treatment with alkaline borohydride (broken line) on a column (2 X 148 cm.) of Sephadex G-15. The column was eluted with 0.1M acetic acid at a flow rate of 0.7 ml. per min.**

were observed after staining the dried papers. The components were separated by preparative paper chromatography and were then eluted and freeze-dried. Analysis of the monosaccharide fraction by GLC revealed a single component (0.027 to 0.030-mg.), identified by its relative retention time as mannitol. The di- and trisaccharide fractions were hydrolyzed with acid, and *O*-trimethylsilyl ether derivatives of the hydrolysate were formed. Carbohydrates in the hydrolysates were identified and analyzed by GLC with the results shown in Table III. The trisaccharide component contained the proper ratios of monosaccharides for a trisaccharide having mannitol at the former reducing end, as would be expected in the product from alkaline borohydride elimination. This confirmed the earlier report that the carbohydrate moieties in both enzymes are *O*-glycosidically linked through mannose to the hydroxyl groups of serine and threonine (8). The ratio of glucose to mannose (1:3) in the trisaccharide component indicates that a heterogeneity of structure must be present; i.e., the ratio does not allow the trisaccharide to contain a single unit of each hexose.

The results from analysis of the disaccharide fraction were unexpected, as the alditol component was so small that it could not account for the former reducing end of a disaccharide unit. The alternative explanation that the reducing end is still present in a reducing form is untenable because, if so, it would be reduced to an alditol in the presence of borohydride. An alternative explanation is an acid-labile linkage in the trisaccharide component which was cleaved in the 0.1M acetic acid used during the chromatographic isolation. As such cleavage would be subsequent to the alkaline borohydride treatment, a monosaccharide (alditol) and a disaccharide having a reducing end could result if the acid-labile bond were between the first and second carbohydrate units. This idea was further investigated. A

TABLE III. RESULTS OF ANALYSES OF FRACTIONS OBTAINED FROM PAPER CHROMATOGRAPHY OF THE CARBOHYDRATE FRAGMENT ISOLATED FROM GLUCOAMYLASE I[a] FOLLOWING TREATMENT WITH ALKALINE BOROHYDRIDE

| | Fractions | | | |
|---|---|---|---|---|
| | Disaccharide | | Trisaccharide | |
| Carbohydrate | mg. | Ratio | mg. | Ratio |
| Mannose | 0.262 | 15.4 | 0.051 | 1.5 |
| Galactose | 0.038 | 2.2 | Trace[b] | ... |
| Glucose | 0.069 | 4.1 | 0.018 | 0.5 |
| Mannitol | 0.017 | 1.0 | 0.033 | 1.0 |

[a]Glucoamylase II gave virtually identical results.

[b]Present, but in insufficient amount to quantitate.

portion of the freeze-dried, chromatographically-pure, trisaccharide fraction was dissolved in 0.1M acetic acid and allowed to stand at room temperature for 60 hr. Paper chromatography of the freeze-dried residue revealed small amounts of components migrating as monosaccharides and disaccharides after staining the dried chromatogram. GLC of the trimethylsilyl ether derivatives of the freeze-dried residue revealed mannitol as the only monosaccharide. An aliquot of the pure trisaccharide fraction dissolved in water remained unchanged after 60 hr. at room temperature. Such evidence indicates an acid-labile linkage in at least a portion of the trisaccharide component, further indicating probable heterogeneity of structure and linkage in the component. Only a small amount of hydrolysis would be expected at this stage, because most of the acid-labile linkages would have been hydrolyzed during previous exposure to 0.1M acetic acid during and after the column-chromatographic procedures.

When the reaction product from treating the enzymes with alkaline borohydride, prior to chromatography on Sephadex G-15, was investigated by paper chromatography, only a single component was observed when the paper was stained with silver nitrate dip reagent. The single component had the mobility of a trisaccharide. The presence of this single component mitigates against the presence of disaccharide or monosaccharide units in the enzyme. If disaccharide or monosaccharide units *O*-glycosidically linked to serine and threonine were present, components with the mobility of a disaccharide or monosaccharide should be observed on the chromatogram. The trisaccharide also was the only component observed when the reaction product was dissolved in water and allowed to stand at room temperature for 60 hr. However, when the product was dissolved in 0.1M acetic acid and allowed to stand for 60 hr., components having the mobilities of mono-, di-, and trisaccharides were clearly evident when the freeze-dried residue was subjected to paper chromatography. The monosaccharide was identified as mannitol by the relative retention time of its *O*-trimethylsilyl ether derivative. Such evidence confirms an acid-labile linkage in the trisaccharide reaction product. The linkage seems to be between the first and second carbohydrate residues. The nature of this linkage is not yet known. The absence of a monosaccharide in the elimination products from the alkaline borohydride treatment is further evidence that the mixture is not formed during the elimination reaction.

Evidence from isolating the carbohydrate fragment eliminated from both enzymes indicates that the carbohydrate moieties in glucoamylases I and II are

trisaccharide units. Thus both enzymes would contain approximately 20 trisaccharide units. Destruction of serine and threonine during the alkaline borohydride treatment argues for smaller units, as 34 residues were destroyed in glucoamylase I and 25 residues in glucoamylase II. The same number of serine plus threonine residues, approximating 20, should be destroyed in both enzymes if the carbohydrate units are trisaccharides. Serine and threonine residues, which are not substituted on the hydroxyl groups with carbohydrate, have been reported to be relatively stable during treatment with mild alkali (0.1 to 0.5N at 0° to 5°C. for several days) (33). The reason for the apparent discrepancy in our investigation is not known. However, evidence for the trisaccharide nature of the eliminated carbohydrate fragment is much stronger on the basis of its isolation and analysis than on the basis of decomposition data obtained from amino acid analyses. Conditions for the amino acid analysis of the alkaline borohydride reaction product were less than ideal because salts were present. Amino acid analyses of glycoproteins must also be regarded as approximate, rather than exact, owing to the destructive interaction of amino acids and carbohydrates in addition to their individual destruction under the hydrolytic conditions. Thus, the conclusion that carbohydrate moieties are present as trisaccharide is regarded as warranted by the isolation and analysis data. The presence of small amounts of disaccharide or monosaccharide moieties cannot be eliminated, owing to the above discrepancy and to the shoulder on the elution diagram (Fig. 2) encountered in two instances. However, their presence is regarded as unlikely since no aldosyl-alditols or alditols were detected under conditions where they should be formed from disaccharide and monosaccharide moieties, respectively.

#### Nature of the Linkages in the Carbohydrate Moieties of Glucoamylases I and II

Purified glucoamylases I and II were treated with the α-mannosidase from jack-bean meal. Mannose was released from both enzymes during incubation and was identified by the relative retention time of its *O*-trimethylsilyl ether. Our results agree with the observation of Pazur et al. (10) that a partially modified glucoamylase I could be prepared by treating the pure enzyme with α-mannosidase. The α-mannosidase from jack-bean meal has been shown to hydrolyze terminal nonreducing α-D-mannopyranose units in certain glycoproteins and polysaccharides (34). Our results indicate that at least a portion of the trisaccharide units in glucoamylases I and II contain an α-D-mannopyranose unit at the nonreducing terminus, which also was confirmed by treating the trisaccharide and disaccharide fragments, isolated from glucoamylases I and II after treatment with alkaline borohydride, with α-mannosidase. GLC analysis of aliquots of the enzyme digest removed at various time intervals revealed that mannose was released from both carbohydrate fragments faster than from the intact enzymes.

The above evidence and the analyses of the carbohydrate fragments show that carbohydrate moieties of glucoamylases I and II are present as trisaccharide units with at least two different sequences:

Man–Man–Man–  Man–Glu–Man–

Other units containing galactose must also be present. Analytical data indicate approximately two galactose units per molecule of enzyme. They could be in either

one or two carbohydrate moieties. A few units containing glucose at the nonreducing terminus may also be present. It is not yet possible to further define the structure of the carbohydrate moieties. It is evident that a heterogeneous structure exists, confirming the suggestion of Pazur et al. (10) that the synthesis of the carbohydrate moieties is apparently not under genetic control.

## Conclusions

Previous studies of glucoamylases I and II have shown that they had the same pH optima when starch was used as a substrate, temperature stability at elevated temperatures, action patterns of maltooligosaccharides, antigenicity, and $NH_2$-terminal amino acids (5). They differed in electrophoretic mobility, isoelectric point, and, to a small degree, in stability at room temperature for prolonged periods. Glucoamylase I was not dissociated into subunits in the presence of urea, acid, or β-mercaptoethanol under the conditions used (5).

The results of our investigation indicate that the two enzymes possess different mol. wts. but have the same carbohydrate content. The different mol. wts. are reflected by difference in amino acid composition, which may account for their electrophoretic difference and their different isoelectric points. If the carbohydrate moieties are involved in stabilizing the enzyme (9), the difference in molecular size of the two enzymes may account for their small differences in stability at room temperature over prolonged time. The carbohydrate units of both enzymes are *O*-glycosidically linked through mannose to hydroxyl groups of serine and threonine in the polypeptide chain. The carbohydrates are present as trisaccharide units in both enzymes, the majority of which have an α-D-mannopyranose residue at the nonreducing terminus. A heterogeneous carbohydrate structure is indicated, with the majority of units either man-man-man- or man-glu-man- and smaller amounts of other structures also present. This is further evidence that biosynthesis of the carbohydrate moieties of these enzymes is not under direct genetic control, as protein synthesis is.

## Acknowledgments

This investigation was supported by a grant from the Agricultural Research Service, U.S. Department of Agriculture, Grant No. 12-14-100-9143 (71), administered by the Northern Utilization Research and Development Division, Peoria, Ill. I. J. Russell, W. E. Baumann, and Mrs. C. Rasmussen assisted in obtaining some of the data. The ultracentrifuge experiments were performed in the Department of Chemistry, University of Nebraska.

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[Received August 3, 1971. Accepted December 14, 1971]