GLYCOENZYMES: STRUCTURE AND PROPERTIES OF THE TWO FORMS OF GLUCOAMYLASE FROM *Aspergillus niger**

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ABSTRACT

Aspergillus niger produces two forms (isoenzymes) of glucoamylase that are separable by electrophoresis or by chromatography on DEAE-cellulose and that are designated glucoamylase I and II. The molecular weight of glucoamylase I is 99,000, and that of glucoamylase II is 112,000. Both forms of the glucoamylase contain covalently linked carbohydrate (containing D-mannose, D-glucose, and D-galactose residues) and are therefore glycoenzymes. The carbohydrate–protein linkage in the glycoenzyme is primarily glycosidic to the hydroxyl group of L-serine and L-threonine residues, but glycosylamine linkages to L-asparagine and L-glutamine may also be present. Glucoamylase I and II possess identical amino acid compositions and, presumably, identical amino acid sequences. However, the two glycoenzymes differ in carbohydrate content, glucoamylase II containing nearly twice as many carbohydrate residues per molecule as glucoamylase I. Accordingly, it is suggested that the two forms of glucoamylase are isoglycoenzymes. The difference in electrophoretic and chromatographic properties of the isoglycoenzymes is probably due to a difference in the number of amide groups or glycosylaminically linked carbohydrate units in the polypeptide chains.

INTRODUCTION

Most fungal glucoamylases contain covalently linked carbohydrate residues in their molecular structures, in arrays characteristic of the individual glucoamylase[1–5]. The principal types of monosaccharide residue that have been identified as structural units of glucoamylases are those of D-mannose, D-glucose, D-galactose, and 2-amino-2-deoxy-D-glucose. The term glycoenzyme has been suggested, and is recommended, as appropriately descriptive of enzymes of this type[6]. With the advent of new methods for the purification and analysis of enzymes, an increasing number of enzymes have been found to contain covalently linked carbohydrate residues. Thus, although the presence of carbohydrate residues in yeast invertase was detected many years ago, by

*Dedicated to Dr. Nelson K. Richtmyer in honor of his 70th birthday.

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Richtmyer, Hudson, and Adams[7,8], it is only recently that such residues have been shown to be integral structural units of the enzyme[9]. Yeast invertase, like fungal glucoamylase, is, therefore, a glycoenzyme. Other hydrolytic enzymes that are glycoenzymes include fungal *alpha*-amylase[10], bovine ribonuclease[11], bovine β-D-glucosiduronase[12], bovine 2′-deoxyribonuclease[13], broad-bean α-D-galactosidase[14], and pineapple bromelain[15].

Glucoamylase was first obtained in pure form by Pazur and Ando[16], by chromatography on DEAE-cellulose of extracts of mycelium of *Aspergillus niger*. In these studies, two forms (isoenzymes) of glucoamylase were obtained, and these isoenzymes were separable by electrophoresis, as well as by chromatography on DEAE-cellulose. Subsequently, the suggestion was advanced that the difference in the isoenzymes was due to differences in the number of carbohydrate residues and, perhaps, in the types of protein–carbohydrate linkages present[1,17]. If this suggestion is correct, the two forms of glucoamylase are isoglycoenzymes containing identical polypeptide chains but differing in the carbohydrate portion of the molecule. Additional data on molecular-weight values, amino acid composition, carbohydrate content, and electrophoretic and immunological properties have now been obtained, and these data substantiate the concept of isoglycoenzymes. Radioactive glucoamylases have proved very valuable in these studies; accordingly, procedures for the preparation and characterization of the ^{14}C-labeled glucoamylases are described.

RESULTS AND DISCUSSION

The differences in the electrophoretic mobility of the two forms of glucoamylase are illustrated in Fig. 1, in which the locations of the enzymes on paper-electrophoretic strips are shown. The nomenclature employed for the two isoenzymes, namely, glucoamylase I and II, is in accord with recommendations by Webb[18]. The glucoamylase activities on the strips shown in Fig. 1 were detected by spraying the strips with starch, D-glucose oxidase, peroxidase, and *o*-tolidine, as described in the Experimental section. Duplicate electrophoretic strips stained to reveal protein components showed that the protein and the enzymic activity migrated at identical rates.

Fig. 2 shows enzymic and radioactivity data for paper-electrophoretic strips of ^{14}C-labeled glucoamylase I. In this experiment, the paper strips were dried and cut into sections; each section was extracted with water, and the extract was assayed for enzymic activity and radioactivity. From Fig. 2, it is apparent that the distribution of radioactivity paralleled the distribution of the enzymic activity on the paper strips. Radioactive glucoamylase II, like the non-labeled enzyme, migrated at a rate lower than that of glucoamylase I. The difference in migration rates is reflected in the isoelectric points of the two isoenzymes, namely, 3.5 for glucoamylase I, and 4.0 for glucoamylase II.

The radioactive isoenzymes of glucoamylase were produced by growing *Aspergillus niger* on a labeled-carbohydrate source (D-glucose-*1*-^{14}C or D-mannose-

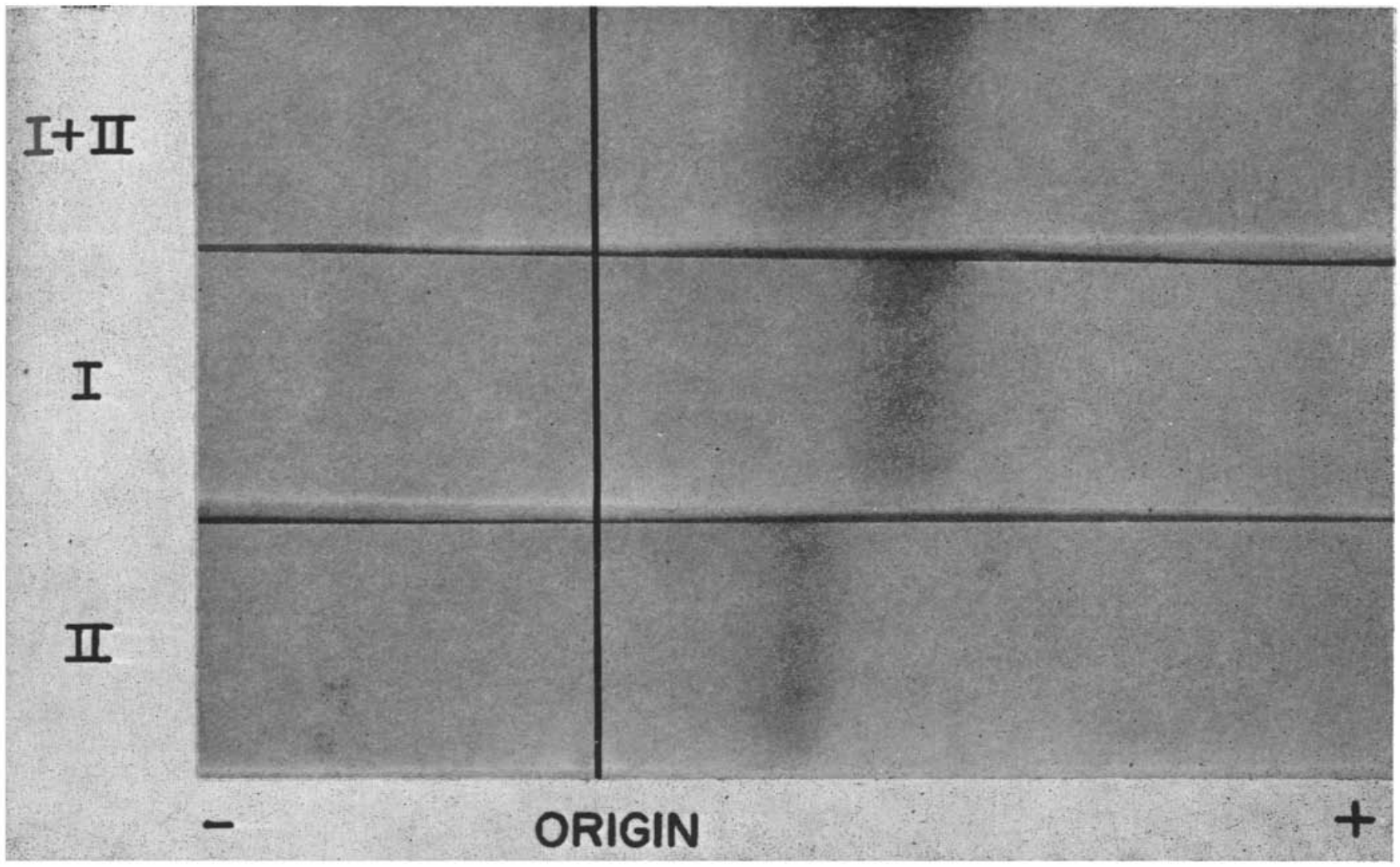

Fig. 1. A photograph of paper-electrophoretic strips of the isoenzymes of glucoamylase. (I, glucoamylase I; II, glucoamylase II; I+II, mixture of the two.)

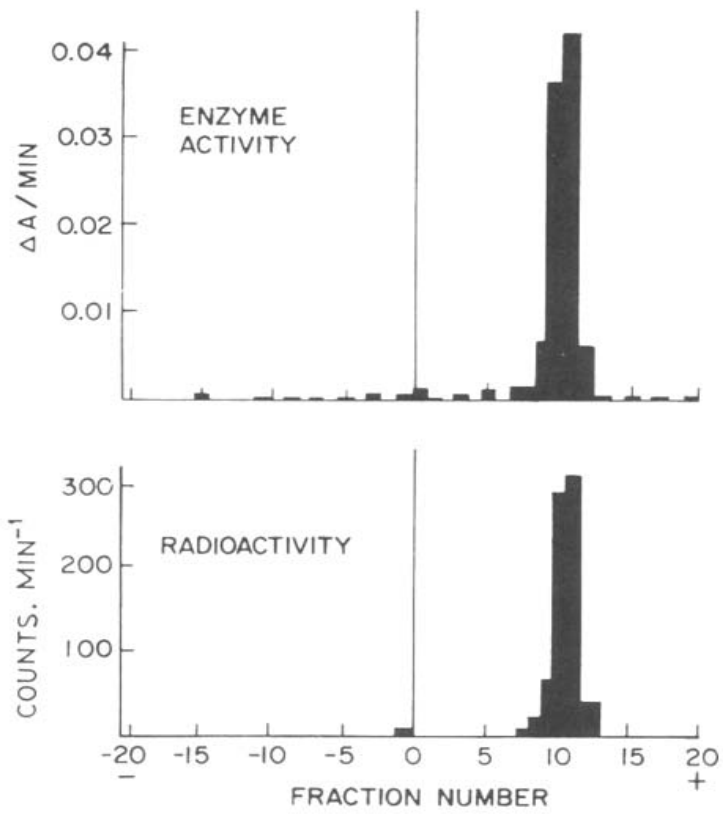

Fig. 2. Enzymic activity and radioactivity of samples of ^{14}C-labeled glucoamylase I extracted from paper-electrophoresis strips.

1-^{14}C). The isoenzymes were isolated from the culture by precipitation with ethanol, and purified by chromatography on DEAE-cellulose. Data on the yields of the glucoamylases, as well as the radioactivities of the isoenzymes for a typical experiment, are recorded in Table I. It should be noted that, under the conditions of this experi-

TABLE I

RADIOACTIVITY AND SPECIFIC ACTIVITY OF ISOENZYMES OF GLUCOAMYLASE FROM *A. niger* GROWN ON D-GLUCOSE-*1-*^{14}C

Treatment	*Radio-activity (total counts. min*$^{-1}$ $\times 10^{-6}$)	*Enzyme activity (total units* $\times 10^{-5}$)	*Specific activity (units/mg)*	*Radio-activity (counts. min*$^{-1}$*/unit)*
Filtrate (I+II)	1,700	2.0	6	
Ethanol ppt. (I+II)	14	1.8	276	
Glucoamylase I	2.0	1.1	2,270	18
Glucoamylase II	0.9	0.3	2,015	32

ment, glucoamylase I was obtained in considerably higher amount than glucoamylase II, perhaps reflecting a difference in the rate of synthesis of the two isoenzymes in the fungal cell. Whereas the specific activities of the two isoenzymes were comparable, the radioactivity of glucoamylase II was almost twice that of glucoamylase I. The higher radioactivity of glucoamylase II may be attributable to the higher carbohydrate content of this isoenzyme.

In order to determine whether nucleotide pathways were utilized for incorporation of the radioactive carbohydrates into the glycoenzymes, the location of the carbon-14 in the carbohydrate residues from labeled glucoamylase I was determined. The labeled glucoamylase was hydrolyzed in acid, and the resulting monosaccharides were separated, and isolated, by paper-chromatographic techniques. Radioautograms of the chromatograms of the hydrolyzates of the isoenzymes showed that the resulting D-mannose, D-glucose, and D-galactose were radioactive. Degradation of the D-mannose isolated from glucoamylase I was effected by a microbiological procedure[19], C-1 being recovered as sodium carbonate, C-2 and C-3 as ethanol, and C-4, -5, and -6 as lactic acid. The data, together with those for appropriate reference compounds, are recorded in Table II. On the basis of these results, it was concluded that the hexose residues are attached to the glycoenzyme mainly as intact residues, most likely *via* a nucleotide pathway. Preliminary evidence has been presented that indicates that the attachment does, indeed, occur subsequent to synthesis of the polypeptide chain[6]. The occurrence, in extracts from mycelium of *A. niger*, of the enzymes of the nucleotide pathway of carbohydrate metabolism has recently been demonstrated[20].

In order to obtain (*a*) additional evidence as to the purity of the isoenzymes, and (*b*) information on molecular weights, the labeled and non-labeled isoenzymes were subjected to density-gradient centrifugation[21]. After centrifugation, the density-

TABLE II

DISTRIBUTION[a] OF ^{14}C IN LABELED MONOSACCHARIDES, AS DETERMINED WITH *Leuconostoc mesenteroides*

Compound	*Initial radioactivity*	*C-1*	*C-2+C-3*	*C-4+C-5+C-6*
D-Glucose-*1*-^{14}C	16,500	14,000	25	40
D-Mannose-*1*-^{14}C	22,400	18,900	80	30
D-Mannose-*2*-^{14}C	17,000	230	11,000	770
D-Mannose-^{14}C[b]	19,900	14,800	435	2,000

[a]All figures expressed in counts. min^{-1}.
[b]Isolated from glucoamylase I.

gradient columns were fractionated, and the fractions analyzed for radioactive, enzymic, and u.v.-absorbing components. Data for an experiment on the sedimentation of ^{14}C-labeled glucoamylase I in a column of cesium chloride are presented in Fig. 3; it is apparent that enzymic activity and radioactivity were present in comparable ratios in the various fractions from the density-gradient column, and that the ^{14}C-labeled enzyme was not contaminated with any radioactive impurities.

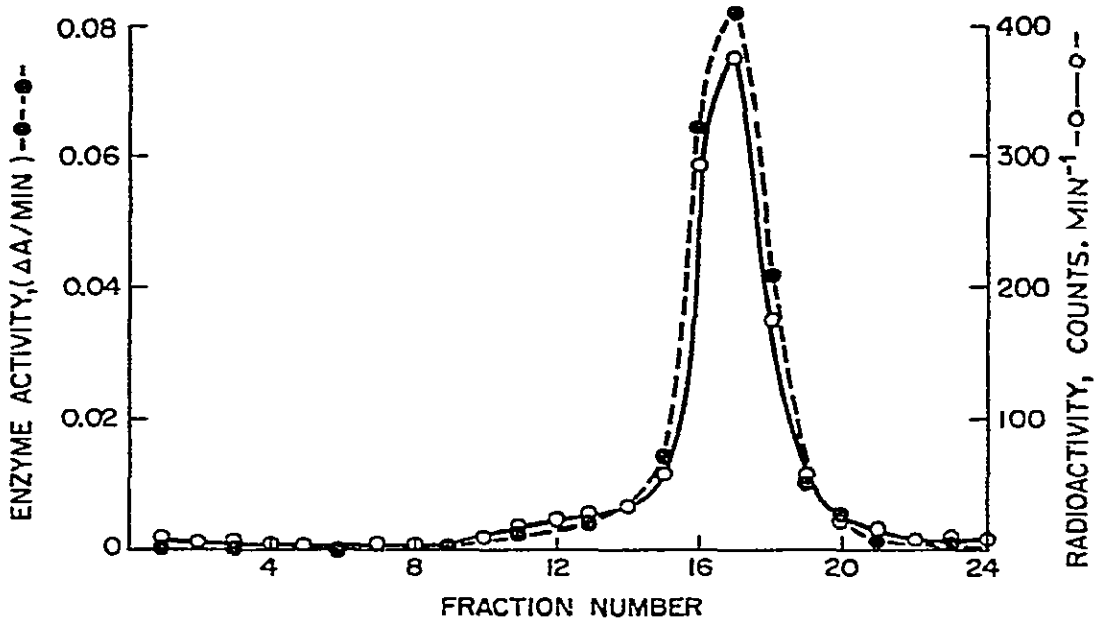

Fig. 3. Distribution of radioactivity and enzymic activity in fractions from density-gradient columns of cesium chloride.

For determinations of molecular weight, labeled and non-labeled isoenzymes, together with reference proteins (serum albumin and D-glucose oxidase), were centrifuged by the density-gradient method. The components in the density-gradient columns were located by measurements of u.v. absorbance, enzyme activity, or radioactivity. After centrifugation for 6 h at 65,000 r.p.m. in sucrose gradients of 5–25%, the distances (in cm) to which the various proteins sedimented were as follows: serum albumin, 1.30; glucoamylase I, 1.72; glucoamylase II, 1.88, and D-glucose oxidase, 2.32. The small difference between the two isoenzymes of glucoamylase was verified by the data in Fig. 4, in which separations of labeled and non-

labeled isoenzymes are shown. Whereas labeled and non-labeled glucoamylase I sedimented at identical rates, glucoamylase II sedimented at a high rate than glucoamylase I in both of the combinations shown in Fig. 4, as well as in several other

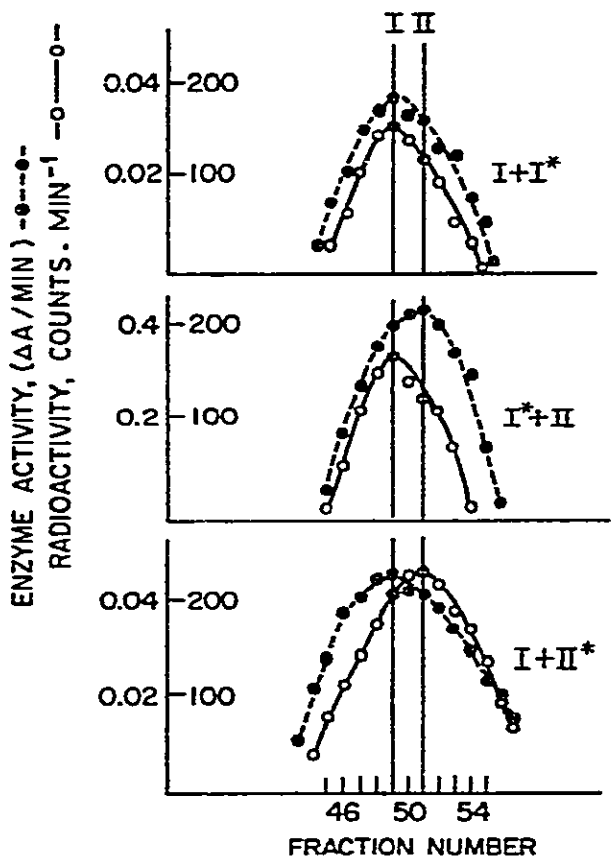

Fig. 4 Distribution of radioactivity and enzymic activity in fractions from density-gradient columns of sucrose; the asterisk indicates the ^{14}C-labeled isoenzyme.

combinations that were tested. The molecular weight of the isoenzyme was calculated from these data by utilizing a formula developed by Martin and Ames[22], together with a molecular weight of 67,000 for serum albumin[23] and of 150,000 for D-glucose oxidase[24]. Values of 99,000 for glucoamylase I and 112,000 for glucoamylase II were obtained. The value for glucoamylase I is in good agreement with that (97,000) calculated from sedimentation and diffusion coefficients obtained from analytical-centrifuge measurements[25].

Quantitative data on the amino acid and carbohydrate compositions of each isoenzyme are presented in Table III. For comparative purposes, data for a glucoamylase from *Rhizopus delemar* are also included. It may be noted that there is a remarkable similarity in the amino acid composition of the two isoenzymes from *A. niger*. In fact, it may be concluded that the two isoenzymes possess the same amino acid composition, and that the small differences are due to experimental error in the amino acid analyses. The amino acid composition of the glucoamylase from *R. delemar* is different from that of the two isoenzymes.

There is, however, a marked difference between the number of carbohydrate residues in the two isoenzymes. Both glucoamylase I and glucoamylase II contain D-mannose, D-glucose, and D-galactose residues, but glucoamylase II contains nearly twice as many hexose residues as glucoamylase I. The glucoamylase from *R.*

TABLE III

NUMBER OF AMINO ACID RESIDUES AND CARBOHYDRATE RESIDUES PER MOLECULE OF GLYCOENZYME

Residues of	*Glucoamylase I*	*Glucoamylase II*	*Glucoamylase R*
Aspartic acid	83	85	104
Threonine[a]	104	103	88
Serine[a]	119	121	108
Glutamic acid	56	54	48
Proline	29	29	31
Glycine	59	63	77
Alanine	78	82	87
Cystine/2	8	8	6
Valine	46	46	58
Methionine	4	4	6
Isoleucine	27	25	36
Leucine	53	56	57
Tyrosine	30	29	44
Phenylalanine	27	28	34
Lysine	16	16	39
Histidine	6	6	8
Arginine	23	22	18
Tryptophan	30	32	36
[Ammonia[b]	21	53	]
Mannose	69	128	67
Glucose	16	20	
Galactose	2	3	
2-Amino-2-deoxy-D-glucose (Glucosamine)			20

[a]Corrected for 13 and 25% loss of threonine and serine, respectively. [b]Corrected for ammonia produced by degradation of threonine, serine, and tryptophan.

delemar contains approximately the same number of carbohydrate residues as glucoamylase I, but these consist only of those of D-mannose and 2-amino-2-deoxy-D-glucose.

Data published[17] on the reductive elimination of the carbohydrate residues from glucoamylase I have been interpreted as indicating many points of attachment of carbohydrate residues to the polypeptide chain of the enzyme. Furthermore, the main points of attachment of carbohydrate residues are at the L-serine and L-threonine residues[17,26]. However, the possibility of presence of other types of linkage of carbohydrate to protein has not been eliminated. One possibility is the occurrence of glycosylamine linkages to the amide groups of L-asparagine or L-glutamine. Glycoproteins are known in which more than one type of carbohydrate–protein linkage are present[27]. Hydrolyzates of glucoamylase II contained, per aliquot, considerably more ammonia than the same aliquot of hydrolyzates of glucoamylase I (see Table III), indicating that this isoenzyme possesses a higher number of amide or, perhaps, glycosylamine linkages. As noted in Fig. 1, on electrophoresis at pH 7.6, glucoamylase II migrates at a lower rate than glucoamylase I, and, accordingly, this isoenzyme must contain a smaller number of negative charges. In molecules having

the same amino acid composition and differing only in the content of neutral carbohydrate, some of the ionizable groups of the amino acids of the protein must be masked in one molecular species if the two species migrate at different rates. As already indicated, this masking may be effected by amide or glycosylamine groups.

In view of the foregoing considerations and the observation that the glucoamylase cannot be dissociated into subunits[28], the hypothesis is advanced that the polypeptide chains of the two isoenzymes of glucoamylase from *Aspergillus niger* are identical in amino acid composition and sequence. This hypothesis is supported by data on N-terminal amino acid residues and on immunological properties of the two isoenzymes. Thus, L-alanine had been found to be the N-terminal amino acid in both isoenzymes, and precipitin tests with antibodies against glucoamylase I and glucoamylase II showed that cross reactivities occur at approximately equivalent concentrations of antigen and antibody. Verification of the suggestion that the two forms of glucoamylase are isoglycoenzymes must await the determination of the complete amino acid sequence in each isoenzyme. Studies on the amino acid sequence of the two glucoamylases are in progress.

EXPERIMENTAL

Enzyme assay. — A rapid, micro procedure for assaying for glucoamylases has been developed; it was used routinely for monitoring activity during synthesis and purification of the glycoenzymes. The procedure is a coupled enzyme assay in which D-glucose oxidase and peroxidase are used to measure the D-glucose liberated from amylose by the glucoamylase. A chromogen (*o*-dianisidine) is used in the reaction mixture, and the u.v. absorption of the reduced chromogen is monitored continuously in a Gilford recording spectrophotometer at 400 nm at 23°. In a typical assay, silica cuvets having a 1-cm light-path were filled with 0.25 ml of aassy mixture consisting of 20 μg of purified D-glucose oxidase, 20 μg of peroxidase, 200 μg of amylose, 80 nmoles of *o*-dianisidine, and 10 μmoles of 40 mM sodium acetate of pH 5.0. A sample (10 μl) of the enzyme solution is injected into the cuvet and the change in color is rapidly monitored. In order to give a change in absorbance of 0.1 to 0.6 per 10 min, it may prove necessary to dilute the enzyme sample being assayed. A unit of enzyme activity was defined as that amount that causes a change in absorbance of 1.0 per 10 min under the conditions just outlined.

Culture of Aspergillus niger. — *Aspergillus niger* (Strain NRRL 330), as well as other strains of *A. niger*, produces the two forms of glucoamylase[16,29]. The spores from a single slant were sufficient for the inoculation of starter cultures.

The *A. niger* strains were maintained on agar slants of the nutrients listed. The medium used for maintaining and culturing *A. niger* had the following composition (figures in g/l): carbohydrate (D-glucose or D-mannose), 30.0; nutrient broth, 8.0; $NaNO_3$, 3.0; K_2HPO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.5; and KCl, 0.5. Each liter of this medium also included 0.1 ml of a trace-element solution, as recommended by Lineback *et al.*[30]; this was composed (in g/l) of $CuSO_4 \cdot 5H_2O$, 1.25; $FeSO_4 \cdot 7H_2O$,

8.0; $ZnSO_4 \cdot 7H_2O$, 6.2; $Na_2MoO_4 \cdot 2H_2O$, 0.31; $MnCl_2 \cdot 4H_2O$, 0.52; and $CaCl_2$, 0.025. The complete medium was autoclaved for 15 min at 120°. When radioactive D-glucose or D-mannose was used, the labeled carbohydrate was added to the autoclaved medium through a microfilter.

The vegetative form of *Aspergillus niger* was grown at room temperature in submerged culture in 500 ml of medium in a 1- or 2-liter, Erlenmeyer flask on a variable-speed shaker (New Brunswick Model 200). The rate of agitation of the culture was found to affect the growth of the organism, and the maximal speed of the shaker was generally employed.

Preparation of ^{14}C-labeled glucoamylases. — *Aspergillus niger* was grown in 500 ml of medium containing 0.5 mCi of D-glucose-*1*-^{14}C. After growth for 85 h, the mycelium was removed by filtration through cheesecloth. The filtrates from two culture-flasks were combined, and mixed with four volumes of 95% ethyl alcohol at room temperature; at this concentration of alcohol, over 90% of the glucoamylase was precipitated from solution. The precipitate was collected by centrifugation, dissolved in phosphate buffer of pH 8.0, and subjected to chromatography on DEAE-cellulose[6,16].

Assays of the eluates from the column showed that glucoamylase activity was present in the fractions of pH 6.0–5.5 and pH 4.5–4.0. These two sets of fractions were combined, and the glucoamylases were precipitated by addition of 95% ethyl alcohol (4 volumes). The precipitates were collected by centrifugation for 15 min at 16,000 *g* and dissolved in a small volume of water, and the solution was lyophilized to dryness. The yield of glucoamylase I (from the fractions of pH 4.0–4.5) was 50 mg, and that of glucoamylase II (from the fractions of pH 5.5–6.0) was 15 mg. The specific activities and radioactivities of these two isoenzymes are recorded in Table I.

Location of ^{14}C in the labeled glucoamylase I. — A sample of purified glucoamylase I (10 mg, total radioactivity 2.7×10^5 counts·min^{-1}) was hydrolyzed in M hydrochloric acid for 4.5 h. at 100°. The hydrolyzate was placed, as a continuous band, on paper chromato-sheets (11 × 14 in.). The chromatograms were developed (two ascents) with 6:4:3 (v/v) butyl alcohol–pyridine–water[31]. Radioautograms of the chromatograms showed that radioactive monosaccharides were present on the chromatograms at R_F values corresponding to those of D-mannose (0.65), D-glucose (0.59), and D-galactose (0.54). The compounds having R_F 0.65 and 0.59 reacted with D-glucose oxidase[24], and that having R_F 0.54 reacted with D-galactose oxidase[32]. The areas containing the carbohydrates were eluted from the chromatograms with water, concentrated to ~0.5 ml, and used in the degradation studies.

Leuconostoc mesenteroides was used as described by Gibbs *et al.*[19] for degrading the D-mannose-*1*-^{14}C and D-glucose-*1*-^{14}C isolated from hydrolyzates of labeled glucoamylase I. The bacterium was adapted to grow on D-mannose by gradually replacing the D-glucose in the medium with D-mannose. At the end of the last 12-h growth-period, the cells were harvested by centrifugation, and washed twice with distilled water. Active cells could be maintained for periods of up to 10 h by storing them, as a paste, under refrigeration; before use, the cells were suspended in water at

a concentration of 1 g (wet weight) per 3 ml of water. Organisms adapted to D-mannose were used for degrading reference samples of D-mannose-*1*-^{14}C, D-mannose-*2*-^{14}C, and D-glucose-*1*-^{14}C.

The degradation mixture consisted of 5 ml of 1.0M KH_2PO_4, pH 6.0 (5 ml), water (5 ml) containing the hexose-^{14}C (total radioactivity ~20,000 counts·min^{-1}), the appropriate non-labeled hexose (90 mg), and the cell suspension of *L. mesenteroides* (3 ml). The degradation was allowed to proceed for 2 h at 30° in a closed reaction-vessel. The carbon dioxide (from C-1 of the hexose) was collected in traps containing 1.0M sodium hydroxide (15 ml). Ethanol (from C-2 and C-3) was obtained by distillation of the degradation mixture, and lactic acid (from C-4, -5, and -6) was obtained by extraction of the residue with diethyl ether. Appropriate aliquots of the sodium carbonate solution, ethanol, and lactic acid were used for measurements of radioactivity in a liquid scintillation counter. Data from typical experiments are recorded in Table II.

Non-labeled glucoamylases. — Samples of purified glucoamylase I and glucoamylase II, prepared by procedures described[16], were available in this laboratory. The non-labeled samples were homogeneous according to their electrophoretic and ultracentrifugational properties, and chromatographic behavior on ion-exchange columns. Purified glucoamylase R, from *Rhizopus delemar*, was also available in this laboratory[4].

Electrophoresis of glucoamylases. — A Spinco Model R electrophoresis cell was used in determining the eletrophoretic properties of the glucoamylases. Samples of 0.1 to 0.03 ml of the enzyme solutions (containing 30 to 90 μg of total protein) were applied to paper strips premoistened with 0.1M potassium phosphate buffer of pH 7.6. Electrophoresis was performed for 18 h at 120 V (D.C.) and 10–15 mamp. The starch-hydrolyzing enzymes were located on the paper by spraying the strip with (*a*) a 2% solution of starch, (*b*) a solution of D-glucose oxidase (1% in 0.2M acetate buffer of pH 5.3) containing a small proportion of peroxidase, and (*c*) a solution of *o*-tolidine (1% in 95% ethanol)[33]. The area of the paper strip containing the glucoamylase appeared as a blue band. A photograph of the electrophoretic strips for a mixture of glucoamylases I and II and for the purified isoenzymes I and II is reproduced in Fig. 1. The protein components on the strips were located by staining duplicate strips with Light-Green SF Yellowish dye (Fisher Scientific Co., Fairlawn, N.J.) as described by Payne and Marsh[34].

The radioactive glucoamylases were also subjected to paper electrophoresis as just described. The dried paper-strips were then sectioned into 0.3-cm sections, beginning at the origin. Each section was extracted with water (0.4 ml). Samples (10 μl) of the extract were used for enzymic assays, and the rest of the sample, for measurements of radioactivity. A plot of the data for glucoamylase I is given in Fig. 2.

In order to determine the isoelectric points of the two glucoamylases, samples of the isoenzymes were subjected to paper electrophoresis in 50 mM citrate buffer over the pH range 3–6 for 18 h at 120 V (D.C.) and 10–15 mamp. The protein components were located by staining the strips with the SF Yellowish dye. Organic dyes K-150 and

K-160 (Kensington Scientific Corp., Berkeley, Calif.) were used as internal standards, in order to determine the amount of electrodiffusion that occurred during the experiment.

Density-gradient centrifugation of glucoamylases. — Samples (~500 μg) of labeled isoenzymes dissolved in 0.2 ml of buffer were each placed, in a layer, on the top of a density-gradient column (1.02–1.10 g/ml) of cesium chloride. The samples were centrifuged for 7 h in a swinging-bucket rotor in a Spinco Model L 65 centrifuge at 58,000 r.p.m. The tube contents were then fractionated (0.2-ml fractions) by use of an automatic fractionator and recorder[21]. Aliquots (4 μl) of each fraction were assayed for glucoamylase activity, and 140-μl aliquots were used for measurements of radioactivity. The data for glucoamylase I are given in Fig. 3.

The method of density-gradient centrifugation was also used for determining the molecular weights of the glucoamylases. Serum albumin (mol. wt.[23] 67,000) and glucose oxidase (mol. wt.[24] 150,000) in various combinations with the glucoamylases were also centrifuged. As it was expected that the molecular weight of glucoamylase II would prove to be only slightly greater than that of glucoamylase I, a series of experiments on the centrifugation of labeled and non-labeled glucoamylases were also performed. In these experiments, mixtures of non-labeled enzymes and radioactive enzymes in the ratio of 49:1 were centrifuged in a sucrose gradient (5–25%) for 6 h at 65,000 r.p.m. One-drop fractions (~0.05 ml) from the column were obtained by use of the fractionator. Aliquots (10 μl) of the fractions were used for determining the enzyme activity, and the rest of the fractions were used for radioactivity measurements. The enzyme-activity peak is indicative of the sedimentation rate for the non-labeled isoenzyme, and the radioactivity peak is indicative of the sedimentation rate for the labeled enzyme. Fig. 4 contains data for experiments with three combinations of non-labeled and labeled isoenzymes. In the sucrose columns (5–25%), after centrifugation for 6 h, the locations from the top meniscus were: serum albumin, 1.30; D-glucose oxidase, 2.32; glucoamylase I, 1.72; and glucoamylase II, 1.88 cm. These values are averages of the values from several different experiments. From these values, and the relationships developed by Martin and Ames[22] for estimating molecular weights, the mol. wt. of glucoamylase I is 99,000 (±3%) and of glucoamylase II, 112,000 (±3%).

Carbohydrate composition of glucoamylases. — For glucoamylase I and II, the total carbohydrate content was measured by the orcinol–sulfuric acid method[35], and the total protein by the method of Lowry *et al.*[36]. In the carbohydrate determinations, the mixtures of enzymes and reagent were heated for 45 min at 80° and the absorbance (at 505 nm) of the cooled solutions was measured. D-Mannose was used as the standard for obtaining the carbohydrate values for the isoenzymes. In the protein determinations, lysozyme was used as the standard for obtaining the protein values. Glucoamylase I was found to contain 14.3%, and glucoamylase II, 23.5%, of carbohydrate.

Solutions of samples (~5 mg) of the isoenzymes, each in 0.2 ml of M hydrochloric acid, were heated in tightly stoppered tubes for 2 h at 100°. Aliquots of the

hydrolyzate were examined for reducing sugars by paper chromatography. The chromatograms revealed that the hydrolyzates from both isoenzymes contained D-mannose, D-glucose, and D-galactose. The identity of each monosaccharide was established by R_F value, use of a specific color-reagent, and effect of a specific glycose oxidase[24,32]. The relative proportions of the monosaccharides in each isoenzyme were determined as follows. A sample (5 ml) of each isoenzyme was dialyzed against running water for 12 to 18 h and then divided into two equal portions. One portion was hydrolyzed in 1.0 ml of M hydrochloric acid in a sealed tube for 3 h at 100°. The carbohydrates in the hydrolyzate were separated by paper chromatography with 6:4:3 (v/v) butyl alcohol–pyridine–water[31]. The D-mannose and D-glucose were eluted from the chromatograms and determined by the diphenylamine procedure[31]. The D-galactose was eluted, and determined by a D-galactose oxidase procedure[32]. The other portion of each isoenzyme was used for measuring the protein concentration of the solution by the method of Lowry *et al.*[36]. Quantitative values for the monosaccharides in each isoenzyme were calculated. Values for the glucoamylase from *R. delemar* have been reported[4]. The data, expressed in residues of monosaccharide per molecule of enzyme, are recorded in Table III.

Amino acid composition of glucoamylase. — For glucoamylase preparations, samples containing ~10 mg of protein were each lyophilized to dryness in a thick-walled, Pyrex tube; constant-boiling hydrochloric acid (10 ml) was added to each sample, and, after the protein had dissolved, the tubes were sealed, and heated for 22 h at 120°. The amino acids in the hydrolyzates were determined by a standard procedure by use of a Beckman or Technicon amino acid analyzer. Correction factors of 1.25 for serine and 1.13 for threonine[37] were used to obtain values for serine and threonine (as these amino acids are partially degraded during the hydrolysis of the protein). The tryptophan contents of the isoenzymes were determined by a spectrophotometric procedure[38]. The amino acid composition for the two isoenzymes and the glucoamylase from *R. delemar* are recorded in Table III. The data on glucoamylase I are based on 6 replicate determinations on 4 different preparations of the isoenzyme, and the data for glucoamylase II are based on 4 replicate determinations on 3 different preparations. The data for the glucoamylase from *R. delemar* are based on 2 determinations on a single preparation of the enzyme.

Identification of N-terminal amino acids. — For glucoamylase I and II, samples containing ~2 μmoles of enzyme were dialyzed against running water for 24 h and then lyophilized to dryness. 2,4-Dinitrophenyl derivatives of the proteins were prepared by procedure 1 of Fraenkel-Conrat *et al.*[39]; the reaction mixture was extracted, and hydrolyzed, and the (2,4-dinitrophenyl)amino acid derivative was identified by two-dimensional paper chromatography. (2,4-Dinitrophenyl)alanine was identified in the hydrolyzate of each derivatized isoenzyme. Estimates from the intensities of the color spots indicated that one mole of (2,4-dinitrophenyl)alanine was produced per mole of isoenzyme.

Immunological properties of the isoenzymes. — Purified solutions of glucoamylase I and II were used as antigens for inducing antibody formation in rabbits

by injecting rabbits with the isoenzyme dissolved in the Freund adjuvant. At the end of the fifth week, the rabbits were sacrificed, and antisera from the blood of the rabbits were prepared by standard immunological techniques. The antisera were divided into aliquots which were then stored at −20° until used. The antigens and antisera were evaluated for homology and cross-reactivity by the microprecipitin test. The isoenzymes were poor antigens; the titer value for I and antisera I was 1:4; for II and antisera I, 1:4; for II and antisera II, 1:32; and for II and antisera I, 1:16.

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