Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

INCORPORATION OF ^{14}C INTO THE CARBOHYDRATE MOIETIES OF A FUNGAL GLUCOAMYLASE*

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(Received March 2nd, 1970; in revised form, April 6th, 1970)

ABSTRACT

Aspergillus niger NRRL 330 was grown in submerged culture in media containing D-glucose-*1*-^{14}C or D-mannose-*1*-^{14}C. Glucoamylase I [α-D-(1→4)-glucan glucohydrolase, E. C. 3.2.1.3] and II were isolated from the culture filtrate in high purity by precipitation with ethanol followed by chromatography on DEAE-cellulose and Bio-Gel P-150. The carbohydrate residues were released from the purified enzymes by acid hydrolysis and were separated by paper chromatography. The hexoses were oxidized to aldonic acids, which were purified by chromatography on an ion-exchange resin. Following oxidation of the purified aldonic acids with sodium metaperiodate, radioactivity was measured in the carbon dioxide (C-1 of the hexose) and formaldehyde (C-6 of the hexose) by liquid-scintillation techniques. A large percentage of the radioactivity was retained in C-1 of the hexose isolated from the enzyme, indicating direct incorporation of the hexose into the glycoprotein without prior breakdown into smaller units followed by resynthesis. Involvement of a nucleotide–hexose pathway in the incorporation of the carbohydrate residues into glucoamylase I and II is consistent with these results. UDP-Glucose pyrophosphorylase (UTP:α-D-glucosyl phosphate uridylyltransferase, E.C. 2.7.7.9) activity was observed in a cell-free extract obtained from mycelia of *A. niger*.

INTRODUCTION

Extracellular glucoamylases [α-D-(1→4)-glucan glucohydrolases, E.C. 3.2.1.3] have been isolated from several fungi, including *Aspergillus niger*[1–7], *A. phoenicis*[8], *A. oryzae*[9–11], *Rhizopus delemar*[12], *Coniophora cerebella*[13], and *Endomycopsis capsularis*[14], and their properties have been studied. Glucoamylases from the first three fungi have been isolated in multiple forms and those from the first four organisms

*This work is published, with the approval of the Director, as Paper No. 2775 Journal Series, Nebraska Agricultural Experiment Station.

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have been shown to be glycoproteins. These glycoprotein enzymes contain combinations of D-mannose, D-glucose, D-galactose, or hexosamine covalently linked to their polypeptide chains.

A. niger produces two forms of glucoamylase, which have been isolated and purified[1-3]. Both forms are glycoproteins containing about 10% (glucoamylase I) and 18% (glucoamylase II) of carbohydrate[6]. D-Mannose is the major carbohydrate constituent[6] and the carbohydrate moieties appear to be *O*-glycosidically linked through this hexose to hydroxyl groups of serine and threonine residues in the polypeptide chain[7]. Smaller proportions of D-glucose and D-galactose are also present in both enzymes. Little is known concerning the function of the carbohydrate moieties in these enzymes or the nature of the pathways involved in their biosynthesis.

In our laboratories, the incorporation of ^{14}C into the carbohydrate moieties of glucoamylase I and II from *A. niger* has now been investigated to obtain information concerning possible pathways for biosynthesis of the carbohydrate portions of the enzyme. Results from the ^{14}C-incorporation studies are consistent with the involvement of a nucleotide–hexose pathway in incorporation of the carbohydrate residues into glucoamylase I and II.

RESULTS AND DISCUSSION

Growth of organism. — *Aspergillus niger* NRRL 330 was grown in submerged culture on a medium of D-mannose or D-glucose containing D-glucose-*1*-^{14}C or D-mannose-*1*-^{14}C, with production of two forms of glucoamylase. The ^{14}C-labelled hexose was added three days after inoculation of the medium with spores of *A. niger*. Barton, Lineback, and Georgi have demonstrated[15] that mycelial growth is nearly complete after 3 days under these conditions and that enzymic activity is nearly maximal. Addition of the ^{14}C-labelled carbohydrate at this time should lessen the relative amount of siphoning of ^{14}C into other synthetic processes that would occur prior to active synthesis of glucoamylase.

Isolation of ^{14}C-labelled glucoamylase I and II. — Glucoamylase I and II were separated from the crude enzyme by chromatography on DEAE-cellulose[3]. A typical pattern showing three major protein components, obtained by elution with a gradient of decreasing pH, is given in Fig. 1. The first component contained D-glucosyl transferase and *p*-nitrophenyl α-D-galactoside-hydrolyzing (α-D-galactosidase) activity. D-Glucosyl transferase activity was identified by formation of isomaltose and panose from a substrate of maltose[3]. The second protein component contained glucoamylase II and 2-acetamido-2-deoxy-α-D-glucosidase (*N*-acetyl-α-D-glucosaminidase) activity. The activity of the latter enzyme was much higher in this study than that encountered with a commercial glucoamylase preparation[3]. This enzyme and the α-D-galactosidase have recently been isolated, purified, and characterized[16]. The third protein component contained primarily glucoamylase I. α-Amylase activity was observed in the region between the first and second major protein components. α-D-Glucosidase activity, as measured by hydrolysis of *p*-nitrophenyl α-D-glucoside, was found in all three

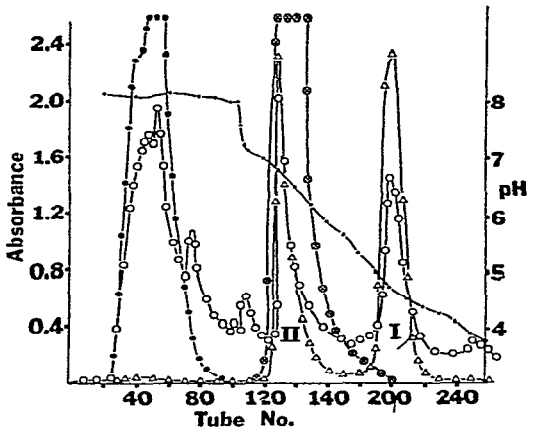

Fig. 1. Chromatography on DEAE-cellulose of crude enzyme obtained from a medium of D-mannose: (○) absorbance at 280 nm; (△) starch-hydrolyzing activity (absorbance at 400 nm) measured by the D-glucose oxidase procedure[3]; (•) pH elution gradient; (●) α-D-galactosidase activity (absorbance at 420 nm) measured by hydrolysis of *p*-nitrophenyl α-D-galactoside; (⊗) 2-acetamido-2-deoxy-β-D-glucosidase activity (absorbance at 420 nm) measured by hydrolysis of *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucoside. The crude enzyme (500 ml) was chromatographed on a column (3.5 × 50 cm) of DEAE-cellulose at a flow rate of 1 ml/min by the gradient-elution procedure previously described[3]. The fractions labelled I and II refer to glucoamylase I and II, respectively.

major components, while starch-hydrolyzing (to D-glucose) activity was confined to the last two protein peaks. Glucoamylase I and II were distinguished from other α-D-glucosidases by its producing only D-glucose from starch or maltose. The glucoamylase II-containing component was also contaminated with a brown-colored material. A much smaller proportion of this material was observed in the glucoamylase I component.

Fractions comprising the glucoamylase II component were combined, concentrated, and fractionated on Bio-Gel P-150. A typical pattern is illustrated in Fig. 2. Glucoamylase II was retarded by the column, whereas the 2-acetamido-2-deoxy-β-D-glucosidase activity was eluted with a K_d of approximately zero, that is, at the void volume of the column. A third major component, having a molecular weight considerably lower than glucoamylase II, was also separated and contained the brown-colored material. This material gave the orange color expected for carbohydrate in the phenol–sulfuric acid assay[17] and produced the color expected for protein in the Lowry procedure[18] but did not yield the color expected for amino derivatives in the Rosen ninhydrin assay[19]. This component is also present in enzyme fractions prepared by stepwise elution from DEAE-cellulose[1], but is present in much smaller proportions in a commercial glucoamylase preparation. Incomplete removal of this material from enzyme samples used for further studies could yield erroneous results, since it would increase both the apparent protein content as determined by the Lowry procedure, and the carbohydrate content as measured by the phenol–sulfuric acid assay.

The combined fractions of glucoamylase I from the DEAE-cellulose column were fractionated further on Bio-Gel P-150. One minor component was eluted before

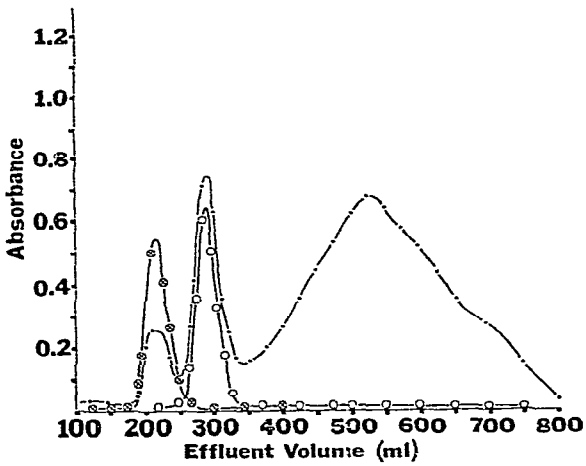

Fig. 2. Gel filtration on Bio-Gel P-150 of the glucoamylase II fraction obtained from chromatography on DEAE-cellulose of the proteins from a medium of D-mannose: (•) absorbance at 280 nm; (○) α-D-glucosidase activity (absorbance at 420 nm) measured by the hydrolysis of *p*-nitrophenyl α-D-glucoside; (⊗) 2-acetamido-2-deoxy-β-D-glucosidase activity (absorbance at 420 nm) measured by hydrolysis of *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucoside. Glucoamylase II (30 ml) was chromatographed on a column (4.5 × 55 cm) of Bio-Gel P-150 by elution with 0.1M NaCl at a flow rate of 0.4 ml/min as described in the experimental section.

glucoamylase I and several minor components appeared after it. Only a single major component (glucoamylase I) was obtained. Brown-colored material present in the minor components was greatly retarded by the column.

The efficiency of the purification procedure at each step is indicated in Table I with values from a typical purification of the crude enzyme derived from a medium of

TABLE I

PURIFICATION OF GLUCOAMYLASE FROM *Aspergillus niger* GROWN ON D-MANNOSE-CONTAINING MEDIA

Procedure	*Volume* (*ml*)	*Enzyme activity*		*Protein* (*mg/ml*)	*Specific activity* (*units/mg*)	*Yield* (%)	*Purification*
		Conc. (*units/ml*)	*Total* (*units* × 10^{-3})				
Crude media	910	5.3	4.8	6.4	0.8	100	1.0
Ethanol fractionation	480	7.9	3.8	2.6	3.0	79	3.8
Chromatography (DEAE-cellulose)							
Glucoamylase II	300	3.3	0.98	0.38	8.6	20	10.1
Glucoamylase I	305	5.2	1.6	0.52	10.0	33	12.5
Chromatography (Bio Gel P-150)							
Glucoamylase II	50	9.0	0.44	0.60	15.0	9	18.8
Glucoamylase I	50	21	1.1	1.4	14.6	23	18.3

D-mannose. The extent of purification and recovery of enzymic activity was based on the activity of the crude enzyme, even though more than one starch-hydrolyzing enzyme was present. The amounts of glucosyl transferase and α-amylase present in the crude enzyme were sufficiently low that their contribution of production of D-glucose from starch was considered negligible under the assay conditions used. The activities of glucoamylase I and II are sufficiently similar to warrant treating them as one in the crude enzyme. The purification procedure provided approximately an 18-fold increase in specific activity for both glucoamylases, with approximately 30% recovery of the original starch-hydrolyzing activity. The specific activities are comparable to those previously reported for glucoamylases purified from a commercial glucoamylase preparation[3]. Growth of the organism on a medium of D-glucose yielded similar results, but the amount of enzymes produced was approximately 3-fold greater than with a medium of D-mannose.

Disc electrophoresis of the crude glucoamylase on 7% polyacrylamide gel indicated the presence of numerous components staining as protein. The same crude preparation on 4% gel clearly revealed the presence of both forms of the glucoamylase. Disc electrophoresis on 7% gel of the glucoamylase II fraction from the DEAE-cellulose column revealed the presence of a single major protein component, also having starch-hydrolyzing activity, and five minor components staining as protein. Under the same conditions, the glucoamylase I fraction possessed a single major protein component, also having starch-hydrolyzing activity, and a minor component staining as protein. Disc electrophoresis of glucoamylase I and II after gel filtration on Bio-Gel P-150 revealed a single protein component for each enzyme, which also possessed starch-hydrolyzing activity. Both of these components were stained by the periodate–Schiff method[20], indicating that they were glycoproteins. The presence of single components in the purified enzyme, responding to assays for protein, glycoprotein, and glucoamylase, indicated that the two glucoamylases were obtained in high purity. The presence of both glucoamylases in the crude culture filtrate demonstrated that the two forms of the enzyme were indeed formed by the organism and were not artifacts of the method of preparation.

Analysis and degradation of hexoses from glucoamylase I and II. — Hexoses were liberated from purified, freeze-dried glucoamylase I and II by hydrolysis in M hydrochloric acid for 6 h at 100°. Preliminary studies indicated that these conditions resulted in maximal release of hexoses with minimal decomposition. The hexoses were separated by paper chromatography. D-Mannose was completely separated from D-glucose and D-galactose by the solvent system used, and was isolated alone. D-Glucose and D-galactose were not completely separated under these conditions, and were isolated together. The small proportions of D-galactose present in the enzymes[6] did not warrant further attempts to separate these two hexoses.

The amounts of hexose and radioactivity isolated from glucoamylase I for each of the four types of labelling experiments are shown in Table II. The activity of the labelled hexoses was measured by liquid-scintillation spectrometric analysis and the carbohydrate concentration was determined by the phenol–sulfuric acid

TABLE II

SPECIFIC ACTIVITY OF CARBOHYDRATES ISOLATED FOLLOWING HYDROLYSIS OF GLUCOAMYLASE I

D-*Hexose in media*	^{14}C-D-*Hexose*	D-*Hexose isolated*	*Weight (mg)*	*Radioactivity*	
				d.p.m.[a] $\times 10^{-4}$	*d.p.m.*[a]*/mg* $\times 10^{-3}$
Mannose	Man-*1*-^{14}C	Man	10.0	3.8	3.9
		Glu+Gal	1.3	0.5	3.9
Glucose	Man-*1*-^{14}C	Man	10.0	6.5	6.9
		Glu+Gal	4.9	1.5	3.1
Mannose	Glu-*1*-^{14}C	Man	17.3	4.6	2.7
		Glu+Gal	3.5	1.4	4.0
Glucose	Glu-*1*-^{14}C	Man	24.2	12.0	5.0
		Glu+Gal	3.8	2.0	5.3

[a]d.p.m. = Disintegrations per minute.

assay[17]. The results indicate that when the labelled and unlabelled hexoses in the growth medium are the same, the specific activities of the two sugar fractions isolated from glucoamylase I are nearly identical. Conversely, when the hexoses in the medium are different, the specific activity of the sugar fractions from the enzyme show marked differences. Similar results were obtained from glucoamylase II. These results indicate the existence of an active mechanism for interconversion of the hexoses in the cell, probably via a hexose phosphate isomerase system. Even when the ^{14}C-labelled and unlabelled sugars are different, interconversion occurs, as shown by incorporation of ^{14}C into both hexose fractions. It should be noted, however, that the ratios of specific activities of the isolated sugar fractions (higher activity/lower activity) were different for the two "mixed source" media, that is, 6.9/3.1 for D-glucose:D-mannose-*1*-^{14}C and 4.0/2.7 for D-mannose:D-glucose-*1*-^{14}C. The smaller ratio for D-mannose:-D-glucose-*1*-^{14}C suggests that D-glucose is more completely interconverted with the other hexoses before incorporation into the enzyme than is D-mannose.

The distribution of the ^{14}C-label in the isolated hexose fractions was determined by an oxidative procedure[21] which yielded C-1 of the hexose as carbon dioxide and C-6 of the hexose as formaldehyde, isolated as its dimedone derivative. Preliminary investigations of the oxidation procedures indicated that the recovery of C-1 was 88% for mannose-*1*-^{14}C and 93% for glucose-*1*-^{14}C. An average value of 90.5% recovery was used for the investigation, and experimental values were multiplied by 1.1 to obtain the distribution of ^{14}C-label. The distribution of ^{14}C in the hexose fractions isolated from glucoamylase I and II is shown in Table III. The values are averages obtained from two separate growth experiments.

The distribution of ^{14}C in the hexose fractions can be explained by carbohydrate interconversions that occur commonly. D-Glucose or D-mannose entering the cell can be phosphorylated by the appropriate kinase to the corresponding hexose 6-phosphate. The hexose 6-phosphates can undergo interconversion via D-fructose

TABLE III

DISTRIBUTION OF ^{14}C IN HEXOSES ISOLATED FROM GLUCOAMYLASE I AND II

D-Hexose in growth media	*^{14}C-D-Hexose administered*	*Glucoamylase*	*D-Hexose isolated*	*^{14}C as % total in hexose*[a]		
				C-1	*C-2–C-5*[b]	*C-6*
Mannose	Man-*1*-^{14}C	I	Man	76	11	13
			Glu+Gal	82	—	20
		II	Man	82	4	14
			Glu+Gal	92	—	13
Glucose	Man-*1*-^{14}C	I	Man	96	—	16
			Glu+Gal	77	2	21
		II	Man	99	—	12
			Glu+Gal	78	3	19
Mannose	Glu-*1*-^{14}C	I	Man	52	28	20
			Glu+Gal	79	9	12
		II	Man	54	25	21
			Glu+Gal	83	7	10
Glucose	Glu-*1*-^{14}C	I	Man	64	16	20
			Glu+Gal	84	6	10
		II	Man	70	10	20
			Glu+Gal	76	7	17

[a]Values are averages of two complete growth experiments corrected as described in the text. [b]The distribution of ^{14}C in C-2–C-5 was obtained by difference.

6-phosphate in the presence of a hexose phosphate isomerase or can be converted into hexose 1-phosphates by phosphohexomutases. D-Fructose 6-phosphate can be siphoned into glycolysis to satisfy the organism's energy requirements. Resynthesis of hexose via the aldolase reaction would then result in the introduction of ^{14}C into C-6 of the hexose from C-1 of the original hexose. Nucleotide sugars formed from hexose 1-phosphates and nucleoside 5'-triphosphates by pyrophosphorylases could be used to incorporate carbohydrates into the glycoprotein enzymes. D-Galactose could be incorporated into the enzyme from UDP-D-galactose*, formed from UDP-D-glucose*.

Under the conditions used for this investigation, D-glucose appeared to be more active metabolically than D-mannose. The percentage of ^{14}C retained in C-1 of the D-mannose incorporated into the glucoamylases was significantly lower with D-glucose-*1*-^{14}C than with D-mannose-*1*-^{14}C. This probably reflects the larger number of metabolic reactions having D-glucose or D-glucose 6-phosphate as substrates relative

*Trivial names for uridine 5'-(α-D-glucopyranosyl pyrophosphate) and the α-D-galactopyranosyl analog.

to those utilizing D-mannose or D-mannose 6-phosphate. Growth of the organism in a medium of D-glucose also resulted in the production of approximately three times as much glucoamylase as did a medium of D-mannose.

The degree of retention of ^{14}C at C-1 in the hexoses of glucoamylase II was higher (with one exception) than those from glucoamylase I. This observation suggests that glucoamylase II may be produced by the organism at an earlier time than glucoamylase I. Although some of the differences are not statistically significant, it would appear that there is a general trend in the direction cited. This seems to indicate that the formation of the two enzymes may be under separate genetic control[9], as has been previously suggested for these two enzymes[22] and for the two glucoamylases of *Candida pelliculosa*[23].

The results of the ^{14}C-incorporation studies are consistent with the existence of a pathway in which the hexoses can be directly incorporated into the two glucoamylases without prior metabolism to smaller fragments followed by resynthesis. All hexose fractions isolated from glucoamylase I or II had most of the ^{14}C retained in C-1 (Table III). In those cases where redistribution of ^{14}C was observed, metabolic demands of the organism, particularly involvement of glycolysis, can easily explain the results. In virtually all cases the occurrence of glycolysis is evidenced by the presence of ^{14}C in C-6 of the hexose isolated from the enzyme. Involvement of a nucleotide–sugar pathway in the incorporation of the hexoses into the two glucoamylases is consistent with the experimental data.

Presence of pyrophosphorylase activity. — Incubation of a crude enzyme extract, prepared from *A. niger* mycelia 4 days after inoculation of the medium with spores, with α-D-glucosyl phosphate and uridine 5′-triphosphate resulted in the formation of a new u.v.-absorbing compound. This compound cochromatographed with authentic UDP-D-glucose on polyethylenimine-impregnated paper[24]. Verification of its identity as UDP-D-glucose was obtained by isolating the compound and observing, by paper and gas-liquid chromatography, a glucose as the only carbohydrate in an acid hydrolyzate. During the 7-h incubation period, uridine 5′-triphosphate in the incubation mixture was almost completely utilized. High UDP-glucose-synthesizing activity was observed in extracts from mycelia grown on media of both D-mannose and D-glucose. No new nucleotide sugar could be demonstrated when α-D-mannosyl phosphate and guanosine 5′-triphosphate were incubated under the same conditions.

Observation of an enzyme system, in crude mycelial extracts, capable of synthesizing UDP-D-glucose from α-D-glucosyl phosphate and uridine 5′-triphosphate lends credence to the above hypothesis of a nucleotide–sugar pathway. Failure to observe formation of GDP-mannose*, from α-D-mannosyl phosphate and guanosine 5′-triphosphate with the crude enzyme, may reflect that appropriate conditions for the synthesis or preparation of the crude enzyme were not used in these experiments, rather than an absence of the requisite enzyme in the mycelia.

Pazur *et al.*[25] have recently reported studies on the biosynthesis of glucoamylase

*Trivial name for guanosine 5′-(α-D-mannopyranosyl pyrophosphate).

I from *A. niger*. They observed that D-mannose isolated from glucoamylase I contained 86% of the ^{14}C in C-1 and 9% in C-6 when *A. niger* was grown on a medium containing D-glucose-*1*-^{14}C. Similar results were claimed when D-mannose-*1*-^{14}C was used in the medium. These authors obtained a crude enzyme system from *A. niger* mycelia capable of synthesizing UDP-D-glucose, TDP-D-glucose*, UDP-D-galactose and GDP-D-mannose† from the appropriate nucleoside 5′-triphosphate and hexose 1-phosphate. The crude enzyme system was also capable of incorporating radioactivity from GDP-D-mannose-^{14}C into a partially modified glucoamylase I. The partially modified glucoamylase I had been prepared by treating glucoamylase I with an α-D-mannosidase to remove part of the D-mannose from the enzyme. These observations were interpreted to indicate that a nucleotide–hexose pathway is involved in the synthesis of the carbohydrate portion of glucoamylase I.

Involvement of nucleotide–hexose pathways in incorporation of the hexoses into the two glucoamylases is consistent with observations concerning the synthesis of the carbohydrate portions of other glycoproteins. Transfer of D-galactosyl residues[26], D-glucosyl residues[27], D-mannosyl residues[25], and D-xylosyl residues[26,28] from the corresponding nucleotide sugars to glycoproteins has been demonstrated with enzyme preparations from different sources.

EXPERIMENTAL

Materials. — D-Mannose-*1*-^{14}C (37.5 mCi/mmole) and D-glucose-*1*-^{14}C (38.5 mCi/mmole) were purchased from New England Nuclear, Boston, Massachusetts. Growth media contained Bacto Dehydrated Nutrient Broth obtained from Difco Laboratories, Detroit, Michigan. All other chemicals used were reagent or analytical grade unless otherwise specified.

Chromatographic procedures. — Chromatography on DEAE-cellulose (reagent grade, capacity 0.85 meq/g, Brown Company, Berlin, New Hampshire) was performed as previously described[3]. Protein components were located in the effluent fractions by determining the absorbance at 280 nm.

Gel filtration was performed at room temperature on a column (4.5 × 55 cm) of Bio-Gel P-150 (100–200 mesh, Bio-Rad Laboratories, Richmond, California, U. S. A.), pre-equilibrated with 0.1M NaCl, with 0.1M NaCl at a flow rate of 0.4 ml/min (maintained with a Mariotte flask) as eluant. Fractions were collected automatically at 30-min intervals. The void volume of the column was determined by using Blue Dextran 2000 (Pharmacia Fine Chemicals, Piscataway, New Jersey, U. S. A.).

Paper chromatography of hexose mixtures was accomplished on Whatman No. 1 chromatography paper (20 × 55 cm) that had been prewashed with distilled

*Trivial name for thymidine 5′-(α-D-glucopyranosyl pyrophosphate).
†Trivial name for guanosine 5′-(α-D-mannopyranosyl pyrophosphate).

water and dried. The papers were developed in a descending 8:2:1 (v/v) ethyl acetate–pyridine–water solvent system[29] for 36 h.

Nucleotide sugars were separated by chromatography on polyethylenimine-impregnated paper[24] by development in a descending system with 0.3M lithium chloride for 5 h. Nucleotide sugars were visualized as blue spots on a white background when viewed under u.v. light.

Enzyme assays. — Glucoamylase activity was determined as previously described[3] by measuring the amount of D-glucose produced from a starch substrate. One unit of glucoamylase activity was defined as the amount of enzyme required to liberate 1 μmole of glucose per min under the reaction conditions defined. Specific activity was expressed as units per mg of protein, determined by the Lowry method[18].

Other glycosidase activities in the fractions obtained by column-chromatographic purifications were assayed by using appropriate *p*-nitrophenyl glycosides as previously described[3], or by incubating aliquots of the fractions with maltose and starch and determining the products by paper chromatography[3].

Disc electrophoresis. — Disc electrophoresis was accomplished[3] on 7% and 4% polyacrylamide gels, with a discontinuous buffer at pH 7.0. Glucoamylase activity was assayed as previously reported[3] by extracting slices of unstained gels and incubating aliquots of the extract with 4% starch.

Glycoproteins were located in the gels with Schiff's reagent[20] after treatment of the gel with periodate. Following electrophoresis, gels were fixed in 7.5% acetic acid for one h, transferred to 2% sodium metaperiodate in 3% acetic acid, and kept overnight at room temperature. Excess periodate was removed by suspending each gel in 5% acetic acid (100 ml) for 8 h. This process was repeated two additional times. The gels were kept in Schiff's reagent at 5° until pink bands, attributed to glycoproteins appeared.

Liquid scintillation spectrometry. — Radioactivity was measured on a Packard Tri-Carb Liquid Scintillation Counting System, model 314EX-2, by the channels-ratio method[30,31]. To increase their solubility, aldonic acids were counted in a xylene–*p*-dioxane-based solution[32]. Other compounds were counted in a toluene solution[33]. All counting was continued until a minimum of 10,000 counts had been recorded.

Growth of the organism. — *Aspergillus niger* NRRL 330 was grown in 125-ml cultures in media containing D-mannose or D-glucose (3%), nutrient broth (2.5%), KH_2PO_4 (0.1%), and trace elements[34]. The spore inoculum (0.5 ml/culture) was prepared by suspending the spores from a single slant-culture, maintained as previously described[34], in 10 ml of distilled water. Flasks (500-ml Erlenmeyer) containing the cultures were aerated at room temperature by shaking on a reciprocating action table (92 cycles/min). On the third day following inoculation, either D-mannose-*1*-^{14}C or D-glucose-*1*-^{14}C was added to each flask (0.5 ml/flask) as a solution containing 0.0125 mCi/ml and 0.06 mg hexose/ml.

Isolation of Glucoamylase I and II. — Six days after inoculation with *A. niger* spores, the mycelia were removed by filtration. The filtrate, designated the crude

enzyme, was dialyzed against running tap water for 4 h. Protein was precipitated at 5° by the addition of abs. ethanol (2.5 volumes).

The ethanol-precipitated material was diluted to 300 ml with distilled water and dialyzed against running tap water for 4 h. The enzyme solution was mixed with sufficient 0.2M citrate–phosphate buffer (pH 8.0) to yield a final buffer concentration of 0.025M, and the pH was adjusted to 8.0 with 0.1M NaOH. Insoluble protein was removed by centrifugation (12,100 × *g* for 20 min at 5°). The supernatant solution was subjected to chromatography on DEAE-cellulose by the gradient-elution procedure previously described[3]. The two protein peaks containing glucoamylase activity were collected individually, dialyzed against running tap water for 4 h, and concentrated to 60 ml by pervaporation at 25°. Insoluble material was removed by centrifugation (27,000 × *g* for 20 min at 5°).

The concentrated glucoamylase samples (30 ml aliquots) were chromatographed on Bio-Gel P-150 as described. Fractions were assayed for protein (absorbance at 280 nm) and enzyme activity. The purified enzyme was dialyzed against 20 volumes of distilled water for 48 h with 4 changes of water and freeze-dried.

Isolation of hexoses from Glucoamylase I and II. — Purified, freeze-dried glucoamylase I and II were each (30 mg) hydrolyzed with M HCl (10 ml) in sealed tubes for 6 h at 100°. The tubes were purged with nitrogen prior to sealing. The hydrolyzate was passed through a column (1 × 5 cm) of Bio-Rad AG 50W-X8 (H^+) resin. Carbohydrates were collected by washing the column with water until the effluent was neutral. The combined effluents were then neutralized to pH 6.0 with Bio-Rad AG 3-X4 (OH^-) resin. The resin was removed by filtration and washed with water. The combined filtrate and washings were freeze-dried.

The residue was dissolved in 0.5 ml of water and the hexoses were separated by paper chromatography. ^{14}C-Labelled components were located by scanning a one-inch strip from the chromatogram with a Vanguard Automatic Chromatogram scanner, Autoscanner 880. Carbohydrates were subsequently located on the same strip by the silver nitrate reagent[35]. Sections corresponding to the two radioactive carbohydrate areas located on the one-inch strip were removed from the chromatogram and the hexoses were eluted with water. The eluted hexose solutions (3–5 ml) were freeze-dried.

Distribution of ^{14}C in the hexoses. — The hexoses were degraded by an oxidative procedure[21]. Oxidation of the freeze-dried hexoses (1–20 mg) was performed on one-third of the reported scale. After oxidation, the methanol was evaporated at 40° with a stream of dry nitrogen. The residue was dissolved in water (1.0 ml) and charged onto a column (1 × 5 cm) of Bio-Rad AG 1-X8 (formate) resin. The resin was eluted with water (20 ml) to remove any unoxidized hexose and 2M formic acid (25 ml) was used to elute the aldonic acid, which was located by monitoring the effluent for radioactivity. The fractions containing the aldonic acid were combined and freeze-dried.

The aldonic acid (1–5 mg) was dissolved in 1.0 ml of 0.5M acetate buffer (pH 4.7). An aliquot (0.05–0.1 ml) of this solution was added to a liquid-scintillation vial,

solubilized in the xylene–*p*-dioxane-based scintillation solution by addition of 2.0 ml of abs. ethanol, and used to determine the total radioactivity in the aldonic acid. A second aliquot (0.2–0.4 ml) of the aldonic acid solution was pipetted into a Thunberg tube and the hydroxide form of Hyamine-10X (0.5 ml, Rohm and Haas Co.) was added to the side arm. The Thunberg tube was evacuated (water aspirator) and 0.3M sodium metaperiodate (1.0 ml) was added. After the sample had been agitated on a wrist-action shaker for 2 h at room temperature, the Hyamine in the trap was dissolved in 1.0 ml of absolute ethanol and transferred quantitatively to a liquid-scintillation vial. Radioactivity was then determined as described.

The solution remaining in the Thunberg tube, containing formaldehyde released by the oxidation, was quantitatively transferred with water to a conical centrifuge tube. The dimedone derivative of formaldehyde was prepared by the method of Reeves[36]. The precipitate was filtered and washed with cold water (1.0 ml). The filter paper containing the derivative was transferred to a liquid-scintillation vial and the filter funnel was rinsed with ethanol (2.0 ml). The ethanol was added to the vial. Radioactivity was measured in the toluene scintillation solution.

Detection of pyrophosphorylase activity. — The presence of pyrophosphorylases was investigated in a manner similar to that used by previous workers[37,38]. Mycelia, obtained by filtration from two flasks of D-mannose or D-glucose media four days after inoculation with *A. niger* spores, were washed with 0.1M phosphate buffer (pH 7.6). A portion of the mycelial mass (3.7 g) was suspended in 3.7 ml of the same buffer, containing 10mM dithiothreitol and 10mM $MgCl_2$, and the mycelia were disrupted in a stainless-steel ball mill for 7 min at 5°. Mycelial debris was removed by centrifugation (27,000 × *g* for 30 min at 3°). The supernatant solution was used as the crude enzyme.

Crude enzyme preparations from D-mannose and D-glucose media were incubated with guanosine 5′-triphosphate or uridine 5′-triphosphate and α-D-glucosyl phosphate or α-D-mannosyl phosphate in the following manner. α-D-Hexose 1-phosphate (0.05 ml of 40 mg/ml), nucleoside 5′-triphosphate (0.05 ml of 40 mg/ml), 30 mM $MgCl_2$ (0.05 ml), and crude enzyme (0.15 ml) were incubated for 7 h at room temperature. An aliquot of the reaction mixture (0.02 ml) and appropriate reference compounds were chromatographed on polyethylenimine-impregnated paper and the formation of new u.v-absorbing compounds was determined by viewing the chromatogram under u.v. light.

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, Illinois, U. S. A.

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