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THE GLYCOPROTEIN NATURE AND ANTIGENICITY OF A FUNGAL D-GLUCOSYLTRANSFERASE*

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ABSTRACT

D-Glucosyltransferase (EC 2.4.1.24) from *Aspergillus niger* has been prepared in pure form by chromatography on DEAE–cellulose. The enzyme transfers D-glucosyl units from maltose and other α-linked D-glucosyl oligosaccharides to glucosyl co-substrates resulting in the synthesis of new types of oligosaccharides. The glucosyltransferase has been found to be a glycoprotein containing 20% of carbohydrate consisting of mannose, glucose, and galactose. The carbohydrate residues are attached as either single units or as short oligosaccharide chains by *O*-glycosyl linkages to the serine and threonine residues of the protein. Antibodies directed against glucosyltransferase have been induced in animals by appropriate immunization regimes. These antibodies combine with the carbohydrate components of the enzyme and, therefore, the carbohydrate residues are the immunodeterminant groups of the glucosyltransferase.

INTRODUCTION

D-Glucosyltransferases (EC 2.4.1.24) from fungal sources transfer D-glucosyl residues from maltose and other α-linked glucosyl oligosaccharides to various glucosyl co-substrates resulting in the synthesis of new glucosyl oligosaccharides[1]. This type of transferase was first detected in extracts from *Aspergillus oryzae*[2] and has been used to prepare glucosyl oligosaccharides having various glucosidic linkages[3]. The transfer of glucose units occurs most frequently to the OH-6 of the accepting glucose unit, but transfers to other positions also occur. The oligosaccharides that are produced from maltose include isomaltose, kojibiose, nigerose, panose (6-*O*-α-D-glucosylmaltose), isomaltotriose (6-*O*-α-D-glucosylisomaltose),

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the tetrasaccharide 6-*O*-α-D-glucosylpanose, and higher-molecular-weight oligosaccharides[1]. Since the initial detection of glucosyltransferase in fungal extracts, other types of transferases capable of transferring D-galactosyl, 2-acetamido-2-deoxy-D-glucosyl, D-glucuronosyl, and D-fructosyl groups have been found in microbial, plant, or mammalian extracts. Some of these enzymes catalyze the synthesis of oligosaccharides[4], others of glycans[5], and still others of glycoproteins[6].

Earlier studies have shown that glucosyltransferase can be isolated and purified by chromatography on adsorbents of the DEAE-cellulose, CM-Biogel, and Sephacryl S-250 types[7–9], and by fractional precipitation methods[10]. In the present study, a glucosyltransferase from *Aspergillus niger* has been purified by chromatography on DEAE-cellulose, and the purified enzyme has been used in structural and immunological studies. The fungal transferase has been found to be a glycoprotein containing galactose, glucose, and mannose. By reductive, alkaline β-elimination, it was shown that the carbohydrate units are attached by *O*-glycosyl linkages to the serine and threonine residues of the enzyme. Following β-elimination, mannitol hexaacetate was identified as an elimination product and amino acid analysis of the native and modified enzyme showed decreases in the threonine and serine values, the appearance of α-aminobutyric acid, and increases in the alanine and glycine values. Methylation analysis data indicated that the transferase possesses a molecular architecture similar to that of glucoamylase[11] with many carbohydrate side-chains of single monosaccharides or hetero-oligosaccharides. Antibodies directed against glucosyltransferase and glucoamylase were prepared and such antibodies were shown to be specific for the carbohydrate components of the individual enzymes. No cross-reactivities of the two antibody and enzyme systems were noted in agar diffusion tests, as apparently significant differences exist in the structure of the carbohydrate components of the glucosyltransferase and the glucoamylase.

RESULTS AND DISCUSSION

Glucosyltransferases from fungi catalyze the transfer of glucosyl units from maltose and other α-linked glucosyl oligosaccharides to glucose and glucosyl oligosaccharides which function as accepting co-substrates. The principal reactions of the fungal transferase on maltose are shown in equations *1–3*

$$\alpha\text{-D-Glc}p\text{-}(1\rightarrow4)\text{-D-Glc} + E \rightarrow Glc \cdot E + Glc \quad (1)$$

$$Glc \cdot E + Glc \rightarrow \alpha\text{-D-Glc}p\text{-}(1\rightarrow6)\text{-D-Glc} + E \quad (2)$$

$$Glc \cdot E + \alpha\text{-D-Glc}p\text{-}(1\rightarrow4)\text{-D-Glc} \rightarrow \alpha\text{-D-Glc}p\text{-}(1\rightarrow6)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow4)\text{-D-Glc} + E \quad (3)$$

where E represents the enzyme molecule.

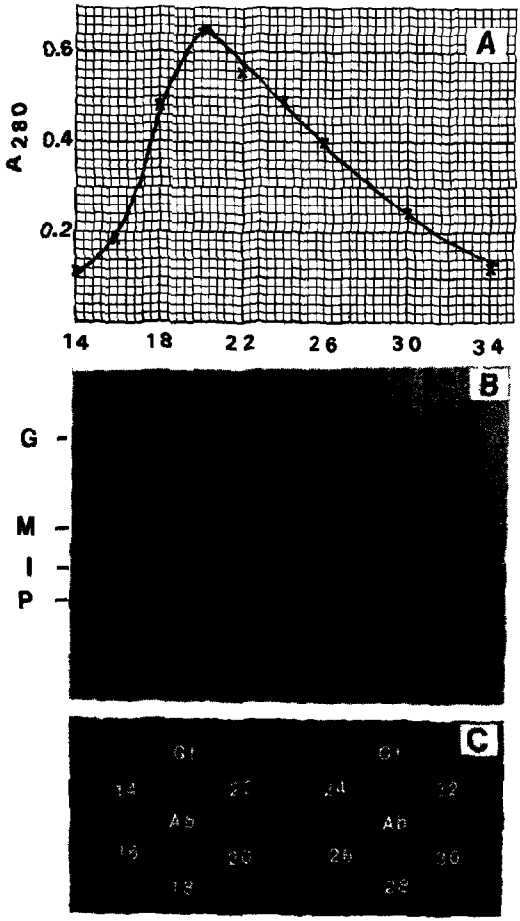

Fig. 1. Fractionation of glucosyltransferase of *A. niger* on DEAE-cellulose Fractions 14-34: (A) Absorbance at 280 nm, (B) paper chromatographic separation of products from maltose, and (C) agar diffusion of glucosyltransferase and antibodies. Abbreviations: G, glucose; M, maltose; I, isomaltose; P, panose; R, reference mixture of the aforementioned compounds; Gt, glycosyltransferase; and Ab, antibody.

Other types of transfer products are produced by the enzyme by transfer of the glucosyl units to positions 2, 3, or 4 of the accepting glucose units. Also, other glucosyl oligosaccharides can function as donors of carbohydrate units to yield additional types of new oligosaccharides. The oligosaccharides produced by the transferase may contain α-D-(1→2), -(1→3), -(1→4), or -(1→6) linkages or any combination thereof. Glucose is a product of enzyme action on all of the substrates and is produced in high proportions. The synthesis of many of these glucosyl oligosaccharides has not been achieved by chemical means and the enzymic route utilizing transferases has been used.

The purification of glucosyltransferase from extracts of *A. niger* was achieved

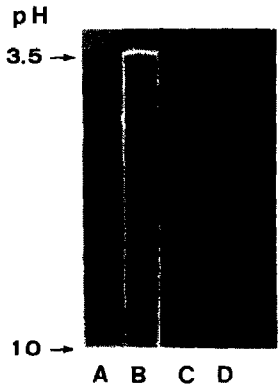

Fig. 2. Isoelectrofocusing in a pH gradient of 3.5–10.0 and agar diffusion of glucosyltransferase: (A) Gel stained for proteins; (B) unstained gel embedded in agar; (C) area of precipitin-band formation; and (D) trough of anti-glucosyltransferase serum.

by two successive adsorptions and elutions from DEAE-cellulose columns. The u.v.-absorbance curve for the fractions from the DEAE-cellulose column is shown in Fig. 1,A. Fractions 16–30 with high u.v.-absorbance were also assayed for glucosyltransferase activity on maltose, and the products of enzyme action were detected by paper chromatography. Results of the assays are shown in Fig. 1,B. The chromatogram in this figure shows that isomaltose and panose (6-*O*-α-D-glucosylmaltose) were produced in highest proportions on incubation of maltose with fractions 18–22. Glucosyltransferase was also identified in these fractions by immunological methods in which samples of the fractions and of antiglucosyltransferase antibodies were subjected to agar-diffusion tests. These results are shown in Fig. 1,C. The fractions containing glucosyltransferase were combined, dialyzed against distilled water for 48 h, and evaporated by lyophilization, to give ~350 mg of purified glucosyltransferase. This sample was dissolved in buffer (pH 8) and subjected to a second chromatography process following the procedure described earlier. The final yield of purified glucosyltransferase was 250 mg, and this material was used as the enzyme source in the subsequent experiments reported herein. The preparation of transferase yielded a single band of protein material on isoelectrofocusing as shown in Fig. 2. A comparable gel stained by the periodate–Schiff method showed that the material in this band was a glycoprotein. Earlier results by ultracentrifugation of the enzyme in an analytical centrifuge[7] indicated molecular homogeneity of the preparation.

The glycoprotein nature of the transferase was confirmed by the positive colorimetric tests for carbohydrate obtained by the phenol–sulfuric acid[12] and the orcinol reactions[13]. The total carbohydrate content of the glucosyltransferase was measured by the phenol–sulfuric acid method and found to be 20%. Acid hydrolysis and chromatographic analysis showed that the glucosyltransferase contained galactose, glucose, and mannose as structural units (see Fig. 3). D-Galactose obtained

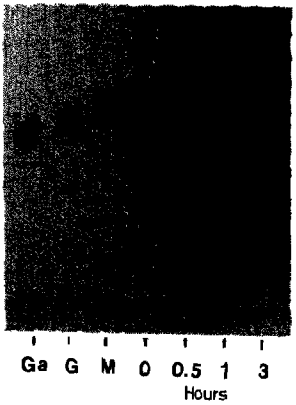

Fig. 3. Paper chromatogram of acid hydrolyzates of glucosyltransferase showing various lengths of hydrolysis. Standards; Ga, D-galactose; G, D-glucose; and M, D-mannose.

from the hydrolyzate moved with a slightly lower R_F value than standard D-galactose and its identity was established by the D-galactose oxidase spray method[14]. The identity of D-glucose was verified by the D-glucose oxidase spray method[14]. A compound having an R_F value higher than mannose was observed but it has not yet been identified.

Methylation analysis on the glucosyltransferase was performed by the Hakomori[15] and the Björndahl *et al.*[16] procedures. Following hydrolysis, reduction, and acetylation of the methylated product, the methylated alditol acetates were identified by g.l.c.–m.s. It was found that the acid hydrolyzate of the methylated transferase contained 2,3,4,6-tetra-*O*-methylmannose or 2,3,4,6-tetra-*O*-methylglucose (not identifiable from retention times and m.s. data), 3,4,6-tri-*O*-methylmannose, 2,3,4-tri-*O*-methylgalactose, 2,3,4-tri-*O*-methylglucose, and 2,4-di-*O*-methylmannose.

Gel electrophoresis of the purified transferase showed a protein band also stained with the periodate–Schiff reagent. The foregoing result, the methylation data, the positive colorimetric tests for carbohydrates, and the results of acid hydrolysis establish that the glucosyltransferase from *Aspergillus niger* contains carbohydrate residues and is therefore a glycoprotein.

In order to determine the type of linkage between carbohydrate and protein, the transferase was subjected to reductive alkaline β-elimination[17]. The products in one-half of this reaction mixture were dried, acetylated with acetic anhydride in pyridine, and then subjected to g.l.c. (see Fig. 4), and the remainder of the mixture was used for amino acid analysis. A major peak of carbohydrate derivative having the same retention time as mannitol hexaacetate was observed. Trace amounts of other acetylated carbohydrate derivatives could be detected (Fig. 4). These results and the methylation data were interpreted as evidence for the presence of single monosaccharides and oligosaccharide chains *O*-glycosyl-linked to threonine and

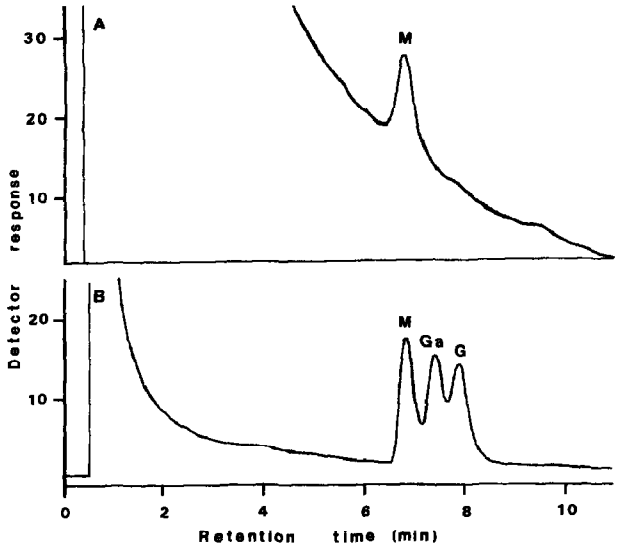

Fig. 4. Gas-liquid chromatogram of alditol acetates of: (A) carbohydrates obtained by reductive, alkaline β-elimination from glucosyltransferase, and (B) reference alditol acetates of hexoses. Abbreviations: M, mannitol hexaacetate; Ga, galactitol hexaacetate; and G, glucitol hexaacetate.

serine residues. The amino acid analysis of hydrolyzates of the transferase before and after β-elimination showed significant decreases (33 and 39%, respectively) in the values for serine and threonine, corresponding increases (22 and 34%, respectively) in values for glycine and alanine, and the appearance of α-aminobutyric acid after reductive elimination. The values for the other amino acids of the transferase remained relatively unchanged (2 to 5%) in the native and modified enzyme.

Antibodies directed against the glucosyltransferase from *Aspergillus niger* were induced in rabbits by immunization with a vaccine of the transferase in Freund's complete adjuvant. Antisera containing these antibodies were prepared by use of standard methods. *Aspergillus niger* also produces a glucoamylase which is used in the commercial manufacture of crystalline dextrose, corn syrups, and high fructose syrups[18]. Antibodies specific for glucoamylase are directed at the carbohydrate components of the enzyme[19]. The anti-glucoamylase antibodies were used in comparative studies with the anti-glucosyltransferase antibodies.

The results of the agar diffusion tests with the transferase, glucoamylase, and corresponding antibodies (Fig. 5,A,B), and with the transferase, periodate-oxidized transferase, and antibodies (Fig. 5C) show clearly that each antibody type is specific for the antigen that has been used in immunization and induction. Cross-reactivity was not obtained with the two enzymes and their antibodies, even though a similarity in structure of the two glycoprotein antigens exists. Microheterogeneity in the carbohydrate chains of fungal enzymes has been noted earlier[20], and such heterogeneity may be responsible for the lack of cross-reactivity in the antigen–

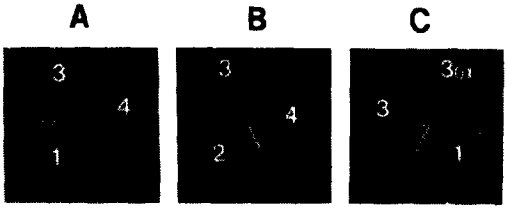

Fig. 5. Agar-diffusion plates for (A) native and (C) periodate-oxidized glucosyltransferase, and (B) native glucoamylase with antisera and antibodies directed against the enzymes: (1) Anti-glucosyltransferase antisera; (2) anti-glucoamylase antibodies; (3) glucosyltransferase; (4) glucoamylase; and (3 ox) periodate-oxidized glucosyltransferase.

antibody reactions. The periodate-oxidized enzyme no longer reacted with the anti-glucosyltransferase antibodies, and a precipitate band was not obtained with the oxidized enzyme (Fig. 5C). However, the native enzyme yielded a strong precipitin band with the antibodies. Periodate does not oxidize internal amino acid residues of a protein and could oxidize serine and threonine residues only if such residues were terminal. Consequently the protein moiety of the transferase is unaltered or altered only slightly by the oxidation, and the loss of antigenicity by the transferase could not be attributed to alterations in the protein moiety of the enzyme. Periodate oxidation oxidizes carbohydrate units that are not substituted at O-3. The methylation data showed that many of the carbohydrate residues of the enzyme are not substituted at O-3. Consequently, it was concluded that the antibodies against glucosyltransferase are directed against the carbohydrate components of the enzyme since periodate oxidation destroyed the antigenicity. Additional data in support of this conclusion could be obtained from experiments on the removal of the carbohydrates with glycosidases and inhibition of the antigen–antibody reaction with carbohydrate chains obtained from the enzyme by alkaline reductive β-elimination.

The nature of the antigen–antibody reaction of the transferase was also investigated by a coupled isoelectrofocusing–agar diffusion method[21,22]. The isoelectrofocusing of the transferase was performed in triplicate in a gradient of pH 3.5 to 10. One finished gel was stained with Coomassie Blue to locate the protein components, another was stained with the periodate–Schiff reagent to locate the glycoprotein component, and the third was embedded in agar in a Petri plate. The glucosyltransferase solution was placed in a trough cut in the agar, and diffusion was allowed to proceed in a moist chamber for an additional 32 h at room temperature (see Fig. 2C,D). A protein component was located ~1.2 cm from the positive pole and it reacted with the antibodies to yield a diffusion band of precipitin complex. Comparisons of the location of the glucosyltransferase band in the polyacrylamide gel with the positions of antibodies of known isoelectric points[23] indicated that the isoelectric point of the transferase was ~4.

EXPERIMENTAL

Preparation of glucosyltransferase. — The glucosyltransferase used in this study was isolated from an enzyme preparation of *Aspergillus niger* that was obtained from the Miles Laboratories, Inc. (Elkhart, IN 56514). The enzyme preparation is available under the trademark DIAZYME and is used as a source of glucoamylase for the commercial production of D-glucose from starch[24]. The preparation also contains a glucosyltransferase that can be conveniently isolated by chromatography on DEAE-cellulose[7]. The DIAZYME preparation (10 g) was subjected to chromatography on DEAE-cellulose (30 g) which had been thoroughly washed with 0.1M citrate–phosphate buffer (pH 8). The enzyme sample was dissolved in distilled water, dialyzed for 24 h, diluted with an equal volume of citrate–phosphate buffer (pH 8), and adsorbed on the DEAE-cellulose column. The column was washed successively with 0.1M citrate–phosphate buffer of pH 8.0, 6.0, and 4.0 (500 mL each). The glucosyltransferase was eluted rapidly from the column by buffer pH 8. The two isozymic forms of glucoamylase that are produced by *A. niger* were eluted with buffer of pH 6.0 and 4.0. Transferase assays were performed on the samples from the column with a maltose substrate and the digests were analyzed by paper chromatography. The fractions that contained the transferase synthesized isomaltose and panose. These fractions were combined, concentrated by lyophilization, and subjected to a second DEAE-cellulose chromatography as described earlier. The second chromatography step removed traces of contaminating glucoamylase from the glucosyltransferase.

The u.v. absorbance of the fractions from the column, the chromatographic results of assays on maltose, and the results of agar diffusion tests with anti-glucosyltransferase antibodies performed as described in a subsequent section are shown in Figs. 1A, 1B, and 1C, respectively. The glucosyltransferase fractions from the second DEAE-cellulose column were combined and lyophilized (yield 250 mg). This sample was used as the source of glucosyltransferase.

Electrophoresis and isoelectrofocusing. — The purified glucosyltransferase was subjected to polyacrylamide gel electrophoresis, following the procedure of Davis[25], in 5% separating and 7.5% stacking gels at room temperature for 3 h and at a constant current of 2.5 mA per gel. At completion of the electrophoresis, one set of gels was stained with the Coomassie Blue dye[25], and a duplicate set was stained by the periodate–Schiff method[26]. The glucosyltransferase yielded a positive reaction with both the protein and glycoprotein stains. Under the condition of the electrophoresis, both components migrated 1.5 cm towards the positive pole.

Isoelectrofocusing was performed[21] in 5% and 7.5% polyacrylamide gels in glycerol–Ampholine solutions of pH gradient of 3.5–10 for 5 h at 4° at a constant voltage of 215 V. One gel was stained with the Coomassie Blue dye, one by the periodate–Schiff method, and a third gel was embedded in agar in a Petri plate for checking the reactivity of the transferase with homologous antisera. After the agar had solidified around the polyacrylamide gel and diffusion had occurred for 18 h, a

trough was cut in the agar about 2 cm from the gel. The trough was filled with serum with anti-glucosyltransferase antibodies prepared as described later. Diffusion was allowed to proceed for an additional 24 h, in which time zones of precipitin complex were formed. The plates and gels were photographed and the results of one experiment are shown in Fig. 2. The enzyme band located at about 1.2 cm from the positive pole stained with protein and glycoprotein reagents and yielded a precipitin complex with the antibodies in the agar-diffusion test.

Carbohydrate analysis. — Since the periodate–Schiff stain of polyacrylamide gels indicated the presence of carbohydrate in the glucosyltransferase, qualitative tests for carbohydrate constituents of glucosyltransferase were performed by the phenol–sulfuric acid and the orcinol methods[12,13]. Positive tests were obtained by both methods. Subsequently, a quantitative analysis for carbohydrate content of the enzyme was made by the phenol–sulfuric acid method. From the absorbance readings at 490 nm for the sample and for standards of D-mannose and D-glucose, it was calculated that the transferase contained 20% of carbohydrate.

The transferase (4 mg) was partially hydrolyzed with 0.1M HCl (0.1 mL) in a boiling water bath. An aliquot of the solution was placed on paper chromatograms after addition of the acid and after heating for periods of 0.5, 1, and 3 h. Four chromatograms were prepared in the above manner and standard solutions of D-galactose, D-glucose, and D-mannose were also deposited. The chromatograms were developed in 6:4:3 (v/v) 1-butanol–pyridine–water[1]. One chromatogram was treated with $AgNO_3$–NaOH for locating reducing sugars; one with hexosamine-detecting reagent; one with D-glucose oxidase, IO_4^-, and *o*-toluidine reagents; and one with D-galactose oxidase, peroxidase, and *o*-toluidine[14]. The carbohydrates released from the enzyme were stained with the $AgNO_3$–NaOH (Fig. 3) but not with the hexosamine-detecting reagent. Also, one of the compounds reacted with D-glucose oxidase and another with D-galactose oxidase.

Methylation analysis. — Purified glucosyltransferase (4 mg) was methylated by the Hakomori method[15] and analyzed for alditol acetates, by g.l.c.–m.s.[16] according to a modified procedure[27], on a column of 3% OV-225 at 190°.

Reductive alkaline β-elimination. — Purified glucosyltransferase (10 mg) was subjected to reductive alkaline β-elimination[17] in 0.1M NaOH containing 0.3M $NaBH_4$ (3 mL) for 280 h at 4°. The excess $NaBH_4$ was decomposed with 0.5M acetic acid, and borate ions were removed by evaporation from methanol several times. The residue was dissolved in water (1 mL), and the solution divided into two equal parts. One part was evaporated under a stream of N_2 and the residue acetylated with 1:1 (v/v) acetic anhydride–pyridine (1 mL). After evaporation, diethyl ether was added to and evaporated several times from the product. The residue was finally dissolved in a small amount of chloroform and analyzed by g.l.c. on a column of OV-225 at 230° (see Fig. 4).

The remainder of the product of the β-elimination reaction was used for amino acid analysis. The dried sample and a duplicate sample of native enzyme (5 mg each) were dissolved separately in 6M HCl (0.5 mL) in 10-mL ampules which

were sealed and heated for 24 h at 110°. Each ampule was opened and the clear solution was evaporated to dryness under a stream of N_2. The residue was dissolved in water (1 mL), and appropriate aliquots of the solution were diluted with citrate buffer (pH 2.2) and analyzed for amino acid content with a Beckman amino acid analyzer. After alkaline, reductive β-elimination, the glucosyltransferase contained 39 and 33% less threonine and serine, respectively, than the native enzyme. Also the values for glycine and alanine were increased by 22 and 34%, respectively, and α-aminobutyric acid was produced in the hydrolyzate. The variations in the other amino acid values were within experimental error; for example, in the β-elimination enzyme, the values for aspartic acid and leucine were 2 and 3% lower, respectively, and for glutamic acid and cysteine 5 and 4% higher, respectively, than in the native enzyme.

Periodate oxidation. — The methylation data indicated that most of the carbohydrate residues of the transferase would be susceptible to periodate oxidation. Glucosyltransferase (5 mg) was dissolved in 20mM $NaIO_4$ (1 mL) and the pH adjusted to 4.5. Oxidation was allowed to proceed for 24 h at 4° in the dark. The excess $NaIO_4$ was eliminated with 1,2-ethanediol. The resultant solution was used as the source of oxidized enzyme. Carbohydrate analysis by the phenol–sulfuric acid method showed that 90% of the carbohydrate residues had been oxidized. In diffusion tests with the native and oxidized transferase and with the glucoamylase, samples (10 μL) of 0.5% solution of the enzymes were deposited in the outer wells of the diffusion plate, and samples (10 μL) of the appropriate antisera in the center wells. The plates were maintained in a moist atmosphere for 24–36 h. The plates that developed precipitin bands were photographed (see Fig. 5,C).

Immunological methods. — Rabbits were immunized by weekly injections of 0.5 mL glucosyltransferase (12 mg) in saline solution (5 mL) mixed with Freund's complete adjuvant (5 mL) into the alternate thigh muscles for a period of 10 weeks. Blood samples were obtained prior to, and after immunization for 4 weeks and subsequent weeks. The antisera were prepared by standard methods and checked for the presence of antibodies by the qualitative precipitin test and by double diffusion in agar. The samples obtained after the 5th week contained anti-glucosyltransferase antibodies. Results of some agar-diffusion tests with glucosyltransferase, periodate-oxidized transferase, anti-glucosyltransferase antibodies, with glucoamylase from the same organism, and anti-glucosyltransferase antibodies are shown in Fig. 5. Antibodies directed against the glucoamylase from *Aspergillus niger* had been isolated earlier by affinity-chromatography methods[19].

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