Purification and Properties of Yeast Amylo-1,6-glucosidase–Oligo-1,4→1,4-glucantransferase*

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ABSTRACT: An enzyme system that debranches glycogen has been isolated from *Saccharomyces cerevisiae* (baker's yeast) and characterized as an amylo-1,6-glucosidase–oligo-1,4→1,4-glucantransferase similar to that found previously only in mammalian systems. The debranching enzyme was purified 1300-fold to a state of homogeneity according to the criteria of ultracentrifugation and disc gel electrophoresis.

The purified enzyme catalyzed the release of 8.4 μmoles of glucose from a glycogen phosphorylase limit dextrin per min per mg of protein at 30°, pH 6.4. Molecular weights of the order of 280×10^3 were obtained by sedimentation equilibrium measurements (with clear indication for dissociation at low protein concentrations) and 210×10^3 by Sephadex chromatography and polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate indicated that the enzyme was made up of at least two, if not three, different subunits. As in the rabbit muscle enzyme both the glucosidase and transferase activities necessary for the debranching process were associated with a single protein species. Relative rates of glucose released decreased from shellfish glycogen phosphorylase limit dextrin (100) to shellfish glycogen (82), rabbit liver glycogen (72), and amylopectin (4.5). Maltotetraose and maltopentaose appear to be donor substrates for a two- (but not a three-) glucose unit transfer and maltohexaose is a donor substrate for both types of transfer. In the presence of glycogen phosphorylase and inorganic phosphate, the yeast debranching enzyme enabled the complete degradation of glycogen to occur.

Debranching enzymes attack the α-1,6-branch linkages of polysaccharides such as glycogen or amylopectin. In mammalian systems, phosphorylase acts on the outer branches of glycogen, leaving a resistant dextrin having outer chains four units long (Walker and Whelan, 1960; Abdullah *et al.*, 1964). For further breakdown of the polysaccharide to occur the mediation of a debranching enzyme is necessary. From extensive work on the rabbit muscle system, we know that the mammalian debranching enzyme consists of two discrete enzymic activities (Cori and Larner, 1951; Brown and Illingworth, 1964; Brown and Illingworth, 1966; Taylor and Whelan, 1968; Nelson *et al.*, 1969). First, there is an oligo-1,4→1,4-glucantransferase activity (EC 2.4.1.25) which preferentially transfers a glucan segment of three residues to another portion of the polysaccharide, leaving the glucose unit involved in the α-1,6 linkage exposed. The α-1,6 linkage is then hydrolyzed by an amylo-1,6-glucosidase (EC 3.2.1.33) which is specific for the glucose stub. Although the system is usually assumed to act on a phosphorylase limit dextrin, it is capable of debranching native glycogen, since successive transfers by the transferase will eventually expose the glucose stub involved in the branch linkage. The two enzymic activities appear to be associated with a single protein, or, alternatively, with two proteins bound together in a tight complex; homogeneous preparations containing both activities have been isolated from rabbit muscle (Brown and Illingworth, 1964; Nelson *et al.*, 1969).

A similar type of debranching enzyme system was unequivocally demonstrated in a nonmammalian system (*Saccharomyces cerevisiae*) by Lee *et al.* (1967). Prior to this report, the only known debranching enzymes in nonmammalian systems were those which acted directly on the α-1,6-branch linkage to set free the unit chains of the polysaccharide. Examples of this type of debranching enzyme are R-enzyme from plants, which acts only on amylopectin (Hobson *et al.*, 1951), pullulanase from *Aerobacter aerogenes* (Bender and Wallenfels, 1966; Abdullah *et al.*, 1966), and isoamylase from baker's yeast (Kjølberg and Manners, 1963; Bathgate and Manners, 1968), which indicates that debranching of glycogen by this latter organism can occur by two distinct mechanisms.

The present paper reports the isolation and some of the properties of yeast amylo-1,6-glucosidase–oligo-1,4→1,4-glucantransferase (glucosidase–transferase).[1] The activity of the enzyme has been characterized, and its action on the structure of glycogen is described.

Materials and Methods

Preparation of Glycogen Phosphorylase. Crystalline rabbit muscle phosphorylase *b* was prepared by the method of Fischer *et al.* (1958), with the modification of Krebs *et al.*

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[1] Abbreviation used is: amylo-1,6-glucosidase (EC 3.2.1.33)–oligo-1,4→1,4-glucantransferase (EC 2.4.1.25), glucosidase–transferase.

(1964). After five recrystallizations, phosphorylase *b* was converted into the *a* form by using purified phosphorylase *b* kinase (Krebs *et al.*, 1964); after two recrystallizations, phosphorylase *a* was found to be free of all traces of α-amylase and glucosidase activity. Phosphorylase activity was assayed according to Hedrick and Fischer (1965), except that 40 mM sodium glycerophosphate, pH 6.8, was used instead of the sodium maleate buffer.

Preparation of Glycogen Phosphorylase Limit Dextrin. Phosphorylase *a* (125 mg, 10,000 units) was incubated at 30° for 5 hr with shellfish glycogen (Mann Research Laboratories, 8 g) in 2 l. of 80 mM sodium phosphate–40 mM mercaptoethanol, pH 6.8. After heating at 100° for 5 min, the digest was dialyzed for 24 hr against distilled water (6 × 4 l.), and concentrated to 300 ml by rotary evaporation. The polysaccharide was precipitated with 4 volumes of ethanol, washed with ethanol, ether, and dried *in vacuo*. The degree of phosphorolysis of the glycogen was estimated to be 31% from the disappearance in inorganic phosphate; the yield of limit dextrin was 4.8 g.

Glucose assays were carried out enzymatically according to the method of Fleming and Pegler (1963), as modified by Abdullah and Whelan (personal communication). Glucose oxidase (30 mg), peroxidase (3 mg), both obtained from Boehringer Mannheim Corporation, and 3,3′-dimethoxybenzidine (10 mg) were dissolved in 100 ml of 0.3 M Tris-HCl in 40% glycerol, pH 7.0. This reagent is stable for several weeks when stored in a dark bottle at 0–5°. The test solution containing 10–50 μg of glucose was mixed with 2 ml of the glucose oxidase reagent and incubated at 30° for 1 hr; 4 ml of 5 N HCl was then added and the solution read at 540 nm. Values obtained were referred to a standard glucose curve.

Glucosidase–transferase activity was measured by the release of glucose from glycogen phosphorylase limit dextrin (Larner and Schliselfeld, 1956; Hers *et al.*, 1967). To 0.2 ml of substrate containing 2.5 mg of glycogen phosphorylase limit dextrin and 125 μg of bovine serum albumin in 30 mM sodium citrate–phosphate buffer, pH 6.4, 0.05 ml of enzyme was added, and the mixture was incubated at 30° for 15 min. The reaction was stopped by addition of the glucose oxidase reagent and the glucose released was determined. One unit of activity represents the amount of enzyme which catalyzes the release of 1 μmole of glucose per min under the above conditions. Glucose release was proportional to the amount of enzyme added provided the latter did not exceed 0.015 unit.

Degree of β-amylolysis of polysaccharides was determined by the method of Walker and Whelan (1960). Total polysaccharide was determined by hydrolysis with amyloglucosidase according to Lee and Whelan (1966). The degree of β-amylolysis was expressed as the percentage of total polysaccharide released as maltose by β-amylase; both maltose and polysaccharide were expressed in terms of their equivalent amounts of glucose for the purpose of the calculation.

Iodine Stains. The polysaccharide solution (usually 0.2 ml) was mixed with 5 ml of iodine solution (0.05% KI–0.005% I_2–0.05 N HCl) to a final concentration of 0.1–0.2 mg/ml. Absorbance was measured at 540 nm.

Paper chromatography of maltosaccharides was performed in a solvent system consisting of ethyl acetate–pyridine–water (10:4:3) (Whistler and Hickson, 1955). The system was capable of the resolution of oligosaccharides from maltose to maltooctaose. Chromatograms were sprayed with the $AgNO_3$–NaOH spray of Trevelyan *et al.* (1950).

Protein was assayed by the method of Lowry *et al.* (1951), using bovine serum albumin as standard.

Ultracentrifuge studies were conducted in a Spinco Model E analytical ultracentrifuge equipped with an electronic speed control and optical system focused at the two-thirds plane of the cell (Svensson, 1954, 1956; Yphantis, 1964). Molecular weight determinations were made according to the high-speed sedimentation equilibrium technique of Yphantis (1964). Centrifuge runs were performed at 5° in a six-channel centerpiece at initial protein concentrations of 0.64–0.97 mg/ml and column heights of 3 mm.

The fringe patterns were read on a modified Nikon microcomparator (Teller, 1967). Points were read throughout until the fringes could no longer be resolved at the base of the cell and data were processed with computer programs developed by Teller *et al.* (1969); a partial specific volume of 0.73 ml/g was used in the calculations. Sedimentation velocity experiments were carried out in a double-sector cell at 5°.

Determination of molecular weight by gel filtration was carried out in 2.5 × 100 cm columns of Sephadex G-200 pretreated in 50 mM Na citrate–phosphate, 0.1 M NaCl, and 1 mM EDTA buffer, pH 7.2. Protein samples (0.5–6 mg/ml) were applied in a volume of 2 ml; fractions were collected at 30-min intervals and absorbance was measured at 280 nm.

Results

Purification of the Enzyme. All operations were carried out at 0–5°; pH values were measured at these temperatures except for the Tris-HCl buffers which were determined at room temperature. Fleischmann's baker's yeast (2 lb) was mixed with 1 l. of ice-cold distilled water. Glass beads (3M Co., 120 μ in diameter, 1200 ml) were added and the slurry was ground for 30 min at 0–5° in an Eppenbach colloid mill (Model MV-6-3) attached to a circulating refrigerated bath according to the procedure of Garver and Epstein (1959). The glass beads were allowed to settle for a few minutes and the supernatant was centrifuged (45 min at 1000*g*) to remove cell debris. In the preparation to be described, a total of 8 lb of yeast in four batches was treated in this manner, to yield approximately 4 l. of combined extract. The extract was brought to pH 4.7 with 1 N acetic acid and stored overnight; the precipitate which formed was centrifuged (30 min at 14,000*g*) and the pellet was resuspended in a minimum volume (*ca.* 800 ml) of ice-cold distilled water. The pH of the suspension was brought to 7.0 with 1 N NaOH, which allowed most of the material in suspension to dissolve. The pH was immediately adjusted to 5.6 with 1 N acetic acid, and the resulting precipitate was removed by centrifugation (15 min at 14,000*g*) and discarded. At this stage, the enzyme preparation was very unstable, losing all activity within a few hours at room temperature; it could be stabilized by addition of protease inhibitors such as diisopropylphosphorofluoridate (Ooms, 1961) or phenylmethylsulfonyl fluoride (Fahrney and Gold, 1963) at concentrations of 0.1 to 1 mM.

Batchwise TEAE-Cellulose Adsorption. The enzyme solution was stirred for 1 hr with 0.1 mM phenylmethylsulfonyl fluoride then dialyzed against two 4-l. changes of distilled

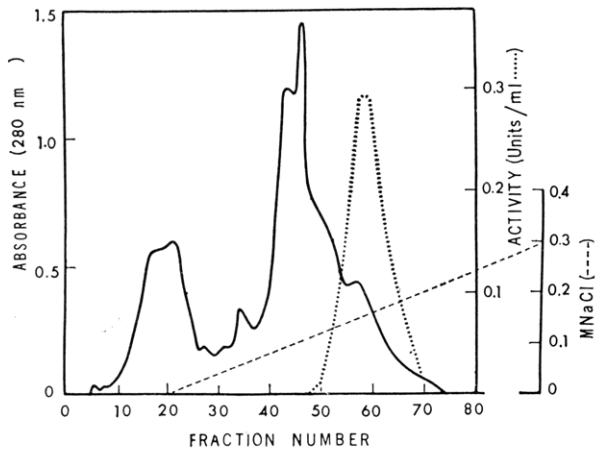

FIGURE 1: Elution profile of a DEAE-cellulose chromatography of yeast glucosidase–transferase. Conditions are described in text.

water for 2 hr each. After adjusting the pH of the dialyzed solution to 7.0 with 2 M Tris, 100 g wet weight of TEAE-cellulose (preequilibrated and washed with 50 mM Tris-HCl, pH 7.0) was added and the suspension stirred for 30 min. The TEAE-cellulose was filtered off under suction on a Büchner funnel and resuspended in 250 ml of 50 mM Tris-HCl, pH 7.0. The suspension was stirred and again filtered. The washed TEAE-cellulose cake was then extracted with two 125-ml portions of 0.1 M NaCl in 0.1 M Na acetate buffer, pH 5.5. The extracts containing the enzyme were combined and dialyzed against 10 mM Tris-HCl, pH 7.0; the solution obtained was free of maltase and α-amylase activities.

DEAE-Cellulose Chromatography. The enzyme solution was concentrated to 100 ml by ultrafiltration in a Diaflo cell using a UM-1 membrane (Amicon Corp.) and chromatographed on a 1.2 × 50 cm column of DEAE-cellulose equilibrated with 50 mM Tris-HCl, pH 7.7. A linear gradient consisting of 1 l. of 50 mM Tris-HCl buffer, pH 7.7, and 1 l. of 0.5 M NaCl in the same buffer was then applied at a flow rate of 40 ml/hr (Figure 1). Fractions 54–68 (8 ml each) containing the enzyme activity were pooled and concentrated to 6 ml by ultrafiltration.

Sephadex G-200 Chromatography. The enzyme solution was chromatographed on a 2.5 × 100 cm column of Sephadex G-200 at a flow rate of 8.6 ml/hr in 0.1 M Tris-HCl, pH 7.0

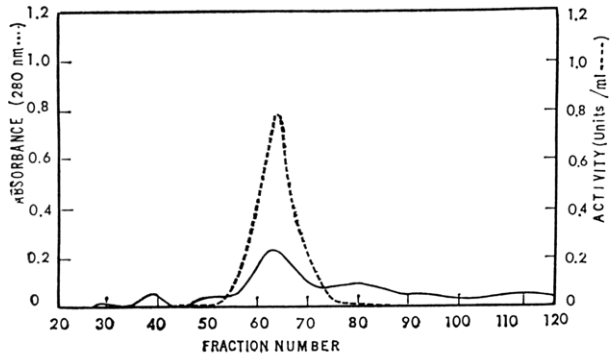

FIGURE 2: Elution profile of a Sephadex G-200 chromatography of yeast glucosidase–transferase. Conditions are described in text.

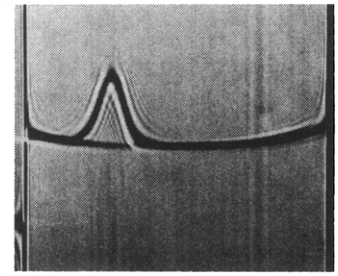

FIGURE 3: Sedimentation velocity pattern of yeast glucosidase–transferase (1.94 mg/ml) in 50 mM Na citrate–phosphate buffer, pH 7.2, containing 1 mM EDTA. Temperature of the rotor was maintained at 3°. Picture was taken at 16 min after attainment of maximum speed (52,640 rpm).

(Figure 2). The active fractions (57–68, 4.3 ml each) were pooled and concentrated by ultrafiltration.

A summary of the purification is shown in Table I. Final products with a specific activity of 6.1 ± 0.1 units per mg of protein were usually obtained. Material prepared to this stage of purity was apparently homogeneous by disc gel electrophoresis. In one experiment, however, rechromatography on Sephadex G-200 gave a material with specific activity of 8.4. This material was used in the experiments on the sedimentation velocity and equilibrium measurements and in the sodium dodecyl sulfate disc gel electrophoresis; all other experiments described below were performed with material having a specific activity of at least 6.0.

Sedimentation Velocity and Equilibrium Measurement. A sedimentation velocity ultracentrifugation pattern of purified yeast glucosidase–transferase is illustrated in Figure 3. Analysis of the pattern with a Dupont 330 curve resolver indicated homogeneity of the preparation; a sedimentation coefficient, $s_{20,w}$, of 10.45 S was calculated.

High-speed sedimentation equilibrium experiments were also carried out; Figure 4 illustrates a representative distribution of number-average (M_n, curve A), weight-average (M_w, curve B), and Z-average (M_Z, curve C) molecular weights as a function of protein concentration. It can be seen that in the concentration range below four fringes (1 mg/ml), the molecular weight of the material decreased sharply, a behavior characteristic for a dissociating system (Hoagland and Teller,

TABLE I: Purification of Yeast Glucosidase–Transferase.

Purification Step	Enzyme Recovery		Specific Activity (Units/mg)	Purification
	Units	%		
Crude extract (8-lb yeast)	360	100	0.0045	
pH 4.7 Precipitate	183	50	0.017	3.8
0.1 M TEAE extract	66	18	0.13	28
DEAE-cellulose chromatography	42	12	1.26	280
Sephadex G-200 chromatography	33	9	6.1	1330

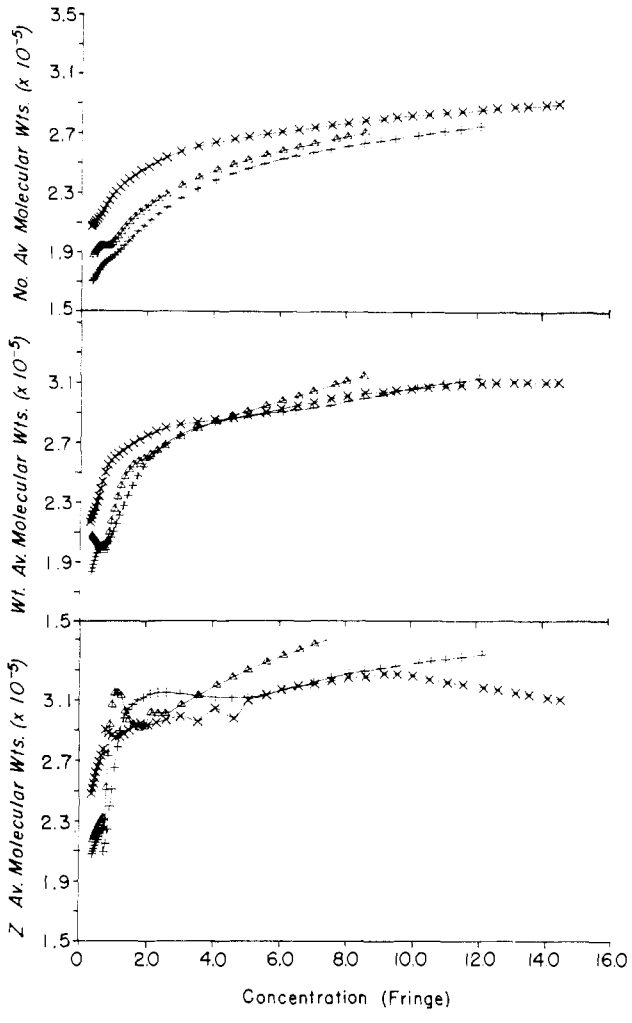

FIGURE 4: Molecular weight distribution as a function of glucosidase–transferase concentration. Four fringes correspond to 1 mg/ml of protein. Initial concentrations of enzyme were 0.64 mg/ml in the first cell (△); 0.73 mg/ml in the second (+); and 0.97 mg/ml in the third cell (×). The buffer was the same as that described in Figure 3.

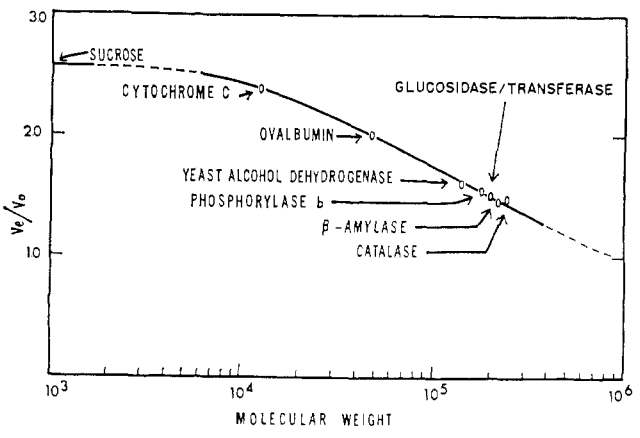

FIGURE 5: Sephadex gel filtration of yeast glucosidase–transferase as compared to that of standard proteins. V_e represents the elution volume of each protein and was taken as the point at which the protein peak emerged from the column; V_0, the void volume, was determined with Blue Dextran (Pharmacia).

1969). Dissociation was also reflected in the appreciable divergence obtained for the number-average, weight-average, and Z-average molecular weights obtained for this enzyme (247×10^3, 279×10^3, and 297×10^3, respectively).

Estimation of Molecular Weight by Sephadex Gel Filtration and Polyacrylamide Gel Electrophoresis. The molecular weight of the yeast glucosidase–transferase was also examined by column chromatography on Sephadex G-200 according to the procedure of Andrews (1965) using a series of proteins of known molecular weight as standards (Figure 5). From the elution to void volume ratio of 1.51 obtained for the enzyme, a molecular weight of the order of 200×10^3 was estimated.

The disc gel electrophoresis procedure of Hedrick and Smith (1968), using gel concentrations of 4, 6, 8, and 10% was further applied to the enzyme system. Figure 6A illustrates the linear relationship obtained for the glucosidase–transferase and one of the protein standards (rabbit muscle phosphorylase *b*) when the relative mobilities of the protein bands to that of the dye front were plotted against gel concentration (Hedrick and Smith, 1968). Similar linear relationships were obtained for five other proteins used as standards. The slopes determined by the method of least squares for each of these proteins are plotted against molecular weight in Figure 6B from which a molecular weight of $215 \pm 10 \times 10^3$ was estimated for the glucosidase–transferase.

At all the concentrations of gels used, the glucosidase–transferase migrated as a single band (Figure 7A,B) satisfying the criterion of homogeneity by this approach. In an attempt to obtain some indication of the subunit structure of the enzyme polyacrylamide gel electrophoreses were also carried out in 0.1% sodium dodecyl sulfate according to the procedure of Shapiro *et al.* (1967). As can be seen in Figure 7C the purified enzyme showed three bands corresponding to a molecular weight of 74×10^3, 94×10^3, and 175×10^3, respectively. On the assumption that the molecule consisted of only two basic subunits of molecular weight 74×10^3 and 94×10^3, and that the species of molecular weight 175×10^3 resulted from the incomplete disaggregation of the molecule, the enzyme was subjected to a drastic treatment with 0.1% sodium dodecyl sulfate for 24 hr at 65°, but no further dissociation to the lower molecular weight species could be observed. From these data it is not possible to assign a molecular weight to the enzyme or propose a definite model for its subunit structure. All that can be said is that at high concentrations ($\geq$1 mg/ml), it has a molecular weight of the order of 280×10^3, that it dissociates readily to smaller molecular weight species at concentrations below 1 mg/ml and that it appears to be made up of at least two and perhaps three different subunits.

Demonstration of α-1,6-Glucosidase Activity. α-Glucosyl Schardinger dextrins are cyclodextrins with a glucosyl stub linked α-1,6 to the dextrin ring; these compounds were developed by Taylor and Whelan (1966) as specific substrates for the α-1,6-glucosidase activity of the debranching enzyme system. The material used in this experiment was a mixture of the α-1,6-glucosyl-substituted cyclohexa- and cycloheptaamylose. When the enzyme (0.036 unit or 6 μg of protein) was incubated at 30° with 12.5 mg of the α-

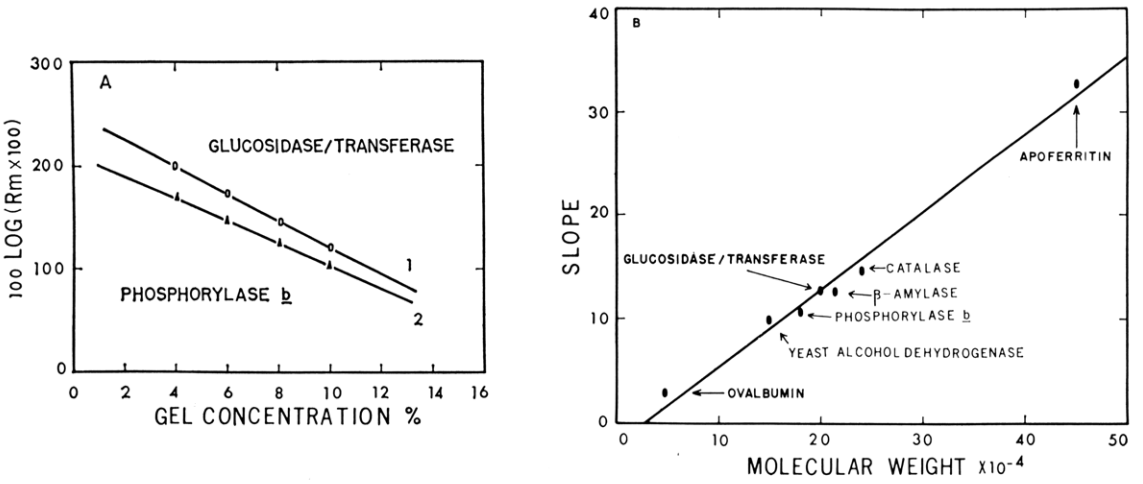

FIGURE 6: Plot of relative mobilities (R_m) of yeast glucosidase–transferase (slope = 13.09) and rabbit muscle glycogen phosphorylase (mo wt 185,000, slope = 10.82) *vs.* acrylamide gel concentration following acrylamide gel electrophoresis according to Hedrick and Smith (1968). In B, the slopes for protein markers and the glucosidase–transferase are plotted as a function of molecular weight.

glucosyl Schardinger dextrin in 1 ml of 50 mM Na citrate–phosphate buffer, pH 6.0, 0.2 mg of glucose was released in 60 min. The reaction was linear with time; the rate of glucose release was 56% of that obtained when glycogen phosphorylase limit dextrin was used as substrate. These data confirm that the purified yeast glucosidase–transferase possesses a specific α-1,6-glucosidase activity.

Demonstration of Oligo-1,4→1,4-glucantransferase Activity. The enzyme preparation was tested for transferase activity by incubation with a series of maltosaccharides (from maltose to maltohexaose), followed by chromatography of the reaction products. Each oligosaccharide (10 mg/ml) was incubated at 30° with the yeast glucosidase–transferase (0.30 unit/ml corresponding to 50 μg of protein/ml) in 8 mM Na citrate–phosphate buffer, pH 6.4. Samples were removed at time intervals (15 and 30 min, 1, 2, 3, and 24 hr), deionized by treatment with a carbonated mixed-bed resin, and chromatographed as described under Methods.

No action on either maltose or maltotriose was detected even after overnight incubation. Only slight action on maltotetraose was detected within 3 hr; after 24 hr the main products were maltose and maltohexaose with traces of maltotriose and maltopentaose, indicative of a two-glucose unit transfer. With maltopentaose as a substrate, maltotriose and maltoheptaose, again as a result of a two-glucose unit transfer, appeared after 30 min. These were followed (1 hr) by maltotetraose and maltooctaose, which were presumed to arise from a three-glucose unit transfer from maltoheptaose to maltopentaose, rather than from a single glucosyl transfer since maltohexaose was absent in the first 3 hr of reaction. During this period maltose was also absent, indicating that a three-glucose unit transfer from maltopentaose had not occurred. With maltohexaose as a substrate, maltotetraose and maltooctaose were produced within 15 min (two-glucose unit transfer) followed at 30 min by maltotriose, maltopentaose, and products larger than maltooctaose. Again, the later products were assumed to arise from a three-glucose unit transfer from maltohexaose and maltooctaose (rather than from a glucosyl transfer) since maltoheptaose was not detected in the first 3 hr of reaction. With maltopentaose and maltohexaose the whole series of oligosaccharides from maltose to maltooctaose and longer chain products were observed after 24 hr; glucose was not detected as a product.

These results demonstrated that the purified enzyme possesses an oligo-1,4→1,4-glucantransferase activity. The enzyme has a glucan (rather than a glucosyl) transferase activity, and two-glucose units were transferred preferentially over three-glucose units. The smallest oligosaccharide which was acted upon was maltotetraose, while maltopentaose and maltohexaose were increasingly superior substrates. While the clear definition of the specificity of the transferase must await a more rigorous study, it would appear from our results that maltotetraose and maltopentaose are donor substrates for a two- (but not a three-) glucose unit transfer and that maltohexaose is a donor substrate for both types

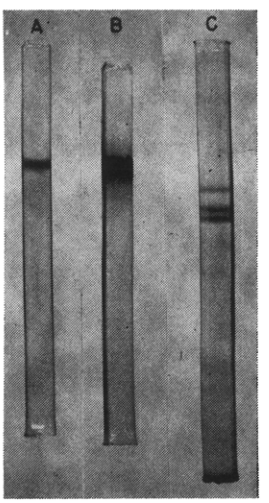

FIGURE 7: (A) Polyacrylamide gel electrophoresis of yeast glucosidase–transferase (25 μg) in 7% gels, 0.38 M Tris-HCl, pH 8.9; (B) same as in A but with 100 μg of enzyme; (C) after denaturation of the enzyme for 3 hr at 37° in 1% sodium dodecyl sulfate–1% mercaptoethanol; gel conditions were those of Shapiro *et al.* (1967).

TABLE II: Combined Action of Yeast Glucosidase–Transferase and Rabbit Muscle Phosphor ylase *a*.[a]

Polysaccharide	Total Polysaccharide[b] (mg/ml, as glucose)	Free Glucose Produced[c] (μg/ml)	Glucose + Glucose-1-P Produced[d] (mg/ml, as glucose)	Degradation (%)	Average Chain Length[e]
Shellfish glycogen	1.03	106	1.00	97	9.7
Shellfish glycogen Limit dextrin[f]	1.90	128	1.90	100	15.8

[a] The incubation mixture contained 0.14 unit of glucosidase–transferase, 1.3 units of phosphorylase *a*, and the polysaccharides listed in 1 ml of 0.1 M Na phosphate buffer, pH 6.5. The digest was incubated at 30° for 2 hr. [b] Determined by hydrolysis with amyloglucosidase (Lee and Whelan, 1966), of a control sample containing only the polysaccharide. [c] Estimated with glucose oxidase. [d] Estimated in terms of glucose equivalent as follows: 0.1 ml of the incubation mixture was diluted to 1.0 ml with water; 0.2 ml of this diluted solution was mixed with 0.2 ml of 0.2 N HCl and heated at 100° for 5 min, then immediately cooled in ice. The solution was neutralized by the addition of 0.2 ml of 0.2 N NaOH and 0.4 ml of 0.5 M Na phosphate buffer, pH 7.0, and assayed for glucose. These conditions are such that complete hydrolysis of glucose 1-phosphate occurred with negligible breakdown of glycogen. [e] Calculated as total polysaccharide divided by free glucose. [f] Produced by the action of yeast glucosidase–transferase, as described under Table III.

of transfer. The yeast transferase differs from the rabbit muscle enzyme in that the latter shows a preference for a three-glucose unit transfer, and maltohexaose is the smallest oligosaccharide acting as a substrate when tested alone with the enzyme (Brown and Illingworth, 1964).

Combined Actions of Glucosidase–Transferase and Phosphorylase a in the Presence of Excess Inorganic Phosphate. Cori and Larner (1951) showed that the muscle debranching enzyme together with phosphorylase (in the presence of inorganic phosphate) would catalyze the complete degradation of glycogen into glucose and glucose 1-phosphate; the amount of glucose produced was directly proportional to the amount of end groups in the polysaccharide, *i.e.*, to the reciprocal of the average chain length of the glycogen. Since phosphorylase acts only upon the outer chains of glycogen, this finding further established the obligatory participation of the debranching enzyme in the complete utilization of glycogen by the phosphorolytic pathway.

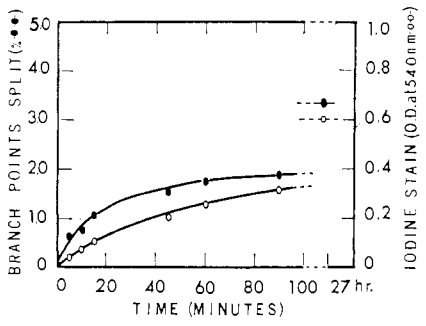

FIGURE 8: Action of yeast glucosidase–transferase on glycogen. The incubation mixture (4.0 ml, 30°) contained shellfish glycogen (20 mg), enzyme (0.6 unit), and 0.1 M Na citrate–phosphate buffer, pH 6.4. Glucose release and iodine stain were measured as described under Methods; the percentage of branch points split was calculated on the basis of a total end-group content of 10% for the shellfish glycogen.

Yeast glucosidase–transferase acting in concert with rabbit muscle phosphorylase *a*, also catalyzed the complete degradation of glycogen (Table II). The two polysaccharides used were shellfish glycogen and the limit dextrin derived from it by the action of yeast glucosidase–transferase. While no other independent method was used to determine the average chain lengths, it seems reasonable to assume that the values obtained are accurate and that the yeast glucosidase–transferase may be substituted for the rabbit muscle debranching enzyme for the purposes of end-group determination of glycogen.

Action of Glucosidase–Transferase on Glycogen. The action of the debranching enzyme system on glycogen may be followed in three ways. First, by the release of glucose: since the latter originates directly from the α-1,6-branch linkages, it is a quantitative measure of the number of branch points split. Second, by the increase in iodine stain, measured at 540 nm: iodine gives characteristic spectra with amylaceous polysaccharides, with λ_{max} ranging from 620 to 650 nm for amyloses, 520 to 580 nm for amylopectins, and 420 to 520 nm for glycogens (Manners, 1957; Whelan, 1958; Bailey and Whelan, 1961). The increase in iodine stain of glycogen by the glucosidase–transferase may be attributed to the effective increase in chain length brought about by the debranching process as will be shown later. Third, by the increase in the degree of degradation by exoenzymes such as β-amylase or phosphorylase.

Figure 8 shows that when yeast glucosidase–transferase acts on glycogen, release of glucose (*i.e.*, the splitting of branch points) occurs concomitantly with an increase in iodine stain. The shellfish glycogen used for the experiment has a very low (<0.05) absorbance over the range of 400–700 nm when stained with iodine. The action of the debranching enzyme ceased when 35% of the total branch points had been split, and the iodine stain reached an absorbance of 0.65 at 540 nm. At this point, the spectrum obtained was both qualitatively and quantitatively similar (λ_{max} 520 nm) to that displayed by amylopectin (Figure 9). It is noteworthy

TABLE III: Degrees of β-Amylolysis of Glycogen and of the Dextrin Formed by Exhaustive Action of Yeast Glucosidase–Transferase.

Polysaccharide	Degree of β-Amylolysis (%)	Average Chain Lengths[b] Total Polysaccharide	Outer Chains	Inner Chains
Glycogen	39	9.7	5.8	2.9
Glycogen dextrin[a]	64	15.8	12.1	2.7
Amylopectin	53	20	12.6	6.4

[a] Prepared as follows: shellfish glycogen (1 g) was dissolved in 50 ml containing 25 mM Na citrate–phosphate buffer, pH 6.4, 1 mM EDTA, and 6.0 units of glucosidase–transferase. The digest was dialyzed at 30° and under agitation in a stoppered erlenmeyer flask against several changes (500 ml every 24 hr) of the same buffer; toluene was added to the digest and to the dialysate to prevent bacterial growth. Both solutions were assayed for free glucose and enzyme activity. The process was continued until no free glucose could be detected. No further addition of enzyme was made since at the end of the incubation period (6 days) the digest still retained 45% of the original activity. After heating the digest for 5 min in boiling water, the polysaccharide was precipitated by addition of two volumes of ethanol, washed with ethanol then ether, then air dried, and finally dried in a vacuum oven at 30° over P_2O_5 (yield, about 0.8 g). [b] Average chain length values for the shellfish glycogen and its dextrin were determined by digestion with glucosidase–transferase and phosphorylase *a* in the presence of an excess of inorganic phosphate (see text). The average chain length value for amylopectin was that determined by Lee and Whelan (1966). Outer chain lengths were calculated from the degree of β-amylolysis as the number of glucose residues removed plus two; inner chain lengths were calculated as (average chain length − average outer chain length − 1).

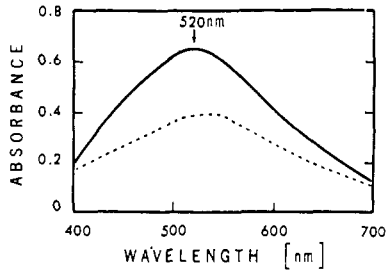

FIGURE 9: Iodine stain spectrum of glycogen after prolonged debranching by glucosidase–transferase; upper solid curve: a sample of glycogen (0.2 ml) taken from the experiment shown in Figure 8 after 25-hr incubation at 30° was mixed with 5 ml of iodine solution (0.2-mg/ml final concentration); lower, broken curve: iodine spectrum of native amylopectin at a concentration of 0.1 mg/ml.

that while pullulanase will also increase the iodine stain, the effects are much less marked (Lee *et al.*, 1967; Bathgate and Manners, 1968; also see Abdullah and Whelan, 1963; and Brown *et al.*, 1963). Of course, the effects of any debranching enzyme on the iodine-staining properties of polysaccharides can only be studied when the preparations are totally free of α-amylase activity.

It thus appears that the yeast debranching enzyme converts glycogen into a polysaccharide similar to amylopectin. Since it only removes the glucosyl residues involved in the α-1,6 branch points, the reorganization of the structure of the polysaccharide is not accompanied by any gross change in size. The nature of the change in terms of the average chain length may be calculated from the number of branch points that are split (*i.e.*, those residues that give rise to a free glucose unit on hydrolysis of the α-1,6 linkage). Thus for a glycogen with an average chain length of 10, 10% of the glucosyl residues are involved at branch points. A loss of 35% of these would leave the polysaccharide with 96.5% of its original molecular weight, and only 6.5% of glucosyl residues involved at branch points. The resultant polysaccharide would have an average chain length of 96.5/6.5 = 14.9 which represents a 50% increase over the original value.

These conclusions were confirmed by the preparation of a glycogen limit dextrin formed by exhaustive action of the debranching enzyme, and determination of the degree of β-amylolysis of this "debranched" material as compared to that of the original polysaccharide (Table III). The limit dextrin obtained had an average chain length of 15.8, which compares well with the value of 14.9 calculated above. Structural analysis showed that it had long outer chains, while the inner chains had lengths similar to that of the parent glycogen. These results support the hypothesis that the debranching enzyme acts primarily on the outer chain, *i.e.*, that only the outer tier of branch points are affected; no rearrangement of the inner structure occurs. It should be noted that the total contribution by weight of the outer chains is 77%. The dextrin produced also resembles amylopectin in its tendency to retrograde from solution: at concentrations of about 20 mg/ml, its solutions precipitated when frozen and thawed, but the precipitate redissolved readily upon gentle heating.

Comparative Actions of Yeast Glucosidase–Transferase on Glycogen, Glycogen Phosphorylase Limit Dextrin, and Amylopectin. The release of glucose from various polysaccharides as a function of time is shown in Figure 10. Relative initial rates (calculated from the 10-min values) decreased from shellfish glycogen phosphorylase limit dextrin (taken as 100), to shellfish glycogen (82), rabbit liver glycogen (72), and amylopectin (4.5). The release of glucose from the phosphorylase limit dextrin was linear with time and proportional to enzyme concentration thus making this polysaccharide the choice substrate for assay of the enzyme. The progressive decrease in rate with time when both glycogens were used as substrates had also been observed with the rabbit muscle enzyme (Brown *et al.*, 1966) and was attributed to an inhibition of the transferase as the outer chain length increases. This is reflected also in the difference in rate observed with shellfish glycogen (with a short average chain length of 9.7) and the rabbit liver glycogen (average chain length of 14, Lee and Whelan, 1966); the slowdown may be due either to an inherent specificity of the transferase

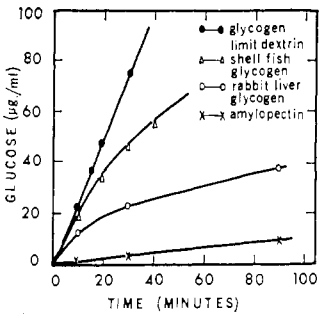

FIGURE 10: Action of yeast glucosidase–transferase (0.014 unit/ml) on shellfish glycogen (16 mg/ml), rabbit liver glycogen (16 mg/ml), shellfish glycogen phosphorylase limit dextrin (10 mg/ml), and amylopectin (8 mg/ml), at 30° in 0.1 M Na citrate–phosphate buffer, pH 6.4.

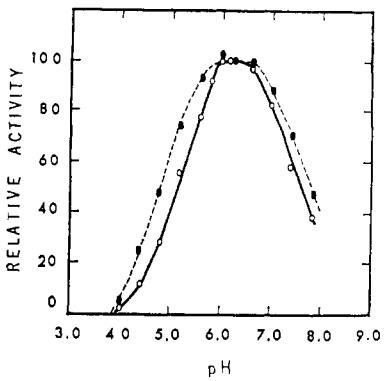

FIGURE 11: pH Dependence of yeast glucosidase–transferase: solid curve, with α-glucosyl Schardinger dextrin (2.5 mg) as substrate, and 0.07 unit of enzyme; broken curve, with glycogen phosphorylase limit dextrin (2 mg) as substrate and 0.035 unit of enzyme. Both digests (0.2 ml) contained 0.5 mg/ml of bovine serum albumin and were incubated at 30° for 30 min. Na citrate–phosphate buffer (50 mM) was used in the range of pH 4.0–6.0 and Na phosphate (50 mM) in the range of pH 6.0–7.8.

for shorter chains or to the likelihood that several transfers may be necessary before the glucose stub on a long chain is finally exposed. The relative rates of reaction on glycogens may then be a function of their average outer chain length; amylopectin also gives a linear (but slow) release of glucose as a function of time and enzyme concentration.

pH Dependence of Activity. The pH optimum of the enzyme displayed a maximum in the region pH 6.0–6.6 when both glycogen phosphorylase limit dextrin and α-glucosyl Schardinger dextrins were used as substrates (Figure 11). Since the Schardinger dextrin substrate reflects only the amylo-1,6-glucosidase activity while the limit dextrin reflects both glucosidase and transferase activities, it would appear that the two activities have the same pH dependence or, alternatively, that the transferase activity is not rate limiting. The above data contrast with similar studies on the rabbit muscle enzyme in which multiple pH optima have been reported (Nelson *et al.*, 1969; Taylor and Whelan, 1968).

Discussion

The purification procedure up to the stage of TEAE extraction was derived from the method of L. D. Nielsen, M. Fosset, and E. H. Fischer (unpublished results) for the preparation of yeast glycogen phosphorylase. It was during this preparation that the debranching enzyme was first observed as an impurity. An essential feature of the purification procedure is the use of phenylmethylsulfonyl fluoride to inactivate proteolytic enzymes which are responsible for the instability of the enzyme. It is known that baker's yeast contains several proteases or peptidases that are active over a broad pH range (5–9), which are inactivated by either phenylmethylsulfonyl fluoride or isopropylphosphorofluoridate, or both (Hata *et al.*, 1967; Lenney and Dalbec, 1967).

Although no specific assay for the "transferase" activity of the enzyme was performed, the evidence presented strongly suggests that the glucosidase and transferase activities implicated in the debranching process are associated with what appears to be a single protein species, as has been found in the rabbit muscle system (Brown and Illingworth Brown, 1966). On the other hand, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate according to the procedure of Shapiro *et al.* (1967) indicates that the protein dissociates into smaller subunits; whether or not separate chains might be responsible for the different activities of the enzyme will have to await further investigation. The activity peaks obtained during DEAE-cellulose and Sephadex G-200 chromatographies were symmetrical with no suggestion of separation of activities; identical elution profiles were obtained when the enzyme was assayed by the increase in iodine stain (a measurement of the overall debranching process) or by the release of glucose from a phosphorylase limit dextrin.

Studies on the mode of action of the yeast debranching enzyme on glycogen and related polysaccharides and its resulting effect on the structure of the polysaccharide show clearly that it is similar to the classical mammalian debranching enzyme. The identification and characterization of this enzyme in yeast, together with the known presence of a glycogen phosphorylase, provides the enzymic elements necessary for a phosphorolytic degradation of glycogen similar to that found in mammalian tissue. Evidence for the separate existence in yeast of another debranching enzyme related to the plant and microbial debranching enzymes, *viz.*, isoamylase, has been presented by Bathgate and Manners (1968); a hydrolytic pathway for the degradation of glycogen involving isoamylase, α-amylase, and maltase may therefore also be suggested. In the purification steps from the TEAE extraction onward, no evidence was obtained for the presence of an isoamylase. Whether one or both of these pathways are operative in yeast remains to be determined.

The specificity of the yeast transferase activity differs from that of the rabbit muscle enzyme in that two rather than three glucose units are preferentially transferred. This would suggest that the specificity of the two debranching systems (Brown and Illingworth, 1964) toward polysaccharide substrates might be different, particularly in the case of a β-limit dextrin; the latter should be as good a substrate as a phosphorylase-limit dextrin for the yeast enzyme.

It was recently observed that the yeast system will catalyze the incorporation of ^{14}C-labeled glucose into glycogen, as

reported for the rabbit muscle system (Hers *et al.*, 1967); a study of this reaction will be presented elsewhere.

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