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BIOSYNTHESIS OF PHYTOGLYCOGEN IN MAIZE ENDOSPERM

THE BRANCHING ENZYME*

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SUMMARY

1. An α-1,4-glucan:α-1,4-glucan 6-glycosyltransferase (EC 2.4.1.18) which synthesizes a phytoglycogen-like polymer from amylose has been isolated in a form free of Q-enzyme, amylases and phosphorylase from a su_1 genotype of maize. A Q-enzyme and an α-amylase were obtained separately in the same procedure.

2. The enzyme is most effective in producing a highly branched product from an unbranched substrate. The intermediate reaction products of the enzymatic branching of amylose appear to consist of a uniform population of partially branched molecules.

3. Kinetic data were obtained. The value of K_m for an amylose substrate at pH 7.6 was 0.2 mg/ml. pH dependence demonstrated an optimum pH of 7.4 and half-optimal values of 5.2 and 9.3 in either of two buffers.

4. The branching reaction is not reversible. It is inhibited by *p*-chloromercuribenzoate and $HgCl_2$, but not by oligosaccharides.

5. The possible role of the enzyme in starch metabolism is discussed.

INTRODUCTION

Phytoglycogen is known as an important reserve polysaccharide of the sugary-1 (su_1) or 'sweet corn' genotype of maize[1]. It has been shown, however, that phytoglycogen is not found in certain mutants containing the su_1 gene yet accumulates in certain other genotypes in which this gene is lacking[2]. These observations bear on the as yet uncertain relationship of phytoglycogen metabolism to starch synthesis. One view, suggested by ERLANDER[3], holds that phytoglycogen through a debranching mechanism is an intermediate in the synthesis of starch, in contrast to the possibility that the material may simply accumulate as a modified by-product of starch synthesis

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in certain genotypes. The synthetic route to phytoglycogen *in vivo* is therefore uncertain, although LAVINTMAN AND KRISMAN[4] have reported the presence of an enzyme in an unspecified strain of sweet corn which has the ability to convert amylose and amylopectin to phytoglycogen *in vitro* by a branching mechanism. LAVINTMAN[5] later described the characteristics of that enzyme, although the experiments appeared to be made in the presence of an interfering Q-type activity which catalyzes the branching of amylose to yield amylopectin as a final product.

A number of closely related and clearly defined maize genotypes are available in our laboratory which show major interactions in the metabolic pathways leading to the accumulation of phytoglycogen. We therefore began a study of the branching enzymes occurring in these mutants in an attempt to clarify their roles. As the initial step we obtained from our basic su_1 genotype a partially purified preparation of its phytoglycogen-forming branching enzyme, an α-1,4-glucan:α-1,4-glucan 6-glycosyltransferase (EC 2.4.1.18), free of contaminating Q-enzyme, amylase and phosphorylase activities. This report describes the isolation and properties of that enzyme, hereafter referred to as the 'branching enzyme'.

MATERIALS AND METHODS

Strains

The maize genotype grown for these studies as a potential source of 'branching enzyme' was recessive for the su_1 allele but otherwise had a background related to the dent hybrid W23/L317, designated normal. It accumulates phytoglycogen in large amounts.

Reagents

All reagents were of A.R. grade or as designated by the manufacturer. DEAE-cellulose for column chromatography was Whatman grade DE-11. Amylose, maltose, melibiose and raffinose were obtained from Calbiochem. Maltotriose was isolated from an acid hydrolysate of amylose by fractionation on a charcoal–celite column[6]. Potato amylopectin was prepared by the butanol fractionation of potato starch. Amylopectin from normal maize starch was prepared by the thymol fractionation procedure[7] and proved to be indistinguishable in these studies from dispersed starch of waxy maize, which contains negligible amylose. Phytoglycogen was obtained from an extract of su_1 endosperm by ethanol precipitation[8].

The I_2–KI reagent used in all kinetic studies was prepared by dissolving 2.6 g of KI and 0.26 g of I_2 in 10 ml of water. 0.5 ml of this solution was diluted to 130 ml of water just prior to use. The $CaCl_2$–I_2 reagent used in product analysis studies was that of KRISMAN[9].

Tissue fractionation

Ears of su_1 and normal (to serve as a control) corn were picked 20 days after hand pollination and immediately frozen in liquid N_2. They were kept frozen at $-25°$ until used. 10 g of frozen kernels of the desired genotype were freed of embryos and pericarp (to reduce amylase levels) and ground in a mortar and pestle with 10 ml of 0.6 M sucrose in 10 mM phosphate–1 mM EDTA buffer (pH 7.6). This buffer (*minus* sucrose) was routinely used in the experiments which follow. The homogenate was

filtered through cheese-cloth and centrifuged 15 min at 25 000 × *g*. An upper lipid layer was removed by filtration through muslin after which the solution was centrifuged at 100 000 × *g* for 2 h to sediment phytoglycogen. The supernatant was dialyzed overnight against buffer. All operations were at 4°.

Column chromatography

DEAE-cellulose column chromatography of the 'branching enzyme' was by a modification of the procedure of LAVINTMAN AND KRISMAN[4]. The entire volume of previously obtained dialysate (10–15 ml) was placed directly on a 10 cm × 1 cm column previously equilibrated with buffer at pH 7.6. The loaded column was washed at 4° with 35 ml of buffer and eluted with 350 ml of a concave NaCl concentration gradient, 0–1 M, buffered as above. Flow rates were 20 ml/h; 10-ml fractions were collected.

Protein determinations

Protein was estimated by the method of LOWRY *et al.*[10]. Absorbance at 280 mμ ($A^{1\,cm}_{280\,m\mu}$) obtained with an ISCO UV flow monitor served as an approximation to protein concentration in eluates from chromatography columns. The method of KALCKAR[11] was sometimes used after it was found to give the same results as the Lowry procedure.

Amylose-active fractions

The generalized activity of the column chromatographic fractions toward an amylose substrate was measured by incubating at 30°, 0.1 ml of each fraction, diluted to 0.5 ml with H_2O, with 1.0 ml of a dialyzed dispersion of amylose (0.4 mg/ml). A 0.2-ml portion was withdrawn after 5 min and added directly to 1.2 ml of the I_2–KI reagent. The loss per min of absorbance at 660 mμ was taken as the activity of the fraction, one unit of activity being defined as a decrease of one absorbance unit/min.

It was found convenient to routinely pre-locate the amylose-active fractions by incubating 0.1 ml of each fraction with 0.2 ml of amylose (0.2 mg/ml) for several h. The addition of 1.8 ml of the $CaCl_2$–I_2 reagent allowed the immediate location of such fractions by their deviation from the dark blue precipitate of amylose in the controls.

'Branching enzyme' characterization; standard assay system

Chromatographic fractions containing the 'branching enzyme' but excluding those containing Q-enzyme activity were pooled and brought to 60% of saturation with $(NH_4)_2SO_4$ by the addition of 1.5 vol. of a saturated solution preadjusted to pH 7.0. The resulting precipitate, containing the enzyme, was centrifuged, dissolved in 10 ml of buffer, dialyzed against buffer and stored at 4° or frozen. This preparation was used directly in the characterization experiments. The enzyme gradually lost activity when stored under these conditions.

Activity of the enzyme was routinely measured in duplicate in all of the characterization experiments by incubating (except where otherwise indicated) 5 μg of protein with 0.1 mg of amylose in a final volume of 1.5 ml of buffer at 30°. Samples of 0.2 ml were withdrawn at 1-min intervals and added to 1.2 ml of the I_2–KI reagent for measurement of ($A^{1\,cm}_{660\,m\mu}$) in a Beckman DU. The reagent immediately stopped the reaction.

Substrate concentrations

Amylose concentrations were determined as glucose by the method of NELSON[12] and correlated with the ($A^{1\,cm}_{660\,m\mu}$) readings obtained with the I_2–KI reagent. An absorbance of 1.0 corresponds to an approximate amylose concentration of 0.2 mg/ml in the 'branching enzyme' reaction mixture and is linearly related to amylose concentration within the ranges encountered in these experiments. The concentrations of other carbohydrates added to the reaction mixture were determined in advance by weight.

EXPERIMENTAL

Purification procedure

Elution of the DEAE-cellulose column and analysis of the fractions from su_1 for their ability to modify an amylose substrate according to the methods described earlier gave the distribution of protein and amylose-active enzymes shown in Fig. 1. NaCl concentrations were determined for the gradient using flame photometry and were completely reproducible. Attempts to find phosphorylase activity in the effluent fractions, using the procedure of CORI AND CORI[13] were negative.

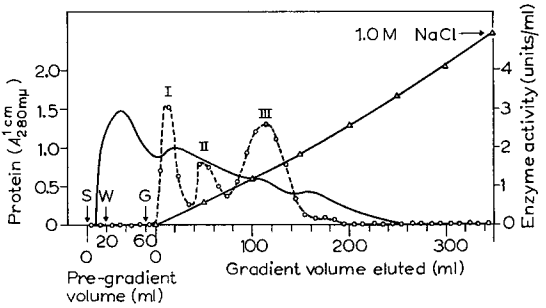


Fig. 1. Chromatography of amylose-active enzymes from the 100 000 × *g* supernatant of su_1 maize endosperm on a 10 cm × 1 cm DEAE-cellulose column as described in the text. Sample application began at S and continued until W. A buffer wash (pH 7.6) was applied until the 0–1 M NaCl gradient was begun at G. ———, protein concentrations as $A^{1\,cm}_{280\,m\mu}$; △—△, NaCl effluent molarity; ○...○, enzymatic activity of the fractions toward amylose (units/ml) were obtained as described.

3 peaks of activity (I, II and III, Fig. 1) were obtained from the su_1 extract, whereas only 2 (II and III based on the elution pattern) were obtained from normal. These were identified as such by product analysis as described in the next section. I, which was present in su_1 but missing in normal, proved to be the 'branching enzyme' sought in these studies; II is apparently a Q-enzyme, and III has α-amylase activity.

It was of considerable concern that the 'branching enzyme' was invariably eluted at concentrations of NaCl far lower than the 0.15–0.2 M previously reported for a similar enzyme[4] and the 0.2 M concentration (as part of a gradient) since described by LAVINTMAN[5]. Recent experiments have shown that both small changes in pH and the total amount of protein loaded on the column may greatly influence the retention of the enzyme reported here, although never to the extent of requiring greater than 0.05 M NaCl for its elution. Several experiments were performed in

which 4 times greater quantities of protein in twice the volume described in this procedure were applied to the same size column at pH 7.5. These showed that nearly 20 bed volumes of buffer wash were required to reduce the protein concentration of the effluent to zero. The 'branching enzyme' activity (I) was eluted in the wash under these conditions. Activities II and III as usual did not appear until the gradient was begun. The light loading conditions of LAVINTMAN[5] were duplicated in another experiment but still resulted in the elution of the 'branching enzyme' from our su_1 genotype at effluent NaCl concentrations of 0.05 M or less. In this instance the enzyme was not clearly separated from the Q-enzyme or α-amylase peaks and identification of separate enzymes was not possible. The use of a heavily loaded column with careful pH control appears to be of advantage in obtaining 'branching enzyme' free of interfering Q-enzyme and amylase activities from our organism.

Identification of amylose-active peaks

The most active fraction from each of I, II and III was selected for analysis and dialyzed against the standard buffer. They were then examined for 'branching enzyme', Q-enzyme and amylase activities by analyzing the products after 2 h and 18 h incubation of each enzyme's action on amylose, potato and maize amylopectin and phytoglycogen. β-Amylolysis limits, spectra with the $CaCl_2$–I_2 reagent, colors

TABLE I

EFFECTS OF ENZYME FRACTIONS I AND II FROM su_1 ENDOSPERM ON AMYLOSE AND POTATO AMYLOPECTIN

Protein concentrations of I and II from chromatography of su_1 extracts (Fig. 1) were determined and adjusted to 0.1 mg/ml. 1 ml was added to 5 mg of substrate in 1 ml of water. After incubating at 30° for 2 h and 18 h under toluene vapor, 2 vol. of methanol were added to the reaction mixture to precipitate the polysaccharides. The precipitate was centrifuged at 15 000 × *g*, washed twice with 70% methanol and redissolved for analysis in 0.5 ml of water. The supernatant from the first precipitation was analyzed by silica gel thin layer chromatography for traces of reducing sugars and oligosaccharides[14,15]. The β-amylolysis limits of the polymers were obtained in triplicate by incubating 0.1 mg of polysaccharide with 0.2 mg of sweet potato β-amylase (Worthington) in a final volume of 0.2 ml of 0.03 M citrate buffer, at pH 6, under toluene vapor at 37° for 18 h. The reducing power was measured after heating the mixture 10 min at 100° to stop the reaction[4]. The λ_{max} for the polysaccharide: $CaCl_2$–I_2 complex was determined on a Cary recording spectrophotometer and the apparent color was noted visually. Substrates *minus* enzyme were also carried through the procedure. The properties of maize amylopectin and phytoglycogen also carried through the procedure are included for comparison (see text). The first of the 2 paired figures separated by dashes in the β-limit and λ_{max} columns was obtained after 2 h incubation, the second after 18 h. In all other cases, including the substrates *minus* enzyme entries and entries in the Apparent Color column, values were identical for both the 2-h and 18-h incubations.

Substrate ± enzyme	*β-Amylolysis limit (%)*	*λ_{max}, $CaCl_2$–I_2 (mμ)*	*Apparent color $CaCl_2$–I_2*	*Reducing sugars or oligosaccharides*
Amylose	88	640	Blue	None
Amylose + enzyme I	40–39	470–465	Red–orange	None
Amylose + enzyme II	45–44	525–520	Purple	None
Potato amylopectin	46	530	Purple	None
Potato amylopectin + enzyme I	42–42	490–485	Red–purple	None
Potato amylopectin + enzyme II	46–45	530–520	Purple	None
Maize amylopectin	43	490	Red–purple	None
Maize phytoglycogen	38	460	Red–orange	None

observed with that reagent and production of reducing sugars and oligosaccharides were determined for each and compared with the starting materials. The effects of enzyme fractions I and II from su_1 on amylose and potato amylopectin are presented in Table I. These data indicate that I converts amylose to a phytoglycogen-like polymer, whereas II produces amylopectin similar to potato amylopectin from this same substrate. The properties of the 4 substrates used in these studies are included for comparison. The properties of amylopectin from normal (and waxy) maize and phytoglycogen from su_1 were not altered by these 2 enzymes, whereas the effect of III on all 4 substrates was pronounced. In all cases III caused the precipitable iodine-staining polymers to completely disappear after 2 h incubation, leaving instead large quantities of maltose, maltotriose and higher oligosaccharides in the supernatant fraction in a manner expected for an α-amylase.

II and III from the normal genotype duplicated the effects of II and III from su_1 except that II from normal had no effect on potato amylopectin. It is of interest for future Q-enzyme studies in maize that II in the su_1 fractions is almost certainly contaminated with small amounts of 'branching enzyme' because of the tailing observed during chromatography on cellulose columns, whereas II from normal should not have such contamination.

Enzymatic conversion of amylose to phytoglycogen

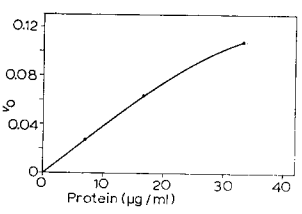
The 'branching enzyme' activity was pooled and concentrated as described under MATERIALS AND METHODS, and the course of the enzymatic conversion of amylose to phytoglycogen was observed in the standard characterization system. Fig. 2a indicates that the loss of ($A^{1\,cm}_{660\,m\mu}$) of the substrate: I_2–KI reagent complex

Fig. 2. Conversion of amylose to phytoglycogen by the 'branching enzyme'. Protein, 10 μg; amylose, 0.2 mg; buffer, 10 mM phosphate –1 mM EDTA, pH 7.6, 3.0 ml. Incubation in duplicate at 30° under toluene vapor. Sample volumes of 0.2 ml were withdrawn at the times indicated and added to 1.2 ml of I_2–KI reagent (a) or 1.2 ml of $CaCl_2$–I_2 reagent (b). ($A^{1\,cm}_{660\,m\mu}$) values were obtained on a Beckman DU, spectra on a Cary recording spectrophotometer. The spectrum of the $CaCl_2$–I_2 complex of an equal mixture by weight of amylose and phytoglycogen, ○— — —○, is also presented in (b).

is linear for approx. 25 min, or until a 40% decrease in absorbance has occurred, and nearly reaches completion in 90 min. No significant change was observed after 18 h and 36 h. A time sequence of absorption spectra of the $CaCl_2$–I_2: product complexes for the same system are presented in Fig. 2b for 30-min intervals. No changes were observed after 90 min. At no time do these spectra approximate the bimodal spectrum observed for an equal mixture of amylose and phytoglycogen. The reaction intermediates rather gave the spectra expected for polymers having structures intermediate between these two extremes.

Effect of enzyme concentration

Fig. 3 indicates that the dependence of reaction rate on enzyme concentration is nearly linear over wide ranges in concentration. The individual assays gave completely linear plots of ($A^{1\,cm}_{660\,m\mu}$) with time for up to 4 min incubation, although the systems containing higher protein levels showed some nonlinearity for the remaining two min of the assay. The presence of the high levels of protein in these preparations reduced the absorbance values of the amylose:I_2–KI complex somewhat (4% for the highest protein level, less than 1% for the lowest) but did not seem to interfere with the linear rates of decrease of absorbance observed with time. The effect was not seen in the unmodified standard assay in which lower protein concentrations (3.3 μg/ml) were used.



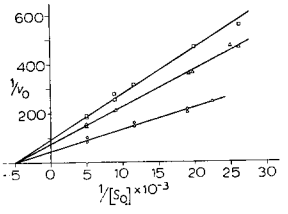


Fig. 3. Initial reaction rates as a function of protein concentration. The standard assay system was used except that protein concentration was varied as indicated, and 0.5 mg of amylose substrate was used in a final volume of 1.5 ml. At 1-min intervals 0.2-ml samples were withdrawn and added to 1.2 ml of I_2–KI reagent. ($A^{1\,cm}_{660\,m\mu}$) values were plotted and extrapolated to zero. The initial slopes were taken as a measure of reaction rates, v_0, expressed as loss of ($A^{1\,cm}_{660\,m\mu}$)/min.

Fig. 4. LINEWEAVER–BURK plot of the dependence of the reaction rates of the 'branching enzyme' on amylose substrate concentration. Initial reaction rates, v_0, are expressed as the decrease in ($A^{1\,cm}_{660\,m\mu}$)/min of the amylose: I_2–KI complex under the standard assay conditions described in the text. Initial substrate concentrations [S_0], are μg of amylose/ml in the reaction mixture; protein was 3.3 μg/ml. ○—○, freshly prepared enzyme; △—△, same preparation stored at −25°, 2 days; □—□, same preparation stored at −25°, 5 days.

Effect of substrate concentration and storage

The dependence of the initial velocity of the 'branching enzyme' reaction on substrate concentration was determined and presented as a LINEWEAVER–BURK[16] plot in Fig. 4. The K_m value for the enzyme-catalyzed branching of amylose is approx. 0.2 mg of amylose/ml or an effective concentration of $1.2 \cdot 10^{-3}$ M anhydroglucose residues in the unbranched amylose suspension under the conditions of the standard assay.

The stability of the preparation to storage at −25° for 2 days and 5 days is indicated by the change in maximal velocity resulting from these conditions. The data show a loss of approx. 50% of the original activity when the preparation was kept frozen for 5 days. Despite this loss of activity, the K_m value for the reaction remained unchanged. This would not have been expected if two enzymes having significantly different stabilities and K_m values were present in the preparation at similar levels of activity.

Later investigations showed that dithiothreitol (or GSH) at concentrations of 10^{-3}–10^{-4} M protect the enzyme against loss of activity through almost any cycle of storage conditions, dialysis, lyophilization or $(NH_4)_2SO_4$ precipitation studied. The protected enzyme was stable for reasonable periods at room temperature as well.

pH–activity curve

Fig. 5 indicates that the optimum pH of the enzyme is 7.4 with activity apparently dependent on ionizable groups having approximate pK values of 5.2 and 9.3, the pH values of half-optimal activity. These groups are presumed to be associated with the enzyme or enzyme–substrate complex, since it is unlikely that the free amylose is involved in an ionization reaction prior to its reaction with the enzyme. No cofactor requirement has been observed for this enzyme. An identical pH-dependent activity curve was obtained using the phosphate–EDTA buffer of the standard assay, although its buffering capacity was negligible in some pH regions.

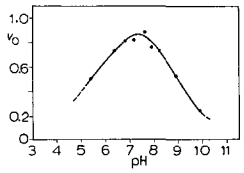


Fig. 5. pH–activity curve. Enzyme, 50 μg, dialyzed against water; amylose, 0.5 mg; buffer, 0.02 M diethyl barbiturate–0.1 M citrate–0.1 M phosphate preadjusted to the desired pH values, 0.2 ml; water to 1.7 ml. Incubation for 6 min at 30°. Samples of 0.2 ml withdrawn at 1-min intervals from the incubation mixture for addition to 1 ml of I_2–KI reagent. Reaction rates, v_0, expressed as decrease of $(A^{1\,cm}_{660\,m\mu})$/min. Absorbance measured on a Beckman DU.

Inhibition and reversibility of 'branching enzyme' activity

The activity of the enzyme in the conversion of amylose to phytoglycogen was not reduced by the presence of added maize amylopectin or phytoglycogen. The standard assay system was used except that the amylose concentration was 0.27 mg/ml (approx. 1.6 mM in anhydroglucose units). Amylopectin or phytoglycogen was added at the same concentration as the amylose substrate but no reduction in the reaction rates occurred, indicating the apparent absence of product inhibition. Incubation of the enzyme with phytoglycogen as substrate gave no observable increase in $(A^{1\,cm}_{660\,m\mu})$ values, suggesting that the branching reaction is effectively irreversible. These observations are consistent with unpublished substrate-binding studies for this enzyme which indicate that a strong affinity exists between amylose and the enzyme but that almost none is present between the enzyme and either su_1 phytoglycogen or the phytoglycogen produced as the product of the enzyme's action on amylose.

Maltotriose, maltose, melibiose and raffinose at a concentration of 3 mM were added individually to the same assay system as possible inhibitors but also without effect.

Inhibitors which were effective under the same conditions were *p*-chloromercuribenzoate (32% inhibition at 0.17 mM, 100% at 1.7 mM) and $HgCl_2$ (100% at 10 mM). The effectiveness of these reagents and the previously mentioned protection afforded the enzyme by sulfhydryl compounds during storage suggest the requirement for a free sulfhydryl group in the enzyme for retention of activity.

DISCUSSION

An understanding of the origin of branched starches requires a clarification of the metabolic roles of those enzymes which are capable of converting amylose to branched products. A comparative study in our laboratory of the properties of such enzymes present in the endosperms of a group of closely related maize genotypes has led to the isolation of a phytoglycogen-synthesizing enzyme free of Q-enzyme, amylases and phosphorylase from our reference su_1 'sweet corn' genotype. Q-enzyme activity was also observed in this phytoglycogen-accumulating mutant. The use of a heavily loaded DEAE-cellulose column and a concave NaCl gradient elution sequence results in appearance of the phytoglycogen-forming 'branching enzyme' at salt concentrations of less than 0.05 M. The normal genotype, lacking phytoglycogen, also lacks this branching enzyme.

A comparison of the bimodal $CaCl_2$–I_2 spectrum of a mixture of amylose and phytoglycogen, with the single peak seen at any one time for the intermediate products formed by the enzyme acting on an amylose substrate, suggests that the substrate is branched in such a manner as to yield a continuously changing and relatively uniform population of transitional products intermediate between amylose and phytoglycogen.

The 'branching enzyme' was most active toward an amylose substrate, which it converted to a phytoglycogen-like product, and less active toward potato amylopectin, which was incompletely branched further by the enzyme to a structure comparable to amylopectin isolated from normal or waxy maize. The possibility of the involvement of this one enzyme in the synthesis of both phytoglycogen and amylopectin *in vivo* cannot be dismissed. The enzyme appears unable to react extensively with potato amylopectin (β-limit, 46%) and not at all with maize amylopectin (β-limit, 43%) suggesting the need for an unbranched substrate as a starting material to permit the introduction of the maximum number of branches in the product.

Lineweaver–Burk treatment of the dependence of enzyme activity on substrate concentration suggested a value of 0.2 mg/ml for K_m at pH 7.6 based on the standard assay procedure. The pH optimum was estimated to be 7.4 with the activity falling off rapidly to half-optimum at pH 5.2 and 9.3. Maize amylopectin, phytoglycogen and certain oligosaccharides do not inhibit the branching activity of the enzyme toward amylose. The reaction appears not to proceed in the reverse direction. Inhibition by *p*-chloromercuribenzoate and $HgCl_2$ suggest the requirement for activity of a free sulfhydryl group on the enzyme.

Attempts to obtain the enzyme in pure form and to compare its properties with similar enzymes isolated from related genotypes are in progress.

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