Corn and Potato \( \alpha-1,4 \)-Glucan: \( \alpha-1,4 \)-Glucan 6-Glycosyltransferase: Evidence for Separate Hydrolytic and Branching Components*

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**Abstract:** The hydrolytic and branching activities of branching enzyme preparations separated on DEAE-cellulose result from the functions of two separate enzymes. Two fractions can be separated from the DEAE-cellulose preparations by zonal ultracentrifugation or by membrane ultrafiltration. A high molecular weight fraction \((M = 70,000)\) hydrolyzes amylose. The nature of this enzyme depends on its source. That from potato branching enzyme, like \( \beta \)-amylase, stops near branch points. That from dent and waxy corn, like \( \alpha \)-amylase, can bypass branch points. A lower molecular weight fraction \((M \approx 20,000)\) introduces barriers to \( \beta \)-amylolysis in amylose without apparent hydrolysis. Composites of the two enzyme fractions will reproduce the effect of the original DEAE-cellulose branching enzymes on the structure of amylose.

The branching enzyme \((\alpha-1,4\text{-glucan:}\alpha-1,4\text{-glucan 6-glycosyltransferase}, \text{EC 2.4.1.18})\) belongs to a group of enzymes involved in the formation of storage polyglucans. This group of enzymes "branches" linear maltodextrins. Amylose, the exclusively \( \alpha-1,4 \)-glucosyl-linked polymer, is converted to amylpectin, an \( \alpha-1,6 \)-glucosyl-branched polymer, by the branching enzyme of the potato (Peat et al., 1953; Barker et al., 1950). The branching enzyme consists of an \( \alpha-1,4 \)-glucosidic hydrolytic activity and an \( \alpha-1,6 \)-glucosidic branching activity which either consecutively (Manners, 1962) or independently (Bourne and Peat, 1945; Barker et al., 1950; Geddes and Greenwood, 1969) accomplish this conversion. Our work has supported the independent conversion.

We examined the physical nature of the associated hydrolytic and branching activities of potato and corn DEAE-cellulose branching enzyme preparations (Griffin and Wu, 1968), that is, whether they are separate functions of one two-headed enzyme or separate enzymes of a functional complex. To accomplish this study, branching enzyme preparations have been isolated from immature potato, dent, and waxy corn by the DEAE-cellulose method of Griffin and Wu (1968). The molecular weight, enzymic action on amylose, and chromatographic behavior of the corn preparations are compared with each other and with those of the DEAE-cellulose potato branching enzyme. Finally, the DEAE-cellulose branching enzymes were fractionated by zonal ultracentrifugation in a linear sucrose gradient and by Diaflo\(^1\) ultrafiltration. We isolated two distinct components. Their effect on the structure of amylose is described and compared to that of the original DEAE-cellulose branching enzyme preparations.

**Experimental Section**

Isolation of Corn Juice Solubles. At 21 days after hand pollination kernels of hybrid waxy and ordinary dent corn (grown by Bear Hybrid Corn Co., Inc.) were cut from cobs immediately after snapping and husking the ear. The kernels were ground to a pulp in a hand corn mill. Cellular debris was removed from the juice by filtering it through a nylon bolting cloth. Starch was removed by centrifuging in an International table top centrifuge. The clarified juice was immediately frozen in a Dry Ice-acetone mixture and stored in Dry Ice while being transported to in-house lyophilization equipment. There the clarified juice was lyophilized at less than 0.1 mm Hg. The lyophilized juice was stored at \(-18^\circ\) for subsequent column chromatography. About 1.0-g portions of the stored corn juice solubles were suspended in 10 ml of aqueous 0.20

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\(^1\) Mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over other firms or similar products not mentioned.
m sodium citrate buffer at pH 7.0 containing 0.1 mg/ml of Cleland's (1964) reagent. After 2-hr gentle agitation at 4° on a rotary mixer (Scientific Instrument Co.) and centrifugation in a S-1 head of a Sorval supercentrifuge to remove insolubles, the sample was applied to and eluted from a (1.9 × 150 cm) column of Sephadex G-25 equilibrated with 0.01 m sodium citrate buffer (pH 7.0) (0.1 mg/ml of Cleland's reagent). That portion of the sample excluded from the Sephadex column was immediately applied to a (1.9 × 40 cm) DEAE-cellulose column equilibrated with the same buffer. The DEAE column was then eluted at 120 ml/hr with one column volume of the equilibrating buffer and then a linear gradient to 0.05 m sodium citrate buffer (pH 7.0) (0.01 mg/ml of Cleland's reagent) through a Beckman Spectrochrom 130 column chromatography system (Bernier and Putman, 1963).

Ultrafiltration. Rapid concentration of protein solutions or the selective removal of microsolutes can be accomplished by ultrafiltration through Diaflo membranes. The general procedures for this technique have been described by Blatt et al. (1965). In our experiments, up to 50 ml of the enzyme preparation was pipetted into an Amicon Ultrafil, Model 50, cell equipped with a UM-1 Diaflo membrane. The UM-1 membrane selectively retains globular proteins above M = 20,000. After loading the cell, 50 psi of argon gas was applied and the charge reduced to 5 ml. This step required about 45 min at 25°. The concentrate was washed by adding more solvent and reconstituting to 5 ml. After repeating this washing operation as often as necessary to obtain a protein-free filtrate, the concentrate was washed quantitatively into a volume greater than 10 ml and made up to 50 ml.

Dextran Density Gradient Zonal Ultracentrifugation. On 4.66 ml of a 5-20% linear sucrose gradient was layered 0.25 ml of the DEAE-cellulose preparation (Martin and Ames, 1961). The tubes were centrifuged 17.5 hr at 30,000 rpm in a swinging-bucket rotor (SW65K) of a Spinco Model L2-65 preparative ultracentrifuge at 15°. After centrifugation the contents of the tubes were fractionated and analyzed for protein (absorbancy at 254 nm) on an Isco density gradient fractionator (Brakke, 1963). When appropriate, the 18-20 fractions (0.250 ml each) were assayed for branching activity.

Molecular Weight Determination. Modifications of the techniques of Siegel and Monty (1966) and of Martin and Ames (1961), adapting the enzymic activity to detect and measure the location of an enzyme in gel filtration and density gradient ultracentrifugation (Griffin and Wu, 1968), were used to determine the molecular parameters of eq 1, where

\[ M = 6\pi a s / (1 - \eta \rho) \]

M is the molecular weight; s, the sedimentation coefficient; \( \eta \), the partial specific volume; a, the viscosity of the medium; \( \rho \), the density of the medium; and \( s \), the Stoke's radius of the enzyme. The sedimentation coefficient was determined by zonal ultracentrifugation in a 5-20% sucrose gradient (Martin and Ames, 1961). The apparent value of the partial specific volume of the enzyme was determined from its buoyant or flotation density in cesium chloride by isodensity zonal ultracentrifugation. Its Stoke's radius was determined by Ackers' (1964) method from the Gelotte parameter, \( K_a \), determined from gel filtration data.

Standard Branching Enzyme Digests. Portions (≤3 ml) of branching enzyme solutions or its fractions were added to a 10-ml volumetric flask containing 5 ml of 0.20% potato amyllose in 0.50 % KCl, pH 4.2, and 2 ml of 0.40 m sodium citrate buffer, pH 7.0. These mixtures were made up to 10 ml and then digested at the enzyme's optimum temperature in a water bath. At appropriate intervals 1 ml of the solution was withdrawn and used to determine iodine affinity, reducing power, and \( \beta \)-amylolysis limit of the products in these digests. For assay of branching enzyme activity in zonal ultracentrifugation this standard digest has been modified. That is, 0.250-ml ultracentrifuge fractions are combined with 0.50 ml of the amyllose solution and 0.200 ml of the citrate and digested at the optimum temperature of the enzyme for 30 min.

Iodine Affinity. One milliliter of the branching enzyme digests containing 1 mg of polysaccharide was pipetted into a 100-ml volumetric flask containing 5 ml of 0.004 % KIO3 and 5 ml of 0.016 % KI acidified with 1 ml 0.5 % HCl, and made up to volume with distilled water. This last step produces I\(^{-}\) ion, which complexes with the polysaccharide. The absorbancy of this mixture in a 5-cm cell was recorded over the wavelength range 400-700 nm against a reagent blank using a Cary 14 recording spectrophotometer. Two parameters are obtained from these data to characterize the products of our branching enzyme digests. First, the absorbancy at 655 nm of the standard digest is used to measure enzymic activity (Igaue, 1963). One unit of enzyme activity is that amount of enzyme which will reduce the absorbancy of the iodine-amylose complex at 655 nm 1% /min in the standard reaction mixture. Secondly, the \( E_{\text{cm}}^\alpha \), calculated at the wavelength of maximum absorption can be used to calculate an apparent amylose content of the product by eq 2. Equation 2 was developed by E. H. Melvin (unpublished data) to determine analytically the amylose content of unmodified cereal starches from spectroscopic data obtained by the method described here. Since the amylose component of our products may not be of the same size or structure as in cereal starches, the percentages of amylose that we have calculated for our samples are apparent values.

\[ \% \text{amylose} = 0.2955 E_{\text{cm}}^\alpha - 7.74 \]  

(2)

Melvin's results indicate that α-amylose digests contain 1.8% amylose, and β-amylase digests contain 1.3% amylose. The percentage conversion to maltose of the polysaccharide was determined by the copper reducing power method. Amylose and amylopectin were converted to their respective \( \beta \)-amylolysis limits in less than 2 hr at 25°. No further action could be noted after extended digestion times.

Reducing Powers. The increase in reducing ends produced in both branching enzyme digests and \( \beta \)-amylose digests of the products of the same digest was determined by the copper-reduction method of Brown modified by Dygert et al. (1965). Duplicate samples containing 100 µg/maltose equivalent were analyzed before and after \( \beta \) amylolysis. The results are calculated as apparent percentage conversion to maltose.

Results

DEAE-cellulose Branching Enzymes of Corn and Potato Not Identical. Branching enzyme activity (Manners, 1962) can be isolated by the DEAE-cellulose method (Griffin and Wu, 1968) from immature potato, dent corn, and waxy corn. The activity always converts amylose to an amylopectin-like product with respect to its iodine affinity and \( \beta \)-amylolysis conversion (Manners, 1962). Depending on the plant source, however, the activity is eluted at different positions from a DEAE-cellulose column equilibrated with 0.01 m sodium citrate buffer, pH 7.0. Potato branching enzyme activity is adsorbed to the column and eluted in several peaks between
Table I: Physical and Enzymic Properties of DEAE-Cellulose Branching Enzymes.

<table>
<thead>
<tr>
<th>Branching Enzyme</th>
<th>Mol Wt ( \times 10^{-3} )</th>
<th>Optimum Temp (°C)</th>
<th>High Enzyme Concentration</th>
<th>Low Enzyme Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dent</td>
<td>70</td>
<td>37</td>
<td>0 - 50</td>
<td>0 - 55</td>
</tr>
<tr>
<td>Waxy</td>
<td>68</td>
<td>37</td>
<td>0 - 42</td>
<td>0 - 55</td>
</tr>
<tr>
<td>Potato</td>
<td>72</td>
<td>23</td>
<td>40-10 - 40-55</td>
<td>85-50 - 30-60</td>
</tr>
</tbody>
</table>

* The branching enzyme preparations were prepared by the DEAE-cellulose method of Griffin and Wu (1968). * Molecular weights were determined by the techniques of Siegel and Monty (1966) and Martin and Ames (1961) using gel filtration and density gradient ultracentrifugation (see Experimental Section). * Temperature at which enzyme causes greatest iodine affinity loss of amylase digested with each branching enzyme in pH 7.0, sodium citrate buffer. * Iodine affinity is reported as percentage apparent amylose (Griffin and Wu, 1968), \( \beta \) limit (experimental), and reducing power (see Experimental Section) of the product formed after 72 hr in digests containing high (0.1 unit/mg of amylose) and low enzyme concentrations (0.001 unit/mg of amylose). Both \( \beta \) limit and the reducing power are reported as apparent conversion to maltose.

0.03 and 0.04 m sodium citrate buffer, pH 7.0 (Griffin and Wu, 1968). Both dent and waxy corn branching enzyme activities are eluted in a single peak with the equilibrating buffer. That is, they are not adsorbed to the column. This behavior most likely reflects differences in the protein components of the two preparations.

Examination of the physical and enzymic properties of dent corn, waxy corn, and potato DEAE-cellulose branching enzyme preparations show several basic differences between the corn and potato preparations (Table I). In each preparation a protein (\( M = 70,000 \)) is isolated that will convert amylase to branched product. However, with respect to the remaining properties of Table I, the DEAE-cellulose branching enzyme preparations of dent and waxy corn are similar, but both differ from that of the potato.

The action of corn and potato DEAE-cellulose branching enzymes on the structure of amylase also differs. DEAE-cellulose potato branching enzyme never completely converts amylase to an amyllopectin-like product, *i.e.*, on the basis of its iodine affinity reported as percentage apparent amylose (Table I). However, the product's \( \beta \)-amylolysis limit is always equal to or less than that of amyllopectin, 55%. The iodine affinity and \( \beta \)-amylolysis limit of the stable product vary with enzyme concentration. Both dent and waxy corn preparations completely convert amylase to branched oligoglucan. Their products at all enzyme concentrations contain no apparent amylose and show the amyllopectin \( \beta \)-amylolysis limit.

**Sedimentation Components.** In Figure 1 are reproduced sedimentation diagrams of potato and dent DEAE branching enzyme in a 5-20% sucrose gradient after being centrifuged 17.5 hr at 50,000 rpm in the swinging-bucket rotor of a Spinco L2-65 preparative ultracentrifuge. The contents of the centrifuge tubes were fractionated and analyzed for branching enzyme activity by the method described in the Experimental Section. Vertical bars mark the position in the tubes of marker proteins sedimenting at 5 S and 2 S in water at 25°. The preparations consist of a protein-like material (absorbancy 254 m\( \mu \)) sedimenting near 2 S; almost all the conventionally assayed branching enzyme activity sedimented near 5 S. As shown in Table II, essentially 90% of the activity is under the single peak around 5 S. Two tubes on either side of the tube containing the marker were combined as fractions designated by the sedimentation coefficient of the marker. The 5S fraction contains most of the protein-like material in addition to a small portion of the original branching enzyme preparation's activity.
The activity in each of the fractions (2S and 5S) will include the branching and/or the hydrolytic activity of their parent branching enzyme preparation. The branching enzyme assay measures the iodine-affinity decrease of amylose which can be the result of branching (Archibald et al., 1961) or hydrolysis (Bailey and Whelan, 1961) of the amylose molecules. Thus more specific analyses are required to determine if there has been a separation of branching and hydrolytic activities in our density gradient fractions.

Only 5S Component Contains Hydrolytic Activity. The reducing power and β-amylolysis limit of the product obtained by digesting amylose and amylopectin with the SS component are compared in Table III to those of the products obtained with the pertinent whole DEAE-cellulose branching enzyme. Whenever the SS component alone is involved, the reducing power of the product is considerably greater than that of the whole DEAE-cellulose preparations. The β-amylolysis results, particularly those with the products obtained from amylose, indicate that unlike the whole preparation, the SS component introduces no barriers to β-amylolysis. That is, the β-amylolysis limit of the product is 100% rather than 55-60% expected of amylopectin. Thus the SS component alone exhibits only a capacity to hydrolyze linear amylose and no branching capacity.

The action of the SS component on amylopectin, which contains α-1,6-branch points, produces two sets of data depending on the plant source of the component. That of the potato converts only 5% of the amylopectin to apparent maltose. The β-amylolysis limit of the product is not significantly greater than that of untreated amylopectin or amylopectin digested with whole DEAE-cellulose potato branching enzyme. Consequently, most of the reducing power increase here must be a measure of the bonds broken in the external branches of the amylopectin. This action is analogous to that of β-amylase. α-Amylase would bypass the branch points and hydrolyze bonds in the interior chains of amylopectin, which action makes more of the molecule available for β-amylase conversion. This action occurs with the SS component isolated from corn. That is, bonds broken in the amylopectin (25% as apparent maltose) increase the β-amylolysis limit of the product considerably.

The evidence presented here indicates that the SS component is only a hydrolytic enzyme. The nature of the enzyme varies with its source. That is, the potato SS component resembles β-amylase and the corn SS component resembles α-amylase on the basis of their respective actions with amylose and amylopectin.

2S Component Contains Branching Factor. Most of the branching enzyme activity isolated by density gradient ultracentrifugation apparently loses its ability to branch, that is, to introduce barriers to β-amylolysis in amylose. Some insight into the fate of the branching activity can be gained from Table IV. Here the action of a composite of the SS and 2S components of dent corn DEAE-cellulose branching enzyme on the structure of amylose is compared with that of the SS and 2S fractions individually. The SS component alone converts amylose to a product with essentially the same iodine affinity as the Table IV: Effect of 5S and 2S Fractions of Dent Corn DEAE-Cellulose Branching Enzyme on the Structure of Amylose.

<table>
<thead>
<tr>
<th>Sedimenting Fraction</th>
<th>Iodine Affinity (%)</th>
<th>β-Amylolysis Limit (%)</th>
</tr>
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<tbody>
<tr>
<td>5 S</td>
<td>28.0</td>
<td>92.0</td>
</tr>
<tr>
<td>2 S</td>
<td>85.0</td>
<td>89.0</td>
</tr>
<tr>
<td>Composite</td>
<td>27.0</td>
<td>58.0</td>
</tr>
<tr>
<td>None</td>
<td>100.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>

a Isolation of the 5S and 2S fractions is described in Figure 1 and Table II. b Product formed from digest of 3 ml of the pertinent fraction for 72 hr in a standard digest with amylose (see Experimental Section). c Equal volumes of the 5S and 2S fractions of dent corn DEAE-cellulose branching enzyme.
digested with the same whole branching enzyme and that progressively increase with the filtrate concentration, a fraction of the product remains unbranched in each digest.

Discussion

The hydrolytic and branching activities of DEAE-cellulose branching enzyme preparations are functions of separable enzymes. The nature of the hydrolytic component varies with plant source, but its molecular size ($M = 70,000$) remains constant. More important, the decrease in iodine affinity of amylose by the action of this component accounts for nearly all the branching enzyme activity of each DEAE-cellulose preparation. Branch points are introduced in amylose by a lower molecular weight component ($M \approx 20,000$) without greatly reducing its iodine affinity—at most 10%. Since not all molecules of an amylose sample are branched, the action of both components is necessary to convert amylose completely to amylpectin. These results question the method used to assay the branching enzyme, the branching enzyme’s involvement in starch synthesis, and specificities of the branching enzyme.

The branching enzyme assay depends on the initial rate of decrease of the iodine affinity of amylose. According to our data, almost 90% of this assay for our DEAE preparations is a measure of hydrolysis rather than branching. Thus the assay may not measure the branching potential of a preparation. Moreover, when modified to detect branching enzyme, the assay could even miss significant amounts of branching component not associated with a hydrolytic component. These potential errors could explain the ambiguous results sometimes obtained when branching enzyme assays are correlated with the structure and proportion of amylpectin in natural starches (Badenhuizen, 1959). More definite results might be expected if the analytical work were based on a rapid assay directly related to the formation of α-1,6-branched points.

The dependence of the branching enzyme assay primarily on the hydrolytic component of the DEAE-cellulose preparations causes some practical problems. Most of the protein associated with the preparations has a lower molecular weight ($M = 20,000$). Estimates of the enzyme’s molecular size (Griffin and Wu, 1968) are based on detection of the enzyme’s movement in a centrifugal field and elution from gel filtration columns by the branching enzyme assay. Thus the value obtained ($M \approx 70,000$) is that of the hydrolytic component. If the molecular weight estimates had been based exclusively on a protein assay, the molecular size would have approached ($M = 20,000$) that of the branching component.

Finally, this work generally supports the observations (Geddes and Greenwood, 1969; Bourne and Peat, 1945) that amylose as such is not a substrate of the branching enzyme. Rather, the enzyme requires specific low molecular weight maltodextrins. The branching component isolated here introduces barriers to β-amyolysis or branches in only a portion of the amylose sample. Significant hydrolysis of the amylose must occur before all the sample is converted to a branched product, implying that some of the linear chains in the amylose sample are not proper substrates for the enzyme because of their large size. Conversely, in a system actively synthesizing starch, some linear maltodextrins could escape branching because of these same specificities and ultimately form amylose.

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