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Corn and Potato α-1,4-Glucan:α-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 β-amylase, stops near branch points. That from dent and waxy corn, like α-amylase, can bypass branch points. A lower molecular weight fraction ($M \approx$ 20,000) introduces barriers to β-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 (α-1,4-glucan:α-1,4-glucan 6-glucosyltransferase, 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 α-1,4-glucosyl-linked polymer, is converted to amylopectin, an α-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 α-1,4-glucosidic hydrolytic activity and an α-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° 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) (0.1 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 reconcentrating 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.

Sucrose 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 50,000 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 mμ) 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

$$M = 6\pi\eta as/(1 - \bar{V}\rho) \quad (1)$$

M is the molecular weight; s, the sedimentation coefficient; $\bar{V}$, the partial specific volume; η, the viscosity of the medium; ρ, the density of the medium; and a, 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_d, 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 amylose in 0.50 N 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 β-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 amylose 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 N KIO_3 and 5 ml of 0.016 N KI acidified with 1 ml 0.5 N HCl, and made up to volume with distilled water. This last step produces I_3^- ion, which complexes with the polysaccharide. The absorbancy of this mixture in a 5-cm cell was recorded over the wavelength range 400–700 mμ 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 mμ 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 mμ 1%/min in the standard reaction mixture. Secondly, the $E_{1\,cm}^{1\%}$ 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.

$$\% \text{ amylose} = 0.2955E_{1\,cm}^{1\%} - 7.74 \quad (2)$$

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.

β Amylolysis. To 1 ml of an aqueous solution of crystalline Mann-assayed sweet-potato β-amylase containing 5.8 units/ml of activity, 1 ml of the branching enzyme digests was added. The percentage conversion to maltose of the polysaccharide was determined by a copper reducing power method. Amylose and amylopectin were converted to their respective β-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 β-amylase digests of the products of the same digests 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 β 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 β-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.

Branching Enzyme[a]	Mol Wt[b] × 10^{-3}	Optimum Temp[c] (°C)	Product with Amylose[d]			
			High Enzyme Concentration		Low Enzyme Concentration	
			% Amylose	β Limit	% Amylose	β Limit
Dent	70	37	0	50	0	55
Waxy	68	37	0	42	0	55
Potato	72	23	40–10	40–55	85–50	30–60

[a] The branching enzyme preparations were prepared by the DEAE-cellulose method of Griffin and Wu (1968). [b] 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). [c] Temperature at which enzyme causes greatest iodine affinity loss of amylose digested with each branching enzyme in pH 7.0, sodium citrate buffer. [d] Iodine affinity is reported as percentage apparent amylose (Griffin and Wu, 1968), β 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 β 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 amylose 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.

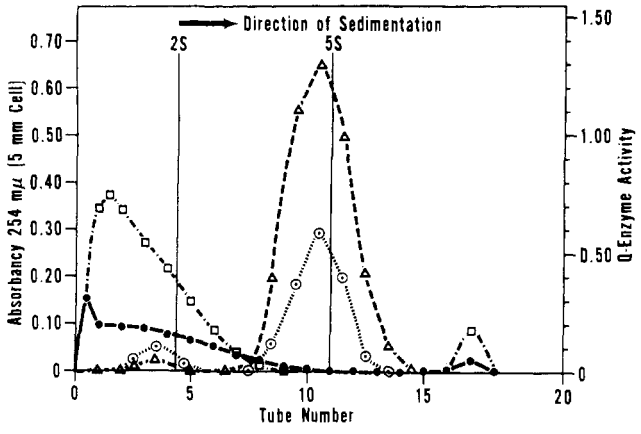

FIGURE 1: Zonal ultracentrifugation of potato and dent corn DEAE branching enzyme preparations in a sucrose gradient. A 0.250-ml sample of each preparation was layered on 4.66 ml of a 5–20% sucrose gradient buffered with 0.05 M citrate buffer, pH 7.0. After the tubes had been centrifuged 17.5 hr at 50,000 rpm and 15°, they were fractionated and analyzed. Each 0.250-ml fraction was assayed for branching enzyme (see Experimental Section). Activity (Δ—Δ, potato; ⊙····⊙, dent corn) is plotted *vs.* tube number. The absorbancy at 254 mμ of each fraction (●—●, potato; □···□, dent corn) is plotted on the same scale. Vertical bars designate the tubes in which peak concentrations of the 5S and 2S marker proteins are found.

The temperatures of optimum activity (Table I, column 2) clearly differentiate the corn and potato preparations. Optimum for the potato branching enzyme preparation is 23°. Barker *et al.* (1949) have reported 21° as optimum for potato branching enzyme activity, which is low compared to that of other plant carbohydrate-metabolizing enzymes. Branching enzyme preparations from broad beans (Barker *et al.*, 1949), green beans (Hobson *et al.*, 1950), and yeast (Gunja *et al.*, 1960) have similar values in the range 20–25°. Branching enzyme preparations from cereal grains, however, show optima nearer 35° (Manners *et al.*, 1968; Igaue, 1963). Likewise, optimum temperature of activity is 37° for our dent and waxy corn preparations.

The action of corn and potato DEAE-cellulose branching enzymes on the structure of amylose also differs. DEAE-cellulose potato branching enzyme never completely converts amylose to an amylopectin-like product, *i.e.*, on the basis of its iodine affinity reported as percentage apparent amylose (Table I). However, the product's β-amylolysis limit is always equal to or less than that of amylopectin, 55%. The iodine affinity and β-amylolysis limit of the stable product vary with enzyme concentration. Both dent and waxy corn preparations completely convert amylose to branched oligoglucan. Their products at all enzyme concentrations contain no apparent amylose and show the amylopectin β-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 mμ) 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 2S fraction contains most of the protein-like material in addition to a small portion of the original branching enzyme preparation's activity.

TABLE II: Yield of Branching Enzyme Activity in 5S and 2S Components of DEAE-Cellulose Branching Enzyme.

DEAE Branching Enzyme[a]		Zonal Ultracentrifugation Components				
		2 S		5 S		Total
Source	Activity (Units)[b]	Units	%[c]	Units	%	Yield (%)
Dent	1.60	0.17	10	1.41	88	98
Waxy	2.00	0.20	10	1.70	85	95
Potato	3.00	0.10	3	2.80	93	96

[a] From each enzyme solution 0.25 ml containing the indicated branching enzyme activity was fractionated by zonal ultracentrifugation (see Experimental Section) into components sedimenting at 5 S and 2 S. All activities are estimated from the absorbancy decrease of the I_3^- complex at 655 mμ of amylose digested 30 min with the branching enzyme or its fractions in a modified standard digest (see Experimental Section). [b] All units are expressed as the absorbancy decrease per minute. [c] All yields are expressed as percentages of the activity in the whole enzyme sample.

TABLE III: Effect of the 5S Density Gradient Fraction[a] of the DEAE-Cellulose Branching Enzyme on the Structure of Amylose and Amylopectin.

	Polysaccharide[b]			
	Amylose		Amylopectin	
Source	RP	β-Amylolysis Limit	RP	β-Amylolysis Limit
No enzyme	0	98.0	0	56.0
Potato	50 (2)	102.0 (58.0)	5 (2)	57.8 (56.4)
Dent	31.0 (9)	92.0 (51)	25 (8)	87 (51)

[a] Isolation of the 5S fraction is described in the Experimental Section and Figure 1. [b] Reducing power (RP) and β limit are reported as apparent conversion to maltose (see Experimental Section). Values in parentheses were obtained with whole DEAE-cellulose branching enzyme source of the 5S fraction.

The activity in each of the fractions (2 S and 5 S) 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 5S component are compared in Table III to those of the products obtained with the pertinent whole DEAE-cellulose branching enzyme. Whenever the 5S component alone is involved, the reducing power of the product is considerably greater than with the whole DEAE-cellulose preparations. The β-amylolysis results, particularly those with the products obtained from amylose, indicate that unlike the whole preparation the 5S 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 5S component alone exhibits only a capacity to hydrolyze linear amylose and no branching capacity.

The action of the 5S 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 amylolysis. 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 5S 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 5S component is only a hydrolytic enzyme. The nature of the enzyme varies with its source. That is, the potato 5S component resembles β-amylase and the corn 5S 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 2S and 5S components of dent corn DEAE branching enzyme on the structure of amylose is compared with that of the 5S and 2S fractions individually. The 5S 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.

	Product[b]	
Sedimenting Fraction[a]	Iodine Affinity (% Apparent Amylose)	β-Amylolysis Limit (% Apparent Maltose)
5 S	28.0	92.0
2 S	85.0	89.0
Composite[c]	27.0	58.0
None	100.0	100.0

[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.

TABLE V: Effect of Filtrate Concentration on the Structure of the Product from Amylose.

Volume[a] UM-1 Filtrate (ml)	Product[b]		Branching Enzyme Digest of Product[c]
	Iodine Affinity (% Apparent Amylose)	β-Amylolysis Limit (% Conversion to Maltose)	Iodine Affinity (% Apparent Amylose)
1	94	92	36
2	92	80	49
3	91	70	59
3 (boiled)[d]	98	102	30
0	100	100	26

[a] Ultrafiltration procedures using Amicon UM-1 membrane are described in the Experimental Section. [b] Product of the pertinent volume of UM-1 dialysate digested 72 hr in a standard digest with amylose. [c] One-milliliter aliquots of the product digested with sufficient whole potato branching enzyme to convert an equivalent amount of amylose to a product with 26% apparent amylose. [d] Dialysate heated 30 min at 99°.

composite, but its β-amylolysis conversion is equivalent to that of amylose. The 2S component alone decreases both the iodine affinity and β-amylolysis of amylose only slightly; therefore it may also branch amylose. However, the composite only will definitely convert amylose to branched product. Although the 2S component may insert branches in amylose unaided, it certainly contains a factor that promotes this activity in conjunction with the 5S component.

Filtrate of 2S Component Introduces Branches in Amylose. Ultrafiltration with a Diaflo UM-1 membrane (Blatt *et al.*, 1965) permits retention and concentration of proteins above $M = 20{,}000$, whereas those having $M < 20{,}000$ are passed in the filtrate. The molecular weight of the protein-like material having branching capability separated by zonal ultracentrifugation has an $s_{20} \leq 2$ and should lie in this range (Edsall, 1953). Therefore the ultrafiltration method should provide a convenient method of obtaining larger quantities of the 2S component than is possible by zonal ultracentrifugation.

The filtrate obtained does introduce barriers to β amylolysis in amylose without greatly decreasing its iodine affinity (Table V). The apparent content of amylose is decreased about 8% at the highest filtrate concentrations used in a digest with amylose. At the same time the β-amylolysis limit of the product progressively decreases with increasing filtrate. This effect is enzymic since it can be stopped by boiling the filtrate before it is added to the digest.

Only a portion of the amylose molecules digested with the filtrate is branched. Table V lists the iodine affinity of the filtrate products of amylose digested with whole potato DEAE branching enzyme. If all the molecules of the amylose were branched, the iodine affinity of the product should not change. If none are branched, as is represented by digests with no filtrate or boiled filtrate, the amylose should be converted to a product with 26% apparent amylose. Because the apparent amylose value of the filtrate products changes to values that are greater than those of unbranched material (amylose) 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 amylopectin. 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 amylopectin 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-branch 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 ($M \approx 20{,}000$). 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 ($M \approx 20{,}000$) 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 amylolysis or branches in only a portion of the amylose samples. 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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Reversible Inactivation of the Potential Pepsin Activity of Pepsinogen by Alcohols*

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ABSTRACT: The effect of a series of aliphatic alcohols, extending from methanol to 1-hexanol, on the native conformation of pepsinogen was investigated by enzymatic and spectral methods. It was found that the presence of an alcohol exerts on pepsinogen a specific conformational change by which it is transformed to a form which is insusceptible to acid treatment under which native pepsinogen is fully converted to pepsin. This was demonstrated by the absence or partial loss of the potential pepsin activity of pepsinogen upon alcohol treatment. The effectiveness of the alcohols on the above transformation was found to increase with chain length and concentration of the alcohol, as determined at pH 8.0 and 35°, and to depend on pH and temperature. Thus, the molarity of the alcohol which causes 50% reduction in the potential pepsin activity of pepsinogen, $M_{1/2}$, is 2.67 for methanol and only 0.024 for 1-hexanol. The alcoholic inactivation is fully reversible and by dialysis or dilution with the employed buffer the potential pepsin activity of pepsinogen can be fully recovered. Moreover, when alcohol-denatured pepsinogen is exposed to acid (pH 1.7 for 2 min), and then transferred to neutral pH, a full regain in its potential pepsin activity is achieved after 1 hr. Alcoholic solutions of pepsinogen display changes in specific optical rotation at 366 nm, $[\alpha]_{366}$, and in CD, absorption, and fluorescence spectra. The changes in $[\alpha]_{366}$ and in CD spectrum which follow ethanolic treatment of pepsinogen were studied in detail, and were found to be reversible and parallel to the apparent change in potential pepsin activity of pepsinogen. Ethanol-treated pepsinogen also shows slower rate of diffusion in immunodiffusion experiments. Based on all the above observations, a kinetic mechanism for the effect of alcohols on pepsinogen is presented. According to this mechanism when n molecules of alcohol are bound to pepsinogen it can be transformed to an acid inactivable form. The values of n were found to be 6 for methanol and ethanol, and 4 for 1-butanol. This study leads to the conclusion that the postulated intramolecular cleavage of the acid-labile bond during the process of activation of pepsinogen requires a definite conformation of the zymogen. It is speculated that this conformation in acid is of high strain energy which is exploited as energy of activation in the pepsinogen-to-pepsin conversion.

At the pH region of 6.5–8.5 and at temperature below 40° pepsinogen is stable and retains full potential pepsin activity (Perlmann, 1963, 1967, 1970; Grizzuti and Perlmann, 1969). At acidic solutions pepsinogen is converted to pepsin whereas above pH 9 its potential pepsin activity is markedly reduced (Ryle, 1970). Inactivation of pepsinogen by a short exposure to pH 10–11 is fully reversible; activity can be recovered by reducing the pH back to neutral. However, alkaline exposure of more than several minutes will cause an irreversible loss of potential pepsin activity of pepsinogen, the extent of which increases with time of exposure. Similarly, heating of a neutral solution of pepsinogen up to 60° is followed by a complete deactivation of the zymogen, yet if the pepsinogen solution is kept at this temperature for only several minutes and then cooled to 25° the potential pepsin activity is completely restored. A series of heating and cooling, however,

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