Isolation and Characterization of the Potato α-1,4-Glucan–α-1,4-Glucan 6-Glucosyltransferase*

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ABSTRACT: A protein having α-1,4-glucan–α-1,4-glucan 6-glucosyltransferase (Q enzyme) activity has been isolated directly from the lyophilized juice of immature potatoes. This procedure involves adsorption of the lyophilized juice on DEAE-cellulose and elution with a linear salt gradient. Cleland's reagent (dithiothreitol) stabilized the activity in the eluate for more than 6 months. The specific activity of the protein can be increased as much as fivefold by using an ultrafiltration technique to remove contaminating peptides. After the protein has been purified, its specific activity is about 17 units/mg of protein. The enzyme's effect on the iodine affinity of amylose and formation of an amylopectin-like product in its reaction mixtures with amylose have been used to characterize the enzyme as a Q enzyme.

The sedimentation coefficient and partial specific volume of the Q enzyme were estimated by zonal and isodensity ultracentrifugation, respectively. The first was carried out in a 5–20% linear density gradient of sucrose; the second, in concentrated cesium chloride. A gel filtration technique has been used to estimate its Stoke's radius. From these parameters, molecular weight (M = 70,000) and frictional coefficient (f/f_0 = 1.24) have been calculated. The procedures selected to isolate, purify, and characterize the potato Q enzyme should prove equally adaptable for similar enzymes from other plant sources.

Plant branching enzymes (α-1,4-glucan–α-1,4-glucan 6-glucosyltransferase) designated by the term Q enzyme (Gunja *et al.*, 1960) in this paper have little effect on the amylopectin fraction of starch but will convert the amylose fraction into an amylopectin-like polysaccharide (Haworth *et al.*, 1944; Bourne and Peat, 1945). The polysaccharide formed resembles amylopectin with respect to chain length, solubility properties, iodine-staining ability, and β-amylolysis limit (Barker *et al.*, 1949a).

Q-enzyme preparations have been obtained from many higher plants including potato (Haworth *et al.*, 1944; Bourne and Peat, 1945), broad beans (Hobson *et al.*, 1950), wrinkled pea (Hobson *et al.*, 1950), green gram (Baum and Giri, 1952), squash (Philips and Averill, 1953), maize (Fuwa, 1957), sweet corn (Lavintman, 1966), and rice (Igaue, 1963). Purification is difficult. Ammonium sulfate precipitation gives unstable preparations, and although improved methods give materials which can be stored as freeze-dried powders (Barker *et al.*, 1950), they contain only about 5% of the active Q enzyme. Gilbert and Patrick (1952) prepared crystalline potato Q enzyme by ethanol–citrate fractionation at low temperature. Improvements over these purification methods have been developed by Baum and Gilbert (1953) and by Peat *et al.* (1959). More recently, Igaue (1963) made a highly purified rice Q enzyme by elution of a Q enzyme prepared by the method of Barker *et al.* (1950) from columns of hydroxylapatite. Except in Igaue's work, the physical properties of the isolated Q enzymes have not been established.

In this paper a column chromatographic procedure is described by which potato Q enzyme can be isolated directly from potato juice without any preliminary salt fractionation and then purified by ultrafiltration. Aqueous solutions of the enzyme are sufficiently stable to permit the estimation of its molecular weight.

Experimental Procedure

Isolation of Potato Juice Solubles. Immature potato tubers (diameter ≤ 25 mm) were harvested just before the plant flowered. These were peeled and then sliced into 0.5% sodium dithionate at room temperature (25°). The slices were washed with distilled water and 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 at 2000 rpm in a no. 266 swinging-bucket rotor of a no. 2, Model K, International[1] centrifuge. The clarified juice was immediately frozen in a Dry-Ice–acetone mixture and lyophilized at less than 0.1 mm. The lyophilized potato juice was stored at −18° for subsequent column chromatography.

Anion-Exchange Chromatography. About 1.0-g portions of the stored potato juice solubles were suspended in 10 ml of aqueous 0.20 M sodium citrate buffer at pH 7.0 containing 0.1 mg/ml of Cleland's (1964) reagent.

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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.

After 2-hr gentle agitation at 4° on a rotary mixer (Scientific Instrument Co.) and centrifugation at 300*g* in an 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 G-25 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 a linear gradient to 0.05 M sodium citrate buffer (pH 7.0) through a Beckman Spectrochrom 130 column chromatography system (Bernier and Putnam, 1963). The effluent was continuously monitored at 250, 260, and 280 mμ in the flow cell of a Beckman D. B. spectrophotometer, which is an integral part of the Spectrochrom 130. Fractions (25 ml) of the effluent were collected and assayed for Q-enzyme activity. The columns, spectrophotometric flow cell, and the fraction collector of the Spectrochrom 130 were thermostated at 4°.

Ultrafiltration. Rapid concentration of protein solutions or the selective removal of contaminating microsolutes can be accomplished by ultrafiltration through Diaflo membranes. The general procedures for this ultrafiltration technique have been described by Blatt *et al.* (1965). In our experiments, up to 50 ml of an enzyme solution was pipetted into an Amicon Ultrafil Model 50 cell equipped with either Diaflo Ultrafil membrane UM-1 or UM-2. The UM-1 membrane selectively retains globular proteins above M = 20,000 and the UM-2 membrane retains peptides and proteins above M = 2000. 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 operation as often as necessary to gain a protein-free filtrate, the concentrate was washed quantitatively into a volume greater than 10 ml. At times it was desirable to reduce the volume of the enzyme solutions to less than 1 ml. For this purpose a syringe ultrafilter fitted with a Teflon-coated membrane holder was used. A screw device moved the plunger of the syringe to develop enough hydraulic pressure for rapid ultrafiltration.

Gel Filtration. Enzyme preparations and protein solutions were passed through a K 25–100 (25 × 100 cm) Sephadex laboratory column containing Bio-Gel P-60 and equipped with a nylon-net bed support and stabilizer. The bed material was prepared, poured, and stabilized according to the general methods described by Siegel and Monty (1966). The effluent from the column was pumped from the bottom of the column at 12 ml/hr with one vein of a multiveined Buchler peristaltic pump. Simultaneously, buffer was pumped at the same rate through another vein of the pump to the top of the column. The effluent flowed through the 5-mm (optical path) flow cell of an Isco chromatographic analyzer to a fraction collector. The analyzer automatically recorded the absorbancy of the effluent at 254 mμ as a function of time, which is essentially equivalent to volume (due to the constant elution rate). This elution diagram was used to detect protein. Fractions (4 ml) were collected, portions of which were assayed for Q-enzyme activity.

Gel filtration data are presented in terms of the parameter K_d defined (Gelotte, 1960) as

$$K_d = \frac{V_e - V_0}{V_t - V_g - V_0} \quad (1)$$

Here V_e = the elution volume corresponding to the peak concentration of the solute; V_0 = void volume of the column (*e.g.*, the elution volume of Blue Dextran 2000 which does not penetrate the space interior to the gel particles); V_t = total volume of the gel; and V_g = volume not accessible to the solvent (*i.e.*, volume due to the component molecules of the gel). The total volume potentially accessible to solutes $V_t - V_g$ is equal to the elution volume of KCl (Pecsok and Saunders, 1966).

Sucrose Density Gradient Centrifugation. Fractions (0.100–0.250 ml) were layered on 4.66 ml of a 5–20% linear sucrose gradient (Martin and Ames, 1961). The sucrose solutions were buffered with 0.05 M sodium citrate buffer (pH 7.0) and contained 0.1 mg/ml of Cleland's reagent. The tubes were centrifuged for 17.5 hr at 50,000 rpm in the swinging-bucket rotor (SW65K) of a Spinco Model L2-65 preparative ultracentrifuge at 15°. Deviations from these conditions are noted in the text. After centrifugation the contents of the tubes were analyzed for protein (absorbancy at 254 mμ) and fractionated on the Isco density gradient fractionator (Brakke, 1963). The 18–20 fractions (0.250 ml each) were assayed for Q-enzyme activity when appropriate.

Isodensity Centrifugation. Fractions (0.250 ml) of Q enzyme and protein solutions were layered on 4.66 ml of a 1.227 g/ml of aqueous cesium chloride solution. The tubes were centrifuged for 24 hr at 60,000 rpm in the swinging-bucket rotor (SW65K) of a Spinco Model L2-65 preparative ultracentrifuge at 15°. After centrifugation 18–20 fractions (0.250 ml each) were collected and analyzed for protein (absorbancy at 254 mμ) in an Isco density gradient fractionator. The fractions were immediately assayed for Q-enzyme activity. Also, the refractive index of each fraction at 15° was determined, from which the cesium chloride density of each fraction could be calculated (Elias, 1964). The density of the cesium chloride solution at the peak concentration of protein or enzyme is the flotation density, $P_{15°}$, of the protein at 15°. The reciprocal of this value is the partial specific volume, $\bar{V}_{15°}$, of the protein.

Q-Enzyme Assay. Q-enzyme activity was determined by an iodine-staining method similar to those reported earlier (Igaue, 1963). The components of a standard reaction mixture were: 5 ml of 0.20 g/100 ml of crystalline potato amylose (Schoch, 1945) in 0.5 N KCl (pH 4.2), 2 ml of a 0.40 M sodium citrate buffer (pH 7.0), up to 3 ml of a Q-enzyme solution, and sufficient distilled water to make 10 ml. The amylose solutions were prepared as follows. The potato amylose (500 mg) on a dry basis was suspended in 50 ml of distilled water, about 10 ml of 45.2% aqueous potassium hydroxide was added, and the

TABLE I: Q-enzyme Yield from DEAE-cellulose Column Chromatography.

	Isolation								
	1[a]			2[b]			3[b]		
		Activity[c]			Activity[c]			Activity[c]	
Fraction	Vol (ml)	Units/ml	Units	Vol (ml)	Units/ml	Units	Vol (ml)	Units/ml	Units
A				525	0.22	113	250	0.22	55
B	1000	0.34	340	625	0.38	237	300	0.37	111
C				375	0.37	138	600	0.34	204
Total units[d] of activity			340			488			370
Weight (g) of lyophilized potato juice			1.00			1.40			1.10
Units/g			340			348			336

[a] Salt gradient (0.001–0.2 M sodium citrate buffer, pH 7.0) completed in 2000 ml; Q-enzyme activity eluted in one peak. [b] Salt gradient (0.01–0.05 M sodium citrate buffer, pH 7.0) completed in 3000 ml; Q-enzyme activity eluted in several peaks. [c] A unit is the percentage decrease in absorbancy per minute at 655 mμ of 1 mg of amylose in a standard reaction mixture at 25° with the designated Q-enzyme fraction. [d] Sum of the total activity in each zone (Figure 1) for each DEAE-cellulose Q-enzyme isolation.

solution was stirred for 1 hr. After this treatment, the solution was neutralized to pH 4.2 with concentrated hydrochloric acid and quantitatively made up to 250 ml so that the final KCl concentration was 0.5 N.

The reaction mixtures were incubated in a 25° water bath. At appropriate intervals a 1-ml aliquot 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 of 0.5 N HCl. This last step produces I_3^- ion, which complexes with amylose or its products from the Q-enzyme digests. The absorbancy of this mixture in a 5-cm cell was recorded in a Cary 14 recording spectrophotometer over the wavelength range 400–700 mμ against a reagent blank. One unit of Q-enzyme activity is defined as that amount of enzyme preparation or its solution which will reduce the absorbancy of the iodine–amylose complex 1%/min at 655 mμ in the standard reaction mixture, and is calculated from the slope of a plot of the percentage decrease of absorbancy of the iodine–amylose complex at 655 mμ *vs.* time. The specific activity is reported as units/mg of protein. Protein was determined by the Lowry modification (Lowry *et al.*, 1951) of the Folin–Ciocalteu method with bovine serum albumin as a standard.

Detection of Q-enzyme activity in chromatographic or density gradient fractions involved scaling down the assay procedure. The 0.20-g/100 ml amylose solution (0.500 ml) was added to 0.200 ml of the sodium citrate buffer (pH 7.0), 0.250 ml of the Q-enzyme fraction, and 0.050 ml of distilled water. After 30 min at 25°, this reaction mixture was transferred quantitatively to the iodine-staining solution described previously. Decrease of the absorbancy of the iodine–amylose complex was expressed as the total percentage change. In some tests, however, the activity could be calculated and expressed in Q-enzyme units by dividing the total percentage decrease by 30 min.

β-Amylolysis. Aliquots (1 ml) of the standard Q-enzyme reaction mixture were added to 1 ml of an aqueous solution of crystalline, Mann Assayed, sweet-potato β-amylase containing 5.8 units/ml of activity. The percentage conversion into maltose was determined by the copper reducing power method of Brown modified by Dygert *et al.* (1965). Under these conditions amylose and amylopectin were converted into their respective β-amylolysis limits in less than 2-hr digestion at 25°. No further action could be noted at more extended digestion times.

Physical–Chemical Methods. A Spinco Model L analytical centrifuge equipped with a Wolter phase plate and a schlieren optical system was used in all sedimentation work. Sedimentation coefficients were calculated from the velocity of the principal peak of the schlieren patterns based on a solvent in the reference cell. Equilibrium weight-average molecular weights were calculated from schlieren patterns according to the methods described by Schachman (1959). The weight-average molecular weight of starch components was also determined by light scattering using a modified Brice-Phoenix light-scattering instrument (Senti *et al.*, 1955).

Results

DEAE-cellulose Chromatography. Three solutions of that portion of 1–1.4 g of lyophilized potato juice soluble in 0.20 M sodium citrate buffer (pH 7.0) and excluded from Sephadex G-25 were adsorbed on and eluted from a DEAE-cellulose column according to the method described in the Experimental Section. A typical elution diagram is seen in Figure 1. Here at least

TABLE II: Purification of the DEAE Q-enzyme Fractions by Diaflo Ultrafiltration.

Fraction[a]		Protein[b]		Activity[c]	
DEAE	Ultrafiltration	μg	%	Total Units	Sp Units/mg
A (0.024 M)[d]	Stock	3430		11.0	3.0
	Concentrate[e]	780	22.8	11.0	14.0
	Filtrate[f]	2700	78.7		
C (0.036 M)[d]	Stock	2188		17.0	7.84
	Concentrate[e]	996	45.6	17.0	17.25
	Filtrate[f]	1216	55.4		

[a] Ultrafiltration and DEAE chromatography described in the Experimental Section. [b] Determined on each ultrafiltration fraction by the Lowry modification of the Folin–Ciocalteu method (Lowry *et al.*, 1951). [c] Percentage decrease per minute of the absorbancy at 655 mμ of the iodine–amylose complex in a standard reaction mixture with the pertinent fraction. Total activity is that in the volume of the zone. Specific activity is the unit per milligram of protein. [d] Molarity of the eluent (citrate buffer, pH 7.0) at the principal peak of the zone. [e] That portion of the protein and activity retained by a Diaflo UM-1 membrane quantitatively made up to 25 ml. [f] Composite of the material passing a Diaflo UM-1 membrane until negative protein assay in filtrate resulted.

three peaks of Q-enzyme activity have been eluted between 0.02 and 0.04 M sodium citrate buffer (pH 7.0) in an area which has very little protein concentration as judged by the absorbancy of the eluate at 280 mμ. The peak of Q-enzyme activity eluted at the higher buffer concentration (zone C) appears to be multiple.

Although the amount of enzyme activity isolated in each of the zones A–C of Figure 1 varies for different experiments, the total amount of activity isolated per gram of lyophilized potato juice is nearly constant. This observation is demonstrated by the yields of activity per gram of lyophilized potato juice recorded in Table I. These yields have been computed from the individual yield of each zone for three different isolations differing only in the amount of lyophilized potato juice applied to the column.

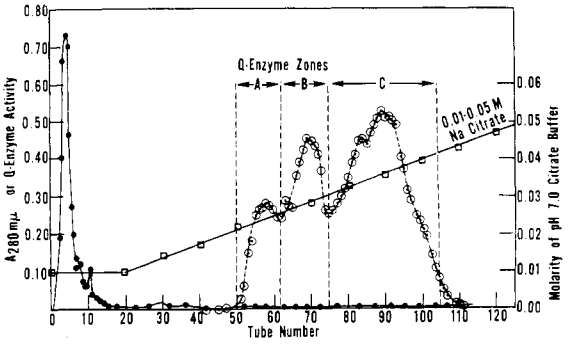

FIGURE 1: DEAE-cellulose column chromatography of the Sephadex G-25 excluded protein from 1.1 g of lyophilized potato juice. The excluded protein was added directly to a 1.9 × 40 cm DEAE-cellulose column equilibrated with 0.01 M sodium citrate buffer (pH 7.0). After washing with 500 ml of the equilibrating buffer the column was eluted with a linear gradient to 0.05 M sodium citrate buffer (pH 7.0) at 4° The absorbancy at 280 mμ (●——●), percentage decrease of the absorbancy at 655 mμ of the iodine–amylose complex of the Q-enzyme reaction mixture (⊙——⊙), and the molarity of the elution gradient (□——□) are plotted as a function of tube number (25 ml/tube). Q-enzyme zones A–C were cut at the vertical broken lines.

The DEAE-cellulose enzyme fractions can be further purified by using the pressure ultrafiltration method described in the Experimental Section, which employs selective molecular diffusion through a Diaflo membrane. By this method the protein having Q-enzyme activity has been separated from a lower molecular weight protein or peptide so that all the biological activity in each zone is retained in a protein having a specific activity of about 17 units/mg of protein. The proportion of low molecular weight protein in the enzyme fractions increases as the molarity of the eluting buffer decreases. In Table II some typical data are presented that demonstrate these points. The amounts of protein and of Q-enzyme activity in each of the ultrafiltration fractions and in the stock solution were determined. From these quantities the proportion of protein in each ultrafiltration fraction relative to that in the stock enzyme fraction could be calculated, as well as its specific activity.

We suggest that the protein in the filtrate may be a peptide. Our suggestion rests on the fact that the material assaying as protein in the filtrate from the Diaflo UM-1 membrane also completely passes a UM-2 membrane. The Diaflo UM-2 membrane retains peptides or proteins with molecular weights greater than $M = 2000$. Therefore, the molecular weight of the protein-like material in our UM-1 filtrates must be less than or equal to 2000. Since its association with the enzyme activity has survived gel filtration from Sephadex G-25, adsorption to the enzyme is indicated. Otherwise, the activity should have been completely excluded in a much smaller elution volume relative to that of the peptide, which should have been completely included in the gel matrix. Further, this association appears to affect the elution behavior of the enzyme on DEAE-cellulose. That is, the proportion of the peptide associated with each zone decreases as the molarity of the eluting buffer increases.

TABLE III: Comparison of the Properties of DEAE-Isolated Q-enzyme-Synthesized and Natural Products.

Polysaccharide[a]	β-Amylolysis[b] Limit (%)	I_3^- Complex,[c] λ_{max} (mμ)	$s_{20,w}$[d] × 10^{13}	M[e] (g)
Potato amylopectin				
This paper	58	560	>200	>40 × 10^6 (LS)
Barker *et al.* (1950)	56	560		
Manners (1962)	50–60	≈540		≈10 × 10^6
Potato amylose				
This paper	100	630	5.0	250,000 (LS)
Nussenbaum and Hassid (1951)	90	630		200,000 (Osm)
Potato amylose + Q enzyme				
This paper	57	540	2.0	22,000 (equilibrium)
Nussenbaum and Hassid (1951)	51		2.1	31,000 ($s_{20,w}$ and f/f_0)
Zevenhuizen (1964)	58	540		

[a] Potato starch fractions isolated by the Schoch (1945) method or a modification of it. The Q-enzyme product formed from amylose was characterized after the absorption spectra of the I_3^- complex became stable. [b] Conversion by crystalline β-amylase expressed as per cent apparent maltose. [c] Wavelength of maximum absorption of the iodine–polysaccharide complex. [d] Sedimentation coefficient expressed as S units and calculated by the method given in the Experimental Section. [e] Molecular weight of the polysaccharide determined by the method indicated in parentheses: LS = light scattering; Osm = osmometry; equil = equilibrium ultracentrifugation; and s and f/f_0 = sedimentation coefficient and an assumed frictional ratio.

The elution of human serum albumin from DEAE-cellulose presents a similar elution diagram. In that instance also, the elution of the protein in several peaks over a wide area of the chromatogram has been correlated with an adsorbed low molecular weight component (James and Stanworth, 1964).

Two additional points are also made because all the Q-enzyme activity is retained by the Diaflo UM-1 membrane. First, the activity of our Q enzyme is not affected by the associated peptide. Secondly, the Q-enzyme activity of the fractions can be safely concentrated by Diaflo ultrafiltration even at 25° without any immediate loss of activity. However, there is some evidence (below) that the resulting concentrated solutions are more unstable than the dilute solutions of the enzyme.

Stability of the Q-Enzyme Activity. The most effective method of retaining the activity of our Q enzyme isolates has been storage at 4° in the original DEAE-cellulose effluent containing 0.1 mg/ml of Cleland's reagent. That is, the activity of an isolate of 300 units dropped to about 240 units in 3 days at 4°. At this point 0.1 mg/ml of Cleland's reagent was added and the activity was maintained for 2 weeks. The portion of the isolate not so protected continued to lose activity. Other samples also stored at 4° in the original effluent containing 0.1 mg/ml of Cleland's reagent have been stable for 6 months. A 60-fold concentration of the enzyme by ultrafiltration with a UM-1 membrane on the third day caused no immediate loss of activity. However, after 10 days all the activity was lost. After 4 days, some of the activity of the 60X concentrate could be regained by adding Cleland's reagent and diluting 60 times. This last solution was stable. Therefore, 0.1 mg/ml of Cleland's reagent has been included in all the buffers used in this isolation procedure and the enzyme solutions have been stored cold (4°) and dilute until used.

Identification of the Enzyme. Three general criteria have been used to identify the enzymic activity of our DEAE fractions as that of a typical Q enzyme (Manners, 1962): the action of the enzyme on the iodine affinity and reducing power of amylose, its temperature of optimum activity, and the amylopectin-like properties of the products formed in its reaction mixtures with amylose.

Action on Amylose. The action of our DEAE fractions on amylose presents the same features as those which characterize the action of the potato Q enzyme on a similar substrate (Hobson *et al.*, 1950). There is an initial 90% decrease in the absorbancy (655 mμ) of the iodine–amylose complex. At the same time a small copper reducing power develops which is typically in the range 2–5% apparent maltose.

Optimum Temperature. The optimum temperature of the potato Q-enzyme activity is distinctive; its value, about 21° (Barker *et al.*, 1949a), is low. At 35°, the usual optimum temperature of plant-metabolizing enzymes, the Q enzyme exhibits less than 50% of its maximum activity. Data from a series of reaction mixtures of our purified fractions with potato amylose at several temperatures from 15 to 40° indicate that the total percentage decrease of the absorbancy of the iodine–amylose complex after 30 min is maximum at 23.5°. This value though (2.5°) higher is nearly that previously reported for the Q enzyme.

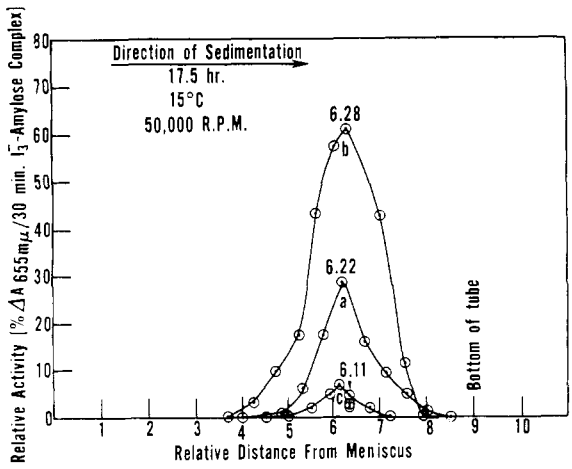

FIGURE 2: Zonal ultracentrifugation of the DEAE-isolated Q-enzyme zones in a sucrose gradient. The 0.250-ml aliquots of Q-enzyme zones concentrated to different extents by ultrafiltration (Diaflo) were layered on 4.66 ml of a 5–20% sucrose gradient buffered with 0.05 M citrate buffer (pH 7.0). After the tubes had been centrifuged 17.5 hr, 50,000 rpm, at 15°, they were fractionated and analyzed. Each 0.250-ml fraction was assayed for Q-enzyme activity according to the method described in the Experimental Section. The percentage decrease of absorbancy of the iodine–amylose complex (% $\Delta A_{655\,m\mu}$)/30 min at 655 mμ in 30 min is plotted *vs.* the distance of each fraction from the meniscus measured on the recorder diagram of an Isco density gradient fractionator (Brakke, 1963). The point of maximum concentration (maximum % $\Delta A_{655\,m\mu}$ of I_3^-–amylose)/30 min is the distance sedimented by the enzyme: curve a is zone A (UM-1 Diaflo membrane concentration 10X), curve b is zone B (UM-1 Diaflo membrane, concentration 20X), and curve c is zone C (UM-1 Diaflo membrane, concentration 5X).

Nature of the Enzyme Product. A third, and last, criterion used to identify the enzymic actions of our purified fractions is the nature of the stable polysaccharide present in the reaction mixture of amylose with our Q-enzyme zones after enzymic action ceased. That is, after the decrease of absorbancy (% $\Delta A_{655\,m\mu}$) of the iodine–amylose complex has ceased, an amylopectin-like product can be isolated from the reaction mixture. In Table III, selected enzymic, spectrophotometric (iodine–polysaccharide complex), ultracentrifugal, and molecular weight data, calculated by the methods outlined in the Experimental Section, are presented for a typical product of our enzyme reaction mixtures with potato amylose compared with those of our substrate amylose and the amylopectin fraction from the same potato starch (Schoch, 1945). Included for comparison are data for similar starch fractions and for the products of other Q-enzyme preparations. Similar chemical and physical properties have been used extensively to differentiate between linear and branched starch-type polysaccharides, as well as between branched polysaccharides, such as amylopectin and glycogen (Manners, 1962).

It can be seen from Table III that in all these categories the polysaccharide products of our enzyme reaction mixtures with potato amylose are the same as those from other Q-enzyme preparations. All products resemble amylopectin with respect to their β-amylolysis limit and their wavelength of maximum absorption of the iodine complex. However the sedimentation coefficient indicates, and the molecular weight confirms, that the product is small; 20–30 × 10^3 rather than 10–40 × 10^6 as reported here for amylopectin isolated from starch.

Molecular Weight Determination. The concentration (<0.05 mg/ml) of our stable enzyme solutions is extremely small. Within this concentration limit the enzyme is most easily detected and its concentration measured by its enzymic activity. It is possible to obtain the proper concentration (2–10 mg/ml) by Diaflo ultrafiltration to determine the molecular size and configuration of the enzyme by the usual light-scattering and ultracentrifugation techniques. However, some of our data suggest that the enzyme may be unstable at these concentrations. Therefore, other techniques (Siegel and Monty, 1966; Martin and Ames, 1961) adapting enzymic activity to detect and to measure the concentration of the enzyme were used to determine the molecular parameters of eq 2 and 3

$$M = 6\pi\eta as/(1 - \bar{V}P) \quad (2)$$

$$f/f_0 = a\Big/\left(\frac{3\bar{V}M}{4\pi N}\right)^{1/3} \quad (3)$$

where M is the molecular weight and f/f_0 is the frictional ratio of a macromolecule in dilute solution. The parameters are s, the sedimentation coefficient; $\bar{V}$, the partial specific volume; and a, the Stoke's radius of the molecule. Also, η is the viscosity of the medium, P its density, and N is Avogadro's number.

Determination of Sedimentation Coefficient. The sedimentation coefficient of our Q-enzyme activity was determined by zonal ultracentrifugation in a 5–20% sucrose gradient (see Experimental Section). The results of this operation are summarized in Figure 2. Here the Q-enzyme activity of 0.250-ml fractions from tubes loaded with 0.20 ml of each zone of activity layered on 4.66 ml of the gradient and centrifuged at the conditions shown on the figure is plotted as a function of the fraction's distance from the meniscus of the contents of the centrifuge tubes.

The distance recorded at the peak of Q-enzyme activity of each zone is the distance the activity had sedimented during centrifugation. This value can be used to calculate a sedimentation coefficient directly. However, the rate of sedimentation of most biological material in a linear 5–20% sucrose gradient at 15° is independent of its position in the gradient (Martin and Ames, 1961). The sedimentation coefficient in water at 20° of several proteins (human serum albumin, bovine serum albumin, ovalbumin, α-chymotrypsin, egg lysozyme, and sperm whale myoglobin) having similar partial specific volumes is a linear function of the distance each has sedimented under our centrifugal conditions. Since these conditions are the same as those used for our Q-enzyme zones (Figure 2), the enzymes sedimentation coefficient in water can be determined from the average distance the Q-enzyme activity had sedimented. Its value is 4.80 S.

The accuracy of this sedimentation coefficient requires that the partial specific volume of the known proteins and that of the unknown Q enzyme be equal. More precisely, the partial specific volume of the known and unknown can vary only 0.03 cc/g without introducing a significant error in the sedimentation coefficient estimated by this method (Martin and Ames, 1961).

Determination of Partial Specific Volume. An apparent value of the partial specific volume of a protein can be calculated from its buoyant or flotation density determined by the isodensity zonal ultracentrifugation method in a concentrated cesium chloride gradient as described in the Experimental Section. The equivalence of this value and the value determined in dilute salt solution can be uncertain. It is known that proteins interact with either the solvent or the gradient material in concentrated cesium chloride. Cox and Schumaker (1961) showed that the partial specific volume of several typical proteins (including bovine serum albumin used here) determined in concentrated cesium chloride are from 0.02 to 0.03 cc/g high compared with those determined in a dilute salt solution. Since this variation could not produce a significant error in the sedimentation coefficient determined by the density gradient ultracentrifugation method (Martin and Ames, 1961), then the partial specific volume of our Q enzyme determined in this manner can be reliably compared with that of the proteins used to obtain the sedimentation coefficient.

In Table IV the flotation density, $P_{15°}$, and the calculated apparent partial specific volume, $\bar{V}_{app}$, of our Q enzyme are compared with the values for bovine and human serum albumin. All are compared in the first column with the range of values of the partial specific volume of the albumins calculated from densities determined in dilute salt solution (Edsall, 1953). Since the apparent partial specific volume of our Q enzyme (0.74 cc/g) is nearly that of the albumins (0.76 cc/g), their actual partial specific volumes, even if solvent interactions have occurred, should be within the limits necessary for the determination of an accurate sedimentation coefficient. Further, the effect of solvent or gradient material interactions appears not to have seriously affected the value of the partial specific volume of either albumin. Their apparent values are in the upper part of the range reported by Edsall (1953). Therefore, we have assumed that the value 0.74 cc/g is the partial specific volume of our Q enzyme.

Determination of Stoke's Radius. Ackers (1964) has proposed a theory explaining the experimentally observed separation of solutes by gel filtration according to their Stoke's radius, a, based on a mechanism of both steric and frictional hindrance to molecular diffusion within the assumed uniform cylindrical pores of radius, r, of the gel matrix of a column material. His relation based on the Renkin (1955) equation (eq 4) expresses

$$K_d = [1 - (a/r)^2][1 - 2.104(a/r) + 2.09(a/r)^3 - 0.95(a/r)^5] \quad (4)$$

the Gelotte parameter, K_d of eq 1, as a function of the Stoke's radius, a, of the solute and the effective pore radius, r, of the gel. Ackers (1964) has calculated and published an extensive table of the theoretical values of the ratio a/r corresponding to experimentally observed values of K_d. From this table it should be possible to calibrate a column with a protein of known Stoke's radius. On the basis of the r value from these data, then the Stoke's radius, a, of any unknown protein not excluded from the column can be determined from its elution position on the same column. This method has been examined and found essentially valid for Sephadex G-200 (Siegel and Monty, 1966; Pecsok and Saunders, 1966).

TABLE IV: Determination of the Partial Specific Volume of the Q Enzyme.

Sample	$\bar{V}$ Lit. Pycnometer[a] (cc/g)	$d_{15°}$ (g/cc)[b]	$\bar{V}$ Density Gradient[c] (cc/g)
DEAE-isolated Q enzyme[d]		1.352	0.74
Human serum albumin	0.729–0.736	1.340	0.75
Bovine serum albumin	0.730–0.740	1.334	0.75

[a] Values from Edsall (1953). [b] Density of CsCl solution in which sample does not sediment. Twenty-four hours at 60,000 rpm in the SW65K swinging-bucket rotor of the Model L2-65 Beckman preparative ultracentrifuge at 15° was required to obtain a 4.66-ml CsCl gradient from a CsCl solution with a nominal density of 1.227 g/cc buffered with 0.05 M citrate at pH 7.0. [c] $\bar{V}_{app} = 1/d_{15°}$. [d] Zone C of Figure 1.

Figure 3A is the elution diagram from Bio-Gel P-60 of Blue Dextran 2000 (excluded macromolecule); three proteins of known Stoke's radius (ovalbumin, bovine hemoglobin, and bovine serum albumin dimer) and KCl. The elution volume of Blue Dextran 2000 and KCl were used to determine the denominator of the K_d ratio (eq 1). Figure 3B is the elution diagram from Bio-Gel P-60 of the Q-enzyme activity of zone C of our DEAE-isolated Q-enzyme preparations. The elution volumes of the proteins and Q enzyme at their maximum concentration are used to determine the numerator of the K_d parameter.

The K_d value of the proteins of known Stoke's radius and that of the Q enzyme are recorded in the first column of Table V. These values were used to interpolate values of the a/r ratio from Ackers' tables (Ackers, 1964) for each protein recorded in the second column. By using the known Stoke's radii (last column, Table V) and a/r ratios of ovalbumin, bovine hemoglobin, and bovine serum albumin given in the last column, values of parameter r can be calculated. The average value of r (7.19 mμ) is then used to calculate the Stoke's radius

TABLE V: Determination of Stoke's Radius by Gel Filtration on Bio-Gel P-60.

Sample	K_d[a]	a/r[b]	r[c] (mμ)	a[d] (mμ)
Ovalbumin	0.121	0.3766	7.24	2.73
Bovine serum albumin	0.018	0.6000	7.25	4.35
Bovine hemoglobin	0.077	0.4350	7.10	3.10
DEAE-isolated Q enzyme	0.057	0.4720	[7.19]	3.39

[a] The K_d parameter (Gelotte, 1960) defined in the text (eq 1). [b] Ratio of the Stoke's radius, a, to the apparent pore size, r, of the Bio-Gel P-60 column interpolated from published tables (Ackers, 1964) for the specific K_d values of this table according to eq 4. [c] Value of r calculated from the value of a/r (column 2) and the values of a given in the last column for the reference proteins (Edsall, 1953). The value in parentheses is the average pore size used to calculate the Stoke's radius of the DEAE-isolated Q enzyme. [d] Stoke's radius for reference proteins (Edsall, 1953) and for the DEAE-isolated Q enzyme calculated from the average value of the pore size, r, of column 3.

of our Q enzyme (3.39 mμ) given in the last column. The slight deviation from the average value of r for each of the known proteins, even though bovine hemoglobin is rather asymmetric, validates the use of Bio-Gel P-60 as a column material for Ackers' method.

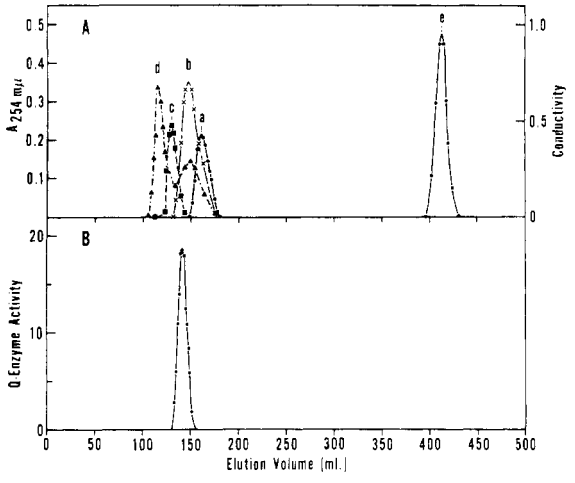

FIGURE 3: Determination of elution volumes from a gel filtration column and its calibration. (A) The absorbancy at 254 mμ (A_{254}) of the effluent from a Bio-Gel P-60 column (2.5 × 100 cm) charged with 5 ml of 0.05 M citrate buffer (pH 7.0) containing: (a) 15 mg of ovalbumin (●——●), (b) 10 mg of bovine hemoglobin (x——x), (c) 10 mg of bovine serum albumin (■——■), (d) 5 mg of Blue Dextran 2000 (▲——▲), and (e) 0.5 M KCl (·——·). (B) The percentage decrease of absorbancy at 655 mμ of an iodine–amylose complex in 30-min reaction mixtures of 0.250-ml aliquots of effluent with a standard reaction mixture (see Experimental Section) from the column charged with 5 ml (1.65 units of Q enzyme) of zone C is plotted *vs.* the volume of eluate. The elution volume of each species was determined from the volume at which its concentration is maximum (protein, maximum $A_{254\,m\mu}$; enzyme, maximum % $A_{655\,m\mu}$ of I_3^-–amylose complex). The excluded volume, V_0, of the column was determined from the elution volume of the Blue Dextran 2000, and the internal volume of the gel matrix, V_i, was calculated from the difference between the excluded volume and the elution volume of KCl. The column was eluted at 12 ml/hr with 0.05 M citrate buffer (pH 7.0) containing 0.1 mg/ml of Cleland's reagent. The eluate was analyzed and fractionated by an Isco chromatographic system (Brakke, 1963). All operations were carried out in a refrigerator at 4°.

Calculation of Molecular Weight and Frictional Ratio. The values for the sedimentation coefficient (s = 4.80 × 10^{-13} sec^{-1}, the partial specific volume ($\bar{V}$ = 0.740 cc/g), and the Stoke's radius (a = 3.39 mμ) were substituted in eq 2 and 3 with the appropriate values of the other constants and parameters, assuming that the medium is water at 20°. The values obtained are M = 70 × 10^3 and f/f_0 = 1.24.

Discussion

A stable enzyme system having about 17 units/mg of protein Q-enzyme activity can be rapidly and reliably isolated directly from lyophilized potato juice employing a relatively mild DEAE chromatographic procedure. Modifications of zonal ultracentrifugation and gel filtration techniques have been used to estimate the sedimentation coefficient, partial specific volume, and Stoke's radius of the protein having this activity (Siegel and Monty, 1966; Martin and Ames, 1961; Ackers, 1964). These values are used to calculate from their definitions (eq 2 and 3) the molecular weight (M = 70,000) and frictional ratio (f/f_0 = 1.24) of the potato Q enzyme and to identify it as a protein having the size and shape characteristic of serum albumins [human (M = 69,000; f/f_0 = 1.28); horse (M = 70,000; f/f_0 = 1.27)] (Edsall, 1953). Although some uncertainty has been reported regarding the accuracy of the partial specific volume determination (Cox and Schumaker, 1961) by zonal ultracentrifugation in concentrated cesium chloride, the values for several proteins and the Q enzyme reported here appear to be accurate. Thus these procedures provide a molecular weight determination of the enzyme from our stable, dilute, DEAE isolates. Uncertainties due to inactivation of the enzyme by concentration procedures or by storage at the relatively high concentrations necessary for conventional determinations of molecular size are minimized.

Our enzyme preparations, like those previously reported (Manners, 1962), synthesize an amylopectin-like product with a higher reducing power and a much lower molecular weight than their amylose substrate. Consequently, unlike current concepts of the action pattern of the Q enzyme (Manners, 1962), more α-1,4-glucosidic

bonds are scissioned than α-1,6-glucosidic bonds are made. If, as has been proposed, this net scissioning is due to an α-amylase-like contaminant rather than the inherent action pattern of the Q enzyme, this contaminant is a minor component of our preparations. This conclusion is supported by both our physical and enzymic studies. All the Q-enzyme activity of our preparations sediments and is eluted by gel filtration as one symmetrical peak. Thus this activity appears to be the property of one molecular species. However owing to the nonspecific nature of the Q-enzyme assay, this evidence is not conclusive. Several enzymes, *e.g.*, α-amylase β-amylase, and phosphorylase normally present in potato juice, also produce an assay similar to that of the Q enzyme. Of these enzymes only α-amylase can be the proper molecular size for the observed sedimentation and elution behavior (Edsall, 1953). However, this α-amylase-like contaminant is only a minor contributor to the observed activity. That is, the net scissioning of bonds in the amylopectin-like product (5% as apparent maltose) is considerably lower than the 20% apparent maltose expected if the 90% decrease of the iodine affinity of the amylose substrate were produced by α-amylase (Barker *et al.*, 1949a). Here also β-amylase and phosphorylase are ruled out since both would have produced a much larger (>50%) apparent conversion into maltose.

In addition to a possible minor enzymic contaminant our DEAE-isolated Q-enzyme preparations contain at least one inactive component, a low molecular weight peptide. Other than its apparent effect on the elution position of the Q enzyme from DEAE-cellulose in several peaks, this component has no effect on the activity of the Q enzyme. Although the highest specific activity reported here (17 units/mg of protein) is less than that reported for waxy rice Q enzyme (155 units/mg of protein) prepared by elution of a Q-enzyme preparation (Hobson *et al.*, 1950) from hydroxylapatite (Igaue, 1963), this activity difference may indicate a real difference between Q enzymes of different plants rather than the presence of large amounts of an inactive low molecular weight material. However, our data do not exclude the presence of inactive protein of at least the same molecular size as the Q enzyme.

A study of the relative amount, enzymic properties, and molecular properties of well-characterized Q enzymes from several plant sources should clarify the somewhat uncertain function of this enzyme in starch synthesis. Some data (Manners, 1962) have correlated the proportion of potato Q enzyme relative to that of the amylose-synthesizing enzymes with the proportion and structure of amylose and amylopectin synthesized *in vitro*. However, other studies with relatively impure Q-enzyme preparations from other plant sources have failed to verify this relation (Fuwa, 1957; Manners, 1962). Modification of the isolation procedures outlined here can provide a rapid, uniform method applicable to the isolation and study of the Q enzyme of other plants. For example, the juice or solvent extract of their fruit can be isolated in the field and immediately frozen. Since potato and broad bean Q enzymes are stable in frozen whole juice (Barker *et al.*, 1949b), the time necessary to reach laboratory lyophilization equipment will not produce significant losses of enzyme activity. After lyophilization, isolation and characterization of the enzyme can be deferred to any convenient time independent of the field isolation of the plant juice.

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Peptide Synthesis in Cell-Free Extracts of *Bacillus brevis* 8185*

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ABSTRACT: The biosynthesis of antibiotic polypeptides by cell-free extracts of *Bacillus brevis* ATCC 8185 is described.

Optimal conditions for tyrocidine biosynthesis were determined. No gramicidin-synthesizing activity was observed, and the only tyrocidine produced in significant amounts was tyrocidine D. It appears that this strain differs in this respect from *B. brevis* ATCC 10068, which produces the same spectrum of polypeptides, but does not appear to synthesize any one of the tyrocidines preferentially under the same conditions.

The recent controversy over the nature of the system that synthesizes gramicidin S has been largely resolved by the agreement of all groups concerned that an enzymatic, nonribosomal mechanism is involved (Yukioka *et al.*, 1965; Berg *et al.*, 1965; Bhagavan *et al.*, 1966; Tomino *et al.*, 1967). It seemed likely that a similar system was involved in the biosynthesis of the antibiotic polypeptides of the tyrocidine and gramicidin families produced by other strains of *Bacillus brevis* and clearly related to gramicidin S in structure and biological activity. A report from Fujikawa *et al.* (1966) suggested that a nonribosomal system was indeed utilized for tyrocidine production. The only other work on cell-free extracts, however, had implicated a ribosomal pathway for this synthesis (Bodley *et al.*, 1964), but in view of the apparently erroneous reports published by this same group of workers on gramicidin S biosynthesis (Hall *et al.*, 1965) and later corrected (Bhagavan *et al.*, 1966), it seemed worthwhile to reexamine the question of tyrocidine biosynthesis, in order to resolve the conflict in the literature.

The present work confirms and extends the findings of Fujikawa *et al.* (1966), that the synthetic system is nonribosomal in nature. The extracts have been characterized in more detail and additional observations on the biosynthesis of tyrocidines and gramicidins were carried out. No technical problems were encountered in the course of this work which could help to explain the earlier erroneous reports, and it seems unlikely at this time that a scientific explanation for these discrepancies will be forthcoming.

As this paper was being prepared for publication, we received a copy of a manuscript from Fujikawa *et al.* (1968) describing their work on the purification and properties of the tyrocidine-synthesizing system. Their preparation appears to be similar in all essentials to the one we describe here, and in addition, the partial purification and fractionation of the active extract is reported.

Materials and Methods

Chemical and Enzymes. Puromycin was obtained from Nutrional Biochemicals Corp. Chloramphenicol was purchased from Parke Davis Co. Gramicidin and tyrocidine were obtained from Mann Chemical Co. Amino acids, PEP,[1] and ATP were supplied by Sigma Chemical Co. CTP, GTP, and UTP were products of Calbiochem. All of the isotopic compounds were obtained from New England Nuclear Corp. and had the following specific activities (millicuries per millimole): L-[U-^{14}C]-alanine, 117; L-[U-^{14}C]aspartic acid, 167; L-[U-^{14}C]-asparagine, 46.9; [1,2-^{14}C]ethanolamine, 4.34; [U-^{14}C]-glycine, 116; L-[U-^{14}C]glutamic acid, 195; L-[U-^{14}C]-glutamine, 52.3; DL-[1-^{14}C]leucine, 31; L-[U-^{14}C]leucine, 251.4; DL-[5-^{14}C]ornithine, 11.0; DL-[3-^{14}C]-phenylalanine, 4.46; DL-[5-^{14}C]proline, 5; L-[U-^{14}C]serine, 120; DL-[3-^{14}C]tryptophan 1.93; DL-[1-^{14}C]tyrosine, 2.4; DL-[1-^{14}C]valine, 18.5; and uniformly labeled

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[1] Abbreviation used that is not listed in *Biochemistry 5*, 1445 (1966), is: PEP, phosphoenolpyruvate.