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THE HYDROLYSIS OF MALTODEXTRINS BY A β-AMYLASE ISOLATED FROM LEAVES OF *VICIA FABA*

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SUMMARY

The action pattern of a β-amylase (α-1,4-glucan maltohydrolase, EC 3.2.1.2) which was extensively purified from extracts of leaves of *Vicia faba* has been investigated The enzyme, which was purified by $(NH_4)_2SO_4$ fraction, precipitation with acetone and chromatography on Sephadex G-200 and Bio-gel P-100 columns had a specific activity of 100 μmoles/min per mg and was obtained in an over-all yield of 13%. The final preparation was homogeneous on the basis of polyacrylamide gel electrophoresis at several pH values, sedimentation in the ultracentrifuge and its elution profile on Sephadex G-200 columns.

A symmetrical peak with $s_{20,w} = 6.5$ was found on ultracentrifugal sedimentation analysis. The molecular weight of the leaf β-amylase, calculated from data obtained by sucrose density gradient centrifugation, chromatography on Sephadex G-200 and ultracentrifugation, was 107 000. The purified enzyme had a Stokes radius of 31.8 Å and a diffusion constant of $6.49 \cdot 10^{-7}$ cm²/s. Information obtained from a complete analysis of the amino acid composition indicated the presence of comparatively large amounts of aspartic and glutamic acids, and the partial specific volume was calculated to be 0.733.

The action pattern of the purified leaf β-amylase upon hydrolysis of a number of maltodextrins was the same and was independent of the molecular size. The only products detected during the course of hydrolysis of maltodextrins with chain lengths of from 9 to 198 glucose residues were maltose and very small amounts of glucose and maltotriose. No evidence for the release of other intermediates was obtained, even when the hydrolysis was carried out essentially to completion. The extent of binding and rate of hydrolysis increased with chain length and reached a maximal level at a chain length of about 40–50 glucose units. The K_m of the enzyme for linear maltodextrins decreased with chain-length up to about 50 glucose units, and with longer chain lengths it remained constant at about $1.4 \cdot 10^{-4}$ M. The maximum velocity of the reaction was not significantly influenced by the chain-length of the maltodextrin used as substrate. The findings reported are consistent with a

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mechanism in which the maltodextrin combined with β-amylase to form a loose complex, which then rearranges to form a tightly bound intermediate which is hydrolyzed completely to maltose and small amounts of glucose and maltotriose.

INTRODUCTION

The role of β-amylases (α-1,4-glucan maltohydrolase, EC 3.2.1.2), which are found mainly in higher plants, in mobilizing starch during germination has been well established. The seeds and tubers of many plants have been shown to contain β-amylase, and the enzyme has been extensively purified from sweet potatoes, barley, soybean and wheat. The kinetic characteristics and action patterns of these enzymes vary somewhat and there are large differences in their molecular weights. The enzyme from sweet potato has a molecular weight of 197 000, the two isozymes of barley β-amylase have molecular weights of 80 000 and 90 000[1], while the enzyme from soybeans has a molecular weight of only 61 700[2]. β-Amylase has been detected in the leaves of some plants[3], however it has not yet been isolated from this tissue. The lack of information on the properties of β-amylase from leaf tissues prompted us to isolate and examine the enzyme from *Vicia faba*.

When *V. faba* leaves were transferred from light to dark conditions, the starch present in this tissue was rapidly hydrolyzed. However, no low molecular weight maltodextrins were formed, even after almost all of the starch was degraded. During studies with crude extracts, it was observed that the rate of hydrolysis of various amylose substrates was not the same, however the action patterns obtained with all the substrates were identical and no detectable intermediate molecular weight maltodextrins accumulated under these conditions. These preliminary results indicated that the enzyme present in leaves might hydrolyze amylose by a mechanism in which the intermediate products of the reaction repeatedly realign themselves on the enzyme surface to form new active complexes without complete dissociation of the enzyme and substrate.

The present communication describes the purification of a β-amylase from leaf tissue, and reports some of its physical and chemical characteristics. Evidence that establishes some of the general kinetic parameters of this enzyme is presented, and a binding and hydrolytic mechanism is proposed to explain the action pattern which was observed during the hydrolysis of a homologous series of maltodextrins.

EXPERIMENTAL PROCEDURE

The activity of β-amylase was assayed according to the procedure of Bernfeld[4] using 3,5-dinitrosalicylic acid as the oxidizing agent. The standard reaction mixture was incubated at 30 °C and contained in a final volume of 1.6 ml: 35 mM sodium acetate buffer, pH 5.0; 1% soluble corn starch and an appropriate amount of enzyme. One unit of activity is defined as the amount of enzyme which will catalyze the formation of 1 μmole of maltose per min under the standard assay conditions and specific activity is expressed as units per mg of protein.

Linear maltodextrins of specific molecular weight were prepared by limited acid hydrolysis of amylose obtained from soluble potato starch. 10-g batches of the

amylose were suspended in 50 ml of 6 M HCl and the resulting mixture was incubated at 30 °C for 2–7 h. After 2.5 and 7 h of hydrolysis the reaction mixtures were adjusted to pH 7.0 with 5 M NaOH. The suspension in each case was filtered, and 3 vol. of 95% ethanol was added to the filtrate. The precipitate was collected by centrifugation, and it was dissolved in 25 ml of distilled water. The maltodextrin mixture was reprecipitated twice with 95% ethanol as described above and the final pellet was dissolved in 15 ml of distilled water. The separation of homologous maltodextrins was carried out by exclusion chromatography on Biogel P-10 columns[5]. 4-ml samples were applied to Bio-gel P-10 columns (2.2 cm × 25 cm) and the columns were eluted with distilled water at a flow rate of 12 ml/h. Fractions of 4 ml were collected and aliquots were assayed for hexose content. The major fractions containing maltodextrins of decreasing molecular weight which were eluted successively from the columns were pooled and precipitated with 3 vol. of 95% ethanol. The precipitate, in each case, was dissolved in 2 ml of distilled water and applied to another Bio-gel P-10 column (2.2 cm × 25 cm). Each of the maltodextrin fractions was eluted as a single peak with the same volume of distilled water which was used in the previous step. Only the fractions from each peak having the highest hexose content were combined in order to eliminate as much contamination as possible from higher molecular weight polymers at the front of the peak and lower molecular weight maltodextrins at the rear of the peak. About 20% of the total hexose present was discarded by removing the leading and trailing edges of the peaks. The fractions from the center of the peak, in each case, were combined and concentrated by precipitation with 95% ethanol. They were then rechromatographed on the same Biogel P-10 columns two more times. The final fractions from the center of each peak were collected as described above and dissolved in 5–10 ml of distilled water. In the final run, the leading and trailing fractions from each maltodextrin peak had essentially the same composition as the major fraction, which indicated that these polysaccharides were as homogeneous as this procedure would permit. The samples were passed through Millipore filters to remove bacteria and they were kept at 3 °C. These substrates were used in various experiments as soon as possible after they were prepared since they underwent retrogradation after a period of time. The amount of maltodextrin in each of the peaks varied with the time of hydrolysis. More high molecular weight maltodextrins were found after 2 h of hydrolysis, whereas longer times favored the formation of maltodextrins with a lower molecular weight.

The average molecular weight and chain length of each of the isolated maltodextrins was determined. The total number of reducing end groups was estimated by a modification of the procedure of Hoffman[6] using ferricyanide as the oxidizing agent. The total amount of carbohydrate, as glucose equivalents, was determined by the anthrone procedure[7]. The average chain length of each of the amylodextrins used in the experiments described in this report was calculated by dividing the total number of glucose equivalents in a sample by the number of reducing end groups. The average chain length of seven maltodextrin samples isolated by this procedure was 9, 16, 20, 24, 31, 50 and 98 glucose equivalents per chain.

Solutions were concentrated under reduced pressure at 26 °C in a rotary evaporator. The following solvent systems were used for the separation and analysis of maltodextrins by paper chromatography: ethyl acetate–acetic acid–water (1:2:1, by vol.)[8], 1-butanol–pyridine–water (6:4:3, by vol.)[9], water–ethanol–nitromethane,

(18:46:35, by vol.)[10]. The $AgNO_3$ and alcoholic NaOH reagent was used to detect maltodextrins on the paper chromatograms[11]. Silica gel plates were irrigated with a solvent containing ethyl acetate–acetic acid–water[8], and the areas containing maltodextrins were developed by spraying with a modified naphthoresorcinol reagent containing 20% trichloroacetic acid instead of 10% H_2SO_4[12].

Polyacrylamide gel electrophoresis was performed by the procedure of Davis[13] with Tris–glycine buffer adjusted to pH 8.9 with HCl and sodium acetate buffer adjusted to pH 4.3. Electrophoresis was carried out until the bromophenol blue tracking dye migrated to the end of the lower gel. The molecular weight of β-amylase was determined by sucrose density gradient centrifugation by the method of Martin and Ames[14]. Centrifugation was carried out at 37 000 rev./min for 16.5 h at 3 °C. Rabbit muscle lactate dehydrogenase and pyruvate kinase were used as standards. Chromatography on Sephadex G-200 columns was carried out according to the method of Andrews[15]. Sedimentation velocity and sedimentation equilibrium experiments were carried out in an analytical ultracentrifuge at 20 °C, and calculations were made according to Schachman[16]. The Stokes radius of β-amylase was calculated by the method of Ackers[17] and the diffusion constant was derived from sedimentation data according to the procedure of Siegel and Monty[18].

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was carried out by the procedure of Shapiro *et al.*[19]. The gels were calibrated with lysozyme (Sigma), D-glyceraldehyde-*P* dehydrogenase (Worthington), chymotrypsinogen (Worthington) and ovalbumin (Sigma).

The amino acid composition of dialyzed samples of the purified enzyme were obtained by the method of Spackman *et al.*[20]. Half-cystine and methionine were estimated by oxidation of the protein with performic acid to convert these amino acids to cysteic acid and methionine sulfone prior to acid hydrolysis[21]. Tryptophan was determined by amino acid analysis of protein samples hydrolyzed in the presence of 4% thioglycollic acid[22]. Protein was determined by the biuret method[23], and at low concentrations it was assayed spectrophotometrically[24], with crystalline bovine serum albumin as the standard.

MATERIALS

Sephadex G-200 was purchased from Pharmacia, Bio-gel P-100 and Bio-gel P-10 were obtained from Calbiochem. Amylose which contained less than 5% amylopectin was isolated from soluble potato starch[25]. Soluble corn starch was purchased from Sigma. Enzyme grade Tris (General Biochemicals) and $(NH_4)_2SO_4$ and sucrose (Mann) were used without purification. Muscle lactate dehydrogenase, pyruvate kinase, aldolase and hexokinase were purchased from Boehringer. Precoated silica gel plates were obtained from Brinkmann Industries. Porcine pancreatic α-amylase (α-1,4-glucan 4-glucanohydrolase, EC 3.2.1.1) was purchased from Worthington and a partially purified α-amylase preparation isolated from seeds of *V. faba* was kindly provided by Dr Abou Issa. ^{14}C-labelled starch which was prepared by exposing tobacco leaves to $^{14}CO_2$, obtained from Calbiochem and other samples were prepared by incubating small amounts of linear low molecular weight maltodextrins with [^{14}C]glucose-1-*P* and purified potato phosphorylase[25].

RESULTS

Subcellular distribution of β-amylase in leaves

The epidermal tissue layer of *V. faba* leaves was found to contain the highest concentration of the enzyme. Strips of the epidermal tissue were separated from the rest of the leaf and they were homogenized in 1 vol. of 0.01 M Tris–HCl, pH 7.0. The resulting suspension was centrifuged and the supernatant was assayed for β-amylase activity. It was found that the activity per g wet weight of tissue in the epidermal layer was 3–5 times greater than the activity found in extracts prepared from other parts of the leaf.

In order to examine the subcellular distribution of β-amylase, chloroplasts were prepared from *V. faba* leaves using either 0.4 M sucrose or 0.35 M NaCl–0.1 M Tris–HCl buffer (pH 7.0) as the suspending medium[26]. The final washed pellet was resuspended in 20 ml of 0.1 M Tris–HCl (pH 7.0) and it was sonicated at 20 kcycles for 3-min intervals for a total time of 30 min. Microscopic examination showed that almost all the chloroplasts in this preparation had been ruptured. The broken chloroplast fraction was assayed and it was found to be devoid of β-amylase activity. When 25 g of leaves homogenized in 5 vol. of the same buffer were subjected to differential centrifugation almost all of the β-amylase activity present in the homogenate was recovered in the soluble 100 000 × *g* supernatant fraction. These data indicate that the enzyme, which is readily extracted from whole leaves with dilute buffer, is probably present in the cytoplasmic fraction of the epidermal cells in these leaves.

Purification of β-amylase from V. faba leaves

A purification procedure was developed which yielded 6 mg of pure enzyme from 250 g of leaves. All of the steps were carried out at 3 °C and the yields and activities through all stages of purification are summarized in Table I. A crude extract was obtained by homogenizing 250 g of leaves in a Waring Blender with 700 ml of 0.01 M Tris–HCl (pH 7.0) 0.05 M 2-mercaptoethanol and centrifuging the

TABLE I

PURIFICATION OF β-AMYLASE FROM 250 g OF *V. faba* LEAVES

Fraction	*Vol. (ml)*	*Protein (mg/ml)*	*Total activity (units)*	*Specific activity (units/mg)*	*Yield (%)*
Crude extract	1000	1.94	4500	2.3	100
First acetone precipitation	206	0.48	3700	37.3	82
Second acetone precipitation	10	4.82	2980	61.8	66
Chromatography on Biogel P-100	24.5	1.42	2523	73.5	56
Chromatography on Sephadex G-200	50	0.59	2445	82.4	54
Third acetone precipitation	2.3	2.6	602	100.0	13

suspension. An equal volume of acetone at −5 °C was added to 1000 ml of the crude extract and the precipitate which formed was collected by centrifugation and suspended in 200 ml of 0.01 M Tris–HCl (pH 7.0)–0.05 M 2-mercaptoethanol. Undissolved protein was removed by centrifugation and the enzyme (Fraction 2, Table I) was further purified by a second precipitation with acetone. Most of the β-amylase was precipitated between 0.35 and 0.5 vol. of acetone at this stage of purification (Fraction 3, Table I) The enzyme, in 10 ml, was precipitated by the addition of 10 ml of saturated $(NH_4)_2SO_4$ at 3 °C and the precipitate was collected by centrifugation and it was dissolved in 3 ml of buffer. The solution was applied to a Bio-gel P-100 column (2.2 cm × 30 cm) and the column was eluted with 0.01 M Tris–HCl (pH 7.0)–0.05 M 2-mercaptoethanol. Fractions of 3 ml were collected at a flow rate of about 5 ml/min. The fractions containing significant β-amylase activity were pooled (Fraction 4, Table I) and the enzyme, in a volume of 24.5 ml, was precipitated by the addition of 24.5 ml of saturated $(NH_4)_2SO_4$ at 3 °C. The suspension was centrifuged and the precipitate was dissolved in 3 ml of buffer and it was layered on the top of a Sephadex G-200 column (2.2 cm × 27 cm). The column was then eluted with 0.01 M Tris–HCl (pH 7.0) and fractions of 3 ml were collected at a flow rate of 0.5 to 2 ml/min. The enzyme was eluted in a nearly symmetrical peak Fractions containing β-amylase activity were combined (Fraction 5, Table I) and 16.7 ml of saturated $(NH_4)_2SO_4$ at 3 °C was added to this solution. The resulting precipitate was removed by centrifugation, and the clear supernatant solution was adjusted to 0.5 saturation by the addition of 33.3 ml of saturated $(NH_4)_2SO_4$. The precipitate was collected and it was dissolved in 2 ml of 0.1 M Tris–HCl, (pH 7.0). 2 ml of acetone at −10 °C was added to this solution and the suspension was centrifuged to remove the inactive precipitate. Then 0.4 ml of acetone was added to 4.0 ml of the clear supernatant solution. A fine precipitate which formed when this mixture was stirred for 15 min at −10 °C was collected by centrifugation and dissolved in 0.1 M Tris–HCl (pH 7.0) and dialyzed against the same buffer for 3 h.

Properties of leaf β-amylase

The final specific activity of the purified enzyme was 100 μmoles/min per mg (Fraction 6, Table I). The purification summarized in Table I was obtained with at least 10 preparations, however, the recovery with many preparations was as high as 30%. The procedure yielded a homogeneous protein when the last acetone precipitation step was included.

The specific activity of the purified enzyme isolated from leaves was considerably lower than those of most β-amylases isolated from grains and tubers. The specific activities of purified sweet potato, barley, and wheat β-amylases under comparable conditions would be about 500–1000 μmoles/min per mg[27]. Moreover, it has been reported that extracts of ungerminated barley contain inactive forms of β-amylase which can be activated by the addition of 2-mercaptoethanol or H_2S[27]. Experiments were carried out to determine if the lower specific activity was a characteristic of the leaf β-amylase or whether it was due to the presence of an inactive form of the enzyme in the purified preparation. Although 2-mercaptoethanol greatly increased the stability of leaf β-amylase at all stages of purification, it did not increase the total activity of β-amylase in any of these preparations even when they were incubated at 25–37 °C for periods of up to 3 h in the presence of 0.2 M 2-mercapto-

ethanol. The activity of β-amylase in crude extracts from 10 different preparations varied between 12 and 18 units per g wet weight of leaves, however, the final specific activity of the purified enzyme obtained, in each case, was 100 μmoles/min per mg. The final specific activity of the homogeneous preparations was also independent of the age of the leaves and the method of extraction. Thus, it seemed unlikely that the lower specific activity was due to the presence of inactive forms of β-amylase in the purified preparation which could be activated by reduction with 2-mercaptoethanol. It must therefore be concluded that β-amylase from this leaf tissue has a lower specific activity than those from most grains and tubers.

The enzyme was most stable when it was stored at 3 °C in the presence of 0.05 M β-mercaptoethanol. About 50% of the activity remained after one week under these conditions Repeated freezing and thawing resulted in a large loss of activity. The activity of the enzyme was strictly linear and proportional to enzyme concentration under the standard assay conditions, when a soluble corn starch was used as the substrate. The rates obtained with 25 μg, 12.5 μg, 6.25 μg and 2.5 μg of purified enzyme were 2.42, 1.19, 0.60 and 0.24 μmoles of maltose formed per min, respectively.

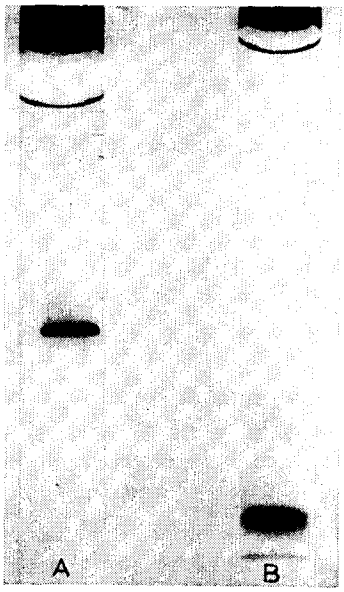
Characteristics of purified β-amylase from V. faba leaves

Polyacrylamide gel electrophoresis of the purified enzyme at pH 8.9 and pH 4.3 showed the presence of only a single component. Approx. 100 μg of the enzyme was applied to the gel, and with some preparations, two very minor diffuse bands were sometimes also observed. These impurities comprised less than 3% of the total protein present and they were completely removed by repeating the last fractionation step with acetone As shown in Fig. 1, only one protein band was detected by development with amido black or by scanning at 280 nm. An unstained gel was sliced and the enzyme was eluted and assayed for activity by the standard procedure as described under Experimental Procedure. β-amylase activity was found only in the area of the gel which also absorbed at 280 nm.

High speed ultracentrifugation of the purified enzyme preparation showed the presence of a peak which had a sedimentation coefficient of 6.5 S in 0.05 M Tris–HCl, pH 7.5 at 20 °C. The enzyme sedimented as a single boundary and there was no significant effect of concentration in the range from 8–3 mg per ml of protein. The molecular weight of the protein determined by sedimentation equilibrium was 110 000. This run was carried out in the same buffer at a protein concentration of 0.3 mg/ml at 20 °C.

The elution pattern of the preparation from Fraction 6, Table I on gel filtration also showed that the enzyme was essentially homogeneous. The enzyme, 5 mg in 5 ml, was carefully layered on the top of a Sephadex G-200 column (2.5 cm × 27 cm), and the column was eluted at flow rates of from 0.5–1.0 ml/min as described previously. Only a single symmetrical protein peak with enzymatic activity was eluted from the column. The fractions taken across the peak had a constant specific activity of 100 μmoles/min per mg protein.

Thus, the results obtained from polyacrylamide gel electrophoresis, high speed ultracentrifugation and chromatography on Sephadex G-200 indicate that the β-amylase isolated from leaves of *V. faba* is homogeneous. The limiting specific activity of the enzyme from leaves is 100 μmoles/min per mg of protein at pH 5.0 and 30 °C,



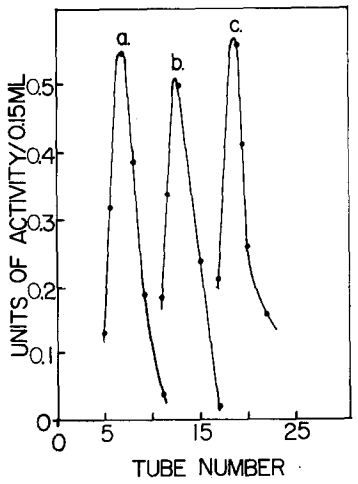


Fig. 1. Polyacrylamide gel electrophoresis patterns of purified β-amylase. Gel A was run at pH 4.3 in sodium acetate buffer, and Gel B was run at pH 8.9 in Tris–glycine buffer. In each case 100 μg of enzyme with a specific activity of 100 units per mg of protein was applied to the gel.

Fig. 2. Sucrose density gradient centrifugation of β-amylase. Approx. 2 units of β-amylase, 5 units of lactate dehydrogenase and 2 units of pyruvate kinase in a total volume of 0.1 ml, were layered on a 5–20% linear sucrose gradient (10.5 ml). After centrifugation at 37 000 rev./min for 16.5 h at 3 °C the solution was fractionated by collecting 0.15-ml samples, and the activity of each enzyme was assayed as indicated in the text. The fractions were numbered starting from the bottom of the gradient. Curve a, the elution profile of pyruvate kinase activity; Curve b, the elution profile of lactate dehydrogenase activity; Curve c, the elution profile of β-amylase.

if it is assumed that the purified preparation does not contain inactive physically identical forms of β-amylase.

Determination of molecular weight by sucrose density gradient centrifugation and chromatography on Sephadex G-200

The molecular weight of purified β-amylase was determined by centrifugation in a sucrose gradient. The relative distribution of β-amylase, lactate dehydrogenase and pyruvate kinase after centrifugation in a 5–20% sucrose gradient is shown in Fig. 2. From several runs a molecular weight of 107 000 was calculated from the ratio of the distance of migration of β-amylase relative to lactate dehydrogenase and pyruvate kinase. The molecular weight was determined by using the relationship $\log d_1/d_2 = 2/3 \log M_1/M_2$ as suggested by Martin and Ames[14]. Similar values were obtained by chromatography on Sephadex G-200 and sedimentation analysis as shown below.

The elution volume of β-amylase on a Sephadex G-200 column (2.2 cm $\times$ 27 cm) was compared with the elution volumes of lactate dehydrogenase, aldolase and hexokinase on the same column. The column had a bed volume of 102.6 ml, and a void volume of 41 ml as determined with Blue Dextran 2000. The elution volume of β-amylase was 68.5 ml. Calculations based on plots of the log of the molecular weight

against elution volume according to the method of Andrews[15] indicated a molecular weight of approx. 110 000 for β-amylase. The Stokes radius calculated by the method of Ackers[17] was 31.8 Å. The effective pore radius of the gel was taken as 187 Å. A molecular weight of 109 000 for β-amylase was derived using a partial specific volume of 0.733 ml/g, a Stokes radius of 31.8 Å and an elution volume of 68.5 ml. This value is in good agreement with the molecular weight determined by sucrose density centrifugation. The diffusion constant of the purified enzyme, which was determined according to the procedure of Siegel and Monty[18], was $6.49 \cdot 10^{-7}$ $cm^2 \cdot s^{-1}$.

The molecular weight of the polypeptide chains of purified β-amylase was determined by electrophoresis on polyacrylamide gels in the presence of 0.1% sodium dodecyl sulfate. The protein was completely dissociated by incubation in the presence of 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol. The monomer migrated as a single band, and the molecular weight was estimated to be 26 000 ± 1500 on the basis of the distance migrated by standard proteins. The semilog plot of molecular weight against distance of migration of lysozyme, D-glyceraldehyde-*P* dehydrogenase, chymotrypsinogen and ovalbumin was linear. On the basis of a molecular weight of 107 000 for the native enzyme it appears that *V. faba* β-amylase is a tetramer of four identical subunits of molecular weight 26 000.

Amino acid composition

Purified β-amylase (specific activity of 100 units per mg) was hydrolyzed, and analyzed for amino acid composition. The thoroughly dialyzed samples were hydrolyzed in evacuated sealed tubes at 100 °C with 6 M HCl for 24 and 44 h. The data obtained from an analysis of the amino acid composition is summarized in Table II.

V. faba β-amylase contained relatively large amounts of aspartic and glutamic acid. If the amide residues of the enzyme were low, then the high level of acidic amino acids could, in part, explain the high mobility of the enzyme at pH 8.9 on disc gel electrophoresis (Fig. 1), and the strong binding of the enzyme to DEAE-cellulose columns. Very high salt concentrations, greater than 0.3 M, were required to elute the enzyme from this cationic resin. The enzyme contained relatively small amounts of half-cystine, methionine and tryptophan. The amino acid composition of the leaf enzyme was found to be quite similar to those of the enzymes isolated from barley, malt and wheat, although the molecular weights of the respective enzymes varied considerably (Table II). After removal of β-mercaptoethylamine by extensive dialysis against 0.2 M Tris–HCl (pH 7.0) the A_{280}:A_{260} ratio of the purified enzyme was found to be 1.7. A partial specific volume of 0.733 ml/g for β-amylase was calculated from the amino acid composition[28].

Using an average molecular weight of 107 000 and a maximum specific activity of 100 μmoles/min per mg, the turnover number of the enzyme was calculated to be 10 700 moles of maltose liberated per min per mole of enzyme under the standard assay conditions.

The pH optimum of V. faba β-amylase

The dependence of enzyme activity on the pH of the reaction mixture was examined at 30 °C (Fig. 3). The standard incubation conditions were used in these experiments and the rate at pH 6.0 was 0.2 μmole/min. The pH optimum of the purified enzyme was found to be 6.0, and the enzyme had approx. 15% and 70% of

TABLE II

AMINO ACID COMPOSITION OF β-AMYLASES ISOLATED FROM *V. faba* LEAVES, BARLEY, MALT AND WHEAT

5-mg samples of β-amylase were dialyzed and hydrolyzed in evaculated sealed tubes for 24, 44, and 72 h at a concentration of 1 mg/ml with 6 M HCl at 105 °C.

Amino acid	*V. faba leaf β-amylase*					
	Residues per 107 000 g[a]	*24 h (g/100 g)*	*44 h (g/100 g)*	*Barley*[e] *(g/100 g)*	*Malt*[e] *(g/100 g)*	*Wheat*[f] *(g/100 g)*
Lysine	57	6.9	6.8	3.7	5.4	4.1
Histidine	16	2.0	2.1	2.0	3.7	4.2
Arginine	35	5.0	4.8	5.3	8.0	7.1
Half-cysteine	9	1.8[b]	—	1.5	0	2.0
Aspartic acid	125	12.9	13.5	9.7	12.1	10.7
Threonine	55[c]	5.1	5.0	4.2	3.2	2.7
Serine	76[c]	6.1	5.9	4.3	3.9	2.9
Glutamic acid	100	12.2	12.1	15.4	13.8	12.0
Proline	48	4.5	4.4	5.2	6.1	5.0
Glycine	88	4.6	4.7	5.7	4.4	4.3
Alanine	32	4.3	4.4	5.0	5.2	4.8
Valine	71[c]	6.0	6.2	6.2	6.9	6.2
Methionine	8	0.81[b]	—	1.3	1.4	2.6
Isoleucine	56[c]	5.2	5.4	3.2	4.0	3.7
Leucine	96	9.6	10.1	8.9	8.1	8.4
Tyrosine	44	6.0	6.7	3.6	5.2	6.2
Phenylalanine	45	5.8	6.2	4.7	4.6	5.5
Tryptophan	8	1.4[d]	—	—	—	3.4

[a] The values are average or extrapolated values calculated from data obtained after 24, 44 and 72 of hydrolysis. The number of residues were calculated using a molecular weight of 107 000 for β-amylase and the values were estimated by taking the nearest whole number. The recoveries after 24 and 44 h of hydrolysis were generally greater than 95% and the total number of residues per mole of enzyme was 969.

[b] The cysteine and methionine were converted to cysteic acid and methionine sulfoxide by oxidation with performic acid prior to hydrolysis.

[c] Extrapolated values to zero time for the hydrolysis of serine and threonine and to infinite time for valine and isoleucine.

[d] Determined on samples hydrolyzed in 6 M HCl in the presence of 4% thioglycollic acid.

[e] Data obtained from Ernst *et al.*[34]. The molecular weights of barley and malt β-amylases were 63 000 and 93 000, respectively.

[f] Data obtained from Tkachuk and Tipples[35]. The molecular weight of crystalline wheat flour β-amylase was 64 200.

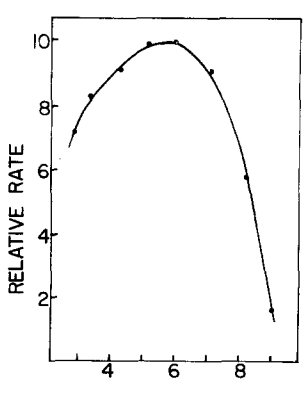


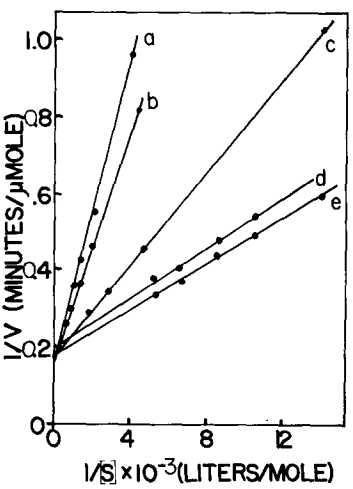
Fig. 3. Influence of pH on the activity of β-amylase from *V. faba*. The reaction mixture was incubated at 30 °C and contained in 1.6 ml: 1% soluble corn starch, 0.05 M buffer and enzyme. Sodium acetate buffer was used from pH 2.9 to 5.2, sodium phosphate buffer was used from pH 6.0 to 7.0 and Tris–HCl buffer was used in the range from pH 7.5 to 9.0.

the maximal activity at pH 9.0 and pH 3.0, respectively. The profile of the activity *versus* pH curve for leaf β-amylases was similar to those isolated from other sources[1].

Influence of substrate composition and concentration on the velocity of the reaction

Essentially stoichiometric quantities of maltose were formed from linear starch substrates when they were incubated with the purified enzyme. Amylopectins and glycogen were converted to maltose and high molecular weight limit dextrins when they were incubated with a large excess of the purified enzyme. The limit dextrins isolated from these reaction mixtures were not further degraded when they were incubated with 1 mg of the purified enzyme, which strongly indicated that the final preparation is completely free of any α-amylase activity.

The influence of substrate concentration on the activity of purified β-amylase as a function of chain length was examined and the results are summarized in Fig. 4. The apparent Michaelis constants calculated by a weighted least squares analysis of data obtained with maltodextrins containing 9, 16, 31, 50 and 98 glucose equivalents per chain were found to be $1.3 \cdot 10^{-3}$ M, $6.7 \cdot 10^{-4}$ M, $3.6 \cdot 10^{-4}$ M, $1.6 \cdot 10^{-4}$ M and $1.7 \cdot 10^{-4}$ M, respectively. The apparent Michaelis constant of amylose type substrates was strongly dependent on the length of the polysaccharide chain below about 50 glucose equivalents per chain as shown in Fig. 5. Amylodextrins with chain lengths greater than 50 residues all had apparent Michaelis constants in the range of $1.3 \cdot 10^{-4}$ M to $1.7 \cdot 10^{-4}$ M. In marked contrast, changes in the chain length of the maltodextrin added to the reaction mixture had practically no effect on the maximum veloc-



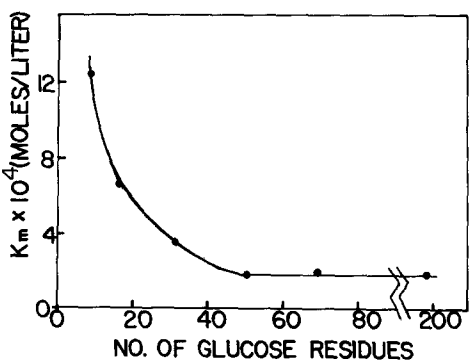


Fig. 4. The dependence of the initial velocity of the reaction on the concentration of maltodextrin as a function of varying average chain lengths. The reaction mixtures were incubated at 30 °C and contained 35 mM sodium acetate buffer, pH 5.0, appropriate amounts of purified enzyme and increasing concentrations of each maltodextrin in a total volume of 1.6 ml. Curve a, was obtained with a maltodextrin which had an average chain length of 9 glucose units; Curve b, was obtained with a maltodextrin which had an average chain length of 16 glucose units; Curve c, with 31 glucose units; Curve d, with 50 glucose units; Curve e, with 98 glucose units.

Fig. 5. Influence of the chain length on the apparent Michaelis constant of linear maltodextrin substrates of β-amylase.

ity. The maximum velocities, expressed as μmoles·min^{-1}·mg^{-1}, were: 9 residues, 98; 16 residues, 97; 31 residues, 94; 50 residues, 90; 98 residues, 95.

It should be noted at this point that data obtained from many experiments similar to those described in Fig. 4, indicate that a change in the chain length of a maltodextrin had almost the same influence on the reciprocal of the velocity of the reaction as a comparable change in the concentration of the maltodextrin based on the total concentration of "maltose equivalents" present. Thus, for example, at a 1/[maltodextrin] value of 8000 l/mole the 1/velocity value for the maltodextrins with 50, 31 and 9 glucose residues were 0.27, 0.45 and 1.50 min/μmole, respectively. If the concentration of the maltodextrins were adjusted so that the number of "maltose equivalents" in the reaction mixtures were equal, then the 1/velocity values for maltodextrins with 50, 31 and 9 glucose residues were found to be 0.27, 0.28 and 0.27, respectively. The corresponding 1/[maltodextrin] values were 1450, 4950 and 8000 l/mole in this experiment. From this evidence it may be tentatively suggested that after the maltodextrin has reached a chain length of at least 50 glucose residues, the collision of a molecule of substrate and enzyme will yield an enzyme–substrate complex which is completely saturated with respect to the binding sites for "maltose equivalents." When substrates of shorter chain length are present a proportionally higher concentration of maltodextrin and more collisions are required to achieve the same degree of saturation of these binding sites.

Mechanism of hydrolysis of maltodextrins by the purified enzyme

The action pattern of the β-amylase isolated from *V. faba* was also examined with maltodextrins of various chain lengths. In these experiments, samples were removed from reaction mixtures at various times and the chain length of the maltodextrins formed during incubation with the enzyme was determined by chromatography on Bio-gel P-10 columns. Amylases may hydrolyze starch by a number of different mechanisms. The enzyme could degrade a linear starch substrate to maltose without releasing lower molecular weight intermediates. Alternatively, the enzyme could remove several maltose units from an amylose chain, and the lower molecular weight intermediate might then dissociate from the enzyme. In preliminary experiments it was found that the β-amylase from *V. faba* appeared to hydrolyze starch by the former mechanism. The data obtained in a typical experiment with a high molecular weight substrate are summarized in Fig. 6. Many maltodextrins of intermediate chain lengths were formed when a linear maltodextrin with 198 glucose units per chain was hydrolyzed with α-amylase obtained from either porcine pancreas or *V. faba* seeds. The results obtained with the *V. faba* α-amylase are summarized in Fig. 6A. The average chain length of the maltodextrin found in the reaction mixture clearly decreased with time of incubation in this case. Since α-amylases are endohydrolases, they would be expected to form high concentrations of lower molecular weight products. When a high molecular weight maltodextrin was hydrolyzed by the purified leaf β-amylase the amount of maltodextrin decreased as maltose accumulated, but the size of the maltodextrin chain did not decrease below the exclusion limit of the Bio-gel P-10 column (Fig. 6B). As seen in the figure, there was no shift in the peak of the curves toward the right which would indicate larger elution volumes and hence lower molecular weights. Instead, only the size of the peak decreased with time of incubation.

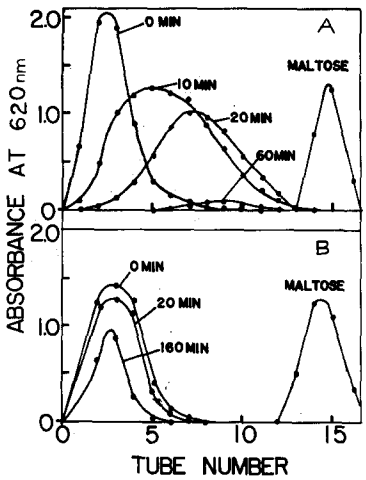


Fig. 6. The time-course and molecular weight of products formed from linear maltodextrins by α-amylase obtained from *V. faba* seeds and by β-amylase isolated from leaves of *V. faba*. The upper figure (A) shows the elution pattern of products formed by α-amylase at various times. The elution volume of maltose in this column is shown by the position of the last peak in A. The lower figure (B) shows the elution pattern of products formed by β-amylase. The standard incubation mixture and assay were used with a maltodextrin substrate of 198 glucose equivalents per chain. The Bio-gel P-10 columns (2.2 cm × 25 cm) were eluted with water and fractions of approx. 6 ml were assayed as described in the text. Only the maltose peak obtained after 10 min of incubation is shown in B.

In order to obtain additional evidence for the possibility that intermediates were being released from the enzyme, experiments were carried out utilizing starch uniformly labelled with ^{14}C and the information obtained in the studies described above. The hydrolysis of ^{14}C-labelled starch was carried out in the presence of maltodextrins with chain lengths of from 9 to 31 glucose units per chain Samples were removed from the incubation mixture at various times until more than 90% of the labelled starch was hydrolyzed to maltose. The reaction mixture was incubated at 30 °C and contained in 5 ml: 35 mM sodium acetate buffer, pH 5.0; 25 μCi of uniformly labelled starch (1 μCi/mg); 0.9 μmole of maltodextrin and an appropriate amount of enzyme. The products of the reaction were chromatographed on Bio-gel P-10 columns as described previously, and they were examined by chromatography on paper and thin-layer plates. The maltodextrins reisolated from the reaction mixture by chromatography on Bio-gel P-10 columns contained only traces of radioactivity. Calculations based on the total amount of ^{14}C found in the maltodextrin fractions indicated that less than 0.01% of the radioactive starch was converted to maltodextrins with chain lengths of 9 to 31 maltose units during the entire course of the reaction. Furthermore, when samples were chromatographed on paper with several solvent systems[8–10] or resolved by chromatography on silica gel plates[8] only radioactive high molecular weight starch, maltose and very small amounts of glucose and maltotriose were detected. The same results were obtained with samples of ^{14}C-labelled starch prepared by several different procedures[25,26]. Thus, these results show that only very small amounts of labelled intermediates were released into non-radioactive pools of maltodextrins during the course of hydrolysis of this substrate

by *V. faba* β-amylase. However, since only very small amounts of lower molecular weight products would be formed by the action of an exohydrolase on a very high molecular weight substrate, other experiments were carried out with maltodextrins of much lower molecular weight.

Additional evidence for the observation that the maltodextrin was being hydrolyzed to maltose without a detectable release of intermediates from the enzyme was obtained by measuring the rate of hydrolysis of maltodextrins which have average chain lengths of 16 and 20 glucose units per mole (Fig. 7). The exclusion volumes of these maltodextrins were well within the exclusion limit of the Bio-gel columns used in these experiments. The results summarized in Fig. 7 indicate that there was still no significant shift in the position of the peaks, in each case, even after more than 80% of the maltodextrin had been hydrolyzed. Since the apparent K_m of linear maltodextrins increased with decreasing chain-length, even a small dissociation of intermediates from the enzyme would be expected to favor the accumulation of low molecular weight compounds since these substrates would be hydrolyzed more slowly by the enzyme. The experiments described in Fig. 6B and Fig. 7 were designed to detect the conversion of various maltodextrin substrates to lower molecular weight intermediates in the range of about 9 to 30 glucose units per chain. In order to detect the possible release of lower molecular weight intermediates aliquots of the reaction mixtures, as well as concentrated samples eluted from the Bio-gel P-10 in the vicinity of the maltose peak were examined by paper and thin-layer chromatography. Only the added maltodextrin, maltose and very small amounts of glucose and maltotriose were found in these samples, which again suggested that sizeable

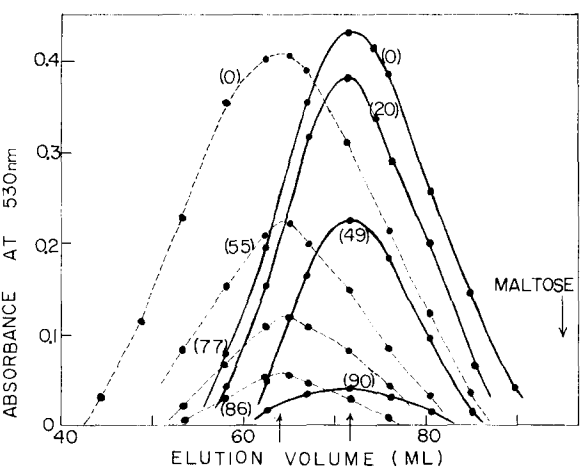


Fig. 7. Chromatography of products formed during hydrolysis of maltodextrins which have average chain lengths of 16 and 20 glucose units. The elution patterns obtained during the hydrolysis of a maltodextrin with 20 glucose units are shown as dotted lines, and the extent of reaction, in each case, is indicated in parentheses. The elution patterns obtained upon hydrolysis of a maltodextrin with 16 glucose units are shown as solid lines and the extent of hydrolysis is indicated in parentheses. The Bio-gel columns (2.2 cm × 25 cm) were eluted with water and fractions of 1.5 ml were collected and assayed as described in the text. The elution volume of maltose in this column is indicated by the arrow.

quantities of lower molecular weight intermediates were not being released during hydrolysis.

Quantitative studies were carried out to compare the rate of formation of maltose with the rate of utilization of maltodextrin as a function of time. The decrease in the concentration of maltodextrin as well as the formation of maltose was measured during the course of a reaction in which a maltodextrin with 16 glucose units per mole was used as the substrate. The amount of maltodextrin present after 0, 20, 49 and 90% hydrolysis was 0.30, 0.25, 0.14 and 0.04 μmole/ml, respectively. The corresponding amount of maltose formed was 0, 0.44, 1.3 and 2.1 μmoles/ml. It was calculated, that the molar ratio of maltose formed to maltodextrin utilized at various times of hydrolysis was about 8:1, and this ratio was essentially constant during the entire course of hydrolysis. Similar results were obtained when the enzyme was incubated for various times with other maltodextrins. The amount of maltose formed, in each case, corresponded to the amount of maltodextrin hydrolyzed, thus supporting the observation that leaf β-amylase binds substrates and hydrolyzes them to maltose without releasing detectable amounts of intermediate molecular weight maltodextrins.

DISCUSSION

The data obtained in the present study may be explained in terms of the relative rates of the following series of binding and hydrolytic reactions as they influence the rate of formation of maltose, and the dissociation of enzyme–substrate intermediates:

$$E + \text{maltodextrin}_n \leftrightharpoons E \cdot \text{maltodextrin}_n \quad (1)$$
$$E \cdot \text{maltodextrin}_n \rightarrow E^1 \cdot \text{maltodextrin}_n \quad (2)$$
$$E^1 \cdot \text{maltodextrin}_n + x\ \text{maltodextrin}_n \leftrightharpoons E^1 \cdot (x + 1)\ \text{maltodextrin}_n \quad (3)$$
$$E^1 \cdot \text{maltodextrin}_n + m\ H_2O \rightarrow E^1 \cdot \text{maltodextrin} + m\ \text{maltose} \quad (4)$$
$$E^1 \cdot \text{maltose (glucose, maltotriose)} \leftrightharpoons E + \text{maltose (glucose, maltotriose)} \quad (5)$$

The formation of enzyme·maltodextrin$_n$ complexes by equilibration of enzyme and maltodextrin (Reaction 1) may be a relatively rapid reaction, and it is possible that a loose enzyme·maltodextrin complex is first formed with all of the maltodextrins tested. The initial binding reaction is then followed by a much tighter essentially irreversible rearrangement of the E·maltodextrin$_n$ complex (Reaction 2). If the chain length of the maltodextrin is less than 50 glucose residues more maltodextrin may be bound (Reaction 3). This possibility is included here because in the present study it was found that an increase in chain length of a maltodextrin had almost the same effect on the velocity of the reaction as an equivalent increase in the concentration of the maltodextrin. It might be tentatively suggested that saturation of binding sites on *V. faba* β-amylase is dependent on the total "maltose equivalents" present in the reaction mixture. The E^1·maltodextrin$_n$ is then hydrolyzed to maltose (Reaction 4), and the final product maltose, glucose or maltotriose then dissociates from the enzyme (Reaction 5). Generally, β-amylases hydrolyze only penultimate linkages starting at the non-reducing end of a maltodextrin chain. They do not usually catalyze the hydrolysis of maltose[29,30]. The ratios of the rates of hydrolysis of maltotriose and maltotetrose are about 1 to 10^3 (ref. 30). The velocities

obtained with higher molecular weight maltodextrins have been reported to be nearly the same as that observed with maltotetrose[31]. These results indicate that maltotetrose is the smallest maltodextrin which is tightly bound and rapidly hydrolyzed by most β-amylases. Accordingly, it is not unexpected that only glucose, maltose and maltotriose are released by the enzyme (Reaction 5).

The kinetic studies on *V. faba* β-amylase show that the K_m of the maltodextrin decreased with increasing chain length up to about 40 to 50 glucose units per chain. Beyond this point the apparent K_m did not vary greatly with increasing chain length. Although the K_m decreased with increasing chain length, the maximum velocity was essentially unaffected. These results show that the chain length of the maltodextrin does not influence the rate limiting step in the sequence of intermediate reactions leading to maltose under conditions of substrate saturation. It is probable that the rate limiting step in this sequence of reactions is the one involving water (Reaction 4). Recently the effect of the molarity of water on the hydrolytic rate of an exo-β-(1 $\rightarrow$ 3) glucanase was studied [32]. A linear relationship between the molarity of water and the rate of hydrolysis was observed in this system, indicating that water was probably involved in the rate limiting step. It has been suggested that some hydrolases may even contain a site for water on the enzyme[30,33].

The hydrolysis of substrate molecules which contain many more maltose units than available binding sites on the enzyme may be explained if it is assumed that the maltose units in the chain of the molecule already bound to the enzyme will be preferentially attracted to binding sites which become available as the amylose molecule is being hydrolyzed. Thus, in experiments similar to those described in Fig. 6B, the enzyme would not be expected to completely bind a maltodextrin with 198 glucose units per chain. In this case, it is proposed that additional binding of "maltose units" in the residual chain occurs as hydrolysis proceeds at the non-reducing end of the molecule. The results obtained in the present study indicate that the area of the enzyme surface which is involved in binding the substrate may be influenced by maltodextrins which contain as many as 50 glucose units per chain. Short chain intermediates of less than 50 glucose units per chain, which must be formed during the breakdown of high molecular weight maltodextrins and remain bound to the enzyme, may be hydrolyzed at a much faster rate than low molecular weight maltodextrins which are added to the reaction mixture. The relatively slow overall rates observed when low molecular weight maltodextrins are being hydrolyzed may be due to rate limiting binding or rearrangement steps which occur in Reactions 1 and 2. These reactions do not appear to be rate limiting when high molecular weight maltodextrins are being hydrolyzed. Furthermore, as indicated in Reaction 3, another molecule of the high molecular weight substrate may be bound to a subsite on the enzyme before the bound low molecular weight intermediate is completely hydrolyzed. As explained previously, this effect may have the same influence on the velocity as increasing the chain length of the maltodextrin which is already undergoing hydrolysis. If the enzyme contains only a single active site, then after each maltose unit is released from the non-reducing end of a maltodextrin chain, the enzyme and the polysaccharide would have to rearrange without completely dissociating in order to bring the next maltose unit into the vicinity of the active site.

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