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THE LIMIT DEXTRINASE FROM MALTED SORGHUM (*Sorghum vulgare*)*

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ABSTRACT

A limit dextrinase, free from contaminating carbohydrases, has been purified from malted sorghum flour. The enzyme readily hydrolysed α-limit dextrins having maltosyl or maltotriosyl side-chains, pullulan, and amylopectin β-limit dextrin. Glycogen β-limit dextrin and amylopectin were more slowly hydrolysed, the detection of the hydrolysis of amylopectin being dependent on enzyme concentration. No significant debranching of glycogen could be detected.

INTRODUCTION

The extensive breakdown of starch to the fermentable sugars D-glucose and maltose forms a major part of the brewing process. This result is achieved by using germinated cereals as a source of both the starch and the enzymes, alpha-amylase, beta-amylase, and limit dextrinase, which are necessary to convert the starch into D-glucose and maltose. The use of germinated rather than ungerminated cereals is necessary since two of these enzymes, alpha-amylase and limit dextrinase, are formed only during germination and are required for the effective degradation of the branched component of starch[2]. The role of limit dextrinase as the enzyme that hydrolyses the (1→6)-α-D-glucosidic linkage is therefore important in achieving the maximal conversion of starch into fermentable sugars.

The action of debranching enzymes towards both oligosaccharide and polysaccharide substrates has now been studied in several plants[1,3,4]. The overall specificity of these enzymes is similar. With oligosaccharide substrates, the enzyme is capable of hydrolysing α-limit dextrins containing at least one (1→4)-α-D-glucosidic linkage on either side of the susceptible (1→6)-α-D-glucosidic linkage. Amylopectin β-limit dextrin is readily attacked with the release of maltose and maltotriose, while amylopectin and glycogen β-limit dextrin are also debranched, but not with the same

*Studies on Debranching Enzymes: Part V. For Part IV, see Ref. 1.

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facility as amylopectin β-limit dextrin. With all these enzymes, however, there is no significant debranching of glycogen. While the enzyme is capable of hydrolysing the (1→6)-α-D-glucosidic linkages in both oligosaccharide and polysaccharide substrates, it is believed that, *in vivo*, small, branched α-dextrins are the true substrate for the enzyme[5].

Kaffircorn, a variety of sorghum (*Sorghum vulgare*), is used extensively in Southern Africa for the manufacture of kaffir beer. Despite its economic importance, research on the biochemistry of this cereal and its germination has been limited. Of the amylolytic enzymes, alpha-amylase[6–8] and beta-amylase[9] have been extensively purified, and the extraction of α-D-glucosidase has been studied[10], whilst the effect of gibberellic acid on amylase formation has been examined[11]. Since an early report of Kneen[12], there appear to have been no further studies on the sorghum debranching-enzyme system. We now describe the purification of this debranching enzyme and its properties. A preliminary account of some of these results has been published[13,14].

MATERIALS AND METHODS

Materials. — The polysaccharide and oligosaccharide substrates were prepared as described previously[1,2,4]. All chemicals were supplied by British Drug House. Chemicals Ltd., Poole, England. Proteins for molecular-weight determination were from the Sigma Chemical Company. Malted sorghum flour was a gift from Dr. L. Novellie of the C.S.I.R., Pretoria, South Africa.

Analytical methods. — Protein was measured by a modification of the Lowry method[15]. Column and electrophoretic fractions were monitored at 280 nm to determine the protein content of the fractions. Reducing sugar was determined by a modified Nelson–Somogyi method[16], either as recommended, or on one-fifth of the scale, so as to give greater sensitivity (mini-Nelson). The reagents were calibrated against maltotriose. Reducing sugars were identified by descending paper chromatography on Whatman No. 1 paper with 1-propanol–ethyl acetate–water[17] (14:2:7). Reducing sugars were located with alkaline silver nitrate[18]. Samples for chromatography were deionised by shaking with a small quantity of Zerolit DM-F resin. The standard solution employed for iodine staining consisted of 0.2% of iodine in 2% potassium iodide (50 ml), made up to 60 ml with 6M hydrochloric acid and then diluted to 500 ml with distilled water.

Ultrafiltration. — This technique was carried out at 4° in Diaflo cells (Amicon Corporation, Lexington, Massachusetts, U.S.A.) fitted with PM-10 or PM-30 membranes.

Enzyme assays. — The purification of the enzyme was followed by measuring its activity in the presence of pullulan (5 mg) and 10mM phosphate–citrate buffer (pH 5.0, 1 ml) at 30°. Samples (0.1 ml) were removed from the digests at suitable intervals for measurement of reducing power. The reducing powers of appropriate enzyme and substrate blanks were also measured after similar incubation. Results are expressed as either the increase in absorbance at 600 nm or as μg of apparent

maltotriose released. One unit of limit-dextrinase activity is defined as the amount of enzyme that releases 1 μmol of equivalent maltotriose from pullulan in 1 min at pH 5.0 and 30° under the above conditions. For convenience, the activities have, in some cases, been expressed as milliunits (mU)[1,4].

The alpha-amylase was also assayed throughout the purification procedure. Digests contained amylopectin β-limit dextrin (2 mg) and enzyme solution (1 ml) suitably diluted in 20mM phosphate–citrate buffer (pH 5.0). Samples (0.1 ml) were withdrawn during incubation at 37° and added to 5 ml of iodine reagent, and the absorbance at 540 nm was measured. One unit of alpha-amylase activity is given by the reciprocal of the time (min) for E_{540} to drop to half of its initial value.

Purification of sorghum limit-dextrinase. — Germinated kaffircorn (*Sorghum vulgare*) flour (500 g) was extracted with 3% potassium chloride (1.6 l) for 4 h at room temperature. The suspension was then centrifuged (6000 *g*; 30 min; 4°) to remove insoluble material. Solid ammonium sulphate (201 g) was stirred into the extract (1150 ml) at 4° to give 30% saturation. Stirring was continued for 1 h, before removing the precipitate by centrifugation (600 *g*; 30 min; 4°). The supernatant solution (1210 ml) was then raised to 50% saturation by the slow addition of ammonium sulphate (151 g). After stirring for a further 1 h, the precipitate was collected by centrifugation (6000 *g*; 30 min; 4°) and the supernatant solution discarded. The precipitate was suspended in distilled water (200 ml, 4°) and dialysed against several changes of distilled water for 72 h at 4°. Any precipitate was removed by centrifugation (30,000 *g*; 30 min; 4°).

Sodium citrate buffer (0.1M; pH 6.0) was added to the enzyme solution (*i.e.*, the 30–50% ammonium sulphate fraction) to give a final buffer concentration of 5mM. The solution was concentrated to 80 ml by ultrafiltration on a PM-10 membrane. It was then applied at 1.5 ml/h to the left-hand, central tab of the paper curtain of a Beckman Spinco model CP continuous-electrophoresis cell, and the separation of proteins was effected at 700 volts and 55 ± 5 mA using 5mM sodium citrate buffer (pH 6.0) to irrigate the paper at 20°. Fractions containing limit-dextrinase activity were combined (see Fig. 1), and 0.2M phosphate–citrate buffer (pH 5.0) was added to give a final buffer concentration of 20mM. The enzyme solution was then concentrated to 4 ml by ultrafiltration on a PM-10 membrane.

Preliminary experiments showed that EDTA, ammonium oxalate, acid treatment, and glycogen precipitation were inadequate in removing or destroying alpha-amylase, a major contaminant in the preparation. A modification of the method reported for the purification of sorghum malt alpha-amylase[6] was used. The enzyme preparation (*i.e.*, the continuous-electrophoresis fraction) was applied to a column (1.5 × 40 cm) of potato starch and Celite (1:2 w/w) equilibrated with 20mM phosphate–citrate buffer (pH 5.0). A single peak of protein containing the limit dextrinase was obtained. These fractions were combined and applied to a freshly prepared, potato starch–Celite column equilibrated in the same manner. The fractions containing limit dextrinase were concentrated to 1 ml by ultrafiltration on a PM-30 membrane and loaded onto a column (40 × 2.5 cm) of Sephadex G-150 (superfine grade) equilibrated

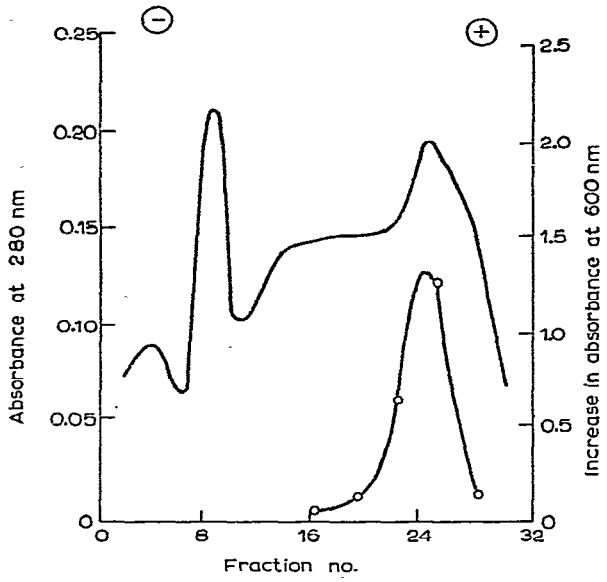

Fig. 1. Continuous electrophoresis of 30–50% ammonium sulphate fraction. For conditions, see text. Key: ————, distribution of protein; O——O, distribution of limit-dextrinase activity.

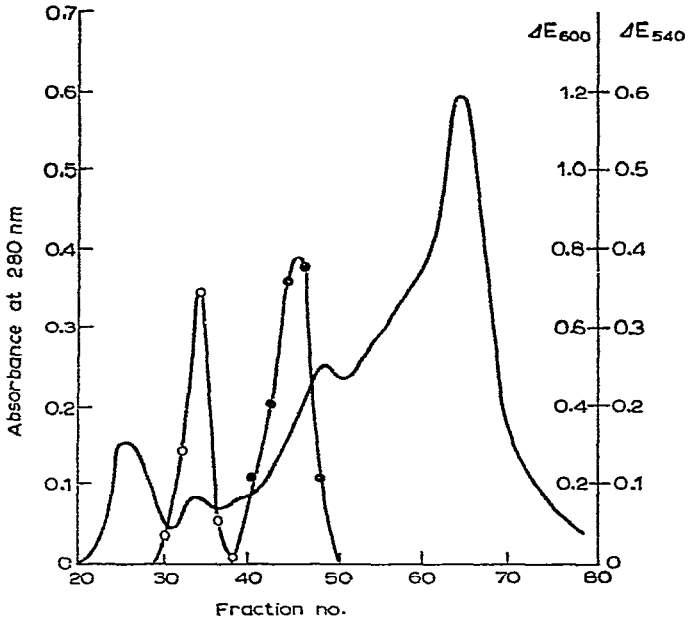

Fig. 2. Sephadex G-150 chromatography of sorghum limit-dextrinase. Key: ————, elution of protein; O——O, elution of limit dextrinase; ●——●, elution of amylase.

with 20mM phosphate–citrate buffer (pH 5.0). The elution patterns of protein, limit dextrinase, and amylase are shown in Fig. 2. At this stage, the enzyme preparation still contained a trace of amylase. The fractions from the single peak of activity were therefore combined and *p*-hydroxymercuribenzoate was added (5 μmol per unit of limit dextrinase) as a selective inhibitor. This treated preparation was used for all further studies. The enzyme solution had a specific activity of ~1.1 unit/mg of protein (Table I) which represented a degree of purification of ~40-fold.

TABLE I

THE PURIFICATION STAGES OF THE LIMIT-DEXTRINASE PREPARATION

Fraction	*Total protein (mg)*	*Total units*	*Specific activity (units/mg)*	*Yield (%)*
1. Initial extract[a]	2275	66	0.029	—
2. 30–50% Ammonium sulphate	765	49	0.064	74
3. Continuous electrophoresis	54	11	0.20	17
4. Starch–Celite	22	7.1	0.33	11
5. Sephadex G-150	2.5	2.7	1.08	4

[a]This represents the protein in solution after centrifuging the homogenate of malted sorghum flour (500 g).

RESULTS

Purity of the enzyme. — Following gel filtration on Sephadex G-150, the enzyme preparation still contained a trace of amylase activity even though there was an apparent separation of the two activities on the column (see Fig. 2). The addition of 5 μmol of *p*-hydroxymercuribenzoate per enzyme unit totally inactivated any contaminating amylase without affecting the limit-dextrinase activity. The action of other possible amylase inhibitors had been previously tested on the enzyme preparation prior to gel filtration and found to be unsatisfactory (Table II).

TABLE II

EFFECT OF VARIOUS INHIBITORS ON THE LIMIT-DEXTRINASE AND AMYLASE ACTIVITIES

Enzyme activity	*Inhibition* (%)			
	EGTA[a]	*EDTA*[b]	*Ammonium oxalate*[c]	p*OHMB*[d]
Amylase	76	0	35	100
Limit dextrinase	98	16	17	0

[a]EGTA = 1,2-Bis(2-aminoethoxy)ethane-N,N,N^1,N^1-tetra-acetic acid (10mM). [b]EDTA = Ethylenediaminetetra-acetic acid (10mM). [c]50mM. [d]*p*-Hydroxymercuribenzoate (0.1mM).

The presence of contaminating carbohydrases was tested using digests containing either maltotriose (5 mg) or linear maltosaccharides (d.p. >15; 5 mg)

and enzyme (1 mU) in 20mM sodium phosphate–citrate buffer (pH 5.0, 0.5 ml). Paper chromatography of the digest after incubation at 30° for 24 h showed no detectable breakdown of these substrates, indicating the absence of alpha-amylase, beta-amylase, α-D-glucosidase, and D-enzyme activities from the enzyme preparation.

General properties of the enzyme. — The pH optimum of the enzyme was determined by using pullulan as substrate in the presence of a range of phosphate–citrate buffers. Maximum activity occurred in the range pH 5.0–5.4, the activity falling off rapidly towards the lower pH values and more gradually towards the higher pH values.

The enzyme was relatively sensitive to heat treatment, being totally inactivated when 15 mU of enzyme in 20mM phosphate–citrate buffer (pH 5.0, 0.5 ml) was heated to 70° for 1 h. However, only 60% inactivation occurred at 60°. These results are consistent with the previous conclusions of Kneen[12].

The molecular weight of the purified enzyme was estimated by molecular-sieve chromatography[19] on a column of Sephadex G-150 which had been calibrated with the proteins cytochrome C, soybean trypsin inhibitor, horseradish peroxidase, and bovine serum albumin. A plot of $V_e - V_0$ (elution volume) against log(molecular weight) showed the limit dextrinase to have a molecular weight of ~90,000.

Action of limit dextrinase on polysaccharide substrates. — Limit-dextrinase activity towards various polysaccharides was monitored from changes in both reducing power and iodine-staining power. Digests contained substrate and enzyme

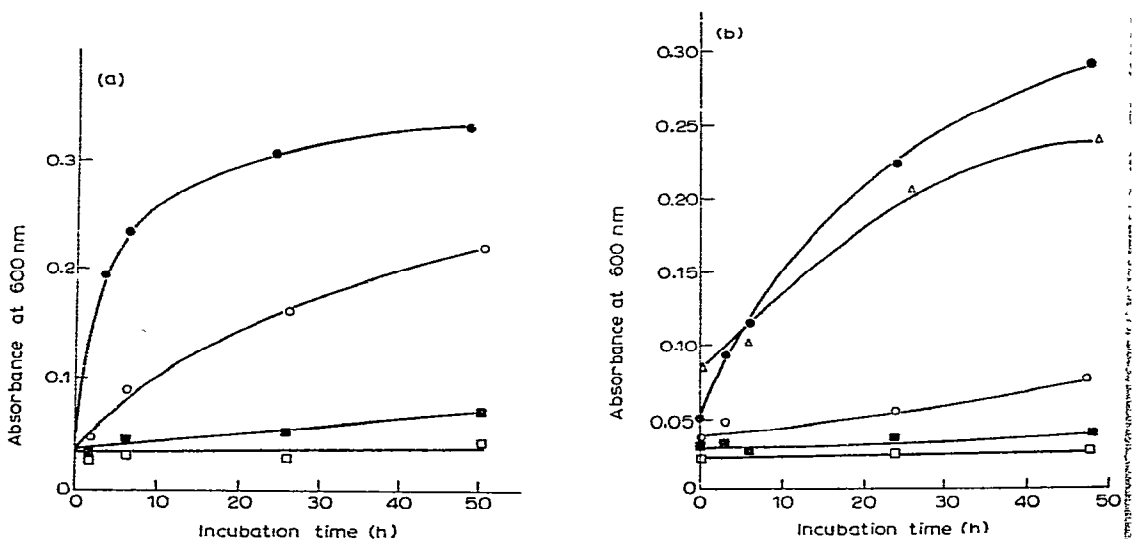

Fig. 3. The production of reducing sugars by purified sorghum limit-dextrinase from polysaccharide substrates with (*a*) 80 mU of enzyme, (*b*) 8 mU of enzyme. Experimental conditions are given in the text. Key: O——O, amylopectin (16 mg in the 6-ml digest); ●——●, amylopectin β-limit dextrin (8 mg); □——□, glycogen (8 mg); ■——■, glycogen β-limit dextrin (4 mg); △——△, amylopectin (16 mg, 1-ml sample analysed).

(80 mU) in 50mM sodium acetate buffer (pH 5.0, 6 ml) and were incubated at 30°. Duplicate samples (0.2 ml) were removed at intervals for the determination of reducing power (see Fig. 3a). Duplicate samples (0.1 ml for amylopectin; 0.2 ml for others) were also removed and added to standard iodine solution (4 ml). For glycogen and glycogen β-limit dextrin, saturated ammonium sulphate (4 ml) was added to the iodine complexes to increase the absorbance[20]. The absorption spectra of the polysaccharide–iodine complexes were then recorded over the range 400–700 nm. The individual absorbances were measured at 680 nm for amylopectin and amylopectin β-limit dextrin, and at 490 nm for glycogen and glycogen β-limit dextrin. The increase in absorbance of the polysaccharide–iodine complexes are detailed in Table III together with the changes in absorption maximum of the complexes.

TABLE III

EFFECT OF LIMIT DEXTRINASE ON THE IODINE-STAINING POWER OF POLYSACCHARIDES

Substrate	*Increase*[a] (%)		*Changes in* λ_{max} (*nm*)
	E_{680}	E_{490}	
Amylopectin	23	—	557 → 576
Amylopectin β-limit dextrin	88	—	536 → 555
Glycogen	—	2	Nil
Glycogen β-limit dextrin	—	14	Nil

[a]Values cited are those after incubation for 25 h at 30°.

TABLE IV

RELATIVE, INITIAL RATE OF ATTACK ON VARIOUS OLIGOSACCHARIDES BY SORGHUM LIMIT-DEXTRINASE

Substrate	*Initial velocity*[a]
Panose	0
6^3-α-Maltosylmaltotriose	220
6^3-α-Maltosylmaltotetraose	300
6^3-α-Maltotriosylmaltotriose	250
6^3-α-Maltotriosylmaltotetraose	400
P-9[b]	250
α-D-Glucosyl-cyclohexaamylose	0
α-Maltosyl-cyclohexaamylose	50

[a]Relative to pullulan = 100. [b]Nonasaccharide isolated from the action of bacterial pullulanase on pullulan.

The previous experiment was repeated at a lower concentration of enzyme (8 mU). In this case, only amylopectin β-limit dextrin produced a significant increase in reducing power (Fig. 3b). All digests still contained active enzyme, since on the addition of pullulan, significant increases in reducing power were obtained. When the digest containing amylopectin and enzyme (8 mU) was scaled-up and larger samples

(1 ml) were removed for analysis, significant increases in reducing power were found.

Action of limit dextrinase on oligosaccharide substrates. — The relative rates of attack of limit dextrinase on oligosaccharides were determined. Digests contained substrate (mM with respect to (1→6)-α-D-glucosidic linkages) and enzyme (10 mU) in 10mM phosphate–citrate buffer (pH 5.0, 1 ml), and were incubated at 30°. Duplicate samples (0.2 ml) were removed at intervals for assay of reducing power. Initial velocities were estimated from plots of reducing power against time of incubation. The values for initial rates are given relative to the value for pullulan (Table IV).

Kinetic studies. — Initial velocities of the reactions were determined at 30° by estimating the rate of increase of reducing power. Reaction mixtures contained, in a total volume of 1 ml, substrate, enzyme (5 mU), and buffer (10mM sodium acetate, pH 5.0). During incubation, aliquots (0.1 ml) were removed, and assayed for reducing sugar by the mini-Nelson method.

TABLE V

KINETIC CONSTANTS OF SORGHUM LIMIT-DEXTRINASE

Substrate	K_m (*mg/ml*)	K_m (*mM*)[a]	V_{max}[b]
Amylopectin	—	—	0.14
Amylopectin β-limit dextrin	2.5	1.5	2.6
Glycogen β-limit dextrin	30	26	3.0
Pullulan	0.2	0.4	1.0
6^3-α-Maltotriosylmaltotetraose	1.7	1.5	5.3

[a]Molarity expressed with respect to (1→6)-α-D-glucosidic linkages using a $\overline{CL}$ of 10 for amylopectin β-limit dextrin and 6 for glycogen β-limit dextrin in these calculations. [b]Relative to V_{max} for pullulan.

The initial velocity data were plotted graphically in double reciprocal form to confirm the linearity of the graphs. The values cited for the kinetic constants (Table V) were obtained from a computer programme[21] which fitted the S and V_0 data to the standard form of the Michaelis–Menten equation. The low rate of attack on amylopectin did not allow an accurate K_m value to be obtained, but it was found that the substrate was effectively saturating the enzyme at 40 mg/ml and the result cited for V_{max} is the initial velocity measured at this substrate concentration.

DISCUSSION

Five enzymes (including four from cereals) have now been purified from higher plants[1,3,4] that catalyse the hydrolysis of the (1→6)-α-D-glucosidic linkages in α-limit dextrins, amylopectin, the β-limit dextrins from glycogen and amylopectin, and pullulan. However, none of the enzymes catalyse any significant debranching of glycogen.

The enzyme in this study, sorghum limit-dextrinase, has been purified 40-fold from an initial extract of malted kaffircorn flour. The results in Table I for the yield and purification are almost certainly underestimates, as the crude extract contained

other enzymes, particularly α-D-glucosidase, which could affect the assay (*cf.* Ref. 22). This enzyme, and also high concentrations of alpha-amylase, hydrolyse maltotriose and hence increase the reducing power of the pullulan-containing digests. The preparation obtained from the sequential application of ammonium sulphate precipitation, continuous electrophoresis, and starch–Celite and Sephadex G-150 chromatography was free from any significant interfering carbohydrases, *e.g.*, alpha-amylase, beta-amylase, α-D-glucosidase, or D-enzyme. The main contaminant during purification was alpha-amylase. The removal of this enzyme using columns of starch–Celite significantly simplified the purification and enabled a more-accurate estimate of limit-dextrinase activity to be made (*cf.* Ref. 2).

The physical properties of the enzyme are generally similar to those of the other plant limit-dextrinases. The elution volume of the enzyme on a calibrated column of Sephadex G-150 showed that it had a molecular weight of ~90,000; this compares closely with those of the enzymes from oats[4] and broad beans[1], which are ~80,000. The sorghum limit-dextrinase is a larger enzyme than the alpha- and beta-amylases, which have molecular weights of ~50,000 and ~54,000, respectively[8,9]. The pH optimum of the limit dextrinase was in the range pH 5.0–5.4 which is similar to all other plant limit-dextrinases so far studied[3,4], with the exception of broad bean[1] (pH 6.6).

The present results show that sorghum limit-dextrinase will hydrolyse some or all of the (1→6)-α-D-linkages in pullulan, amylopectin, the β-limit dextrins of glycogen and amylopectin, α-maltosyl-cyclohexaamylose, and α-limit dextrins containing at least one (1→4)-α-D-glucosidic linkage on either side of the susceptible (1→6)-α-D-glucosidic linkage. This aspect of its specificity is therefore similar to the other plant limit-dextrinases[1,3,4], as well as to the pullulanase from *Klebsiella aerogenes*[23].

This study also confirms the results obtained with potato limit-dextrinase[24] in that the activity towards amylopectin selectively disappears on dilution of the enzyme. This does not represent a real change in specificity, since when larger samples are used for the analysis of reducing sugars, the release of reducing groups from amylopectin is detectable even with low concentrations of enzyme. The apparent change in specificity can therefore be accounted for by the fact that the small amounts of reducing sugar released from amylopectin by low concentrations of enzyme may be below the detection limit of the analytical method[13].

The relative rates of hydrolysis of oligosaccharides are of the same magnitude as those obtained for other plant limit-dextrinases. The rate of hydrolysis increases with the degree of polymerisation of both chains of the oligosaccharide so that, of those tested and having only one (1→6)-α-D-glucosidic linkage, 6^3-α-maltotriosyl-maltotetraose was hydrolysed at the greatest rate. All the α-limit dextrins were hydrolysed at a greater rate than pullulan. It is also significant that 6^3-α-maltotriosyl-maltotriose and P9 were hydrolysed at a rate greater than the parent polysaccharide pullulan, from which they were prepared. The size of the substrate must therefore, at some stage, become a limiting factor in the rate of hydrolysis of the oligosaccharide.

Compared with the rates of hydrolysis for the α-limit dextrins and pullulan-derived oligosaccharides, α-maltosyl-cyclohexaamylose is hydrolysed at a relatively low rate. This result could be explained if cyclohexaamylose, one product of the reaction, was an inhibitor of the enzyme. It has already been reported[25] that Schardinger dextrins are strong inhibitors of both *Klebsiella aerogenes* pullulanase, which resembles the plant limit-dextrinases in many respects, and sweet-corn limit-dextrinase; it is reasonable to assume that cyclohexaamylose might also inhibit sorghum limit-dextrinase.

Kinetic results on polysaccharide substrates have to be approached with some caution, as amylaceous polysaccharides are polydisperse and contain numerous susceptible linkages which may be in different stereochemical environments. In particular, the kinetic constants cannot be expressed in terms of simple, individual, rate constants. Nevertheless, the results do give some insight into the nature of the enzyme specificity. Of the two substrates examined that could be hydrolysed *in vivo*, namely amylopectin and 6^3-α-maltotriosylmaltotetraose, it appears from the kinetic data that the latter would be the preferred substrate *in vivo*. Amylopectin β-limit dextrin is a good substrate having a Michaelis constant similar to that of 6^3-α-maltotriosylmaltotetraose and a V_{max} of ~50% that of the oligosaccharide. It is interesting to note that the V_{max} for the β-limit dextrins from both glycogen and amylopectin are very similar. However, K_m for glycogen β-limit dextrin is 10 times that of the amylopectin β-limit dextrin. One possible interpretation is that the V_{max} or "catalytic" function is determined largely by the length of the side-chains, shorter chains being preferred, whereas the K_m or "binding" function is affected more by the degree of branching. A high degree of branching may result in steric hindrance to the formation of the enzyme–substrate complex and hence result in a high K_m value. Pullulan and 6^3-α-maltotriosylmaltotetraose, for which one would expect little or no steric hindrance to binding, have the lowest K_m values.

The present work, together with that of Novellie and co-workers[8,9], completes the initial purification studies on the major starch-degrading enzymes of sorghum. As proposed by Dunn[26], the key enzyme in this process is alpha-amylase which can act on starch granules. The resulting mixture of linear and branched α-dextrins are then hydrolysed to the disaccharide level by beta-amylase and limit dextrinase, respectively, acting in conjunction with alpha-amylase.

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