Carbohydrate Research, 83 (1980) 109–118
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

PURIFICATION AND CHARACTERISATION OF LIMIT DEXTRINASE FROM *Pisum sativum* L.

DAVID YELLOWLEES
Department of Chemistry and Biochemistry, James Cook University of North Queensland, Townsville (Australia)

(Received October 11th, 1979; accepted for publication, October 19th, 1979)

ABSTRACT

A limit dextrinase has been purified 2,700-fold from ungerminated peas by affinity chromatography. The enzyme hydrolyses (1→6)-α-D-glucosidic linkages in alpha-limit dextrins containing at least one α-(1→4)-linked D-glucose residue on either side of the susceptible linkage. The limit dextrinase also hydrolyses the polysaccharides amylopectin, amylopectin beta-limit dextrin, glycogen beta-limit dextrin, and pullulan, but has no activity towards glycogen.

INTRODUCTION

The role that enzymes play in the mobilisation of starch during the germination of cereal seeds has been well documented[1]. In contrast, the mode of starch hydrolysis, and its control, in the cotyledons of legume seeds during germination is not as well understood. Marked differences exist between pea seeds, the most studied legume, and those of cereals, both in structure and germination pattern. In cereals, the starch is endospermic in origin and thus structurally isolated from the embryo, whereas the cotyledonary reserves of peas are an integral part of the embryo. It is of interest to establish if these differences extend to the enzymes involved in the mobilisation of starch reserves during germination and their control.

Alpha-amylase and beta-amylase are both present in the cotyledons of germinating peas, and their role in the hydrolysis of the (1→4)-α-D-glucosidic linkages in starch is well established[2]. Swain and Dekker[3] presented evidence for the existence of debranching activity in peas, based upon the erroneous assumption that debranching enzymes cause the disappearance of the coloured iodine complex formed with amylopectin beta-limit dextrin. Shain and Mayer used the same assumption to show the presence of an "amylopectin-(1→6)-glucosidase" in peas[4] and that this was produced during germination by the activation of a zymogen[5]. This evidence cannot be used as proof of the existence of a debranching enzyme in peas.

Debranching enzymes in plant tissues are known by the synonyms limit dextrinase and R-enzyme. The former, trivial name is preferred in this report, as it describes more accurately the function of the enzyme *in vivo*. There have been several

0008-6215/80/0000–0000/$ 02.25,

studies of the purification and characterisation of these enzymes from cereal seeds[6-9]. All those so far studied have similar activity and are capable of hydrolysing the (1→6)-α-D-glucosidic linkages in the polysaccharides amylopectin and amylopectin beta-limit dextrin as well as the oligosaccharide alpha-limit dextrins containing at least one (1→4)-α-D-glucosidic linkage on either side of the susceptible (1→6)-α-D-glucosidic linkage. In addition, the polysaccharide pullulan is hydrolysed by limit dextrinase. The ability to hydrolyse this polysaccharide has been important in the detection and assay of limit dextrinases, due to the inability of other amylolytic enzymes to hydrolyse it to any significant extent[10]. The enzyme from pea seeds was detected in this manner and we now describe the purification of this debranching enzyme and its properties. A preliminary account of these results has been presented[11].

MATERIALS AND METHODS

Materials. — Pullulan, amylopectin, amylopectin beta-limit dextrin, glycogen, and glycogen beta-limit dextrin were prepared by standard methods. 6^3-α-Maltotriosylmaltotriose was prepared by the action of *Aerobacter aerogenes* pullulanase on pullulan. 6^3-α-D-Glucosylmaltotriose (B4), 6^3-α-maltosylmaltotriose (B5), 6^3-α-maltosylmaltotetraose (B6), and 6^3-α-maltotriosylmaltotetraose (B7) were prepared from salivary alpha-limit dextrins by paper chromatography on Whatman No. 17 paper with 1-propanol–ethyl acetate–water (14:2:7). The Schardinger dextrins α-D-glucosyl-, α-maltosyl-, and α-maltotriosyl-cyclohexa-amylose were prepared by the method of Manners and Yellowlees[12]. Linear maltosaccharides were prepared from a partial, acid hydrolysis of amylose. Cyclohexa-amylose and 2-mercaptoethanol were supplied by Sigma Chemical Company. Epoxy-activated Sepharose-6B and Sephadex G-150 were purchased from Pharmacia Fine Chemicals, and the protein molecular weight standards were products of Boehringer Mannheim. Pea seeds, *Pisum sativum* (L.) var. Greenfeast, were purchased from Yates and Co. Pty. Ltd., Brisbane.

Analytical methods. — Reducing sugar was determined by a modified Nelson–Somogyi method[13] on one-half the scale recommended. The absorbance was read at 600 nm and the reagents were calibrated against both glucose and maltotriose. Protein was determined by a modified Lowry method[14]; where 2-mercaptoethanol interfered, a further modification was used[15]. Column fractions were monitored at 280 nm, to determine the protein content of the fractions. The standard solution employed for iodine staining consisted of 0.2% of iodine in 2% aqueous potassium iodide (50 ml), acidified with 6M hydrochloric acid (10 ml) and then diluted to 500 ml with distilled water. Reducing sugars were separated by descending paper-chromatography on Whatman No. 1 paper with ethyl acetate–pyridine–water (10:4:3), and detected with alkaline silver nitrate[16].

Enzyme assays. — Limit dextrinase was measured by incubation (30°) of a suitably diluted enzyme solution with pullulan (5 mg) in 20mM sodium phosphate–citrate buffer (pH 6.0) in a total volume of 2 ml. Aliquots (0.5 ml) were removed at suitable intervals for measurement of reducing power. The reducing powers of

appropriate enzyme and substrate blanks were also measured after similar incubation. One unit of enzyme-activity is defined as the amount of enzyme that will produce 1 μmol of equivalent maltotriose from pullulan in 1 min at pH 6.0 and 30° under the above conditions.

Total amylolytic activity was monitored throughout the purification. Digests (1 ml) containing amylopectin (2 mg) and enzyme solution, suitably diluted in 20mM sodium phosphate–citrate buffer (pH 5.0), were incubated at 37°. Aliquots (0.1 ml) were taken at suitable intervals and added to 5 ml of iodine reagent, and the absorbance was measured at 540 nm.

Germination and preparation of extracts of pea enzyme. — The seeds were washed with water, to remove any copper sulphate, before soaking in distilled water for 7 h and surface-sterilising in 1% aqueous sodium hypochlorite for 5 min. The seeds were then washed in sterile water and germinated on moist filter-paper in Petri dishes kept in the dark.

Peas (10) harvested at daily intervals were homogenised in 15 ml of 20mM sodium citrate buffer (pH 6.0), after excision of the developing plumule and radical. After extraction at room temperature for 10 min, the extracts were centrifuged (10,000 *g*, 4°, 10 min), and the supernatant solutions were dialysed for 24 h at 4° against several 1-litre changes of distilled water. The dialysed extracts were used to measure enzyme activity.

Coupling of cyclohexa-amylose to Sepharose 6B. — Cyclohexa-amylose was coupled to epoxy-activated Sepharose 6B by modifying the procedure of Vretblad[17]. Epoxy-activated Sepharose 6B (4 g) was suspended in distilled water (25 ml) and then washed with a further 500 ml of distilled water on a sintered-glass funnel. After being washed with 0.1M sodium hydroxide (25 ml), the gel was added to a solution of cyclohexa-amylose (300 mg) in 0.1M sodium hydroxide (12 ml). The mixture was slowly shaken in a water bath at 45° for 20 h, and then washed in sequence with distilled water (200 ml), aqueous D-glucose (25 mg/ml, 200 ml), distilled water (200 ml), 0.1M sodium borate buffer (pH 8.0, 100 ml) containing sodium chloride (0.5M), and 0.1M sodium acetate buffer (pH 4.0, 200 ml) containing sodium chloride (0.5M). The suspension was filtered between washes. The coupled gel was then equilibrated in 20mM sodium phosphate–citrate buffer (pH 7.0) made 10mM with respect to 2-mercaptoethanol (buffer *A*).

The difference between the amount of cyclohexa-amylose used for activation and that found in the first distilled-water wash after coupling indicated that ~40 μmol of cyclohexa-amylose was immobilised per gramme of epoxy-activated Sepharose 6B.

Purification of the enzyme. — Peas (250 g) were swollen overnight in water before being homogenised at high speed in 3% aqueous potassium chloride (1 litre) for 15 sec in a Waring Blendor. The homogenate was then extracted at room temperature (22–24°) for 2 h before straining through cheese-cloth and then centrifuging (10,000*g*, 10 min, 4°). All further manipulations were carried out in a cold-room at 4°.

Solid ammonium sulphate was stirred into the extract (970 ml) to 30% w/v. After stirring for 2 h, the mixture was centrifuged (10,000*g*, 10 min, 4°) and the supernatant solution discarded. The precipitate was suspended in 20mM sodium phosphate–citrate buffer (pH 7.0) made 0.1M with respect to sodium chloride and 10mM to 2-mercaptoethanol (200 ml). After removal of insoluble material by centrifugation (40,000*g*, 20 min, 4°), the supernatant solution (200 ml) was fractionated with acetone. Cold, redistilled, analytical-grade acetone (−30°) was added slowly to the stirred solution at 0°, to a final concentration of 40% (v/v). The solution was stirred for a further 30 min before centrifugation (10,000*g*, 10 min, 4°). The precipitate was discarded and the supernatant solution brought to a final concentration of 70% of acetone. The resulting precipitate was collected by centrifugation and then dissolved in 50 ml of buffer *A*. Insoluble material was removed by centrifugation (40,000*g*, 5 min, 4°).

The 40–70% acetone fraction was applied to a column (0.8 × 5 cm) of cyclohexa-amylose–Sepharose 6B prepared as described above. The column was washed with buffer *A* until the absorbance at 280 nm stabilised, when it was eluted with buffer *A* containing cyclohexa-amylose (2 mg/ml). Little cyclohexa-amylose, as measured by the phenol–sulphuric acid method[18], was present in the active fractions and was removed by passage of the combined fractions through a column (10 × 1 cm) of Sephadex G-15 previously equilibrated with 20mM sodium phosphate–citrate buffer (pH 6.0) containing 0.1M sodium chloride and 10mM 2-mercaptoethanol. The combined, active fractions were used for all subsequent work.

Polyacrylamide gel electrophoresis. — Polyacrylamide gels (5%) were prepared by using ammonium persulphate and 3-dimethylaminopropionitrile as accelerator. The gel and electrode buffer were either 50mM Tris–hydrochloric acid (pH 8.6) or 50mM glycine–sodium hydroxide (pH 10.0). The enzyme sample (10 μl) was applied in 10% aqueous sucrose in electrode buffer. Electrophoresis was performed with a Gradipore electrophoresis apparatus at a current of 2 mA per gel. The gels were stained[19] for protein with Coomassie Brilliant Blue R250.

Effect of pH on enzyme activity. — The activity of the purified enzyme towards pullulan and amylopectin beta-limit dextrin, as measured by increase in reducing power, was measured at various pH values, using 50mM sodium phosphate–citrate buffer (pH 3.6–7.6).

Products of hydrolysis of oligosaccharides and polysaccharides with purified enzyme. — Substrate (2 mg) was incubated at 37° with enzyme (10 mU) in a total volume of 0.1 ml. Samples were removed at intervals and examined by paper chromatography, as described previously.

RESULTS

Limit-dextrinase assay. — The action of pea limit-dextrinase towards pullulan was characterised by a lag phase during the initial stages of hydrolysis (Fig. 1). This lag phase was exhibited by the enzyme during all stages of the purification procedure

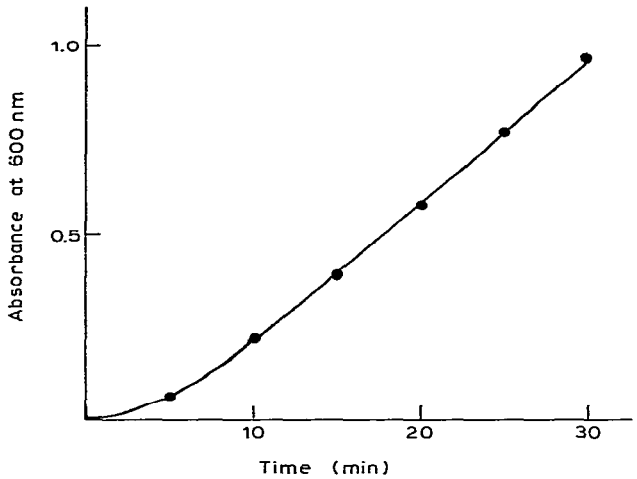

Fig. 1. Increase in reducing power with time from the action of pea limit-dextrinase on pullulan.

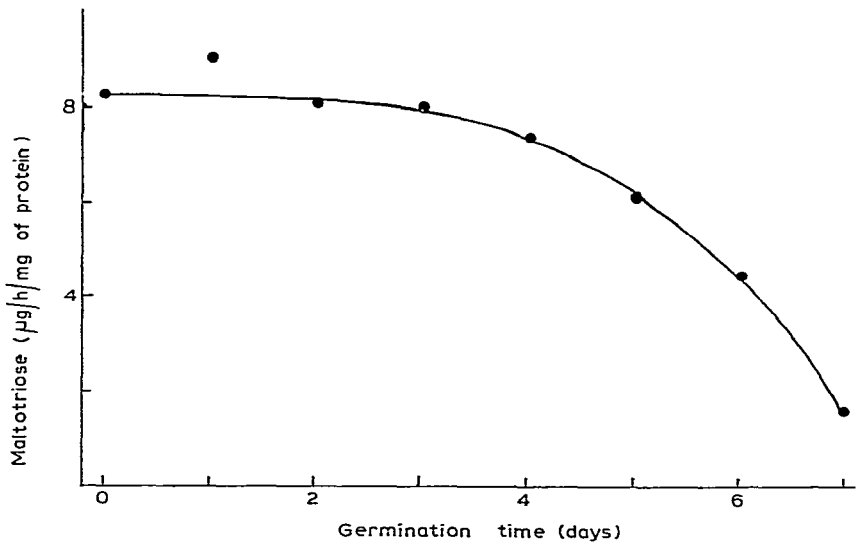

Fig. 2. Level of limit-dextrinase activity in germinating peas.

and, for any fixed concentration of substrate, its duration was dependent upon enzyme concentration. All enzyme activities were calculated from reaction rates, following the assumption of the steady-state. The relationship between protein concentration and enzyme activity was linear.

That the lag phase may be a function of the chain length of the substrate was tested for by heating the reaction mixture at 100° for 5 min after the steady-state had been attained. The digest was then cooled to 30° before the addition of the required amount of enzyme calculated to achieve the same rate. There was no lag

phase after the initiation of this reaction, and the rate was the same as that prior to heating.

Limit-dextrinase levels during germination. — To optimise the conditions for the isolation of the pea enzyme, the level of limit-dextrinase activity in the seeds was followed during germination. The results are illustrated in Fig. 2. As a consequence, ungerminated peas were used as the starting material for purification.

Preparation and purity of the enzyme. — A summary of the limit-dextrinase activity of the fractions obtained at each stage of the purification procedure is given in Table I. On electrophoresis on polyacrylamide gel, the final preparation showed a single band of protein at pH 8.6 and 10.0. The band corresponded in both cases to the limit-dextrinase activity that was detected following sectioning (1 mm) and assay of an unstained gel.

TABLE I

PURIFICATION OF PEA LIMIT-DEXTRINASE

Fraction	*Protein (mg)*	*Total activity (U)*	*Specific activity (U/mg)*	*Yield (%)*
Extract	26993	10.9	0.0004	100
0–30% Ammonium sulphate	4696	7.9	0.0017	73
40–70% Acetone	1280	1.0	0.0008	9
Affinity chromatography	0.39	0.36	1.08	3.3

The purified, limit dextrinase showed a single peak of activity when chromatographed at the rate of 8 ml/h on a column (2.5 × 88 cm) of Sephadex G-150 that had been equilibrated with 20mM sodium phosphate–citrate buffer (pH 7.0) containing 0.1M sodium chloride and 10mM 2-mercaptoethanol. The specific activity of each fraction containing activity was constant. After calibration of the column using cytochrome C (M_r 13,000), chymotrypsinogen A (M_r 25,000), ovalbumin (M_r 45,000), bovine serum albumin (M_r 67,000), rabbit muscle aldolase (M_r 158,000), and catalase (M_r 230,000), it was determined that pea limit-dextrinase has a molecular weight of 180,000 ±5,000.

The presence of contaminating alpha-amylase, beta-amylase, and α-D-glucosidase, at levels not detectable on polyacrylamide gels, was tested for by using a digest containing linear maltosaccharides (d.p. >12; 5 mg) and enzyme (5 mU) in 20mM sodium phosphate–citrate buffer (pH 6.0, 0.5 ml). Paper chromatography of the digest, after incubation at 37° for 24 h, showed no detectable breakdown of the maltosaccharides, indicating the absence of these enzymes.

pH Optimum. — The pH optimum for limit dextrinase was determined with both pullulan and amylopectin beta-limit dextrin as substrates. Similar profiles of

TABLE II

ACTION OF PEA LIMIT-DEXTRINASE TOWARDS OLIGO- AND POLY-SACCHARIDE SUBSTRATES

Substrate	*Oligosaccharide products*[a]
Pullulan	G3, P6, P9, P12
Amylopectin	G7 and higher oligosaccharides
Amylopectin beta-limit dextrin	G2, G3, G6, and higher oligosaccharides
B5	G2, G3
B6	G2, G4
P6	G3
B7	G3, G4
α-Maltosylcyclohexa-amylose	G2, cyclohexa-amylose
α-Maltotriosylcyclohexa-amylose	G3, cyclohexa-amylose

[a]G2, G3, G4 ... represent linear maltosaccharides of d.p. 2, 3, 4...; and P6, P9, and P12 represent maltotriose oligomers of d.p. 6, 9, and 12, the maltotriose residues being joined by (1→6)-α-D-glucosidic linkages.

pH activity were obtained with each substrate. Both showed a broad pH-optimum in the region of pH 5.8–6.2.

Substrate specificity. — The substrate specificity of the enzyme was determined qualitatively by paper chromatography of the digests, and quantitatively by following the increase in reducing power. Products of the action of the enzyme on a range of oligo- and poly-saccharides are reported in Table II. The enzyme had no activity towards isomaltose, panose, 6^3-D-α-glucosylmaltotriose, or α-D-glucosylcyclohexa-amylose.

The relative rates of hydrolysis of various oligo- and poly-saccharide substrates by limit dextrinase were determined by using a digest containing substrate [mM with respect to the content of (1→6)-α-D-glucosidic linkages] and enzyme (10 mU) in

TABLE III

RELATIVE RATES OF ATTACK ON VARIOUS OLIGO- AND POLY-SACCHARIDE SUBSTRATES

Substrate	*Initial velocity*[a]
6^3-α-Maltosylmaltotriose	90
6^3-α-Maltosylmaltotetraose	230
6^3-α-Maltotriosylmaltotriose	170
6^3-α-Maltotriosylmaltotetraose	280
Pullulan	100
Amylopectin	35
Amylopectin beta-limit dextrin	130
Glycogen	0
Glycogen beta-limit dextrin	15

[a]Relative to pullulan (100).

20mM sodium phosphate–citrate buffer (pH 6.0) containing 0.1M sodium chloride (1 ml). Digests were incubated at 30°, samples (0.2 ml) being removed at suitable intervals for the determination of reducing power. Initial velocities were calculated from plots of reducing power against time. The values of the initial rates are given relative to pullulan (Table III).

The debranching of amylopectin, glycogen, and their respective beta-limit dextrins was also studied by following the iodine-staining power of the products. Digests containing amylopectin (4 mg), amylopectin beta-limit dextrin (4 mg), glycogen (10 mg), or glycogen beta-limit dextrin (10 mg) and enzyme (25 mU) in 20mM sodium phosphate–citrate buffer (pH 6.0) containing 0.1M sodium chloride (1 ml) were incubated at 37° for 24 h. Samples (0.1 or 0.2 ml) were withdrawn at intervals and added to iodine reagent (5 ml). With amylopectin, there was a 55% increase in extinction at 580 nm, accompanied by a change in λ_{max} from 550 to 580 nm. Amylopectin and glycogen beta-limit dextrins showed increases of 70 and 15% in iodine-staining power at 550 and 480 nm, respectively. Both substrates also showed increases in the λ_{max} of their iodine complexes.

Inhibition by cyclohexa-amylose. — The steady-state velocities of the pea limit-dextrinase were determined in the absence and presence of various concentrations of cyclohexa-amylose. The concentrations of amylopectin beta-limit dextrin ranged from 0.25 to 7.5 mg/ml, using 15 mU of enzyme in 20mM sodium phosphate–citrate buffer (pH 6.0). The K_m value for amylopectin beta-limit dextrin was 5.4 $\pm$0.5 mg/ml. When the data were plotted in double-reciprocal form, all lines intersected at the same point on the ordinate. On replotting the slopes against the cyclohexa-amylose concentration, a K_i of 8×10^{-5}M was obtained.

DISCUSSION

Results presented herein indicate that the debranching-enzyme activity in peas is present in the dormant seed and that its level does not increase during germination. The activity remains constant for the first three days of germination, after which it gradually decreases. This finding is in conflict with the results of Shain and Mayer[4], who showed an increase in activity with time of germination. However, their assay system, which measured a decrease (not increase) in iodine-staining power in the reported absence of other amylolytic activity, does not measure debranching activity, but must represent an amylolytic activity other than the hydrolysis of (1→6)-α-D-glucosidic linkages.

The constitutive nature of limit dextrinase and its decrease in activity after day 3 of germination raise questions as to its role in the breakdown of starch *in vivo*. Starch is degraded slowly in pea seeds during the first 6 days of germination[2] when the level of alpha-amylase is low, although significant beta-amylase is present. Alpha-amylase activity does not reach a maximum level in pea cotyledons until about the 16th day of germination[20]. This finding implies that, to play a significant role in the breakdown of starch, the limit dextrinase would have to be capable of

hydrolysing the (1→6)-α-D-glucosidic linkages in intact starch granules, since it is reported that beta-amylase is unable to attack these linkages[21]. Studies of the *in vivo* breakdown of starch have shown that the amylose/amylopectin ratio increases during germination[22,23]. One interpretation of these results is that limited debranching of amylopectin occurs[24]; however, Manners and Bathgate[25] considered that the changes in ratio could be explained solely by limited alpha-amylolysis. However, preliminary results with intact starch granules and pea limit-dextrinase indicate that no significant debranching occurs. These results are in accord with those obtained with barley limit-dextrinase and starch granules[9].

The purification procedure reported in this communication enables the preparation of limit dextrinase from peas. The specific activity of the purified enzyme is 1.08 U/mg, representing a 2,700-fold purification from the initial extract. The initial steps of ammonium sulphate and acetone fractionation were necessary for the removal of other amylolytic activity, since alpha-amylase, beta-amylase, and amyloglucosidase are all inhibited[26] by, and reversibly bind to, immobilised cyclohexa-amylose[27]. Contamination by these enzymes, although they possess higher inhibition constants, would affect the affinity-chromatography step. The purified enzyme was devoid of these activities and was homogeneous as judged by polyacrylamide gel electrophoresis.

The inhibition experiments with cyclohexa-amylose demonstrate that the inhibition of limit dextrinase is competitive, as with other debranching enzymes[26]. The inhibition constant was 8×10^{-5}M, more than an order of magnitude greater than that obtained for cyclohexa-amylose on pullulanase.

The results of gel-filtration studies indicated that the pea limit-dextrinase (M_r 180,000) is larger than other limit dextrinases studied so far. The cereal enzymes[6-8] vary between 80,000 and 100,000, and that[28] purified from the legume *Vicia faba* is of the order of 80,000.

The pea enzyme is very similar in specificity to all other plant limit-dextrinases studied. Oligo- and poly-saccharides containing single D-glucosyl groups attached by an α-(1→6) linkage to the rest of the molecule are not hydrolysed. However, oligosaccharides containing maltosyl or larger side-chains are readily hydrolysed, in addition to the polysaccharides amylopectin, amylopectin beta-limit dextrin, and glycogen beta-limit dextrin. As with limit dextrinases from other sources, glycogen is not a substrate.

The establishment of the steady-state velocity with pullulan as substrate was preceded by a lag phase, the duration of which was dependent upon the enzyme concentration. This situation is probably due to the higher affinity of the enzyme for substrates of lower molecular weight. This possibility is backed up by the fact that, following the assumption of the steady-state and subsequent denaturation of the enzyme, the addition of a proportional amount of enzyme to the digest does not lead to any lag phase and the reaction assumes the same steady-state velocity. The dependence of the lag-phase duration on enzyme concentration would also seem to support this suggestion.

The relative rates of hydrolysis of oligo- and poly-saccharides are of the same magnitude as those demonstrated for other limit dextrinases. For the oligosaccharide substrates, the rate of hydrolysis increases with the degree of polymerisation of both chains of the substrate. The optimum substrate tested was therefore the hepta-saccharide 6^3-α-maltotriosylmaltotetraose, which was isolated from a mixture of alpha-limit dextrins. The rate of hydrolysis of all oligosaccharide substrates tested, with the exception of 6^3-α-maltosylmaltotriose, was significantly higher than that of amylopectin beta-limit dextrin, and several-fold higher than that for amylopectin. Therefore, the preferred, natural substrates for limit dextrinases from plant tissues appear to be alpha-limit dextrins rather than amylopectin or its partial degradation products of high molecular weight.

ACKNOWLEDGMENT

The author is grateful to Professor D. J. Manners, Heriot-Watt University, Edinburgh, in whose Department part of this work was done.

REFERENCES

1 J. J. Marshall, *Wallerstein Lab. Commun.*, 35 (1972) 49–98.
2 B. O. Juliano and J. E. Varner, *Plant Physiol.*, 44 (1969) 886–892.
3 R. R. Swain and E. E. Dekker, *Biochim. Biophys. Acta*, 122 (1966) 87–100.
4 Y. Shain and A. M. Mayer, *Physiol. Plant.*, 21 (1968) 765–776.
5 A. M. Mayer and Y. Shain, *Science*, 162 (1968) 1283–1284.
6 E. Y. C. Lee, J. J. Marshall, and W. J. Whelan, *Arch. Biochem. Biophys.*, 143 (1971) 365–374.
7 G. Dunn and D. J. Manners, *Carbohydr. Res.*, 39 (1975) 283–293.
8 D. G. Hardie, D. J. Manners, and D. Yellowlees, *Carbohydr. Res.*, 50 (1976) 75–85.
9 I. Maeda, Z. Nikuni, H. Taniguchi, and M. Nakamura, *Carbohydr. Res.*, 61 (1978) 309–320.
10 D. J. Manners and D. Yellowlees, *J. Inst. Brew. London*, 79 (1973) 377–385.
11 D. Yellowlees, *Proc. Aust. Biochem. Soc.*, 11 (1978) 26.
12 D. J. Manners and D. Yellowlees, *Staerke*, 23 (1971) 228–234.
13 J. F. Robyt and W. J. Whelan, in J. A. Radley (Ed.), *Starch and its Derivatives*, Chapman and Hall, London, 4th edn., 1968, pp. 432–433.
14 G. Miller, *Anal. Chem.*, 31 (1959) 964.
15 M. Higuchi and F. Yoshida, *Anal. Biochem.*, 77 (1977) 542–547.
16 W. E. Trevelyan, D. P. Procter, and J. S. Harrison, *Nature (London)*, 166 (1950) 444–445.
17 P. Vretblad, *FEBS Lett.*, 47 (1974) 86–89.
18 M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Anal. Chem.*, 28 (1956) 350–356.
19 W. Diezel, G. Kopperschläger, and E. Hofmann, *Anal. Biochem.*, 48 (1972) 617–620.
20 A. P. Brown and J. L. Wray, *Biochem. J.*, 108 (1968) 437–444.
21 G. Dunn, *Phytochemistry*, 13 (1974) 1341–1346.
22 T. Fukui and J. Nikuni, *J. Biochem. (Tokyo)*, 43 (1956) 33–40.
23 C. T. Greenwood and J. Thomson, *J. Inst. Brew. London*, 65 (1959) 346–353.
24 S. Kiribuchi and M. Nakamura, *Nippon Nogei Kagaku Kaishi*, 47 (1973) 341–345.
25 D. J. Manners and G. N. Bathgate, *J. Inst. Brew. London*, 75 (1969) 169–175.
26 J. J. Marshall, *FEBS Lett.*, 37 (1973) 269–273.
27 A. Hoschke, E. Laszlo, and J. Hollo, *Staerke*, 28 (1976) 426–432.
28 R. W. Gordon, D. J. Manners, and J. R. Stark, *Carbohydr. Res.*, 42 (1975) 125–134.