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# THE LIMIT DEXTRINASES FROM UNGERMINATED OATS (*Avena sativa* L.) AND UNGERMINATED RICE (*Oryza sativa* L.)*

GRAHAM DUNN AND DAVID J. MANNERS

*Department of Brewing and Biological Sciences, Heriot-Watt University, Edinburgh EH1 1HX (Great Britain)*

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ABSTRACT

The limit dextrinases from ungerminated oats and rice have been purified, and their substrate specificity compared with a bacterial isoamylase preparation. Both cereal enzymes could hydrolyse (1→6)-α-D-glucosidic linkages in oligosaccharide α-dextrins, pullulan, amylopectin, and the β-limit dextrins of amylopectin and glycogen. However, under comparable conditions, they were unable to attack glycogens.

## INTRODUCTION

During the germination of cereals, the α-amylolytic degradation of the starch granules present in the endosperm yields a mixture of products, which include branched oligosaccharides. The latter contain the (1→6)-α-D-glucosidic bonds which were present as inter-chain linkages in the original amylopectin. These linkages may be hydrolysed by debranching enzymes termed limit dextrinases [EC 3.2.1.41] to give linear maltosaccharides. The enzymic degradation of the starch is then completed by β-amylase and α-D-glucosidases, which hydrolyse maltose and other linear maltosaccharides to D-glucose[2]. Since the limit dextrinases have an important *in vivo* role, we have undertaken a comparative survey of the properties of these enzymes from various higher plants, and now describe the purification of limit dextrinase from ungerminated oats (*Avena sativa* L.) and from ungerminated rice (*Oryza sativa* L.). The former cereal was selected since, in contrast to barley, rye, and wheat, the ungerminated grain contains substantial amounts of limit dextrinase[3], whilst rice is a particularly active starch-metabolising plant.

The action of plant debranching enzymes on amylopectin or amylopectin β-limit dextrin results in a limited increase in iodine-staining power and β-amylolysis limit, and a decrease in viscosity[4], but in the original work, these changes were not directly correlated with the number of (1→6)-α-D-glucosidic linkages hydrolysed. In the earlier studies on these enzymes[4–6], alternative means of assay were not available,

*Studies on Debranching Enzymes: Part III. For Part II, see Ref. 1.

so that the purification of the enzyme could only be followed by purely qualitative means; in studies of enzyme specificity, preparations of unknown specific activity had to be used. With the discovery that pullulan [a linear polymer of α-(1→6)-linked maltotriose units] is hydrolysed by plant debranching enzymes[7], a convenient substrate is now available for the quantitative assay of the enzyme[3] and has been used in the present study. The specificity of the cereal limit-dextrinases has also been compared with that of a commercial, bacterial isoamylase preparation.

## METHODS AND MATERIALS

*Analytical methods.* — The protein content of enzyme preparations was estimated by the Miller modification of the Lowry method[8], and of column fractions from the absorbance of the solution at 280 nm. Reducing sugars were estimated by the Nelson method[9], which was calibrated against maltotriose. Total carbohydrate was estimated by using a phenol–sulphuric acid reagent[10]. For iodine staining, the standard solution consisted of 0.1% of iodine in 1% potassium iodide.

*Enzyme assays.* — Unless stated otherwise, all enzyme digests were carried out at 30°, in the presence of sodium acetate buffer (pH 5.0). The activity of the oat and rice enzymes was optimum in the region of pH 5.0.

For the assay of column fractions, a sample (0.1 ml) was added to 0.5% pullulan solution (0.4 ml in buffer). After incubation, the digests were diluted to 1 ml with water, and the reducing power was determined. The reducing powers of appropriate enzyme and substrate controls were also measured after similar incubation. Results are normally expressed as the increase in absorbance at 600 nm. The unit of limit-dextrinase activity is that amount of enzyme which will produce 1 μmole of equivalent maltotriose from pullulan per min at pH 5.0 and 30° in the presence of excess substrate and is identical with that used to assay bacterial pullulanase preparations[11], For convenience, the activities have, in some cases, been expressed as milliunits (mU).

Column fractions were also assayed for amylase activity by replacing pullulan with amylopectin β-limit dextrin. After incubation at 30°, M hydrochloric acid (0.1 ml) and standard iodine reagent (0.1 ml) were added, followed by water (2.0 ml). The absorbance at 680 nm was measured and compared with that of an appropriate substrate blank.

For other iodine-staining measurements, M hydrochloric acid (0.5 ml) was added to 0.5-ml samples of enzyme digests, to stop the reaction, followed by standard iodine reagent (0.5 ml) and water (2 or 10 ml, for glycogen or amylopectin derivatives, respectively).

*Separation methods.* — Proteins were fractionated on columns of DEAE-Sephadex A-50 and Sephadex G-100 and G-150, using the procedures recommended by Pharmacia Ltd. Oligosaccharides were separated by t.l.c., using ethyl acetate–methanol–water (52:36:13) as solvent[12].

*Substrates.* — The polysaccharide and oligosaccharide substrates were laboratory samples available from previous work[3,7].

*Enzyme sources.* — Flour was obtained by passing whole oat grains (*Avena sativa* cv Blenda) through a Casella electric mill fitted with a 2.5-mm sieve. Flour was also obtained by milling whole rice grains (huskless, purchased as Riceland Long Grain Rice from Riceland Foods, Stuttgart, Arkansas, U.S.A.) through the mill fitted with a 1-mm sieve.

## RESULTS

*Preparation of oat limit dextrinase.* — Freshly prepared oat flour (500 g) was extracted with 0.2M sodium acetate buffer (pH 5.0, 1.5 litres) for 3 h at room temperature (18–20°), and insoluble material was then removed by centrifugation (1500 *g*; 40 min). Solid ammonium sulphate was then stirred into the extract (1.2 l) to ~40% saturation (*i.e.*, 220 g per litre of extract). After stirring for 2 h, the mixture was centrifuged as above, and the supernatant solution discarded. The pellet was suspended in 0.01M Tris–hydrochloric acid buffer (pH 7.5; 0.1M with respect to sodium chloride; 200 ml) and dialysed overnight at 4° against this buffer. After removal of insoluble material by centrifugation, the dialysed solution was loaded onto a column of DEAE-Sephadex A-50 (12 × 4 cm) equilibrated with the same buffer. The column was then eluted with a linear gradient of sodium chloride to 0.5M in 0.01M Tris–hydrochloric acid buffer (pH 7.5). The active fractions were combined and concentrated to ~3 ml, using an Amicon ultrafiltration cell fitted with a PM-30 membrane.

The partially purified limit dextrinase was then fractionated at 4° on a column of Sephadex G-150 (60 × 2 cm) equilibrated with 0.05M sodium acetate buffer (pH 5.0). The active fractions were again combined and concentrated by using the Amicon ultrafiltration cell. The final preparation had a specific activity of 800 mU/mg, which represents a 400-fold purification compared to the original extract which had a specific activity of 2 mU/mg. Individual fractions at the peak of enzymic activity had a specific activity substantially greater than 1 unit/mg.

Preliminary experiments had shown[13] that extracts of ungerminated oats contain β-amylase but little or no α-amylase. The β-amylase was not completely separated from the limit dextrinase by the above purification procedure, but was completely inhibited by the addition of an equal volume of 0.1mM parahydroxymercuribenzoate to the final preparation. The enzyme preparation now had no action on amylose or linear maltosaccharides, and the inhibitor had no effect on the limit-dextrinase activity.

Preliminary gel-filtration studies, using a column of Sephadex G-100 which had been calibrated with urease (mol. wt. 480,000), bovine serum albumin (67,000), peroxidase (40,200), pepsin (35,200), and cytochrome C (12,400), indicated that the oat limit dextrinase had a molecular weight in the region of 80,000.

*Properties of oat limit dextrinase.* — (*a*) *Action on oligosaccharide substrates.* The ability of oat limit dextrinase to hydrolyse various oligosaccharides was examined by incubating the substrate [1mM with respect to (1→6)-α-D-glucosidic linkages] with enzyme (10 mU) in buffer at 30°. After 0, 0.5, and 1.0 h, samples were analysed for

reducing power, and the initial rate of hydrolysis of the oligosaccharides was then calculated relative to the initial rate of hydrolysis of pullulan under identical conditions. The results are shown in Table I.

TABLE I

ACTION OF LIMIT DEXTRINASES ON OLIGOSACCHARIDES

| *Substrate* | *Relative velocity*[a] | |
|---|---|---|
| | *Oat enzyme* | *Rice enzyme* |
| Panose | 0 | 0 |
| $6^3$-α-D-Glucosylmaltotriose (B4) | 0 | 0 |
| $6^3$-α-Maltosylmaltotriose (B5) | 120 | 50 |
| $6^3$-α-Maltosylmaltotetraose (B6) | 290 | 210 |
| $6^3$-α-Maltotriosylmaltotriose (P6) | 220 | 170 |
| $6^3$-α-Maltotriosylmaltotetraose (B7) | 380 | 260 |

[a]Relative to pullulan = 100.

The effect of varying the substrate concentration on the initial rate of hydrolysis was also investigated. For the substrates B5, B6, P6, and B7, it was found that the relative rates given in Table I represent the corresponding values for $V_{max}$. Enzymic hydrolysis obeyed Michaelis–Menten kinetics with a $K_m$ for the four oligosaccharides of ~0.2mM (*e.g.*, 0.23 mg/ml for B7).

Oat limit dextrinase had no action on panose under the above conditions, or when the enzyme concentration was increased 10-fold.

(*b*) *Action on polysaccharide substrates.* The substrate specificity of the oat limit dextrinase was examined in four digests containing polysaccharide (16 mg), and enzyme (80 mU) in buffer (2 ml), in a total volume of 6 ml. Duplicate samples (0.2 ml) were removed at intervals up to 48 h for measurement of iodine staining and reducing power. For the former, the absorption spectra of the polysaccharide–iodine complex was measured over the range 400–700 nm. On debranching, the following changes must occur: (*a*) an increase in the iodine-staining power, particularly at wavelengths greater than $\lambda_{max}$; (*b*) an increase in $\lambda_{max}$ of the polysaccharide–iodine complex; (*c*) a significant increase in reducing power. By these criteria, amylopectin, amylopectin β-limit dextrin, and glycogen β-limit dextrin were substrates, of which, amylopectin β-limit dextrin was the best. Glycogen was not hydrolysed. The changes in the absorption spectra of the iodine complexes of amylopectin and phytoglycogen β-limit dextrin are shown in Fig. 1.

Confirmation that debranching of amylopectin had actually taken place was obtained by gel filtration of a digest (3 ml) containing substrate (10 mg) and enzyme (40 mU) which had been incubated for 48 h. The digest was fractionated on a column of Sephadex G-50 (90 × 1.5 cm) which was eluted with water, fractions being analysed for carbohydrate by the phenol–sulphuric acid method[10]. The elution profile is shown in Fig. 2.

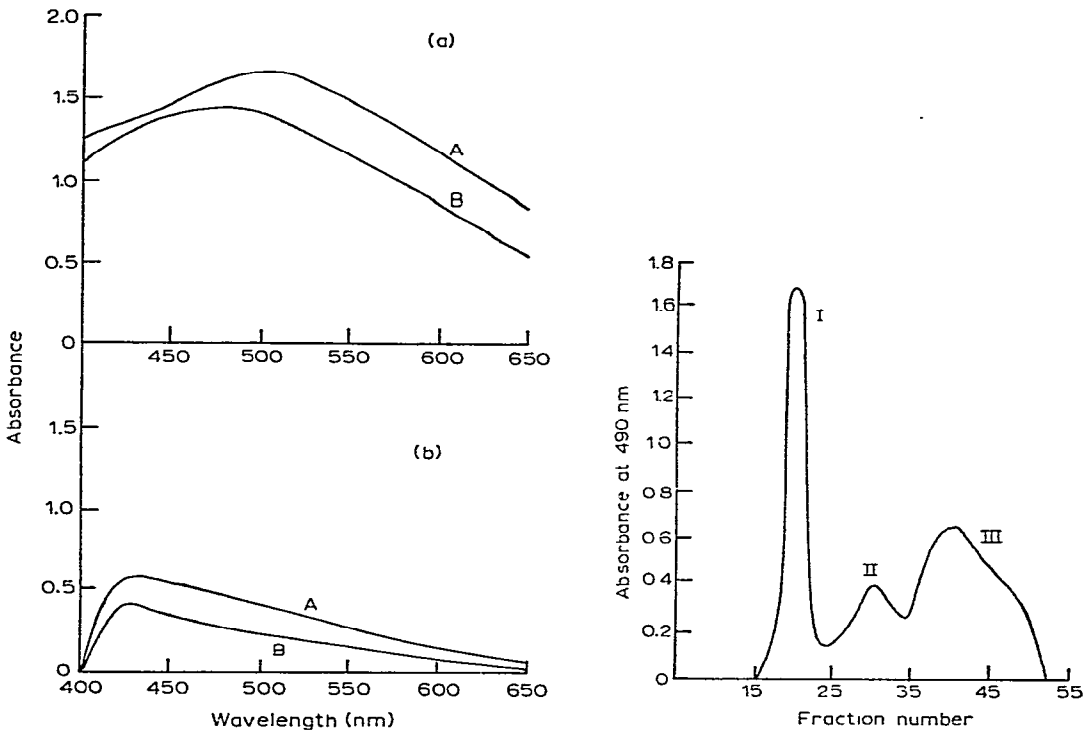



Fig. 1. Absorption spectra of polysaccharide–iodine complexes of (*a*) amylopectin, (*b*) phytoglycogen β-limit dextrin, before (B) and after (A) debranching by oat limit dextrinase.

Fig. 2. Fractionation of the products of debranching of amylopectin by oat limit dextrinase on Sephadex G-50. The amount of carbohydrate present in each fraction was measured by the phenol–sulphuric acid method[10].

Since the $\lambda_{max}$ of the iodine complex of a linear maltosaccharide increases[14] with degree of polymerisation (d.p.) and the elution volume of a maltosaccharide should be proportional to the log of its molecular weight[15], a plot of log ($\lambda_{max}$) against elution volume should be linear. Any branched maltosaccharides will be differentiated from linear maltosaccharides of the same d.p. since their iodine-staining power will be lowered. The iodine-staining properties of the gel-filtration fractions were examined (Fig. 3); those from peaks II and III show a linear relationship between log ($\lambda_{max}$) and elution volume, and therefore contain linear maltosaccharides. Fractions from peak I differ; this result is not unexpected, since peak I presumably consisted of residual polysaccharide which had not been debranched.

The debranching of amylopectin and its β-limit dextrin was also followed by reducing-power measurements, and found to obey Michaelis–Menten kinetics. The appropriate values for $V_{max}$ and $K_m$ are given in Table II.

*Preparation of rice limit dextrinase.* — Freshly prepared rice-flour (100 g) was extracted with 0.1M sodium acetate buffer (pH 5.0, 400 ml) for 3 h at room tempera-

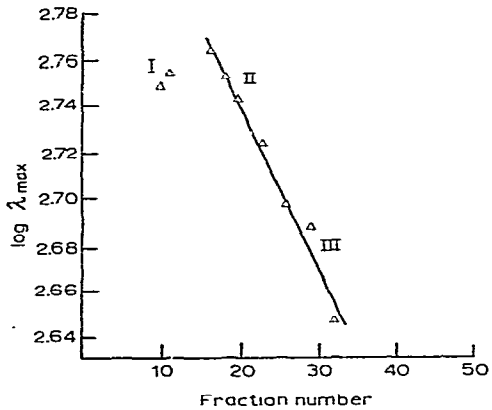



Fig. 3. Iodine-staining properties of the maltosaccharides released on the debranching of amylopectin by oat limit dextrinase. Fractions I, II, and III refer to the peaks shown in Fig. 2.

TABLE II

ACTION OF LIMIT DEXTRINASES ON POLYSACCHARIDES

| *Substrate* | *Oat enzyme* | | *Rice enzyme* | |
|---|---|---|---|---|
| | $V_{max}$[a] | $K_m$ (*mg/ml*) | $V_{max}$[a] | $K_m$ (*mg/ml*) |
| Amylopectin | 26 | 1.4 | 35 | 6 |
| Amylopectin β-limit dextrin | 160 | 1.5 | 150 | 4 |
| Rabbit-liver glycogen β-limit dextrin | — | — | 55 | 7 |
| Phytoglycogen β-limit dextrin | — | — | 135 | 10 |
| Pullulan | 100 | 0.17 | 100 | — |

[a]Relative to pullulan.

ture, and insoluble material was removed by centrifugation. Solid ammonium sulphate was then stirred into the extract to ~80% saturation, and stirring continued for 2 h. The precipitated protein was collected by centrifugation, and suspended in 0.05M Tris–hydrochloric acid buffer (pH 7.5; 0.05M with respect to sodium chloride; 50 ml). After dialysis overnight at 4° against this buffer, and clarification by centrifugation, the dialysed solution was loaded onto a DEAE-Sephadex column (12 × 4 cm) which had been equilibrated at 4° with the same buffer. The column was eluted with a linear gradient of sodium chloride to 0.5M in 0.05M Tris–hydrochloric acid buffer (pH 7.5) (in a total volume of 600 ml). The elution of protein and limit dextrinase is shown in Fig. 4. The fractions from the single peak of activity were combined and concentrated to ~2 ml (Amicon ultrafiltration cell and PM-30 membrane).

The enzyme preparation was further fractionated on a column of Sephadex G-150 (50 × 2 cm) equilibrated at 4° with 0.05M sodium acetate buffer (pH 5.0), and the active fractions were again pooled and concentrated (see Fig. 4). The final specific

activity was 0.6 unit/mg, representing a 15-fold purification. The rice flour is an extremely useful source of limit dextrinase, since from 100 g, 20 units can be extracted, whereas from oats, the corresponding yield was 2.4 units.

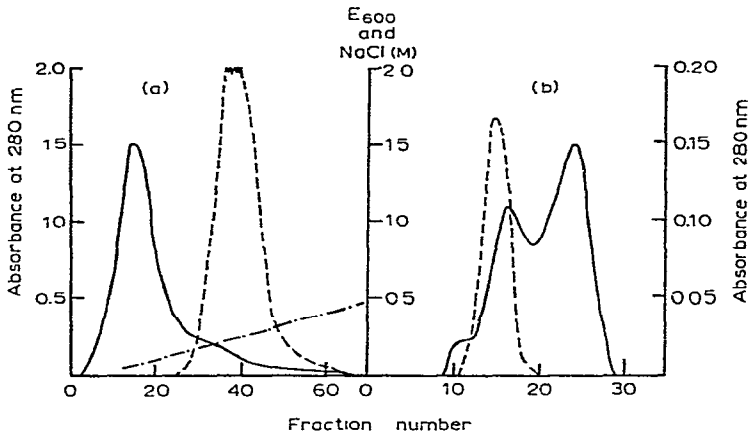



Fig. 4. Elution patterns of rice limit dextrinase through columns of (*a*) DEAE-Sephadex followed by (*b*) Sephadex G-150: ——, elution of protein; — — —, enzymic activity; and —·—·—·—, sodium chloride concentration.

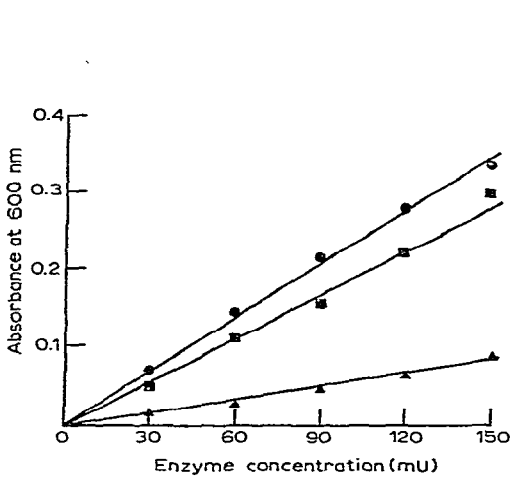
*Properties of rice limit dextrinase — (a) Action on oligosaccharide substrates.* These substrates were tested in a manner identical to that for the oat enzyme, and the results are shown in Table I. In similar digests containing variable amounts of $6^3$-α-maltotriosylmaltotetraose (B7), the $K_m$ was 0.6mM (*i.e.*, 0.67 mg/ml).

(*b*) *Action on polysaccharide substrates.* Digests were prepared containing amylopectin or its β-limit dextrin (4 mg), enzyme (30 mU), and buffer (0.1 ml) in a total volume of 1 ml. Samples (0.1 ml) were removed at intervals up to 1 h for iodine staining. With both substrates, there was an increase in iodine-staining power and an increase in the $\lambda_{max}$ of the polysaccharide–iodine complex. The results with amylopectin were similar to those shown in Fig. 1.

Further digests containing rabbit-liver glycogen and phytoglycogen and their β-limit dextrins (20 mg), enzyme (150 mU), and buffer (0.5 ml) in a total volume of 5 ml were prepared. The enzyme had no effect on the iodine-staining power of the glycogens, but with the β-limit dextrins, there were significant increases. With phytoglycogen β-limit dextrin, they were similar to those shown in Fig. 1; the products of enzyme action were identified (t.l.c.) as maltose and maltotriose. The rate of debranching of rabbit-liver glycogen β-limit dextrin was significantly slower.

The debranching of polysaccharides was also followed by reducing-power measurements. Digests containing enzyme (0–150 mU), substrate (20 mg), and buffer (0.5 ml) in a final volume of 5 ml were prepared, and samples (0.5 ml) removed at intervals up to 1 h for analysis. The results obtained with pullulan, amylopectin,

and amylopectin β-limit dextrin are shown in Fig. 5, and indicate that there is no selective loss of activity towards amylopectin when the enzyme concentration is reduced, as suggested by Drummond *et al.*[16].



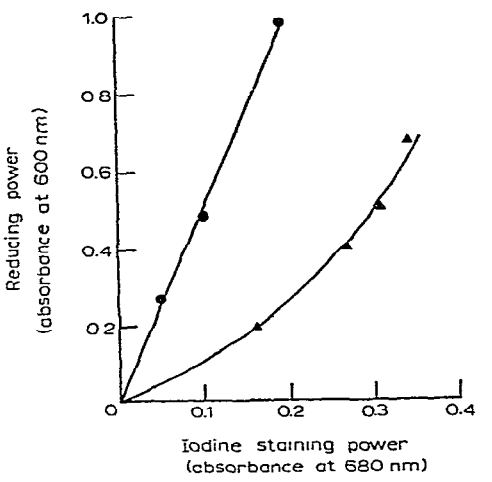




Fig. 5. Effect of enzyme concentration on the rate of debranching of polysaccharides. The increase in reducing power produced by the action of rice limit dextrinase on pullulan (■), amylopectin (▲), and amylopectin β-limit dextrin (●), on incubation for 10 min, is plotted against the amount of enzyme (mU) contained in the corresponding digest.

Fig. 6. Debranching of amylopectin (▲) and its β-limit dextrin (●) by rice limit dextrinase. The increases in reducing power (absorbance at 600 nm) are plotted against the increase in iodine-staining power (absorbance at 680 nm).

The relationship between the increase in iodine-staining power and reducing power during limit-dextrinase action on amylopectin and its β-limit dextrin was examined. Using 1-ml digests whose composition was similar to those used in the previous iodine-staining experiments, the results shown in Fig. 6 were obtained. It is therefore possible, under these conditions, to relate the increase in iodine-staining power to the number of (1→6)-α-D-glucosidic linkages actually hydrolysed.

Using digests of this composition, the kinetics for debranching of four polysaccharides were examined. Those of amylopectin and its β-limit dextrin were examined by iodine staining and those of the other polysaccharides by reducing-power measurements. The results are shown in Table II.

The effect of various oligosaccharides on the rate of debranching of amylopectin and its β-limit dextrin was also investigated. Enzyme action was measured by iodine staining to avoid interference by the substantial amounts of reducing sugars added. The results are given in Table III.

*Properties of bacterial isoamylase.* — A commercial, cell-free preparation from a bacterium (originally described as a *Cytophaga* sp., but more correctly classified as a *Flavobacterium* sp., and purchased from B.D.H. Ltd as L1 Lytic Enzyme) contains

TABLE III

EFFECT OF OLIGOSACCHARIDES ON THE RATE OF DEBRANCHING OF AMYLOPECTIN AND ITS β-LIMIT DEXTRIN BY RICE LIMIT DEXTRINASE[a]

| *Oligosaccharide* | *Inhibition* (%) | |
|---|---|---|
| | *Amylopectin* | *Amylopectin β-limit dextrin* |
| D-Glucose | 0 | 0 |
| Maltose | 50 | 50 |
| Maltotriose | 75 | 75 |
| Maltotetraose | 75 | 75 |
| Maltopentaose | 100 | 85 |
| Maltohexaose | 100 | — |
| Maltoheptaose | 100 | — |
| Panose | 0 | 0 |
| $6^3$-α-D-Glucosylmaltotriose (B4) | 75 | 40 |
| $6^3$-α-Maltosylmaltotriose (B5) | 75 | — |
| $6^3$-α-Maltosylmaltotetraose (B6) | 100 | 85 |
| $6^3$-α-Maltotriosylmaltotriose (P6) | 100 | 90 |
| $6^3$-α-Maltotriosylmaltotetraose (B7) | 100 | 95 |

[a]Digests contained enzyme (30 mU), substrate (4 mg), oligosaccharide (5 mg), and buffer (0.1 ml) in a total volume of 1 ml, and duplicate samples (0.1 ml) were analysed by iodine staining at intervals up to 1 h. Controls without oligosaccharides were also prepared and analysed.

a number of carbohydrases including β-D-glucanases[17] and isoamylase[18]. The latter enzyme catalyses the extensive debranching of glycogen and amylopectin.

The action of the L1 preparation (10 mg) on various oligosaccharides (5 mg) in 5mM sodium acetate buffer (pH 5.5, 1 ml) was examined by t.l.c. After incubation for 2 h, there was no action on B4, B5, B6, or $6^3$-α-maltotriosylmaltotriose; B7 was partly hydrolysed to a mixture of maltotriose and maltotetraose.

The changes in the iodine-staining power of amylopectin and its β-limit dextrin produced on debranching by the L1 preparation were qualitatively the same as those produced by the cereal limit dextrinases.

## DISCUSSION

The present results show that extracts of ungerminated oats and rice contain limit dextrinases which debranch amylopectin, the β-limit dextrins of glycogen and amylopectin, and a range of α-dextrins containing side chains of two or more D-glucose residues. The enzymes also hydrolyse pullulan, but have no significant action on glycogen under comparable conditions.

In an earlier, preliminary study of oat limit dextrinase[13], an approximately 90-fold purification was obtained by a combination of ammonium sulphate fractionation, continuous curtain electrophoresis, and gel filtration on Sephadex G-100. The replacement of the electrophoresis step by chromatography on DEAE-Sephadex has led to an improved procedure, resulting in a 400-fold purification. In contrast to the previous study in which debranching of amylopectin could not be demonstrated[13],

the purified enzyme debranched amylopectin. In general, debranching enzymes have a much lower affinity for amylopectin than for pullulan or α-dextrins, and the apparent ability to act on amylopectin is dependent on (*a*) the enzyme concentration, (*b*) the substrate concentration, and (*c*) the method of assay. As discussed elsewhere[19], dilute solutions of debranching enzymes may hydrolyse amylopectin so slowly that the resultant increase in reducing power may not be measurable, unless larger samples are removed for analysis. Our earlier experiments on oat limit dextrinase were carried out with dilute solutions.

Flour from ungerminated rice is a good source of limit dextrinase, and application of the methods used for the purification of the oat enzyme gave a preparation of comparable specific activity. Control experiments showed that amylase and α-D-glucosidase impurities were absent.

The relative activity of the two cereal enzymes on various oligo- and polysaccharide substrates was very similar (Tables I and II). Neither enzyme could hydrolyse α-dextrins with (1→6)-linked α-D-glucosyl side-chains, and the relative velocity increased with the length of the side chain (A-chain) and main chain (B-chain). Of the α-dextrins, B7 [*i.e.*, maltotriose (1→6)-linked to maltotetraose] was the most readily attacked.

With the polysaccharides, amylopectin was hydrolysed at ~30% of the rate of pullulan, and ~20% of the rate of the corresponding β-limit dextrin. Since glycogen was not a substrate, but glycogen β-limit dextrins were attacked, it follows that the length of both the exterior and interior chains are relevant factors. Maltosyl or maltotriosyl side-chains are readily removed, irrespective of the interior chain-length, but for the hydrolysis of longer maltosaccharide side-chains, the interior chain-length is a limiting factor. Presumably with glycogen, a combination of the outer chains of ~8 D-glucose residues, and the high degree of branching on the periphery of the molecule together hinder the formation of active enzyme–substrate complexes. The fact that phytoglycogen β-limit dextrin is hydrolysed at ~2.5 times the rate of rabbit-liver glycogen β-limit dextrin may indicate structural differences between the plant and animal glycogens which are not revealed by measurements of average chain-length and β-amylolysis limit. Differences between the two types of glycogen have also been detected by examination of the elution profiles obtained by extensive debranching with bacterial isoamylase followed by gel filtration[20].

The extent of inhibition of rice limit dextrinase by various oligosaccharides (Table III) is consistent with the specificity studies recorded in Table I, and suggests that the enzyme contains binding sites for at least two, and probably more, adjacent α-(1→4)-linked D-glucose residues. It should be emphasised that the topography of the enzyme in the vicinity of the active site must be able to accept a linear α-D-glucan (pullulan), a highly-branched α-D-glucan (amylopectin or glycogen β-limit dextrin), and oligosaccharides of low molecular weight (α-dextrins).

The overall conclusions on the specificity of the two cereal limit dextrinases are supported in related studies on limit dextrinases from malted barley and sorghum[21], broad beans[22], potatoes[16], and sweet corn[23]. In all of these studies, it now seems

certain that the same debranching enzyme will act on amylopectin, α-dextrins, and pullulan. Previous evidence[5,24] for a plant debranching enzyme, originally termed R-enzyme or amylopectin 6-glucanohydrolase, which could act on amylopectin, but not an α-dextrins, has not been confirmed.

The cereal limit dextrinases differ from the bacterial (*Flavobacterium* or *Cytophaga* sp.) isoamylase[18] since the latter cannot hydrolyse B5, B6, or pullulan, but can readily debranch glycogen. However, extreme caution is required in defining the absolute specificity of a debranching enzyme, since this may apparently vary with enzyme concentration[16,19]. Moreover, if a debranching enzyme (*e.g.*, *Aerobacter* pullulanase) attacks rabbit-liver glycogen at ~2% of the rate at which it hydrolyses pullulan[25], it is then debatable as to whether or not glycogen should be regarded as a significant substrate. We consider that the natural substrates for the limit dextrinases are intermediate α-dextrins arising from the α-amylolytic breakdown of starch and that pullulan and amylopectin are alternative substrates of no *in vivo* significance.

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REFERENCES

1 D. G. HARDIE AND D. J. MANNERS, *Carbohyd. Res.*, 36 (1974) 207–210.
2 G. DUNN, *Phytochem.*, 13 (1974) 1341–1346; D. J. MANNERS, *Essays in Biochem.*, 10 (1974) 37–71.
3 D. J. MANNERS AND D. YELLOWLEES, *J. Inst. Brew.*, 79 (1973) 377–385.
4 P. N. HOBSON, W. J. WHELAN, AND S. PEAT, *J. Chem. Soc.*, (1951) 1451–1459.
5 I. C. MACWILLIAM AND G. HARRIS, *Arch. Biochem. Biophys.*, 84 (1959) 442–454.
6 I. D. FLEMING AND D. J. MANNERS, *Chem. Ind. (London)*, (1958) 831–832.
7 D. J. MANNERS, J. J. MARSHALL, AND D. YELLOWLEES, *Biochem. J.*, 116 (1970) 539–541.
8 G. L. MILLER, *Anal. Chem.*, 31 (1959) 964.
9 N. NELSON, *J. Biol. Chem.*, 143 (1944) 375–380; J. F. ROBYT AND W. J. WHELAN, in J. A. RADLEY (Ed.), *Starch and its Derivatives*, Chapman and Hall, London, 4th edition, 1968, pp. 431–2.
10 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350–356.
11 H. BENDER AND K. WALLENFELS, *Methods Enzymol.*, 8 (1966) 555–559.
12 C. N. HUBER, H. D. SCOBELL, AND HAN TAI, *Cereal Chem.*, 43 (1966) 342–346.
13 D. J. MANNERS AND J. J. MARSHALL, unpublished work; J. J. MARSHALL, Ph.D. Thesis, Heriot–Watt University, Edinburgh, 1969.
14 D. J. MANNERS AND J. R. STARK, *Staerke*, 26 (1974) 78–81.
15 P. ANDREWS, *Biochem. J.*, 91 (1964) 222–233.
16 G. S. DRUMMOND, E. E. SMITH, AND W. J. WHELAN, *FEBS Lett.*, 9 (1970) 136–140.
17 D. J. MANNERS AND G. WILSON, *Biochem. J.*, 135 (1973) 11–18.
18 Z. GUNJA-SMITH, J. J. MARSHALL, E. E. SMITH, AND W. J. WHELAN, *FEBS Lett.*, 12 (1970) 96–100.
19 G. DUNN, D. G. HARDIE, AND D. J. MANNERS, *Biochem. J.*, 133 (1973) 413–416.
20 Z. GUNJA-SMITH, J. J. MARSHALL, C. MERCIER, E. E. SMITH, AND W. J. WHELAN, *FEBS Lett.*, 12 (1970) 101–104.
21 D. G. HARDIE AND D. J. MANNERS, unpublished work.
22 R. GORDON, D. J. MANNERS, AND J. R. STARK, *Carbohyd. Res.*, (1975) in press.
23 E. Y. C. LEE, J. J. MARSHALL, AND W. J. WHELAN, *Arch. Biochem. Biophys.*, 143 (1971) 365–374.
24 D. J. MANNERS AND K. L. SPARRA, *J. Inst. Brew.*, 72 (1966) 359–365.
25 K. YOKOBAYASHI, H. AKAI, T. SUGIMOTO, M. HIRAO, K. SUGIMOTO, AND T. HARADA, *Biochim. Biophys. Acta*, 293 (1973) 197–202.