Observations on the Action of Limit Dextrinases on Amylopectin-Like Polysaccharides

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The rate of hydrolysis of amylopectin by three different limit dextrinase preparations is only about 15–23% of that of amylopectin β -limit dextrin under similar conditions. On dilution of the enzymes there was no change in specificity. The factors controlling the specificity of the enzyme and the possible significance *in vivo* of the results are discussed.

Previous studies (Manners *et al.*, 1970) have shown that limit dextrinase preparations from germinated barley and from ungerminated oats catalyse the debranching of pullulan, amylopectin β -limit dextrin and a range of α -dextrins possessing α -maltosyl or α -maltotriosyl side chains. However, the barley limit dextrinase had apparently no action on amylopectin, glycogen or glycogen β -limit dextrin, as shown by iodine-staining-power measurements, after incubation of these substrates with the enzyme for periods of up to 40 h. Debranching enzyme preparations from broad beans and malted barley were shown by MacWilliam & Harris (1959) to be a mixture. One enzyme acted on amylopectin and its β -limit dextrin, but not on oligosaccharide α -dextrins, and was termed R-enzyme, in accord with the earlier nomenclature of Hobson *et al.* (1951). The second enzyme apparently had no action on amylopectin, but readily debranched α -dextrins, and was therefore called limit dextrinase. These general conclusions on the barley enzyme system were later confirmed (Manners & Sparra, 1966; Manners & Rowe, 1971). Drummond *et al.* (1970) have not accepted the evidence for the existence of a separate limit dextrinase and an R-enzyme in the same plant extract. They reported that, when debranching-enzyme preparations from potatoes or from *Aerobacter aerogenes*, which normally acted on amylopectin, pullulan and α -dextrins, were diluted, the activity towards amylopectin selectively disappeared, and the remaining activity towards pullulan and α -dextrins then resembled that ascribed to 'limit dextrinase'. Although no explanation for the apparent change in specificity on dilution was offered, these authors suggested that the term 'R-enzyme' could be used to describe debranching activity towards both amylopectin and the other substrates. We have therefore re-examined the action of plant limit dextrinases on amylopectin and related polysaccharides.

Experimental

Materials. The polysaccharide and oligosaccharide substrates were laboratory samples used in previous

studies (Manners *et al.*, 1970; Manners & Yellowlees, 1971). Limit dextrinase was prepared from extracts of malted sorghum, ungerminated oats and ungerminated rice by methods similar to those used for germinated barley (Manners & Rowe, 1971; Manners & Yellowlees, 1971). An extract of malted sorghum was fractionated with ammonium sulphate, and the protein precipitated between 30 and 50% saturation was collected, dialysed and subjected to continuous curtain electrophoresis with a Beckman model CP cell (Beckman Instruments, Palo Alto, Calif., U.S.A.). Those fractions showing limit dextrinase activity were combined, freed from α -amylase by passing twice through a starch-Celite column and finally purified by gel filtration on Sephadex G-150 (Pharmacia, Uppsala, Sweden). Limit dextrinase was also prepared from extracts of ungerminated oats and rice, by precipitation with ammonium sulphate (0–40% and 0–80% saturation respectively) followed by chromatography on columns of DEAE-Sephadex and Sephadex G-150. At each stage, active fractions were combined and concentrated by ultrafiltration, as appropriate. Limit dextrinase activity was assayed with pullulan as substrate (Bender & Wallenfels, 1966), the unit of activity being defined as the amount of enzyme which in 1 min at 30°C and pH 5.0 liberates 1 μ mol of maltotriose. The final limit dextrinase preparations, which were essentially free from other interfering carbohydrases such as α -amylase and α -glucosidase, had specific activities of approx. 1.0, 0.8 and 0.6 unit/mg of protein respectively. For convenience, the enzyme concentration in the various digests is expressed as munits.

Methods. Enzyme action was followed as described previously (Manners & Sparra, 1966) by iodine staining or by measurement of the increase in reducing power by using a colorimetric alkaline copper reagent (Nelson, 1944; Somogyi, 1952). With the sorghum limit dextrinase, digests contained enzyme (80 or 8 munits), substrate (8 mg of amylopectin, 4 mg of amylopectin β -limit dextrin or glycogen, or 2 mg of glycogen β -limit dextrin) and buffer (0.15 M-sodium acetate, pH 5.0) in a total volume of 6 ml.

Duplicate samples (0.2 ml) were removed at intervals for analysis of reducing sugars. Samples (0.1 ml or 0.2 ml) were also removed for measurement of iodine staining power, at 680 nm for amylopectin and its β -dextrin and at 490 nm for glycogen and its β -dextrin. In one experiment with amylopectin, the digest volume was increased to 10 ml and duplicate samples (1.0 ml) were removed for determination of reducing power.

With the oat limit dextrinase, digests contained either a standard amount of enzyme (8 munits/ml), buffer and various amounts of amylopectin (0–8 mg), or various amounts of enzyme (0–8 munits), buffer and a fixed amount of amylopectin (2 mg). After incubation at 30°C for 2 h, duplicate samples were removed for measurement of reducing sugar.

With the rice limit dextrinase, digests containing enzyme (30 munits), buffer and substrate (0–4 mg) in a total volume of 1 ml were incubated at 30°C and duplicate samples (0.1 ml) were removed at intervals for analysis of reducing power or iodine staining. Control experiments had shown that the latter technique can also be used to assay debranching activity.

Where appropriate, initial rates of hydrolysis were obtained from tangents of the progress curves.

Results and discussion

When relatively high concentrations of sorghum limit dextrinase (80 munits/digest) were incubated for 50 h with the four polysaccharide substrates, there was a significant increase in reducing power with amylopectin and its β -limit dextrin, but not with glycogen (Fig. 1a). There were also similar increases in iodine staining power; after 25 h the percentage increases with amylopectin β -dextrin, amylopectin, glycogen β -dextrin and glycogen were 88, 23, 14 and 2 respectively. The relative initial rates of hydrolysis of amylopectin β -limit dextrin, amylopectin and glycogen β -limit dextrin were 36, 5 and 1 respectively. The digests were repeated with 8 munits of enzyme, but significant increases in reducing power (see Fig. 1b) and iodine staining power (45%) were observed only with amylopectin β -limit dextrin. At the end of the incubation period, the digests still contained active enzyme, since addition of pullulan followed by further incubation at 30°C led to significant increases in reducing power. However, since the rate of hydrolysis is dependent on enzyme concentration, it seemed possible that, with the diluted enzyme preparation, the production of new reducing groups might be so low as to be below the sensitivity of the colorimetric alkaline copper reagent if the digest was analysed in the usual way (with duplicate 0.2 ml samples). That this possibility was correct is shown in Fig. 1(b), where analysis of 1.0 ml samples from a digest with amylopectin showed a significant increase in reducing power. We have therefore confirmed the observations made by

Fig. 1. Action of sorghum limit dextrinase on various polysaccharides

Enzyme action was followed by measurement of the increase in reducing power of 0.2 ml samples removed from digests containing amylopectin (○), amylopectin β -limit dextrin (●), glycogen (□) or glycogen β -limit dextrin (■) and either 80 munits (a) or 8 munits (b) of enzyme. The broken line shows the increase in reducing power of amylopectin when 1.0 ml samples were analysed. All experimental points represent the average of duplicate analyses.

Drummond *et al.* (1970), but conclude that there is no real change in the specificity of the enzyme on dilution, and that the effect is due to the limit of sensitivity of the analytical reagent, at low rates of enzymic hydrolysis.

The action of oat limit dextrinase on amylopectin was also examined. This polysaccharide was incubated with various concentrations of the enzyme for 2 h. The results (Table 1) show that the rate of debranching of amylopectin is directly proportional to enzyme concentration. This result is also not in accord with the suggested change in specificity on dilution (Drummond *et al.*, 1970). The reaction followed normal Michaelis–Menten kinetics; the relative initial rates of hydrolysis of pullulan, amylopectin and amylopectin β -limit dextrin were 100, 25 and 160 respectively. For amylopectin, the K_m value was about 10 mg/ml. The above results contrast with those of Marshall (1969), who reported that, at unspecified

Table 1. *Effect of the concentration of oat limit dextrinase on the rate of debranching of amylopectin*

Digests contained amylopectin (2mg), buffer (0.3ml) and enzyme (0.2ml) in a total volume of 1.0ml, and were analysed for reducing sugars after incubation at 30°C for 2h.

Enzyme concentration (munits/digest)	0.8	2.0	4.0	6.0	8.0
Extent of hydrolysis (increase in E_{600})	0.01	0.02	0.05	0.08	0.12

concentrations, oat limit dextrinase did not act on amylopectin.

Generally similar results have also been obtained with a limit dextrinase preparation from ungerminated rice. The relative initial rates of hydrolysis of pullulan, amylopectin and amylopectin β -limit dextrin were 100, 35 and 150 respectively, and for amylopectin the K_m value was about 6mg/ml.

The present demonstration that limit dextrinases have a small but significant affinity for amylopectin probably has little importance *in vivo*, since in plant tissues only α -amylases of the commonly occurring starch-metabolizing enzymes are able to attack the native starch granules. Hence the action of α -amylase will degrade at least part of the amylopectin to the oligosaccharide level. Further degradation of the latter to linear maltosaccharides will require the action of limit dextrinase. It should be noted that limit dextrinases have a high 'affinity' for α -dextrans; for example, the rice enzyme has K_m 6.5×10^{-4} M for a branched heptasaccharide and the oat enzyme K_m 2.0×10^{-4} M for a branched pentasaccharide. The results also need not have implications with respect to enzyme nomenclature, in spite of recent suggestions (Lee & Whelan, 1971) that the name 'limit dextrinase' should be abandoned. The substrates *in vivo* are most probably oligosaccharide α -dextrans derived from amylopectin, although the parent polysaccharide and pullulan represent alternative substrates that are useful for studies *in vitro*. It should be emphasized that the term 'limit dextrinase' has been firmly established in the literature for almost 30 years.

Previous results (Manners & Yellowlees, 1971) have suggested that barley limit dextrinase had no significant action on amylopectin under conditions in which hydrolysis by debranching enzymes from potatoes and *A. aerogenes* had been demonstrated by Drummond *et al.* (1970). This suggestion was based on an insignificant increase in reducing power observed after incubation for 4.5 h in a digest containing 7.2mg of substrate/ml and 5.8 munits of enzyme/ml, compared with significant increases observed (Drummond *et al.*, 1970) after incubation for 5 h in digests containing 4mg of substrate/ml and 2.5 munits of enzyme/ml. It is possible that, if the period of incubation with the barley enzyme had been increased or the relative concentration of enzyme

increased (cf. Fig. 1), a significant increase in reducing power would have been observed.

The above results also emphasize the need for caution in attempting to define, in absolute terms, the specificity of debranching enzymes. In a digest containing 0.67mg of glycogen/ml and 13.3 munits of the sorghum enzyme/ml it was not possible to demonstrate significant hydrolysis, although at these concentrations amylopectin β -limit dextrin was readily attacked (Fig. 1a). It is, however, possible that at a higher concentration of enzyme glycogen might also be a substrate.

We have previously suggested that the specificity of debranching enzymes may be controlled partly by the length of the side chain that is released and partly by the relative position of attachment of the side chain to the adjacent main chain (Manners, 1970, 1971). The results given in Fig. 1 support this view. For the plant limit dextrinases, amylopectin β -limit dextrin appears to be the optimum polysaccharide substrate at present available with side chains of α -maltosyl or α -maltotriosyl units, and with about 6 glucose residues between branch points on the periphery of the molecule. The increased length of the side chains in amylopectin results in a decreased rate of hydrolysis. A similar relationship exists with glycogen and its β -dextrin, but with these substrates the relatively short inner chains of about 3–4 glucose residues apparently hinder enzyme action so that the rate of hydrolysis is only a fraction of that of the corresponding amylopectin-type substrates.

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