ON THE SPECIFICITY OF STARCH DEBRANCHING ENZYMES

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1. Introduction

An enzyme preparation from broad beans that hydrolyses the 1 → 6-branch linkages of amylopectin, amylopectin β-limit dextrin and amylopectin α-limit dextrins was discovered by Hobson, Whelan and Peat [1] and termed R-enzyme. Subsequently MacWilliam and Harris [2] described the fractionation of bean and malted barley extracts on alumina such that separate 1 → 6-bond hydrolases were found. One debranched amylopectin and its β-dextrin and not oligosaccharide α-limit dextrin. The second had the reverse specificity. These separated activities were given the respective names R-enzyme and limit dextrinase. Manners and coworkers [3, 4] have subsequently confirmed the MacWilliam and Harris finding, with the same and with different plant extracts.

In our own work, when further purifying potato R-enzyme, we have never observed a separation of the activities. Moreover, as we shall report here, a bacterial R-enzyme, pullulanase, has all the activities of R-enzyme, even when purified to homogeneity.

Our inability to correlate our own results with those of MacWilliam and Harris [2] and Manners and coworkers [3, 4] has perhaps been overcome following a recent report by Manners, Marshall and Yellowlees [5], where it is stated that amylopectin β-limit dextrin is a substrate for "limit dextrinase". This is contrary to the report by MacWilliam and Harris [2] but now permits an explanation of what limit dextrinase might be, since under its new definition, it has all the activities originally ascribed to R-enzyme [1], save that of attacking amylopectin. We report here that when R-enzyme and pullulanase are diluted, the activity towards amylopectin selectively disappears, while the activities towards the β- and α-limit dextrins are retained.

2. Materials and methods

Amylopectin was prepared from waxy maize as by Schoch [6] and its β-limit dextrin by exhaustive and repeated degradation by β-amylase [7]. A mixture of hexa- and hepta-saccharide α-limit dextrins was separated by charcoal-column chromatography [8] of the products of the action of salivary α-amylase on amylopectin [9]. Pullulan was prepared with the aid of *Pullularia pullulans* [10].

Reducing sugars were measured by the method of Nelson [11]; total carbohydrate was determined with glucose oxidase [12] following the total hydrolysis of polysaccharides as by Pirt and Whelan [13]. Protein concentrations were measured using the Lowry procedure [14]. The iodine-staining powers of digests were determined by adding portions to an acidified solution of 0.02% I_2 and 0.2% KI and measuring the absorbance at 680 nm in a Coleman Junior II spectrophotometer.

The action of R-enzyme on various substrates was followed at 30° in digests containing substrate (4 mg/ml), 50 mM sodium citrate buffer, pH 7.0, and enzyme as indicated. The action of pullulanase was followed in similar digests containing 40 mM sodium citrate-phosphate buffer pH 5.0. Samples were removed at intervals and the increases in reducing power and in iodine-staining power were determined. Reducing power was expressed as μg equivalent of maltose.

Potato R-enzyme cochromatographed with Q-enzyme when an ammonium sulphate concentrate was

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fractionated on DEAE-cellulose [15]. The eluate was concentrated ten-fold in an Amicon ultrafiltration 400 cell containing a Diaflo membrane W.M.–1 (M.W. cut-off, 10,000) and was dialysed overnight at 4° against 30 mM sodium citrate-1 mM dithiothreitol, pH 7.0. The concentrate was twice treated with three volumes of a 0.5% suspension of retrograded potato amylose [16] for 3 hr at 0°. The suspended amylose was removed each time by centrifugation for 10 min at 12,000 *g*. Only 0.2% (0.03 units/ml) [15] of the original Q-enzyme activity remained after the two treatments and more than 90% of the debranching activity was recovered. Pullulanase was prepared from *A. aerogenes* by the method of Wallenfels et al. [17], as modified by Frantz [18]. R-Enzyme and pullulanase activities were assayed at 30° in digests (2 ml) containing 0.2% amylopectin β-dextrin and 40 mM sodium citrate, pH 7.0, or 40 mM sodium citrate-phosphate, pH 5.0, buffers, respectively. The amount of pullulanase that released 10 μg equivalents of maltose/hr under these conditions corresponded to 2.5×10^{-3} IU when pullulan was used as substrate [18].

3. Results

Our observations stemmed from studies of the purification of the amylopectin synthesizing enzyme of potato (Q-enzyme) and tests for its freedom from α-amylase [15]. It was noted that various Q-enzyme preparations hydrolysed amylopectin β-dextrin. Sometimes amylopectin was also hydrolysed, sometimes not. The hydrolytic activity was therefore not likely to be α-amylase. Suspecting that hydrolysis of the 1 → 6-bonds was occurring, the hydrolytic activity was separated from Q-enzyme by adsorption of the latter on retrograded amylose and then tested for activity towards appropriate poly- and oligosaccharide substrates. The concentrated hydrolytic activity was found to hydrolyse amylopectin β-dextrin, α-limit dextrins, pullulan and also amylopectin (fig. 1). However, on dilution, and with amylopectin and its β-dextrin and α-limit dextrins as substrates, the activity towards amylopectin was no longer apparent, while the actions on β-dextrin and α-dextrins were retained (fig. 2). The enzyme attacking β-limit dextrin remained active in the amylopectin digest (see fig. 2).

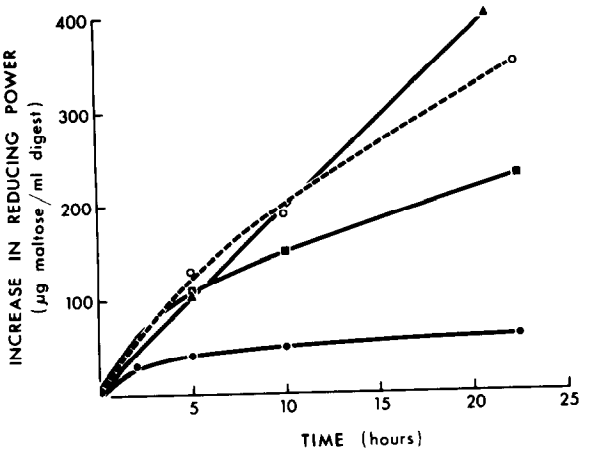

Fig. 1. Hydrolysis of poly- and oligosaccharides by potato R-enzyme (2.5×10^{-3} IU per ml, pullulan substrate). Experimental details are in the text.
●——●: amylopectin;
■——■: amylopectin β-dextrin;
▲——▲: amylopectin α-dextrins;
○- - -○: pullulan.

Bacterial pullulanase was then found to display similar behaviour. At an activity equal to that of the concentrated potato enzyme acting on β-dextrin, all three polysaccharide and the oligosaccharide sub-

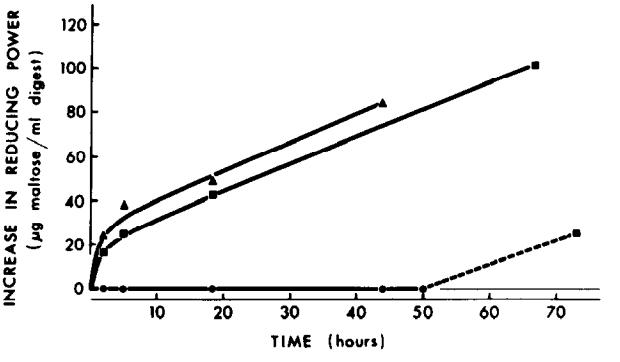

Fig. 2. Hydrolysis of poly- and oligosaccharides by diluted potato R-enzyme. The enzyme concentration was a quarter of that in fig. 1.
●——●: amylopectin;
■——■: amylopectin β-dextrin;
▲——▲: amylopectin α-dextrins.
At 50 hr β-dextrin was added to the amylopectin digest.

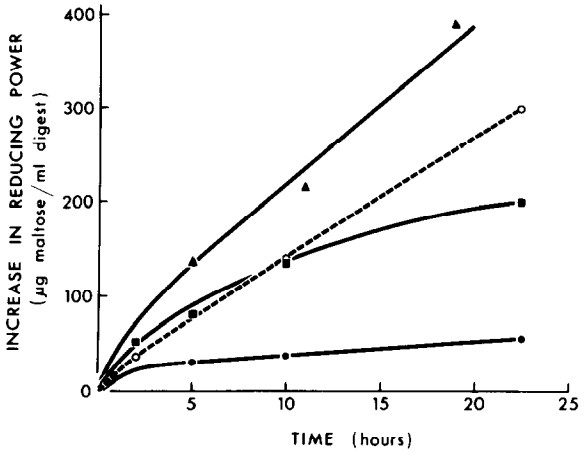

Fig. 3. Hydrolysis of poly- and oligosaccharides by pullulanase (2.5 × 10^{-3} IU per ml, pullulan substrate).
●——●: amylopectin;
■——■: amylopectin β-dextrin;
▲——▲: amylopectin α-dextrins;
○- - -○: pullulan.

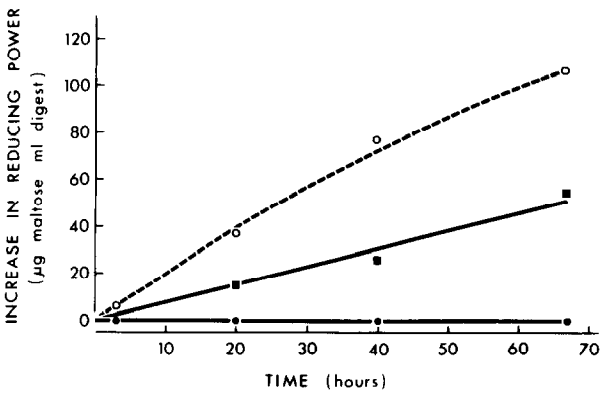

Fig. 4. Hydrolysis of polysaccharides by diluted pullulanase. The enzyme concentration was one-tenth of that in fig. 3.
●——●: amylopectin;
■——■: amylopectin β-dextrin;
○- - -○: pullulan.

strates were hydrolysed (fig. 3). On dilution, the activities on pullulan and β-dextrin were retained, while that on amylopectin was no longer apparent (fig. 4). The pullulanase was a preparation displaying a single protein band, coincident with pullulanase activity, on disc-gel electrophoresis [19].

The potato and pullulanase preparations caused equal increases in the intensities of iodine stain of amylopectin and amylopectin β-dextrin, concomitant with the release of reducing groups (table 1). A thin-layer chromatogram of pullulan after partial hydrolysis by the potato preparation showed maltotriose and its 1 → 6-linked oligomers, as already reported for pullulanase [20]. The hydrolysis of the pullulan was therefore occurring by splitting of the 1 → 6-bonds, and by an endo mechanism.

Table 1
Actions of R-enzyme and pullulanase on poly- and oligosaccharide substrates.

Substrate	1 → 6-Bonds hydrolysed (%)		Increase in iodine stain, 680 nm (%)	
	R-Enzyme	Pullulanase	R-Enzyme	Pullulanase
Amylopectin	21	21	26	28
Amylopectin β-limit dextrin	47	43	40	41
Pullulan	35	35	–	–
Amylopectin α-limit dextrins	36	40	–	–

The activities of the two enzymes were equal (2.5 × 10^{-3} IU per ml, pullulan substrate). The time of incubation was 50 hr. Experimental details are in the text.

4. Discussion

We have noted above that MacWilliam and Harris [2] seemed to have separated the 1 → 6-bond hydrolases of malted barley into two fractions, one acting on polysaccharides (amylopectin, amylopectin β-dextrin) and one on oligosaccharides (α-limit dextrins). Subsequent investigations by Manners et al. [5] have now included polysaccharides, i.e. amylopectin β-dextrin and pullulan among the substrates for this "limit dextrinase". It is clear from our results that a potato enzyme preparation can be made to behave like barley limit dextrinase under its new definition, but that the same potato preparation will also debranch amylopectin if used in more concentrated form. We do not know why there is a selective disappearance of the amylopectin debranching activity on dilution, but would point out that even in the original description of R-enzyme by Hobson et al. [1], the more rapid action on β-limit dextrin was recorded, and the enzyme evidently is able to debranch short side chains (β-dextrin) more easily than long side chains (amylopectin). The same enzyme preparation can therefore be made to behave both as an "R-enzyme" in the original definition of Hobson et al. [1], and as a "limit dextrinase" as most recently defined by Manners et al. [5]. The same is true for the bacterial enzyme pullulanase, a homogeneous protein. We therefore suggest that limit dextrinase as newly defined [5] and R-enzyme as originally defined [1] are one and the same entity, that the term limit dextrinase be abandoned, and that R-enzyme should retain its original definition. We have no opinion on what is the activity in malted barley referred to by Manners and Sparra [4] as R-enzyme, and which is described as acting on amylopectin and its β-dextrin, as judged by increases in intensity of iodine stain and degree of β-amylolysis, but not acting on pullulan and α-limit dextrins. It is this activity that is in need of further investigation and renaming.

The dilution effects demonstrated with potato R-enzyme and pullulanase stress the requirements that the specificities of debranching enzymes be defined (a) by direct measurement of debranching activity, such as increase in reducing power, and (b) not only in terms of their relative rates of action on various substrates, but in absolute terms under standardized incubation conditions at as high an enzyme concentration as possible. A conclusion that an enzyme preparation does not attack a particular substrate really applies only to a given set of experimental conditions and cannot be taken as definitive under all circumstances. The examples of α-and β-amylase in relation to maltotriose may be cited. Enzyme levels may be chosen where maltotetraose is rapidly hydrolysed but maltotriose is apparently not. At higher enzyme concentrations, hydrolysis of the latter is seen [21]. The same may be true for R-enzyme with potential substrates such as glycogen where so far hydrolysis has not been observed [22].

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