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Note

A viscometric assay for pullulanase-type, debranching enzymes*

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Bacterial pullulanases (E.C. 3.2.1.41) specifically hydrolyse the α-(1→6)-D-glucosidic bonds which form the inter-chain linkages in certain α-(1→4)-linked D-glucose polymers such as amylopectin[2]. The limit dextrinases, which serve a similar function in higher plants, are now known to be qualitatively similar in specificity to these bacterial enzymes[3,4]. Both groups of enzymes may be routinely assayed by following the release of reducing groups from the fungal polysaccharide pullulan[5], which consists of maltotriose units connected through the terminal residues by α-(1→6) linkages to form a linear polymer[6]. Pullulanases or limit dextrinases hydrolyse the α-(1→6) linkages to give maltotriose as the end product. As a substrate, pullulan has the great advantage that it is not attacked by alpha- or beta-amylases, and only very slowly by α-D-glucosidases. Unfortunately, however, the oligosaccharides that are released by pullulanase action form good substrates for α-D-glucosidases. Thus, if the reaction is monitored by using a reductometric method and α-D-glucosidases are present, which is usual, for example, in many plant extracts, an erroneous estimate of debranching activity will be obtained.

Viscometry has been used to monitor the degradation of polysaccharides, especially β-D-glucans[7], and it gives a very sensitive assay for enzymes which hydrolyse internal linkages in an endo-fashion. Since bacterial pullulanase has been reported to have an endo-mechanism for the degradation of pullulan[8], and hydrolysis of the maltotriose released or exo-attack by α-D-glucosidases would have a negligible effect on viscosity, the use of viscometry for the assay of a pullulanase-type, debranching enzyme has been investigated.

EXPERIMENTAL

Limit dextrinase was purified from germinated barley[4,9]. The preparation was free of amylases or α-D-glucosidases, and had a specific activity of 3 units/mg of protein. One unit releases one μmole of apparent maltotriose per min at 30°, from pullulan[5].

Amyloglucosidase (a gift from Dr. I. D. Fleming of Glaxo Research Ltd.) was

*Studies on debranching enzymes: Part II. For Part I, see ref. 1.

a purified preparation from *Aspergillus niger*, and had a specific activity of 20 international units per mg.

Pullulan was a laboratory sample and its preparation had been described previously[10]. Assays of reducing power were performed by a modified Nelson–Somogyi procedure[11].

Viscometry was carried out at 37°, and solutions, pipettes, and viscometer tubes were pre-warmed to this temperature. The reaction was started by mixing 1 ml of enzyme solution with 1.5 ml of 1% pullulan in 100mM sodium acetate buffer (pH 5.3). A portion (2 ml) of the mixture was rapidly pipetted into an Ostwald No. 1 B.S. viscometer tube, and the flow time recorded at intervals of 2 min during the incubation. These conditions gave an initial flow-time of 50–60 sec.

RESULTS

Pullulan can be regarded as a homopolymer of repeating maltotriose units, so that the treatment of Bryce and Greenwood[12] can be used. They showed that for random degradation of a homopolymer at a constant rate, and assuming that the specific viscosity at any particular degree of degradation is proportional to the number-average degree of polymerisation, the reciprocal of specific viscosity should rise at a constant rate. When the viscometric assay was performed using several different concentrations of barley limit-dextrinase, this relationship was found to hold for each concentration tested (Fig. 1). For convenience, zero time was arbitrarily taken as the time when the first measurement of flow time was started.

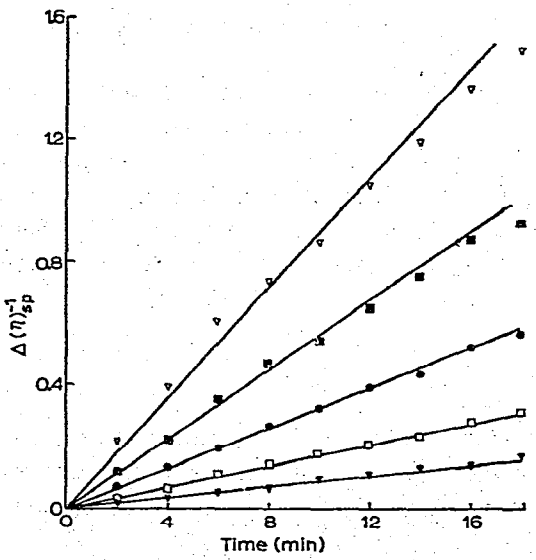

Fig. 1. The increase in reciprocal specific viscosity as a function of time at several different concentrations of enzyme. For conditions, see Experimental section. Key ▼, □, ●, ■, and ▽ represent digests containing, respectively, 1.62, 3.25, 6.5, 13.0, and 19.0×10^{-3} units of barley limit-dextrinase.

The rate of increase of reciprocal specific viscosity is a useful measure of enzyme activity, since when the slopes of the lines in Fig. 1 were plotted against enzyme concentration, a straight line was obtained (Fig. 2).

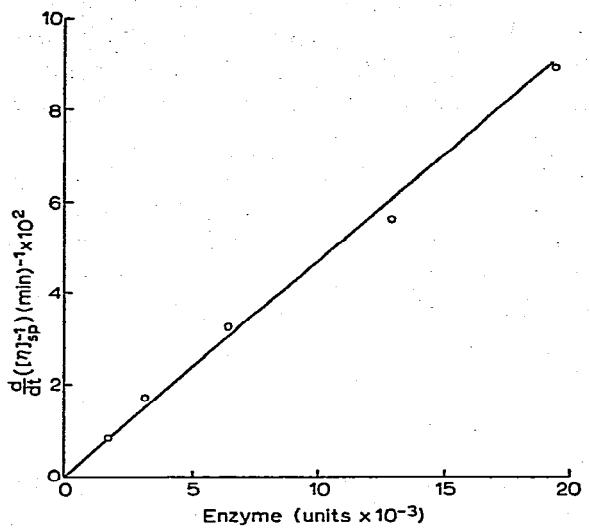

Fig. 2. The rate of increase of reciprocal specific viscosity as a function of enzyme concentration. Data calculated from Fig. 1.

The linear rise in reciprocal specific viscosity obtained is consistent with a random, internal cleavage of pullulan by barley limit-dextrinase. Below a specific viscosity of ~0.5, the relationship became non-linear (results not reported). This could be due to a breakdown in the viscosity–average d.p. relationship at low d.p., or to a change in action pattern of the enzyme.

TABLE I

EFFECTS OF AMYLOGLUCOSIDASE ON THE REDUCTOMETRIC AND VISCOMETRIC ASSAYS OF LIMIT DEXTRINASE[a]

Enzyme	*Reductometric assay* (ΔE_{600})	*Viscometric assay* ($\Delta [\eta]_{sp}^{-1}$ *per min*)
Limit dextrinase	0.171	0.0061
Amyloglucosidase	0.011	—
Limit dextrinase + amyloglucosidase	0.398	0.0064

[a]Digests contained 2.5×10^{-3} units of barley limit-dextrinase or 5×10^{-3} units of amyloglucosidase, or both. Incubations for the reductometric assay contained enzyme(s), and 5 mg of pullulan in 1 ml of 20mM citrate buffer (pH 5.3); 100-μl samples were assayed for reducing power. Results are expressed as the increase in extinction at 600 nm after incubation for 4 h at 30°. The viscometric assay is as described in the text.

The method is also insensitive to the presence of α-D-glucosidases (Table I). When a mixture of limit dextrinase and amyloglucosidase was assayed by the reductometric method, the value obtained was much more than the sum of the values using the two enzymes separately, indicating hydrolysis, by the amyloglucosidase, of the products released by limit dextrinase. In contrast, the presence of amyloglucosidase had an insignificant effect on the viscometric assay of pullulan. It may also be seen from Table I that viscometry is by far the more sensitive method. The increase in reducing power was measured over a period of 4 h, whereas a significant decrease in viscosity was obtained in only 2 min.

In the absence of automated equipment, this method is more laborious than the conventional reductometric technique. It also has the disadvantage that absolute units cannot be obtained unless a purified debranching enzyme is available for calibration purposes. In view of its high specificity and sensitivity, however, it is to be recommended for the assay of crude plant-extracts where the presence of other carbohydrases, particularly α-D-glucosidases, is suspected.

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REFERENCES

1 D. J. MANNERS AND D. YELLOWLEES, *J. Inst. Brewing*, 79 (1973) 377.
2 E. Y. C. LEE AND W. J. WHELAN, in P. BOYER (Ed.), *The Enzymes*, Vol. 5, Academic Press, London and New York, 3rd edition, 1972, p. 192.
3 G. S. DRUMMOND, E. E. SMITH, AND W. J. WHELAN, *FEBS Letters*, 9 (1970) 136.
4 G. DUNN, D. G. HARDIE, AND D. J. MANNERS, *Biochem. J.*, 133 (1970) 413.
5 H. BENDER AND K. WALLENFELS, *Methods Enzymol.*, 8 (1966) 555.
6 B. J. CATLEY AND W. J. WHELAN, *Arch. Biochem. Biophys.*, 143 (1971) 138.
7 D. J. MANNERS AND G. WILSON, *Biochem. J.*, 135 (1973) 11.
8 G. S. DRUMMOND, E. E. SMITH, AND W. J. WHELAN, *FEBS Letters*, 5 (1969) 85.
9 D. G. HARDIE AND D. J. MANNERS, unpublished work.
10 D. J. MANNERS AND D. YELLOWLEES, *Staerke*, 23 (1971) 228.
11 J. F. ROBYT AND W. J. WHELAN, in J. A. RADLEY (Ed.), *Starch and its Derivatives*, Chapman and Hall, London, 4th edition, 1968, p. 432.
12 W. A. J. BRYCE AND C. T. GREENWOOD, *J. Polym. Sci.*, 25 (1957) 480.