Carbohydrate Research, 61 (1978) 419–424
© Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

Note

An assay for pullulanase in the presence of other carbohydrases*.

BRIAN J. CATLEY
Department of Brewing and Biological Sciences, Heriot–Watt University, Edinburgh (Great Britain)
(Received July 1st, 1977; accepted for publication with revisions, September 5th, 1977)

Enzymes that catalyse the hydrolysis of α-D-(1 → 6)-glucosidic bonds (EC 3.2.1.41) are of interest both as reagents in the elucidation of poly- and oligosaccharide structures and as catalysts controlling the cellular catabolism of α-D-glucans. Crude preparations of (1 → 6)-α-D-glucanohydrolase activity, whether originating from an intra- or extra-cellular location, are accompanied by other catalytic activities, some of which may mediate the hydrolysis of glucosidic bonds other than the α-D-(1 → 6) linkage. It is the accurate assay of the (1 → 6)-α-D-glucanohydrolase activity in the initial stages of enzyme extraction that presents a problem. The favoured choice of substrate is the microbial polysaccharide pullulan[1]. It is a linear α-D-glucan, the structure of which may be described as a polymer of α-maltotriosyl units linked by α-(1 → 6)-bonds at the two terminal D-glucosyl residues of the triose[2]. One third of the α-D-glucosidic bonds are therefore in the (1 → 6) position, the rest being in the (1 → 4). It appears to be comparatively resistant to carbohydrases other than pullulanase (alternatively known as R-enzyme or limit dextrinase), the systematic name of which is pullulan 6-glucanohydrolase[3]. Thus, hog pancreatic alpha amylase is without action, and human salivary alpha amylase attacks the molecule only at the points where maltotetraose has replaced maltotriose[4]. As the occurrence of this exchange is infrequent (1 in 15) the polymer is virtually resistant to this enzyme. Beta amylase is without action on pullulan[2]. The action pattern of the pullulanase derived from *Klebsiella aerogenes* is to cleave the (1 → 6)-α-D-glucosidic bonds in a random fashion to yield a series of oligosaccharides, eventually producing a complete conversion into maltotriose[5]. Pullulan is also hydrolysed by isopullulanase[6], a carbohydrase derived from *Aspergillus niger* which yields 6-*O*-α-maltosyl-D-glucose. The enzyme is therefore termed a pullulan 4-glucanohydrolase. However, whereas macromolecular pullulan might resist the catalytic hydrolysis of carbohydrases, the pullulanolysis products of pullulan do not. Maltotriose is subject to fragmentation by both beta and alpha amylase, yielding, in both cases, maltose and D-glucose, and by

*Dedicated to Professor Dexter French on the occasion of his 60th birthday.

α-D-glucosidase to produce D-glucose. Thus the situation may be imagined where both pullulanase and α-D-glucosidase are present in a crude preparation and whereas the glucosidase may be able to attack the pullulan in a limited fashion, it readily causes the hydrolysis of the liberated maltotriose to 3 molar equivalents of D-glucose. As the easiest method of monitoring the hydrolysis of a glycosidic bond is to measure the appearance of reducing power of the liberated hemiacetal, it is seen that the presence of α-D-glucosidase, if sufficiently active, would triple the apparent pullulanase activity when measured in this way. Thus, for every α-D-(1 → 6) bond present in pullulan that is hydrolysed by pullulanase two α-D-(1 → 4) bonds, susceptible to attack by the amyloglucosidase, are exposed and hydrolysed. A measure of the rate of disappearance of the macromolecular substrate, in place of an assay of the rate of appearance of products of hydrolysis, might avoid this problem; the circumstances in which the polymer pullulan may be enzymically modified, other than by pullulanase, are considerably less frequent than the further hydrolysis of fragments released from the macromolecule by pullulanase digestion. One property of pullulan that has been exploited in this fashion is the decrease in specific viscosity upon pullulanolysis[7]. Another property, described in this communication, is the insolubility of pullulan, and the solubility of the products of pullulanolysis, in aqueous ethanol.

EXPERIMENTAL

Materials. — Pullulanase derived from *Klebsiella aerogenes* was purchased from Boehringer, Mannheim, and amyloglucosidase, derived from *Rhizopus* mould, from the Sigma Chemical Company, London.

Preparation of [^{14}C]pullulan. — *Aureobasidium pullulans* (ATCC 9348) was grown in a medium of the following composition: D-glucose, 250mM; potassium chloride 5.0 mM; magnesium chloride, 4.0mM; sodium sulphate 4.0mM; sodium hydrogenphosphate and dihydrogenphosphate, 20 mM; ammonium chloride, 20 mM; ferric chloride hexahydrate, 2.0μM; manganese chloride tetrahydrate, 2.0μM; zinc chloride, 2.0μM; calcium chloride, 50μM; copper(II) sulphate pentahydrate, 0.2μM; and yeast extract (Oxoid) 0.04%. The inoculated medium (100 ml) was agitated on a gyratory shaker at 200 rev.min^{-1} for 24 h at 23°, at which point 25 μCi of uniformly labelled D-[^{14}C]glucose (Radiochemical Centre Ltd., Amersham) were added and the medium shaken for a further 24 h. Cells were removed by centrifugation at 25,000*g* for 15 min and the radioactively labelled, extracellular polymer precipitated from the clear supernatant liquid by the addition of two volumes of ethanol. After storage overnight at 4°, the white precipitate was washed with 66% aqueous ethanol, ethanol and methanol, and finally diethyl ether. It was then dried *in vacuo* for 24 h at 40°. The yield was 1.47 g from 4.50 g of D-glucose originally present in the medium. The glucan content of this amorphous powder, determined by acidic hydrolysis for 6 h at 100° in 0.75M sulfuric acid[8], followed by measurement of the released D-glucose by the D-glucose oxidase reagent[9], was 104%. Measurement of the ^{14}C isotope by liquid-scintillation spectrometry showed the incorporated radioactivity to be 1.2 μCi

g^{-1}. Exhaustive pullulanolysis, followed by paper chromatography in ethyl acetate–pyridine–water (10:4:3, by volume)[10], revealed a single spot of radioactivity coincidental with maltotriose. There was no radioactivity remaining at the origin.

Estimation of pullulanase activity. — A typical digest incubated at 37°, comprised [^{14}C]pullulan, 4 mg; citrate (0.05 mmol)phosphate (0.1 mmol) buffer, pH 5.0, and pullulanase in a final volume of 1.0 ml. The concentration of [^{14}C]pullulan present in the digest was estimated by applying 100-μl aliquots onto squares (2 × 2 cm) of Whatman No. 17 chromatography paper, which were immediately placed in 66% aqueous ethanol to stop the hydrolysis. At the end of the hydrolysis, the accumulated squares were extracted at room temperature with 3 × 150 ml stirred volumes of 66% aqueous ethanol, each wash lasting 10 min. The squares were then dried, placed in vials of scintillation fluid containing 2,5-diphenyloxazole (0.5%) and 1,4-bis[2(5-phenyloxazolyl)]benzene (0.01%) in toluene, and counted by standard procedures of liquid-scintillation spectrometry.

The activity of pullulanase on pullulan and amyloglucosidase on amylose was measured by the appearance of reducing power[11] and is calculated as the rate of release of maltotriose equivalents or D-glucose, respectively, at 37°. They are presented in terms of the Katal unit (Kat), a catalytic activity capable of liberating 1 mol of product per sec.

RESULTS AND DISCUSSION

Fig. 1 displays the rate of loss of [^{14}C]isotope from labelled pullulan during the pullulanase-catalysed hydrolysis of the polysaccharide at three different concentrations of enzyme. The plot of radioactivity against time is apparently linear for at least the first 40% of the total hydrolysis, and it is this slope that is used as a measure of enzyme activity. A plot of these slopes, determined for a variety of pullulanase concentrations extending over a 50-fold range of activities, is linear and is shown in Fig. 2. The assay for [^{14}C]pullulan is that used for labelled glycogen synthesised from a radioactive sugar nucleotide[12], and depends on the difference in solubility of substrate and products in aqueous ethanol. Examining the consequences of using different concentrations of aqueous ethanol to remove the products of pullulanolysis, activities of 14, 20, and 27 units (see Table I for a definition of the unit) were obtained from assays of identical concentrations of pullulanase but where extraction of the products was into 80, 66, and 50% aqueous ethanol respectively. As shown by Drummond *et al.*[13] the action pattern of pullulanase derived from *Klebsiella aerogenes* is to cleave the (1 → 6)-α-D-glucosidic bonds in an endo fashion to yield a series of oligosaccharides and eventually producing maltotriose. It is the decreased solubility of the larger fragments in the more-concentrated ethanol that must account for these observed differences in activity. For these reasons care must be taken in the dilution of the ethanol used for the extraction procedure.

The aim, however, of the present communication is not only to provide an alternative assay for pullulanase, but to devise a method that will be unaffected by

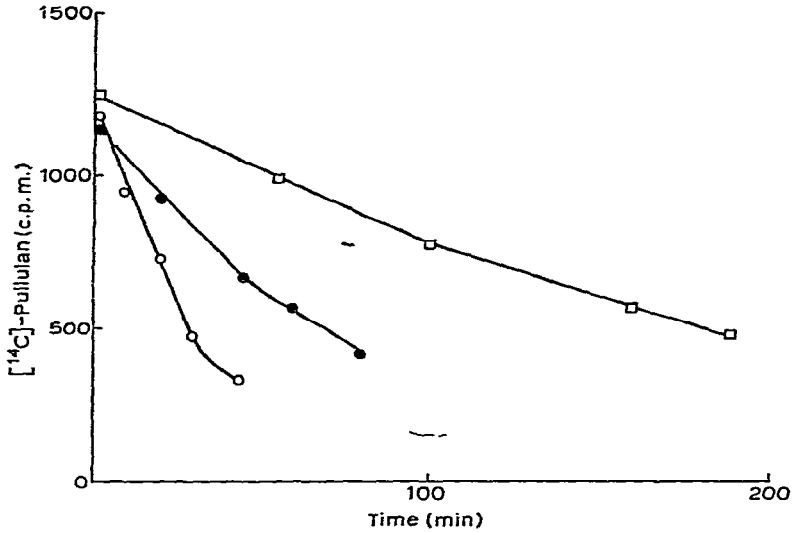

Fig. 1. The assay of pullulanase activity, measured as the rate of hydrolysis of [^{14}C]pullulan. Three different concentrations of pullulanase were used: 1.8nKat, ○; 0.72nKat, ●; and 0.36nKat, □; per ml of digest. The assay of [^{14}C]pullulan is described in the experimental section.

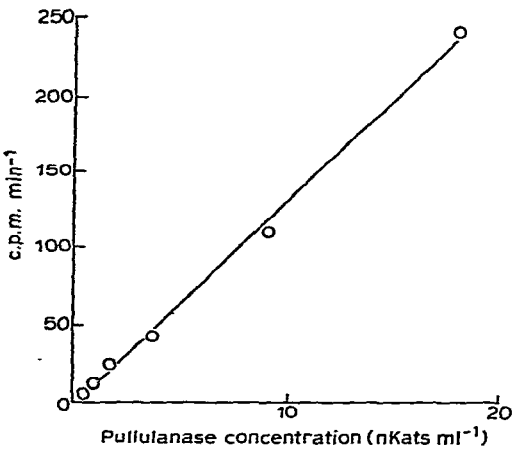

Fig. 2. The relationship between pullulanase concentration (nKat.ml^{-1} digest) and catalytic activity, as determined by the radioactive method described in the experimental section and illustrated in Fig. 1. The hydrolase activity is recorded as the rate of decrease of [^{14}C]pullulan (c.p.m. min^{-1}) measured on 100-μl samples.

the presence of other carbohydrases. Measurements of pullulanase activity (1.4 nKat) were made, by using the established reductometric method[1,14] and the radioactive technique described, in the presence and absence of amyloglucosidase activity (130 nKat). The amyloglucosidase used is a carbohydrase preparation derived from a genus of *Rhizopus*, and catalyses the hydrolysis of both (1 → 6)-α- and (1 → 4)-α-D-glucosidic bonds. A comparison of the methods of assessment is presented in Table I,

TABLE I

ASSAY OF PULLULANASE ACTIVITY (1.4 nKAT) IN THE PRESENCE AND ABSENCE OF AMYLOGLUCOSIDASE (130 nKAT)

Assay	*Enzyme Activity*[a,b]		
	Pullulanase	*Pullulanase and amyloglucosidase*	*Amyloglucosidase*
Reductometric[a]	0.0039	0.0122	0.0028
Radioactive[b]	13.5	16.0	2.0

[a]Determined by a modified Nelson–Somogyi procedure[11] and recorded as the release min^{-1} of μmol of maltotriose equivalents (pullulanase, pullulanase, and amyloglucosidase) or D-glucose (amyloglucosidase alone) measured in 100-μl samples. [b]Determined by the extraction method described in the experimental section and recorded as the rate of decrease of [^{14}C]pullulan (c.p.m. min^{-1}) measured in 100-μl samples.

where it may be seen that the reductometric assay of the pullulanolysis that was augmented with amyloglucosidase provides a measure of activity that is three-fold in excess of its true value. In contrast, the measure of activity derived from the radioactive assay was increased by only 18% in the presence of a carbohydrase concentration that is 130 times more effective in cleaving (1 → 4)-α-D-glucosidic bonds in amylose than the pullulanase is in hydrolysing α-D-(1 → 6)bonds in pullulan.

The advantages of the radioactive assay have been demonstrated. The disadvantage is that it does not produce a direct assessment of the catalytic power as a measure of the rate of hydrolysis of D-glucosidic bonds. The isotopically labelled pullulan substrate would have to be calibrated, with a purified pullulanase, by its incorporation into a pullulanase-assay digest that was monitored both by the reductometric and the radioactive methods. As mentioned in the introduction, the assay described may also be used to measure isopullulanase activity, as the method cannot distinguish between the fragmentation of pullulan at the (1 → 4) or the (1 → 6)bond.

The preparation of [^{14}C]pullulan is simple and is described in the experimental section. The delay in the addition of D-[^{14}C]glucose after the commencement of growth of *A. pullulans* is based on the observation that cellular growth precedes pullulan synthesis[15], and addition of the isotope during the logarithmic phase results in a more efficient incorporation into the extracellular polymer. The disadvantage of an assay where the measure is of a decrease in some parameter is compensated for in the sensitivity and accuracy of measurements that are possible by means of liquid-scintillation spectrometry. Thus, whilst not seeking to displace the conventional, reductometric method for the assay of pullulanase, the radioactive method offers clear advantages in the measurement of low activities and activities in the presence of other carbohydrases.

REFERENCES

1 H. BENDER AND K. WALLENFELS, *Biochem. Z.*, 334 (1961) 79–95.
2 K. WALLENFELS, G. KEILICH, G. BECHTLER, AND D. FREUDENBERGER, *Biochem. Z.*, 341 (1965) 433–450.
3 E. Y. C. LEE AND W. J. WHELAN, *Enzymes*, 5 (1971) 191–234.
4 B. J. CATLEY AND W. J. WHELAN, *Arch. Biochem. Biophys.*, 143 (1971) 138–142.
5 K. WALLENFELS AND I. R. RACHED, *Biochem. Z.*, 344 (1966) 524–526.
6 Y. SAKANO, M. HIGUCHI, AND T. KOBAYASHI, *Arch. Biochem. Biophys.*, 153 (1972) 180–187.
7 D. G. HARDIE AND D. J. MANNERS, *Carbohydr. Res.*, 36 (1974) 207–210.
8 S. J. PIRT AND W. J. WHELAN, *J. Sci. Food Agric.*, 2 (1951) 224.
9 J. B. LLOYD AND W. J. WHELAN, *Anal. Biochem.*, 30 (1969) 467–470.
10 G. O. ASPINALL AND R. J. FERRIER, *J. Chem. Soc.*, (1957) 4188–4194.
11 N. NELSON, *J. Biol. Chem.*, 153 (1944) 375–380.
12 J. A. THOMAS, K. K. SCHLENDER, AND J. LARNER, *Anal. Biochem.*, 25 (1968) 486–499.
13 G. S. DRUMMOND, E. E. SMITH, AND W. J. WHELAN, *FEBS Lett.*, 5 (1969) 85–88.
14 M. ABDULLAH, B. J. CATLEY, E. Y. C. LEE, J. ROBYT, K. WALLENFELS, AND W. J. WHELAN, *Cereal Chem.*, 43 (1966) 111–118.
15 B. J. CATLEY, *J. Gen. Microbiol.*, 78 (1973) 33–38.