Carbohydrate Research, 76 (1979) 203–213

PARTIAL PURIFICATION AND PROPERTIES OF A BACTERIAL ISOAMYLASE*

RICHARD M. EVANS, DAVID J. MANNERS, AND J. ROGER STARK

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

(Received March 5th, 1979; accepted for publication, March 12th, 1979)

ABSTRACT

Isoamylase has been prepared by affinity chromatography of a commercial enzyme-preparation from a strain of *Cytophaga* (also known as a *Flavobacterium* or *Polyangium*). The enzyme was not very stable, but the stability could be improved by calcium ions. The enzyme had a very low but significant activity on pullulan and on alpha-dextrins having maltosyl side-chains. This observation, which is contrary to previous reports, has been related to the specificity of isoamylase and other bacterial debranching-enzymes.

INTRODUCTION

Debranching enzymes, which hydrolyse the (1→6)-α-D-glucosidic inter-chain linkages in certain branched α-D-glucans, are extremely useful for the detailed structural analysis of amylopectins and glycogens, and of various dextrins derived from these polysaccharides. In particular, pullulanase from *Aerobacter aerogenes* has been widely used for the analysis of amylopectins[2–4]. However, the action of this enzyme on glycogens is variable. Some samples, for example, rabbit-liver glycogen and its beta-limit dextrin, are hydrolysed to a limited extent[5], whereas other samples, for example, human-liver glycogen, are apparently resistant to enzyme action[6]. Hence, the value of pullulanase in structural studies of glycogens appears to be limited.

In 1970, an extracellular debranching-enzyme from a commercial *Cytophaga* preparation was reported to hydrolyse all of the inter-chain linkages in both amylopectin and glycogen[7]. This enzymic activity, termed isoamylase, apparently had no action on pullulan. Isoamylase has been used to determine the average chain-length of glycogens[7,8] and to examine the distribution of chain lengths[9]. It should be noted that although the organism (N.C.I.B. 9497) was originally described as a *Cytophaga*[7], and then as *Flavobacterium*[10], more-recent work indicates[11] that it may be a *Polyangium* sp. The term "bacterial isoamylase" will therefore be used, wherever possible.

*Studies on Debranching Enzymes: Part VII. For Part VI, see Ref. 1.

Moreover, the commercial enzyme-preparation is a mixture of various enzymes, including β-D-glucanases[12] and proteolytic enzymes.

Although the action of the bacterial isoamylase on glycogen was complete, it was unable to debranch glycogen beta-limit dextrin completely. This was due to the apparent inability of the enzyme to hydrolyse inter-chain linkages attaching side-chains of two D-glucosyl residues to the polysaccharides, whereas side-chains of three D-glucosyl residues were readily released to give maltotriose[7]. This property forms the basis of an enzymic method for determining the ratio of A-chains to B-chains* in glycogens and amylopectins[14]. This ratio is an important parameter to be considered in relation to the overall molecular structure of the polysaccharides, *i.e.*, in deciding between Haworth-, Staudinger-, and Meyer-type structures[3].

Although the bacterial isoamylase has been widely used[3,6,7,9,14], full details of its purification and properties have not been published. In the original work[7], the commercial enzyme-preparation was extracted with 100mM sodium acetate buffer (pH 5.5), and the centrifuged extract used either directly, or after DEAE-cellulose chromatography at pH 8.0, which gave a 10-fold purification. By a combination of ion-exchange chromatography and molecular-sieve chromatography on polyacrylamide gel, a 100-fold purification has been reported, but in abstract form only[15]. We now describe a purification of the bacterial isoamylase based on affinity chromatography, which was an essential preliminary to our studies of the structural analysis of amylopectin and glycogen using this enzyme.

MATERIALS AND METHODS

Substrates. — Amylopectin (waxy-maize starch), amylopectin beta-limit dextrin, rat-liver glycogen, rat-liver glycogen beta-limit dextrin, and pullulan were laboratory samples, prepared by standard methods. Branched oligosaccharides were prepared by the salivary alpha-amylolysis of amylopectin, followed by preparative paper chromatography on Whatman No. 17 paper with 1-propanol–ethyl acetate–water (14:2:7), as described previously[16].

Chromatographic supports. — DEAE-Cellulose (DE 52) was supplied by W. and R. Balston Ltd. (Maidstone, England), ConA-Sepharose 4B by Pharmacia (Great Britain) Ltd. (London), and Biogel P-60 by Bio-Rad Laboratories Ltd. (Watford, England). All supports were prepared for use as recommended by the manufacturers.

Analytical methods. — Descending paper chromatography was performed with the solvent system described above. The papers were developed for 18–24 h, and the sugars detected with a silver nitrate reagent[17]. Reducing sugars were determined by a modified Nelson–Somogyi reagent[18] scaled down to half the recommended volumes, absorbances being measured at 600 nm. Total carbohydrate was determined by the phenol–sulphuric acid method[19]. Protein was estimated by the Miller[20] modification

*An A-chain is attached to the macromolecule by a single linkage from its potential reducing-group, whereas B-chains are linked to two or more other chains[13].

of the procedure of Lowry *et al.*[21]. Iodine staining of portions of enzyme digests (0.1 ml) was performed by the addition of 5 ml of iodine reagent (0.02% iodine and 0.2% potassium iodide in 0.01M sulphuric acid), the spectrum of the polysaccharide–iodine complex being recorded against an iodine–water control, using a Unicam SP800 spectrophotometer.

Assay for isoamylase activity. — The method of Gunja-Smith *et al.*[7] was used, in which suitably diluted, enzyme solution (0.05 ml) was incubated with glycogen (5 mg/ml) in 100mM sodium acetate buffer (pH 5.5) at 37° for 20 min. The reducing power of the digest, and of substrate and enzyme controls, was determined by the modified Nelson–Somogyi procedure. One unit of isoamylase activity is defined[7] as the amount of enzyme that releases 1 μmol glucose equivalent per min.

When isoamylase digests were to be incubated for several hours, toluene and 20mM calcium chloride were also present (see Results).

Purification of isoamylase. — All operations were performed at 0–5°, the apparatus being cooled before use, and column fractionations were performed in a cold room at 0–2°.

The original work[7] was carried out on a Lytic enzyme-preparation L1 produced by a *Cytophaga*, and purchased from British Drug Houses Ltd. (Poole, England). The present work was carried out on another batch of L1, kindly provided by Glaxo Research Ltd. (Stoke Poges, England), but produced by a different strain of the organism.

The freeze-dried culture filtrate was suspended in 10 ml of 10mM Tris–hydrochloric acid buffer pH 8.0 (concentration 50 mg/ml) and centrifuged at 1,500*g*, and insoluble material was discarded. The supernatant solution was applied to a column (6 × 2 cm) of DEAE-cellulose previously equilibrated with the same buffer, and eluted from the column with buffer. Appropriate fractions were combined and concentrated to ~5 ml by using an Amicon ultrafiltration-cell (Amicon Corp., Lexington, Mass., U.S.A.) fitted with a PM-10 membrane.

The affinity-chromatography procedure was based on the affinity of glycogen for concanavalin A, and the affinity of isoamylase for glycogen. The column was prepared by saturating a column (30 × 1.5 cm) of ConA-Sepharose with glycogen, by applying a 2% solution (3 ml) of glycogen in 250mM sodium acetate (pH 5.5) and eluting with this buffer. The concentrated solution of isoamylase was then applied to the column, and eluted with the same buffer. Under these conditions, the isoamylase was initially bound to the glycogen, which was then degraded, releasing maltosaccharides which no longer had a strong affinity for the ConA-Sepharose. Once the glycogen was substantially degraded, the isoamylase was eluted from the column. A typical elution profile is shown in Fig. 1. The point of elution of the enzyme was dependent on the flow rate, the ratio of glycogen to enzyme, and the temperature. In the absence of glycogen, the isoamylase was not bound to the column.

The fractions containing isoamylase activity were combined, concentrated to 1–2 ml, and applied to a column (40 × 1.5 cm) of Biogel P-60 which had been equilibrated with 100mM sodium acetate buffer (pH 5.5). The column was then

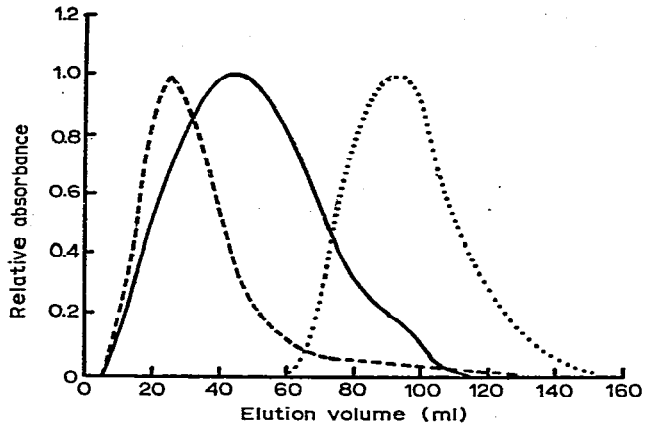

Fig. 1. Elution profile of isoamylase from a glycogen–ConA–Sepharose column: ---, protein (absorbance at 280 nm); ——, carbohydrate (phenol–sulphuric acid reagent); ...., isoamylase activity.

eluted with the same buffer. This procedure removed any maltosaccharides which had been introduced during the affinity procedure. Appropriate column fractions were combined and concentrated to 2–3 ml. The final enzyme solution had a specific activity of ~80 units per mg of protein, representing ~20-fold purification, with a yield of ~20%.

The isoamylase preparation was free from alpha-amylase, beta-amylase, and α-D-glucosidase activities, since, on prolonged incubation (up to 48 h) with either maltopentaose or maltoheptaose, there was no change in the maltosaccharide (paper chromatography), or in the reducing power of the digest.

RESULTS

Properties of the enzyme. — *(a) pH Optimum.* The enzyme was incubated with glycogen in the presence of 80mM acetate buffer (pH 3–6) or 80mM phosphate buffer

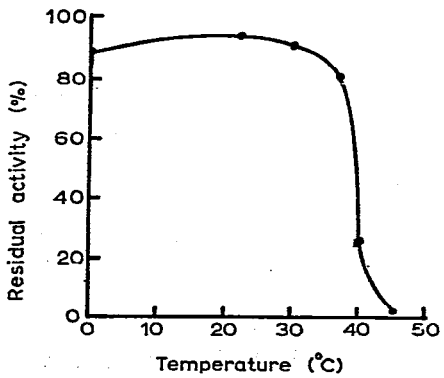

Fig. 2. Effect of temperature on the stability of isoamylase in the absence of substrate.

(pH 5–8). Maximum activity occurred over a relatively broad range, the optimum being between pH 5–6.

(*b*) *Temperature optimum.* The enzyme was incubated with glycogen in the presence of acetate buffer (pH 5.5) for 20 min. Under these conditions, the temperature optimum was 45°, and there was a rapid drop in activity above this temperature.

(*c*) *Stability of the enzyme.* The enzyme was incubated in 0.1M acetate buffer (pH 5.5), in the absence of substrate, at different temperatures in the range 0–45°. Enzyme activity was measured initially and after 30 min. The results (Fig. 2) showed that, in the absence of substrate, the enzyme was particularly unstable at temperatures above 37°.

(*d*) *Cation requirements for stability.* Incubation of the enzyme with 10mM EDTA caused a decrease in the stability, which became more marked with increasing temperature so that, at 37°, all activity was lost in less than 30 min. This suggested that a divalent cation was required for stability. The enzyme was therefore incubated with various divalent cations (10mM) in 50mM acetate buffer (pH 5.5) at both 30° and 37°. The activity was measured initially and after incubation for 18 h, the final concentration of cation in the assay digests being 0.1mM. The results (Table I) showed that the enzyme had some requirement for calcium ions; however, further experiments showed that the addition of calcium ions would not completely stabilise the enzyme, nor would they reverse the inactivation by EDTA.

Specificity of the enzyme. — (*a*) *Action on glycogen.* The action of the enzyme on rat-liver glycogen was examined in a digest containing enzyme (0.02 unit/ml), substrate (5 mg/ml), and 80mM acetate buffer (pH 5.5). After incubation at 37°, samples were removed at intervals for analysis by iodine staining and measurement of reducing power. The results showed that there was a change in the λ_{max} to higher wavelengths, and large increases in the maximum absorbance (Fig. 3) and in reducing power (Table II).

TABLE I

EFFECT OF VARIOUS DIVALENT CATIONS ON THE STABILITY OF ISOAMYLASE IN THE ABSENCE OF SUBSTRATE[a]

Cation	*Initial activity*	*Activity after 18 h at*	
		30°	*37°*
None	100	24	0
Ca^{2+}	100	74	37
Mg^{2+}	101	18	0
Zn^{2+}	101	0	0
Ba^{2+}	101	21	0
Cd^{2+}	91	3	0
Mn^{2+}	98	11	0
Hg^{2+}	0	0	0

[a]All activities are expressed as a percentage of the initial activity in the absence of added cations.

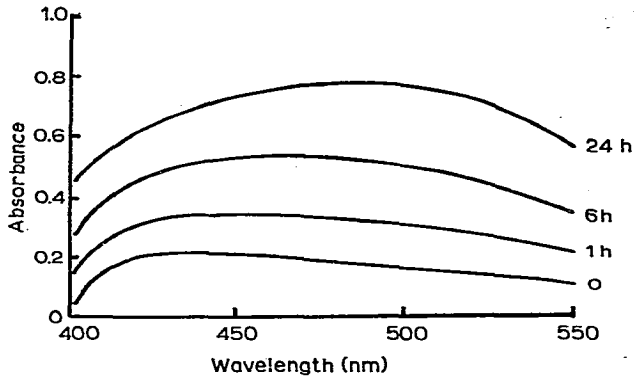

Fig. 3. Effect of isoamylase on the iodine-staining properties of glycogen.

TABLE II

INCREASE IN REDUCING POWER DURING ISOAMYLASE ACTION ON POLYSACCHARIDES[a]

Substrate	*Time (h)*					
	0.5	*1*	*2*	*6*	*18*	*24*
Rat-liver glycogen	—	0.37	—	0.60	—	0.82
Rat-liver glycogen beta-limit dextrin	0.33	0.38	0.45	—	0.61	—
Amylopectin beta-limit dextrin	0.16	0.21	0.28	—	0.45	—

[a]Results are shown as the increase in absorbance at 600 nm. Experimental conditions are given in the text. The final absorbance figures correspond to 7.0, 10.5, and 7.8% conversion into apparent glucose, respectively.

(*b*) *Action on the beta-limit dextrins of glycogen and amylopectin.* Digests containing either glycogen beta-limit dextrin (5 mg/ml) or amylopectin beta-limit dextrin (5 mg/ml) in 80mM acetate buffer (pH 5.5) and isoamylase (0.17 unit/ml) were incubated at 37°. The reducing power of both digests increased rapidly with time (Table II). The initial rates of attack on these substrates, relative to the rate of attack on glycogen, are shown in Table III. Paper-chromatographic analysis showed the presence of small proportions of maltose and larger proportions of maltotriose in the digests.

(*c*) *Comparison of the action of the bacterial isoamylase and* Aerobacter aerogenes *pullulanase on various substrates.* Glycogen, pullulan, and various alpha-limit dextrins were incubated with either isoamylase or pullulanase (Boehringer, from *Aerobacter aerogenes*) in 0.1M acetate buffer (pH 5.5) and 50mM calcium chloride at 37°. The substrate concentration was adjusted to 2mM with respect to (1→6)-α-D-glucosidic linkages. The isoamylase concentration was 6 units/ml for the branched oligosaccharides and pullulan, and 0.01 unit/ml for glycogen. The pullulanase concentration was 0.01 unit/ml for the oligosaccharides and pullulan, and 2.5 units/ml

TABLE III

A COMPARISON OF THE ACTION OF THE BACTERIAL ISOAMYLASE AND *Aerobacter aerogenes* PULLULANASE ON VARIOUS SUBSTRATES

Substrate[a]	*Isoamylase initial rate*[b]	*Isoamylase products*[c]	*Pullulanase initial rate*[d]
Glycogen	100		n.d.a.
Glycogen beta-limit dextrin	32	G2, G3 (⩾ G4)	—
Amylopectin beta-limit dextrin	13	G2, G3 (⩾ G4)	—
Pullulan	0.04	G3, G6, G9, *etc.*	100
6^3-α-Maltosylmaltotriose	0.04	G2, G3	110
6^3-α-Maltosylmaltotetraose	0.27	G2, G4 (G_3[e])	270
6^3-α-Maltotriosylmaltotriose	0.17	G3	104
6^2-α-D-Glucosylmaltose	n.d.a.		n.d.a.
6^3-α-D-Glucosylmaltotriose	n.d.a.		n.d.a.

[a]Substrate concentrations were equivalent to 2mM (1→6)-α-D linkages, except for the beta-limit dextrins which was 5 mg/ml: n.d.a., no detectable action. [b]Rate relative to glycogen = 100. [c]G2, G3, and G4 represent linear maltosaccharides of d.p. 2, 3, and 4; minor products are shown in parenthesis. G6 and G9 are oligosaccharides derived from pullulan containing two or three maltotriose units, respectively. [d]Rate relative to pullulan = 100. [e]The trace of G3 came from traces of 6^3-α-maltotriosylmaltotriose which contaminated this substrate.

TABLE IV

KINETIC PROPERTIES OF THE BACTERIAL ISOAMYLASE WITH VARIOUS SUBSTRATES

Substrate	K_m[a]	K_m[b]	V_{max}[c]
Glycogen	0.65	0.33	100
Pullulan	7.6	15.6	0.7
6^3-α-Maltosylmaltotetraose	1.6	1.6	0.4
6^3-α-Maltosylmaltotriose	5.6	5.8	0.3

[a]Results expressed as mg/ml. [b]Results expressed as mM with respect to (1→6)-α-D linkages. [c]Results relative to glycogen = 100.

for glycogen. Samples were removed at intervals for analysis by paper chromatography and for the measurement of reducing power. From the latter, the initial rate of attack relative to glycogen or pullulan was calculated. The results (Table III) show that isoamylase has a very low but detectable action on pullulan and on two oligosaccharides containing maltosyl side-chains. In all cases, the products were those to be expected from debranching; the results also confirm the absence of contaminating enzymes in the isoamylase preparation. Both debranching enzymes had no action on alpha-limit dextrins having α-D-glucosyl side-chains.

(*d*) *Kinetic studies.* The kinetic constants K_m and V_{max} were determined[22] with two polysaccharide and two oligosaccharide substrates. The results are given in Table IV.

DISCUSSION

The present investigation has shown that affinity chromatography on a column of ConA–Sepharose saturated with glycogen provides a convenient method for the purification of bacterial isoamylase. The strain of *Cytophaga* used was specifically selected for its debranching activity. The specific activity of the initial extract (~4 units/mg of protein) was much higher than that of a B.D.H. L1 preparation (~0.02 unit/mg of protein). Enzyme from this latter source was used in the initial studies[7] of the properties and specificity.

Our enzyme preparation showed pH and temperature properties similar to those previously reported[7], and its action on glycogen and the beta-limit dextrins of amylopectin and glycogen is generally in accord with the earlier studies. The substantial activity towards glycogen distinguishes isoamylase from other debranching enzymes, for example, pullulanase from *Aerobacter aerogenes*[6] and the plant limit-dextrinases[1].

In agreement with previous observations[7], the results show that the bacterial isoamylase is not a stable enzyme even at the optimum pH and temperature. Although the rate of loss of activity can be decreased by calcium ions (Table I), the mode of action of these cations is not apparent. These results were obtained on incubation of the enzyme in the absence of substrate, which normally has a protective action against inactivation. Hence, the possibility that the enzymic activity may be substantially decreased during prolonged incubation must be borne in mind.

The present results also indicate that the specificity of the enzyme may not be quite as clear cut as suggested by Whelan and his co-workers[7], who reported that the enzyme had no action on substrates having α-maltosyl side-chains[7,8] or on pullulan[7]. With 6^3-α-maltosylmaltotriose and 6^3-α-maltosylmaltotetraose, a low initial rate of hydrolysis can be measured (Table III). Whether this finding can be extrapolated to beta-limit dextrins of high molecular weight is, perhaps, a matter of opinion. Nevertheless, paper-chromatographic analysis has shown the presence of both maltose and maltotriose in enzyme digests with glycogen and amylopectin beta-limit dextrins*. Moreover, the initial rate of hydrolysis of the α-maltosyl substrates, although very low, is sufficient for K_m and V_{max} values to be determined (Table IV). The enzyme has a greater affinity for the hexasaccharide than for the pentasaccharide, indicating the influence of the molecular size of the substrate.

An isoamylase has been isolated from a *Pseudomonas* sp. strain SB-15 (later named *Pseudomonas amylodermosa*[23]) by Harada and his co-workers[24]. This enzyme readily hydrolysed alpha-limit dextrins having maltotriosyl side-chains, but paper-chromatographic analysis failed to detect the release of maltose from alpha-dextrins, showing an apparent similarity with the original findings[7] on the *Cytophaga* iso-

*The photograph of the paper chromatogram of the original digest of isoamylase action on glycogen beta-dextrin (Ref. 7, Fig. 3E) also shows a trace of maltose. Whether this is a true product of enzyme action, or represents a maltose contaminant of the beta-dextrin preparation, is not clear.

TABLE V

A COMPARISON OF THE RELATIVE RATES OF THE ACTION OF VARIOUS BACTERIAL DEBRANCHING-ENZYMES ON SOME OLIGO- AND POLY-SACCHARIDES

Substrate	*Bacterial debranching-enzyme*						
	A	*B*	*C*	*D*	*E*	*F*	*G*[a]
Glycogen	100	—	—	<1	—	—	—
Glycogen beta-limit dextrin	32	—	—	39	—	—	—
Amylopectin	—	—	—	113	15	100	—
Amylopectin beta-limit dextrin	13	—	—	164	—	—	—
Pullulan	0.04	100	100	100	100	<1	78
Alpha-limit dextrins	—	—	—	172	—	—	—
6^2-α-D-Glucosylmaltose	0	0	0	—	0	0	0.12
6^3-α-D-Glucosylmaltotriose	0	0	0	—	0	0	0.17
6^3-α-Maltosylmaltotriose	0.04	110	55	—	22	2.8	6
6^3-α-Maltosylmaltotetraose	0.27	270	171	—	43	6.9	18
6^3-α-Maltotriosylmaltotriose	0.17	104	91	—	162	9.7	100

[a]Key: A, *Cytophaga* isoamylase, this paper. B, *A. aerogenes* pullulanase, this paper. C, *A. aerogenes* pullulanase, Ref. 5. D, *A. aerogenes* pullulanase, crystalline form, Ref. 26. E, *A. aerogenes* pullulanase, Ref. 25. F, *Pseudomonas* isoamylase, Ref. 25. G, *Streptococcus mitis* pullulanase, Ref. 27.

amylase. However, when acting on amylopectin or glycogen beta-limit dextrins, some maltose was released although in much smaller amounts than maltotriose. The molar ratio of maltose to maltotriose was 1:13.4 and 1:9.7, respectively, indicating that at least some of the maltosyl side-chains could be released[24]. More recently, Harada and co-workers have reported[25] reducing-power determinations which show that the *Pseudomonas* isoamylase does, in fact, have a small but measurable activity towards 6^3-α-maltosylmaltotriose and 6^3-α-maltosylmaltotetraose (see Table V).

Our results also show a very slow action of *Cytophaga* isoamylase on pullulan. It is possible that our enzyme preparation was contaminated with a pullulanase, although, in a control experiment, the pullulanase from *Aerobacter aerogenes* had no affinity for the glycogen–ConA–Sepharose column. The hydrolysis of pullulan may be due to the resemblance of this substrate with the outermost regions of a

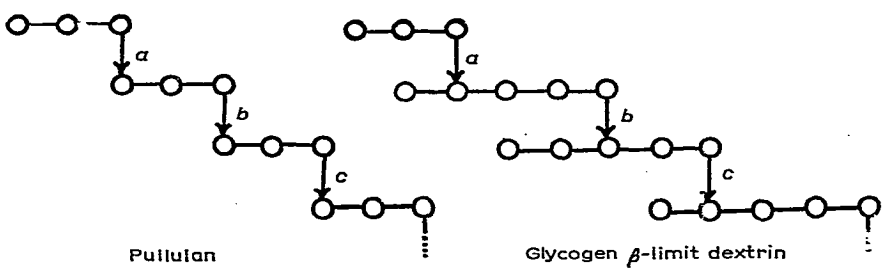

Fig. 4. Structural relationship between pullulan and a segment of a glycogen beta-limit dextrin.

beta-limit dextrin in which the A-chain is a maltotriosyl unit (Fig. 4). The hydrolysis of linkage *a* would yield maltotriose from both substrates, whilst hydrolysis of linkages *b* and *c* would yield branched oligosaccharides containing one or two (1→6)-α-D-glucosidic linkages.

In their original paper, Whelan and his co-workers[7] stated that the enzyme has no action on pullulan, although their numerical results indicate that 1.8% and 12.7% of the glucosidic bonds in pullulan and glycogen beta-dextrin were hydrolysed under certain conditions. It is not clear whether the figure of 1.8% is experimentally significant, but at its face value, it represents 14% of the activity towards a susceptible substrate. In our experiments, although the initial rate was admittedly very low, it was sufficient to yield oligosaccharide products that could be detected by paper chromatography, and gave measurable kinetic constants (Table IV).

We believe that the precise definition of the specificity of a debranching enzyme is extremely difficult. Whilst susceptible substrates can be readily identified, the hydrolysis of other substrates at rates of 1% or less cannot be ignored, particularly if these latter hydrolyses have structural implications with respect to the substrate specificity.

In Table V, various findings on the relative rates of debranching of certain polysaccharide and oligosaccharide substrates are summarised. Four sets of results refer to different samples of pullulanase from *Aerobacter aerogenes*. In general, there is only qualitative agreement in the trends of some of the results. For the isoamylases from *Cytophaga* and *Pseudomonas*, the results with oligosaccharide substrates show some qualitative similarities, but differ markedly in their relative activities towards beta-limit dextrins. It should be noted that the enzymes may not always have been saturated with substrate in the various experiments. Nevertheless, caution is clearly required in defining the specificity of debranching enzymes, particularly those of bacterial origin, where different strains of micro-organisms having the same taxonomic name may have been used.

ACKNOWLEDGMENTS

This work forms part of a programme supported by the Scottish Home and Health Department, to whom we are indebted for a research fellowship (to R.M.E.). We also thank Glaxo Research Ltd. for the sample of L1 enzyme-preparation.

REFERENCES

1 D. G. HARDIE AND D. J. MANNERS, *Tech. Q., Master Brew. Assoc. Am.*, 14 (1977) 120–125.
2 E. Y. C. LEE, C. MERCIER, AND W. J. WHELAN, *Arch. Biochem. Biophys.*, 125 (1968) 1028–1030.
3 W. J. WHELAN, *Biochem. J.*, 122 (1971) 609–622.
4 R. W. BAILEY, E. Y. C. LEE, AND W. J. WHELAN, *Int. Congr. Biochem. Abstr., 7th, Tokyo*, (1967) D36.
5 M. ABDULLAH, B. J. CATLEY, E. Y. C. LEE, J. ROBYT, K. WALLENFELS, AND W. J. WHELAN, *Cereal Chem.*, 43 (1966) 111–118.
6 C. MERCIER AND W. J. WHELAN, *Eur. J. Biochem.*, 16 (1970) 579–583.

7 Z. GUNJA-SMITH, J. J. MARSHALL, E. E. SMITH, AND W. J. WHELAN, *FEBS Lett.*, 12 (1970) 96–100.
8 Z. GUNJA-SMITH, J. J. MARSHALL, AND W. J. WHELAN, *FEBS Lett.*, 13 (1971) 309–311.
9 G. WOBER AND W. J. WHELAN, *Fed. Proc.*, 30 (1971) 1064 Abstr.
10 T. G. MITCHELL, M. S. HENDRIE, AND J. M. SHEWAN, *J. Appl. Bacteriol*, 32 (1969) 40–50.
11 National Collection of Industrial Bacteria, Catalogue of Strains, 3rd edition, 1975, p. 151.
12 J. J. MARSHALL, *Carbohydr. Res.*, 26 (1973) 274–277; D. J. MANNERS AND G. WILSON, *Biochem. J.*, 135 (1973) 11–18.
13 S. PEAT, W. J. WHELAN, AND G. J. THOMAS, *J. Chem. Soc.*, (1952) 4546–4548.
14 J. J. MARSHALL AND W. J. WHELAN, *Arch. Biochem. Biophys.*, 161 (1974) 234–238.
15 J. J. MARSHALL, Z. GUNJA-SMITH, AND E. E. SMITH, *Fed. Proc.*, 30 (1970) 1064.
16 D. J. MANNERS AND D. YELLOWLEES, *Staerke*, 23 (1971) 228–234.
17 W. E. TREVELYAN, D. P. PROCTER, AND J. S. HARRISON, *Nature (London)*, 166 (1950) 444.
18 J. F. ROBYT AND W. J. WHELAN, in J. A. RADLEY (Ed.), *Starch and its Derivatives*, Chapman and Hall, London, 4th edition, 1968, pp. 432–433.
19 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350–356.
20 G. L. MILLER, *Anal. Chem.*, 31 (1959) 64.
21 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265–275.
22 R. EISENTHAL AND A. CORNISH-BOWDEN, *Biochem. J.*, 139 (1974) 715–720.
23 T. HARADA, *Mem. Inst. Sci. Ind. Res., Osaka Univ.*, 34 (1977) 49–61.
24 K. YOKOBAYASHI, A. MISAKI, AND T. HARADA, *Biochim. Biophys. Acta*, 212 (1970) 458–469.
25 K. KAINUMA, S. KOBAYASHI, AND T. HARADA, *Carbohydr. Res.*, 61 (1978) 345–357.
26 C. MERCIER, B. M. FRANTZ, AND W. J. WHELAN, *Eur. J. Biochem.*, 26 (1972) 1–9.
27 G. J. WALKER, *Biochem. J.*, 108 (1968) 33–40.