STUDIES ON CARBOHYDRATE-METABOLISING ENZYMES

PART XXVII*. A COMPARISON OF THE ACTION OF YEAST ISOAMYLASE AND BACTERIAL PULLULANASE ON AMYLOPECTIN

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ABSTRACT

The action of purified yeast isoamylase on amylopectin, like that of bacterial pullulanase, results in the hydrolysis of the outermost inter-chain linkages with the liberation of linear maltosaccharides having an average degree of polymerisation of approximately 15 D-glucose residues. This hydrolytic action distinguishes yeast isoamylase from yeast amylo-(1→6)-glucosidase, which acts by a combination of transferase and glucosidase activities. The products of enzyme action on amylopectin are discussed in relation to the probable molecular structure of the polysaccharide.

INTRODUCTION

Extracts of both brewer's and baker's yeast contain an enzyme which causes a limited increase in the iodine-staining power and beta-amylolysis limit of amylopectin and glycogen[2,3]. This activity was due to an enzyme named isoamylase, which was believed to catalyse a hydrolytic debranching of the polysaccharides, with the release of the linear, exterior chains. In 1967, Lee, Nielsen, and Fischer[4] showed that baker's yeast contained a debranching enzyme whose action was similar to that of mammalian muscle amylo-(1→6)-glucosidase. By a combination of transferase and glucosidase activities, it caused an increase in the iodine-staining power and beta-amylolysis limit of amylopectin and its beta-limit dextrin. The products of enzyme action were D-glucose and a modified polysaccharide having longer exterior chains. The changes in the properties of the polysaccharides were different from those resulting from the action of bacterial pullulanase; this latter enzyme shows only a hydrolytic debranching activity towards susceptible substrates[5]. Lee and co-workers therefore suggested[4] that the action of yeast amylo-(1→6)-glucosidase could account for the activity formerly ascribed to isoamylase.

We now describe the results of an examination of the products of the action of yeast isoamylase and bacterial pullulanase on amylopectin. The results lead to the conclusion that both enzymes are hydrolytic α-(1→6)-D-glucosidases and therefore differ from amylo-(1→6)-glucosidase. A preliminary account of this work was presented in 1970 at the Fifth International Carbohydrate Symposium held in Paris.

*For part XXVI, see Ref. 1.

MATERIALS AND METHOS

Materials. — The following polysaccharides were prepared by standard methods: rabbit-liver glycogen, potato amylose and amylopectin, and pullulan. Amylopectin beta-limit dextrin was prepared by prolonged incubation of amylopectin with beta-amylase, followed by dialysis and gel filtration on Sephadex G-75 to remove maltose. Maltose, isomaltose, and panose were chromatographically homogeneous. Higher maltosaccharides, having a degree of polymerisation (d.p.) >15, were isolated by Dr. J. R. Stark from a partial, acid hydrolysate of amylose. Samples of pure α-D-glucosylcyclohexaamylose and 6^3-α-maltosylmaltotriose were kindly provided by Dr. V. Thambyrajah and Dr. D. Yellowlees, respectively.

Analytical methods. —— Reducing sugars were determined by the Nelson–Somogyi reagent[6], calibrated against D-glucose, maltose, and maltotriose. Total carbohydrate was measured by a phenol–sulphuric acid method[7]; protein was estimated by using a slightly modified Lowry reagent[8].

The d.p. of maltosaccharides was determined enzymically with sorbitol (D-glucitol) dehydrogenase[9]. For iodine-staining measurements, a standard solution was prepared from 0.2% iodine in 2.0% aqueous potassium iodide (50 ml) which was diluted with 4M hydrochloric acid (10 ml) and water (440 ml). Descending paper chromatograms were normally prepared by using propyl alcohol–ethyl acetate–water (14:2:7) as solvent. The beta-amylolysis limits of polysaccharides were determined as described previously[10], using 100 units of enzyme per mg of polysaccharide.

Assay of isoamylase. — Digests were prepared containing freeze-dried enzyme preparation (10–20 mg) in 50mM citrate buffer (pH 6.0; 1 ml) and 1% rabbit liver glycogen (1 ml) and were incubated at 20°; enzyme controls were also prepared. Samples (0.1 ml) were withdrawn after 0, 24, and 48 h and added to standard iodine solution (5 ml), and the extinction at 440 nm was measured on a Unicam SP800 spectrophotometer. The unit of isoamylase activity is defined[11] as the amount of enzyme causing an increase in extinction of 2×10^{-2} spectrophotometric units in 24 h under the above conditions.

Assay of pullulanase. — Digests were prepared containing 5% pullulan (0.2 ml), 20mM citrate buffer (pH 5.0, 0.7 ml), and enzyme (0.1 ml). After incubation at 37°, the reducing power of samples (0.1 ml) was measured at 0, 10, 20, and 30 min by the Nelson–Somogyi method. The unit of pullulanase activity was originally defined[12] as the amount of enzyme which will liberate 1 μmole of maltotriose in one minute at 30°. Using the above conditions, but at 37°, various pullulanase preparations had activities in the range 1.0–2.1 units per ml.

Assay of phosphorylase. — The conditions of Hers[13] were used, in which the amount of inorganic phosphate liberated during the transfer of D-glucose from α-D-glucose 1-phosphate to glycogen is measured.

Preparation of isoamylase. — Commercial baker's yeast (D.C.L., vacuum packed under nitrogen; 80 g) was extracted with 0.1M sodium hydrogen carbonate (pH 8.0, 700 ml) at 20° with continuous stirring for 2.5–3.0 h. All subsequent opera-

tions were carried out at 0°. The extract was centrifuged (10,000 *g*; 20 min) and, after cooling to below 0°, an equal volume of redistilled acetone (at −10°) was added slowly over a period of 2–3 h. The precipitate was collected by centrifugation (15,000 *g*; 20 min) and extracted overnight with 0.1M citrate–phosphate buffer (pH 6.0, 200 ml). The extract was centrifuged (15,000 *g*; 20 min), and the solid residue was retained as Fraction I. The supernatant solution was then subjected to acetone fractionation, and the protein precipitated by acetone concentrations of 0–16, 17–32, and 33–38% (by volume) was collected and dissolved in ice-cold, distilled water. An equal volume of 0.1M citrate buffer (pH 6.0) was then added, and the solutions were freeze-dried to give fractions II, III, and IV, respectively.

Some properties of these fractions are given in Table I.

TABLE I

PROPERTIES OF ACETONE FRACTIONS OF BAKER'S YEAST EXTRACT

Fraction	*II*	*III*	*IV*
Yield, g	1.06	2.45	2.35
Protein content, %	0.50	1.70	1.35
Effect on iodine-staining power of glycogen, %[a]	−1.6	+38.4	+25.2
Isoamylase, specific activity		12.2	9.4

[a]After incubation for 24 h.

Preliminary experiments showed that the four fractions had phosphorylase activities of 0.44, 2.02, 0.38, and 2.00 units per mg of protein, respectively. Qualitative tests showed that fractions III and IV, which contained the bulk of the isoamylase activity, showed some hydrolytic activity towards maltose, isomaltose, and panose, but none towards α-D-glucosyl Schardinger dextrin or higher maltosaccharides. A trace of D-glucose was released from 6^3-α-maltosylmaltotriose and from pullulan.

Preliminary experiments had shown that fractions III and IV contained branching enzyme since they reduced the iodine-staining power of amylose without producing oligosaccharides of low molecular weight. This activity can be selectively inhibited by 0.5mM mercuric chloride[14]. When fractions III or IV were incubated with amylopectin beta-limit dextrin, there was no increase in iodine-staining power, but in the presence of the inhibitor there was a small but significant increase. The inhibitor had no effect on isoamylase activity towards glycogen.

Since attempts to purify fraction III by gel filtration on Sephadex G-200 were not successful, a second acetone precipitation was carried out. A solution of the isoamylase fraction (1.5 g) in water (100 ml) was cooled and fractionated by the addition of redistilled acetone (at −14°). From fraction III, sub-fractions *a*, *b*, and *c* were obtained by the addition of acetone between the limits of 16–21, 22–27, and 28–32% by volume; fraction IV yielded subfractions *d* and *e* at acetone concentrations of 32–35 and 36–38%, respectively. Each precipitate was collected by centrifuga-

tion, dissolved in distilled water, dialysed overnight at 2°, and then freeze-dried after the addition of an equal volume of 0.1M citrate buffer (pH 6.0). The properties of the sub-fractions are recorded in Table II.

TABLE II

PROPERTIES OF ACETONE SUB-FRACTIONS OF BAKER'S YEAST EXTRACT

Subfraction	a	b	c	d	e
Yield, g	1.8	1.9	2.8	2.4	3.0
Protein content, %	0.49	0.29	0.60	0.59	0.32
Change in iodine-staining power of glycogen, %[a]	+5	+50	+15	+5	0
Isoamylase, specific activity	5.1	78.0	8.6	3.4	0
Change in iodine-staining power of amylopectin beta-limit dextrin, %[b]					
(*a*) absence of mercuric chloride	−11	−6	−6	−10	
(*b*) presence of mercuric chloride	+7	+10	+7	+6	

[a]After 24 h. [b]After 4 h.

Qualitative experiments showed that fraction *b*, which had the highest isoamylase activity, showed no activity towards maltose, isomaltose, panose, or 6^3-α-maltosylmaltotriose, and released maltose and maltotriose from amylopectin beta-limit dextrin. Fraction *b* also caused a greater increase in the iodine-staining properties of potato amylopectin than either fractions *a* or *c*, in the presence of 0.5mM mercuric chloride.

Preparation of pullulanase. — This enzyme was prepared from *Aerobacter aerogenes*, as described by Bender and Wallenfels[12], maltose being used as the carbon source for growth. The enzyme was precipitated at 0° from the centrifuged culture medium by the addition of 1.5 vol. of redistilled acetone (at −14°). The precipitate was dissolved in the minimal volume of distilled water at 0° and freeze-dried. Pullulanase was obtained from the freeze-dried preparation by two successive extractions at 0–5° with 20mM sodium phosphate buffer (pH 6.8, containing 10mM EDTA) for 10–15 h; the extracts were centrifuged (11,000 *g*, 20 min), and the supernatant solutions were dialysed against the same buffer at 0–5° for 24 h and then stored at 2°. The solutions were free from alpha-amylase since, on incubation with higher maltosaccharides (d.p. >15), sugars of lower molecular weight were not produced, and with amylopectin beta-limit dextrin there was an increase in iodine-staining power which reached a maximum after 24 h and remained constant even after incubation for 48 h. On incubation with rabbit-liver glycogen (10 mg) at pH 5.0 and 37°, the enzyme (0.32 unit) caused the extinction of the iodine complex at 440 nm to increase by 42% during 48 h.

RESULTS

Action of isoamylase on amylopectin. — Amylopectin (189 mg) dissolved in water (10 ml), fraction *b* (200 mg) dissolved in 50mM citrate buffer (pH 6.0, 8 ml),

and 5mM mercuric chloride (2 ml) were incubated at 20° for 21 h. The extinction at 550 nm of a sample (0.05 ml) examined by iodine staining increased from 0.91 to a maximum value of 1.15, and the λ_{max} changed from 550 to 560 nm. After heating (10 min at 100°), the digest was cooled, centrifuged to remove denatured protein, concentrated to *ca.* 4 ml, and applied to a column (41 × 2.5 cm) of Sephadex G-50. The rate of elution of carbohydrate by water was followed by the phenol–sulphuric acid method[7], and is shown in Fig. 1. The contents of the tubes containing residual polysaccharide, intermediate fraction, and linear maltosaccharides were combined and freeze dried. The yields are given in Table III.

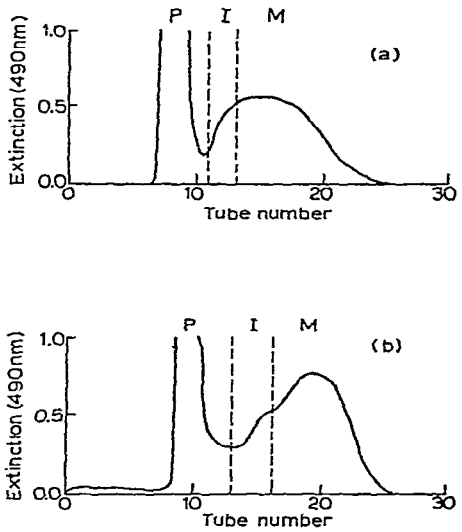

Fig. 1. Elution patterns from Sephadex G-50 of amylopectin after debranching with (*a*) yeast isoamylase (*b*) bacterial pullulanase. P, I, and M denote residual polysaccharide, intermediate fraction, and maltosaccharide fraction, respectively.

Properties of the residual isodextrin. — The beta-amylolysis limit was 72%, compared with a value of 56% for the original amylopectin. The isodextrin also had a greater iodine-staining power than amylopectin (see Table III); the latter had λ_{max} 550 and ε_{max} 1.13 in water, and λ_{max} 550 and ε_{max} 1.18 in half-saturated ammonium sulphate. Incubation of the isodextrin with pullulanase caused the extinction of the iodine complex to increase from 1.37 to 1.43 during 48 h, showing that further, limited debranching had taken place.

Properties of the intermediate fraction. — When incubated with beta-amylase, maltose was the only reducing sugar which could be detected by paper chromatography. In a quantitative experiment, incubation of the carbohydrate (2 mg) with beta-amylase (10 units) at pH 4.8 and 37° gave 78, 89, and 90% conversion into maltose after 1, 3, and 5 h, respectively. Evidence that this fraction contained polysaccharide rather than oligosaccharide was obtained by iodine staining (see Table III).

This view was confirmed by measurement of the degree of polymerisation with D-glucitol dehydrogenase, which gave a value of 91.

TABLE III

COMPARISON OF PRODUCTS FROM ACTION OF ISOAMYLASE AND PULLULANASE ON AMYLOPECTIN

	Isoamylase-treated amylopectin	*Pullulanase-treated amylopectin*
Residual polysaccharide		
Tube Nos.	5–10	4–12
Yield, mg	98	82
Beta-amylolysis limit, %	72	70
Iodine staining		
(*a*) in water ε_{max}	1.43	1.41
λ_{max}	570	572
(*b*) in half-saturated ammonium sulphate, ε_{max}	1.40	1.38
λ_{max}	566	568
Intermediate fraction		
Tube Nos.	11–13	13–16
Yield, mg	20	21
Beta-amylolysis limit, %	90	96
Iodine staining		
(*a*) in water, ε_{max}	1.99	2.09
λ_{max}	560	560
(*b*) in half saturated ammonium sulphate ε_{max}	2.30	2.39
λ_{max}	582	582
Degree of polymerisation	91	97
Linear maltosaccharides		
Tube Nos.	14–29	17–27
Yield, mg	62	77
Iodine staining		
(*a*) in water, ε_{max}	0.82	0.80
λ_{max}	452	452
(*b*) in half-saturated ammonium sulphate ε_{max}	1.16	1.16
λ_{max}	450	446
Degree of polymerisation	15.4	15.2

Properties of the debranched carbohydrate. — Paper-chromatographic analysis, using propyl alcohol–ethyl acetate–water (14:2:10) as solvent and multiple development over 8 days, showed the presence of a series of maltosaccharides ranging from maltooctaose to material having a d.p. of at least 21. By the enzymic method, the average d.p. of this carbohydrate was 15.4. Incubation with beta-amylase gave maltose and a small proportion of D-glucose, but no other reducing sugars. The iodine-staining properties were similar to those of authentic maltosaccharides having a d.p. of 13–20.

Action of pullulanase on amylopectin. — Amylopectin (197 mg) was treated with pullulanase (3.2 units) at pH 5.0 and 37°. The extinction of the iodine complex at 550 nm of a sample (0.05 ml) increased from 1.05 to 1.21 and 1.22 after 17 and 21 h.

The enzyme digest was inactivated by heating, and the products were fractionated by gel filtration as for isoamylase. The yields and properties of the three carbohydrate fractions are given in Table III.

Properties of the residual dextrin. — Under the above conditions, pullulanase action was not entirely complete since, on incubation with fresh pullulanase, the iodine-staining power of a sample increased from 1.30 to 1.38 and 1.40 after 24 and 48 h. The beta-amylolysis limit of the dextrin (70%) was significantly greater than that of the parent amylopectin (56%), and there was a similar increase in iodine-staining power (see Table III).

Properties of the intermediate fraction. — This material, which had a d.p. of 97, gave 83, 91, and 96% conversion into maltose on beta-amylolysis for 1, 3, and 5 h, respectively. The iodine-staining properties were similar to those of the product from isoamylase action (see Table III).

Properties of the debranched carbohydrate. — This fraction also contained a series of maltosaccharides ranging from d.p. 8 to 21. The average d.p., determined enzymically, was 15.2, and beta-amylolysis gave maltose and a small proportion of D-glucose. The iodine-staining properties were again similar to those from the isoamylase experiment.

DISCUSSION

Enzyme preparations from yeast which show isoamylase activity are frequently contaminated with other carbohydrases. In the present work, repeated fractionation with acetone gave an isoamylase preparation which had no action on maltose, isomaltose, panose, α-D-glucosylcyclohexaamylose, or 6^3-α-maltosylmaltotriose, *i.e.* it was free from α-D-glucosidase, amylo-(1→6)-glucosidase, and limit dextrinase. However, traces of branching enzyme were still present, but their effect could be minimised by selective inhibition with 0.5mM mercuric chloride. In previous studies of isoamylase action on glycogen[3,15], the presence of this possible impurity was of no consequence, since yeast branching-enzyme has no action on glycogen[14].

Incubation of purified isoamylase with amylopectin gave the expected increase in iodine-staining power and beta-amylolysis limit. These changes were due to the hydrolysis of a proportion of the α-(1→6)-D-glucosidic inter-chain linkages, since by gel filtration, it was possible to separate a series of linear maltosaccharides from the residual polysaccharide (isodextrin). The average d.p. of these maltosaccharides (15.4) was in good agreement with the calculated, exterior chain-length (14–15) of the amylopectin. The majority of the A-chains (side chains) had therefore been removed. The shortest A-chains contained 8 D-glucose residues, and the longest had a d.p. >21. If isoamylase action had involved a transferase–glucosidase mechanism, the products of enzyme action would have been D-glucose and a polysaccharide having elongated B-chains (main chains).

Although the isolation of pullulanase from culture filtrates of *A. aerogenes* is well documented[5,12], it is not apparent from the literature that alpha-amylase may be

an undesirable impurity in such preparations. The degree of contamination is erratic and cannot be directly related to the strain of the organism or to the conditions of growth[16]. In the present work, only pullulanase preparations that did not cause the liberation of chromatographically mobile oligosaccharides from higher maltosaccharides (d.p. >15) were used, since any limited alpha-amylolysis would alter the change in iodine-staining power and increase the apparent beta-amylolysis limit.

The action of purified pullulanase on amylopectin was very similar to that of isoamylase. Linear maltosaccharides having a d.p. of 15.2 were released, *i.e.* the majority of the outermost α-(1→6)-D-glucosidic interchain linkages had been hydroysed. With both debranching enzymes, not all of the more-accessible inter-chain linkages had been hydrolysed, since the residual branched-polysaccharide was partly degraded on further incubation with pullulanase. The extent of debranching is related to enzyme concentration, and for the present purpose, it was not necessary to attempt maximal fragmentation of the amylopectin molecules by having an excess of debranching enzyme present.

Since both glycogen[15] and amylopectin are degraded by hydrolytic debranching, isoamylase is clearly distinguished from amylo-(1→6)-glucosidase. The presence of the latter enzyme in yeast was first described by Lee *et al.*[4] and confirmed by Bathgate and Manners[15]. Since the present work was completed, a detailed account of the purification and properties of yeast amylo-(1→6)-glucosidase has been published[17]. It is now accepted[17] that yeast contains two distinct debranching enzymes, although their relative importance *in vivo* is not known.

The specificity of yeast isoamylase, purified by a combination of column chromatography and gel filtration, has been examined by Kobayashi and his co-workers[18]. The purified enzyme had no action on maltose, isomaltose, panose, isopanose, and 6^3-α-D-glucosylmaltotriose, showing that it was unable to hydrolyse α-(1→6)-D-glucosidic linkages attaching a single D-glucose residue to either D-glucose or a maltosaccharide. However, enzyme action on an alpha,beta-limit dextrin (having a d.p. of 9.6 and prepared by the combined action of barley beta-amylase and malt alpha-amylase on amylopectin) yielded D-glucose, maltose, and higher oligosaccharides. This release of D-glucose, which is not due to α-D-glucosidase activity, is interpreted by the authors as evidence that the enzyme can hydrolyse terminal α-(1→6)-D-glucosidic linkages; this conclusion is inconsistent with the inability of the enzyme to hydrolyse panose or 6^3-α-D-glucosylmaltotriose. An alternative explanation is that their isoamylase preparation was contaminated with amylo-(1→6)-glucosidase. The shape of the elution profile of the enzyme during the final gel-filtration step indicates some heterogeneity, and the protein fractions were not tested against α-D-glucosylcyclohexaamylose, which is a specific substrate for detecting the hydrolytic activity of amylo-(1→6)-glucosidase[19]. The results of Kobayashi *et al.*[18] illustrate the difficulty in rigorously characterising the specificity of debranching enzymes.

Our previous studies[15] have shown that yeast isoamylase can liberate maltosaccharides having d.p. 5–9 from glycogen, and the present results show that chains

ranging from 2–21 D-glucose residues may be released from amylopectin-type polysaccharides. Although the enzyme cannot release single D-glucose residues from polysaccharide substrates, it nevertheless shows a much wider specificity than the two plant debranching-enzymes, amylopectin 6-glucano-hydrolase and limit dextrinase[1]. The former acts only on amylopectin and its beta-limit dextrin, whilst for the latter, amylopectin beta-limit dextrin and pullulan appear to be the only satisfactory polysaccharide substrates.

The ability of yeast preparations to release reducing sugars from oligosaccharide alpha-limit dextrins prompted the suggestion that a limit dextrinase resembling the plant enzyme might also be present[15]. The earlier work[3,20] was carried out by using a mixture of branched oligosaccharides as substrate, and enzyme action was followed by measurement of the increase in reducing power or by paper chromatography. These results could be due to amylo-(1→6)-glucosidase action on the alpha-limit dextrins, which would give D-glucose as one of the products. Purified, baker's yeast isoamylase has no action on 6^3-α-maltosylmaltotriose, which is readily hydrolysed by cereal limit-dextrinases[1]. It is therefore not necessary to postulate the existence of debranching enzymes other than amylo-(1→6)-glucosidase and isoamylase to account for the observed action on alpha-limit dextrins.

It is usually accepted that amylopectin has a multiply branched, "tree-type" structure as originally proposed by Meyer[21]. Isoamylase and pullulanase action involves the release of A-chains, together with some B-chains. Enzyme action on amylopectin beta-limit dextrin gives maltose and maltotriose in a yield which approaches that calculated for a polysaccharide containing equal number of A- and B-chains[22]. This fact, and evidence from other enzymic studies[23,24], renders the singly branched structure proposed by Haworth[25] and the comb-type structure postulated by Staudinger[26] as less likely alternatives. A new model structure for amylopectin has been proposed by Frey-Wyssling[27] in an attempt to explain the close packing of amylose and amylopectin molecules within the starch granule. The model is essentially a variant of the comb-type, and consists of a single main-chain which has a helical conformation; to every third D-glucose residue, a short, linear side-chain (A-chain) is attached. An amylopectin molecule of d.p. 5×10^5 would carry about 22,000 side chains, each containing on the average about 23 D-glucose residues. The resulting structure would permit interpenetration of the side chains from adjacent amylopectin molecules to give close packing. Although this model is in accord with certain physical dimensions observed in starch granules by X-ray diffraction, it is inconsistent with the results of enzymic debranching. The action of either isoamylase or pullulanase would liberate more than 90% of the molecule as linear A-chains, leaving as a residual polysaccharide an amylose-type molecule which would be completely degraded by beta-amylase. The results in Table III show that only 30–40% of amylopectin is released as linear side-chains, and the residual polysaccharide still has a highly branched structure. The multiply branched structure of amylopectin has still to be reconciled with the physical characteristics of the starch granule.

Although the intermediate fraction (Table III) is clearly not homogeneous, the results indicate that enzymic debranching liberates a proportion of linear chains which are considerably longer than the average chain-length of 22, and are presumably B-chains. Whelan and co-workers[28] have also obtained evidence for a significant predominance of chains containing 40 or more D-glucose residues in various debranched amylopectins. These observations could reflect some deviation in the amylopectin from a symmetrical and regularly branched structure, which could be a consequence of biosynthesis in a sterically confined environment within an amyloplast. This would lead to increased growth in certain directions, and result in an asymmetrical molecule; the marked differences in hydrodynamic properties between amylopectin and glycogen support this view. Other evidence for some variation in the interior chain-lengths has already been provided by the detection of doubly branched alpha-dextrins[29] and macrodextrins of d.p. 35–46[30] amongst the products of alpha-amylolysis.

Since this work was completed and a preliminary account published[31], Whelan and his co-workers[32] have suggested that the generally accepted structure for amylopectin should be revised. Experimental evidence obtained by the use of debranching enzymes indicates that only half of the B-chains may carry A-chains, in contrast to the original Meyer formulation in which all the B-chains carried an A-chain. Our comments on this revised structure will be given elsewhere, but as far as the present work is concerned, the proposed revision of the structure for amylopectin in no way alters the conclusions on the mode of action on yeast isoamylase.

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