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Preliminary communication

The amylase of *Pseudomonas stutzeri* as a probe of the structure of amylopectin*

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The cluster model[1–4] of the structure of amylopectin rests[5–7] on (*a*) the isolation of ~ 35% of branch points in multiply branched oligosaccharides in which (1→6)-linked units are separated by only one glucose residue, (*b*) the formation, by acid hydrolysis of ungelatinised starch, of residual "Nageli" and "Lintner" amylodextrins, (*c*) the distribution of the lengths of enzymically debranched chains into discrete ranges, (*d*) the formation of macrodextrins on partial degradation with alpha-amylase or cyclodextrin glycosyltransferase, and (*e*) the observation of discrete periodicities of 50–100 Å by electron microscopy and X-ray diffraction. The sizes and arrangements of the clusters remain uncertain, although the models present them as intermolecularly hydrogen-bonded aggregates of 5–10 chains of d.p. 15–20, corresponding to 30 000–80 000 entities of relative molecular mass (RMM) 12 000–30 000 per amylopectin molecule. Hizukuri[8] suggested 22–25 chains of d.p. 12–16 corresponding to 15 000–23 000 clusters of RMM 43 000–66 000 per molecule of RMM 10^8. Burchard and Thurn[9] demonstrated excellent agreement of the angular dependence of scattered light with a modified Robin–Mercier[3] model containing clusters of d.p. ~ 50 with 3–4 branches and connected by chains carrying 1.4 clusters on average. Bertoft[10], from the results of partial alpha-amylolysis of waxy maize amylopectin, described two types of cluster containing 19 or 26 chains and 256 or 348 "anhydroglucose" units, respectively.

We now propose that the unusual pattern of action of the maltotetraose-forming amylase from *Pseudomonas stutzeri* may provide a new approach to analysing sizes and distributions of clusters in amylopectins.

P. stutzeri amylase[11,12] catalyses the removal of maltotetraose units from the non-reducing chain ends of amylopectins and has[11] an amylolysis limit towards potato amylopectin of 52% (*cf.*, 57% for beta-amylase). However, the enzyme possesses some endo-activity as shown by its ability to release soluble coloured products from insoluble dyed starch[13–15] and amylose[16]. We now report that the limit dextrins, obtained by the

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action of the enzyme on amylopectins, are consistent with cluster models and are characteristic of the source of the polysaccharide.

A solution of wheat amylopectin (prepared[17] by Dr. P. A. Leonard from wheat "A" starch, blue value 0.054, 1 g) in 50mM imidazole–HCl buffer pH 7.0 (20 mL) was treated at 37° with *P. stutzeri* amylase [purified[11,16,18] from an NCIB 11359 culture filtrate, 5 U; 1 U is the amount of enzyme which liberates 1 μmol of *p*-nitrophenol from *p*-nitrophenyl α-maltoheptaoside per min at 25° (Boehringer Mannheim C-System)]. The reducing power of the mixture became constant after 22 h, the original, slightly turbid solution had become clear, and the enzyme was still active. After 28 h, all the soluble carbohydrate present could pass through a 0.45-μm filter (unlike amylopectin and beta-limit dextrins), and liquid chromatography (Chrompak TSK G2000SW, elution with water, refractive index detection) showed (Fig. 1a) a single broad peak with an apex corresponding to an RMM of ~ 15000 (54% of the carbohydrate), together with one other sharp peak corresponding to maltotetraose. A sample (13.2 mL, 2.04 mmol of glucose equiv.), after removal of the enzyme (95°, 2 min, centrifugation), was diluted with ethanol (3 vol.) dropwise with stirring. The precipitate which formed overnight was collected by centrifugation, washed with ethanol, and lyophilised from

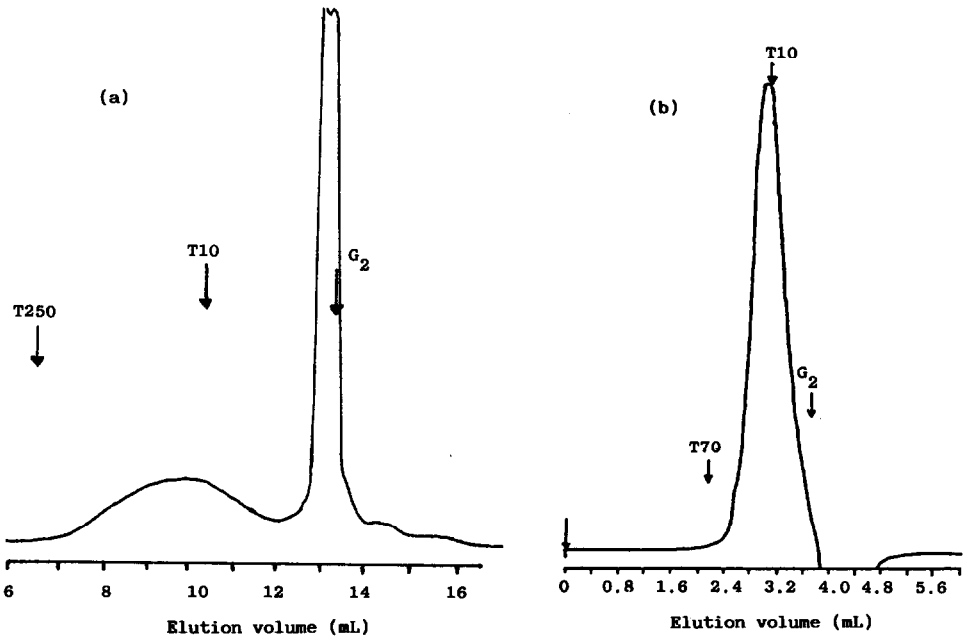

Fig. 1. H.p.l.c. of the limit dextrins obtained by the action of *Pseudomonas stutzeri* amylase on wheat amylopectin: (a) hydrolysate on Chrompack TSK G2000SW (30 × 0.75 cm) and (b) ethanol precipitate on Synchropak GPC100 (30 × 0.45 cm); T10, T70, and T250 are dextrans of weight-average molecular mass ~ 10000, ~ 70000, and ~ 250000, respectively, and obtained from Pharmacia, Uppsala, Sweden; G_2 is maltose.

water. Chromatography (2.6% solution in water, TSK G2000SW column) showed a single symmetrical peak with an apex at RMM ~ 14 000.

A further sample of the *P. stutzeri* amylase limit dextrin was obtained from wheat amylopectin by two sequential amylolysis–ethanol precipitation cycles. The combined ethanol-supernatant solutions contained 57% of the original carbohydrate, mainly, but not entirely, as maltotetraose. Liquid chromatography (Synchropak GPC100, water eluant) of the final ethanol precipitate showed (Fig. 1b) a single symmetrical peak of apparent RMM ~ 12 000 (dextran standards). ^{1}H-N.m.r. analysis at 400 MHz and integration of the signals for (C-1–*H*)–(O–C-4), (C-1–*H*)–(O–C-6), (C-1–*H*)–OHα, and (C-2–*H*)–(C-1–OHβ) showed[16] the limit dextrin to consist of ~ 88 glucose residues on average, corresponding to an RMM of 14 300, with 12 branches and an average chain length of 7.

The unique, limited endo-activity of the *P. stutzeri* amylase was demonstrated further by its ability to degrade amylopectin beta-limit dextrins (prepared using *Ipomoea batatas* beta-amylase, Boehringer). Wheat amylopectin beta-limit dextrin was further degraded by 11% with the production of a new limit dextrin and a mixture of glucose, maltose, maltotriose, and maltotetraose in approximately equal proportions. For the beta-limit dextrin from potato amylopectin (blue value 0.18), the extent of degradation was 17% and the main low molecular weight product was maltose with smaller proportions of glucose and maltotriose. This activity was not due to the presence of other contaminating amylases because the same, limited degradation was observed using different preparations of *P. stutzeri* amylase. It is not likely that the oligosaccharides are produced from the maltosyl and maltotriosyl stubs on the limit dextrins, since Sakano *et al.* showed[19] that *P. stutzeri* amylase does not remove maltosyl stubs from branched gluco-oligosaccharides. Also, different distributions of products from the two limit dextrins would be unlikely.

The average chain lengths of the limit dextrins obtained by sequential treatment of amylopectins with beta-amylase and *P. stutzeri* amylase were estimated by ^{1}H-n.m.r. spectroscopy to be 6.4 (wheat) and 7.7 (potato). In liquid chromatography (Synchropak column), the dextrins were eluted as well-defined peaks with apparent RMM values of ~ 7 600 (wheat) and ~ 23 000 (potato).

The observed patterns of degradation of amylopectins by *P. stutzeri* amylase are consistent with simultaneous exo-attenuation of external chains to produce maltotetraose and limited endo-cleavage of inter-cluster chains. The properties of the dextrins are in agreement with models of amylopectins in which a high proportion of the glucose chains are arranged in clusters of a relatively uniform size. Furthermore, the differences observed between the wheat and potato amylopectins, in terms of the extents of degradation and the nature of the products, characterise important differences in their fine structures. Wheat amylopectin has a smaller average chain length than potato amylopectin (26 *versus* 29 in this work) and a similar degree of beta-amylolysis (~ 55%), but the degree of G_4-amylolysis of the wheat amylopectin was lower (~ 54% *versus* 69%). Although wheat amylopectin has shorter external chains than potato amylopectin (13 *versus* 15[20–21]), the more extensive degradation of potato amylopectin beta-limit

dextrin confirms that there must have been a greater degree of inter-branch hydrolysis in potato amylopectin. Thus, wheat amylopectin may contain more highly branched clusters separated by fewer, longer, inter-cluster chains. This proposal is borne out in part by the analyses of chain-length distributions carried out by Hizukuri and Maehara[22]; compared to potato amylopectin, that from wheat has a much higher proportion of A-chains which are also shorter on average (11 units *versus* 16).

The data presented here allow the estimation of the sizes of the macrodextrins in the original amylopectins if they are represented by the beta,G_4-limit dextrins prior to attenuation (55%) by beta-amylase. These macrodextrins (clusters?) in wheat and potato amylopectins are therefore proposed to have RMM values of 14 000 (85 units) and 42 000 (260 units), respectively.

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