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Review Paper

Recent Developments in Our Understanding of Amylopectin Structure

David J. Manners*

Department of Brewing and Biological Sciences, Heriot-Watt University, Edinburgh EH1 1HX, UK

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ABSTRACT

The molecular structure of amylopectin can be described in terms of various parameters, e.g. average chain length, exterior and interior chain lengths, ratio of A-chains to B-chains and chain profile. Enzymic methods for the measurement of these parameters are critically discussed. The overall results are then considered in relation to the cluster model for amylopectin, which has emerged in recent years as the most probable molecular model.

INTRODUCTION

Starch, the reserve carbohydrate of most plants, occurs as water-insoluble granules, the size and shape of which vary with the species and maturity of the plant. The majority of starch granules contain a mixture of two polysaccharides, amylose and amylopectin. The latter is the major component (70–80%) and comprises branched macromolecules (molecular weight $\sim 10^7$) in which linear chains containing, on average, 20–25 (1→4)-α-linked D-glucose residues are interlinked by (1→6)-α-D-glucosidic linkages to form a multiply branched structure. The presence of the 4–5% of inter-chain linkages has a profound effect on the physical and biological properties of the amylopectin.

*Present address: 165 Mayfield Road, Edinburgh EH9 3AY, UK.

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Amylose, the minor component of starch (20–30%) consists of much longer linear chains containing, on average, several hundred (1→4)-α-linked D-glucose residues. The majority of the chains are interlinked in the same way as amylopectin to form a very lightly branched structure. The degree of branching in amylose is, of course, very much less than in amylopectin; for example, four samples of amylose ranging in degree of polymerisation (DP) from 1590 to 6340 contained between 9 and 20 chains per molecule (Hizukuri *et al.*, 1981). These comments apply to laboratory prepared samples which, in contrast to many commercial preparations, have not been inadvertently degraded. It is now known that most samples of amylose are lightly branched, but this is not sufficient to affect the physical properties of amylose either in neutral solution, where it behaves as a random coil, or in the solid state.

The branched nature of amylose accounts for the incomplete degradation by β-amylase (about 70% conversion into maltose). This fact has been known for more than 36 years, and it is surprising therefore to continue to read in recent reviews that 'amylose is a linear molecule'.

The amylose content of most starches is 20–30%, so that fractionation of a dispersion of the starch in hot water is required to give pure samples of amylose and amylopectin. This fractionation is effected by the addition of a polar organic molecule such as thymol or *n*-butanol which gives an insoluble complex with the amylose; the amylopectin can then be recovered from the supernatant solution. Many fractionations are imperfect, and further purification is required to obtain amylopectin entirely free from amylose. In addition, some starches, e.g. wrinkled pea and amylomaize, contain a third component — an intermediate fraction — which is neither a normal amylopectin nor an amylose, and this material makes fractionation more difficult. In the waxy varieties of some cereals, e.g. maize, sorghum and rice, the amylose content of the starch is about 1%, so that the whole waxy starch can be used as a convenient source of amylopectin.

STRUCTURAL STUDIES ON AMYLOPECTIN

Although the main structural features of amylopectin have been known for some 50 years, details of the fine structure are still lacking.† Indeed, there is still some uncertainty about the detailed arrangements of the constituent linear chains of (1→4)-linked α-D-glucose residues, and during the period 1970–79, three different molecular structures for amylopectin were proposed. One of these molecular structures, the so-

†For details of the earlier chemical studies, the reviews by Williams (1968) and Banks & Greenwood (1975) should be consulted.

called cluster model, has emerged as the most probable structure, although there are some variations of the cluster model, and it is not yet clear whether it applies to all amylopectins, irrespective of the botanical source of the starch.

The fine structure of amylopectin represents something of a paradox. In purely chemical terms, it could scarcely be simpler. It is composed of only one monosaccharide residue, the great majority of which are joined together by (1→4)-α-D-glucosidic linkages to form chains of varying lengths. The minute proportions of α-D-glucose 6-phosphate and/or α-D-glucose 3-phosphate residues reported in some potato amylopectins, perhaps one phosphate group per 300 glucose residues (Takeda & Hizukuri, 1982), can be disregarded in the present context. Amylopectin also contains about 4–5% of (1→6)-α-D-glucosidic linkages which interlink the chains to form a three dimensional macromolecule. The problems arise from the very high molecular weight of amylopectin (~ 10^7) giving a DP of ~ 10^5. Since the average chain length is usually 20–25 glucose residues, it follows that each macromolecule contains several thousand individual chains.

From a chemical point of view, three types of glucose residue can be described. There are 4–5% of non-reducing end-groups, which give rise to tetra-*O*-methyl-D-glucose on methylation analysis, or to formic acid on periodate oxidation. There are an equal number of branch point residues which are triply linked by two (1→4)- and one (1→6)-α-D-glucosidic linkages to adjacent residues. These give rise to di-*O*-methyl-D-glucose on methylation analysis (ideally, the 2,3-isomer, but in practice, other isomers may be obtained due to undermethylation), and to the relatively minute amounts of isomaltose and panose which are present in partial acid hydrolysates. However, some 90% of the glucose residues are indistinguishable from each other by chemical techniques. They yield 2,3,6-tri-*O*-methyl-D-glucose on methylation analysis, and are oxidised by periodate to the corresponding dialdehyde but do not yield formic acid, formaldehyde or any other low molecular weight compound.

Substantial progress in investigating the fine structure of amylopectin has become possible by the use of highly purified amylolytic enzymes. An elegant survey of much of the pioneering work in this area was provided by Whelan (1971) in his CIBA Medal Lecture. The present review will describe more recent results, with particular emphasis on the use of debranching enzymes. These enzymes specifically hydrolyse the (1→6)-α-D-glucosidic inter-chain linkages in amylopectin and derived dextrins, but have no action on the major (1→4)-α-D-glucosidic linkages. Their specificity is therefore the opposite to that of the conventional α- and β-amylases.

THE STRUCTURAL PARAMETERS OF AMYLOPECTIN

A number of terms are now frequently used to describe the structural parameters of amylopectin-type molecules.

Exterior chains are those parts of a chain between the non-reducing end-group and the outermost branch point, whilst interior chains represent parts of a chain between branch points in the interior of the molecule (see Fig. 1). A-Chains are linked to the molecule only by the potential reducing group, whilst B-chains are similarly linked, but also carry one or more A-chains. The C-chain carries the sole reducing group in the molecule, although for some purposes, the possible presence of a reducing group is not significant, and the C-chain can be regarded as a B-chain. The concept of A, B and C-chains was introduced by Peat *et al.*, (1952, 1956) and the degree of multiple branching may be expressed as the ratio of A-chains to B-chains.

It must be emphasised that analytical methods for measurement of the chain length (CL), exterior chain length (ECL) and interior chain length

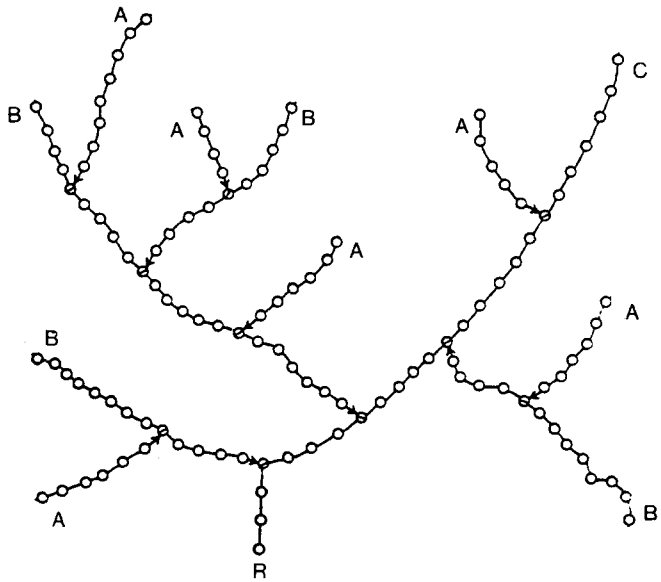

Fig. 1. Segment of a hypothetical branched (1→4)-α-D-glucan. In amylopectins, the exterior chains may contain 12–16 glucose residues, but in phytoglycogen and animal glycogens, they are about half this length. O—, a (1→4)-linked α-D-glucose residue; O←, a (1→6)-linked α-D-glucose residue; R, a free reducing group; A, B and C, types of chain.

(ICL) give average values, and that individual chains can vary considerably in length. From the above definitions and Fig. 1, it can be seen that an amylopectin molecule contains roughly equal numbers of exterior and interior chains. If the molecule has an A:B chain ratio of 1:1, then each A-chain is also an exterior chain, and each B-chain, on the average, consists of one exterior and two interior chains. In fact, many B-chains are now known to consist of one exterior chain and only one interior chain. Other B-chains contain one exterior chain and three or more interior chains. The proportion of very long B-chains will decrease as their CL increases. Nevertheless there is now evidence (see p. 105) for a small proportion of B-chains containing perhaps 50 or more glucose residues which play a key role in the overall molecular structure.

If an amylopectin molecule has an A:B chain ratio of 1·5:1, the basic assumptions made above remain, but the quantitative aspects require minor adjustments.

THE ACTION PATTERN OF STARCH DEGRADING ENZYMES

Four classes of enzyme have been widely used in enzymic studies of the fine structure of amylopectin. α-Amylases hydrolyse non-terminal $(1\rightarrow4)$-α-D-glucosidic linkages by a partly random action to give eventually a mixture of maltose, branched oligosaccharides (α-dextrins) and either maltotriose or glucose depending on the relative concentrations of enzyme and substrate. The α-dextrins contain the original $(1\rightarrow6)$ inter-chain linkage(s) and two or more adjacent $(1\rightarrow4)$-α-D-glucosidic linkages. The α-dextrins are a heterogeneous mixture with DPs ranging from four to more than 10, and individual α-dextrins may contain one, two or even three $(1\rightarrow6)$ inter-chain linkages, depending on their proximity to each other in the original macromolecule, and whether the enzyme can hydrolyse $(1\rightarrow4)$-α-D-glucosidic linkages in very short interior chains containing perhaps only two, three or four glucose residues (Enevoldsen & Schmidt, 1974). There is some evidence that α-amylases require a linear segment of at least five glucose residues for rapid hydrolysis. It should also be noted that α-amylases from different biological sources show some variation in specificity towards the $(1\rightarrow4)$-α-D-glucosidic linkages in the vicinity of an inter-chain linkage, as shown by the different structures of the smallest α-dextrins which can be isolated (see Fig. 2).

β-Amylases hydrolyse, in stepwise fashion, alternate linkages in their substrates, so that linear molecules are completely converted into

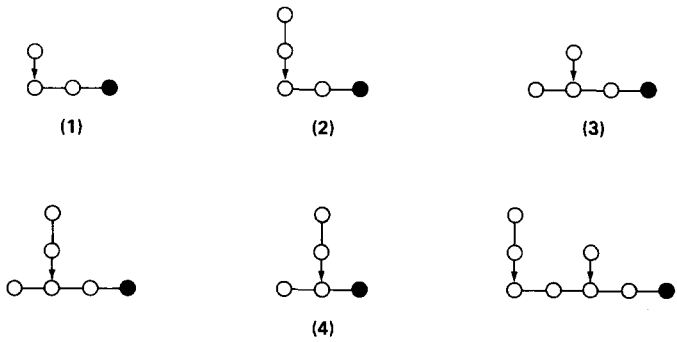

Fig. 2. Typical structures of α-dextrins. (1) 6^3-α-D-glucosylmaltotriose; (2) 6^3-α-maltosylmaltotriose; (3) 6^3-α-D-glucosylmaltotetraose; (4) 6^2-α-maltosylmaltotriose. α-Dextrins (1) and (4) are produced by cereal α-amylases and *Bacillus subtilis* α-amylase respectively. o —, a (1→4)-linked α-D-glucose residue; ↓, (1→6)-α-D-glucosidic inter-chain linkage; ●, a free reducing group. From Manners (1985*a*), reproduced by permission of Academic Press, Orlando.

maltose. (It should be noted that chains containing an odd number of residues will give one molecule of maltotriose or glucose, depending on the concentration of enzyme, in addition to the many molecules of maltose.)

With branched substrates, enzyme action is confined to the exterior chains, since β-amylase cannot hydrolyse or by-pass (1→6) inter-chain linkages. Enzyme action ceases leaving 'stubs' containing an average of two glucose residues per chain. With A-chains, the stubs actually contain either two or three residues per chain, whilst with B-chains, either one or two residues are present in the stub. Assuming equal number of A and B-chains, the exterior chain length is therefore the number of glucose residues removed by β-amylase plus two. The interior chain length is given by

$$\mathrm{ICL} = \mathrm{CL} - \mathrm{ECL} - 1$$

Thus an amylopectin with a CL of 20 and a β-amylolysis limit of 55% will have an ECL of 13 and an ICL of 6. The actual branch point residue is regarded as separate from the exterior and interior chains.

Although these definitions have been used by the majority of starch biochemists for some decades, Inouchi *et al.* (1987) have used a different definition of exterior and interior chains. This means that their results cannot be directly compared with those of other workers. Thus, phytoglycogen with a CL of 10 and β-amylolysis limit of 40% is said to have an ECL of 2·9, which is less than the number of glucose residues (four) actually removed by β-amylase.

Amylopectins are also degraded by plant and animal phosphorylases. In the presence of inorganic phosphate, enzyme action results in a stepwise cleavage of adjacent linkages in the exterior chains to give about 40% of α-D-glucose 1-phosphate. Like β-amylase, the phosphorylases cannot degrade or by-pass the inter-chain linkages, and enzyme action ceases giving 'stubs' of about four glucose residues. ECL may therefore be calculated from the number of glucose residues removed by phosphorylase plus four, although it is more difficult to ensure that phosphorolysis is complete, as compared to β-amylolysis. Whilst the plant phosphorylases are of considerable physiological significance, most of the structural studies have been carried out using the well-characterised enzyme from rabbit muscle.

Debranching enzymes which selectively hydrolyse the (1→6)-D-glucosidic inter-chain linkages in amylopectin and derived α- and β-dextrins, have been extensively used in the exploration of the amylopectin macromolecule. Debranching activity was first reported in the 1940s in sorghum malt preparations, as an amylolytic activity which hydrolysed the inter-chain linkages in α-dextrins, and was described as 'limit dextrinase' (Kneen & Spoerl, 1948). Some years later, the enzymic debranching of amylopectin by R-enzyme was described, which resulted in an *increase* in both the iodine staining power and β-amylolysis limit of amylopectin and its β-limit dextrin (Hobson *et al.*, 1951). For many years, it was believed that R-enzyme and limit dextrinase were separate enzymes, but it is now clear that both activities were due to a single enzyme (Drummond *et al.*, 1970), which will be referred to as limit dextrinase (Manners & Yellowless, 1973). Although this enzyme is present in the cereals, and many higher plants, purification is not easy, due amongst other factors to the difficulty in removing the last traces of α-amylase impurities. The latter would, of course, *decrease* the iodine staining power of the substrate. Consequently, debranching enzymes from microbial sources, which are more readily purified, have been widely used for structural analyses. The first of these enzymes was pullulanase, originally isolated from *Aerobacter aerogenes* (now known as *Klebsiella aerogenes*) which completely converted pullulan (a linear polymer of (1→6)-linked α-maltotriosyl residues) into maltotriose, and partially debranched both amylopectin and glycogen, as shown by increases in both iodine staining power and β-amylolysis limit (Abdullah *et al.*, 1966). Pullulanase has also been isolated from other bacteria, e.g. *Streptococcus mitis* and an alkalophilic *Bacillus* (Walker, 1968; Nakamura *et al.*, 1975).

Isoamylase has proved to be an even more useful debranching enzyme. It was originally isolated from a *Pseudomonas* (Yokobayashi *et*

al., 1969) and could completely debranch both glycogen and amylopectin (Akai *et al.*, 1971). The enzyme would also readily debranch α-dextrins with maltotriosyl side chains, although substrates with maltosyl side chains were only slowly hydrolysed. This action on α-dextrins represents a significant difference from pullulanase which readily removes both maltosyl and maltotriosyl side chains. An isoamylase was also described by Gunja-Smith *et al.* (1970*a*) from a strain of *Cytophaga* (also known as a *Flavobacterium* or *Polyangium*), with a specificity similar to that of the *Pseudomonas* enzyme. Initial reports that it had no action on substrates with maltosyl side chains could not be confirmed, when a low but significant activity towards α-dextrins with maltosyl side chains was observed (Evans *et al.*, 1979).

Isoamylase-type enzymes have also been reported in yeast (Gunja *et al.*, 1961), and in certain higher plants, e.g. sweet corn and potato, but difficulties with their purification and their instability have prevented their widespread use as analytical tools (Manners & Rowe, 1969; Ishizaki *et al.*, 1983).

THE ENZYMIC ANALYSIS OF AMYLOPECTIN

Determination of average chain length

Since isoamylase hydrolyses all the (1→6)-α-D-glucosidic linkages in amylopectin, and also in glycogen, accurate measurement of the increase in reducing groups provides a simple method for the determination of CL (Gunja-Smith *et al.*, 1970*a*). The method is routinely used on the milligram scale, and the increase in reducing power is measured after 24 h. Isoamylase from *Pseudomonas amyloderamosa* is now available commercially. The *Cytophaga* isoamylase may also be used, after a preliminary purification. In all cases, it is essential to ensure that sufficient isoamylase is present to completely debranch the amylopectin.

Determination of exterior and interior chain lengths

The use of β-amylase to determine the ECL and ICL of amylopectins has already been described (p. 92).

The enzyme preparation, which is also commercially available, must be free of α-amylase and α-glucosidase impurities, and used at a concentration to ensure complete degradation of the exterior chains within a few hours.

The calculation

$$ECL = (CL \times \beta\text{-limit}) + 2{\cdot}0$$

is based on the assumption that the amylopectin contains equal numbers of A- and B-chains. If the ratio should actually be different from 1:1, there will be a small change in the average size of the exterior chain 'stub' in the β-limit dextrin. If the A:B ratio was 1·5:1, the average size of the stub would be 2·10 glucose residues; if the A:B ratio was 2·0:1, the corresponding figure would be 2·17 glucose residues. These increases are, in fact, so small in comparison to the experimental errors in the determination of CL ($\pm$ 1 glucose residue) and β-amylolysis limit ($\pm$ 1%) that to a first approximation, they can be ignored. For the rest of this review, an average 'stub' size of two glucose residues will be assumed for β-limit dextrins.

Determination of A:B chain ratios

Several enzymic analyses of A:B chain ratios have now been reported (see Table 1). Some are based on direct methods in which an appropriate dextrin, usually the β-limit dextrin, is treated with one or more debranching enzymes. Other analyses are indirect and are based on the examination of chain profiles following complete debranching. The latter results will be considered in the following section.

The first experimental determination of the A:B chain ratio involved measurement of the amount of maltose and maltotriose liberated by the action of a broad bean debranching enzyme on waxy maize starch (Peat *et al.*, 1956). These oligosaccharides arise only from the A-chains. The experiment was performed on a gram scale and the oligosaccharides were separated by charcoal–Celite chromatography. The yield indicated an A:B ratio of about 1·5:1. In later, smaller-scale experiments, using pullulanase as the debranching enzyme and quantitative paper chromatography to separate the products, β-dextrins of potato amylopectin and waxy sorghum starch gave A:B ratios of 1·3:1 and 1·2:1 respectively (Bathgate & Manners, 1966). In more recent experiments, quantitative gel filtration chromatography and HPLC have been used to determine the amount of maltose and maltotriose released by the debranching of an amylopectin β-dextrin (Enevoldsen, 1980; Bender *et al.*, 1982).

An alternative method was devised by Marshall & Whelan (1974), which depended on the digestion of a β-dextrin (a) with isoamylase, which would release reducing groups equivalent to all the B-chains and half the A-chains, and (b) with a mixture of pullulanase and isoamylase, which liberated reducing groups from all the A- and B-chains. The dif-

TABLE 1
Properties of Some Amylopectins[a]

Sample	*CL*	*β-Amylolysis limit (%)*	*ECL*	*ICL*	*A:B ratio*	*Reference*
Waxy maize starch	24	52	14–15	8–9	1·5:1	Peat *et al.* (1956)
Potato amylopectin	24	55	15	8	1·3:1	Bathgate & Manners (1966)
Waxy sorghum starch	24	58	16	7	1·2:1	Bathgate & Manners (1966)
Potato amylopectin	24	56	15–16	7–8	1·06:1	Bender *et al.* (1982)
Maize amylopectin	23	58	15	7	1·22:1	Bender *et al.* (1982)
Waxy maize starch[b]	19	57	13	5	1·3:1	Inouchi *et al.* (1987)
Phytoglycogen[b]	10	40	6	3	1·0:1	Inouchi *et al.* (1987)
Rice amylopectin	18	56	12	5	1·5:1	Enevoldsen (1980)
Waxy rice starch	19	56	12–13	5–6	1·2:1	Enevoldsen (1980)

[a]Exterior chain lengths (ECL) and interior chain lengths (ICL) calculated by the method described on p. 92.
[b]Inouchi *et al.* (1987) give ECL 10 and 3, and ICL 9 and 7, respectively by their method of calculation.

ference in reducing power corresponded to one-half of the A-chains. The method is based on the assumption that isoamylase cannot release maltosyl side chains from β-dextrins, but this assumption is not entirely valid since in other experiments (Evans *et al.*, 1979), a slow hydrolysis of substrates with maltosyl side chains was observed. Moreover, the method is very sensitive to small experimental errors in the measurement of reducing power (Atwell *et al.*, 1980*a*); since isoamylase is unstable, inadequate amounts of the enzyme can also lead to erroneously high ratios of A:B chains (Manners & Matheson, 1981). Marshall & Whelan (1974) reported that in eight samples of amylopectin, the A:B chain ratios ranged from 1·5:1 for wheat amylopectin to 2·6:1 for waxy maize starch. An A:B chain ratio of 2:1 or more would have substantial implications with respect to any molecular model for amylopectin. However, analysis of waxy maize starch, with due allowance to the factors mentioned above, gave an A:B chain ratio of 1·0:1 (Manners & Matheson, 1981).

The overall results (Table 1) indicate that in most samples of amylopectin there are rather more A-chains than B-chains, the ratios ranging from about 1·0:1 to 1·5:1.

Examination of chain profile

An important technique which is now widely used involves the complete debranching of amylopectin followed by fractionation of the linear

chains by gel filtration. The resultant elution pattern reveals the size distribution of the constituent chains, i.e. the chain profile. The technique was first introduced by Lee *et al.* (1968) and has been developed and refined by others, particularly Hizukuri (1985, 1986).

The first experiments using pullulanase with waxy maize starch followed by Sephadex G50 gel filtration revealed two peaks of chains having a DP <20 and DP about 40 (Lee *et al.*, 1968). In later experiments using isoamylase and amylopectins from potato, waxy rice and waxy maize starches, the chain profiles also showed two characteristic peaks with DP values of about 20 and 50 respectively (Akai *et al.*, 1971). In both investigations, the chain profile of waxy maize starch β-limit dextrin showed the presence of three peaks. Akai *et al.* (1971) reported that these had DP values of about 4, 14 and 40. The low molecular weight peak would include maltose and maltotriose released from A-chain stubs. Both experiments showed that the original peak of DP about 20 contained a mixture of A-chains and some B-chains, and that there were, in fact, two populations of B-chains, the longest having chains with DP values 40–80.

In some experiments, the chain profiles also showed a small peak of high molecular weight material which was excluded from the gel. This was amylose impurity resulting from an incomplete fractionation of the original starch, and can therefore be ignored in the following discussion.

Many experiments with amylopectins from several starches showed a bimodal distribution of their constituent chains. However, MacGregor & Morgan (1984) reported that amylopectin from large and small granules of normal and waxy barley gave a trimodal distribution, with three peaks of chains corresponding to DP values of 45–50, 18–20 and 10–12 (Fig. 3). The latter could represent the A-chains in the original molecule. These workers used Biogel P-6 for the gel filtration instead of the Sephadex G-50 or G-75 used by other workers, and the resolving power of the two types of gel support could well be different.

More recently, the chain profile technique has been refined by Hizukuri (1985) who has used gel permeation HPLC with monitoring by a low angle laser-light-scattering photometer and a differential refractometer. In an extensive survey of amylopectins, 17 samples (including potato, rice and maize) showed a bimodal distribution of chains, whilst wheat, tapioca and tulip amylopectins gave a trimodal distribution. It is therefore clear that amylopectin structure is related to the botanical source of the starch.

The HPLC resolution has been further improved (Hizukuri, 1986) and this has revealed tetramodal distribution profiles for potato, tapioca and kuzu amylopectins (Fig. 4). The lowest molecular weight peak was

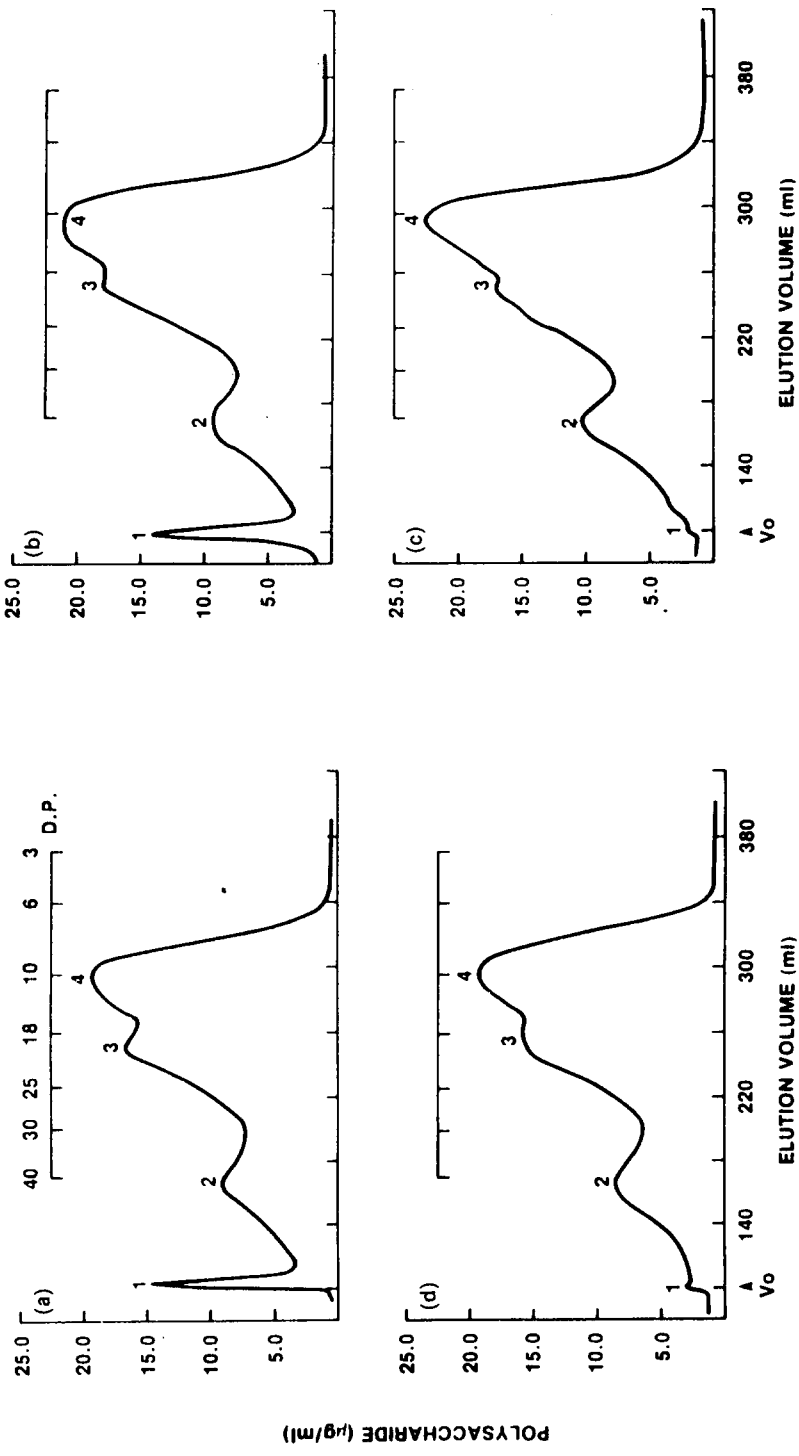

Fig. 3. Fractionation on Biogel P-6 of debranched amylopectins from: (a) Large normal starch granules; (b) small normal starch granules; (c) large waxy starch granules; and (d) small waxy starch granules. From MacGregor & Morgan (1984) reproduced by permission of the American Association of Cereal Chemists.

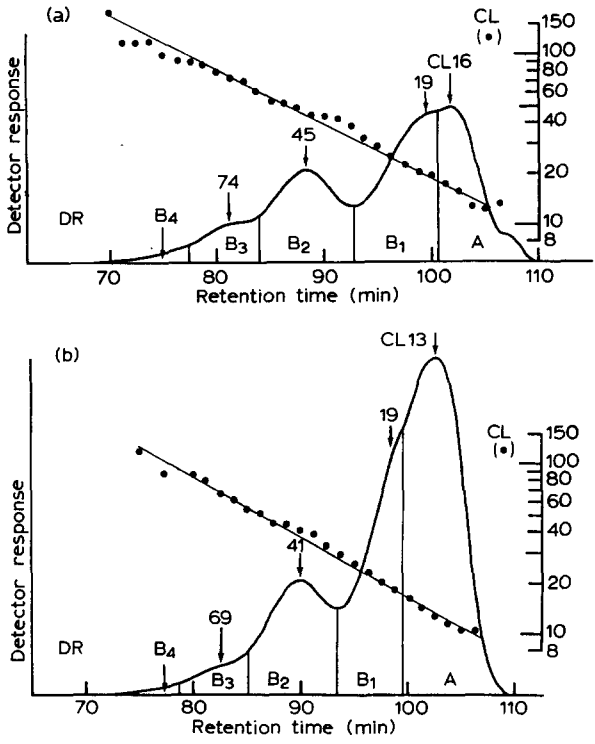

Fig. 4. Gel permeation HPLC of (a) potato amylopectin and (b) waxy-rice amylopectin. From Hizukuri (1986), reproduced by permission of Elsevier, Amsterdam.

divided into two fractions (A and B1) and the other three peaks have been designated B2, B3 and B4 in reverse order of elution. The implications of this polymodal distribution on the fine structure of amylopectin will be discussed later (see p. 105).

The polymodal distribution of the chain profile of debranched β-limit dextrins has been used by Inouchi *et al.* (1987) to calculate the A:B chain ratio. The elution patterns gave fractions II, III, and IV corresponding to the longer B-chains, the shorter B-chains and maltose plus maltotriose respectively. The ratio of

$$\frac{\text{fraction IV}}{\text{fraction II} + \text{fraction III}}$$

corresponded to A/B, and the experimental results indicated A:B chain ratios of 1·3:1 for waxy maize starch, and from 1·3:1 to 1·7:1 for a

series of mutant starches. Phytoglycogen, the sugary-1 mutant, had an A:B ratio of 1·0:1.

In all the above work, the chain profiles have been calculated on a weight basis. Palmer *et al.* (1983) have suggested that the results should be plotted on a numerical basis, since this will provide the most correct interpretation. When the chain profile of phytoglycogen is examined, the weight distribution shows polydispersity with two distinct populations of component chains having peak DP values of 9 and 25–35 respectively. When plotted on a numerical basis, only one peak is obtained, so that the apparent polydispersity is an artefact and not structurally significant. With waxy maize starch, Palmer *et al.* (1983) observed two distinct chain populations irrespective of whether the results were calculated on a weight or numerical basis. It follows that caution is required in the interpretation of chain profiles and that minor peaks on profiles plotted on a weight basis may not always be structurally significant.

A further note of caution arises from the demonstration by Atwell *et al.* (1980*b*) that the patterns of degradation of wheat amylopectin by pullulanase and isoamylase are not identical, especially at intermediate stages of debranching. It is therefore essential to specify the particular debranching enzyme used in any study of chain profiles.

THE ENZYMIC SYNTHESIS OF AMYLOPECTIN

Before considering the detailed organisation of amylopectin, and particularly within a starch granule, it is necessary to briefly consider the enzymic synthesis of the macromolecule.† The $(1\rightarrow4)$-α-D-glucosidic linkages arise from a chain-lengthening reaction, catalysed by starch synthase, which repetitively transfers α-D-glucosyl residues from adenosine diphosphate glucose (ADPG) or uridine diphosphate glucose (UDPG) to an existing chain of $(1\rightarrow4)$-linked α-D-glucose residues $[G]_x$:

$$\text{ADPG} + [G]_x \rightarrow G-(1\rightarrow4)-[G]_x + \text{ADP}$$

The mode of synthesis of the glucosyl-acceptor $[G]_x$ is the subject of continuing investigations. The resultant linear chains are converted into branched chains by Q-enzyme, a transglucosylase whose mode of action is shown below (Fig. 5). During in-vitro experiments, linear chains of DP > 50 were required to act as effective substrates, but a simple branched substrate-amylodextrin, with a DP of about 25 was also

†For details of the enzymic synthesis of amylopectin, the reviews by Preiss & Levi (1980), and Manners (1985*a*) should be consulted.

effective (Borovsky *et al.*, 1979; see Fig. 5). The close association of the A- and C-chains in amylodextrin, probably in a double helical conformation, could provide a favourable environment for rapid Q-enzyme action. It was therefore concluded that the branch points in amylopectin

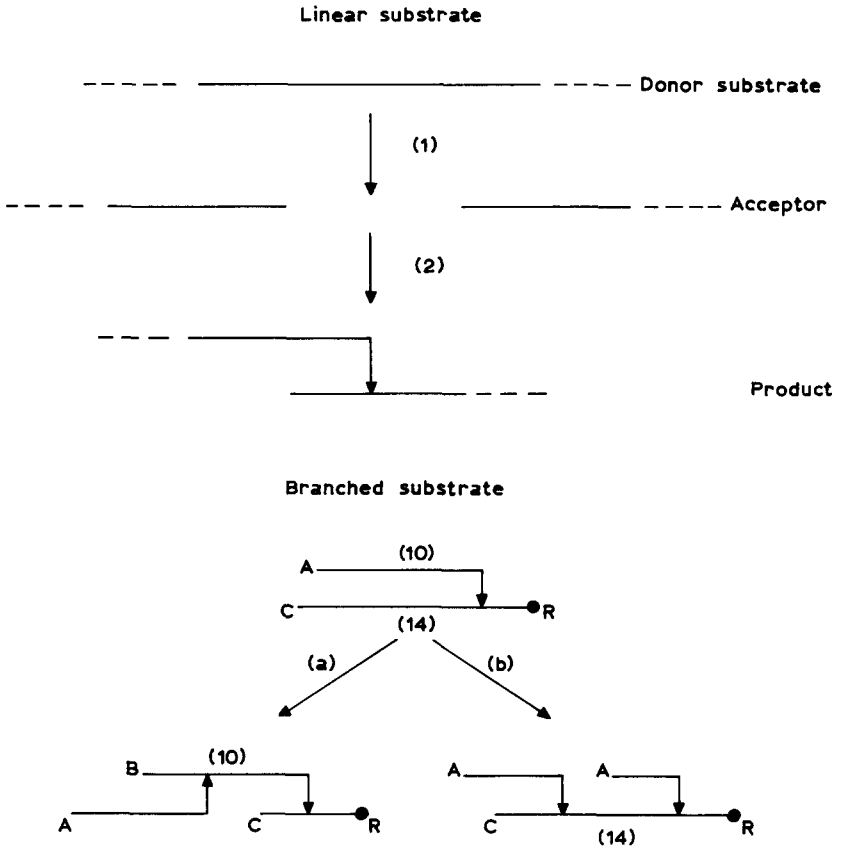

Fig. 5. Action of Q-enzyme on a linear substrate, and a branched substrate, from Manners (1985*a*) and Borovsky *et al.* (1979). With the linear substrate, a short chain of glucose residues is transferred from the donor substrate to an acceptor, which may be part of the original chain, i.e. intra-chain transfer, or part of an adjacent chain, i.e. inter-chain transfer. With potato Q-enzyme, inter-chain transfer appears to predominate. With the branched substrate, transfer of a segment of the original C chain is shown in (a), and transfer in the opposite direction is shown in (b). ——, linear chain of (1→4)-linked α-D-glucose residues; ↓, (1→6)-α-D-glucosidic inter-chain linkage; (1) scission of a (1→4)-α-D-glucosidic linkage; (2) formation of a (1→6)-α-D-glucosidic linkage. A, B and C, types of chain; R, reducing group. The approximate number of D-glucose residues in the A and C chains is shown in brackets.

are synthesised by a random action of Q-enzyme on a pair of $(1 \rightarrow 4)$-α-D-glucan chains. This action pattern formed the basis of one possible molecular model for amylopectin (see p. 103), although for other reasons, this model is not generally accepted.

Both starch synthase and Q-enzyme occur in multiple forms. The former enzyme also exists in insoluble and soluble forms, and it is believed that a granule-bound insoluble form is responsible for the synthesis of amylose. This enzyme is missing from the waxy cereals, where the synthesis of amylopectin results from the combined action of one or more soluble starch synthase and Q-enzyme complexes. Full details of the interactions between these two enzymes, and the resultant branched products are not yet available, although in general terms, it is known that the degree of branching is related to the relative amounts of the two types of enzyme. This interaction between the chain-lengthening enzyme and Q-enzyme cannot be identical in all starches, since there are variations in chains profiles and physical properties, particularly the X-ray diffraction patterns.

STRUCTURAL MODELS FOR AMYLOPECTIN

Several molecular models have been proposed for amylopectin during the past 50 years or so (see Fig. 6). The original models suggested by Haworth *et al.* (1937) and by Staudinger & Husemann (1937) are not compatible with the enzymic evidence for a macromolecule containing approximately equal numbers of A- and B-chains.

The model of Meyer & Bernfeld (1940) was the first to include multiple branching as a feature. It has been redrawn as a regularly rebranched structure (Lee *et al.*, 1968), but whether this is entirely compatible with Meyer's original views is not certain. There is no obvious evidence in the literature of the 1940s for regular branching, and indeed, there is much later evidence to support a non-regular branching, e.g. the presence of high molecular weight dextrins (macrodextrins) and multiply branched α-dextrins during the α-amylolysis of amylopectin (Babor *et al.*, 1968; Kainuma & French, 1970).

A revision of the Meyer structure was suggested by Gunja-Smith *et al.* (1970*b*), and some years later, the same research group also proposed an elongated model having some of the features of the original Haworth and Staudinger structures (Borovsky *et al.*, 1979). Both of these novel structures (Fig. 6) are based on subtle enzymic analyses, the basis of which is now less certain (Manners, 1985*b*).

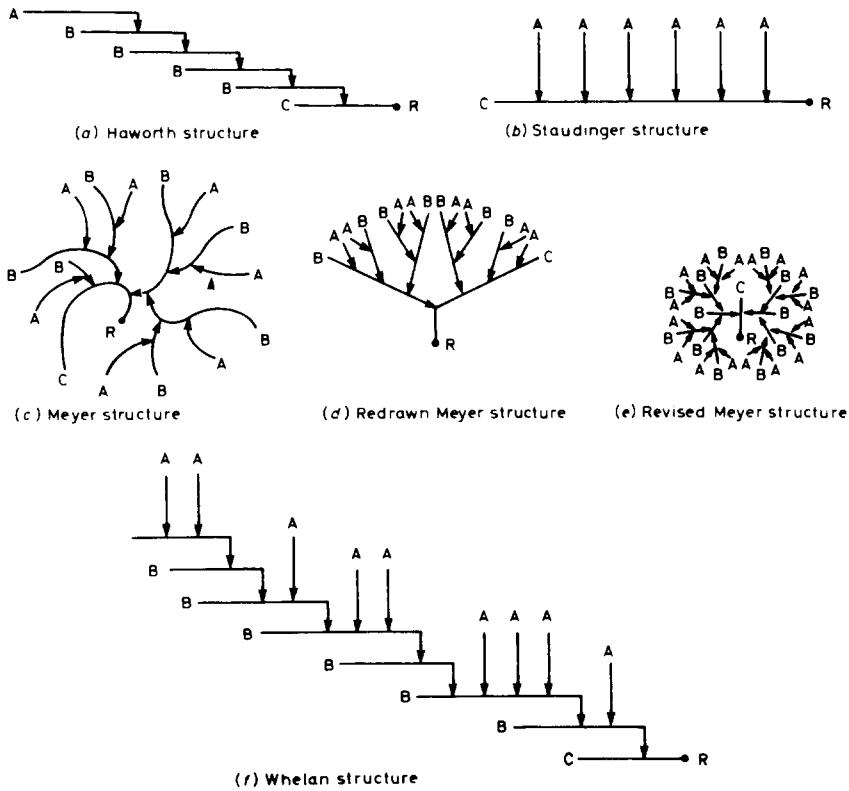

Fig. 6. Diagrams of the molecular structure of amylopectin, as proposed by (a) Haworth; (b) Staudinger; and (c) Meyer; (d) the Meyer structure redrawn as a regularly branched structure; (e) the Whelan revision of the Meyer structure; (f) the Whelan elongated structure. From Manners (1985*b*), reproduced by permission of Cereal Foods World.

The general view that has emerged during the last 15 years or so is that amylopectin has a cluster type structure (see Fig. 7). This concept arose independently in the laboratories of Nikuni and French (for reviews see Nikuni (1975) and French (1984)). The cluster model is in accord with the relatively high viscosity of amylopectin (compared to glycogen, which has an even higher molecular weight), the crystallinity of the macromolecule as revealed by X-ray analysis, and the relative resistance of parts of the molecule to attack by acid and by amylolytic enzymes. This resistance forms the basis of the cluster model proposed by Robin *et al.* (1974), who studied the sequential degradation by β-amylase and pullulanase of amylopectin, its β-limit dextrin and a derived acid-resistant amylodextrin. In their original paper, Robin *et al.* (1974) defined A- and B-chains in a different sense to those used by other work-

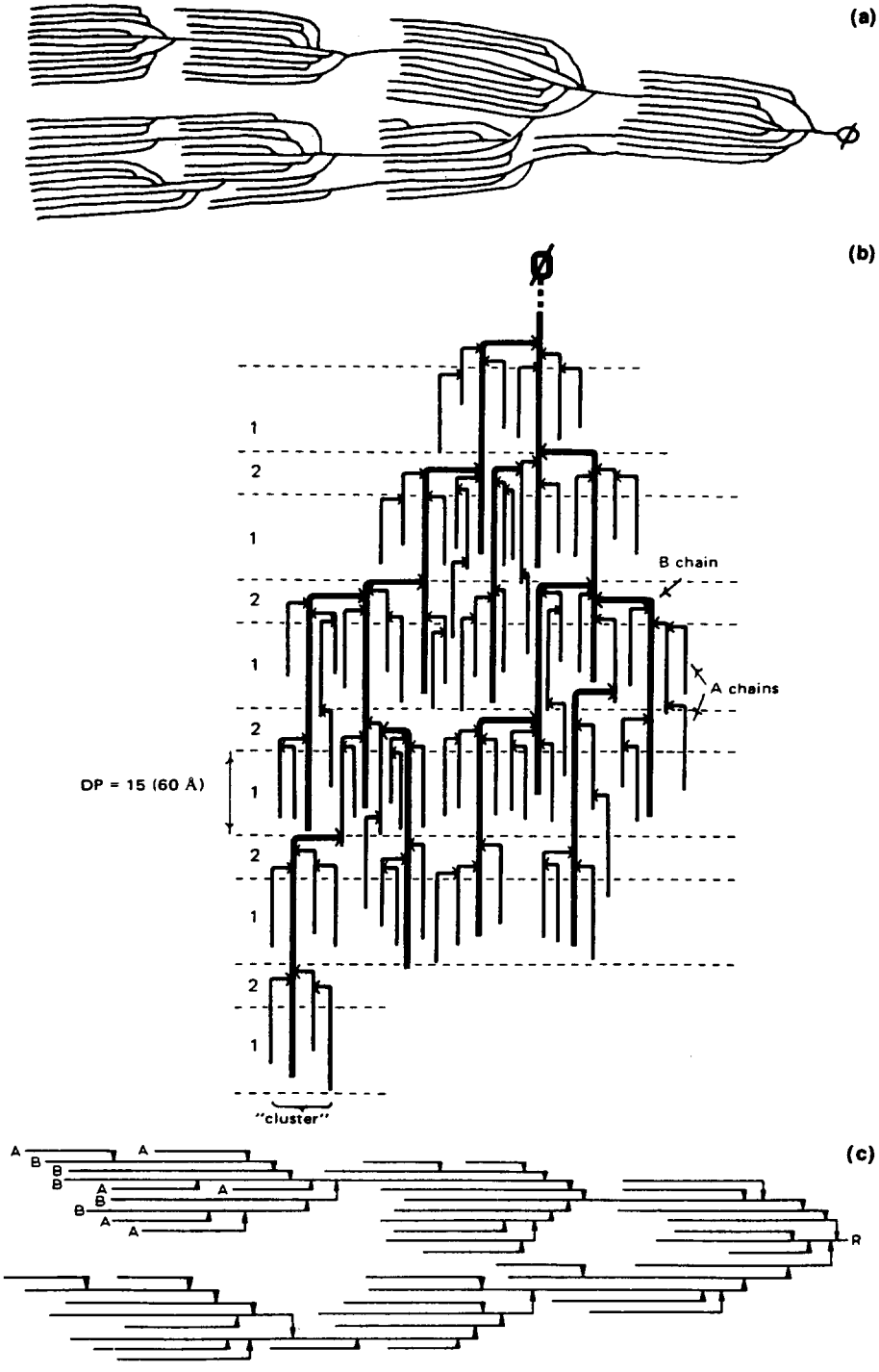

Fig. 7. Structural cluster-type models for amylopectin as proposed by (a) French; (b) Robin and co-workers; (c) Manners & Matheson. From Manners (1985*b*), reproduced by permission of Cereal Foods World.

ers. Nevertheless, the results showed the presence of populations of chains having CL values of 15–20 which were in highly ordered clusters. These clusters were linked to each other by much longer chains, which would correspond to the longer B-chains found in elution profiles.

A variant of the cluster model is shown in Fig. 7, based in part on the change in the ratio of A:B chains when waxy maize starch is partly debranched with pullulanase. An amylopectin molecule with the elongated structure proposed by Borovsky *et al.* (1979) would have an A:B-chain ratio of 0·3:1 after 35% debranching. The observed value was 0·6:1 (Manners & Matheson 1981). This observation also provides evidence that a proportion of the B-chains carry more than one A-chain, which is, of course, one structural feature of the elongated model of Borovsky *et al.* (1979).

A further refinement of the cluster model was proposed by Hizukuri (1986) and is based on the polymodal distribution of the chain profile (Fig. 8). The fraction A quoted on p. 99 represents the A-chains while fractions B1–B4 are the various B-chains. It is proposed that single clusters are composed of fractions A and B1, that the chains in fraction B2 extend into two clusters, those in fraction B3 into three clusters, and the 1% of chains in fraction B4 could even extend into four clusters. The average CL of the fractions B1, B2 and B3 were 20–24, 42–48 and 69–75 respectively in four different amylopectins, the relative lengths being 1:2:3. The A-chains contained 12–16 glucose residues. The sums of the chains in fractions A and B1 were about 80–90% of the total, and constitute single clusters. The remaining 10–20% of chains are mainly involved in inter-cluster connections. The overall size of a single cluster is similar to that obtained by X-ray diffraction analysis of the crystalline domains of starch granules.

AMYLOPECTIN AND THE STARCH GRANULE

Since a detailed discussion of starch granule structure could form the basis of a separate review, the present account will be limited to certain aspects.

Firstly, starch granules are resistant to attack by most amylolytic enzymes, with the notable exception of α-amylase, so that enzymic degradation studies have been of limited value, and most information has been obtained by physical methods such as X-ray diffraction, electron microscopy, nmr spectroscopy and small-angle light scattering. Details of many of these studies have been provided by Blanshard (1987).

Secondly, amylopectin in the solid state is responsible for the crystalline regions in the granules of two component starches (Lineback, 1984).

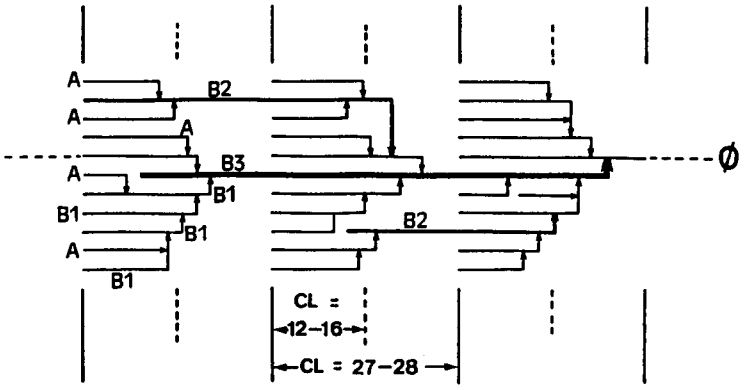

Fig. 8. Cluster model of amylopectin proposed by Hizukuri (1986). For definition of A, B1, B2 and B3, see text. The reducing end-group is shown by ϕ. Reproduced by permission of Elsevier, Amsterdam.

For example, waxy maize starch exists as granules which are similar to those of normal maize starch, so that amylose cannot be the crystalline material.

Waxy maize starch granules have been examined by electron microscopy (Yamaguchi *et al.*, 1979), and the results were consistent with an extended cluster model for amylopectin. Granules that had been mashed in water showed some remnants of granule organisation with rippled fibrous structures, the ripples being about 70 Å apart. These could be alternations between amorphous and crystalline regions, in which the latter were due to the side-by-side association of parallel clusters of A- and shorter B-chains. The presence of growth rings at irregular intervals of 1200–1400 Å was also shown and a possible relationship between these and other structures is shown in Fig. 9.

The detailed arrangement of amylose and amylopectin within a starch granule continues to be the subject of investigation and speculation. It is known that at least some of the amylose can be leached out of a granule with warm water (Cowie & Greenwood, 1957), leaving an amylopectin-rich granule, indicating a weak association between some of the amylose and amylopectin. In addition, the relatively insoluble sac remaining after the rupture of starch granules has the iodine staining power of an amylopectin (Stark *et al.*, 1983) showing the presence of an amylopectin-rich zone near the surface of the granule. This surface may not be smooth and Lineback (1984) has suggested a boundary with the appearance of a 'hairy billiard ball' with some exterior chains of amylopectin, perhaps in

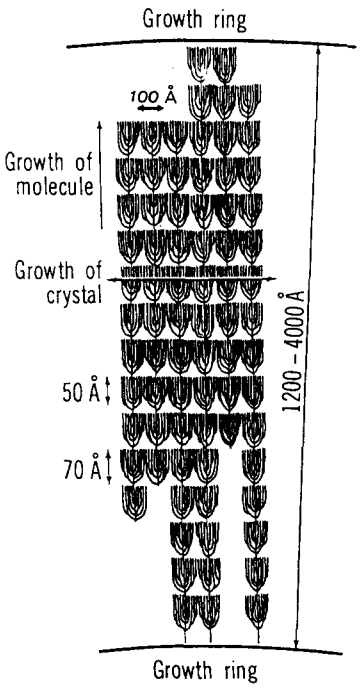

Fig. 9. Schematic representation of the arrangement of amylopectin molecules within a growth ring of a waxy starch granule. Diagram by courtesy of K. Kainuma. From French (1984), reproduced by permission of Academic Press, Orlando.

a double helical conformation, protruding from the surface. Possible models for amylose and amylopectin interactions within a granule have been proposed by Lineback (1984) and Blanshard (1987). It is clear that further details of the associations between amylose and amylopectin, and between individual amylopectin and individual amylose molecules are required.

Finally, the relationship between the chain profiles of amylopectin and the crystalline structure of starch granules has been investigated by Hizukuri (1985). Native starch granules give distinctive X-ray diffraction patterns according to the botanical source of the starch. A-Starch patterns are characteristic of cereal starches; B-patterns of tuber starches, and C-patterns are intermediate between A and B. Eleven samples of A-type amylopectin had CL values in the range 23–29 (average 26), seven B-type amylopectins had CL values of 30–44 (average 36) and three C-type amylopectins had CL values of about 28. Chain profile studies showed that in general, the amylopectins from A-type starches had shorter chains in both the long (F1) and short (F2) chain fractions, and larger amounts of the short-chain fractions, than in amylopectins from B-type starches (see Table 2). The C-type starches gave

intermediate values, and it was suggested that these starches could yield a varying type of crystalline structure depending on the environmental temperature and other factors, whereas the A- and B-type starches were insensitive to temperature. It was concluded that the chain length and chain profile of the amylopectin was an intrinsic factor in determining the crystalline structure of starch granules.

TABLE 2
Chain Length Distributions in Amylopectins[a]

Type of starch	*Number of samples*	*Average CL*	*CL of F1*	*CL of F2*	*Ratio F2/F1 (weight basis)*	*Ratio F2/F1 (molar basis)*
A	11	26	54	17	3·2	10
B	6	36	62	19	1·8	6
C	3	28	55	18	2·8	8

[a]Data from Hizukuri (1985), reproduced by permission of Elsevier, Amsterdam.

The factors affecting the crystalline type (A–C) of native starches have been examined by Gidley (1987). Using linear malto-oligosaccharides as model compounds, the crystallisation of an A-type polymorph over a B-type was favoured, *inter alia* by shorter CL values, higher temperature, higher concentrations and the presence of salts and water-soluble alcohols. In the A-type structure, there is a close-packed arrangement of double helices, whereas in the B-type, the structure is more open, with a greater amount of inter-helical water. The effect of chain length on the polymorphic form may be rationalised from entropy considerations, since with longer chains, the entropy change on crystallisation will become larger and favour the polymorph of highest entropy, i.e. the B-type.

OTHER ENZYMIC ANALYSES OF AMYLOPECTIN

In addition to the debranching enzymes, three additional types of amylolytic enzyme have been used in structural studies. In some cases, these have revealed differences in the fine structure of amylopectins from different starches which were not revealed by other methods of analysis.

α-Amylase

The initial stages of the α-amylolysis of large and small barley starch granules, and of waxy maize starch, have been followed by gel chromatography on Sepharose CL4B or CL6B (Bertoft & Henriksnas, 1982; Bertoft, 1986). The results indicate a non-random hydrolysis of amylopectin with the formation of intermediate products with a molecular weight > 30 000. These arise from the preferential hydrolysis of certain (1→4)-α-D-glucosidic linkages in the amylopectin which join together two, three or more clusters in the macromolecule. The size of the intermediate products varies considerably and includes 'super clusters' with molecular weights ~ 10^5, arising from highly ordered regions of amylopectin. On prolonged α-amylolysis, the intermediate products are converted into the low molecular weight α-dextrins. Overall, the pattern of α-amylolysis is fully in accord with the cluster model for amylopectin.

Cyclodextrin glycosyl-transferase

This enzyme (CGT), from *Klebsiella pneumoniae*, catalyses the simultaneous cyclisation (exo-attack) and chain shortening (endo-attack) of amylopectin with the formation of a mixture of cyclic and non-cyclic degradation products (Bender *et al.*, 1982). Analysis of maize and potato amylopectin using CGT has revealed significant differences in their fine structures. 57% of the maize amylopectin and 64% of the potato amylopectin was recovered as non-cyclic products which had β-amylolysis limits ranging from 24 to 37% respectively. These and other results indicated that the long B-chains in maize amylopectin were more susceptible to attack by CGT than those in potato amylopectin. The products from potato amylopectin contained marked amounts of longer B-chains, suggesting that the clusters were not so tightly packed. By contrast, in maize amylopectin, which has a similar CL to potato amylopectin (23–24), the macromolecule is composed of tightly packed clusters connected by relatively long B-chains.

Maltotetraohydrolase

The bacterium *Pseudomonas stutzeri* produces a maltotetraohydrolase which releases maltotetraose from starch-type polysaccharides (Robyt & Ackerman, 1971). Enzyme action is not arrested by the outermost branch points so that (1→4)-α-D-glucosidic linkages in the interior of an amylopectin molecule can also be attacked, and the enzyme can

therefore degrade amylopectin β-limit dextrins. Wheat amylopectin β-limit dextrin gave a mixture of maltotetraose and lower maltosaccharides, together with higher molecular weight dextrin products (Sebesta, 1987; Finch & Sebesta, 1988). By contrast, potato amylopectin β-limit dextrin gave, in addition to high molecular weight dextrins, mainly maltose with some glucose and maltotriose; maltotetraose was, however, absent. This suggests that the interior of wheat amylopectin contains some relatively long interior chains which can be readily attacked by the maltotetraohydrolase, and that similar chains are absent from potato amylopectin. These results, like those with CGT, suggest that there are significant differences in the detailed arrangements of the clusters between potato and cereal amylopectins. Further investigation of these differences is one of the next targets for the starch biochemist.

CONCLUSIONS

This review has surveyed the use of enzymic methods for the examination of the fine structure of amylopectin. These methods have yielded information which is not available by purely chemical and physical techniques, and have revealed subtle differences between amylopectins from starches from different botanical sources. Overall, a cluster model is becoming increasingly accepted for amylopectin. However, present knowledge of the organisation of amylopectin within a starch granule is still limited, and remains for further examination by a combination of physical, enzymic and other methods of analysis.

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