Structure of Amylopectins Isolated from Large and Small Starch Granules of Normal and Waxy Barley[1]

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ABSTRACT

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Debranching, followed by gel-permeation chromatography on Biogel P-6, showed that amylopectins from large and small starch granules of normal and waxy barley contained three groups of unit chains having approximate degrees of polymerization (DPs) of 45–50, 18–20, and 10–12. Gel-permeation chromatography on Biogel P-4 separated the 18–20 and 10–12 DP groups into individual unit chains, showing that both groups were heterogeneous. Maltohexaose was the smallest unit chain found in the amylopectins studied. During β-amylolysis, the 45–50 DP peak was degraded, and the predominant chain length was reduced to approximately 38 glucose units. The other two peaks were extensively degraded to give one heterogeneous peak having a predominant DP of 10–12. A new peak, composed of maltose and maltotriose stubs, was detected in the β-limit dextrins. Amylopectins from the different types of starch granules studied appeared to have identical structures.

Starch granules from mature barley kernels can be separated into two distinct populations on the basis of granule size. The minor fraction (10% by weight) contains granules smaller than 5 μm in diameter, but the major fraction contains granules 10–20 μm in diameter (May and Buttrose 1959, MacGregor et al 1971a). Large and small granules appear to contain similar proportions of amylose and amylopectin (Goering and DeHaas 1974, Kano 1977a, MacGregor and Ballance 1980), but they differ significantly in enzyme susceptibility and gelatinization temperature (Bathgate and Palmer 1972, Palmer 1972). These results suggest that the components of these two types of starch granule differ structurally.

Starches from normal and waxy types of barley also differ in physical properties and in enzyme susceptibility (Goering et al 1973). Such findings may be readily attributed to the large difference in amylose content of starches from these two barley types. However, differences in amylopectin structure could also contribute significantly to those properties.

This study was initiated to determine whether there were significant differences in the fine structure of amylopectins isolated from different types of barley starch.

MATERIALS AND METHODS

Starch Isolation

Starch granules from near-isogenic lines of normal and waxy Hector barley were extracted, purified, and fractionated into large- and small-granule populations by methods described previously (MacGregor 1979).

Starch Analysis

Amylose contents were determined by an amperometric titration technique (Coton et al 1955, Larson et al 1953). Protein contents were determined by the method of Mitcheson and Stowell (1970) for sample digestion, followed by nitrogen determination with Nessler reagent (Williams 1964). Nitrogen values were multiplied by a factor of 6.25 to give protein values.

Starch Fractionation

Starch fractionation was performed as described previously (Banks and Greenwood 1967).

Preparation of β-Amylase

A sound sample of barley (*Hordeum distichum* cv. Klages) that did not contain detectable amounts of α-amylase was used. One kilogram of barley was extracted with 2 L of 1% NaCl containing $10^{-3}M$ thioglycerol, and the extract was centrifuged (10,000 $\times g$, 10 min). The pellet was re-extracted with 1 L of the NaCl solution and, after centrifugation, the two supernatant solutions were combined and made to 40% with $(NH_4)_2SO_4$. The precipitate was dissolved in 1% NaCl (620 ml, $10^{-3}M$ thioglycerol), dialyzed extensively against this solution, and the β-amylase was precipitated between $(NH_4)_2SO_4$ concentrations of 20 and 35%. Crude β-amylase was dissolved in acetate buffer (0.02M, pH 4.75, $10^{-3}M$ thioglycerol), dialyzed against this buffer, and fractionated on carboxymethyl cellulose (CMC) as described previously (MacGregor et al 1971b). Active fractions were pooled, concentrated (Diaflo PM10 membrane) to 20 ml, and run on a Biogel P-150 column (100 $\times$ 2.5 cm) with acetate buffer (0.02M, pH 4.75, $10^{-3}M$ thioglycerol). Fractions containing β-amylase were pooled, concentrated, dialyzed against acetate buffer (0.02M, pH 5.5, $10^{-3}M$ thioglycerol), and applied to a Sepharose-β-cyclodextrin column (30 $\times$ 1 cm), as described previously (Weselake and Hill 1982, 1983). β-Amylase was eluted in the void volume. This treatment removed a small amount of contaminating α-amylase, but a trace of limit dextrinase remained. This was removed by eluting the enzyme from a CMC column (90 $\times$ 2 cm) with the following linear gradient: 1 L of 0.05M acetate buffer (pH 4.75, $10^{-3}M$ thioglycerol); and 1 L of 0.18M acetate buffer (pH 4.75, $10^{-3}M$ thioglycerol). Active fractions were pooled, concentrated, and saturated with $(NH_4)_2SO_4$. β-Amylase was stored at 5°C as an $(NH_4)_2SO_4$ precipitate.

The β-amylase did not hydrolyze pullulan (no increase in reducing power detected) or amylopectin β-limit dextrin (no increase in reducing power or change in iodine color), indicating that the enzyme did not contain detectable amounts of α-amylase or limit dextrinase. No glucose was detected in β-amylase-amylopectin digests, showing the absence of α-glucosidase activity.

Debranching of Polysaccharides

Isoamylase (amylopectin 6-glucanohydrolase, E.C.3.2.1.68) from *Pseudomonas amyloderamosa* and pullulanase (pullulan 6-glucanohydrolase, E.C.3.2.1.41) from *Aerobacter aerogenes* (ATCC 9621) were obtained from Hayashibara Biochemical Labs. Inc., Okayama, Japan. These enzymes neither decreased the viscosity nor increased the reducing power of solutions of linear amylose. Therefore, they did not contain detectable amounts of α-amylase.

Amylopectin. Digests containing amylopectin (25 mg) and isoamylase (10 μl, 590 units of activity as determined by Yokobayashi et al [1970]) in acetate buffer (5 ml, 0.01M, pH 3.8) were incubated at 35°C for 24 hr and were then boiled for 5 min. A known volume of digest containing 3 mg of polysaccharide was loaded onto a column of Biogel P-6. The remainder of the digest was frozen and freeze-dried for further analysis on a column of Biogel P-4.

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Amylopectin β-limit dextrin. Digests containing amylopectin (20 mg) and purified barley β-amylase (20 μl, 50 nanokatal) in acetate buffer (15 ml, 0.1*M*, m*M* thioglycerol, pH 5.5) were incubated at 35°C for 20 hr and were then boiled for 5 min. Digests were dialyzed exhaustively at 4°C against the acetate buffer in the absence of thiol. Pullulanase (10 μl, 50 nanokatal) was added, and the digests were incubated at 35°C for 20 hr and boiled for 5 min. A portion of the digest (approximately 3 mg of polysaccharide) was analyzed on a column of Biogel P-6, and the remainder was freeze-dried for further analysis on Biogel P-4.

Gel-Permeation Chromatography

Three milligrams of debranched polysaccharide was loaded onto a Pharmacia column (90 × 2.5 cm) packed with Biogel P-6. The column was maintained at 70°C and was eluted with degassed water at 70°C. Flow rates were maintained at 30 ml/hr, and 4-ml fractions were collected. Fractions were assayed for total carbohydrate by an automated orcinol-sulfuric acid procedure (LaBerge et al 1973). Recovery of polysaccharide was always at least 95%.

Freeze-dried samples of debranched amylopectin and β-limit dextrin were taken up in a small volume of water (approximately 2 ml), and portions (20 μl, 150–200 μg of polysaccharide) were analyzed on two Beckman columns (160 × 0.9 and 90 × 0.9 cm) connected in tandem and packed with Biogel P-4 (−400 mesh). These columns were maintained at 70°C and eluted with degassed, deionized water at the same temperature. The effluent was continuously monitored by an automated orcinol-sulfuric acid procedure (LaBerge et al 1973). Polysaccharide recoveries were at least 95%. All gel-permeation chromatography experiments were replicated.

β-Amylolysis Limits of Amylopectins

Digests contained 25 mg of amylopectin and 20 μl of barley β-amylase (50 nanokatal) in 20 ml of acetate buffer (0.02*M*, pH 5.5). These were incubated at 35°C for 20 hr. Total carbohydrate was measured by the orcinol-sulfuric acid method (LaBerge et al 1973) and maltose with neocuproine reagent (Dygert et al 1965).

Lengths of Unit Chains

Fractions obtained from the elution of debranched samples of amylopectin and β-limit dextrin on the Biogel P-6 column were assayed for total carbohydrate (orcinol-sulfuric acid method) and reducing sugar by the Nelson (1944) copper reagent, essentially as described by Robyt and Whelan (1968). Both methods were calibrated against D-glucose. The length or degree of polymerization (DP) of unit-chains in each polysaccharide fraction was determined by dividing the total amount of carbohydrate present by the reducing sugar content (Gunja-Smith et al 1971).

Average chain lengths of debranched samples of amylopectin and amylopectin β-limit dextrin were determined by dividing the total amount of polysaccharide present (glucose equivalents) by the reducing power (glucose equivalents).

RESULTS AND DISCUSSION

The samples of barley starch used in this study were similar to other barley starches in having the well-established bimodal distribution of granule sizes (May and Buttrose 1959, MacGregor et al 1971a). By repeated decantation and sedimentation in saline solution, large granules (10–25 μm in diameter) were separated from small granules (less than 5 μm in diameter). Care was taken to minimize losses of the small granules, which usually accounted for about 10% of the total starch recovered. Examination by both light and scanning electron microscopy showed that samples of large granules were essentially free of small granules and vice versa.

Properties of these starch granules (Table I) were similar to those of granules isolated from isogenic lines of Manchurian barley used in a previous study (MacGregor and Ballance 1980). Small granules from normal starch had a slightly lower amylose content than did large granules (Table I). This agrees with previous findings on a number of barley cultivars (Goering and DeHaas 1974, Kano 1977a). Large granules from waxy starch contained detectable amounts of amylose, but only a trace was found in the small granules, in agreement with previous findings (Banks et al 1970). Protein content of the starch samples was low, indicating that the starch granules were reasonably clean. The small amounts of protein remaining may be an integral part of the granule and not just impurities adsorbed by the granule surface.

Small differences were found in the degree of degradation by β-amylase of amylopectins isolated from these different types of starch granules (Table II). In general, these values are similar to those reported for amylopectins isolated from a number of plant species, including cereal grains (Greenwood and Thomson 1962, Banks et al 1970, Kano et al 1981, Biliaderis 1982) and do not indicate any significant difference in the amylopectin structures. Similarly, the average chain lengths found (21–23) are similar to values reported for amylopectins from waxy maize (Biliaderis 1982, Palmer et al 1983) and manioc (Hood and Mercier 1978) starches.

Small amounts of amylose were present in amylopectins from normal starch granules, but only trace amounts were found in amylopectins from waxy starches. Normal starch was fractionated several times, and a small amount of amylose was always detected in amylopectin fractions. This amylose may be low-molecular-weight material that does not complex efficiently with thymol during starch fractionation. After fractionation, 65–70% of the normal and 80–85% of the waxy starch used was recovered as amylopectin.

When amylopectins, debranched by isoamylase, were fractionated on a column of Biogel P-6, one minor and three major peaks of carbohydrate were obtained (Fig. 1). The minor peak was eluted sharply at the void volume of the column and so had a DP of at least 60. It represented 4–5% of the debranched amylopectins from normal barley starch (Fig. 1A and B), but only a trace was present in amylopectins from waxy barley starch (Fig. 1C and D). This material, which had a λ_{max} similar to that of amylose and was highly degraded by β-amylase (Fig. 4), was probably amylose impurity. It has been detected previously in amylopectins prepared from waxy maize starch (Akai et al 1971) and in various wheat starches (Lii and Lineback 1977, Atwell et al 1980). Profiles of the major peaks were very similar for all amylopectins. Peak 2, the first major peak, contained material having a DP of 40–50 and corresponds to the DP 42 peak obtained by Kano (1977b) for other barley amylopectins. Slightly higher DP values (45–60) have been reported for a similar peak in amylopectins isolated from a number of other cereal grains (Gunja-Smith et al 1970, Mercier 1973, Akai et al 1971, Lii and Lineback 1977, Hood and Mercier 1978, Atwell et al 1980, Ikawa et al 1981, Sargeant 1982, Matsukura et al 1983). Such variations in DP values are probably within experimental

TABLE I
Properties of Starch Granules

	Normal		Waxy	
	Large	**Small**	**Large**	**Small**
Granule size (μm)	10–25	5	10–25	5
Amylose (%)	22.1	19.0	3.6	1.8
Protein (%)[a]	0.23	0.43	0.06	0.15

[a]Protein = N × 6.25.

TABLE II
Properties of Amylopectins

	From Normal Starch Granules		From Waxy Starch Granules	
	Large	**Small**	**Large**	**Small**
β-Amylolysis limit (%)	59	55	54	57
Amylose (%)	4.0	2.6	1.1	1.1
Average length of unit-chains (cl)[a]	21	23	23	22

[a]cl = chain length.

error and are unlikely to represent a significant difference between the amylopectins of barley and other cereals.

Several published reports have shown that debranched amylopectins have a bimodal distribution of unit chains. However, the occasional appearance of a shoulder near the apex of the lower-DP peak (DeHaas and Goering 1972, Kano et al 1981, Atwell et al 1980) suggests that this peak may contain two groups of chains. The results shown in Fig. 1 confirm this idea. Peaks 3 and 4 were not well separated, but they clearly show the presence of two groups of chains. One had a maximum DP of 18–20 (peak 3) and the other a DP of 10–12 (peak 4). Elution patterns for all four debranched amylopectins were similar. The relative proportions of groups 2 and 3 + 4 in terms of weight and molarity were calculated for each amylopectin, assuming an average DP of 42 for peak 2 and a DP of 15 for peaks 3 + 4. Results are shown in Table III. Amylopectin from large normal starch granules had a slightly higher proportion of peak 2 compared to the other amylopectins. However, because of the errors inherent in these determinations, the difference is unlikely to be significant. Ratio values of 3.7 obtained for the other amylopectins are identical to the value of 3.7 obtained by Kano et al (1981) for an amylopectin from a different barley cultivar. The molar ratios of peaks 3 + 4/peak 2 were similar to the value of 10.6 reported for waxy maize amylopectin (Biliaderis 1982) but slightly higher than that (7.5) found for manioc amylopectin (Hood and Mercier 1978).

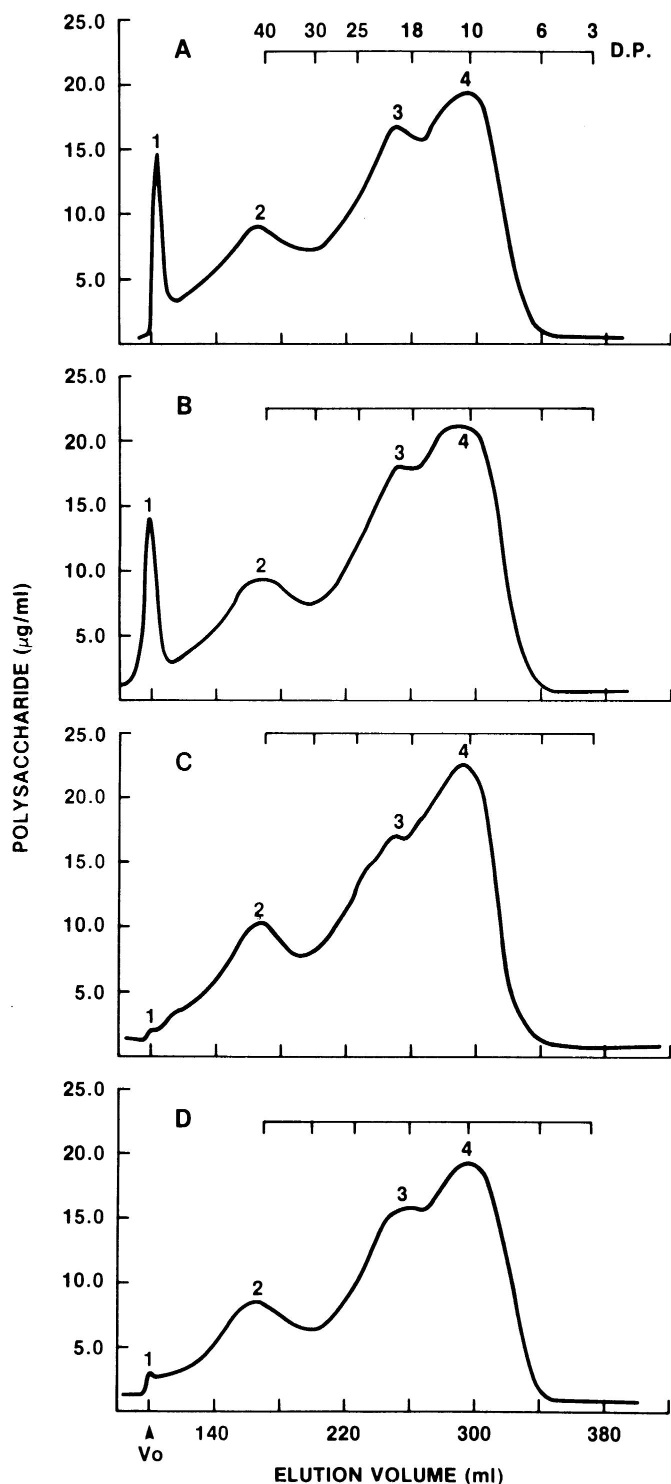

Fig. 1. Fractionation on Biogel P-6 of debranched amylopectins from: **A,** large normal, **B,** small normal; **C,** large waxy; and **D,** small waxy starch granules.

Amylopectins from banana and breadfruit starches have a bimodal distribution of the higher DP chains (Loos et al 1981, Kayisu and Hood 1981). This distribution suggests that the unit chains of amylopectins from different botanical sources may have different size distributions.

Debranched amylopectins were analyzed further on columns of Biogel P-4 (Fig. 2). This gel was not able to resolve maltodextrins of DP 30 or higher, so amylopectin peaks 1 and 2 (Fig. 1) were eluted close to the void volume of the column. Individual components of peaks 3 and 4 shown in Fig. 1 were well resolved to show the heterogeneous nature of these peaks. Each contained a wide distribution of chain sizes with chains of DP 21–22 and DP 12 predominating in peaks 3 and 4, respectively. These DP values are in good agreement with those shown in Fig. 1. Although separation of maltodextrins up to DP 26 was obtained, a quantitative determination of these dextrins could not be made. However, all amylopectins gave similar qualitative patterns.

No chains smaller than maltohexaose were detected, indicating that amylopectin does not contain unit chains having fewer than six glucose units. This confirms previous findings of Kano et al (1981) and agrees well with analytical results of Borovsky et al (1979) on amylopectins prepared in vitro.

When debranched amylopectins were treated with β-amylase complete conversion to maltose was obtained, thus confirming the completeness of amylopectin debranching by isoamylase from *Pseudomonas amyloderamosa*. Chromatography of the products on Biogel P-4 showed only maltose with a small amount of maltotriose. The profile for amylopectin from large normal starch granules is shown in Fig. 3A. All amylopectins gave similar profiles. The amount of β-amylase used was not sufficient to hydrolyze maltotriose. Addition of excess β-amylase led to the complete hydrolysis of maltotriose, leaving maltose and a trace of glucose as the only products.

Debranched amylopectin β-limit dextrins were fractionated on Biogel P-6 (Fig. 4). Although chromatography was done with 3 mg of each β-limit dextrin, the profiles obtained were adjusted to represent the amount of dextrin obtained from 3 mg of the respective amylopectin. The β-amylolysis limits shown in Table II

TABLE III
Relative Sizes of Groups of Unit Chains from Debranched Amylopectins[a]

Source of Amylopectin	Peak No. 1	Peak No. 2	Peak No. 3 + 4	Peak 3 + 4/ Peak 2[b]	Peak 3 + 4/ Peak 2[c]
Large normal starch	5.4	22.2	72.4	3.3	9.1
Small normal starch	5.4	20.0	74.5	3.7	10.4
Large waxy starch	···	21.2	78.8	3.7	10.4
Small waxy starch	···	21.4	78.6	3.7	10.4

[a] As a percentage of total material eluted from columns of Biogel P-6.
[b] Proportions by weight.
[c] Molar proportions.

were used to make this adjustment. Therefore, profiles shown in Figs. 1 and 4 are directly comparable.

Beta-amylase extensively degraded the material forming peak 1 in amylopectin (Fig. 1), so that the corresponding peak in the β-limit dextrin (Fig. 4, peak 1) was much smaller in quantity. It was no longer eluted in the void volume and so had been reduced in size as well. Amylopectin peak 2 was also reduced in quantity, and the chains comprising this peak were shortened. The predominant chain length fell from approximately DP 42 in amylopectin to DP 38 in the β-limit dextrin. Amylopectin peaks 3 and 4 (Fig. 1) were extensively degraded by β-amylase to give one peak in the β-limit dextrin (Fig. 4) with a wide distribution of chain lengths centered around a DP of 10–12. Comparison of Figs. 1 and 4 shows clearly that amylopectin peaks 3 and 4 were significantly more extensively hydrolyzed than peak 2 by β-amylase. This strongly suggests that the unit chains comprising peaks 3 and 4 tend to be on the outside of the amylopectin molecule, whereas the longer chains of peak 2 are in the interior. This is in agreement with current ideas on amylopectin structure (Robin et al 1974, 1975).

Peak 4 in the β-limit dextrin profile (Fig. 4) contained small molecular weight dextrins consisting largely of maltose and maltotriose (Fig. 5). These are the small remnants of the outer chains of amylopectin left after hydrolysis by β-amylase.

All four amylopectin β-limit dextrins appeared to give similar profiles (Fig. 4), and no significant differences were detected in the distribution of peak sizes obtained for each dextrin (Table IV). However, the large change in the ratio of combined peaks 3 and 4 to peak 2 in amylopectin (Table III) to the ratio of the corresponding peaks (peak 3/peak 2) in the β-limit dextrin (Table IV) can be seen clearly. Similar results have been obtained for amylopectin β-limit dextrins from maize, wheat, and manioc starches (Mercier 1973, Hood and Mercier 1978, Robin 1981). Also, average chain length values of 8–10, reported for amylopectin β-limit dextrins from these starches, were similar to values for β-limit dextrins from waxy barley starches (Table IV).

Debranched amylopectin β-limit dextrins were analyzed also on columns of Biogel P-4 (Fig. 5). The higher DP peaks of the β-limit dextrin (Fig. 4, peaks 1 and 2) were not resolved, but individual chains in peaks 3 and 4 have been separated. Peak 3 (Fig. 4) consists of chains of DP 4 to approximately 23, with chains of DP 10–11 predominating. As discussed earlier, peak 4 consists of maltose and maltotriose, and equimolar proportions of these two dextrins were found in all samples. This is consistent with the idea that amylopectin contains equal numbers of odd- and even-numbered chains (Peat et al 1956, Liddle and Manners 1957). Beta-amylolysis of amylopectin would reduce even-numbered chains to maltose and odd-numbered chains to maltotriose. Thus, under the experimental conditions used, no hydrolysis of maltotriose stubs in β-limit dextrins was detected. Glucose stubs were not found in samples of debranched β-limit dextrins analyzed.

The predominance of maltohexaose amongst the DP 4–8 chains agrees with the findings of Robin (1981). If these results were to be expressed on a molar basis, this feature would be even more pronounced. The possible significance of six-unit chains to the structure of cereal amylopectins has been discussed previously (Robin 1981), but a definitive answer requires a more detailed investigation of the interior structure of amylopectin.

Amylopectin β-limit dextrins debranched with pullulanase were hydrolyzed completely to maltose and maltotriose by β-amylase. A

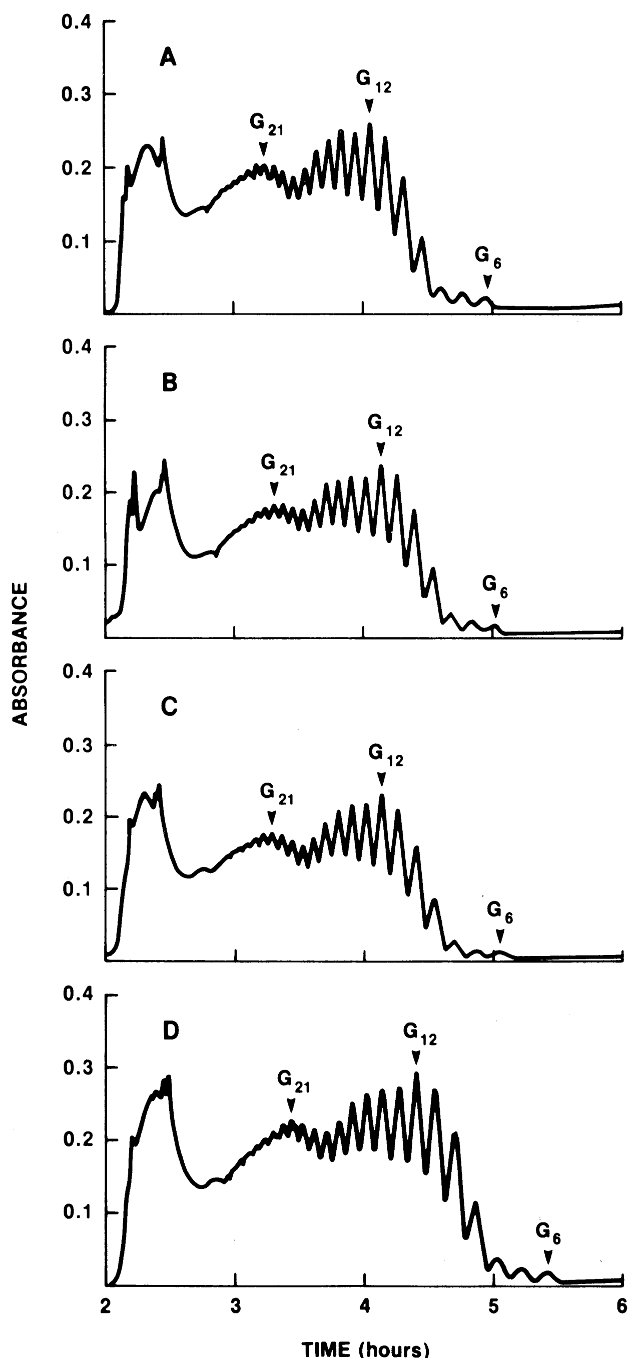

Fig. 2. Fractionation on Biogel P-4 of debranched amylopectins from: **A,** large normal; **B,** small normal; **C,** large waxy; and **D,** small waxy starch granules.

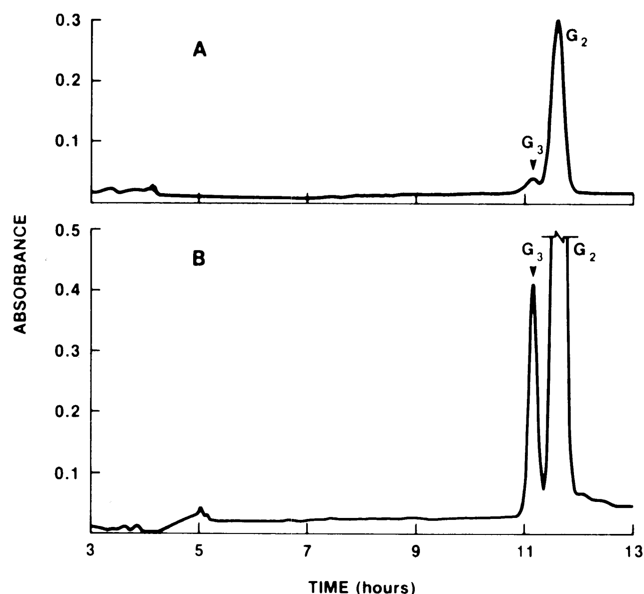

Fig. 3. Fractionation on Biogel P-4 of: **A,** β-amylase treated debranched amylopectin, and **B,** β-amylase treated debranched amylopectin β-limit dextrin from large normal starch granules.

TABLE IV
Relative Sizes of Groups of Unit Chains from Debranched Amylopectin β-Limit Dextrins[a]

Source of β-Limit Dextrin	Peak No. 1	2	3	4	Peak 3/ Peak 2	Average Length of Unit Chains (cl)[b]
Large normal starch	1.4	33.8	46.6	18.2	1.4	7.5
Small normal starch	Trace	36.2	44.8	18.9	1.2	7.5
Large waxy starch	0	35.6	43.6	20.7	1.2	9.5
Small waxy starch	Trace	35.7	42.6	21.7	1.2	9.0

[a] As a percentage of total material eluted from columns of Biogel P-6.
[b] cl = chain length.

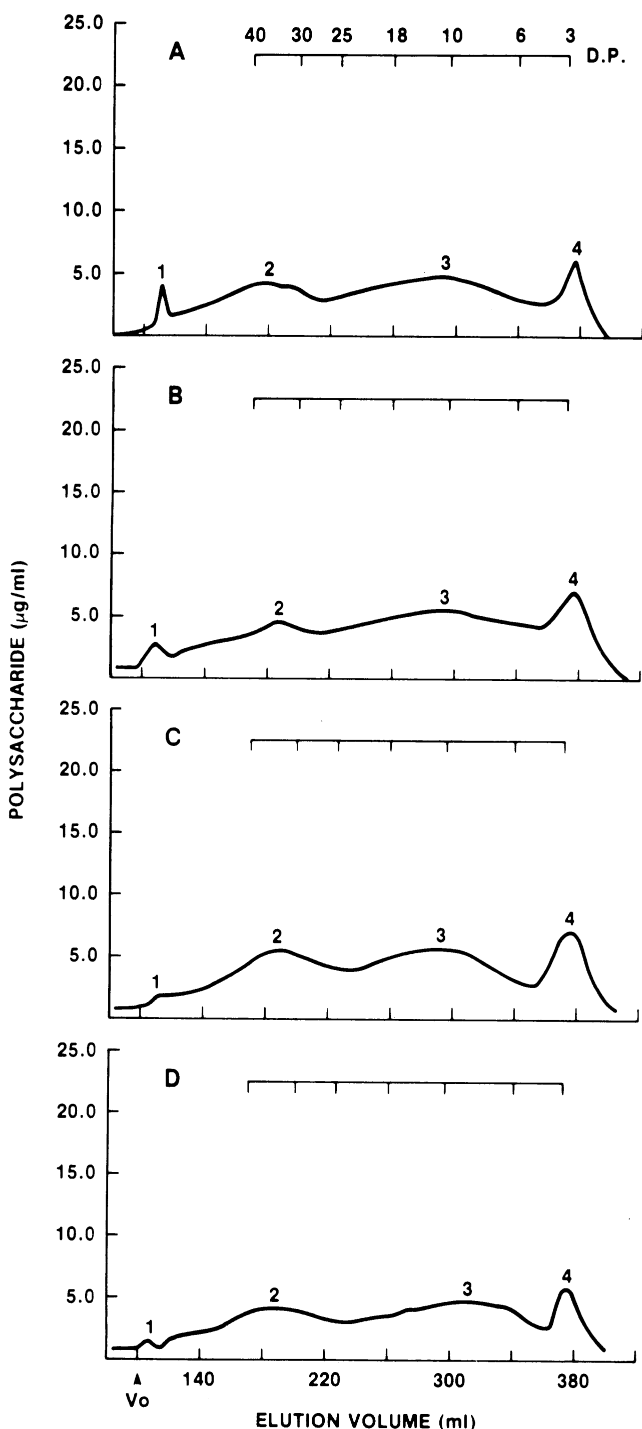

Fig. 4. Fractionation on Biogel P-6 of debranched amylopectin β-limit dextrins from: A, large normal; B, small normal; C, large waxy; and D, small waxy starch granules.

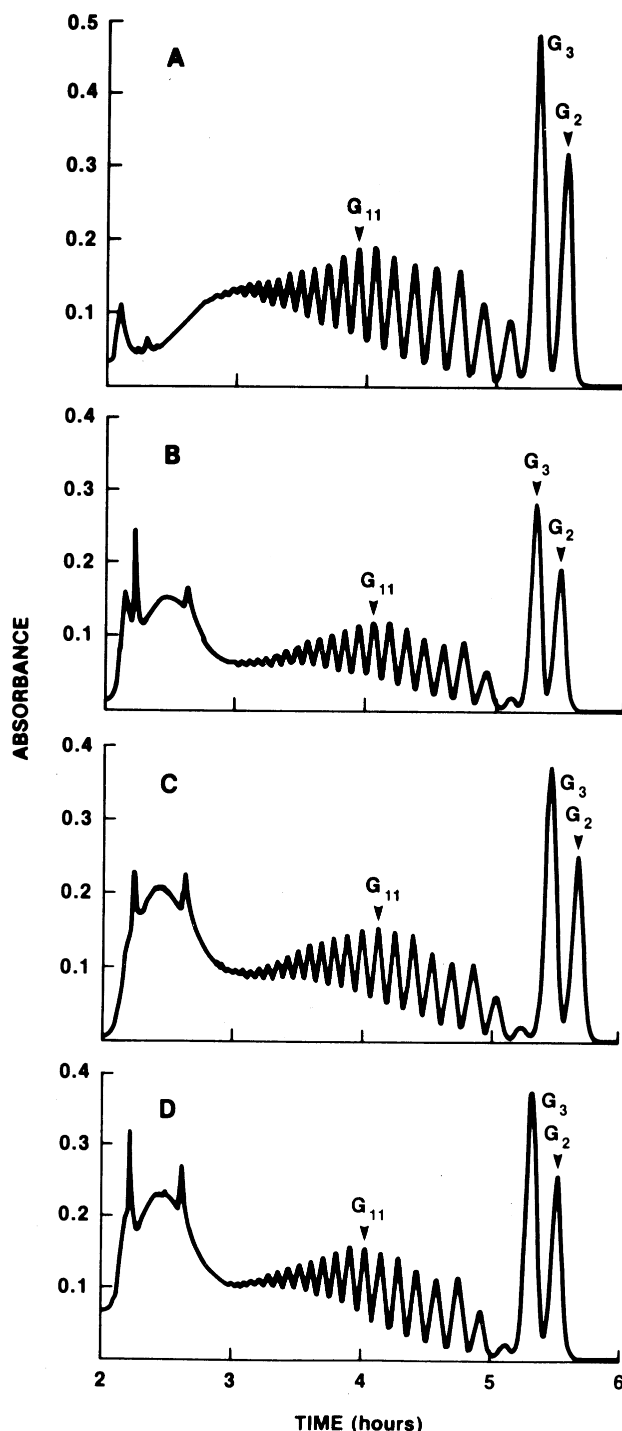

Fig. 5. Fractionation on Biogel P-4 of debranched amylopectin β-limit dextrins from: A, large normal; B, small normal; C, large waxy; and D, small waxy starch granules.

typical profile is shown in Fig. 3B for the dextrin from large normal starch granules. When debranching was performed with isoamylase, however, a series of maltodextrins in addition to maltose and maltotriose was obtained after β-amylolysis. Furthermore, decreased yields of maltose were obtained, supporting the findings of Kainuma et al (1978) that isoamylase is less efficient than pullulanase for hydrolyzing small branched dextrins.

These studies show that the unit-chain profiles of amylopectins from different types of barley starch are similar to each other and to profiles reported by other workers for amylopectins from a number of cereal grains. However, there appear to be three distinct groups of unit chains, not two, as has been widely reported. Two of these groups are hydrolyzed extensively by β-amylase and presumably are composed mainly of A-chains. It is tempting to speculate that these groups may represent the internal and external A-chains discussed by Robin (1981). The high-DP group is less extensively hydrolyzed by β-amylase and so must contain a higher proportion of B-chains. These results are consistent with the cluster type of amylopectin structure first proposed by French (1972) and developed further by Robin et al (1974, 1975) and Manners and Matheson (1981).

The remarkable similarity of the unit chain profiles of the amylopectins and corresponding β-limit dextrins analyzed in this work indicates the precise control excercised over the biosynthesis of these macromolecules in different types and sizes of starch granules.

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