Structural Characterization of Amylopectin and Intermediate Material in Amylomaize Starch Granules^{†,††}

Tadashi BABA* and Yuji ARAI

Laboratory of Metabolic Chemistry,
Institute of Applied Biochemistry,
University of Tsukuba, Ibaraki 305, Japan

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The fine structures of amylopectin and intermediate material characteristic of amylomaize starch were investigated by chemical and enzymatic means. In comparison with waxy-maize amylopectin, that of amylomaize starch was found to possess a $\overline{CL}$ approximately 10 glucose units longer. Unit-chain profiles of waxy and amylomaize amylopectins revealed that the clear difference lay simply in the relative amounts of two unit-chain fractions. By fractionations of debranched β -limit dextrins, it was demonstrated that the $\overline{CL}$ of the internal chains in amylomaize amylopectin was 9 glucose units longer than that in waxy-maize amylopectin. In addition, the proportion of maltose and maltotriose fractions in the debranched dextrin for amylomaize amylopectin was noticeably smaller than found for waxy-maize amylopectin. These data suggest a lesser branching frequency of outer branches in amylomaize amylopectin, confirming the previous proposal that this amylopectin has longer inner and outer branches than those of normal amylopectin.

As for amylomaize intermediate material, the average degree of polymerization was estimated to be 250 to 300 glucose units per molecule. It was also indicated that there were 5 or 6 glucose residues corresponding to the non-reducing end in the molecule. The unit-chain profile of the intermediate material implied that this molecule was mainly composed of branches with $\overline{CL}$ around 50. Moreover, the presence of only small amounts of maltose and maltotriose fractions was demonstrated by the unit-chain distribution of this β -limit dextrin. These findings indicate that amylomaize intermediate material is totally consistent with a branched glucan having a low molecular weight, proposing that this anomalous glucan has such a fine structure that four or five branches with $\overline{CL}$ around 50 are linked to a main linear chain of 100 to 150 glucose units.

As compared with normal maize (*Zea mays* L.), amylomaize endosperm produces a starch which possesses both a high proportion of amylose¹⁾ and a unique granular property.^{2~5)} The fine structure of amylomaize amylopectin (AAP) has been the subject of controversy for over twenty-five years.

In 1955, Wolff and his co-workers⁶⁾ first separated a 50% amylomaize starch into two components by 1-butanol, examined each

component, and demonstrated that AAP had longer inner and outer branches than those of normal amylopectin, postulating that the abnormal amylopectin was intermediate in structure between normal amylose and amylopectin fractions. Greenwood and Thomson⁷⁾ also obtained the long-branch amylopectin from 70% amylomaize starch, but pointed out that the amylopectin fraction could be further split into two components by differential ultracentrifugation.

[†] Dedicated to Professor Tatsuro Itoh on the occasion of his retirement from our laboratory.

^{††} Structural Features of Amylomaize Starch, Part III. For Part I, see ref. 29.

* Present address: Department of Dental Public Health, Nihon University School of Dentistry at Matsudo, Matsudo-shi, Chiba 271, Japan.

Abbreviations: $\overline{CL}$, average chain length; BNF, 1-butanol non-complexable fraction; AAP, amylomaize amylopectin; AIM, amylomaize intermediate material; RLG, rabbit-liver glycogen; WMS, waxy-maize starch; DMSO, dimethyl sulfoxide; GLC, gas-liquid chromatography.

gation. Their later investigations^{8~10)} demonstrated that the two components were equivalent to normal amylopectin and amylose of short chain length. However, the proposal of Greenwood *et al.*^{8~10)} was disputed by Montgomery and her co-workers,¹¹⁾ who separated amylose-free amylopectin from five different preparations of amylomaize starch, and proposed that AAP was less highly branched than normal amylopectin, confirming Wolff's finding⁶⁾ and not supporting those of Greenwood.^{7~10)} Although the fine structure of AAP has not been completely clarified, there are many reports demonstrating the presence of the abnormal amylopectin in amylomaize starch.^{12~15)}

Recently, enzymological studies on starch biosynthesis in amylomaize endosperm have been reported.^{16~23)} Boyer and Preiss¹⁷⁾ observed that amylomaize kernels were deficient in one of three forms of branching enzyme (EC 2.4.1.18) found in normal maize. It was also reported that a near-linear relationship existed between the dosage of the dominant *Amylose-extender* allele and the enzyme activity of the deficient form.²¹⁾ On the basis of these observations, they have concluded that the absence of branching enzyme may result in fewer branch points in AAP. We also found that the total content of branching enzymes in amylomaize endosperm was approximately one-third of that present in normal maize during endosperm development.²³⁾ Thus, the recent investigations on branching enzymes of amylomaize endosperms imply that the enzymes affect amylopectin component in the original starch qualitatively and/or quantitatively.

It is well known that glycogen-storage disease (Type IV) is due to the absence of a branching enzyme, leading to an accumulation of a low-molecular-weight glycogen with an anomalous structure.^{24~28)} Since considerable reduction of the enzyme activity was found in amylomaize endosperms, our attention was directed to the third abnormal molecule. Interestingly, our recent report²⁹⁾ confirmed the presence of a fairly high proportion of a

branched glucan with a lower molecular weight than that of normal amylose, in a fraction of amylomaize starch which could not form a complex with 1-butanol (BNF). It was considered that the unknown molecule was similar to the so-called "intermediate material" proposed by Greenwood *et al.*³⁰⁾ and Whistler.³¹⁾ Also, we have concluded either that a 50% amylomaize starch is composed of approximately 30% normal amylose, 40% amylomaize-characteristic amylopectin (AAP), and 30% intermediate material (AIM), or that the abnormal starch contains only negligible amounts of amylopectin with normal properties and short-chain amylose, if they exist.²⁹⁾ Thus, amylomaize starch can be regarded as being qualitatively heterogeneous in its components compared with normal-maize starch. We therefore think that the postulated structures of AAP have become complicated because many workers have believed that the anomalous starch as well as normal-maize starch is composed only of amylose and amylopectin entities.

In order to elucidate structural features of amylomaize starch it is necessary to isolate and characterize both AAP and AIM. This paper describes the fine-structural differences among rabbit-liver glycogen (RLG), waxy-maize starch (WMS), AAP, and AIM, and provides evidence of the abnormal characteristics of AAP and AIM, which differ in several respects from the usual form.

MATERIALS AND METHODS

Materials. Waxy and amylomaize starches were prepared from 36-day-old endosperm tissues³²⁾ and defatted with 80% MeOH.³³⁾ BNF was obtained from a 2% DMSO-amylomaize starch dispersion by the method of Adkins and Greenwood,¹⁰⁾ as described previously.²⁹⁾ Crystalline *Pseudomonas* isoamylase (EC 3.2.1.68) and *Aerobacter* pullulanase (EC 3.2.1.41) were the products of Hayashibara Biochemical Laboratories Inc. (Okayama, Japan). Sweet-potato β -amylase (EC 3.2.1.2) and RLG were purchased from Sigma. Since the commercial β -amylase was appreciably contaminated with the activities of α -glucosidase (EC 3.2.1.20) and α -amylase (EC 3.2.1.1), the β -amylase was re-purified according to the method of Marshall and Whelan.³⁴⁾ All other reagents were of the

highest purities available.

Analytical procedures. Amperometric iodine affinities of polysaccharides were measured at 10°C as described previously.³²⁾ Absorption spectra of D-glucan-iodine complex were recorded on a Shimadzu UV-240 spectrophotometer over the range of 400 to 700 nm according to the procedure of Krisman³⁵⁾ with minor modifications.²³⁾ Amounts of total carbohydrates and reducing-end groups were measured by the phenol-sulfuric acid method³⁶⁾ and the Somogyi-Nelson procedure,³⁷⁾ respectively, using maltose as the standard.

Descending paper chromatography was conducted on Toyo filter paper No. 50 developed with 1-butanol-pyridine-water (6 : 4 : 3, by volume) at room temperature.³⁸⁾ The chromatogram was then treated with glucoamylase (EC. 3.2.1.3)-acetone aqueous solution to enhance the spot intensity of the highest oligosaccharides,³⁹⁾ and visualized by the silver-nitrate dip method.⁴⁰⁾

Gas-liquid chromatography (GLC) was performed on a Shimadzu Model GC-4B equipped with a hydrogen flame-ionization detector, stainless-steel column (3 mm × 150 cm), and Shimadzu Chromatopac C-E1B computer. Partially methylated sugars were converted into their corresponding alditol acetates and separated on the column packed with Gas Chrom Q (100~120 mesh) coated with 3% ECNSS-M at 170°C. For GLC of Smith-degradation products, the acetylated samples were also separated on the same column at 150°C or at 100 to 190°C programmed at a rate of 10°C per min. The flow rate of the carrier nitrogen gas was 50 ml/min.

Enzymatic hydrolyses of D-glucans. The hydrolysis of D-glucan with β -amylase was conducted at 30°C in a mixture (pH 4.8) of 50 mM acetate buffer, 25 IU of β -amylase, and 2 mg of sample, which had been dispersed with 90% DMSO to give a final concentration of 2% (w/v), in a total volume of 2 ml. When the hydrolysis ceased (12 hr), the extent of β -amylolysis of the glucans was determined by measuring the amounts of reducing power and total carbohydrates in the digest.

The α -1,6-branch linkages of polysaccharides (5 mg/ml) were hydrolyzed at 37°C with isoamylase (295 units⁴¹⁾/ml) in 2 ml of 50 mM sodium acetate-HCl buffer (pH 3.5), or at 30°C with pullulanase (2 IU/ml) in 2 ml of 50 mM acetate buffer (pH 5.5). Both hydrolysates gave no further increase in the copper reducing power after 3 hr of the incubations and these were maintained for 12 hr. The digests were then heated at 100°C for 5 min to inactivate the enzymes. In case of the successive hydrolysis with both enzymes, polysaccharides were treated first with isoamylase for 6 hr. After the digest was heated at 100°C for 5 min and adjusted to pH 5.5 with 1 M NaOH, the hydrolysis was followed by the treatment with pullulanase for 12 hr. $\overline{\text{CL}}$ of the enzymatically debranched glucan was calculated from the ratio of reducing ends and total

carbohydrates.

For experiments of unit-chain distribution, DMSO-dispersed samples (20 or 40 mg) were dialyzed for 6 hr at room temperature against 50 mM acetate buffer (1 liter). The dialysate was diluted to 6 ml with the same buffer and treated with the debranching enzyme as described above. The debranched products were freeze-dried and then stored at -20°C until processing. All dialysis procedures were carried out using a cellulose tube (MW cut-off 3,500, Nakarai Chemical Co.) unless otherwise stated.

Periodate oxidation and Smith-degradation. Periodate oxidation and Smith-degradation of polysaccharides were effected by the procedures reported previously.^{42~44)} DMSO-dispersed polysaccharides (20 mg/ml) were dialyzed overnight against 1 liter of water. To 1 ml of the aqueous glucan solution (5 to 10 mg of polysaccharide), the same volume of 50 mM sodium periodate was added, and the mixture was incubated at 20°C in the dark. The consumption of periodate was measured at suitable intervals by the method of Avigad.⁴⁵⁾ After oxidation for 8 days, 40 μ l of ethylene glycol was added to decompose the residual periodate. The mixture was left for 20 min and then reduced with sodium borohydride (20 mg) in 1 ml of 0.01 M aqueous NaOH.⁴⁴⁾ The excess borohydride was destroyed by adjusting the pH to 5.5 with 1 M acetic acid. The mixture was dialyzed overnight against water (1 liter) and freeze-dried. The D-glucan polyalcohol was hydrolyzed with 0.5 M sulfuric acid (1 ml) at 100°C for 10 hr. The hydrolysate was neutralized with barium carbonate, filtered, and passed through a column of Dowex 50 × 8 (H⁺ form, 1 ml), and the resin was washed with water (2 ml). The effluent and washings were combined, brought to dryness, and acetylated by heating with 2 ml of pyridine-acetic anhydride mixture (1 : 1) at 100°C for 30 min, as described by Lindberg.⁴⁶⁾ The resulting product was dissolved in chloroform and analyzed by GLC.

Methylation analyses. Polysaccharides were methylated four times by the method of Hakomori⁴⁷⁾ as described by Lindberg.⁴⁶⁾ D-Glucans (20 mg) were dissolved under a nitrogen atmosphere in dry DMSO (5 ml) and treated for 6 hr at room temperature with 2 M methylsulfinyl carbanion. The resulting polysaccharide alkoxide was then methylated by the dropwise addition of methyl iodide (2 ml) in an ice-water bath. The reaction mixture was stirred overnight at room temperature, dialyzed against tap water, and freeze-dried. No infrared band at 3,400 to 3,600 cm⁻¹ as hydroxyl group was found in the final products. The methylated polysaccharide (10 mg) was dissolved in 4 ml of 90% formic acid and kept at 100°C for 2 hr. The solution was brought to dryness *in vacuo* at 40°C, dissolved in 0.25 M sulfuric acid (2 ml), and heated again at 100°C for 12 hr. After cooling to room temperature, the solution was neutralized with barium carbonate, filtered, and applied onto a column of Dowex

50 × 8 (H⁺ form, 1 ml). The resin was washed with water (2 ml), and the effluent and washings were combined and concentrated to dryness. The partially methylated sugars were converted into their corresponding alditol acetates according to the procedure of Lindberg,⁴⁶⁾ and these were analyzed by GLC.

Preparations of β -limit dextrin. β -Limit dextrans of polysaccharides were prepared by the method described previously.⁴⁸⁾ The DMSO-dispersed polysaccharide (400 mg/20 ml) was dialyzed for 6 hr against 2 liters of 0.1 M acetate buffer (pH 4.8) containing 0.02% sodium azide and a trace amount of precipitate formed was removed by centrifugation. The dialysate was made up to 60 ml with the same buffer and treated at 30°C with sweet-potato β -amylase (1,500 IU). The β -amylolysis was nearly complete after incubation for 12 hr, as measured by the reducing power, and then the enzyme was inactivated by heating the digest at 100°C for 15 min. After dialyzing overnight against the same buffer (4 liters) to eliminate maltose, the resulting dextrin was submitted to a second β -amylolysis. In the final incubation (third amylolysis), the dialysis was carried out against water, and the product was then freeze-dried.

Gel-permeation chromatography. Procedures of gel-permeation chromatography were essentially similar to those previously described.²⁹⁾ Debranched products or untreated polysaccharides were suspended with water (0.5 ml) and dissolved by addition of 1 M NaOH (0.5 ml), and the resulting solution was made up to 5 ml with water. A portion (3 ml) of the glucan solution was then applied onto a column (1.6 × 76 cm) of Sepharose CL-4B, and Sephadex G-150 and G-50 (Pharmacia) previously equilibrated with 0.05 M NaOH containing 0.02% sodium azide, and eluted with the same solution at room temperature. Fractions (1.9 ml) were collected at a flow rate of 10 ml/hr and neutralized with 1 M HCl. The recovery of carbohydrates applied on the column was more than 95% under these conditions. The complete debranching was confirmed by measuring quantitative conversion into maltose when a portion of the debranched product was subjected to β -amylolysis.

RESULTS

Isolations of AAP and AIM

BNF (150 mg) obtained from 50% amylo-maize starch was suspended in water (4 ml) and heated in a boiling water bath for a few minutes. After cooling to room temperature, the suspension was dissolved by an addition of 5 M NaOH (1 ml), diluted to 10 ml with water, and then filtered through a Sepharose CL-2B column (3.2 × 87 cm). As previously report-

FIG. 1. Elution Profile of BNF from Amylo-maize Starch on Sepharose CL-2B.

The glucan solution (150 mg) was applied onto a Sepharose CL-2B column (3.2 × 87 cm) previously equilibrated with 0.05 M NaOH containing 0.02% sodium azide, and eluted with the same solution at room temperature. Fractions (8 ml) were collected at a flow rate of 30 ml/hr and neutralized with 5 M HCl. The carbohydrate content (●—●) and $\lambda_{\max}$ of the glucan-iodine complex (lateral bar) in each fraction were measured as described in the MATERIALS AND METHODS.

ed,²⁹⁾ the elution profile of carbohydrate content revealed that the BNF was composed of high- and low-molecular-weight molecules (Fig. 1), being regarded as AAP and AIM, respectively. The corresponding fractions were pooled and precipitated with five volumes of MeOH, and then allowed to stand overnight at room temperature. The precipitates were collected by centrifugation at 10,000 × *g* for 10 min, washed with acetone and ether, and dried *in vacuo*. The same isolation procedure was repeated seven times, and from 1.05 g of the original BNF finally yielded 305 mg AAP and 422 mg AIM.

General properties of AAP and AIM

As shown in Table I, the properties of AAP and AIM were compared with those of RLG and WMS. When solubilities of polysaccharides in 90% DMSO were examined at a concentration of 2% (w/v) at room temperature, WMS and AAP were dissolved with much difficulty, whereas AIM and RLG were readily soluble in the same solvent up to the concentration of 5%. Although WMS showed only a negligible amount of iodine binding capacity, a noticeable amount of iodine

TABLE I. CHARACTERIZATIONS OF AAP AND AIM

	RLG	WMS	AAP	AIM
Solubility in 90% DMSO ^a	++	+	+	++
I.B.C. ^b (mg I ₂ /100 mg of sample)	n.d. ^c	0.1	3.4	5.1
Iodine complex, $\lambda_{\max}$ (nm)				
Native	452	536	573	595
After debranching ^d	510	553	591	603
β -Amylolysis limits (%)				
Native	35.2	58.0	59.6	57.2
After pullulanase treatment	64.3	99.3	97.4	93.8
After isoamylase treatment	97.3	101.1	100.1	98.7
After both enzyme treatments ^d	104.3	105.1	104.9	100.4
Average chain length ($\overline{CL}$) ^d	13.3	20.3	29.4	40.6
External chain length (ECL) ^e	7.2	14.3	20.0	25.7
Internal chain length (ICL) ^f	5.1	5.0	8.4	13.9

^a +, dissolved with much difficulty; ++, dissolved readily.

^b Iodine binding capacity measured at 10°C.

^c Not determined.

^d Obtained by the successive debranching treatment with isoamylase plus pullulanase.

^e $ECL = (\% \text{ of } \beta\text{-amylolysis limit}/100) \times \overline{CL} + 2.5$.

^f $ICL = \overline{CL} - ECL - 1.0$.

affinity was observed on AAP and AIM. It was important to note that AIM had more iodine affinity than found in normal-maize starch (its iodine binding capacity is 4.3, corresponding to 23.5% amylose content). Furthermore, both curves of the amperometric iodine titration for AAP and AIM were inflexionless (not shown), as pointed out by Banks and Greenwood.³⁰⁾ As expected for the values of iodine binding capacity, AIM had a higher wavelength of $\lambda_{\max}$ of the glucan-iodine complex than found in WMS and AAP. However, the increment of $\lambda_{\max}$ due to the debranching treatment was observed in all samples.

No remarkable differences of degree of β -amylolysis could be found among native polysaccharides of WMS, AAP, and AIM. The AIM has only around 60% of β -amylolysis limit. This result evidently indicates that AIM is not a linear molecule but a branched one. The extents of β -amylolysis after debranching treatment either with isoamylase or with isoamylase plus pullulanase were more than 97% in all samples, demonstrating the nearly complete debranchings of the glucans. The $\overline{CL}$ s per branch for RLG and WMS agreed well with

those reported by many workers,^{49~51)} while those for AAP and AIM were 9 and 20 glucose units longer than found in WMS, respectively. External and internal chain lengths per one branch were theoretically calculated from the values of $\overline{CL}$ after debranching and β -amylolysis limit.⁵¹⁾ It was clearly expected that AAP would be consistent with an amylopectin with anomalous structure having longer external and internal chains than those of normal amylopectin (WMS). The lengths of these chains were estimated to be longer for AIM than calculated for AAP. However, there is considerable difficulty involved in interpreting this data, because it is not evident whether or not this AIM with a low molecular weight is a highly branched molecule as well as amylopectin.

In addition, an estimation of the molecular weight of AIM was performed on a column of Sepharose CL-4B which had been calibrated with standard dextrans (Sigma) having molecular weights of 500,000, 70,000, and 40,000 (Fig. 2). The molecular weight of the AIM was found to be 40,000~50,000, being equivalent to an approximate degree of polymerization of 250 to 300 glucose units per molecule.

FIG. 2. Estimation of Molecular Weight of AIM by Gel Filtration on Sepharose CL-4B.

Calibration was performed using standard dextrans with molecular weights of 40,000, 70,000, and 500,000. The value obtained for AIM is indicated by the open circle.

Smith-degradation of AAP and AIM

AAP and AIM were oxidized with sodium periodate and the course of periodate consumption was measured (not shown). The periodate consumption remained relatively constant in all samples after the treatment for two days. The oxidized polysaccharides were further treated by the Smith-degradation procedure^{43,44} involving borohydride reduction and complete hydrolysis with acid. In the treatment of glycogen and amylopectin with this procedure, erythritol would result from all glucose units except those at the non-reducing ends of chains, where glycerol would be found. It is also thought that the ratio of non-reducing ends to reducing ends is, in general, very low in these highly branched glucans with high molecular weight. Consequently, the ratio of erythritol to glycerol is a direct measurement of the number of glucose units per branch. As summarized in Table II, the molar ratio of erythritol to glycerol for three independent runs was 11.3, 20.7, 36.0, and 48.2 for RLG, WMS, AAP, and AIM, respectively.

Methylation analyses of AAP and AIM

Methylated sugar components of RLG, WMS, AAP, and AIM were analyzed by GLC as their alditol acetates. The methylated sugars from all polysaccharides gave peaks corresponding to 2,3,4,6-tetra- (peak I), 2,3,6-tri- (peak II), and 2,3-di-*O*-methyl-D-glucose (peak

TABLE II. PERIODATE OXIDATION AND SMITH-DEGRADATION OF AAP AND AIM

The consumption of periodate was measured after the oxidation of polysaccharide for eight days, and the Smith-degradation products were analyzed by GLC as their alditol acetates.

Polysaccharides	Periodate oxidation	Smith-degradation
	Periodate consumed (mol/glucose residue)	Molar ratio (glycerol : erythritol)
RLG	1.00	1.0 : 11.3
WMS	0.98	1.0 : 20.7
AAP	0.98	1.0 : 36.0
AIM	1.18	1.0 : 48.2

III) on the GLC profiles, each compound being derived from residues of non-reducing-end glucose, α -1,4-linked glucose, and both α -1,4- and α -1,6-linked glucose in the original polysaccharides, respectively. As listed in Table III, molar proportions of the peaks I, II, and III were: RLG, 1.0 : 8.4 : 0.9; WMS, 1.0 : 17.5 : 1.1; AAP, 1.0 : 26.8 : 1.0; AIM, 1.0 : 44.0 : 1.0. The result indicates that $\overline{CL}$ per branch is estimated to be 10.4, 19.5, 28.8, and 46.0 for RLG, WMS, AAP, and AIM, respectively. The value for AIM was obtained from the sample which was only methylated once. When this molecule was methylated four times repeatedly as well as the other samples, the molar proportion was found to be 1.0 : 31.6 : 0.6. This data is converted into 1.7 : 52.7 : 1.0 on the basis of 2,3-di-*O*-methyl-D-glucose, implying that the $\overline{CL}$ of AIM is also estimated to be nearly 50.

Fractionations of AAP and AIM with or without debranching treatment on Sepharose CL-4B

Figure 3 illustrates the elution patterns of WMS, AAP, and AIM with or without isoamylase treatment on a Sepharose CL-4B column. A single peak of WMS and AAP in the high-molecular-weight region disappeared due to the debranching of these polysaccharides,

TABLE III. METHYLATION ANALYSES OF AAP AND AIM

Alditol acetates from	R _T ^a	Molar ratio ^b			
		RLG	WMS	AAP	AIM ^c
2,3,4,6-Tetra- <i>O</i> -methyl-D-glucose	1.00	1.0	1.0	1.0	1.0
2,3,6-Tri- <i>O</i> -methyl-D-glucose	2.43	8.4	17.5	26.8	44.0
2,3-Di- <i>O</i> -methyl-D-glucose	5.25	0.9	1.1	1.0	1.0

^a Retention times of the corresponding alditol acetates, relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

^b Calculated from the molar-response factors for partially alditol acetates (see reference⁵²).

^c Obtained from once-methylated sample. When AIM was methylated four times repeatedly like the other samples, the molar proportion was estimated to be 1.0:31.6:0.6 (1.7:52.7:1.0 on the basis of the 2,3-di-*O*-methyl-D-glucose).

FIG. 3. Elution Profile of WMS (A), AAP (B), and AIM (C) on Sepharose CL-4B.

The glucan solution (20 mg) with (●—●) or without (○—○) isoamylase debranching treatment was filtered through a column (1.6 × 76 cm) of Sepharose CL-4B at a flow rate of 10 ml/hr. The lateral bar indicates $\lambda_{\max}$ of the glucan-iodine complex in each fraction (1.9 ml) after the debranching treatment.

and a new peak appeared in a much lower molecular weight position. Likewise, one new single peak of debranched AIM was observed in the low-molecular-weight region, whereas

no such peak was found when the debranching treatment was not carried out. In some experiments, a typical amylose of normal-maize starch was filtered through the same column. There was found a peak of the amylose with $\lambda_{\max}$ of 653 nm near the elution volume of 95 ml, demonstrating that AAP and AIM isolated were free from an amylose component in the original amylomaize starch.

Unit-chain distributions of AAP and AIM on Sephadex G-150

AAP and AIM after successive debranching treatment with isoamylase plus pullulanase were distributed on a column of Sephadex G-150, and the unit-chain profiles are shown in Fig. 4. The debranched RLG indicated a unit-chain profile consisting only of a single peak with $\overline{\text{CL}}$ of 17, whereas the profiles of WMS and AAP were composed of two unit-chain groups of a longer $\overline{\text{CL}}$ fraction (Fr. I) and a shorter fraction (Fr. II). The peaks of the Frs. I and II in AAP shifted to a position of higher molecular weight than those in WMS. The unit-chain profile, which was mainly constituted of a peak with $\overline{\text{CL}}$ around 50, was observed at AIM, although there was a small peak having $\lambda_{\max}$ of 640 nm near the void volume. The percentages of Frs. I and II of the debranched WMS and AAP are given in Table IV. In contrast with WMS, AAP had both a noticeably smaller unit-chain ratio of Frs. I and II, and longer $\overline{\text{CL}}$ s of these fractions.

TABLE IV. UNIT-CHAIN DISTRIBUTIONS OF WMS AND AAP ON SEPHADEX G-150

Amylopectins	Fr. I (%)	Fr. II (%)	Peak of Fr. I (CL)	Peak of Fr. II (CL)	Ratio of unit-chains ^a (Fr. I:Fr. II)
WMS	27.0	73.0	38	16	1.0:6.4
AAP	53.9	46.1	55	20	1.0:2.4

^a Calculated as follows: unit-chain ratio of Fr. II =

$$\frac{(\text{carbohydrate percentage of Fr. II}) \div (\overline{\text{CL}} \text{ of peak of Fr. II})}{(\text{carbohydrate percentage of Fr. I}) \div (\overline{\text{CL}} \text{ of peak of Fr. I})}$$

FIG. 4. Unit-chain Profiles of RLG (A), WMS (B), AAP (C), and AIM (D) on Sephadex G-150.

Successively debranched polysaccharides (40 mg) with isoamylase plus pullulanase were filtered through a column (1.6 × 76 cm) of Sephadex G-150. The amounts of carbohydrates and reducing-end groups in each fraction (1.9 ml) were determined as described in the MATERIALS AND METHODS, and CL was calculated from the ratio of total carbohydrates and reducing ends. The lateral bar indicates $\lambda_{\max}$ of the glucan-iodine complex in each fraction.

Characterizations of β -limit dextrins from AAP and AIM

Properties of β -limit dextrins from AAP and

AIM were compared with those from RLG and WMS, as shown in Table V. Measurement of the ratio of total carbohydrates and reducing-end groups gave an average degree of polymerization of 95 ± 29 (five determinations) for AIM β -limit dextrin. The dextrins of WMS, AAP, and AIM were found to have CLs of 10.0, 17.2, and 23.9, respectively, after the pullulanase treatment. The extent of β -amylolysis of RLG β -limit dextrin with the pullulanase treatment was only 66.1%, indicating the limited action of pullulanase on the dextrin.⁵³⁾ However, the successive treatment of this β -limit dextrin with isoamylase plus pullulanase gave the CL of 7.9 and the degree of β -amylolysis of 109.8%. The extent of β -amylolysis of debranched dextrin from AIM was somewhat lower than those from the other samples. This may be due to a presence of fairly long linear chains in the debranched dextrin rather than the incompleteness of the debranching treatment. In fact, a slight retrogradation occurred during the hydrolysis of the debranched dextrin with β -amylase.

The debranched β -limit dextrins were then fractionated on a Sephadex G-50 column, as shown in Fig. 5. Similar profiles were observed at the chromatographic separations on Sephadex G-75 (not shown). Generally, β -amylase hydrolyzes polysaccharides exowise to form maltose from the non-reducing ends, and the reaction stops near the branch points, reducing the external chains into maltosyl and maltotriosyl stubs. Consequently, maltose and maltotriose would be liberated by debranching the resulting dextrin. The unit-chain profile of

TABLE V. CHARACTERIZATIONS OF β -LIMIT DEXTRINS FROM AAP AND AIM

β -Limit dextrins from	Average degree of polymerization ^a	$\lambda_{\max}$ of iodine stain before debranching (nm)	$\overline{CL}$ ^b	β -Amylolysis limits after debranching ^b (%)
RLG	n.d. ^c	452	7.9	109.8
WMS	n.d.	534	10.0	106.3
AAP	n.d.	566	17.2	104.1
AIM	95 $\pm$ 29	583	23.9	98.0

^a Determined by the Somogyi-Nelson method.³⁷⁾

^b Examined by the debranching treatment with pullulanase except that β -limit dextrin from RLG was successively debranched with isoamylase plus pullulanase.

^c Not determined.

FIG. 5. Unit-chain Profiles of β -Limit Dextrins from RLG (A), WMS (B), AAP (C), and AIM (D) on Sephadex G-50.

The debranched β -limit dextrins (20 mg) with pullulanase (isoamylase plus pullulanase for RLG) were filtered through a Sephadex G-50 column (1.6 $\times$ 76 cm). Details are the same as in Fig. 4.

AIM β -limit dextrin revealed that a peak including maltose and maltotriose was much lower than found in the other dextrins. The $\overline{CL}$ of the internal chains in the original poly-

saccharide was estimated to be 14 and 36 for RLG and WMS, respectively. These peaks for AAP and AIM shifted to a higher molecular weight region than that for WMS. This result suggests that AAP has its own structure with longer internal chains than those of the similar structure of normal amylopectin.

As shown in Table VI, recovery of carbohydrates applied onto the Sephadex G-50 column was more than 97% in all samples, indicating that any debranched materials were scarcely stocked in the column during the unit-chain distributions. As compared with WMS, the carbohydrate percentage of the maltose and maltotriose fractions in AAP was noticeably lower (approximately two-third of that in WMS). The much smaller amount of these fractions was observed at AIM. In some experiments, the debranched products were quantitatively examined by descending paper chromatography. The proportion of maltose and maltotriose in the debranched dextrin agreed very closely with those indicated in Table VI.

In order to obtain a clear unit-chain profile, the debranched β -limit dextrins of AAP and AIM were subjected to gel-permeation chromatography on Sephadex G-150, as shown in Fig. 6. The $\overline{CL}$ of the internal chain in the original molecule was found to be 45 and 80 for AAP and AIM, respectively (Table VI). The chain length of AIM was substantially longer than those of RLG, WMS, and AAP.

TABLE VI. FRACTIONATIONS OF DEBRANCHED β -LIMIT DEXTRINS FROM AAP AND AIM ON SEPHADEX G-50^a

β -Limit dextrins from	Recovery of charged sample (%)	Maltose and maltotriose fractions (%)	Internal chain fraction (%)	Peak of internal chain fraction ($\overline{\text{CL}}$)
RLG	97.1	17.6	82.4	14
WMS	98.0	15.5	84.5	36
AAP	98.0	10.3	89.7	45 ^b
AIM	98.4	6.4	93.6	80 ^b

^a Examined by the debranching treatment with pullulanase except that β -limit dextrin from RLG was successively debranched with isoamylase plus pullulanase.

^b Obtained by fractionations on Sephadex G-150 (see Fig. 6).

FIG. 6. Unit-chain Profiles of β -Limit Dextrins from AIM (A) and AAP (B) on Sephadex G-150.

The debranched β -limit dextrins were filtered through a Sephadex G-150 column (1.6 $\times$ 76 cm). Details are the same as in Fig. 4.

DISCUSSION

The structural investigation for AAP clearly indicates that this molecule differs in several respects, such as high wavelength of λ_{max} of the iodine stain, longer degree of $\overline{\text{CL}}$ per branch, and unit-chain profile of the debranched products, from other high-molecular-weight polymers (Tables I, II, and III, and Fig. 4). As summarized in Table VII, the $\overline{\text{CL}}$ of AAP, whose value was determined by three different methods, was approximately 10 glucose units longer than that found in WMS. The lengths

of external and internal chains per one branch for AAP were also estimated to be about 6 and 3 glucose units longer than those of WMS, respectively. The unit-chain profiles of WMS and AAP indicated that the clear difference between these amylopectins lay simply in the relative amounts of the two unit-chain fractions; where for the normal amylopectin Fr. II was in excess, for AAP Fr. I was in excess (Fig. 4 and Table IV). In addition, it was demonstrated that the $\overline{\text{CL}}$ of Frs. I and II in AAP was longer than found in WMS. Therefore, the present data seem to support the proposal by Wolff *et al.*⁶⁾ that AAP has longer inner and outer branches than those of normal amylopectin, and lead to the hypothesis that the outer branches (A-chains in the Whelan's amylopectin model⁵⁴⁾) of AAP molecule are fewer in number than found in the normal form.

The above possibilities are firmly confirmed by the direct examination of unit-chain distribution of debranched β -limit dextrins (Figs. 5 and 6, and Table VI). The unit-chain profiles of the dextrins showed that $\overline{\text{CL}}$ of the internal chains in AAP molecule was 9 glucose units longer than that in WMS. Moreover, the proportion of maltose and maltotriose fractions in the debranched dextrin was noticeably smaller in AAP as compared with WMS, demonstrating the lesser branching frequency of outer branches in the AAP. Similar experimental results have been already reported by Mercier.¹²⁾ She fractionated the debranch-

TABLE VII. CHEMICAL AND ENZYMATIC DETERMINATIONS OF $\overline{CL}$, AND THE THEORETICAL ESTIMATES OF EXTERNAL AND INTERNAL CHAIN LENGTHS PER ONE BRANCH

Treatments	WMS			AAP		
	$\overline{CL}$	ECL ^a	ICL ^b	$\overline{CL}$	ECL ^a	ICL ^b
Enzymatic debranching	20.3	14.3	5.0	29.4	20.0	8.4
Smith-degradation	20.7	14.5	5.2	36.0	24.0	11.0
Methylation	19.5	13.8	4.7	28.8	19.7	8.1

^a External chain length = (% of β -amylolysis limit/100) $\times \overline{CL}$ + 2.5.

^b Internal chain length = $\overline{CL}$ - ECL - 1.0.

ed β -limit dextrans of normal-, waxy-, and amylomaize starches using gel-permeation chromatography, and showed that the dextrin from amylomaize starch had a much greater proportion of long unit-chain fraction than those found in both normal- and waxy-maize starches. This finding can be interpreted by proposing that amylomaize starch has a branched material with an internal chain length greater than that associated with normal amylopectin, although it should be noted that, in her work,¹²⁾ an excessive retrogradation of chains occurred during the chromatographic separation of debranched dextrin from amylomaize starch, as pointed out by Banks and Greenwood.³⁰⁾ Combining the results of the present study, it may be concluded that the fine structure of AAP is totally identical with that of amylowaxy amylopectin, namely, a loosely branched structure with longer internal and external chains than those of normal amylopectin.⁵⁵⁾

Another structural investigation of AIM demonstrates that this molecule is in accord well with a branched glucan having a low molecular weight. The AIM is also found to have entirely anomalous characteristics in the fine structure by contrast with the other high-molecular-weight glucans. The structural information obtained for this molecule is as follows:

(1) The extent of the β -amylolysis limit of AIM is only 57%, lower than would be expected for its high wavelength of $\lambda_{\max}$ of the iodine stain (Table I). This result clearly demonstrates that AIM is a branched glucan.

(2) The molecular weight of AIM is estimated to be 40,000 ~ 50,000 (Fig. 2), indicating that this AIM is constructed with approximately 250 to 300 glucose units per molecule.

(3) The treatments of AIM with chemical and enzymatic means give the $\overline{CL}$ of 40 to 50 per branch for the molecule (Tables I, II, and III).

(4) The analyses of Smith-degradation and methylation on AIM especially reveal that the molar ratio of α -1,4- or α -1,6-linked glucose residue to non-reducing-end glucose residue is 1:40 to 50 (Tables II and III). Since this AIM consists of 250 to 300 glucose units, the result demonstrates that there are 5 or 6 glucose residues corresponding to the non-reducing end per molecule.

(5) The unit-chain profile of AIM implies that this material is mainly composed of branches with $\overline{CL}$ around 50 (Figs. 3 and 4).

(6) The average degree of polymerization of β -limit dextrin from AIM is estimated to be around 100 glucose units (Table V). The unit-chain profile of this dextrin is characterized by the presence of only a single peak of the internal chain fraction with $\overline{CL}$ close to 80 (Figs. 5 and 6), and by a much smaller amount of maltose and maltotriose fractions than found in WMS and AAP (Table VI). These data indicate that the β -limit dextrin of AIM is constituted of an essentially linear chain of approximately 80 glucose units, and suggest the absence of a branch carrying the other branches and/or a possibility of further branching near the branch point. Thus, these results propose that AIM itself

has such a fine structure that four or five branches having $\overline{CL}$ around 50 are linked to a main linear chain of 100 to 150 glucose units.

Furthermore, AIM was readily soluble in DMSO up to the concentration of 5%, and possessed a noticeable amount of iodine binding capacity with an inflexionless curve (Table I), as would be expected for the fine structure. Therefore, the existence of the unusually high proportion of AIM in amylomaize starch granules²⁹⁾ may explain the well-known changes of the physical properties for the abnormal starch, such as high iodine affinity, strong retrogradation in water, and easy solubility in DMSO.

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