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Note

Examination of molar-based distribution of A, B and C chains of amylopectin by fluorescent labeling with 2-aminopyridine

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Abstract

A method for determination of a molar-based distribution of A, B and C chains of amylopectin was developed. Labeling with fluorescent 2-aminopyridine was proportional to the number-average degree of polymerization (dp_n) of the chains in the range of 6–440. Number-average chain lengths (cl_n) of amylopectins from six different plant sources (rice, maize, wheat, potato, sweet potato and yam) determined by the labeling method were in good agreement with values obtained by determination of non-reducing residues. The molar-based distributions were polymodal (A, B_1 and $B_2 + B_3$ fractions) and characteristic to botanical sources. Amylopectins from starches with A-crystalline type had higher amount of $A + B_1$ chains (90–93% by mole) than starches with B-type (68–87%). Molar ratios of $(A + B_1)/(B_2 + B_3)$ were 8.9–12.9 for the A-type starches and 2.1–6.5 for the B-type starches, suggesting that amylopectins of A-type starches had 1.5–2 times more branches per cluster than B-type. The distributions of C chains, except for amylomaize, showed a broad, asymmetrical profile from dp $\sim$10 to $\sim$130 with a peak at dp $\sim$40 and were very similar among botanical sources, suggesting that the biosynthetic process for C chains is similar in different plant species.

Keywords: Starch; Amylopectin; Structure; Fluorescent labeling; 2-Aminopyridine; C chain

Starch is composed of essentially two kinds of α-glucans that have distinctive structure. Amylose is small, linear and slightly branched molecules whereas amylopectin is a large, highly branched molecule and their structures have been shown to be characteristic to botanical sources.[1] The chain-length distribution of amylopectin has been of particular interest because of its relation to physicochemical properties of starch, such as crystalline structure.[2] It is also informative for studies on starch biosynthesis since activities of respective enzymes involved in the biosynthetic pathway, such as starch synthases, branching enzymes and debranching enzymes, have potential to alter the chain-length distribution. High-performance size-exclusion chromatography (HPSEC) with a differential refractometer and high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD) have been frequently used for the examination of the distribution. These methods measure essentially a weight-based distribution, and molar-based information can be obtained only indirectly in combination with other means such as calibration of the columns. Recently labeling techniques with a fluorophore have been introduced to the structural analysis of the starch components.[3,4] The pre-column labeling enables on-line, direct measurement of a molar-based distribution of the molecules.

In this study a quantitative method for examination of a molar-based distribution of unit chains of amylopectin was developed and the structure of amylopectins from various botanical sources was re-evaluated on a molar-basis. Also, C chains, the only unit chain having a free reducing terminal in a molecule, were examined in terms of their size distributions, using the same labeling technique.

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Labeling of malto-saccharide chains with dp found in native amylopectin was compared using maltohexaose (G_6) and synthetic amyloses. The amyloses used and their dp_n values were: AS–10, 52; AS–30, 141; AS–70, 440. The dp_n was obtained by the determination of reducing residues by the modified Park–Johnson method.[5,6] These specimens (4 mg each) were labeled with 2-aminopyridine (2-AP) and analyzed by HPSEC separately. The ratio of peak areas, refractive index (RI) response/fluorescence response (RI/F_{area}), showed linear relationship with dp_n in the range 6–440 (Fig. 1), indicating that the amount of the fluorescent label incorporated to the saccharides was proportional to the number of reducing residues and independent on dp_n of the malto-saccharide chains in the range examined. Thus the labeling method is satisfactory for the analysis of chain-length distribution of amylopectin. A similar conclusion was drawn from the experiments with different fluorophore/labeling condition.[3]

Debranched amylopectins were analyzed as above

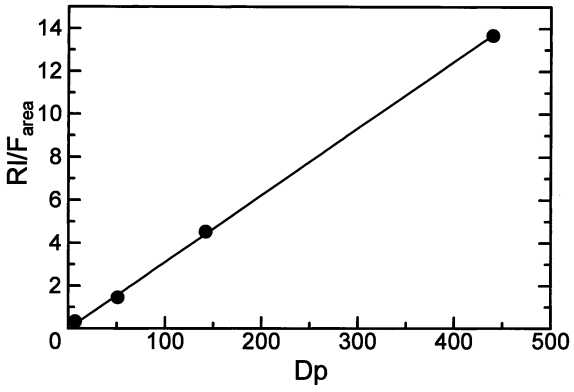

Fig. 1. Relationship between dp_n and the RI/F ratio of peak areas.

Table 1
Number-average chain lengths (cl_n) of amylopectins

Source[a]	Cl_n	
	Labeling	Smith degradation
Wheat (No)	19	20
Wheat (R)	20	20
Rice (A)	18	19
Rice (Ni)	18	19
Maize	21	22
Amylomaize	33	32
Sweet potato (J)	23	22
Sweet potato (K)	20	22
Potato (E)	22	23
Potato (B)	22	24
Yam	25	24

[a] The letters in parenthesis denote cultivar's names.

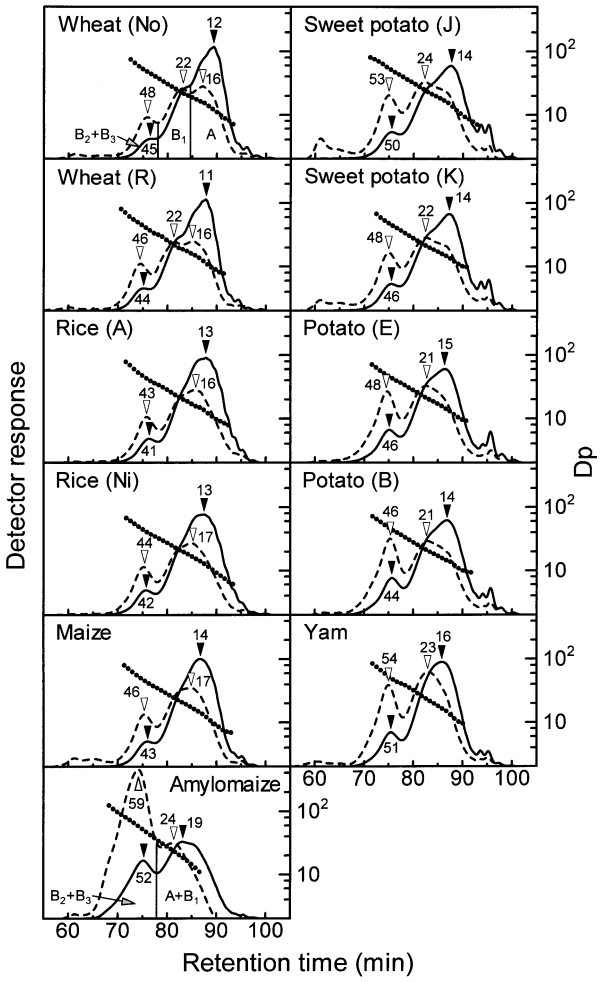

Fig. 2. Molar- and weight-based distributions of unit chains of amylopectins from various botanical sources: —, fluorescence response;-- -, RI response; ●, dp. Numbers with arrowheads indicate dp. Dp at given elution position was calculated using the ratio of the two detector responses measured as height, RI/F_{height}, at the position.

and the number-average chain length (cl_n) was estimated. The cl_n was calculated from an RI/F_{area}, using that of amylose with known dp_n (AS-10, dp_n 52) as a standard. The cl_n values were in good agreement with the values determined by the Smith degradation method[7] for all the amylopectins (Table 1), showing that the labeling method is reliable to estimate cl_n of amylopectin.

Molar- and weight-based distributions of chain length of amylopectins are shown in Fig. 2. Peat et al.[8,9] classified the unit chains into three types, A, B and C chains, according to their involvement in α-1,6-branch linkages. The A chain carries no chains and links to another chain at the reducing terminal via α-1,6-linkage. The B chain carries other chains and links to another chain in the same manner as the A chain. The C chain carries other chains but is not connected to another chain, thus it has a free reducing terminal

residue. Provided dp_n of 10,000 (the value is relatively small among those reported) and cl_n of 20, one out of 500 unit chains is the C chain. Therefore, the contribution of the C chain to the results shown in Fig. 2 is negligible.

The molar-based distributions in Fig. 2 were polymodal, similar to the well-known feature for weight-based distributions, but there was a difference in that the relative peak area of long chains (B_2 and longer; B_2, B_3 and B_4 denotes a B chain that spans two, three and four clusters, respectively. See Ref. 10) was much less in a molar basis than in a weight basis. Major portions of the fluorescence profiles comprised short- and long-chain fractions with peak dp of 11–19 and 41–52, which corresponded to $A + B_1$ and $B_2 + B_3$ fractions,[10] respectively. As expected, the fluorescence response from long chains, B_4 or longer, was hardly detected. For some amylopectins, such as wheat and sweet potato, an inflection point by which short-chain fraction was divided into A and B_1 fractions was clearly observed in elution profiles by a fluorescence detector as well as by a refractometer. It has been shown from HPAEC–PAD analysis that dp 6–8 is the region in which most notable differences by source are observed.[11,12] Small peaks at the end of the fluorescence profiles were apparent for potato and sweet potato amylopectins. Determination of dp of these small peaks by the RI/F_{height} values was impossible because of the incorrect RI response caused by salts. The dp of these peaks were confirmed as 6 and 7 by comparison of retention time using authentic malto-oligosaccharides as standard. Such patterns, a peak at dp 6 and/or 7 and a hollow at 8, were consistent with the results by HPAEC–PAD.[12]

The elution profiles were divided into three or two fractions as shown in Fig. 2 for wheat or amylomaize as examples. Due to the absence of clear inflection points in fluorescence profiles, the second major peak around 75 min was designated as $B_2 + B_3$ fraction. Cl_n and percentage in mole for each fraction are listed in Table 2. A periodicity in weight-average chain length of B_1, B_2 and B_3 chains[10] supports the cluster structure, and analysis on a molar basis indicated that A chains also conform to the rules. Starches of A-type crystalline appeared to have higher proportion of A-chain fraction (57.7–68.3%) than those of B-type ($\sim$ 51% for potato), being consistent with the previous studies.[2,12] Assuming that the ratio of the A chain to the B_1 chain (both of which participate in formation of single cluster) falls into a narrow range, the increased amount of A chain can be interrupted as an increase in the number of chains per cluster. The sum of the A and B_1 chains amounted to 90–93 and 68–87% for amylopectins from starches of A- and B-crystalline type, respectively. Thus, the values indicated that one cluster of A-type starch contains more chains than that of B-type, being in agreement with the report by Gérard et al.[13] Similarly, the ratio of $(A + B_1)/(B_2 + B_3)$ suggested that a B_2 or B_3 chain of amylopectin from A-type starch carried more chains, apparently twice as many, than that of B-type starch. Overall, the molar-based distributions presented further evidence for the previous findings obtained by weight-based detection system[2,10] or by different approach with α-amylolysis.[13] Such information on a molar basis will be valuable for modeling of a cluster structure.

Much less attention has been paid to C chains of amylopectin molecules due to their low amount and lack of appropriate methods to separate or distinguish C chains from A and B chains. Here amylopectins were labeled with 2-AP according to the method previously reported for amylose.[4] The previous study showed that the RI/F_{area} is proportional to the dp_n up to 4,400 and in an analysis of labeled potato amylose (dp_n, 4,400) by

Table 2
Chain-length distributions of amylopectins on a molar basis

Source	Cl_n			Amount in mole (%)			$(A+B_1)/(B_2+B_3)$	Crystalline type
	A	B_1	B_2+B_3	A	B_1	B_2+B_3		
Wheat (No)	12.7	24.3	57.3	65.3	27.5	7.2	12.9	A
Wheat (R)	13.0	25.2	52.6	64.5	28.0	7.5	12.3	A
Rice (A)	12.6	24.1	47.2	68.3	23.2	8.5	10.8	A
Rice (Ni)	12.2	24.2	47.7	67.7	23.3	9.0	10.1	A
Maize	13.2	25.3	59.8	65.1	25.8	9.1	10.0	Ca
Sweet potato (J)	12.8	26.9	65.0	61.7	28.8	9.5	9.5	A
Sweet potato (K)	12.4	23.9	61.8	57.7	32.2	10.1	8.9	A
Potato (E)	12.5	24.0	54.3	52.3	34.4	13.3	6.5	B
Yam	18.5		59.4	86.4		13.6	6.4	B
Potato (B)	12.6	23.4	53.2	51.3	33.0	15.7	5.4	B
Amylomaize	17.1		64.7	67.8		32.2	2.1	B

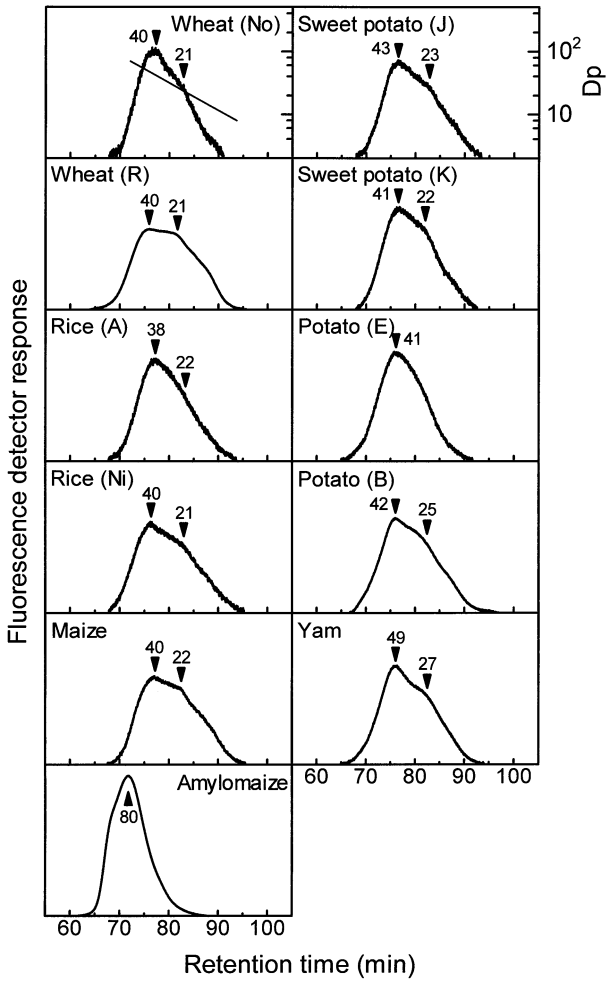

Fig. 3. Molar-based distributions of C chains of amylopectins from various sources. Numbers with arrowheads indicate dp. Dp was determined using a calibration line obtained by analyses of unit-chain distributions of respective sources (Fig. 2). Gain of the fluorescent detector was set to be 100 times higher than that for analyses shown in Fig. 2. Detector output was normalized to show the same peak area.

HPSEC, dp plots at given elution time gave straight line up to dp ~20,000. Therefore, we presumed that amylopectins could be similarly labeled. The molar-based distributions of C chains are shown in Fig. 3. The C chains showed an asymmetrical, broad distribution from dp ~10 to ~130 with a peak at dp 38–49 and a shoulder at dp 21–27 except for amylomaize (peak dp of 80). Also, other than amylomaize, the distributions were very similar regardless of the botanical origin of amylopectins. The result implied that the biosynthetic process for C chains is similar in different plant species. Roughly two thirds of C chains appeared to be long enough to span two clusters and the rest would participate in the formation of a single cluster. The reason for the relatively defined length for the C chains is unknown, but recently Commuri and Keeling proposed that intrinsic properties of starch synthases determine and set the limitations on the length of unit chains.[14] In spite of the absence of evidence for the involvement of starch synthase(s) in C chain synthesis, the proposal is attractive since the same mechanism can be applicable to all the three types of unit chains. When labeled C chains (wheat) were subjected to hydrolysis with β-amylase, 35% of the molecules remained within the elution position of the C chains without the β-amylase treatment (data not shown). The result indicated that the majority of the molecules in the labeled C chain fraction was linear, thus the profiles shown in Fig. 3 was justified in being considered as distribution of C chains. Prior treatment with pullulanase caused a slight decrease in the amount of undigested C chains (29%). Identification of the structure resistant to the sequential enzymatic hydrolysis is now under investigation.

1. Experimental

Materials.—Amylopectins used were rice (japonica, cv. Akitakomachi and cv. Nihonbare), maize (white dent), amylomaize (Hylon7, Ref. 15), wheat (cv. Rosella, Ref. 16 and cv. Nohrin-61), sweet potato (cv. Koganesengan and cv. Joy white), potato (cv. Eniwa and cv. Benimaru) and yam (*Dioscorea batatas* Decne., Ref. 17). The amylopectins were fractionated from defatted starches by the method of Lansky et al.[18] with some modifications.[19] Debranched amylopectin was prepared as previously described.[12] 2-Aminopyridine (2-AP, a grade for fluorescent labeling) was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Sodium cyanoborohydride ($NaCNBH_3$) was obtained from Aldrich Chemical Co. Inc. (Milwaukee, WI, USA). Synthetic amyloses were products of Nakano Vinegar Co., Ltd. (Aichi, Japan). Maltohexaose and *Pseudomonas* isoamylase were obtained from Hayashibara Biochemical Laboratories Inc. (Okayama, Japan). Other reagents were of the highest grade commercially available.

Fluorescent labeling of debranched amylopectin and C chain with 2-AP.—Reducing residues of debranched amylopectin were labeled with 2-AP by the method that was originally developed for amylose[4] with a slight alteration. Debranched amylopectin (4 mg) was dissolved in 111 μL of 90% Me_2SO and 89 μL of H_2O and then labeled with 200 μL each of 2-AP and $NaCNBH_3$. The labeled specimen was filtered through a 0.22-μm filter then used (30 μL) for chromatography without removal of excess 2-AP. Fluorescent labeling of C chain of amylopectin was

performed as follows. Amylopectin (5 mg) was dissolved and labeled in the same manner as for amylose.[4] The labeled amylopectin was precipitated by addition of 900 μL of EtOH and the mixture was left at −20 °C overnight. The precipitate was collected by centrifugation at 2,000 rpm for 10 min at 4 °C then washed three times with 3 mL of 75% EtOH containing 0.1 M NaCl. The washed precipitate was dissolved in 1 mL of Me_2SO by heating and 3 vol. of EtOH was added. The precipitate was collected and washed twice as above. The washed amylopectin was dissolved in H_2O by heating and debranched by isoamylase (in total of 1 mL of 10 mM acetate buffer, pH 3.5; 0.03 U/mg amylopectin) at 45 °C for 12 h. The debranched amylopectin (30 μL) was then injected into a chromatograph after filtration.

High-performance size-exclusion chromatography (HPSEC).—The system used consists of a liquid chromatograph (PU-1580, Jasco, Tokyo, Japan), a Shodex OHpak SB-803 HQ and two Shodex OHpak SB-802.5 HQ analytical columns (8 × 300 mm each, Showa Denko, Tokyo, Japan) connected in series, a fluorescent detector (FP-920, Jasco, exciting and measuring wavelengths were 315 and 400 nm, respectively), and a differential refractometer (ERC-7512, Erma, Tokyo, Japan). Aqueous Me_2SO (50%) containing 50 mM NaCl was used as eluent with flow-rate of 0.25 mL/min. Column temperature was kept at 50 °C.

Other analytical methods.—Total carbohydrate was determined by the phenol–sulfuric acid method.[20] Reducing sugar was determined by either the Somogyi–Nelson method[21,22] or the modified Park–Johnson method.[5,6] Non-reducing residues were determined as previously described.[7]

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