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A periodic distribution of the chain length of amylopectin as revealed by high-performance anion-exchange chromatography

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Abstract

The chains of amylopectin over dp 80 were separated on baseline resolution by high-performance anion-exchange chromatography with pulsed amperometric detection under improved conditions, and the chain-length (cl) distributions of amylopectins from eleven plant sources were analyzed. The differences in amount of each chain between arrowhead (*Sagittaria trifolia* L. var. *sinensis* Makino) and other kinds of amylopectins exhibited periodic waves which divided the abscissa at intervals of dp 12, except those of edible canna and yam amylopectins, where the interval was of the order of dp 15. Accordingly, chain-length distributions were fractionated into fa, dp 6–12; fb_1, dp 13–24; fb_2, dp 25–36, and fb_3, dp > 37. Amylopectin with short and long $\overline{cl}_n$ had large and small amounts of the fa fraction, respectively, and showed A and B type X-ray diffractions of starch granules. The amount of the fa fraction was suggested to play an important role in the determination of starch crystalline polymorphs.

Keywords: Amylopectin chain-length; High-performance anion-exchange chromatography; Starch polymorphism

1. Introduction

Amylopectin is a branched macromolecule consisting of many (1 → 4)-α-glucan chains which are interlinked by (1 → 6)-α-glucosidic linkages. To describe the structural characteristics of amylopectin molecules from various botanical sources, several parame-

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ters have been used [1]. Chain-length (cl) distribution is one of the key parameters, and size-exclusion chromatography (SEC) has been generally employed to estimate the distribution. The chain length of amylopectin shows basically bimodal distribution, F_1 (long B chain) and F_2 (short B and A chains) fractions by SEC. Hizukuri [2] has found a good correlation between the weight-average chain length ($\overline{cl}_w$) and the ratio of the weight fractions of F_2/F_1. This implies that the average chain length is primarily dependent on the amounts of the two fractions. Later, the F_1 and F_2 fractions were separated into A, B_1, B_2, B_3, and B_4 fractions by selection of better columns for SEC [3]. The average chain lengths of these fractions have provided evidence for the cluster structures proposed by Nikuni [4] and French [5]. However, SEC could not separate individual chains. High-performance liquid chromatography (HPLC) on an NH_2-bonded silica column using a refractive index (RI) detector has been demonstrated to be a useful method for separating chains up to dp ~ 26 (ref. [6]). However, this is not satisfactory for analysis of the cl distribution of amylopectin since the main portion of the chains is distributed up to dp ~ 100.

In 1989 Koizumi et al. [7] reported that high-performance anion-exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) was useful for the analysis of several linear homoglucan series. (1 → 4)-α-Glucan members with a dp of up to 50 or 60 have been separated by this technique, and subsequently cl distributions of amylopectins from several plant sources have been characterized [8]. Since then, this technique has been utilized widely for the characterization of the cl distributions of amylopectin [9–12], glycogen [13] and α-glucan synthesized by transformed *Escherichia coli* [14]. Recently, Wong and Jane [15] recommended nitrate as a better eluting agent than acetate.

The purpose of this study was to expand the detectable range of amylopectin chains by HPAEC-PAD to characterize the cl distributions of amylopectins from various botanical sources, and to obtain precise information on the structure of amylopectin.

2. Materials and methods

Materials.—Waxy rice (Mezuru mochi) starch was prepared by an alkaline leaching method [16] from polished rice which was a gift from Dr. Maruyama (Ministry of Agriculture and Forestry). Normal maize (white dent) starch was kindly donated by Sanwa Denpun Kogyo Co. Ltd. (Nara). Sweet potato (Koganesengan) starch was prepared in this laboratory [17]. Edible canna starch was extracted from the rhizomes harvested in January using water by the same method for potato starch [18]. Amylopectins were fractionated from these starches by butanol complexing [19]. Amylopectins from rice (IR42) [16], wheat (Norin-61) [20], barley (Bomi) [21], potato (Benimaru) [18], lotus (*Nelumbo nucifera* Gaertn.) [10], arrowhead (*Sagittaria trifolia* L. var. *sinensis* Makino) [22] and yam (*Dioscorea batatas* Decne.) [23] were the same specimens as used previously.

Crystalline *Pseudomonas* isoamylase was purchased from Hayashibara Biochemical Laboratories, Inc. (Okayama).

Preparation of amylopectin isoamylolyzates.—Amylopectin was dissolved in 10 mM acetate buffer, pH 3.5, at concentration of 2.5 mg/mL, and was debranched with 0.03 U of isoamylase/mg of amylopectin for 12 h at 45 °C. After termination of the reaction by heating at 100 °C for 5 min, the digest was filtered through a G4 glass filter and lyophilized. One unit of the activity was the amount of enzyme which hydrolyzes the one branch linkage of amylopectin at 45 °C and pH 3.5 per min.

Preparation of non-phosphorylated chains of amylopectin.—Amylopectin was debranched under the conditions described above, then non-phosphorylated and phosphorylated chains were separated by ion-exchange chromatography [24].

HPAEC.—HPAEC was performed with a Dionex series 4500i gradient chromatography system equipped with an Eluant Degas Module, and a Model 2 PAD (all from Dionex, Sunnyvale, CA). The working and reference electrodes were gold and silver–silver chloride, respectively. The pulse potentials and durations at range 2 were as follows: $E_1 = 0.1$ ($t_1 = 480$); $E_2 = 0.6$ ($t_2 = 120$); $E_3 = -0.6$ V ($t_3 = 120$ ms). The response time and the sensitivity of a detector was set to 1.0 s and 10 K nA, respectively. Two columns of Dionex CarboPac PA-1 (4 mm diameter, 250 mm long) were connected in series. Chromatocorder 12 integrator (System Instruments, Tokyo) was used for calculation of peak areas. Eluents were 150 mM sodium hydroxide solution as eluent A and 150 mM sodium hydroxide solution containing 500 mM sodium acetate as eluent B. The eluents were prepared with 18 MΩ cm deionized water and filtered through a 0.2-μm membrane filter. Each run was carried out at ambient temperature with a flow-rate of 1 mL/min. Amylopectin isoamylase hydrolyzate (10 mg) was dissolved in 1 mL of 150 mM sodium hydroxide, and was filtered through a 0.2-μm membrane filter, and 25 μL of the solution was chromatographed. The cl distribution is represented as a percentage of the total peak area and the detector response varying with dp was disregarded. The data were shown as the average values of three experiments.

General analytical methods.—Total carbohydrate was determined by the phenol–sulfuric acid method [25]. Determination of reducing residue was carried out by Somogyi's method [26] using Nelson's reagent [27], but the heating time was extended to 30 min [28]. The number-average degree of polymerization ($\overline{dp}_n$) was calculated from the values of the total carbohydrate and the reducing residue.

3. Results

Examination of conditions for HPAEC-PAD.—Table 1 shows the gradient program which gave the best separation among those tested. The program took the longest time to reach 70% of the eluent B. The extension of the duration to increase eluent B from 70 to

Table 1
Gradient elution program for analysis of chain-length distribution

Time (min)	0	6	17	34	53	113	213	223
150 mM NaOH (%)	70	60	50	40	35	30	10	0
150 mM NaOH–500 mM NaOAc (%)	30	40	50	60	65	70	90	100

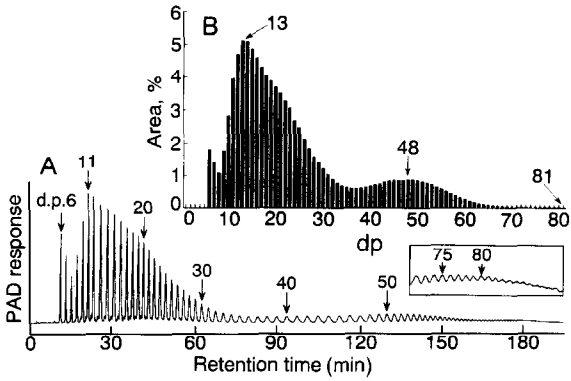

Fig. 1. HPAEC of non-phosphorylated chains of potato amylopectin. The elution program is shown in Table 1. A, original chromatogram; B, chain-length distribution by peak areas.

90% of the program reduced the detectable range of the longer chains over dp 50 by broadening the peaks. The column was pre-equilibrated for 30 min with 30% eluent B. Fig. 1 is the original chromatogram (Fig. 1A) and the histogram of the cl distribution by peak areas (%) of non-phosphorylated potato amylopectin (Fig. 1B) obtained by the program. Chains of up to dp 81 were detected with baseline separation. The amount of sample for loading was increased twice by increasing the concentration of the solution but the separation range increased only a few dp. The chromatogram had a hollow at dp 8 and the highest peak at dp 11 with a shoulder at dp 20. The hollow at dp 8 has been observed previously as a characteristic for potato and some other amylopectins of root origin [8]. The cl distribution represented by relative area showed a peak at dp 13. The distribution was similar to those reported by Koizumi et al. [8].

Fig. 2 shows the standard deviation of relative area and relative standard deviation (RSD) for 7 runs on a normal maize amylopectin. Up to dp 51, most of the RSD values were less than 5% (average, 3.9) but increased to 13% above dp 53, probably because of low sensitivity. Consequently, the graph above dp 50 was hard to interpret.

Chain-length distributions of amylopectins.—Chain-length distributions of amylopectins from various sources were revealed by HPAEC-PAD under the above condi-

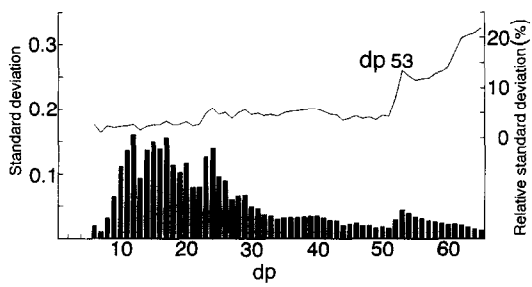

Fig. 2. The relative standard deviation of the relative area and the absolute value of the standard deviation ($n = 7$; specimen, normal maize). Bar, standard deviation; line, relative standard deviation.

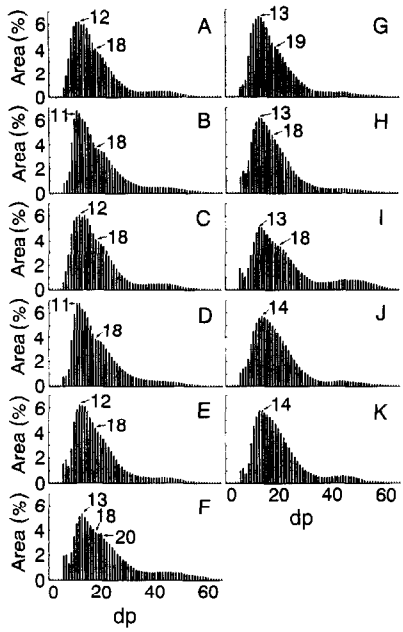

Fig. 3. Chain-length distributions of amylopectins from different sources. A, waxy rice; B, wheat; C, rice; D, barley; E, arrowhead; F, sweet potato (non-phosphorylated chains); G, normal maize; H, lotus; I, potato (non-phosphorylated chains); J, edible canna; K, yam.

tions (Fig. 3). The phosphorylated chains of potato and sweet potato amylopectins were removed by ion-exchange resin because they may have interfered with the chromatography. These chains appear to bind by their phosphate groups more tightly than non-phosphorylated chains and the separation of these phosphorylated chains are under investigation. The amounts of the removed phosphorylated chains of potato and sweet potato amylopectins were 13 and 3% by weight, respectively. Each distribution showed a characteristic pattern. Waxy and non-waxy rice amylopectins had the highest peak at dp 12 and a shoulder at dp 18, showing very similar distributions to each other. Non-phosphorylated chains of potato and sweet potato amylopectins were analogously distributed with the highest peak at dp 13. It was characteristic for potato amylopectin that the chains of dp 6–8 decreased in the order as mentioned above. The amount of relatively long chain with dp > 37 of potato amylopectin was the largest among the specimens and was actually much more than the measured value because long phosphorylated chains [29] with $\overline{dp}$ 42 had been removed. In sweet potato, the amount of chains of dp 7 was the same as that of dp 6 and a shoulder at dp 20 was observed. The distribution of wheat amylopectin resembled that of barley amylopectin, showing a peak at dp 11 and a shoulder at dp 18. Normal maize amylopectin had a peak at dp 13 and a shoulder at dp 19. The increments at dp 6–8 were small (similar to wheat and barley) but the distribution of normal maize amylopectin was unique and distinguishable from other cereal amylopectins, as described later. Arrowhead amylopectin showed a distribution with a peak at dp 12, a shoulder at dp 18 and a hollow at dp 8. Lotus amylopectin

showed a peak at dp 13, a shoulder at dp 18 and a slightly lower peak at dp 8 than at dp 7. The chains of edible canna and yam amylopectins were distributed with peaks at dp 14 and no shoulder at ~ dp 20. Edible canna did not show the lowest value at dp 8 but yam showed a similar shape to arrowhead, sweet potato and potato having the lowest value in the range of dp 6–9 at dp 8.

Fig. 3 is a series of histograms of cl distribution up to dp 65. However, the maximum dp values of chains detected were as follows: waxy rice, 71; rice, 68; potato, 81; sweet potato, 70; wheat, 67; barley, 66; normal maize, 68; arrowhead, 69; lotus, 69; edible canna, 71; yam, 70.

4. Discussion

The detector responses per molecule or OH group are dependent on the dp of malto-oligosaccharides and have only been documented [8] up to dp 17, so the real amount of each chain could not be compared over the entire range. To characterize the distribution over a wide range, we compared simply the relative area of each peak from various amylopectins. The differences in percentage distribution of each chain were compared to that of arrowhead as a standard (Fig. 4) for which the average cl was the mid value of the eleven specimens. The difference was estimated by subtraction of the relative area of each chain of arrowhead from those of other specimens. Fig. 4 shows the increasing order of the sum of the absolute values of the differences as follows: normal maize, 5.9; lotus, 6.3; rice, 9.4; barley, 10.4; wheat, 11.5; waxy rice, 11.8; edible canna, 14.1; yam, 15.2; sweet potato, 19.7; potato, 23.1. These values may be considered to be a measure of similarity in cl distribution. The values of sweet potato and potato amylopectins were much higher than the others, implying that their distributions were distinctive and that there were considerable differences from that of arrowhead amylopectin. The values for cereal amylopectins were closely ranged from 9.4 to 11.8, except for normal maize (5.9), suggesting that these distributions were similar but that normal maize amylopectin was a little different from the other cereal amylopectins examined in this study and was rather similar to lotus amylopectin.

It is of interest that a periodicity has been found in the distribution on the abscissa of Fig. 4. The intercepts on the axis of abscissa appeared at a multiple of dp 12, that is, 12, 24, 36, and 48. At or near these dp values, a shoulder (potato), a hollow (sweet potato), and a trough (potato and sweet potato) in the distribution were observed except edible canna and yam for which the period might be dp 15. The periodic patterns of cl distributions of amylopectins have been observed [3] by size-exclusion chromatography of chains and a cluster structure has been proposed. However, the boundaries of the peaks were somewhat arbitrary. It is of interest that the periodicity of dp 12 seemed to be common for most amylopectins although a slightly longer period also had been suggested in edible canna and yam amylopectins.

According to the periodic pattern mentioned above, the chains were fractionated into four fractions as follows: fa, dp 6–12; fb_1, 13–24; fb_2, 25–36; fb_3, > 37. Fractions fa, fb_1, fb_2, and fb_3 probably correspond to A, B_1, B_2, and B_2 or B_3 and longer chains by previous classifications. The cl distributions are summarized by the sum of the relative

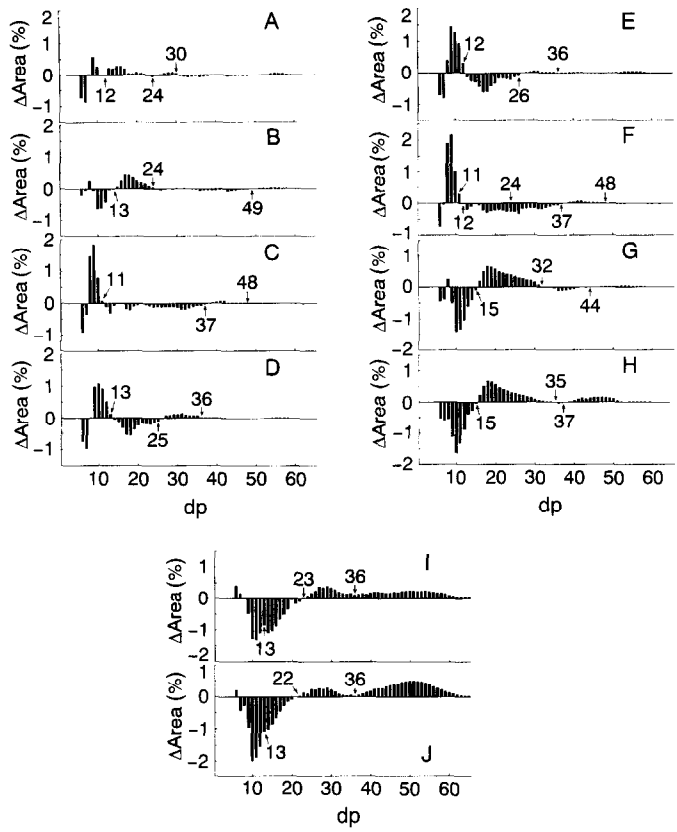

Fig. 4. Differences in chain-length distributions by source. The differences were obtained by subtracting the peak areas of arrowhead from those of others. A, normal maize; B, lotus; C, rice; D, barley; E, wheat; F, waxy rice; G, edible canna; H, yam; I, sweet potato (non-phosphorylated chains); J, potato (non-phosphorylated chains).

area in Table 2. Tentatively, edible canna and yam amylopectins were also classified by the same ranges. The distributions appear to be specific for each source. Cereal amylopectins had larger proportions of fraction fa (24–29%) and smaller proportions of fraction fb_3 (9–10%), while potato amylopectin had a larger proportion of fraction fb_3, which would be much more if the phosphorylated chains were counted (see above). The other amylopectins showed intermediate distributions between the cereal and potato. The molar ratios of these fractions determine the number-average chain length. The amylopectins with high $\overline{cl}$ had larger proportions of the longer chain fractions. Potato amylopectin had high amounts of fractions fb_2 and fb_3, and edible canna and yam amylopectins had high amounts of fractions fb_1 and fb_2. The summarized distribution (Table 2) showed a clear relationship between crystalline polymorphs, A, B, and C type, of starch granules and the cl distribution as observed previously [2,30] but in a little more detail. Namely, the A type had a relatively abundant fa fraction, the B type had the least and the C type had intermediate amounts, while fractions fb_1, fb_2, and fb_3 seemed

Table 2
Differences in chain-length distributions by source [a]

Source	dp_n of isoamylolyzate	Crystalline type	Relative area (%)				fa/fb_1	fa/fb_{1-2} [b]	fa/fb_{1-3} [b]
			fa	fb_1	fb_2	fb_3			
Waxy rice	18.9	A	29	50	11	9	0.58	0.47	0.41
Wheat	20.1	A	27	49	14	10	0.56	0.44	0.38
Rice	21.6	A	27	52	12	9	0.53	0.44	0.38
Barley	19.3	A	27	50	14	9	0.53	0.41	0.36
Arrowhead	20.5	Ca	25	53	13	9	0.47	0.37	0.33
Sweet potato	22.1	A	21	47	17	15	0.45	0.33	0.27
Normal maize	22.0	Ca	24	54	13	9	0.44	0.35	0.31
Lotus	22.6	Cc	23	55	13	9	0.41	0.33	0.30
Potato	23.6	B	18	48	15	18	0.37	0.28	0.22
Edible canna	25.7	B	20	56	15	9	0.35	0.28	0.25
Yam	23.2	B	18	56	15	11	0.32	0.25	0.22

[a] Fractionation was as follows: fa, dp 6–12; fb_1, 13–24; fb_2, 25–36; fb_3, > 37.
[b] fb_{1-2}, $fb_1 + fb_2$; fb_{1-3}, $fb_1 + fb_2 + fb_3$.

to be less related. The ratio fa/fb_1, fa/$fb_1 + fb_2$, and fa/$fb_1 + fb_2 + fb_3$ decreased from the A type with low $\overline{dp}_n$ toward the B type with high $\overline{dp}_n$. Thus, the crystalline type transition from the A to the B type is well correlated to the cl distribution and the amount of the fa fraction appears to be a key factor for determining the crystalline polymorphs of starch granules. We classified normal maize as Ca (Table 2) because it gave a faint but detectable 15.8 Å diffraction line under wet conditions [31].

In general, the biosynthesis of amylopectin molecules is explained by the concerted action of the multiple forms of starch synthase and branching enzyme. It is conceivable that two or more branching enzymes with different action specificities regarding the chain length of the products [32] play a key role in the formation of a characteristic cl distribution by sources. The same periodicity from different sources, as revealed by the present study, may suggest the respective isoforms of branching enzymes from wide sources have the same or close action specificities and the characteristic cl distribution may be caused by variable proportions of the enzyme isoforms by sources.

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