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Estimation of the distributions of chain length of amylopectins by high-performance liquid chromatography with pulsed amperometric detection☆

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ABSTRACT

High-performance anion-exchange chromatography for a detailed estimation of the distribution of chain length of amylopectins was developed using a pulsed amperometric detector under alkaline conditions. As maltosaccharides having different degrees of polymerization, which were produced by debranching of amylopectin with isoamylase, exhibit different pulsed amperometric detector responses, the individual maltosaccharides (degree of polymerization 6–17) were isolated by high-performance liquid chromatography on an amino column and an octadecylsilane column to use as quantification standards. By the use of this high-performance anion-exchange chromatography the chain length distributions of some typical amylopectins were characterized in detail.

INTRODUCTION

The estimation of the chain length distribution is of primary importance for characterizing the molecular structure of amylopectin. Previously, Hizukuri [1] characterized the polymodal distribution of the chain length of the several kinds of amylopectin by size-exclusion chromatography using a differential refractometer and a small-angle laser light-scattering photometer. This technique is useful for this purpose but cannot separate individual members of the components of debranched amylopectins.

Recently, Koizumi *et al.* [2] showed that some homoglucan series could be well separated into the individual members [degree of polymerization (DP) up to over 50] by high-performance anion-exchange chromatography (HPAEC) with pulsed amperometric detection (PAD). However, it was impossible to determine the individual glucans directly by use of their peak areas in the chromatogram, as the responses of a pulsed amperometric detector to glucans having different DPs were different.

The purpose of this study was to develop a detailed method for quantitative analysis of maltosaccharides, the components of debranched amylopectin, and to characterize the chain length distributions of some typical amylopectins by HPAEC–PAD.

EXPERIMENTAL

Chromatography

HPAEC was performed with a Model 4000i Dionex BioLC system and a Model 2 PAD system

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(Dionex, Sunnyvale, CA, USA). The pulse potentials and durations used were identical to those described in a previous paper [2]. The column used was a Dionex HPIC-AS6 (the same type of column is now called CarboPac PA-1) (250 × 4 mm I.D.) (10 μm) equipped with an AG6 guard column (50 × 4 mm I.D.). An 807 IT digital integrator (Jasco, Tokyo, Japan) was used to calculate peak areas.

High-performance liquid chromatography (HPLC) for isolation of individual maltosaccharides was conducted with a Jasco 880-PU pump, a U6K universal injector (Waters, Milford, MA, USA) and an SE-61 RI monitor (Showa Denko, Tokyo, Japan). The columns used were an Asahipak NH2P-50 (250 × 10 mm I.D.) (5 μm) (Asahi Kasei, Tokyo, Japan) and a YMC-Pack SH-343-5 AQ (250 × 20 mm I.D.) (5 μm) (YMC, Kyoto, Japan). For preparative chromatography at constant temperature a CO-1093C column oven (Uniflows, Tokyo, Japan) was used. Degassing of eluents on line was performed using Degasys DG-1200 (Uniflows).

Materials

Amylopectins tested were of wheat [3], rice (Sasanishiki [4] and Nihonbare, both Japonica), waxy rice (Hiyokumochi, Japonica) [5], corn [6], tapioca [5], edible canna, sweet potato (Minamiyutaka) [7], and potato [5]. Some of them were used in previous studies as cited. Edible canna and rice (Nihonbare) amylopectins were prepared from their corresponding starches by the same procedures as described elsewhere [4]. The individual maltosaccharides (DP 6–17) used as quantification standards were isolated from short-chain amylose EX-1 ($\overline{DP} \approx 17$) [a mixture of (1→4)-α-D-glucans] (Hayashibara, Okayama, Japan). All reagents were of analytical-reagent grade. The eluents for HPAEC were prepared in the same manner as those in a previous paper [2]. Reagent-grade organic solvents used for preparative chromatography were dried and freshly distilled before use. Water used in solvent preparations was distilled, deionized, redistilled and degassed by sonication.

Preparation of amylopectin isoamylolyzates

Amylopectin (100 mg) was dissolved in 25 m*M* acetate buffer, pH 4.5 (40 ml), and was debranched with isoamylase (30 I.U.) for 12 h at 45°C. The resulting linear chains were lyophilized after inactivating the enzyme by boiling. The lyophilized sample (2 mg) was dissolved in 1 ml of 150 m*M* sodium hydroxide solution and the aliquots of 20–30 μl were analyzed.

RESULTS AND DISCUSSION

Separation of individual members in the amylopectin isoamylolyzates

In order to achieve an effectice separation of individual members of a series of chains, several gradient programmes were examined. The gradient programme selected was as follows: 40% eluent B at 0 min, 50% at 2 min, 60% at 10 min and 80% at 40 min. All separations on a HPLC column were carried out at ambient temperature with a flow-rate of 1 ml/min. The HPAEC elution profile of the components of debranched wheat amylopectin is shown in Fig. 1 as an example. The number on each peak, indicating its DP, was confirmed by adding maltooligosaccharides of known DP. A baseline separation of chains up to DP ≥ 55 was achieved as individual peaks. The smallest chain appears to be DP 6, which was common to amylopectins of other sources. The most abundant chain of the wheat amylopectin was apparently DP 11, with a shoulder at DP 18 and 19. Thus, the apparent distribution of

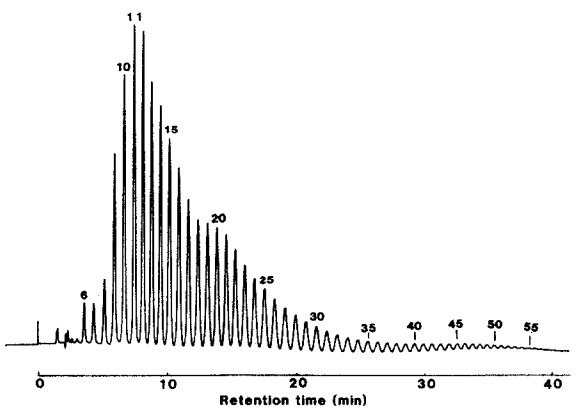

Fig. 1. HPAEC elution profile of the components of debranched wheat amylopectin. The number on each peak indicates its DP. Chromatographic conditions: column, HPIC-AS6 (250 × 4 mm I.D.); eluent A, 150 m*M* sodium hydroxide solution; eluent B, 150 m*M* sodium hydroxide solution containing 500 m*M* sodium acetate; gradient program, 40% eluent B at 0 min, 50% at 2 min, 60% at 10 min and 80% at 40 min; flow-rate, 1 ml/min; detector, PAD 2; meter scale, 10 000 nA; temperature, ambient.

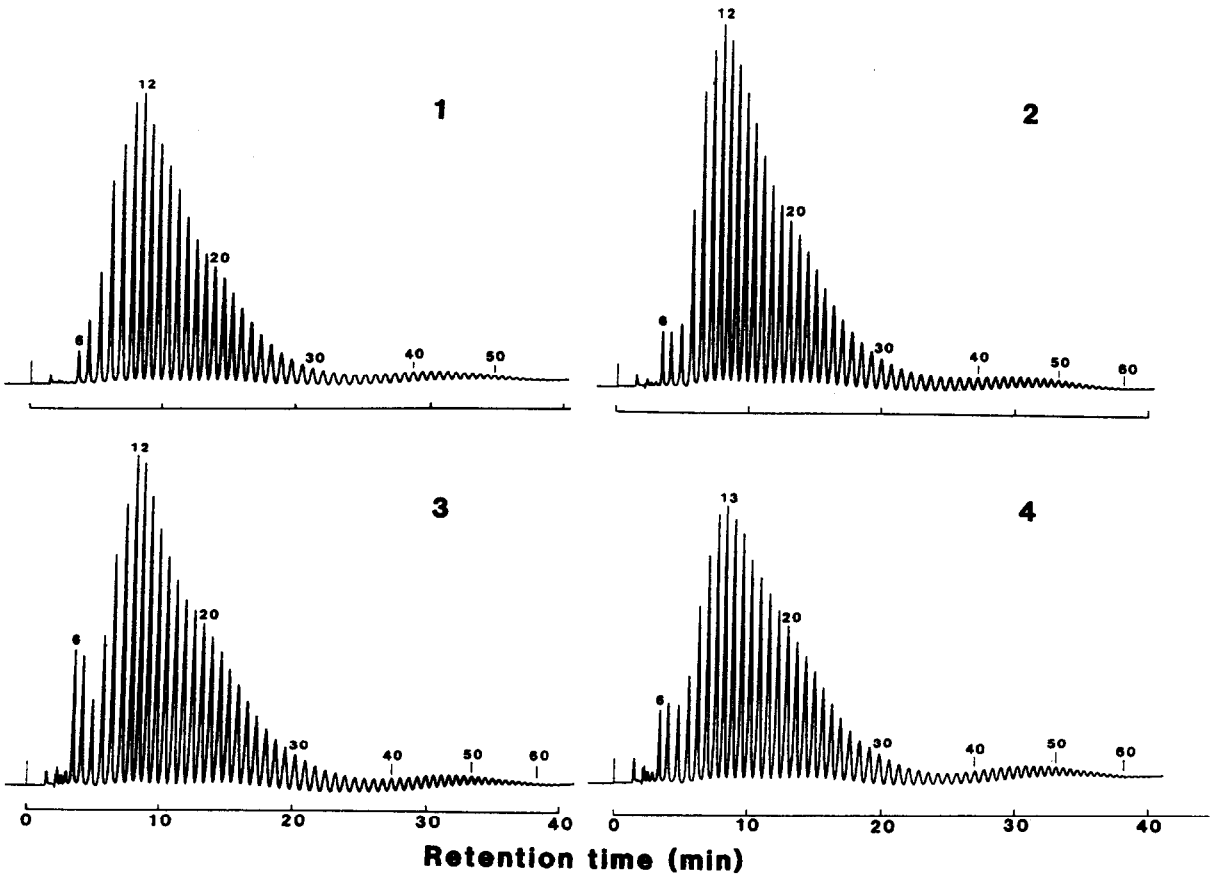

Fig. 2. Chromatograms of isoamylolyzates of some typical amylopectins. 1 = rice (Nihonbare); 2 = corn; 3 = sweet potato; 4 = edible canna. Chromatographic conditions as in Fig. 1.

chain length could be clearly characterized. Besides the peak top DPs, characteristic distributions by species were generally observed in the range DP 6–10 (Fig. 2).

Isolation of individual maltosaccharides (DP 6–17)

Previously we have found that PAD responses to a series of glucans increase with increasing DP [2]. Although this is favorable for detection of higher oligo- and polysaccharides, such differences in the PAD response to saccharides with different DPs require a quantification standard for each maltosaccharide in amylopectin isoamylolyzates to be determined. Therefore, isolation of the individual maltosaccharides was attempted.

Usually HPLC for the separation of oligosaccharides is conducted by using aminopropyl-bonded silica with acetonitrile–water as the eluent. However, amino columns have limited lifetimes, since the bonded phase is readily hydrolyzed. Recently, several attempts have been made to improve the packing stability. One of them, an Asahipak NH2P-50 column, packed with chemically polyamine-bonded vinyl alcohol copolymer gel (5 μm), was selected in this work for the isolation of each maltosaccharide.

Using a semipreparative-size column (250 × 10 mm I.D.) of Asahipak NH2P-50, a 3% aqueous solution of short-chain amylose EX-1 was first roughly separated into several fractions with acetonitrile–water (55:45, v/v) at a flow-rate 2 ml/min (Fig. 3), and then each fraction was purified by repeated rechromatography with gradually decreasing concentrations of acetonitrile (from 62 to 55%) with

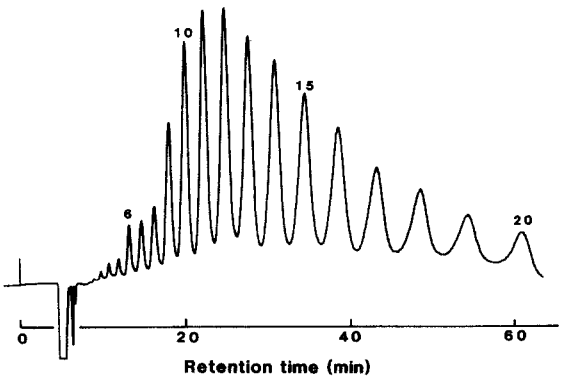

Fig. 3. Separation of maltosaccharides [short-chain amylose EX-1 ($\overline{DP} \approx 17$] on an Asahipak NH2P-50 (250 × 10 mm I.D.). Chromatographic conditions: eluent, acetonitrile–water (55:45, v/v); flow-rate, 2 ml/min; temperature, 33°C.

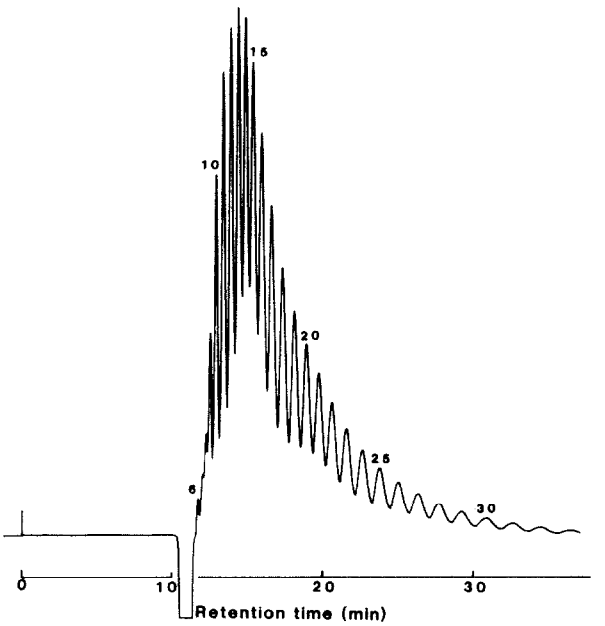

Fig. 4. Separation of maltosaccharides on a YMC-Pack SH-343-5 AQ (250 × 20 mm I.D.). Chromatographic conditions: eluent, 6.0% (v/v) methanol in water; flow-rate, 6 ml/min; temperature, 33°C.

increasing DP. However, the solubility of maltosaccharides, especially above DP 14, was not sufficient to allow chromatography on this column with acetonitrile–water, and, consequently, saccharides of higher DP frequently precipitated on the column. An advantage of this column is that a wider pH range (2–13) can be used, and hence polysaccharides precipitated on the column could be removed by washing with 50 m*M* sodium hydroxide solution, after which there was no change in column performance. The next attempt, in which polysaccharide samples were dissolved in 10% ethylenediamine solution (pH 12.7) and eluted with acetonitrile–water containing 10% ethylenediamine, did not give any satisfactory chromatograms, though precipitation of polysaccharides on the column could be prevented.

Fig. 4 shows the elution profile of short-chain amylose EX-1 on an octadecylsilane (ODS) column with 6.0% methanol at 33°C. In general, ODS columns are not used for the separation of oligosaccharides, because they lead to an undesirable resolution between the α- and β-anomers of the individual oligosaccharides in HPLC at room temperature and with pure water as the eluent. However, a chromatogram obtained under the conditions in Fig. 4 was similar to the elution profile of the same sample on an aminopropyl-bonded silica column obtained previously [8]. The loading capacity of this column (~60 mg) was much larger than that of the amino column described above (~2 mg) and, moreover, the eluent used for this HPLC, a few per cent methanol, was relatively favorable for dissolving higher saccharides.

Separation of maltosaccharides on this ODS column was performed as follows: fractions I (DP 6–13) and II (DP 14–20) were collected under the conditions in Fig. 4, fraction I was rechromatographed with 2.0% methanol (the methanol concentrations of the eluents were accurately adjusted using whole pipettes and a volumetric flask to obtain the best separation of a pair of α- and β-anomers from other pairs) (Fig. 5) to isolate individual maltosaccharides of DP 6–13, and each saccharide was purified by HPLC on the same column with a suitable concentration of methanol (1.0% for DP 6 and 7, 1.2% for DP 8, 1.4% for DP 9, 1.6% for DP 10, 1.8% for DP 11, and 2.0% for DP 12 and 13). As shown in Fig. 5, the α- and β-anomers of all maltosaccharides are resolved by using 2.0% methanol as the eluent. To ensure that two adjacent peaks arose from the same saccharide, the two peaks were separately collected and analyzed on an Asahipak NH2P-50 column and an HPIC-AS6 column. These two fractions

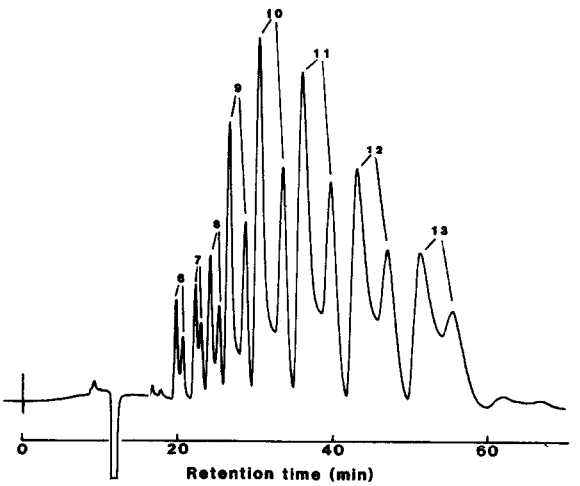

Fig. 5. Separation of maltosaccharides (DP 6–13) on a YMC-Pack SH-343-5 AQ. Chromatographic conditions: eluent, 2.0% (v/v) methanol in water; other conditions as in Fig. 4.

each gave a single peak with the same retention time on an Asahipak NH2P-50, whereas on an HPIC-AS6 the former fraction showed a single peak but the latter fraction showed another small peak immediately in front of the main peak having the same retention time as that of the former fraction. The small peak may correspond to a glucan having the same DP as that of the main peak and containing one (1→6)-α-linkage, since the (1→6)-α-D-glucan moves more slowly on the ODS column [9] and faster on the HPIC-AS6 column [2] than the (1→4)-α-D-glucan. A comparison of the chromatographic behavior of maltohexaose and 6^3-O-α-maltotriosylmaltotriose, which is a hydrolyzate of pullulan with DP 6, substantiated this prediction. This result revealed that short-chain amylose EX-1 does not consist of homogeneous (1→4)-α-D-glucans, but contains a small amount of (1→4)-α-D-glucans containing (1→6)-α-linkages, and the latter can be separated from the former on the ODS column but not on an Asahipak NH2P-50 column. Although individual maltosaccharides of DP 14–17 were also isolated from fraction II on the ODS column with 2.3% methanol, those of DP over 18 could not be purified, as their solubilities are too small to allow solutions to be made, even in pure water.

Relative detector responses of maltooligosaccharides and maltopolysaccharides

Relative detector responses (RDRs) of individual isolated maltosaccharides are summarized in Table I. The RDRs of maltosaccharides of DP 8–13 increase approximately in proportion to the number of hydroxyl groups in the molecule, and the responses per hydroxyl group in maltosaccharides of over DP 14 decrease little by little, whereas those of DP 6 and 7 are slightly higher. These facts may suggest that undetectable hydroxyl groups increase with increasing molecular weight, owing to the formation of random coil.

The individual peak area obtained from chromatograms of amylopectin isoamylolyzates such as in Figs. 1 and 2 was corrected by dividing by the relative detector response. Using the corrected peak area, the exact distributions of chain length (DP 6–17) of some typical amylopectins were compared (Fig. 6). These chain length distributions were characterized by sources and could be regarded as fingerprints. Wheat amylopectin exhibited a somewhat sharp peak with three chains (DP 10, 11 and 12), which was maximal at DP 10 and decreased gradually above DP 13. Tapioca amylopectin gave a similar pattern but peaked at DP 11. These characteristics agreed well with those found by gel-exclusion HPLC [1,3]. Three rice amylopectins (Sasanishiki, Nihonbare and Hiyokumochi) showed similar pat-

TABLE I

RELATIVE PAD RESPONSES OF MALTOSACCHARIDES

Chromatographic conditions as in Fig. 1. The amounts of maltosaccharides used were 1.5 nmol each. RDR = relative PAD response on molar basis.

DP	No. of HCOH	RDR	RDR per HCOH unit
6	20	0.74	1.08
7	23	0.82	1.03
8	26	0.89	0.99
9	29	1.00	1.00
10	32	1.10	1.00
11	35	1.20	1.00
12	38	1.31	1.00
13	41	1.38	0.99
14	44	1.46	0.97
15	47	1.55	0.96
16	50	1.59	0.92
17	53	1.65	0.90

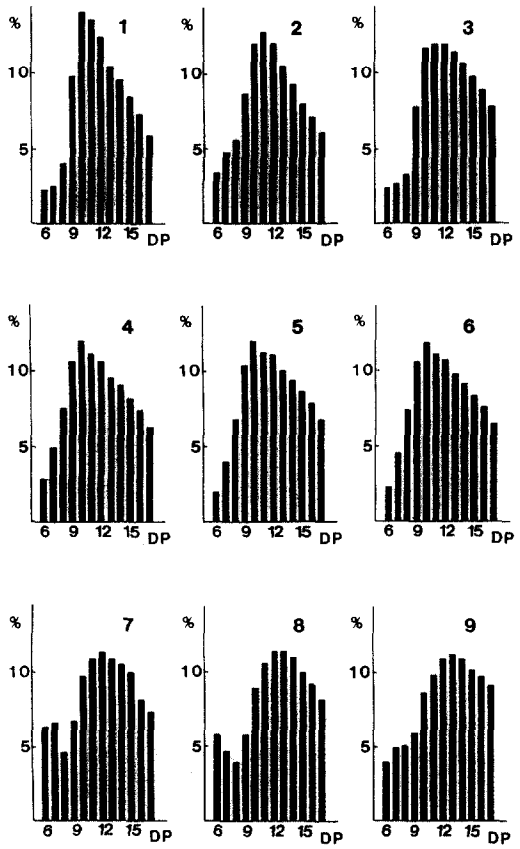

Fig. 6. Comparison of exact distributions of chain length (DP 6–17) of some typical amylopectins. 1 = wheat; 2 = tapioca; 3 = corn; 4 = rice (Sasanishiki); 5 = rice (Nihonbare); 6 = waxy rice (Hiyokumochi); 7 = sweet potato; 8 = potato; 9 = edible canna.

terns, with a slightly broader peak (maximum at DP 10) and gradually decreasing at chains longer than DP 10. Corn amylopectin consisted of only tiny amounts of DP 6–8 chains and showed a dull peak with chains of DP 9–12 (maximum at 10 and 11). Sweet potato and potato amylopectins gave characteristic patterns in the range of DP 6–9, having a hollow at DP 8 and peaks at DP 12, and 12 and 13, respectively. Small amounts of phosphorylated chains at C-6 or C-3 [10,11] in these specimens were not detectable under the experimental conditions and were left for future studies. Edible canna amylopectin exhibited a gradual increase and decrease to and from the peak at DP 13. These minute chain length distributions between DP 6 and 17, although a very limited range, are informative for characterization of amylopectin by sources. We are attempting to expand the range for better elucidation and characterization of the amylopectin structure.

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