Comparison of the Structures of Amylopectins from Different Wheat Varieties[1]

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ABSTRACT

Amylopectins from ten varieties (five classes) of wheat were isolated by high-performance size-exclusion chromatography (HPSEC). All ten purified amylopectin samples contained a single polysaccharide peak with similar retention time and peak shape. Debranching of each sample with isoamylase produced four linear carbohydrate components. HPSEC was useful in rapidly monitoring debranched fragments from amylopectins. The average degree of polymerization ($\overline{DP}$) and proportion (percent of total peak areas) for each fragment was 86 (4.0), 39 (19.1), 19 (30.5), and 12 (46.4%), respectively. Calculated peak area [($\overline{DP}$ 19 + $\overline{DP}$ 12)/$\overline{DP}$ 39] and molar ratios of debranched fragments were slightly higher than those previously reported. All wheat amylopectins studied were identical in their debranching patterns, indicating no detectable differences in their structure.

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Starches from different classes of wheat differ in their functional properties. Prime starches from durum wheat differed significantly in breadmaking properties in comparison to those from hard wheat (Hoseney et al 1971). Medcalf and Gilles (1965) reported that starches from durum and spring wheats had markedly different gelatinization characteristics. These findings led several investigators to consider the relationships between starch structure and functional properties.

Detailed information on the structure of amylopectin has been obtained using debranching enzymes (pullulanase or isoamylase) to selectively hydrolyze the alpha-1,6-glycosidic linkages at the branch points. Gel-filtration column chromatography has been used to separate the debranched carbohydrate fragments (Lee et al 1968, Akai et al 1971) and a bimodal (DeHaas and Goering 1972, Lii and Lineback 1977, Atwell et al 1980, Kano et al 1981) or trimodal distribution (MacGregor and Morgan 1984) of carbohydrate components was observed.

Structural studies of amylopectin have been slow in developing because of the time (one to several days) needed to analyze a single sample by conventional methods. We have developed a rapid, high-performance size-exclusion chromatographic method (HPSEC) to separate and monitor starch components and debranched fragments (Kobayashi et al 1985). The method is effective because small samples (10 μg –1 mg) can be analyzed within 30 min, thus permitting the analysis of a number of samples within a reasonable time with less day-to-day variation than traditional techniques.

The purpose of this study was to determine whether the structures of amylopectins from several types of wheat differ significantly, as part of an effort to determine relationships between starch component or granule structure and functional properties.

MATERIALS AND METHODS

Starch was prepared from wheat flour (10 varieties from five types, Table I) following the method described by Wolf (1964). Glucose and dimethylsulfoxide (Me_2SO, 0.2% water content, Fisher certified ACS) were purchased from Fisher Scientific Co. (Raleigh, NC). Maltose (hydrate, grade 1) was purchased from Sigma Chemical Co. (St. Louis, MO). Maltohexaose was donated by Nikon Shokuhin Kako Co. Ltd. (Shizuoka, Japan). Amylose (EX-III, DP 100, Lot no. 71063008) and isoamylase (59,000 units/mg protein, 1 mg protein/ml, Lot no. 30600) were purchased from Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan). Before use, the buffer in the enzyme suspension was replaced with acetate buffer by adding 10 ml (5 ml twice) of 0.1 *M* acetate buffer, pH 3.80, to 50 μl of the enzyme solution in a collodion bag (Fisher Scientific Co.) at 4°C. The diluted buffer-enzyme solution (5 ml) was allowed to concentrate to 0.5 ml and diluted to 5 ml with acetate buffer for use. Sweet potato β-amylase (crystalline suspension, 27.6 mg protein/ml, 970 units/mg protein, Type 1-B) was purchased from Sigma Chemical Co. Before use, ammonium sulfate in the enzyme suspension (500 μl) was replaced with 0.1 *M* acetate buffer, pH 5.0, as described for isoamylase. These enzyme solutions can be stored for three months without significant loss of activity.

Distilled and deionized water was used. The water and Me_2SO were filtered through a fritted disk funnel (pore size, 10–15 μm) and degassed at room temperature. All other chemicals used were chemically pure grade.

HPLC Apparatus

A model 510 pump equipped with a model U6K universal liquid chromatograph injector and differential refractometer R401 (Waters Associates Inc., Milford, MA) were used. The data were collected on an Apple II+ computer equipped with an ADALAB data acquisition/control card and CHROMATOCHART chromatography software for peak area integration (Interactive Microware, Inc., State College, PA). Two DuPont (Wilmington, DE) Zorbax PSM 60S HPSEC columns (6.2 mm × 30 cm) were connected in series. The columns were protected with a guard column (Waters Assoc., 3.9 mm × 2.5 cm) packed with Co:Pell ODS (Whatman Inc., Clifton, NJ). The columns were immersed in a 40° C water bath. The refractometer was also maintained at 40° C using a circulating water bath.

Preparation of Amylopectin

Starch samples (40 mg) were dissolved in 2 ml of 90% Me_2SO in a boiling water bath for 5 min, and the solutions were centrifuged at 3,000 × *g* for 5 min. An aliquot (400 μl) of the supernatant solution, removed carefully from the top of the solution, was loaded onto the injection loop and fractionated by eluting with Me_2SO (containing 0.2% H_2O) at a flow rate of 0.4 ml/min. Detector attenuation was set at 32×. Aliquots (50 μl) of all collected fractions (200 μl) were analyzed by HPSEC to check their composition and purity. Acetone (5 volumes) was added to the fractions that showed only a single amylopectin peak. The mixture was centrifuged (3,000 × *g*, 5 min) to precipitate amylopectin. The precipitate was dissolved in 100 μl of 90% Me_2SO by heating in a boiling water bath for 5 min. These amylopectin solutions were used for subsequent debranching experiments.

Debranching of Amylopectin

Amylopectin solution (100 μl), 100 μl of 0.1 *M* acetate buffer (pH 3.80), 100 μl of water, and 100 μl of isoamylase solution were thoroughly mixed and allowed to stand for two days at 40°C. A control of the same mixture, with 0.1 *M* acetate buffer replacing the

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isoamylase solution, was used with each debranching experiment. After completion of the reaction, 100 μl of each mixture was analyzed by HPSEC.

Treatment of Debranched Fragments with β-Amylase

Following isoamylase treatment, the debranched fragments were precipitated by adding acetone (5 volumes) and centrifuged. The precipitate was dissolved in 100 μl of 90% Me_2SO. One hundred microliters of 0.1 *M* acetate buffer (pH 5.0), 100 μl of water, and 100 μl of β-amylase solution were added and thoroughly mixed. The mixture was held for 15 hr at 40° C. Eighty microliters were analyzed by HPSEC.

Preparation of β-Limit Dextrins

To 40 mg of amylopectin (obtained by HPSEC) dissolved in 2.0 ml of 90% Me_2SO was added 2.0 ml of water, 3.0 ml of 0.1 *M* acetate buffer (pH 5.0), and 1.0 ml of β-amylase solution. The solution was mixed and held at 40° C for two days. β-Amylase enzyme was inactivated by heating (100° C) for 10 min, centrifuging the sample, and removing the supernatant. The solution was concentrated to 2.0 ml by heating (100° C) while flowing a stream of N_2. Acetone was added (10 ml) to precipitate the carbohydrate, and the sample was centrifuged. The precipitate was dissolved in 1.0 ml of 90% Me_2SO, and 1.0 ml of water, 1.5 ml of 0.1 acetate buffer, and 0.5 ml of β-amylase solution were added and mixed. The mixture was incubated at 40° C for two days. β-Amylase was inactivated (100° C) and the solution centrifuged. The supernatant was concentrated to 1.0 ml by heating under a stream of N_2. Acetone (5 ml) was added to precipitate the β-limit dextrin, and the formed maltose was discarded in the supernatant. The β-limit dextrin was dissolved in 1.0 ml of 90% Me_2SO and the acetone precipitation procedure repeated until all the maltose was removed. Maltose was detected by HPSEC.

Determination of Average Degree of Polymerization ($\overline{DP}$) of Amylose

Amylose (50 mg, Hayashibara) was dissolved in 2 ml of 90% Me_2SO in a boiling water bath for 5 min, and the solution was centrifuged (3,000 × *g*, 5 min). The supernatant solution (1.8 ml), removed carefully from the top of the solution, was used for determinations of total sugar and reducing value. To 200 μl of the supernatant solution was added 50 μl of 90% Me_2SO and diluted to 100 ml with water. Total sugar in a 1.0 ml aliquot was determined by the phenol-sulfuric acid method (Dubois et al 1956). Maltose hydrate (10 mg) was dissolved in 100 ml of water containing 0.225% Me_2SO, and the solution was used as a standard. This quantity of Me_2SO was used because it corresponds to the concentration of Me_2SO present in the diluted amylose sample before $\overline{DP}$ determination.

For reducing value determinations, 100 μl of the supern tant solution, 400 μl of water, and 0.5 ml of Somoygi-Nelson D sol tion were mixed. Reducing values were measured following the Somoygi-Nelson method (Nelson 1944) except that 0.5 ml of C solution and 8 ml of water was used. The colored solution was centrifuged (3,000 × *g*, 5 min) before measuring the absorbance at 520 nm. As a standard, the reducing value of maltose hydrate dissolved in water containing an equal amount of Me_2SO as present in the amylose sample was also measured.

HPSEC Column Calibration

The HPSEC column system was calibrated by using glucose, maltose, maltohexaose, and amylose (Hayashibara). A plot of the weight average molecular weight versus retention time produced a smooth curve, which was used to estimate the $\overline{DP}$ of the debranched fragments.

RESULTS AND DISCUSSION

The wheat starches used in this study are listed in Table I. The HPSEC profiles of all the starches were similar to that of Scout starch shown in Figure 1. The two peaks have been previously identified as amylopectin and amylose (Kobayashi et al 1985). Debranching wheat starch with isoamylase resulted in the carbohydrate pattern shown in Figure 2. Five carbohydrate components are evident, three of which presumably originate from amylopectin. The amylose fraction appears intact. The change in peak area of amylose was negligible after isoamylase treatment. However, the possibility exists that a debranched fragment could elute with the amylose; therefore, pure amylopectin was isolated, using HPSEC, for this study.

Figure 3 illustrates the chromatograms of the polysaccharide fractions obtained from overloading the analytical columns with a large quantity (400 μl, concentration 20 mg/ml) of whole starch. The resolution of amylopectin and amylose is minimal under these conditions.

By collecting amylopectin, fractions containing various quantities of amylose are obtained (Fig. 3). Using this method, approximately 1–2 mg of chromatographically pure amylopectin (Fig. 3, Fraction 1) can be isolated from a single injection.

The chromatographic profiles of debranched, pure amylopectin preparations from all varieties studied were similar in peak size and shape to those illustrated in Figure 4 for amylopectins from Scout, Era, and Omar varieties. The profiles have four peaks (labeled I, II, III, and IV). After two days of reaction, debranching was complete because the elution profiles at two and four days were identical; further treatment of the debranched fragments with fresh isoamylase did not show any detectable change in the profile.

Previous workers have reported a bimodal distribution for debranched amylopectin fragments (DeHaas and Goering 1972, Lii and Lineback 1977, Atwell et al 1980, Kano et al 1981). Recently, MacGregor and Morgan (1984) reported a trimodal distribution of fragments formed after debranching barley amylopectin with

TABLE I
Relative Amount (peak area % ± standard deviation[a]) of Carbohydrate Components Obtained from Debranched Amylopectins

		Carbohydrate Component[b]				
Type	Variety	I	II	III + IV	III	IV
Hard red winter	Scout	4.00 ± 0.36	19.1 ± 0.5	76.8 ± 0.2	30.5	46.3
	Triumph	4.46 ± 0.84	19.3 ± 1.2	76.2 ± 1.7	30.0	46.2
Hard red spring	Era	4.48 ± 0.83	21.4 ± 0.4	74.1 ± 0.5	29.0	45.1
	Waldron	4.44 ± 0.94	18.8 ± 0.2	76.8 ± 1.1	28.6	48.2
Soft red winter	Throne	4.21 ± 0.58	18.7 ± 1.3	77.1 ± 2.1	31.9	45.2
	Yorkstar	3.27 ± 0.40	18.5 ± 0.7	78.2 ± 0.7	30.4	48.2
Club	Paha	3.83 ± 0.93	18.4 ± 0.5	77.8 ± 1.4	30.2	47.6
	Omar	3.41 ± 0.69	18.2 ± 1.9	78.4 ± 2.3	32.1	46.3
Durum	Ward	3.44 ± 0.76	19.6 ± 1.0	77.0 ± 1.8	29.3	47.7
	Wells	4.14 ± 0.42	19.3 ± 0.5	76.6 ± 0.7	30.6	46.0
Average		3.97	19.1	76.9	30.3	46.7

[a] Deviations calculated from triplicate analyses except for components III and IV.
[b] See Figure 4.

isoamylase. The tetramodal distribution of fragments found in this study is similar to the profiles shown by others. However, the high resolution capabilities of the HPSEC, particularly within the high-molecular-weight range of the column system, allow for detection of the four fragments shown in Figure 4.

An estimate of the $\overline{DP}$ present in each of the four peaks was determined using a calibration curve prepared from standard solutions of glucose, maltose, maltohexaose, and amylose.

The $\overline{DP}$ value for the amylose obtained from Hayashibara was determined to be 90 by measuring the total carbohydrate and reducing values of the sample as described in the Methods section. In these determinations, Me_2SO affected the phenol-sulfuric acid method by contributing slightly to absorbance readings, and therefore the concentrations of Me_2SO should be constant in both control and experimental samples. The Somogyi-Nelson method was found not to be affected by the presence of Me_2SO. A similar result was found by Lineback and Sayeed (1971). During boiling, when the color intensity develops in proportion to the reducing power of the sample, the solutions are clear. However, upon dilution with water, a precipitate develops that must be removed by centrifugation before measuring the absorbance. Presumably, the formed precipitate is amylose that is not soluble upon dilution. The formation of the precipitate was found not to interfere with the absorbance readings.

The $\overline{DP}$ of fragments I, II, III, and IV (Fig. 4) was estimated as 86, 39, 19, and 12, respectively. In addition to the four standards used to obtain the calibration curve, three points ($\overline{DP}$ 10, 20, and 40) were inserted for reference. These three points were extracted from calibration-curve data.

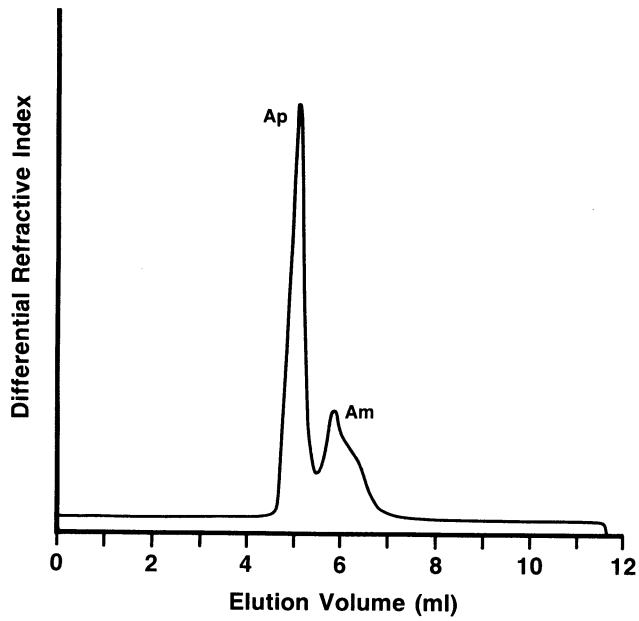

Fig. 1. HPSEC elution profile of native Scout prime starch: **Ap** = amylopectin, **Am** = amylose.

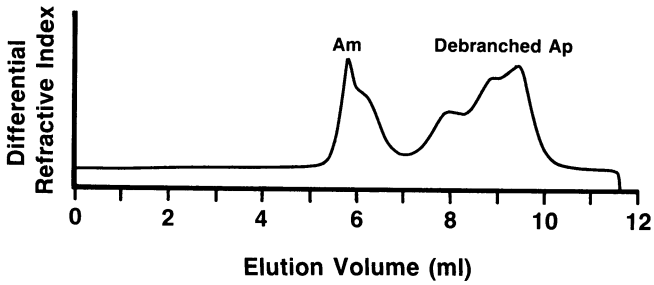

Fig. 2. HPSEC elution profile of isoamylase-treated Scout prime starch. Debranched **Ap** = debranched fragments from amylopectin; **Am** = amylose.

The elution volume of peak I is close to that of amylose. To detect this fragment, pure amylopectin is needed. If whole starch were used, amylose would elute with component I formed from amylopectin. The elution volume of wheat amylopectin was found to be 5.1 ml and that of peak I was 6.3 ml. The remaining debranched fragments (peaks II, III, and IV) were clearly differentiated from intact amylopectin.

Table I lists the relative amounts of the carbohydrate components from the debranched amylopectins of each starch. Each sample was injected in triplicate after two, three, and four days of debranching. The data shown are mean values from three different profiles and the calculated standard deviation. Data shown for peaks III and IV are single determinations and were

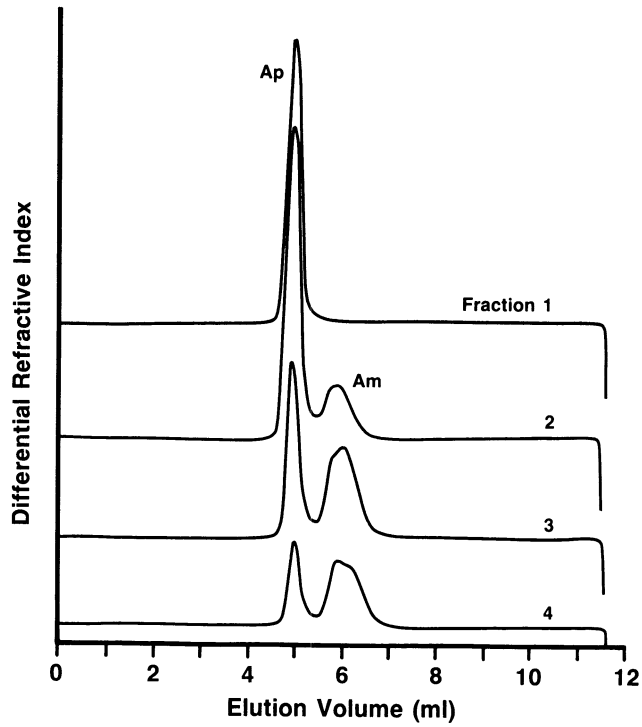

Fig. 3. Purity of fractions collected from preparative HPSEC eluate: **Ap** = amylopectin, **Am** = amylose.

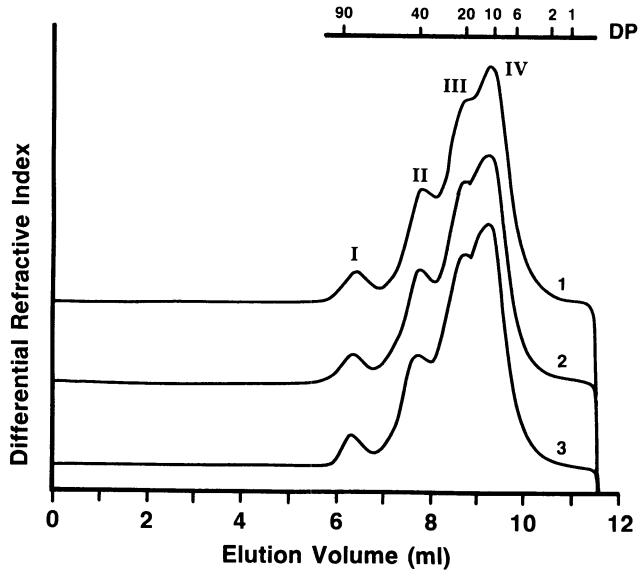

Fig. 4. Profiles of debranched Scout (**1**), Era (**2**), and Omar (**3**) amylopectins. $\overline{DP}$ scale obtained using glucose, maltose, and $\overline{DP}$ 90 amylose. $\overline{DP}$ points 10, 20, and 40 are estimates from calibration curve data and are inserted for reference.

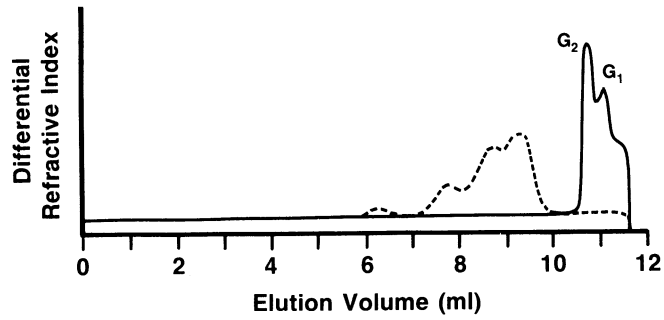

Fig. 5. Beta-amylase-treated debranched amylopectin. Dotted line, before treatment; solid line, after treatment. G_1 = glucose, G_2 = maltose.

determined by inserting lines from the apparent separation perpendicular to the baseline. The results indicate that no significant differences in the relative amounts of debranched fragments were found between the starch samples.

The percentage of components I, II, III, and IV as peak areas was measured as 3.97, 19.1, 30.5, and 46.4%, respectively. The relative quantities of each component are proportional to peak area, because the differential refractive index response factors for linear fragments in this region of the chromatogram were found to be identical. These response factors were measured by calibrating the instrument with known quantities of corn amylose and maltohexaose (G_6). Identical response factors were found for both samples. Maltohexaose elutes at an elution volume of 9.9 ml. Therefore, it was assumed that the refractive index response for linear polysaccharides eluting between amylose (elution volume 5.9 ml) and maltohexaose would be similar. Calculating the molar ratio of material in the fragments resulted in approximate values of 1, 10, 30, and 90 for peaks I, II, III, and IV, respectively.

The area ratio of material in peaks (III + IV)/peak II was 4.03, which corresponds to a molar ratio of 12.2. MacGregor and Morgan (1984) reported a weight ratio of 3.7 corresponding to peaks (III + IV)/peak II for debranched barley amylopectin. Kano et al (1981) observed a similar result for pullulanase-treated barley starch. Hood and Mercier (1978) reported a molar ratio of 7.5 for manioc amylopectin and Biliaderis (1982) calculated the molar ratio of the pullulanase debranched fragments of waxy maize starch and reported a value of 10.6. The values found in this study are slightly higher than previous studies. These differences may be attributed to differences in the amylopectins isolated from various starch sources. In addition, the different chromatographic methods used to separate these fragments could account for variation.

To determine whether each of the debranched fragments was linear, the mixture was treated with β-amylase. The chromatograms illustrated in Figure 5 show the carbohydrate profiles before and after treatment with β-amylase. Only maltose and a small amount of glucose should be formed from β-amylolysis of linear polysaccharides; however, our chromatograms (Fig. 5) indicate that large quantities of glucose, in addition to maltose, were also formed. The shoulder that appears after glucose arises from the injected solvent. A possible explanation for the presence of glucose could be that the β-amylase preparation contained contaminating α-amylase or α-glucosidase (maltase) activity.

The presence of α-amylase activity in the β-amylase enzyme was checked by incubating a β-limit dextrin with the β-amylase preparation. No alterations in the β-limit dextrin were observed by HPSEC and, during the incubation period, no detectable malto-oligosaccharides were found. Therefore, the β-amylase preparation did not contain α-amylase. Low levels of maltase activity were confirmed using maltose as a substrate. It is concluded that the debranched carbohydrates are all linear, because the fragments were completely hydrolyzed by β-amylase resulting in the formation of maltose. Subsequent enzymatic hydrolysis of maltose by the contaminating maltase resulted in the glucose found in these chromatograms. Glucose was found in these samples only after long incubation with β-amylase and/or when high concentrations of the enzyme were used. Therefore, only limited amounts of maltase are believed to be present in the β-amylase preparation.

In conclusion, the similarity in the patterns and amounts of debranched fragments from a total of ten different wheat amylopectins indicates that the structures of the amylopectins are closely similar. These results suggest that the structures of amylopectin will not predominantly affect the differences in functional properties of wheat starches. In addition, it was found that HPSEC is a useful method to rapidly monitor and estimate the quantities of debranched fragments formed from treatment of amylopectins with isoamylase.

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