

Separation and Characterization of Barley Starch Polymers by a Flow Field-Flow Fractionation Technique in Combination with Multiangle Light Scattering and Differential Refractive Index Detection

S. You,¹ S. G. Stevenson,² M. S. Izydorczyk,^{2,3} and K. R. Preston²

ABSTRACT

Cereal Chem. 79(5):624–630

Flow field-flow fractionation (flow FFF) with frit inlet and frit outlet mode (FIFO) was coupled online to multiangle light scattering (MALS) and refractive index (RI) detectors to investigate the molecular characteristics of normal and zero amylose barley starch polymers. Application of two different cross-flows, 0.35 mL/min followed by 0.1 mL/min, and constant channel and frit flows of 0.1 and 1.0 mL/min, respectively, permitted a complete separation of amylose and amylopectin. The improved signals from the detectors due to application of the FIFO mode enabled the proper characterization of the small molecular weight species, as well as significantly enhanced the reproducibility of

the measurements. The weight-average molecular weight (M_w) and z-average root-mean-square (RMS) radii of gyration (R_g) values for amylose and amylopectin in the normal starch samples were 2.3×10^6 and 280×10^6 , and 107 and 260 nm, respectively. The M_w and R_g of amylopectin in the zero amylose starch samples were 360×10^6 and 267 nm, respectively. The slopes (α) obtained by plotting $\log M_w$ versus $\log R_g$ for amylose and amylopectin were 0.6 and 0.3, respectively. These results are in good agreement with the theoretical prediction of the molecular conformation of amylose and amylopectin.

Starch is composed of two primary components, amylose and amylopectin. Although both polymers are built up of the same sugar unit, they differ significantly in solubility, molecular structure, molecular weight, and conformation (Hizukuri 1996). Amylose is a mostly linear polymer, composed of α -D-glucopyranose residues linked through 1 \rightarrow 4 linkages. Amylopectin is a large branched molecule with side chains attached to the linear α -(1 \rightarrow 4) polymer by α -(1 \rightarrow 6) linkages. The average molecular weight (M_w) of amylose may range from 1×10^5 to 1×10^6 g/mol, whereas that of amylopectin ranges from 1×10^7 to 50×10^7 g/mol (Banks et al 1972). Amylopectin and amylose have been successfully separated using size-exclusion chromatography (SEC). However, amylopectin, due to its extremely high molecular weight, elutes in the exclusion volumes of the SEC columns (Sullivan et al 1992). This makes the use of calibration standards for the estimation of its molecular weight not feasible. The absolute molecular weight of both starch polymers has been successfully determined by combining a multiangle light scattering (MALS) detector with SEC columns (Aberle et al 1994; Fishman et al 1996; Bello-Perez et al 1998; You et al 1999; You and Lim 2000; You and Izydorczyk 2002). This technique may provide accurate determination of the molecular weight of the starch components, provided that adequate starch solubilization and separation of amylose and amylopectin have been achieved, and that complete recovery of starch after the chromatographic elution can be ensured. Despite continuous efforts to enhance the resolving power of SEC columns, improvements in separation of starch polymers are still possible and remain desirable. Also, the SEC technique fails to provide information about the distribution of molecular weight in amylopectin because this polymer elutes as a relatively narrow peak in the void volume of all SEC columns. Even when pore diameters of the SEC stationary phase are relatively large compared with the sample particle diameter, and even when the largest molecules might theoretically show differing migration due to a combination of hydrodynamic chromatography and size-exclusion chromatography effects (Stegeman et al 1991), to the best of our knowledge, a good separation of amylopectin polymers has not yet been achieved. Possible shear degradation-alteration of large

molecules such as amylopectin inside the SEC column is another consideration that encourages the search for a more appropriate separation method for the starch polymers.

Recently, another separation technique, flow field-flow fractionation (flow FFF), has been developed as an alternative to SEC and has proven especially applicable to separation of very high molecular weight polymers (Giddings et al 1992; Giddings 1995). In contrast to the tubular and packed SEC column, FFF consists of a thin, open, ribbonlike channel with no packing material. Fractionation takes place in a completely liquid medium. Less shearing of large molecules and no interactions between the eluting species and the packing material occur in the FFF channel. The technique is, therefore, more suitable for characterization of very large macromolecules. One wall of the channel, called the accumulation wall, is permeable to liquids. A liquid flow with a parabolic velocity profile is pumped through the channel during separation. An external field, perpendicular to the channel flow, called the cross-flow, is also applied during separation. The actual separation of macromolecules is a function of their differential diffusion coefficient against the cross-flow and the parabolic profile of the channel flow. The coupling of flow FFF and the MALS detector makes possible the determination of absolute molecular weight and size of the fractionated molecules from flow FFF. The flow FFF-MALS system is a powerful tool for separation and characterization of large macromolecules such as polyvinyl pyrrolidone (Jiang et al 2000), dextran and pullulan (Wittgren and Wahlund 1997).

Recently, flow FFF has been successfully applied to separate and characterize such water soluble polymers as gum arabic (Picton et al 2000), dextrans and pullulans (Viebeck and Williams 2000a), κ -carrageenan (Wittgren et al 1998), and xanthan (Viebeck and Williams 2000b). To our knowledge however, only a few studies have utilized the FFF technique to separate and characterize starch polymers. Lou et al (1994) obtained only a partial separation of amylose and amylopectin using thermal FFF and dimethyl sulphoxide as a solvent. Sedimentation FFF fractionated only very large macromolecules (50–500 nm) and, therefore, was applicable to amylopectin only (Hanselmann et al 1995). Most recently, Roger et al (2001) indicated that fractionation of starch polysaccharides using flow FFF coupled to MALS and RI detectors was a promising alternative to the SEC/MALS/RI system. The FFF/MALS/RI system was used to separate corn starch polymers with varying amylose-amylopectin ratios. The authors initially used a high cross-flow to elute amylose then reduced cross-flow to elute amylopectin. A frit outlet was used to remove 50% of the channel flow between the end of the channel and detector to increase detector signal. This allowed

¹ Dept. of Food Science, University of Manitoba, Winnipeg, MB R3T 2N2, Canada.
² Grain Research Laboratory, 1404-303 Main Street, Winnipeg, MB R3C 3G8, Canada. Contribution 829 of the Grain Research Laboratory.
³ Corresponding author. E-mail: mizydorczyk@grainscanada.gc.ca. Phone: 204-983-1300.

measurement of the average molecular weight and radius of gyration for amylopectin, but no data could be obtained for amylose due to the low signal that was generated by the amylose. Also, the repeatability of the results was not confirmed in their study. In our laboratory, we have developed an automated frit inlet-frit outlet (FIFO) flow FFF procedure for the fractionation of wheat proteins (Stevenson et al 1999). Using recycled frit and cross-flow, this procedure eliminates the need for stop-flow relaxation, gives superior sensitivity and reproducibility (higher detector signal-to-noise ratio), and provides much higher potential throughput relative to conventional flow FFF techniques. In this article, we describe the application of this procedure to the fractionation and characterization of barley starch amylose and amylopectin.

MATERIALS AND METHODS

Normal and zero amylose (waxy) barley starches were isolated from hull-less barley cvs. Falcon and CDC Alamo, respectively, according to the recently published procedure (You and Izydorczyk 2002). Bovine serum albumin (BSA, Sigma Chemical, St. Louis, MO) was used to normalize the photo diodes located around the scattering cell in the MALS detector and to obtain the delay volume between MALS and RI. Three pullulan standards, P-200 (molecular weight 212,000), P-800 (molecular weight 788,000), and P-1600 (molecular weight 1.66×10^6) (Shodex Standards P-82, Showa Denko, Tokyo, Japan), were used to examine the flow FFF-MALS-RI system. All water was purified through a water purification system (Millipore, Mississauga, ON, Canada) before use.

Sample Preparation

Pullulans were dissolved in distilled water (2 mg/mL), filtered through 0.45- μ m membrane (Pall Gelman Laboratory, Ann Arbor, MI) and injected into a flow FFF-MALS-RI system.

Granular normal and zero amylose barley starches were gelatinized in 90% DMSO and precipitated with ethanol (Jane and Chen 1992). Various methods for starch solubilization were considered, particularly microwave heating at 140–150°C (Bello-Perez et al 1998) and autoclaving at 121°C (You and Lim 2000). Because of previous positive experience with dissolution of starch by autoclaving (You and Izydorczyk 2002), purified starches (4 mg) were redissolved in boiling water (8 mL) for 5 min, autoclaved at 121°C for 20 min, and centrifuged for 5 min at $8,000 \times g$. This treatment resulted in 75–80% solubilization as determined by measuring the total carbohydrate content (Dubois et al 1956) in the supernatant.

Apparatus and Procedures

Flow-FFF was performed using a model 1000-FIFO universal fractionator (PostNova Analytics USA, Salt Lake City, UT) as described previously (Stevenson et al 1999). The frit inlet (FI) was used for hydrodynamic relaxation to replace stop-flow relaxation and the frit outlet (FO) was used to remove eluent at the channel outlet to concentrate sample going to the detector (Fig. 1). Eluent removed through the frit outlet was recirculated through a pump back to the frit inlet, while cross-flow through the membrane was recycled through a pump back into the cross-flow to maintain pressure stability. The channel dimensions were length 27.7 cm, width 2.0 cm, and thickness 0.0254 cm. A YM-10 cellulose membrane (Amicon, M_w cutoff 10,000 Da) was placed on top of the accumulation wall inside the flow FFF channel. Sample solutions were injected through an injection valve (model 7725, Rheodyne) with a 20- μ L sample loop. The fractionated macromolecules from flow FFF were monitored by MALS (Dawn DSP, Wyatt Technology, Santa Barbara, CA) and RI (Waters 410, Waters, Mississauga, ON, Canada) detectors, which were calibrated using toluene and five different concentrations of NaCl solution, respectively. Calculations of the weight-average molecular weight (M_w), z-average root-mean-square (RMS) radii of gyration (R_g) were performed using the Astra 4.72 software (Wyatt Technology, Santa Barbara, CA). The Berry extrapolation (first-order) was used to calculate M_w and R_g . The dn/dc values of 0.148 and 0.146 mL/g were used for pullulan standards and starches, respectively. Water containing 0.002% FL-70 and 0.005% sodium azide was used as a mobile phase. It was filtered through 0.2- μ m and then 0.1- μ m nylon membranes (Osmonics Inc., MN), and degassed with stirring under vacuum for at least 2 hr. Three Shimadzu LC-10AD dual-piston pumps (Man-Tech Sci., Guelph, ON) were used to provide channel, cross, and frit flows for the flow FFF-MALS-RI system.

The effect of various cross-flows (0.5–1.0 mL/min) and frit flows (1.4–2.7 mL/min) at a fixed channel flow of 0.2 mL/min on the sample profile was examined using pullulan P-1600. The flow conditions applied to investigate the separation of various pullulan standards were channel flow 0.2 mL/min, cross-flow 1.0 mL/min, and frit flow 1.4 mL/min. A mixture (1:1) of pullulans (P-200 and P-1600) was also injected into flow FFF-MALS-RI system with the flow conditions of channel flow 0.2 mL/min, cross-flow 0.6 mL/min, and frit flow 1.4 mL/min.

The effect of various cross-flows (0.1–0.4 mL/min) on the eluting profiles of normal and zero amylose starches with a channel flow of 0.1 mL/min and frit flow of 1.0 mL/min was

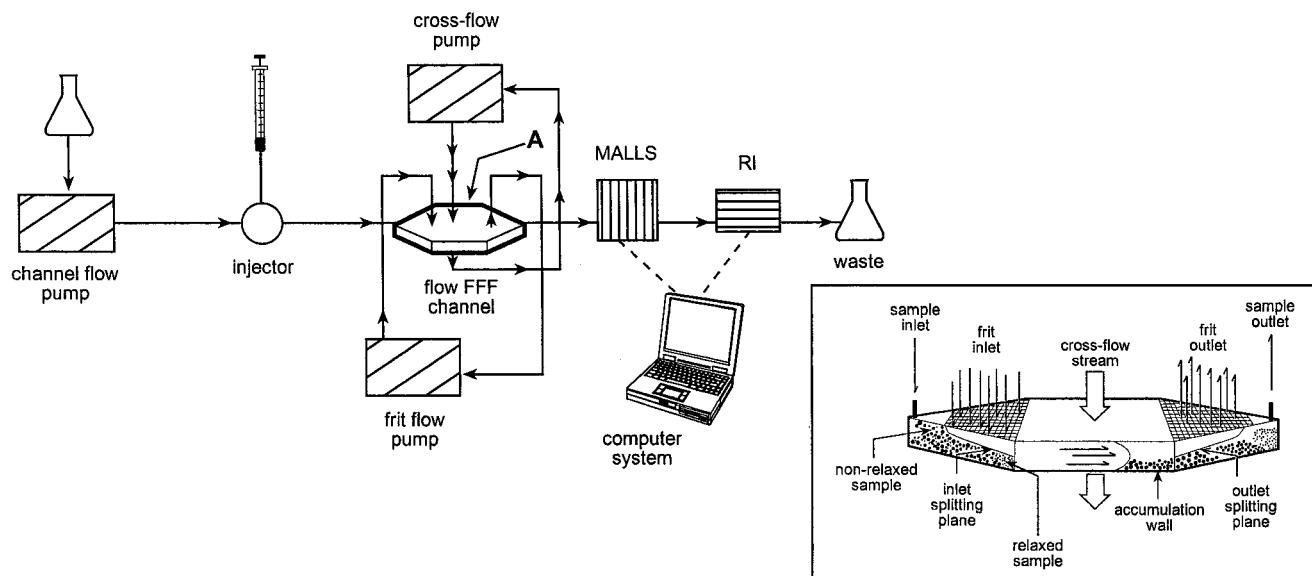


Fig. 1. Flow FFF-MALS-RI system and diagram of flow FFF channel (inset).

examined. To separate amylose and amylopectin molecules in a solution of normal barley starch, two different cross-flows were applied during the measurement. The initial cross-flow was 0.35 mL/min, and then, after the elution of amylose molecules (12 min), the cross-flow was reduced abruptly to 0.1 mL/min to allow amylopectin molecules to elute. This procedure was also applied to zero amylose starch. The amylopectin fractions of normal and zero amylose starches were examined with various cross-flows from 0.08–0.2 mL/min after the initial cross-flow of 0.35 mL/min for 12 min.

Sample recoveries were calculated from the ratio of the mass eluted from the channel as determined by the RI detector and the mass injected as determined by measuring the total carbohydrates (Dubois et al 1956). All treatments were done a minimum of two times on different days.

RESULTS AND DISCUSSION

Pullulan standards with average molecular weight of 0.2×10^6 , 0.78×10^6 , and 1.6×10^6 were initially used as model carbohydrates

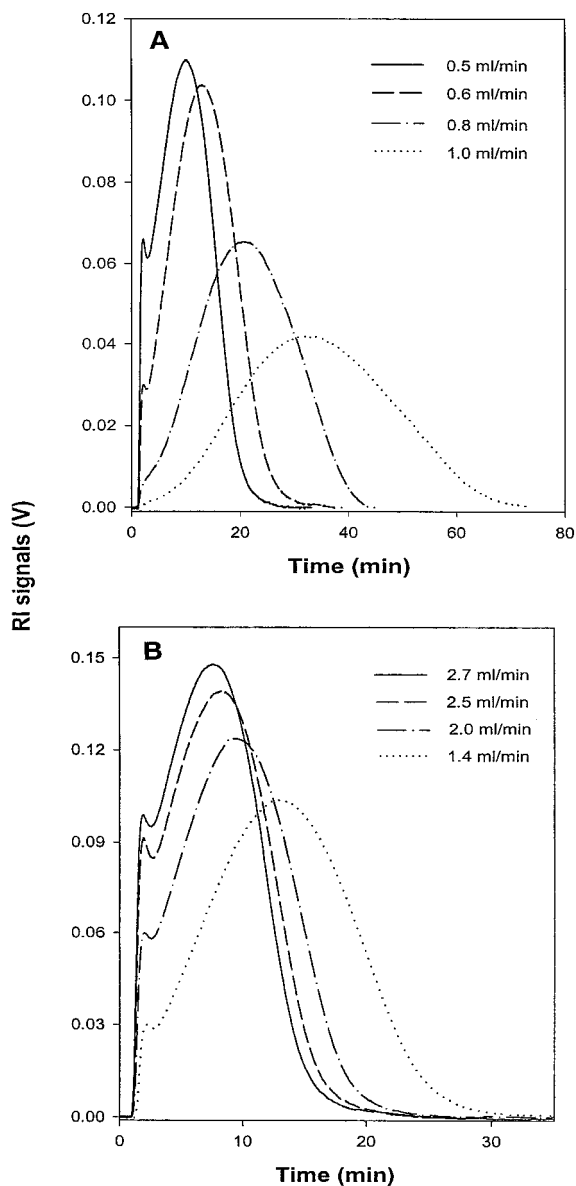


Fig. 2. A, Effects of various cross-flows on elution profiles of pullulan standard (P-1600); channel and frit flows 0.2 and 1.4 mL/min, respectively. **B,** Effects of various frit flows on elution profiles of pullulan standard (P-1600); channel and cross flows 0.2 and 0.6 mL/min, respectively.

to assess the efficiency of the FIFO flow FFF/MALS/RI system and to aid in designing conditions for separating starch polymers. Preliminary studies showed that a channel flow of 0.2 mL/min and a frit flow of 1.4 mL/min provided the best resolution without overly long running times (data not shown). A value of 7 for the ratio of frit to channel flows gave efficient hydrodynamic relaxation at the entrance to the channel and concentrated the pullulans in the channel outlet by removing most of the eluent through the frit outlet. This increase in component concentration greatly increased signal-to-noise ratios of the RI and MALS detectors allowing straightforward determination of concentration and molecular size parameters.

The effects of various cross-flows on the elution profiles of the largest pullulan standard (P-1600) are shown in Fig. 2A. As the cross-flow increased from 0.5 to 1.0 mL/min with constant channel (0.2 mL/min), and frit (1.4 mL/min) flows, the elution of pullulan molecules was delayed. In agreement with theory (Giddings and Caldwell 1989), the magnitude of the applied cross-flow determines the relative position of the molecules in the FFF channel and, consequently, their elution time. Symmetrical peaks with normal (Gaussian) distribution were obtained with higher rates of cross-flow; however, the elution time was longer. Lower cross-flows significantly reduced the elution time and caused the appearance of a small shoulder peak at the lowest retention time. This shoulder peak may be an overloading phenomenon where some of the components are displaced into faster moving regions where elution occurs earlier than expected (Benincasa 2000). Different rates of

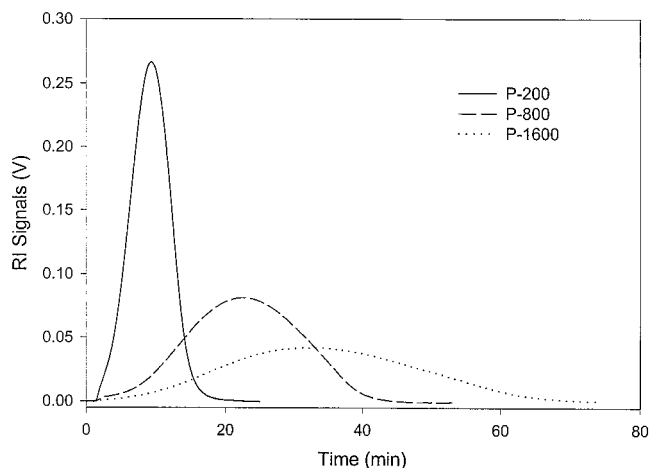


Fig. 3. Elution profiles of different pullulan standards (P-200, P-800, and P-1600) with channel, frit, and cross-flows of 0.2, 1.4, and 1.0 mL/min, respectively.

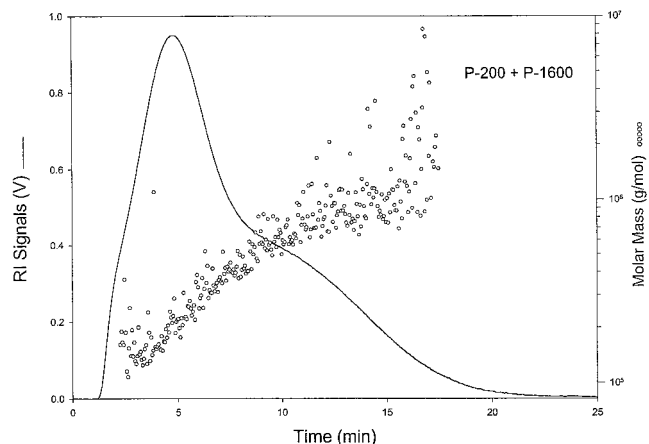


Fig. 4. Elution profile of a mixture of pullulan standards (P-200 and P-1600) with channel, frit, and cross-flows of 0.2, 1.4, and 0.6 mL/min, respectively.

the cross-flow also affected the accuracy of the calculated average molecular weights. With the cross-flows of 1.0 and 0.8 mL/min, the calculated M_w were in good agreement with the expected value, while less accurate values were obtained with lower cross-flows, probably because of the presence of the void peak. The effects of various frit flows with fixed channel (0.2 mL/min) and cross flows (0.6 mL/min) on the elution profiles of P-1600 are shown in the Fig. 2B. The higher frit flow facilitated faster elution of pullulan molecules, but it was accompanied by the appearance of the void peak. Again, higher rates of the frit flow lowered the accuracy of M_w determination.

Figure 3 shows the elution pattern of three different pullulan standards, run separately, using a cross-flow of 1.0 mL/min with a channel flow of 0.2 mL/min, and a frit flow of 1.4 mL/min. P-200 eluted first with a retention time (t_r) of 9.3 min, followed by P-800 with t_r of 23 min and P-1600 with t_r of 34 min. Calculated molecular weights of 224,000, 868,000 and 1.6×10^6 g/mol for P-200, P-800, and P-1600 pullulan standards, respectively, were in good agreement with the values provided by the manufacturer. Sample recoveries were >97%.

The fractogram of a pullulan mixture containing P-200 and P-1600 is presented in Fig. 4. Although it appears that only partial separation of the two pullulans was obtained under the conditions described above, calculated average molecular weights of the species eluting at a t_r of 2–8 min (206,000 g/mol) and 8–25 min (1.2×10^6 g/mol) corresponded relatively well with the expected values.

Separation of Starch Components

Because of the large differences in the molecular weight between pullulan and starch polymers, it was necessary to change the elution conditions for the latter. Upon application of a cross-flow ≥ 0.5 mL/min, quantitative elution of starch molecules was not achievable within acceptable analysis time, as indicated by continuous but low signal from the RI detector (results not shown). It was necessary, therefore, to apply a lower cross-flow. Recently,

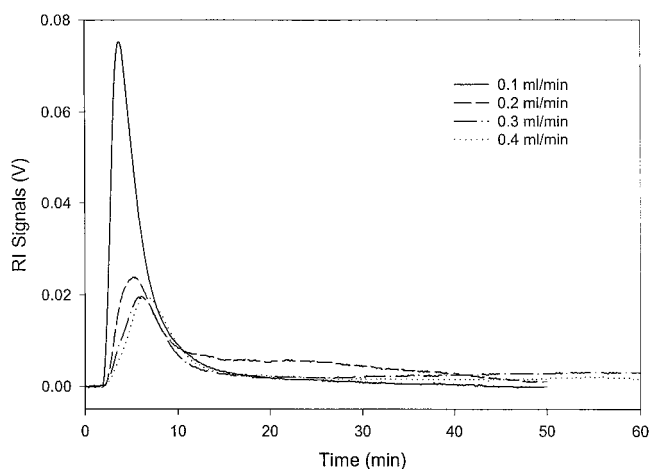


Fig. 5. Effects of various cross-flows on elution profiles of normal barley starch with channel and frit flows of 0.1 and 1.0 mL/min, respectively.

Jiang et al (2000) also reported that lower cross-flows were more suitable for elution and measurement of high molecular weight water-soluble polyvinyl pyrrolidone (M_w up to 10^7). To maintain good resolution, the authors also implemented lower channel flow to compensate for the lower cross-flow. In our studies, the ratio of frit to channel flows was kept within the range of 7–10, as recommended by the FFF manufacturer (Post Nova Analytics USA, Salt Lake City, UT), and frit and channel flows of 1.0 and 0.1 mL/min, respectively, were applied. Figure 5 shows the effects of various cross-flows (0.1–0.4 mL/min) on the RI profile of the eluting polymers in normal starch under these conditions. No separation of amylose and amylopectin was achieved with 0.1 mL/min cross-flow, as indicated by the appearance of one fairly symmetrical peak with an elution time of 2–10 min. Average values of M_w of 133×10^6 g/mol and R_g of 183 nm (Table I) for this material have no real meaning because no separation of amylose and amylopectin was accomplished. A significant decrease in the size of this peak (to $\approx 35\%$ of the original size) and the appearance of peak tailing were observed with increasing cross-flows from 0.1 to 0.3 mL/min. A further increase of the cross-flow to 0.4 mL/min caused only a small shift of the major peak's retention time. Generally the retention time (t_r) of the major peak slightly increased with increasing rate of cross-flow (Table I).

It appears that higher cross-flows delayed the elution of the high molecular weight starch polymers. This was demonstrated by the substantial decrease of the average M_w and R_g of molecules eluting at t_r 2–10 min with increasing cross-flows from 0.1 to 0.35 mL/min (Table I). Only a slight decrease of M_w was observed with the increase of the cross-flow to 0.4 mL/min. The M_w and R_g of the species eluting at t_r 2–10 min on application of the cross-flow ≥ 0.35 (Table I) correspond to the molecular dimensions of amylose polymers (You and Lim 2000; You and Izydorczyk 2002).

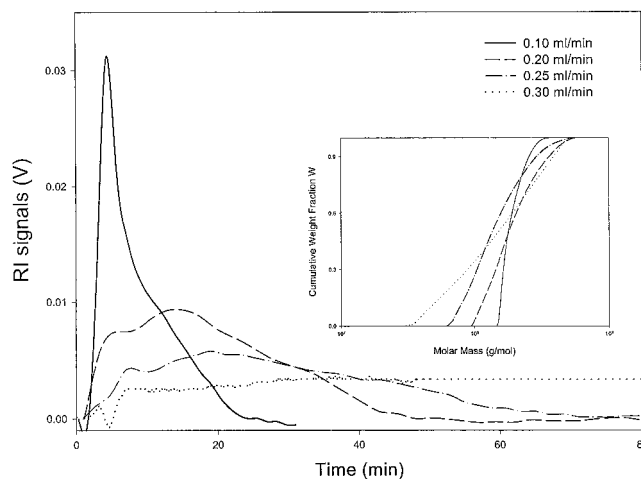


Fig. 6. Effects of various cross-flows on elution profiles of zero amylose starch; channel and frit flows of 0.1 and 1.0 mL/min, respectively. Cumulative molar mass distribution of zero amylose starch is shown in the inset.

TABLE I
Weight Average Molecular Weights (M_w) and Root Mean Square Radii of Gyration (R_g) of Normal and Zero Amylose Starches After Application of Various Cross-Flows

Cross-Flow (mL/min)	t_r (min)	Normal Starch		Zero Amylose Waxy Starch	
		M_w (g/mol $\times 10^{-6}$)	R_g (nm)	M_w (g/mol $\times 10^{-6}$)	R_g (nm)
0.1	6	133 ± 7.1	183 ± 9.9	187 ± 8.5	197 ± 5.4
0.15	6.8	58 ± 9.9	192 ± 7.1	156 ± 36	205 ± 1.4
0.2	7.5	30 ± 3.5	178 ± 15	191 ± 27	234 ± 0.7
0.25	8	11 ± 2.3	189 ± 13	164 ± 25	240 ± 5.7
0.3	8.3	2.9 ± 0.5	163 ± 7.1	216 ± 43	270 ± 22
0.35	8.5	2.1 ± 0.3	117 ± 27		
0.4	8.9	1.9 ± 0.1	123 ± 18		

It is likely, therefore, that application of the cross-flow ≥ 0.35 mL/min causes an initial retention of most of the amylopectin molecules inside the FFF channel while allowing the complete elution of amylose molecules. Eventually, slow elution of amylopectin occurs, but the process is very long, as indicated by elevated RI baselines (Fig. 5). Application of substantially lower cross-flow (0.1 mL/min), on the other hand, causes coelution of both small and large molecules.

Various cross-flows were also applied to elute the waxy barley starch containing no amylose (Fig. 6). With a very low cross-flow (0.1 mL/min), the elution of amylopectin was achieved within 24 min. The peak, however, was rather asymmetrical, with a portion of the material being eluted at a later stage. With increased cross-flow (0.2 mL/min), the elution of amylopectin was obtained within 50 min. The elution profile showed a broader distribution of molecular weights (Fig. 6, insert) at increased cross-flow rates. At higher cross-flow rates, the elution of the entire population of amylopectin molecules was not achieved within a reasonable time. These results indicate, therefore, that on application of appropriate cross-flow, it is feasible to obtain information about the molecular weight distribution in amylopectin. This has not been possible with size-exclusion chromatography because with the majority of SEC packing materials, amylopectin elutes as a narrow peak in the void volume. These results also support the contention that on application of a cross-flow ≥ 0.3 mL/min the species eluting at t_r 2–10 min contain mainly amylose.

The results presented above make it evident that efficient separation of amylose and amylopectin cannot be achieved with the application of any single rate of the cross-flow. The experimental conditions were, therefore, altered to allow application of an initial cross-flow of 0.35 mL/min for the first 12 min, followed by substantial reduction of the cross-flow to 0.1 mL/min. A clear separation of polymers was obtained when a sample of normal starch was subjected to these elution conditions, as indicated by the

appearance of two well-resolved peaks, one at t_r 3–12 min, and the other eluting only a few minutes after the change of the cross-flow at t_r 15–35 min (Fig. 7, top). Strong signals were obtained from both the RI and MALS detectors (Fig. 7). The average M_w and R_g of the polymers eluting in the first peak were 2.3×10^6 g/mol and 107 nm, respectively. These values clearly imply the presence of amylose polymers in the first peak. The average M_w and R_g of amylopectin polymers eluting in the second peak were 280×10^6 g/mol and 260 nm, respectively (Table II). Previous studies, which employed the SEC-MALS-RI system for the separation of amylose and amylopectin in barley starches, estimated the M_w of the fractions corresponding to amylose and amylopectin at 5.67×10^6 g/mol and 226×10^6 g/mol, respectively (You and Izydorczyk 2002). Considerable difference in the M_w of the amylose fraction in particular, as estimated on the basis of the SEC and the flow

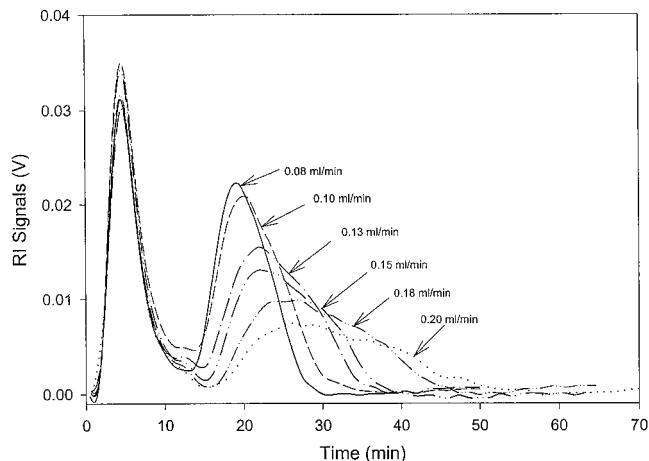


Fig. 8. Elution profiles of normal barley starch with constant initial cross-flow (0.35 mL/min) followed by various second cross-flows (0.08–0.2 mL/min).

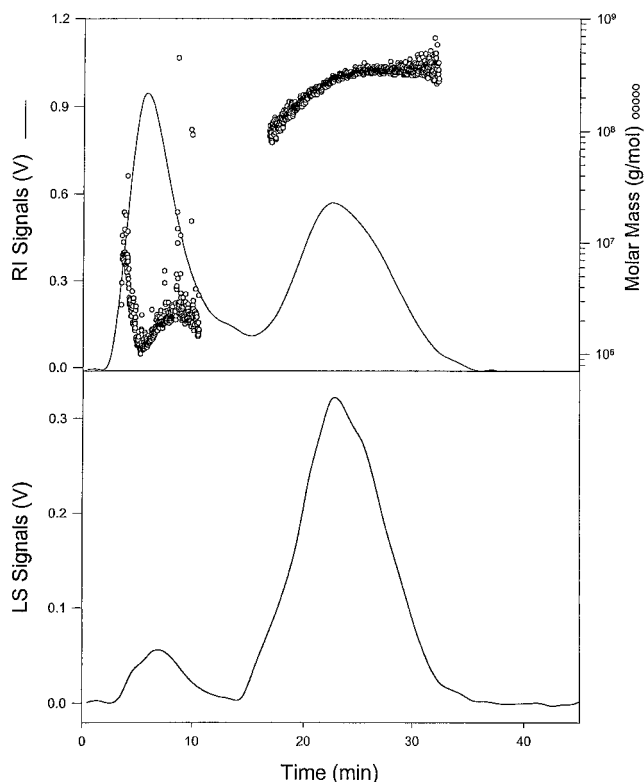


Fig. 7. Refractive index (RI) (top) and light scattering (LS) (bottom) fractograms of normal barley starch. Separation of amylose and amylopectin on application of two cross-flows: 0.35 mL/min (12 min) followed by 0.1 mL/min.

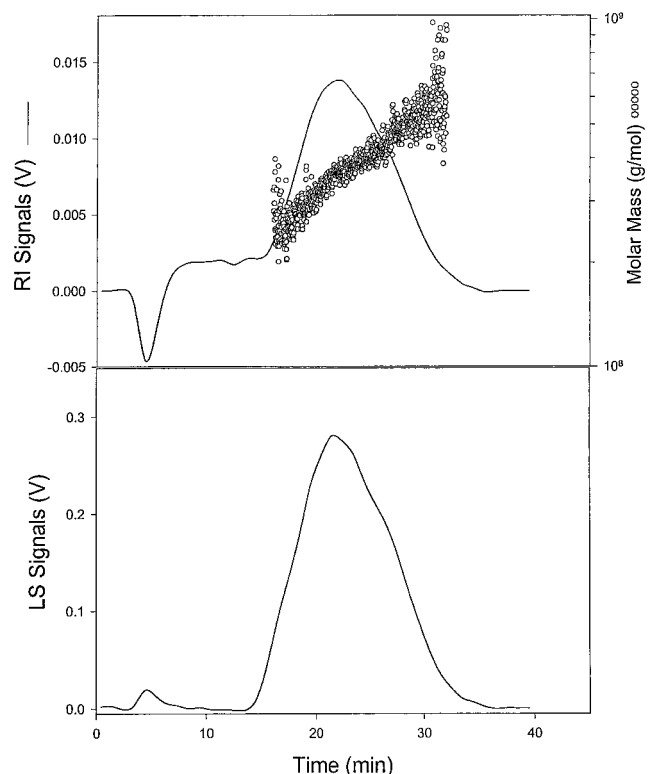


Fig. 9. Refractive index (RI) (top) and light scattering (LS) (bottom) fractograms of zero amylose starch: initial cross-flow 0.35 mL/min (12 min) followed by 0.1 mL/min.

FFF, may reflect substantial differences in the efficiency of separation of starch polymers achieved by these two techniques. In the SEC-MALS-RI system, the estimation of M_w of amylose may be affected by amylopectin polymers eluting in the same region as the amylose fractions. Similar discrepancies in the M_w of gum arabic polysaccharides estimated with SEC-MALS-RI and flow FFF-MALS-RI systems were recently reported by Picton et al (2000). Recently, Roger et al (2001) have also used two different rates of the cross-flow, 0.6 mL/min followed by a stepwise decrease to 0.1 mL/min, to achieve separation of amylose and amylopectin by flow FFF. Elution of polymers was obtained faster (11 min) than in our experiments (35 min). However, the elution of amylose was obscured by the presence of a void peak; also, the signal from the light scattering detector was too low to afford characterization of this polymer.

Comparison of the areas under the two peaks indicated that the low molecular weight species eluting in the first peak constituted 46.8% of the entire population eluted under the above conditions. These values are not in good agreement with the total amylose content (26%) in the normal barley starch as measured previously by potentiometric iodine titration (You and Izydorczyk 2002). Because only 70.3% of the total sample was recovered, it is possible that a portion of the amylopectin was retained in the FFF channel and was not eluted under the experimental conditions. It is also possible that, due to problems with incomplete solubilization of starch, the material applied to the FFF channel contained a different proportion of amylose and amylopectin compared with that in the native starch samples.

To optimize the elution of amylopectin, different rates of the second cross-flow (0.08–0.20 mL/min) were tested using a sample of normal barley starch (Fig. 8). A decrease of the second cross-flow to 0.08 mL/min slightly improved the amylopectin recovery and shortened the elution time. On the other hand, increasing the rates of the second cross-flow progressively extended the elution of amylopectin over a broader range of volume and afforded a better insight into the distribution of molecular weights in amylopectin. Recovery of amylopectin was, however, progressively reduced.

Another barley starch sample containing no amylose was also eluted under the two different cross-flow conditions used for the separation of amylose and amylopectin. As shown in Fig. 9, only one peak corresponding to the high molecular weight amylopectin fraction was observed. The average M_w and R_g were 360×10^6 g/mol and 267 nm, respectively (Table II). The sample recovery, however, was only 50%. Slightly lower values of M_w (299×10^6 g/mol) and R_g (262 nm) for the same sample were obtained using the SEC/MALS system (sample recovery was 71%) (You and Izydorczyk 2002). The low sample recovery obtained with the FFF technique is not fully understood, although we have shown before that the high rate of cross-flow has a particularly negative effect on the recovery of amylopectin (Fig. 8). Such low sample recovery raises a question whether the M_w obtained from this experiment was representative of the whole population of amylopectin chains injected into the channel. Because the average M_w of amylopectin obtained with the FFF technique was higher than that obtained for the same sample with SEC (which had substantially

higher recovery), it is rather unlikely that the high molecular weight amylopectin chains were specifically retained in the channel and therefore not eluted and measured by the detectors. These results show, however, that the recovery problems in the FFF technique are mostly associated with amylopectin. Similar observations have been made in SEC studies (Bello-Perez et al 1998; Yokoyama et al 1998). It appears that the low recovery of amylopectin in the SEC column or FFF channel is associated with difficulties in solubilizing and maintaining this polymer in solution. The behavior of amylopectin in solution and its tendency for aggregation, especially under conditions of shear stress as generated by the combining effects of channel, frit, and cross flows, is not sufficiently understood. More research is needed to optimize the FFF conditions (especially finding the optimum ratio of the channel to frit to cross flows) to obtain better sample recovery without loss of resolution. Repeated gelatinization of amylopectin in DMSO followed by precipitation with ethanol might improve solubilization and dispersity of amylopectin in solution.

Molecular conformations of amylose and amylopectin polymers from normal starch were examined by plotting $\log R_g$ versus $\log M_w$ (Fig. 10). The slope of the line corresponds to the exponent α in the equation $R_g^{1/2} = KM_w^\alpha$, and is related to the conformation of polymers in solution (Astra for Windows User's Guide). Theoretically, the values of α are assigned to sphere, random coils, and rigid rods, and are 0.33, 0.50, and 1.0, respectively (Astra for Windows User's Guide). For amylose, rather scattered data points were obtained when $\log R_g$ versus $\log M_w$ was plotted but the calculated α value of 0.6 for the slope suggested a very different conformation than that obtained for amylopectin ($\alpha = 0.3$). The above results are in a good agreement with theoretical predictions

TABLE II
Weight Average Molecular Weights (M_w), Root Mean Square Radii of Gyration (R_g), and Sample Recoveries of Separated Amylose and Amylopectin from Normal and Zero Amylose Starches by Two Cross-Flows

	Normal Starch		Zero Amylose Waxy Starch	
	Amylose	Amylopectin	Amylose	Amylopectin
M_w (g/mol $\times 10^{-6}$)	2.3 ± 0.1	280 ± 7.8	–	360 ± 9.9
R_g (nm)	107 ± 5.6	260 ± 14	–	267 ± 2.8
Recovery (%)	70.8 ± 2.0		50.0 ± 4.0	

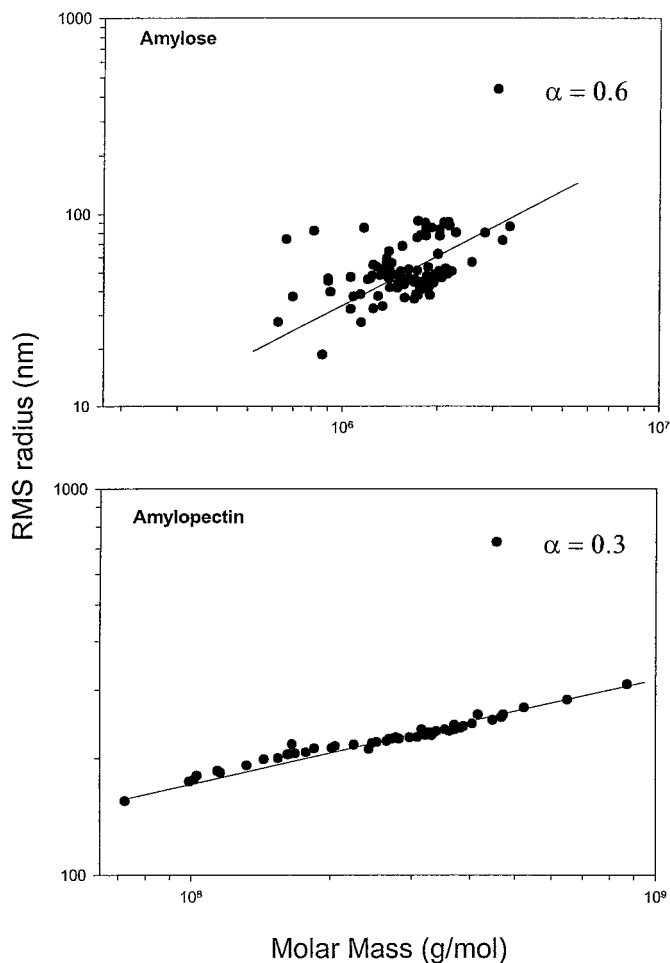


Fig. 10. Distribution of R_g and M_w for amylose and amylopectin polymers.

because amylopectin, due to its branching characteristics, assumes a more compact conformation in solution than amylose.

CONCLUSIONS

The results of the present study indicate that flow FFF-MALS offers the means for separation and size characterization of starch polymers. The key factor determining good polymer resolution is using two appropriate rates of the cross-flow; the initial higher rate of the cross-flow allows elution of only amylose, and the subsequent lower cross-flow affords elution of amylopectin. Appropriate rate of the second cross-flow allows better insights into the distribution of molecular weights in amylopectin. The use of the FIFO mode eliminated the requirements for stop-flow relaxation and pressure balancing and resulted in a more stable baseline, enhanced resolution, and better reproducibility of the molecular weight measurements. The enhanced signals obtained due to the application of the FIFO mode enabled the proper characterization of amylose. In addition, recycling the eluent through the FIFO mode creates the potential for automation of the system. However, further optimization is still needed to improve the recovery of the amylopectin molecules in particular. Also, development of appropriate strategies ensuring complete solubilization of starch in water is required to obtain valid characterization of both polymers, amylose and amylopectin, using the flow FFF/MALS/RI system.

ACKNOWLEDGMENTS

We thank the Natural Science and Engineering Research Council and the Canada Foundation for Innovation for their financial support. We also thank A. W. MacGregor for his valuable advice during the course of this study and M. Ramesh for his contribution during the experiments.

LITERATURE CITED

Aberle, Th., Burchard, W., Vorwerg, W., and Radosta, S. 1994. Conformational contributions of amylose and amylopectin to the structural properties of starches from various sources. *Starch* 46:329-335.

Banks, W., Geddes, R., Greenwood, C. T., and Jones, I. G. 1972. Physico-chemical studies on starches. 63. The molecular size and shape of amylopectins. *Starch* 24:245-251.

Bello-Perez, L. A., Roger, P., Baud, B., and Colonna, P. 1998. Macromolecular features of starches determined by aqueous high-performance size exclusion chromatography. *J. Cereal Sci.* 27:267-278.

Benincasa, M. A. 2000. Synthetic polymers—Water soluble. Pages 407-432 in: *Field-flow Fractionation Handbook*. M. Schimpf, K. Caldwell, and J. C. Giddings, eds. John Wiley and Sons: New York.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* 28:350-356.

Fishman, M. L., Rodriguez, L., and Chau, H. K. 1996. Molar masses and sizes of starches by high-performance size-exclusion chromatography with on-line multi-angle laser light scattering detection. *J. Agri. Food Chem.* 44:3182-3188.

Giddings, J. C. 1995. Measuring colloidal and macromolecular properties by FFF. *Anal. Chem.* 59A:592A-598A.

Giddings, J. C., and Caldwell, K. D. 1989. Field-flow fractionation. Pages 867-938 in: *Physical Methods of Chemistry*, 2nd Ed. B. W. Rossiter and J. F. Hamilton, eds. Wiley-Interscience: New York.

Giddings, J. C., Benincasa, M. A., Liu, M.-K., and Li, P. 1992. Separation of water soluble synthetic and biological macromolecules by flow field-flow fractionation. *J. Liq. Chromatogr.* 15:1729-1747.

Hanselmann, R., Ehart, M., and Widmer, H. M. 1995. Sedimentation field flow fractionation combined with multiangle laser light scattering applied for characterization of starch polymers. *Starch* 46:345-349.

Hizukuri, S. 1996. Starch: Analytical aspects. Pages 347-430 in *Carbohydrates in Foods*. A.-C. Eliasson, ed. Marcel Dekker: New York.

Jane, J., and Chen, J. F. 1992. Effect of amylose molecular size and amylopectin branch chain length on paste properties of starch. *Cereal Chem.* 69:60-65.

Jiang, Y., Miller, M. E., Li, P., and Hansen, M. E. 2000. Characterization of water-soluble polymers by flow FFF-MALS. *Am. Lab.* Feb:98-108.

Lou, J., Myers, M. N., and Giddings, J. C. 1994. Separation of polysaccharides by thermal field-flow fractionation. *J. Liq. Chromatogr.* 17:3239-3260.

Picton, L., Bataille, I., and Muller, G. 2000. Analysis of a complex polysaccharide (gum arabic) by multi-angle laser light scattering coupled on-line to size exclusion chromatography and flow field flow fractionation. *Carbohydr. Polym.* 42:23-31.

Roger, P., Baud, B., and Colonna, P. 2001. Characterization of starch polysaccharides by flow field-flow fractionation-multi-angle laser light scattering-differential refractometer index. *J. Chromatogr. A.* 917:179-185.

Stegeman, G., Kraak, J. C., and Poppe, H. 1991. Hydrodynamic and size-exclusion chromatography of polymers on porous particles. *J. Chromatogr.* 550:721-739.

Stevenson, S. G., Ueno, T., and Preston, K. R. 1999. Automated frit inlet/frit outlet flow field-flow fractionation for protein characterization with emphasis on polymeric wheat proteins. *Anal. Chem.* 71:8-14.

Sullivan, C. R., Corona, A., and Rollings, J. E. 1992. Chromatographic technologies for macromolecular starch characterization. Pages 193-238 in: *Developments Carbohydrate Chemistry*. R. J. Alexander and H. F. Zobel, eds. Am. Assoc. Cereal Chem.: St. Paul, MN.

Viebke, C., and Williams, P. 2000a. The influence of temperature on the characterization of water-soluble polymers using asymmetric flow field-flow-fractionation coupled to multiangle laser light scattering. *Anal. Chem.* 72:3896-3901.

Viebke, C., and Williams, P. A. 2000b. Determination of molecular mass distribution of κ -carrageenan and xanthan using asymmetrical flow field-flow fractionation. *Food Hydrocolloids* 14:265-270.

Wittgren, B., Borgstrom, J., Piculell, L., and Wahlund K.-G. 1998. Conformational change and aggregation of κ -carrageenan studied by flow field-flow fractionation and multiangle light scattering. *Biopolymers* 45:85-96.

Wittgren, B., and Wahlund, K.-G. 1997. Fast molecular mass and size characterization of polysaccharides using asymmetrical flow field-flow fractionation-multiangle light scattering. *J. Chromatogr. A* 760:205-218.

Yokoyama, W., Renner-Nantz, J. J., and Shoemaker, C. F. 1998. Starch molecular mass and size by size-exclusion chromatography in DMSO-LiBr coupled with multiple angle laser light scattering. *Cereal Chem.* 75:530-535.

You, S., Fiedorowicz, M., and Lim, S.-T. 1999. Molecular characterization of wheat amylopectins by multiangle laser light scattering analysis. *Cereal Chem.* 76:116-121.

You, S., and Izydorczyk, M. S. 2002. Molecular characteristics of barley starches with variable amylose content. *Carbohydr. Polym.* 49:33-42.

You, S., and Lim, S.-T. 2000. Molecular characterization of corn starch using an aqueous HPSEC-MALLS-RI system under various dissolution and analytical conditions. *Cereal Chem.* 77:303-308.

[Received September 20, 2001. Accepted April 19, 2002.]