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Simple Purification (Desalting) Procedure To Facilitate Structural Analysis of an Alkali-Solubilized/Neutralized Starch Solution by Intermediate-Pressure Size-Exclusion Chromatography

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A technique was established to remove impurities (e.g., salts) from starch dissolved in strong alkali and neutralized with acid to accommodate starch structural analysis via intermediate-pressure size-exclusion chromatography (IPSEC). Starch (corn and wheat) subjected to an alkaline-microwave dissolution scheme (35 s microwave heating in a mixture of 6 M urea and 1 M KOH) was either treated with ion-exchange resin or passed through a desalting column to remove salt/urea contaminants. Control (untreated) starch solution analyzed by IPSEC displayed a significant interfering peak (attributable to salt/urea), which coeluted with the starch amylose peak. The interfering peak was most efficiently eliminated by first passing the starch solution through a desalting column, which process effectively removed impurities (e.g., salts/urea) without appearing to adversely impact the starch structural analysis. This simple technique coupled with the rapid alkaline-microwave starch dissolution procedure greatly expedites structural investigation of starch by facilitating analysis by IPSEC.

KEYWORDS: Alkaline-microwave starch dissolution; desalting; intermediate-pressure size-exclusion chromatography (IPSEC); starch structure; corn starch; wheat starch

INTRODUCTION

Starch, one of the most abundant biomaterials in the world, is a homopolysaccharide of D-glucose and consists of two distinct polymers: amylose and amylopectin. Amylose is predominantly a linear-chain molecule comprised of anhydroglucose units (AGU) connected via $\alpha(1 \rightarrow 4)$ linkages with a molecular weight of 10^5 to 10^6 g/mol (*1*, *2*). Amylopectin possesses a branch-on-branch structure and a molecular weight ranging from 10^7 to 10^9 g/mol. Linear regions of branch and main chains of amylopectin are connected via $\alpha(1 \rightarrow 4)$ glycosidic bonds, while branch chains themselves are attached to the amylopectin molecule via $\alpha(1 \rightarrow 6)$ linkages with branch points occurring every 20−30 AGU on average (*3*−*5*). Starch structural characteristics have been commonly analyzed by low-pressure, intermediate-pressure, or high-performance size-exclusion chromatography (LP-, IP-, or HP-SEC, respectively) using refractive index (RI) and/or other varied modes of detection (*1*−*4*, *6*−*17*).

One of the most important steps for starch structural characterization involves the dissolution of semicrystalline starch granules using an appropriate solvent to solubilize the individual polymers. Most traditional dissolution methods involve pretreatment of starch granules (heating in 90% aqueous dimethyl sulfoxide (DMSO), followed by precipitation) and redissolution of the starch precipitate in water via physical (e.g., stirring, homogenization, sonication) and thermal (e.g., boiling, autoclaving) processes (*3*, *6*−*11*). However, these methods are time- and labor-intensive, often requiring 12−24 h to achieve complete dissolution.

Strong aqueous alkali is also considered a good solvent for dissolution (*12*, *13*) and has been used to expedite starch dissolution and prevent molecular aggregation of starch molecules. However, starch dissolution in alkali most often requires neutralization prior to SEC analysis, generating neutral salts that can coelute and interfere with the amylose peak during SEC-RI or SEC-MALLS-RI analysis (*14*, *15*). Jackson et al. (*14*) noted that negatively charged ions (e.g., Cl^-), resulting from neutralization with HCl, eluted through the SEC column faster than would be predicted on the basis of their molecular weights and tended to overshadow the amylose peak. Several schemes have been employed to minimize the interference of salts with the SEC-RI analysis of starch. One of the most common methods involves the dissolution of starch in a minute volume of strong alkali, followed by neutralization with a small volume of acid, and a final dilution with mobile phase (up to 11 times) (*4*, *10*, *16*) to reduce the relative size of salt peaks and to minimize interference with the amylose peak. Another scheme

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consists of adjusting the alkali concentration of the starch solution (dissolved in strong alkali) to that of the alkaline mobile phase without a neutralization step (*1*, *2*, *12*, *13*, *15*, *17*). Both schemes eliminate the possibility of conducting SEC using a pure water mobile phase, while the latter exposes both the starch (potential degradation of structure in alkali) and the HPSEC system (harsh operating conditions) to a caustic environment for extended periods of time. Efforts still are needed to further optimize post-treatment of starch solutions dissolved in strong alkali to minimize or eliminate the interference of salts associated with starch SEC analysis.

Most recently, a rapid dissolution scheme has been reported that utilizes microwave heating of starch in a mixture of strong alkali and urea for 35 s to achieve starch solubilization (*18*). Following neutralization, the rapid starch dissolution scheme provides a good separation between amylopectin and amylose molecules on a preparative (low-pressure) size-exclusion column, which is not adversely impacted by the presence of salts. However, starch fractionation on a preparative column requires lengthy run times (24 h) and labor-intensive analyses (e.g., determination of carbohydrate contents and blue values for individual fractions), which undermine the benefits of a rapid dissolution method. This problem could be circumvented by combining the rapid dissolution method with more quick and automated IP- or HP-SEC analysis methods. However, the issue of interfering salt peaks previously discussed for alkali starch dissolution methods precludes this possibility. The objective of this work was to investigate an effective procedure for the removal of salts and urea from the solubilized starch solution to facilitate use of the rapid microwave starch dissolution method (and potentially any alkaline dissolution method) with an IPSEC system. Such a procedure would permit both dissolution and structural analysis of starch to be accomplished in just a matter of a few hours for a single sample.

MATERIALS AND METHODS

Starch Sources. Waxy and normal soft wheat starches were isolated from straight-grade flours of wheat cultivars (Leona and Madsen, respectively) according to the modified protease-digestion isolation method outlined by Shinde et al. (*19*). Commercial waxy and normal corn starches were provided by Tate and Lyle (Decatur, IL), while high-amylose corn starch (Hylon-V, ≈55% amylose) was obtained from National Starch and Chemical Co. (Bridgewater, NJ). Corn starches were used without further purification.

Starch Solubilization. Starches were solubilized according to an alkaline-microwave dissolution method, which was previously validated through direct comparison to the traditional aqueous DMSO dissolution procedure (*18*). Starch (36 mg, dry basis (d.b.) was suspended in 0.5 mL of 6 M urea and 10 mL of 1 M KOH in a 25 mL glass tube and vortexed mildly. The suspension was transferred to a 90 mL Teflon·PFA jar (Savillex, Minnetonka, MN) and heated for 35 s in a 2450 MHz microwave oven AR732 (Emerson Radio Co., North Bergen,
NJ). After cooling to ambient temperature (15 min), the starch solution was neutralized (pH 7−7.6) with 4 M HCl and passed through a 5 μm nylon syringe filter (National Scientific Co., Duluth, GA) prior to injection onto an IPSEC system. The solubility (%) of starch was calculated as the percent of the carbohydrate content (mg/mL) of the starch solution after and before filtration (*11*, *15*). The total carbohydrate values were determined using the phenol-sulfuric acid colorimetric method (*20*).

Removal of Salt/Urea Contaminants. Purification of the starch solution subjected to the alkaline-microwave dissolution method was investigated using both an ion-exchange (IEX) resin and a pre-packed desalting column. IEX resins have been utilized by others (*21*, *22*) for the removal of buffer ions from debranched starch solutions to eliminate interference during starch SEC analysis. For treatment with IEX, a cooled starch solution (10 mL, described in the previous section) was mixed with resin beads (1−5 g) (IONAC NM-60 H^+/OH^- form, J. T. Baker, Phillipsburg, NJ) and shaken on a Wrist Action Shaker (Model 75, Burrell Co., Pittsburgh, PA) at room temperature for 30 min. Following this process, the mixture was passed through a 5 μm nylon syringe filter (National Scientific Co., Duluth, GA) to remove IEX beads and subjected to IPSEC analysis.

Desalting was also investigated using a column (1.6 cm × 5.0 cm) packed with Sephadex G-25 medium gel (model PD-10, exclusion range M_r 1000−5000, Amersham Biosciences, Piscataway, NJ). The desalting column was first eluted with 10 mL of mobile phase (deionized water containing 0.02% (w/v) sodium azide) prior to loading the starch solution onto the column. The cooled starch solution (2.5 mL) was then loaded directly onto the desalting column via gravity flow. Sample loading proceeded until 2.4 mL of eluent (representing the column void volume) was collected from the column. Starch was then eluted from the column in two successive volumes (2.6 and 2 mL) with the addition of subsequent mobile phase based on preliminary trials with blue dextran. The initial 2.6 mL (fraction I, purified starch/free of contaminants) was set aside. The last 2 mL of collected eluate (fraction II, contained both starch and urea/salt contaminants) was retained for further purification (additional passage through the desalting column).

The column was washed with 10 mL of mobile phase to remove retained urea/salts from the column, after which the previously collected 2 mL fraction (fraction II) was loaded onto the column as previously described. Additional mobile phase was added to elute the starch from the column. As previously described, the initial 2.4 mL of eluate (including that collected during sample loading) was discarded (void volume), while the next 2.4 mL (purified fraction II, starch/free of contaminants) was collected. The 2.4 mL volume endpoint for fraction II represented the point at which no more carbohydrate was detected in the eluate (starch had been eluted from the column). The final starch fractions I and II were combined, and the resultant mixture was subjected to IPSEC analysis. Starch recovery (%) through the desalting column was calculated as the percent of the total carbohydrate content (mg) of starch solution after (collected 5.0 mL) and before (original 2.5 mL) passage through the PD-10 column. A dilution factor for the procedure was calculated and defined as the ratio of the carbohydrate concentration (mg/mL) of the starch solution before (original 2.5 mL) and after (collected 5.0 mL) passing through the desalting column. The total carbohydrate content and concentration were determined using the phenol-sulfuric acid colorimetric method (*20*).

IPSEC. The starch solution was injected onto an IPSEC system comprised of a model 1525 Binary HPLC pump, a Rheodyne 7725i manual sample injector with a 200 μL sample loop (Waters Co., Milford, MA), and a model 2410 refractive index detector (Waters Co., Milford, MA). Separation was achieved using HR 16/50 and Tricorn 10/300 columns packed with Sephacryl S-500 HR gel (70 cm in gel height, exclusion range M_r 4×10^4 to 2×10^7, Amersham Biosciences, Piscataway, NJ) operated in series at room temperature similar to that described by Han et al. (*23*). The mobile phase consisted of filtered, deionized water containing 0.02% (w/v) sodium azide (flow rate = 0.8 mL/min). The mobile phase was filtered offline by passing through a 0.2 μm membrane filter and further deaerated by sonication for 20 min. Peak areas (%) of amylopectin and amylose from chromatograms were automatically calculated by Breeze HPLC system software (Waters Co., Milford, MA).

Statistical Analyses. All experiments were repeated twice, and chromatograms representing each treatment were depicted using the mean values of replicate analyses. Starch solubilities, desalting column recoveries, and dilution factors were analyzed by analysis of variance (ANOVA) and a least significant difference (LSD) test at $p < 0.05$. All statistical computations and analyses were conducted using SAS version 9.1 for Windows (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Removal of Salt/Urea Contaminants. The normal soft wheat starch solution prepared according to the alkaline-microwave dissolution scheme (35 s microwave heating in a mixture of 1 M KOH and 6 M urea) was directly injected onto an IPSEC system without any purification/desalting. The chromatogram

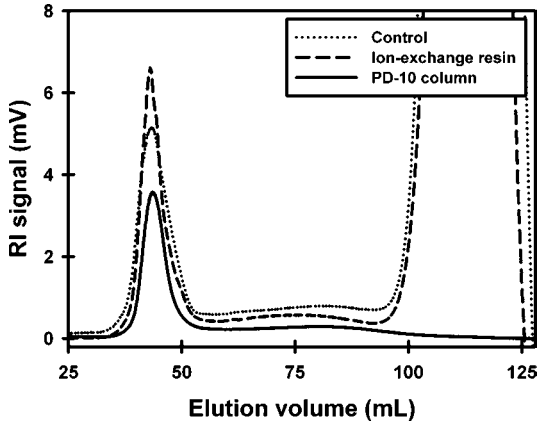

Figure 1. Chromatograms for normal soft wheat starch solubilized by the alkaline-microwave dissolution scheme subjected to no treatment (control), treatment with an ion-exchange resin, and passage through a PD-10 desalting column prior to fractionation by IPSEC.

revealed peaks for amylopectin and amylose, as well as a huge interfering peak that coeluted with amylose (**Figure 1**, control). While the amylopectin peak was symmetrical and clearly resolved, the amylose peak was compromised by the presence of the overshadowing peak, which precluded appropriate determination of the amylose/amylopectin ratio. By running a blank (subjected to the same alkaline-microwave dissolution scheme without added starch), the interfering peak was confirmed to be induced from residual urea and salts remaining from starch dissolution and neutralization steps. The dissolution/neutralization solution alone (without added starch) displayed a sharp and symmetrical peak that eluted at an elution volume of approximately 120 mL (data not shown). However, in the presence of starch, this same peak began to elute at around 90 mL (elution volume), interfering with accurate analysis of the amylose peak. As noted earlier, Jackson et al. (*14*) observed that salts, particularly those possessing Cl^- ions, had a tendency to elute faster than would be anticipated (based on molecular weight) in the presence of starch. This phenomenon may be due in part to ionic/polar interactions between starch and salt molecules, preventing adequate separation of these two molecular fractions within the SEC column. The same authors further suggested that salts be removed prior to analysis of alkali-solubilized/neutralized starch by SEC.

In an attempt to remove salts and urea contaminants, the starch solution subjected to the alkaline-microwave dissolution scheme was treated with IEX beads containing both anion and cation exchange resins, followed by fractionation on the IPSEC system. The intensity of the interfering salt/urea peak decreased proportionally with the amount of IEX beads added (data not shown), but the confounding peak was nevertheless still observed even after treatment with up to 5 g of IEX beads (**Figure 1**, ion-exchange resin). As a result of IEX treatment, the amylose peak became somewhat more distinguishable, and the amylopectin peak was intensified as compared to the control chromatogram (untreated starch solution). The increased intensity of the amylopectin peak was likely due to the concentration of starch in solution, as a function of water uptake by IEX beads. Overall, the IEX treatment (at practical levels of addition employed in our experiments) was not completely successful in eliminating the interfering peak, most likely due to the fact that uncharged urea was not effectively sequestered by IEX resin.

A freshly prepared starch solution from the alkaline-microwave dissolution scheme was also passed through a desalting column (packed with Sephadex G-25), commonly used for desalting or buffer exchange of proteins and/or enzymes (*24*), prior to analysis on the IPSEC system. The resulting chromatogram no longer exhibited the confounding salt/urea peak, eliminating previously observed interference with the amylose peak (**Figure 1**, PD-10 column). While amylose and amylopectin peak intensities decreased as a result of treatment (due to dilution), both peak shapes appeared to approximate those of the control (untreated) and IEX treated starch preparations. The amylopectin retention volume following passage through the desalting column (44.0 mL) did not significantly differ from that of the control (untreated) starch solution (43.9 mL). Therefore, the use of the desalting column appeared to represent a reasonable means for removal of interfering salt/urea contaminants from the starch solution prior to analysis by IPSEC.

Further Validation of the Desalting Column Procedure. To further investigate the effectiveness of the desalting column procedure, starches from both wheat (waxy and normal) and corn (waxy, normal, and high-amylose) encompassing a broad range of amylose contents were analyzed. Solubilities (%) of starches ranged from 91.3 ± 0.3 to 94.1 ± 0.0% (**Table 1**), which were comparable to those reported for waxy and normal starches (*18*) and exceeded that previously reported for the solubilization of high-amylose corn starch (*25*). Resulting chromatograms for solutions of all starches passed through the desalting column were free of the confounding salt/urea peak in contrast to control samples (no treatment with desalting column), which possessed the interfering peak (**Figures 1** and **2**). Han and Lim (*15*) have also shown that confounding salt peaks can be avoided in low-pressure SEC chromatograms of alkali-solubilized starch by adjusting the alkali concentration of the starch solution to that of the mobile phase. While their method was reported to be effective for normal and waxy corn starches, they noted that it did not appear to achieve adequate separation between high-amylose starch and salt molecules. In addition to functioning well with high-amylose starches, the desalting column procedure reported here has the added option and advantage of being able to investigate the starch composition and structure using a water mobile phase.

Recoveries based on the total carbohydrate contents of starch solutions before and after passage through the desalting column ranged from 90.9 ± 0.2 to 93.1 ± 2.8% for all starch samples evaluated, indicating a high recovery of starch through the desalting process. Retention volumes for amylopectin peaks of all evaluated starches did not differ between the control and the desalting column treated samples. Nevertheless, chromatogram peak intensities for the desalted starch solutions did decrease as compared to those of control starch solutions, although this phenomenon was largely ascribable to the dilution of the starch solution during elution through the desalting column (total carbohydrate recoveries through the desalting column exceeded 90%). A dilution factor constant of 2.2, based on the carbohydrate concentrations before and after passage through the desalting column, was consistent across all starches evaluated (**Table 1**). Amylose contents calculated from chromatogram peak areas of starch solutions eluted through the desalting column were in close agreement with those reported in the scientific literature (**Table 1**) and Kim et al. (*18*), who obtained a similar amylose peak area (25.5 vs 25.8% in this study) using the same normal soft wheat starch and the starch dissolution scheme (except that the starch samples did not undergo desalting and were fractionated on a preparative Sepharose CL-2B column).

Table 1. Mean[a] Solubilities, Recoveries, Dilution Factors, and Amylopectin/Amylose Peak Areas for Chromatograms of Soft Wheat and Corn Starches[b]

starch sources	solubility[d] (%)	desalting column recovery[e] (%)	dilution factor[f]	peak area[c] (%) AP[g]	peak area[c] (%) AM[g]	amylose content[h] (%)
			Soft Wheat Starch			
waxy	92.5 ± 0.2[A]	93.1 ± 2.8[A]	2.2 ± 0.1[A]	100.0 ± 0.0	0.0 ± 0.0	3.4 (*26*)
normal	91.3 ± 0.3[A]	92.3 ± 0.7[A]	2.2 ± 0.0[A]	75.5 ± 0.3	24.5 ± 0.3	25.8 (*19*)
			Corn Starch			
waxy	91.6 ± 0.1[A]	92.5 ± 2.1[A]	2.2 ± 0.0[A]	96.5 ± 5.0	3.5 ± 5.0	1.3 (*27*)
normal	91.4 ± 1.6[A]	91.1 ± 1.7[A]	2.2 ± 0.0[A]	73.8 ± 0.7	26.2 ± 0.7	25.5 (*27*)
high-amylose	94.1 ± 0.1[A]	90.9 ± 0.2[A]	2.2 ± 0.0[A]	47.7 ± 3.8	52.3 ± 3.8	53.3 (*27*)

[a] Mean values with the same letter within a column (for a specific type of analysis) are not significantly different ($p < 0.05$). [b] Solubilized according to the alkaline-microwave dissolution protocol, followed by elution through the desalting column, and analysis by IPSEC. [c] Calculated from chromatogram peak areas using HPLC system software. [d] Calculated as the percent of the carbohydrate content (mg/mL) of the starch solution after and before filtration through a 5 μm syringe filter. [e] Calculated as the percentage of the total carbohydrate content (mg) of the starch solution after (collected 5.0 mL) and before (original 2.5 mL) elution through a PD-10 desalting column. [f] Ratio of carbohydrate concentration (mg/mL) of starch solution before and after passing through a PD-10 desalting column. [g] AP: amylopectin and AM: amylose. [h] Literature reported values. Number in parentheses corresponds to the cited reference number.

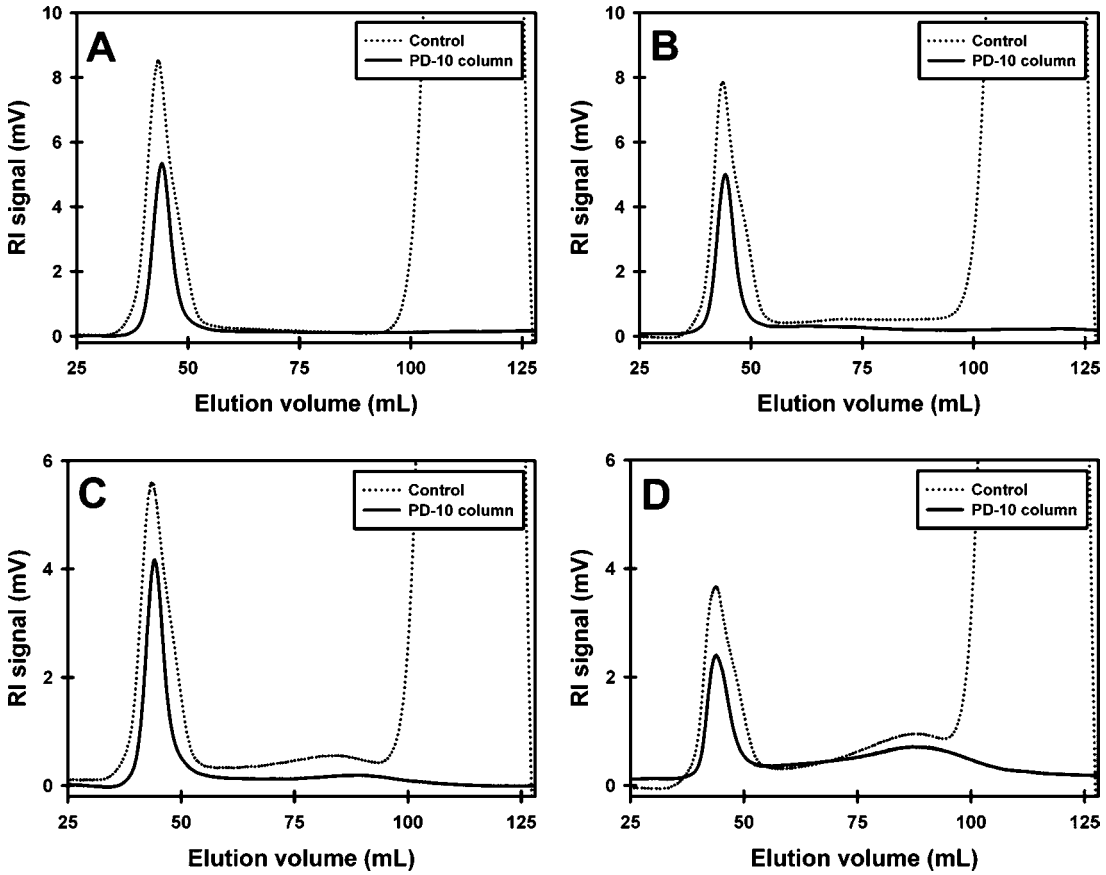

Figure 2. Chromatograms for waxy soft wheat (**A**), waxy corn (**B**), normal corn (**C**), and high-amylose corn (**D**) starches solubilized by the alkaline-microwave dissolution scheme subjected to no treatment (control) or passage through a PD-10 desalting column prior to fractionation by IPSEC.

In summary, this work investigated the removal of contaminants from alkali-solubilized/neutralized starch solution in preparation for analysis by IPSEC. Results demonstrated that the use of a PD-10 desalting column was an effective means of excluding contaminants (e.g., salts/urea that might otherwise compromise starch SEC analysis) from starch solutions to facilitate IPSEC analysis. This study extends the application of the desalting column technique, which has been previously employed for protein or enzyme work (e.g., buffer exchange), to the purification of starch solutions. The desalting technique described here requires only a short time to conduct (≈15 min) and would appear to be useful for purification of almost any alkali-solubilized/neutralized starch system intended for SEC. However, combined with the rapid alkaline-microwave starch dissolution method (*18*), this technique provides a concise and effective means of sample purification needed to expedite starch structural analysis by IPSEC, allowing a single starch sample to be analyzed from start to finish within a 3 h period.

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