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Alkaline Dissolution of Starch Facilitated by Microwave Heating for Analysis by Size-Exclusion Chromatography

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A rapid, straightforward starch dissolution method was developed to facilitate analysis of starch by size-exclusion chromatrography (SEC). Soft wheat starch was dispersed in 6 M urea and 1 M KOH and subjected to heating for 35, 45, or 60 s in a microwave oven as a potential means of starch dissolution. An increase in microwave heating time enhanced starch solubility but resulted in a steady decrease in amylopectin peak area and a consequent, artifactual increase in the amylose peak area due to molecular degradation. However, microwave heating for 35 s afforded a reasonable separation of starch fractions by SEC while minimizing molecular degradation of starch in comparison to a traditional starch dissolution procedure. This procedure provides a fast (<30 min), simple, and reproducible starch dissolution method for preparation of starches for SEC analysis and represents the first successful report of direct dissolution of granular starch via microwave heating.

KEYWORDS: Size-exclusion chromatography; starch dissolution; microwave heating; urea; KOH; starch solubility; soft wheat starch

INTRODUCTION

Complete starch dissolution with minimal molecular degradation of amylopectin and amylose is a prerequisite for valid structural analysis of starch by size-exclusion chromatography (SEC). Starch dissolution is commonly achieved by aqueous dimethyl sulfoxide (DMSO) or strong inorganic alkali (e.g., KOH, NaOH) in combination with physical (e.g., stirring, homogenization, sonication) and thermal (e.g., boiling, autoclaving, microwave heating) treatments (*1*−*9*). However, significant variability in reported molecular weights and other structural data suggests that dissolution conditions may significantly impact starch structural characteristics (*7*).

One of the most common dissolution methods involves heating of starch in 90% aqueous DMSO, followed by precipitation and redissolution of starch in water (with further heating) to produce reconstituted starch, which is then subjected to SEC analysis (*2*, *6*, *7*). However, this approach is both labor- and time-intensive and requires subjection of starch to extensive shear and thermal treatment. Han and Lim (*9*) noted different molecular weights and gyration radii for amylopectin and amylose, depending on the extent of stirring and heating during starch solubilization. Strong alkali has also been used to facilitate starch dissolution and prevent molecular aggregation of starch molecules. Grant et al. (*10*) demonstrated that structural characteristics and amylose content estimation of starch solubilized in a mixture of 1 M KOH and 6 M urea (9:1 v/v) could be analyzed by high-performance size-exclusion chromatography (HPSEC). However, a possible limitation of this solubilization method is that it requires extended heating of starch in the presence of strong alkali (90 min), which has potential to induce degradation of starch molecules (*1*, *8*). Thus, efforts are yet needed to further optimize dissolution methods to minimize the physical and thermal treatment of starch to avoid degradation of polymer molecules during solubilization within an appropriate solvent.

Microwave heating has been explored as a rapid means for dissolution of reconstituted starch in water following initial dissolution in 90% aqueous DMSO (*3*−*5*). Bello-Pérez et al. (*4*) reported that reconstituted starch subjected to increasing lengths of microwave heating in water exhibited increased solubility but a decreased molecular weight due to molecular degradation. However, Bello-Pérez et al. (*5*) showed that complete dissolution of starch in water could be achieved by microwave heating (35−90 s), provided that starch had been previously solubilized in aqueous DMSO. However, there are no reports in the literature of the successful use of microwave heating to facilitate initial dissolution of granular starch. The objective of this work was to investigate the use of alkali combined with microwave heating as an alternative to traditional starch dissolution methods for an appropriate and a more rapid starch dissolution procedure for SEC analysis.

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MATERIALS AND METHODS

Starch Source. Normal (8.9% moisture, 26.5% total amyloses) and waxy (8.8% moisture, 3.4% total amylose) soft wheat starches were isolated from straight-grade flours of Madsen and Leona cultivars, respectively, according to the method outlined by Shinde et al. (*11*). Total amylose values of native starches were determined iodometrically (*12*). A portion of normal wheat starch was also defatted by Soxhlet extraction with 75% (v/v) aqueous *n*-propanol for 8 h (*13*) to accommodate analysis of both native and defatted starches by SEC. Phospholipid levels of native and defatted starches were approximated by starch phosphorus content via inductively coupled plasma−atomic emission spectroscopy (ICP-AES) (*14*).

Starch Solubilization. Starch (36 mg, dry basis) 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, 45, or 60 s in a 2450 MHz AR732 microwave oven (Emerson Radio Co., North Bergen, NJ). After cooling to ambient temperature for 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 the column.

For comparison purposes, a traditional starch dissolution method (*15*) was also conducted as a standard reference. Starch (100 mg, d.b.) was suspended in 10 mL of 90% aqueous DMSO and heated for 1 h in a boiling water bath with continuous stirring. The starch−DMSO solution was further stirred at ambient temperature (22 °C) for 24 h, after which 4 mL of starch−DMSO solution was mixed with five volumes of ethanol to precipitate starch molecules. The precipitate was collected by centrifugation at 3500*g* for 30 min and washed twice with ethanol to remove residual DMSO. The resultant starch pellet was redissolved in 10 mL of boiling water and stirred for 30 min in a boiling water bath, after which the starch solution was cooled to ambient temperature and passed through a 5 μm nylon syringe filter (National Scientific Co., Duluth, GA) prior to injection onto the column.

The solubility (%) of starch following dissolution was calculated as the difference in carbohydrate content of starch solution before and after filtration (*8*, *9*). Total carbohydrate values were determined by the phenol−sulfuric acid colorimetric method (*16*).

Size-Exclusion Chromatography. Filtered starch solution (5 mL) was fractionated on a Sepharose CL-2B (Pharmacia Biotech, Piscataway, NJ) column (2.6 × 82 cm) in ascending mode with a mobile phase of deionized water containing 0.02% (w/v) sodium chloride and 0.02% (w/v) sodium azide at a flow rate of 30 mL/h. The carbohydrate content of each collected vial (4.8 mL) was determined by the phenol−sulfuric acid method (*16*). Blue value was measured at 620 nm by adding 0.5 mL of 0.2% iodine solution (2 g of I_2 and 20 g of KI per liter of deionized water) to 1 mL of each even-numbered vial. Reducing-end analysis was conducted for separated starch molecular fractions (amylopectin, intermediate, and amylose) according to the modified 2,2′-bicinchoninate (BCA) method outlined by Zhang and Lynd (*17*). Protein residues of each collected vial were assessed by the ninhydrin test (*18*).

Analysis of SEC Data. All SEC experiments were repeated three times, and chromatograms representing each treatment were depicted as the mean values of replicate analyses. Recovery (%) was defined as the percent ratio of the carbohydrate content of each vial (4.8 mL) to that of the initial starch solution (5 mL) injected onto the column, and the cumulative recovery was the sum of all vial recoveries. The amylose content was calculated on the basis of either the relative area of the amylose peak or the percent ratio of the weight of total carbohydrate in the amylose fraction to that recovered through the Sepharose CL-2B column. The amylose peak was identified by the blue value chromatogram. Peak areas (amylopectin, intermediate, and amylose) of chromatograms were calculated by use of SAS version 9.1 for Windows (SAS Institute, Cary, NC). Average reducing-end content (%) was defined as the percent ratio of the weight of reducing ends (based on glucose) of each starch molecular fraction to the weight of total carbohydrates recovered through a Sepharose CL-2B column.

For the various treatments, starch solubilities, peak area values, recoveries, and reducing-end contents (all expressed as percent) of each starch molecular fraction 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 by use of SAS version 9.1 for Windows (SAS Institute, Cary, NC).

Table 1. Mean[a] Solubilities, Amylose Peak Area, Intermediate Peak Area, and Cumulative Recoveries of Normal Soft Wheat Starch[b]

heating time,s	solubility,[c] %	amylose peak area,[d] %	intermediate peak area,[e] %	cumulative recovery,[f] %
35	91.2 ± 2.0[b]	25.5 ± 0.3[c]	2.8 ± 0.4[c]	58.9 ± 5.4[a]
45	97.3 ± 1.9[a]	34.3 ± 0.9[b]	5.3 ± 0.3[b]	58.6 ± 3.8[a]
60	98.1 ± 0.1[a]	50.8 ± 1.0[a]	8.6 ± 0.1[a]	58.2 ± 2.8[a]

[a] Mean values are based on three SEC runs. Values with the same letter within a column are not significantly different ($p < 0.05$). [b] Normal soft wheat starch was subjected to varied heating times in a microwave oven in the presence of aqueous KOH/urea. [c] Calculated as the difference in carbohydrate content of the starch solution before and after filtration through a 5 μm syringe filter. [d] Calculated from the relative peak area of the amylose peak to that of the total chromatogram. [e] Percent ratio of the area of intermediate peak to that of the total chromatogram. [f] Cumulative recovery is the sum of all vial or fraction recoveries. Recovery is the percent ratio of the carbohydrate content of each vial or fraction (4.8 mL) to that of the initial starch solution (5 mL) injected onto the column.

RESULTS AND DISCUSSION

Method Development. Soft wheat starch was dissolved in the presence of 6 M urea and 1 M KOH (1:20 v/v) by microwave heating for 35, 45, and 60 s. Solubilities of normal soft wheat starch were 91.2%, 97.3%, and 98.1% for 35, 45, and 60 s of microwave heating, respectively (**Table 1**). These values compared favorably to those reported for reconstituted starch (90−100%), which was dissolved in 90% aqueous DMSO, precipitated with ethanol, and redissolved in water with 35−90 s of microwave heating (*5*), and exceeded those for granular starch (67.3%) subjected directly to microwave heating in water for 90 s (*3*). Differences in solubility may be due in part to different dissolving solvents. While others have used water, the present work utilized a mixture of 6 M urea and 1 M KOH (1:20 v/v). In comparison to the incomplete starch solubilization achieved by microwaving in just water (*3*), urea likely facilitated breakage of hydrogen bonds between starch molecules (*10*), while KOH introduced intermolecular repulsion of starch chains due to formation of the alkoxide ion at alkaline pH, thus enhancing starch solubility (*8*, *19*). Brief pressurization within the microwave bomb during rapid heating also may have assisted the disruption of the native granular structure and aided solubilization of starch molecules. Thus, microwave heating of starch in the presence of urea and KOH proved capable of facilitating starch solubilization, which tended to increase with greater lengths of heating (**Table 1**).

Chromatograms for recovery and blue value of normal soft wheat starch dispersed in urea and KOH solution and subjected to different lengths of microwave heating are shown in **Figure 1**. An increase in heating time from 35 to 60 s led to a decrease in the amylopectin peak area and a corresponding increase (from 2.8% to 8.6%, **Table 1**) in the baseline region (intermediate peak area) between amylopectin and amylose peaks and also in the amylose peak itself (**Figure 1A**). This phenomenon was likely due to the degradation of amylopectin during microwave heating in KOH/urea. Jackson et al. (*1*) showed that amylopectin was depolymerized when starch was extensively heated in alkali. Furthermore, Bello-Pérez et al. (*4*) also demonstrated that increasing length of microwave heating of starch in water resulted in a reduction in molecular weight for amylopectin.

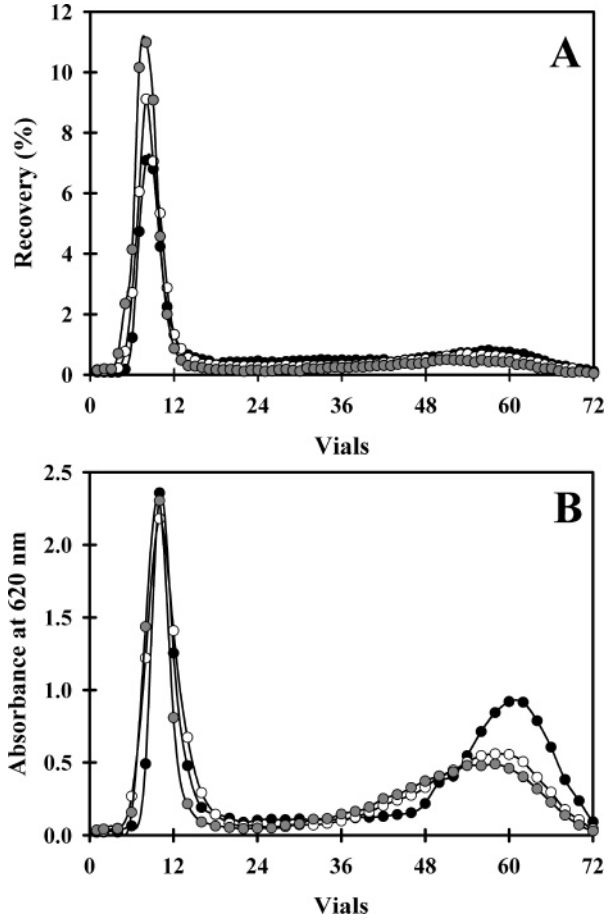

Figure 1. SEC chromatograms (**A**, recovery; **B**, blue value) of normal soft wheat starch subjected to 35 (gray circles), 45 (white circles), or 60 s (black circles) of heating time in a microwave oven in the presence of aqueous KOH/urea.

Thus, the increased starch solubility achieved with increasing lengths of microwave heating (**Table 1**) appeared to come at a cost of molecular degradation.

The blue value curve also appeared to suggest the potential degradation of amylose (**Figure 1B**), whose peak was shifted downstream in the chromatogram as the length of microwave heating increased. Thus, extensive microwave heating of starch in strong alkali may also induce amylose degradation. This finding parallels the report of Bello-Pérez et al. (*5*), who showed that subjection of aqueous starch dispersions to increasing lengths of microwave heating times depolymerized both amylose and amylopectin. Amylose values derived from molecular fraction peak areas significantly increased from 25.5% to 50.8% over the course of 35−60 s of microwave heating (**Table 1**). The amylose content calculated from the starch recovery chromatogram prepared with 35 s of microwave heating (25.5%) was in close agreement with the total amylose value determined via iodometric means (26.5%). Cumulative recoveries did not differ between microwave treatments (**Table 1**) and thus were not responsible for observed differences in molecular fraction peak areas observed for the various microwave heating times. In short, microwave heating of starch for 35 s in a mixture of urea and KOH provided a reasonable measure of amylose content and good separation of amylopectin and amylose starch fractions.

Furthermore, the dissolution protocol (35 s of microwave heating in KOH/urea solution) was also applied to waxy soft wheat starch. The recovery chromatogram of waxy wheat starch

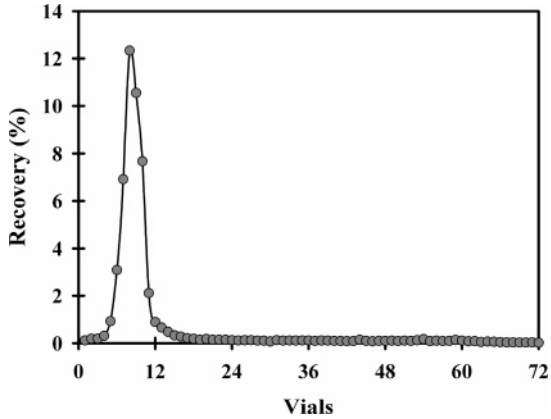

Figure 2. SEC chromatogram for recovery (%) of waxy soft wheat starch subjected to 35 s of heating time in a microwave oven in the presence of aqueous KOH/urea.

exhibited minimal shouldering and tailing of the amylopectin peak (**Figure 2**), further demonstrating that the present starch dissolution method is capable of facilitating accurate structural analysis of starch by SEC. The same dissolution protocol (35 s of microwave heating in KOH/urea solution) has been also successfully applied to tapioca, potato, and teff starches (data not shown), indicating a broader application to starches of varied botanical origin.

Further Validation of the Microwave Dissolution Method. The SEC column was repacked to investigate whether the separation of starch molecules was dependent on the initial column condition. Soft wheat starch subjected to the established dissolution protocol (35 s of microwave heating in KOH/urea solution) was fractionated on the repacked SEC column. Chromatograms and results (e.g., solubility, cumulative recovery) for starch fractionation were very comparable to the original data, and the derived amylose content (25.0%) for the repacked column was in good agreement with the total amylose value (26.5%) determined by iodometric means (**Table 2**), suggesting that the present dissolution protocol was reproducible and independent of column state.

In addition, it is known that native starch granules possess trace levels of protein (*20*), which would not be removed by the present microwave solubilization method due to the lack of a precipitation step characteristic of traditional methods. It was investigated whether trace levels of protein, which were tracked by the ninhydrin test, would overlap or interfere with starch fractionation on a Sepharose CL-2B column. As shown in **Figure 3**, detectable levels of protein did not coelute with amylopectin or amylose but instead eluted immediately after the amylose peak. Thus, molecular fractionation of starch did not appear to be compromised by the presence of residual protein retained in the starch solution.

Our microwave starch dissolution method was also compared to a traditional starch dissolution method with respect to solubility, cumulative recovery, and relative reducing-end content within the individual starch molecular fractions (amylopectin, intermediate, and amylose). Solubilities and cumulative recoveries were comparable for the traditional and microwave dissolution methods (**Table 2**). However, relative to the microwave dissolution procedure, the chromatogram of the traditional method exhibited a decreased intensity, a slightly later elution time for the amylopectin peak, and an increase in amylose peak intensity, resulting in the apparent overestimation of amylose content (31.5%) (**Figure 4** and **Table 2**). It is possible that the more lengthy heating and stirring conditions characteristic of the traditional method led to possible molecular

Table 2. Mean[a] Solubilities, Amylose Peak Areas, Cumulative Recoveries, and Reducing-End and Total Carbohydrate Contents of Individual Starch Molecular Fractions

				reducing-end content,[c] % (total carbohydrate content,[d] %)		
starch source[b]	dissolution method	solubility[c,e] (%)	cumulative recovery[c,f] (%)	AP[g]	IN[g]	AM[g]
native	traditional[h]	90.9 ± 2.5^{b}	54.7 ± 7.4^{a}	0.20 ± 0.03^{a} *(61.9 ± 0.3[b])*	$0.18 \pm 0.01^{a,b}$ *(6.6 ± 0.3[a])*	0.77 ± 0.14^{b} *(31.5 ± 0.2[b])*
native	microwave[i]	92.0 ± 1.4^{a}	55.1 ± 4.1^{a}	0.15 ± 0.00^{b} *(69.9 ± 1.3[a])*	0.13 ± 0.01^{b} *(5.1 ± 0.3[b])*	0.60 ± 0.01^{c} *(25.0 ± 1.1[c])*
defatted[j]	microwave[i]	91.3 ± 0.4^{a}	54.4 ± 1.6^{a}	0.20 ± 0.01^{a} *(58.6 ± 0.0[c])*	0.21 ± 0.04^{a} *(6.2 ± 0.2[a])*	1.01 ± 0.02^{a} *(35.2 ± 0.2[a])*

[a] Mean values are based on three SEC runs. Values with the same letter within a column (for a specific type of analysis) are not significantly different ($p < 0.05$). [b] Native or defatted soft wheat starch was solubilized according to different dissolution schemes, followed by fractionation on a Sepharose CL-2B column. [c] Defined as the percent ratio of the weight of reducing ends (based on glucose) in each starch molecular fraction (amylopectin, intermediate, amylose) to the weight of total carbohydrates recovered through a Sepharose CL-2B column. [d] Defined as the percent ratio of the weight of total carbohydrates in each starch molecular fraction (amylopectin, intermediate, amylose) to that recovered through a Sepharose CL-2B column. [e] Calculated as the difference in carbohydrate content of the starch solution before and after filtration through a 5 μm syringe filter. [f] Cumulative recovery is the sum of all vial or fraction recoveries. Recovery is the percent ratio of the carbohydrate content of each vial or fraction (4.8 mL) to that of the initial starch solution (5 mL) injected onto the column. [g] AP = amylopectin; IN = intermediate; AM = amylose. [h] Starch solubilized via heating in 90% aqueous DMSO, followed by precipitation in ethanol and redissolution in boiling water with mechanical stirring (*15*). [i] Starch solubilized via microwave heating for 35 s in the presence of 1 M KOH and 6 M urea. [j] Defatted starch prepared by Soxhlet extraction with 75% aqueous *n*-propanol for 8 h (*13*).

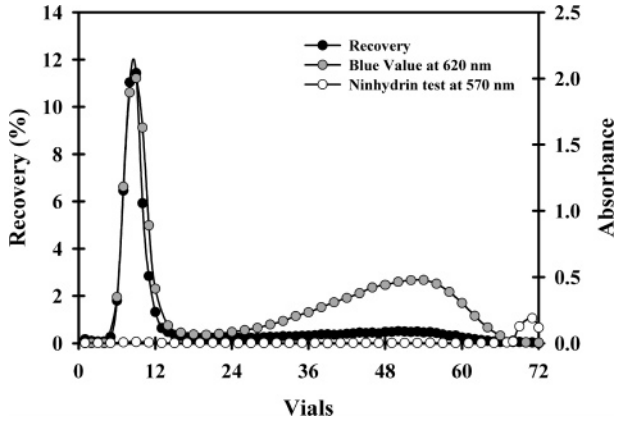

Figure 3. SEC chromatograms for recovery (%), blue value, and ninhydrin test of normal soft wheat starch subjected to 35 s of heating time in a microwave oven in the presence of aqueous KOH/urea.

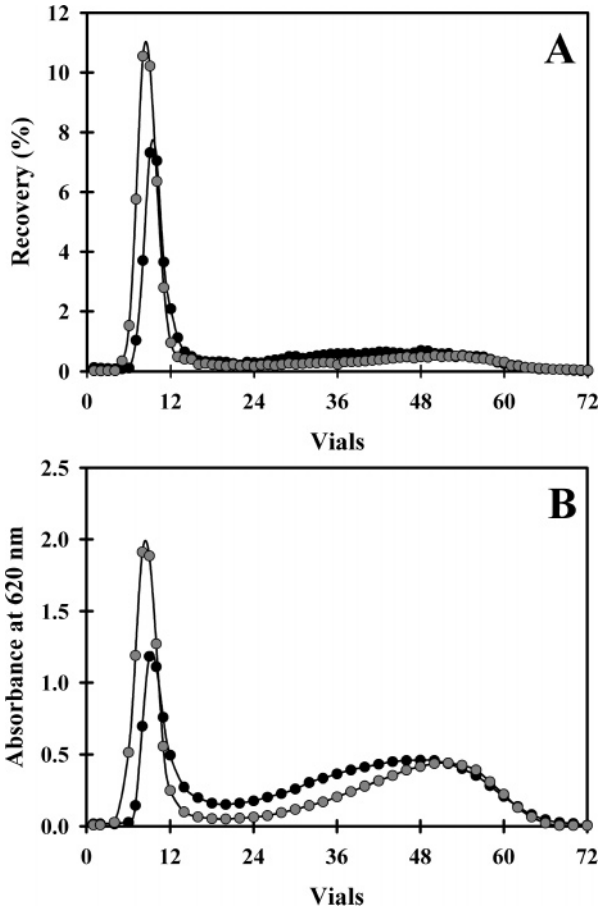

Figure 4. SEC chromatograms (**A**, recovery; **B**, blue value) of normal soft wheat starch subjected to a traditional dissolution method (black circles) and 35 s of heating time in a microwave oven in the presence of aqueous KOH/urea (gray circles).

degradation of amylopectin and amylose molecules. This scenario is indirectly supported by differences in reducing-end and total carbohydrate contents of individual starch molecular fractions obtained via the two dissolution methods. Relative reducing-end contents for two of the three starch molecular fractions (amylopectin and amylose) obtained via the traditional method were significantly higher compared to those obtained by the microwave method (**Table 2**), suggesting a higher degree of molecular degradation for the traditional dissolution method. Also, the total carbohydrate content of the amylopectin fraction for the traditional method (61.9%) was significantly lower than that of the microwave method (69.9%). The reverse trend was observed for the total carbohydrate contents of the intermediate and amylose fractions, which were higher for the traditional method (6.6% and 31.5%, respectively) compared to the microwave method (5.1% and 25.0%, respectively) (**Table 2**). For the traditional (relative to the microwave) method, molecular degradation of amylopectin would appear to explain the lower carbohydrate content of the amylopectin peak and the relatively higher values for the intermediate and amylose fractions. Both the reducing-end and total carbohydrate content data for the individual starch fractions appear to suggest that there is greater potential for molecular degradation of amylopectin with the traditional compared to the microwave method. In addition, it has been previously noted that the traditional dissolution method, while useful for determination of weight-average molecular weight (M_w), has limitations with being able to provide in-line amylose content determination based on amylose peak area (*2*, *9*, *14*, *21*).

One advantage of the traditional dissolution method is that it involves a precipitation step whereby starch granule phospholipids, which have been known to interfere with determination of amylose content via iodometric means (*12*), are effectively removed from the starch. In contrast, the microwave dissolution

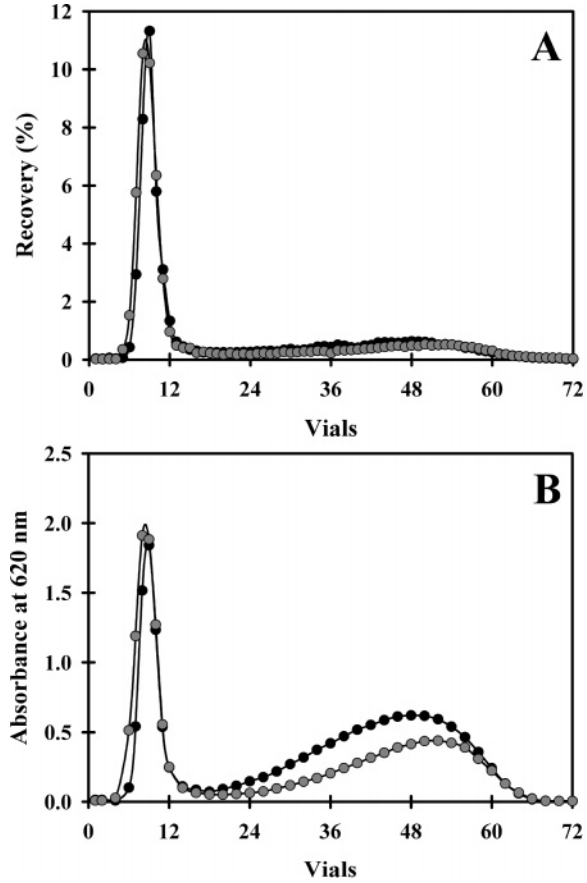

Figure 5. SEC chromatograms (**A**, recovery; **B**, blue value) of native (gray circles) and defatted (black circles) normal soft wheat starch subjected to 35 s of heating time in a microwave oven in the presence of aqueous KOH/urea.

method directly dissolves native starch granules without further purification for injection onto the column. To determine the potential influence of starch granule lipids on the fractionation of starch prepared via microwave dissolution, both native and defatted wheat starches were analyzed. Phosphorus contents of the native and defatted starches were 506.7 (±4.7) and 83.0 (±2.8) ppm, respectively, in approximation of starch phospholipid contents. Solubilities and cumulative recoveries for the native and defatted starches were not significantly different from each other (**Table 2**). The recovery chromatogram of the defatted starch differed only very slightly from that of native starch as shown in **Figure 5**. However, this subtle difference led to a significant relative increase in amylose content (35.2%) for the defatted starch calculated from chromatogram. This observation is thought to be more ascribable to molecular degradation of starch caused by exposure to hot solvent extraction (8 h) rather than an increased ability to quantify amylose due to removal of polar lipids. Observed differences between the native and defatted starch chromatograms appeared to be indirectly explained by differences in reducing-end and total carbohydrate contents (**Table 2**), based on the same rationale previously set forth in explaining differences between the traditional and microwave dissolution methods. This result would suggest that defatting via Soxhlet extraction may induce molecular changes to the starch in addition to extraction of lipids. The microwave starch dissolution method would not necessarily require a defatting step, based on the report of Grant et al. (*10*), who demonstrated that a defatting step was not necessary for HPSEC while employing a similar aqueous urea/KOH dissolution medium (instead heated at 100 °C for 90 min).

This work investigated microwave heating of starch in the presence of urea and KOH as a means for starch dissolution prior to SEC analysis. Results showed that the reduced heating time and physical treatment afforded by the microwave dissolution procedure minimized the potential degradation of amylopectin and amylose for SEC analysis. This report appears to be the first successful application of microwave heating to facilitate primary dissolution of native starch granules (without first having dissolved granules in aqueous DMSO). The microwave dissolution procedure appears to represent at least a comparable (if not more favorable) means for starch dissolution (relative to the traditional method) while at the same time requiring reduced time and effort. Advantages of the microwave starch dissolution protocol (35 s of microwave treatment in a mixture of urea and KOH) are as follows: (1) starch dissolution is achieved while minimizing observable molecular degradation, (2) the procedure requires only 30 min to solubilize starch for injection onto a SEC column (up to 24 h is standard for most traditional methods), and (3) further purification steps (protein, lipid extraction) are not likely necessary. This procedure offers a fast, simple, and reproducible means for dissolution of starch to facilitate structural analysis and characterization of starch polymers.

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