DOI:10.1021/jf903200h

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Size Exclusion Chromatography for the Quantitative Profiling of the Enzyme-Catalyzed Hydrolysis of Xylo-oligosaccharides

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High-performance size exclusion chromatography (HPSEC) is a widely used method for the qualitative profiling of oligosaccharide mixtures, including, for example, enzymatic hydrolysates of plant biomass materials. A novel method employing HPSEC for the quantitative analytical profiling of the progress of enzymatic hydrolysis of different xylan substrates was developed. The method relies on dividing the HPSEC elution profiles into fixed time intervals and utilizing the linear refractive index response (area under the curve) of defined standard compounds. To obtain optimal HPSEC profiles, the method was designed using 0.1 M CH_3COONa both in the mobile phase and as the sample solution matrix, after systematic evaluation of the influence of the mobile phase, including the type, ionic strength, and pH, on the refractive index detector response. A time study of the enzyme-catalyzed hydrolysis of birchwood xylan and wheat bran by a *Bacillus subtilis* XynA xylanase (GH 11) was used as an example to demonstrate the workability of the HPSEC method for obtaining progress curves describing the evolution in the product profile during enzyme catalysis.

KEYWORDS: Size exclusion; quantitative profiling; xylanase; xylo-oligosaccharides

INTRODUCTION

Various xylans are currently under intensive study as carbohydrate sources for fermentation to biofuels or as sources of novel health-promoting food ingredients (*1*−*6*). Xylan basically consists of a backbone of β-1,4-linked D-xylopyranosyl residues that may be substituted to various degrees with arabinofuranosyl, glucuronate, or acetyl groups. The hydrolysis of the xylan backbone to a mixture of xylo-oligosaccharides can be catalyzed by endo-1,4-β-xylanases (xylanases, EC 3.2.1.8) (*7*). With respect to investigation of the action pattern of endo-1,4-β-xylanases and in relation to the production and evaluation of potentially bioactive, prebiotic xylo-oligosaccharides, it is crucial to quantitatively assess the molecular size profile of the oligosaccharides. High-performance size exclusion chromatography (HPSEC) has been employed by several authors for analyzing the molecular weight distribution of different plant oligo- and polysaccharides in solution (*5*) and to separate polydisperse materials into discrete molecular weight distributions (*8*). HPSEC is based on permeation of a solute through a column packed with inert porous particles (*9*). The separation principle relies on the differentiation in the time of permeation of the polysaccharides into and out of the pores of the column packing, which is related to their molecular size. The elution of the polysaccharide is then determined by the time it takes for the polysaccharides to travel through the pores (*10*). To calculate the molecular weight of the polysaccharides, calibration curves can be constructed by measuring the retention volumes (or retention times) of synthetic polymer standards such as pullulan or polyethylene glycol molecules having a known narrow molar mass distribution (*11*).

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As seen in **Table 1**, HPSEC analysis of the molecular weight distribution of xylan-containing substrates has been used over a broad range of mobile phases at different concentrations and pH values. Sodium chloride, sodium acetate, and sodium nitrate appear to be the most commonly used mobile phases in this application. Furthermore, the sample solutions also differ with respect to type, molar concentration, and pH. Some use the same mobile phase and sample solution, whereas others just inject from any sample matrix. Also seen in **Table 1** is that different detectors have been employed for HPSEC analysis of xylo-oligosaccharide profiles, but differential refractometry detectors are the most widely used (*31*). Their operation is based on the continuous measurement of the difference in the refractive indices (RI) of the pure mobile phase and the mobile phase containing the substance (*32*). Generally, the area obtained underneath a RI curve represents the amount of sugars present (*22*).

When the methods employed are evaluated in detail (**Table 1**), it appears that no common HPSEC method exists and no quantification has been used for xylo-oligosaccharide profiling by HPSEC. This makes it difficult to compare the results obtained by different groups. Moreover, the question is whether the methodology employed has any significance on the results and whether it is possible to use the HPSEC profiles for quantitative purpose. The objectives of the work reported in the present paper were therefore (1) to evaluate the influence of specific parameters such as type and concentration of the mobile phase on the chromatographic profile from HPSEC and (2) to evaluate the possibilities for using HPSEC as a quantitative method to assess xylo-oligosaccharide profiles. As an example, we focused on analyzing the enzyme-catalyzed hydrolysis of different xylan substrates by a wild type *Bacillus subtilis* XynA xylanase. This

 Published on Web 12/08/2009

Table 1. Various HPSEC Methods That Have Been Used for Assessment of the Molecular Weight of Various Xylan-Containing Substrates[a]

substrate	mobile phase	concn (M)	sample solution	concn (M)	detector	ref
AX (flour)	NaCl	0.05	NaCl	0.05	RI	*12*
WEAX, WUAX, xylan	NaCl	0.05	CH_3COONa, pH 6	0.025	RI	*13*
dough and batter extract	NaCl	0.05	water		RI	*14*
WEAX and WUAX	NaCl	0.05	CH_3COONa, pH 4.6	ns	RI	*15*
extracts of dough and bread	NaCl	0.05	water		RI	*16*
AX (rye)	NaCl	0.05	ns	ns	UV	*17*
WEAX (wheat and rye flour and dough)	NaCl	0.1	NaCl	0.1	RI, UV	*18*
SAX	CH_3COONa, pH 3	0.4	water		RI	*19*
water-unextractable cell wall material	CH_3COONa, pH 3	0.4	water		RI	*20*
WEAX	CH_3COONa	0.4	water		RI	*5*
AX (wheat)	CH_3COONa, pH 5	0.05	CH_3COONa, pH 5	0.05	RI	*21*
liquors after pretreatment of straw	$NaNO_3$	0.2	water		RI	*22*
AX (rye bran)	$NaNO_3$	0.1	water		RI, UV, MALLS	*23*
water-extractable pentosans	$NaNO_3$	0.1	water		RI	*24*
WEAX (wheat)	$NaNO_3$	0.05	ns	ns	RI, UV, MALLS	*25*
maize bran	$NaNO_3$	0.05	water		RI, UV, MALLS	*26*
water-extractable cell wall polysaccharides	$NaNO_3$ + 0.02% NaN_3	0.1	$NaNO_3$ + 0.02% NaN_3	0.1	RI, MALLS	*27*
WEAX (wheat flour)	NH_4HCO_3	0.002	NH_4HCO_3	0.002	RI	*28*
cell wall fractions (rye grain)	$NaNO_3$	0.05	$NaNO_3$	0.05	RI, MALLS	*29*
WEAX	NaN_3	0.02%	water		ns	*4*
WEAX and WUAX	water		$NaHPO_4$−citric acid, pH 5	0.05	ns	*30*

[a] AX, arabinoxylan; WEAX, water-extractable arabinoxylan; WUAX, water-unextractable arabinoxylan; SAX, solubilized arabinoxylan; RI, refractive index; MALLS, multiangle laser light scattering; ns, not specified.

particular enzyme has been widely used to study the hydrolysis of arabinoxylan and as a template for evaluating the influence of wheat xylanase inhibitors on xylanase actitivy (*33*). The hypothesis was that it was possible to exploit the HPSEC profiles to obtain a quantitative, or at least semiquantitative, insight into the evolution of the xylo-oligosaccharides during endoxylanase treatment of xylan and, in this way, use HPSEC to quantitatively assess different xylo-oligosaccharide product profiles.

MATERIALS AND METHODS

Chemicals and Enzymes. Pullulans with the following average molecular masses, 0.13×10^4, 0.6×10^4, 1×10^4, and 40×10^4 g mol^{-1}, were from Fluka Chemie AG (Buchs, Switzerland) and D-(+)-xylose was from Merck (Darmstadt, Germany). Xylohexaose was obtained from Megazyme (Bray, Ireland).

For the destarching of wheat bran, α-amylase (Termamyl SC, Novozymes A/S (Bagsværd, Denmark)) and amyloglucosidase (Spirizyme Plus FG, Novozymes A/S) were used.

The *B. subtilis* xylanase (BsX) was obtained from Danisco A/S (Brabrand, Denmark). BsX carries a Swiss Prot entry P18429, XynA_BACSU and has been classified as a glycosyl hydrolase family 11 xylanase according to sequence homology (*34*). The xylanase was purified from a Grindamyl H 640 enzyme preparation and was purified according to the method of Sørensen and Sibbesen (*33*). BsX had a molecular mass of 20 kDa, pH optimum at 6, and temperature optimum at 50 °C.

All other reagents used were of at least analytical grade and supplied by Sigma-Aldrich (Bornem, Belgium), unless specified otherwise.

Substrates and Substrate Pretreatment. Water-unextractable arabinoxylans (WUAX) (arabinose/xylose (A/X) = 0.61) from wheat were obtained from Megazyme. Birchwood xylan was purchased from Sigma-Aldrich and was solubilized with ethanol as recommended in the product sheet. Wheat bran was a commercial product from Natur Drogeriet A/S (Hørning, Denmark). The wheat bran was destarched via sequential treatment with α-amylase (Termamyl SC) (95 °C, pH 6, 60 min) and amyloglucosidase (Spirizyme Plus FG) (60 °C, pH 4.5, 30 min) followed by ethanol precipitation. Acid hydrolysis (0.4 M HCl, 2 h, 100 °C) (*5*) of birchwood xylan and wheat bran showed that the monosaccharide composition of the birchwood xylan was 1.0 wt % galactose, 1.9 wt % glucose, and 97.1 wt % xylose and that of the destarched wheat bran was 3 wt % galactose, 10 wt % glucose, 32 wt % arabinose, and 55 wt % xylose (measured by HPAEC according to the method of Arnous and Meyer (*35*)).

Enzymatic Hydrolysis. Enzyme-catalyzed hydrolysis of WUAX, birchwood xylan, and wheat bran by BsX was assessed according to the following general procedure: The substrate (1 wt %) was dissolved in 0.1 M sodium acetate, pH 6, with 0.02 wt % sodium azide. The substrate was preincubated for 10 min at 40 °C under continuous stirring before a suitably diluted enzyme solution, to give a final reaction concentration of 0.12 μmol of enzyme g^{-1} dry matter substrate, was added. For birchwood xylan and wheat bran, samples were withdrawn after 0, 4, 8, 15, 30, 60, 120, and 240 min and for WUAX only after 24 h. The enzymatic hydrolysis was stopped immediately by heating the samples at 100 °C for 10 min. After centrifugation (10000*g*, 10 min), the hydrolysates were analyzed for molecular weight distribution by HPSEC.

High-Performance Size Exclusion Chromatography. HPSEC was carried out using a system consisting of a P680 HPLC pump, an ASI-100 automated sample injector, and an RI-101 refractive index detector (Dionex Corp., Sunnyvale, CA). Samples were separated on a Shodex SB-806 HQ GPC column (300 × 8 mm) with a Shodex SB-G guard column (50 × 6 mm) from Showa Denko K.K. (Tokyo, Japan). Different mobile phases were used with a flow rate of 0.5 mL min^{-1}, and the injection volume was 0.05 mL. All chromatographic experiments were conducted at 40 °C in a thermostated heater, and the columns were equilibrated overnight with various mobile phases prior to the start of any experiment.

Molecular mass markers (0.5 mg mL^{-1}) were pullulans with molecular masses of 0.13×10^4, 0.6×10^4, 1×10^4, and 40×10^4 g mol^{-1}, xylohexaose, and xylose. Furthermore, dextran with a molecular mass of 11×10^4 g mol^{-1} was used.

RESULTS

Effect of the Mobile Phase. *Type of Mobile Phase*. **Figure 1** displays the chromatograms of the sample solution (0.1 M CH_3COONa, pH 6) (**Figure 1A**) and hydrolysates of arabinoxylan (solubilized arabinoxylan, SAX) in sample solution (**Figure 1B**). When only the sample solution was loaded on the HPSEC system, chromatographic peaks still appeared that corresponded to the salt ions in the sample solution (**Figure 1A**). An eluent of 0.4 M CH_3COONa, pH 3, gave a relatively large negative peak, whereas the other three eluents employed gave a positive peak, but with various elution times and shapes. Elution with water and 0.4 M CH_3COONa, pH 3, resulted in the appearance of a peak in the chromatogram after approximately 23.5 min corresponding to SAX (**Figure 1B**). However, when a mixture of 0.05 M NaCl and CH_3COONH_4 was used as the

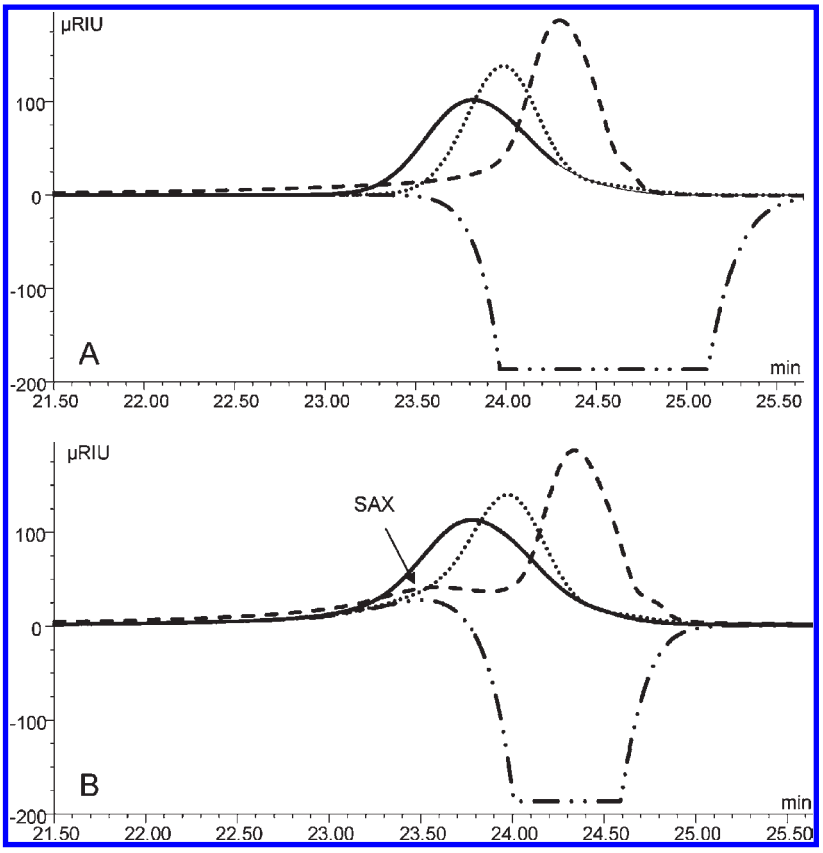

Figure 1. Size exclusion chromatograms of (**A**) sample solution (0.1 M CH_3COONa, pH 6) and (**B**) hydrolysate of arabinoxylan (SAX) in sample solution. The samples are eluted with the following different types of mobile phases: water (— — —), 0.05 M NaCl (—), 0.05 M CH_3COONH_4 (· · ·), and 0.4 M CH_3COONa, pH 3 (— · · —).

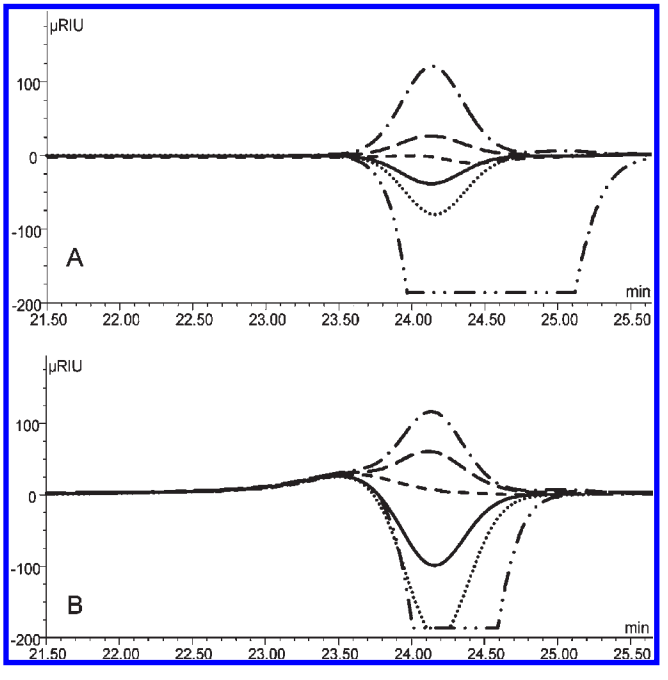

Figure 2. Effect of the ionic strength of the mobile phase (CH_3COONa, pH 6): chromatographic profiles of (**A**) sample solution (0.1 M CH_3COONa, pH 6) and (**B**) hydrolysate of arabinoxylan (SAX). The samples were eluted with the following concentrations of CH_3COONa as the mobile phase: 0.05 M (— · —), 0.075 M (— — —), 0.1 M (- - -), 0.15 M (—), 0.2 M (· · ·), and 0.4 M (— · · —).

eluent, the peak from SAX was shielded by the chromatographic peak from the sample solution.

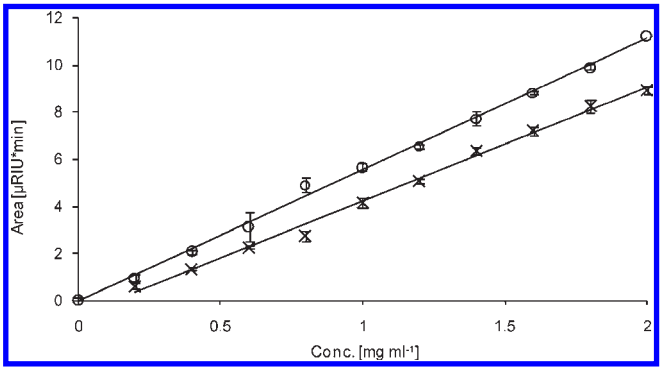

Figure 3. Standard curve (0.1−1 mg mL^{-1}) for pullulan (1.0×10^4 g mol^{-1}) (○) and xylohexaose (0.081×10^4 g mol^{-1}) (×). Values represent the mean of three independent measurements and are shown ±1 standard deviation.

Concentration of the Mobile Phase. To assess the effect of the ionic strength of the mobile phases on the chromatographic profiles, the influence of different concentrations (0.05−0.4 M) of one type of mobile phase (CH_3COONa, pH 6) on the chromatograms was studied (**Figure 2**). In all of the profiles obtained, a relatively large peak appeared after approximately 24 min, which corresponded to the sample solution. The polarity of this peak could be correlated to the ionic strength of the mobile phase: Mobile phases with low ionic strength (0.05−0.075 M) resulted in a positive peak and those with high ionic strength (0.1−0.4 M) in negative peaks. When SAX (**Figure 2B**) was eluted with different concentrations of the mobile phase, a second peak appeared at t = 23.5 min corresponding to the eluted molecules. When using 0.1 M CH_3COONa, pH 6, as mobile phase, it was

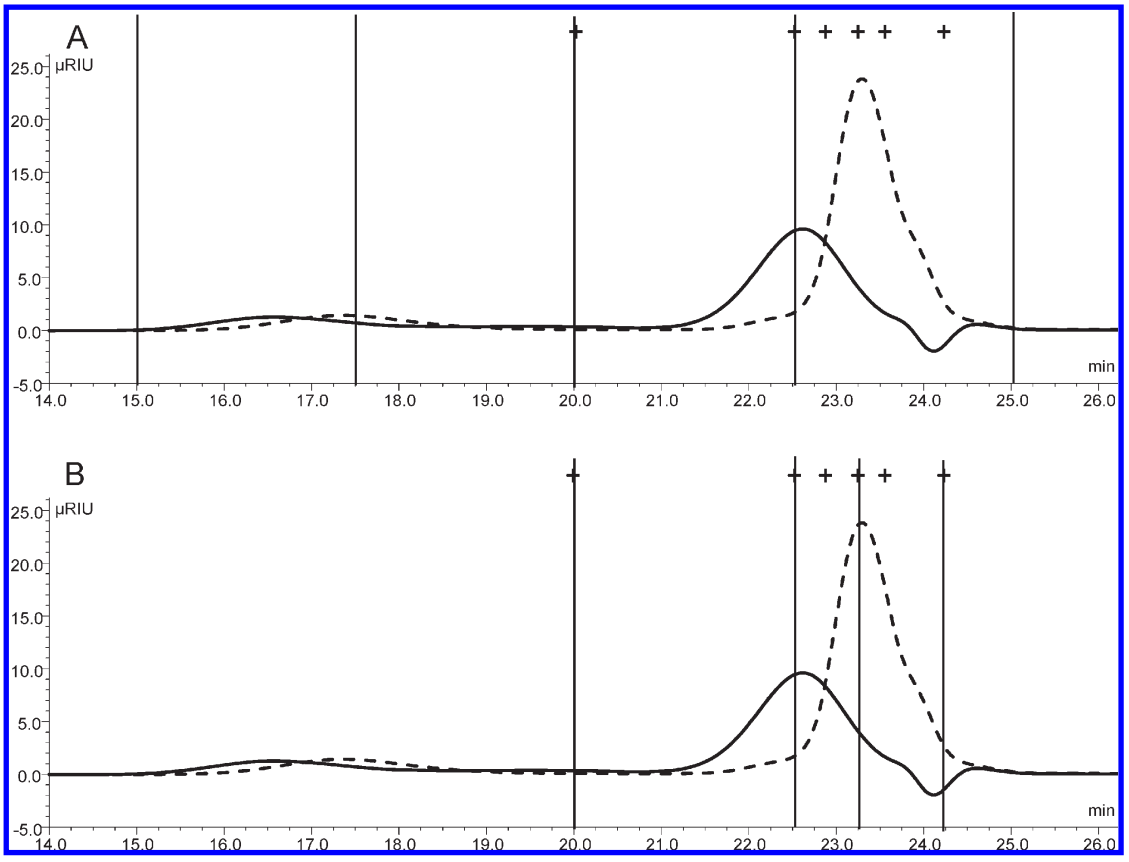

Figure 4. Two strategies for calculating the area of chromatographic peaks: (**A**) calculation of area based on fixed time intervals; (**B**) calculation of area based on time intervals made from the retention time of specific standards. Molecular mass markers (+) from left to right are 40×10^4, 1×10^4, 0.6×10^4, 0.13×10^4, xylohexaose, and xylose. The vertical lines in both panels represent time intervals.

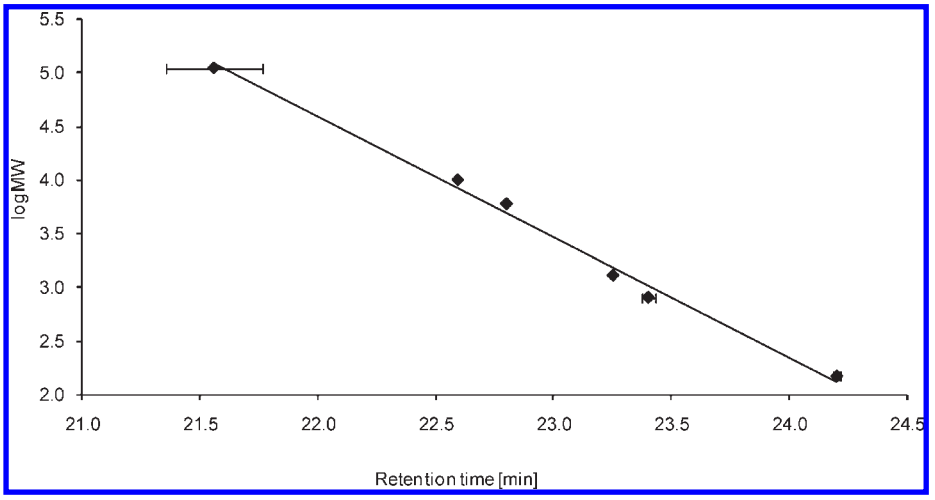

Figure 5. HPSEC calibration plot using the following molecular mass markers (0.5 mg mL^{-1}): pullulans with molecular masses of 0.13×10^4, 0.6×10^4, 1×10^4, and 40×10^4 g mol^{-1}, dextran (11×10^4 g mol^{-1}), xylohexaose (0.081×10^4 g mol^{-1}), and xylose (0.015×10^4 g mol^{-1}). Values represent the mean of three independent measurements and are shown ±1 horizontal standard deviation.

possible to almost eliminate the chromatographic peak from the sample solution. At this concentration the interference from the sample solution peak was so small that it was acceptable. On the basis of the HPSEC profile the molecular mass of SAX was determined to be 650 g mol^{-1}, equivalent to an average degree of polymerization (DP) in the SAX of ~4.8.

The pH of the mobile phase was decreased to 3 to assess its effect on the chromatographic profiles. An extra peak at 24.3 min occurred with both the sample solution and SAX (data not shown). This indicated that when the pH was lowered, the ionic strength of the mobile phase was increased, and therefore a second peak appeared.

Quantification. To verify that the area beneath a refractive index curve profile could be correlated to the concentration, a standard curve was measured for pullulan (1.0×10^4 g mol^{-1}) and xylohexaose (0.081×10^4 g mol^{-1}), both at concentrations of 0.2−2 mg mL^{-1}. R^2 values of 0.998 for pullulan and 0.994 for xylohexaose were obtained, indicating a linear correlation between the area and the concentration (**Figure 3**). The values for the area of xylohexaose at various concentrations were in general

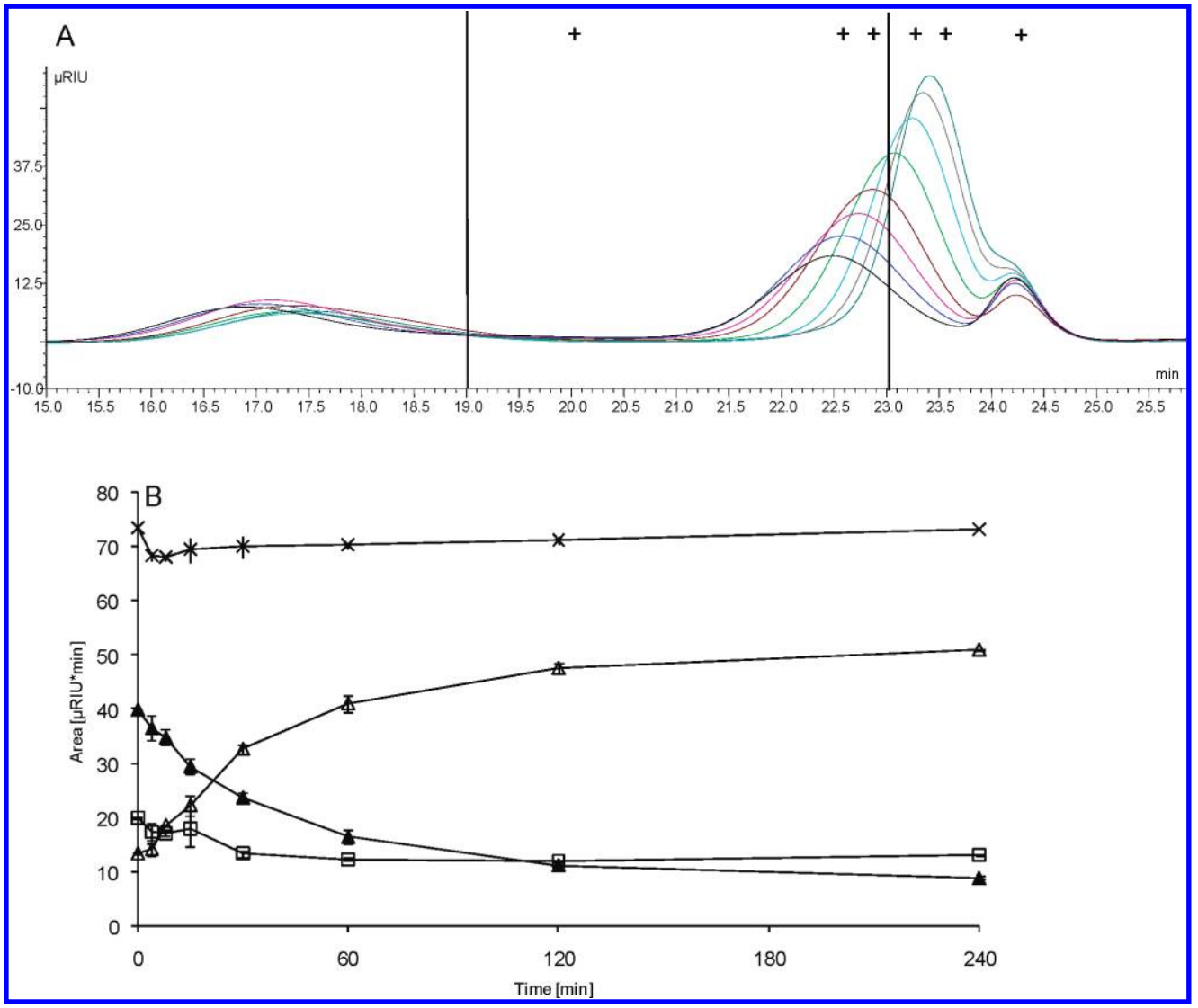

Figure 6. Catalyzed hydrolysis of birchwood xylan by BsX. (**A**) HPSEC chromatographic profile after 0 min (black), 4 min (dark blue), 8 min (pink), 15 min (brown), 30 min (green), 60 min (light blue), 120 min (gray), and 240 min (gray blue). Molecular mass markers (+) from left to right are 40×10^4, 11×10^4, 1×10^4, 0.6×10^4, 0.13×10^4, xylohexaose, and xylose. (**B**) Progression curve of the area in the following time intervals: 15−19 min (□), 19−23 min (▲), 23−27 min (△), total area 15−27 min (×). Values represent the mean of three independent measurements and are shown ±1 standard deviation. The vertical lines represent time intervals.

slightly lower than those obtained for pullulan, and therefore the tendency line does not go through the origin. Both standards were solubilized in water, and a relatively large negative peak corresponding to the water interfered with the chromatographic peak of xylohexaose, making the xylohexaose peak slightly smaller (data not shown). The negative peak appeared because the ionic strength of the sample was lower than that of the mobile phase. This was equivalent to when the sample solution (0.1 M CH_3COONa, pH 6) was eluted with CH_3COONa, pH 6, mobile phases at 0.15, 0.2, and 0.4 M, resulting in a negative peak (**Figure 2A**).

The slopes of both standard curves appeared to be similar: 5.5 μRIU min mL mg^{-1} for pullulan and 4.8 μRIU min mL mg^{-1} for xylohexaose. However, a comparison of the 95% confidence intervals showed that there was a significant difference between the two slopes. Hence, for further quantification, pullulan was used, because the molecular mass was more equivalent to the assessed hydrolysis of birchwood xylan and wheat bran.

In principle, two different strategies may be applied when the area of the chromatographic profiles is calculated to quantify molecules with a specific molecular weight distribution. First, the profile could be divided into fixed time intervals that are not directly correlated to the retention time of specific standards (**Figure 4A**). The area of the profile could then be calculated for each time interval. A second strategy could be to use time intervals based on the retention time of specific standards (**Figure 4B**). However, most likely, the time intervals would then have different lengths and therefore have to be standardized by dividing the calculated area by the time interval. This would imply that much information would be lost. We therefore continued with fixed time intervals to make a quantification of the different molecular weight distributions.

Calibration with Molecular Weight Standards. The apparent molecular mass of a sample was determined by comparison of the chromatographic behavior of the sample to that of standards with known molecular weights. Pullulan, dextran, xylohexaose, and xylose standards ranging in molecular mass from 0.015×10^4 to 11×10^4 g mol^{-1} were used for the calibration (**Figure 5**). An R^2 value of 0.993 was obtained, indicating a linear relationship between the retention time and the logarithm to the molecular weight, at least in the range of the used standards.

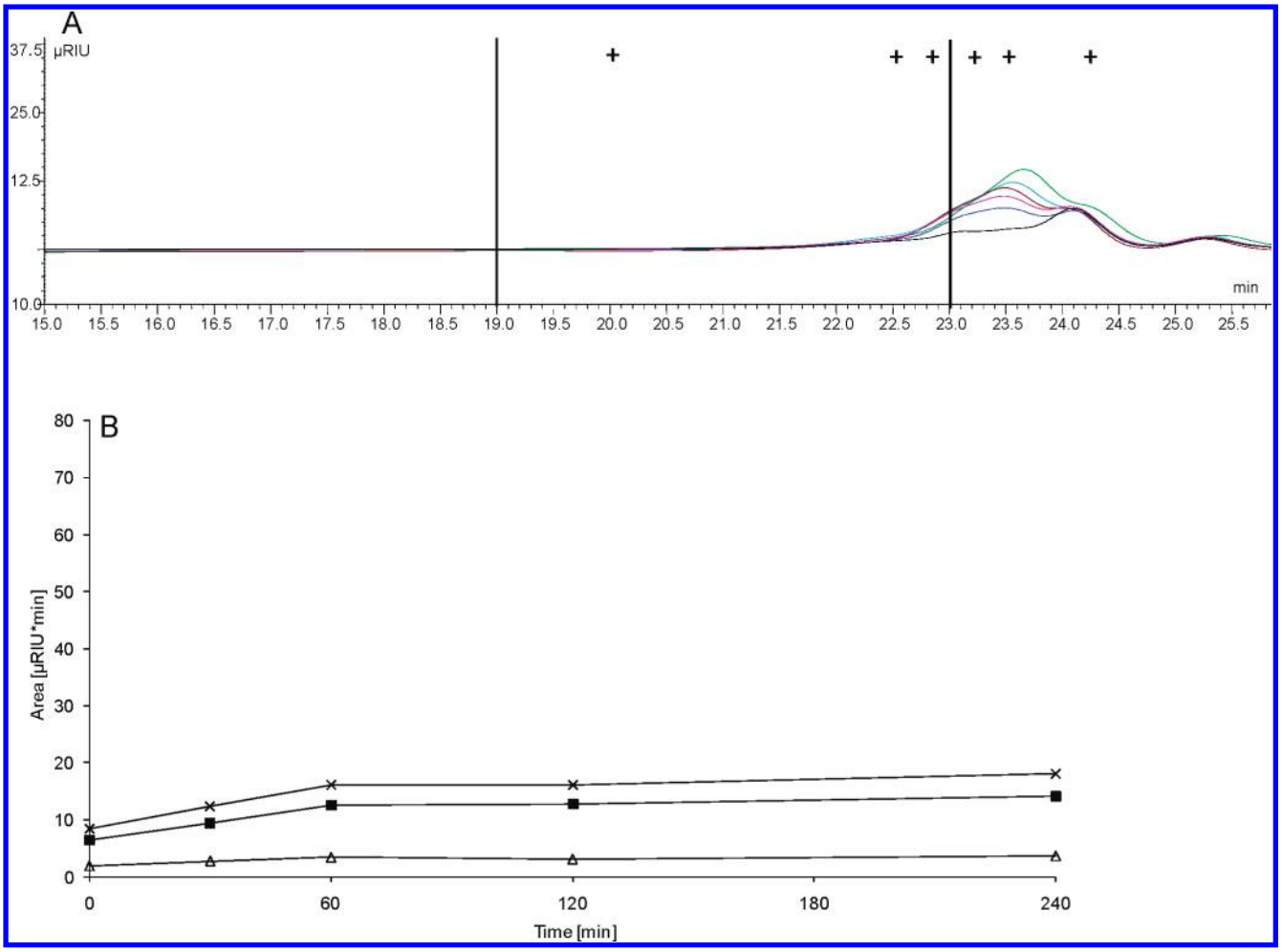

Figure 7. Catalyzed hydrolysis of wheat bran by BsX. (**A**) HPSEC chromatographic profile after 0 min (black), 30 min (dark blue), 60 min (pink), 120 min (brown), 240 min (light blue), ánd 24 h (green). Molecular weight markers (+) from left to right are 40×10^4, 11×10^4, 1×10^4, 0.6×10^4, 0.13×10^4, xylohexaose, and xylose. **B**. Progression curve of the area in the following time intervals: 19−23 min (△), 23−27 min (■), total area 15−27 min (×). Values represent the mean of three independent measurements and are shown ±1 standard deviation. The vertical lines represent time intervals.

Enzymatic Hydrolysis. The strategy with fixed time intervals was applied on a time study of the catalyzed hydrolysis of birchwood xylan by BsX (**Figure 6A**). Because this substrate was water-soluble, the initial profile of birchwood xylan after 0 h of incubation showed its molecular weight distribution (black line, **Figure 6A**). The initial chromatographic profile was composed of three peaks, where the first peak appeared after 16.8 min. Because this was earlier than the elution of the 40×10^4 g mol^{-1} molecular mass standard, these fragments had a molecular mass larger than 40×10^4 g mol^{-1}. The birchwood xylan also contained smaller fragments, eluting at 22.5 min, and the molecular mass of these fragments was estimated to be 1.1×10^4 g mol^{-1} using the calibration curve (**Figure 5**). Because a third peak appeared at the same time as the standard xylose (24.2 min), some xylose was also present in the substrate solution. During the incubation period, the molecular mass of the large and small fragments decreased as a result of the xylanase activity, where the molecular mass of the small fragments reached approximately 0.09×10^4 g mol^{-1} after 4 h. The apparent molecular mass of the large fragments decreased only relatively little during the incubation period and was still larger than 40×10^4 g mol^{-1}. This relatively small change might be explained by the HPSEC setup where only one HPSEC column, being able to separate low molecular mass fragments and not molecules larger than 40×10^4 g mol^{-1}, was used.

The HPSEC profiles were divided into three time intervals that resulted in an equal time division of 4 min, and the area underneath the curve in each interval was calculated (**Figure 6B**). The selection of the number of intervals was made as a compromise between obtaining detailed information and having a manageable overview. The calibration curve in **Figure 5** was used to convert retention times into molecular weights and the standard curve for pullulan in **Figure 3** for converting areas to concentrations.

For the time interval 15−19 min, which represented molecules larger than 40×10^4 g mol^{-1}, the area decreased only a little and mainly during the first 30 min of enzymatic treatment (**Figure 6B**). On the basis of the areas in this time interval, the concentration of these relatively large fragments was 3.59 at 0 min and 2.37 mg mL^{-1} after 240 min. For the time interval 19−23 min representing fragments with a molecular mass between 0.29×10^4 and 40×10^4 g mol^{-1}, the area decreased mainly during the first 120 min. The concentration of these relatively smaller fragments prior to the enzymatic hydrolysis was 7.15 mg mL^{-1} and, after 240 min, 1.62 mg mL^{-1}. In contrast to the other two intervals, the area in the period 23−27 min representing fragments smaller than 0.29×10^4 g mol^{-1} increased during the whole incubation period, however, leveling off after 120 min. At the beginning of the incubation period, the concentration was 2.41 mg mL^{-1}, and it increased to 9.13 mg mL^{-1} after 240 min.

The rate of attack of the xylanase could tentatively be evaluated by calculating the initial rate of decrease or increase in each time interval of the area curves for the first 15 min (**Figure 6B**). When the slopes were compared, the slope of the 15−19 min curve

(0.14 μRIU) was smaller than the one of the time interval 19−23 min (0.70 μRIU). The rate of decrease for the 19−23 min time interval was similar to the rate of increase in the time interval 23−27 min (0.59 μRIU). This indicated that the concentration of the smaller fragments (0.29×10^4−40×10^4 g mol^{-1}) decreased more quickly than that of the large fragments (4×10^5 g mol^{-1}). It could therefore be inferred that the xylanase appeared to have a higher affinity for the smaller fragments, but the rate difference might also be a result of the differences in the concentration of the two different molecular size (substrate) profile groups.

When wheat bran was hydrolyzed by BsX, no molecules above 40×10^4 g mol^{-1} were measured (**Figure 7A**). Because wheat bran was insoluble and therefore was removed by centrifugation after the enzymatic hydrolysis, the HPSEC profiles represent only the soluble fragments released from wheat bran by the BsX action. On the basis of **Figure 7A**, the molecular mass of the released fragments was smaller than 0.55×10^4 g mol^{-1}. During the incubation period the molecular mass of the fragments decreased to approximately 470 g mol^{-1} after 24 h of hydrolysis. This indicated that the released fragments apparently were the preferred substrate for the xylanase.

As in **Figure 6A**, the profile in **Figure 7A** was divided into the same fixed time intervals to be able to compare results from both reactions. However, only the intervals 19−23 and 23−27 min were used, because no fragments eluted earlier. As seen in **Figure 7B**, the area increased in both intervals mainly for the first hour and then only increased slightly. The initial concentration of the fragments with a molecular mass between 0.29×10^4 and 40×10^4 g mol^{-1} (eluting in the interval 19−23 min) was 0.36 mg mL^{-1} and increased to 0.66 mg mL^{-1} after 240 min of incubation. For fragments smaller than 0.29×10^4 g mol^{-1}, the concentration increased from 1.17 mg mL^{-1} after 0 min to 2.55 mg mL^{-1} after 240 min of incubation. The initial rate of increase in the area in the time interval 23−27 was relatively larger (0.101 μRIU) than that in the 19−23 min period (0.028 μRIU). This, together with the larger area, again reflected the apparent preference of the xylanase for the smaller, or at least the soluble, fragments.

DISCUSSION

On the basis of **Figures 1** and **2**, it is seen that the type, concentration, and pH of the mobile phase largely influence the chromatographic peak of the sample solution with respect to size, retention time, and polarity. When SAX was eluted, the peak from the sample solution interfered with the peak of interest, either decreasing or increasing the area and thereby making the concentration estimation less precise. The size and polarity of the sample solution peak was correlated to the ionic strength of the mobile phase, indicating that the RI detector reacted on differences in ionic strength between the sample and the mobile phase. To reduce the chromatographic peak from the sample solution, the ionic strength difference between the sample and the mobile phase had to be minimized. These results correspond with the recommendations from the HPSEC column manufacturer (Showa, Munich, Germany) that samples should be dissolved in the same sample solution as the mobile phase when a differential refractometer is used. However, our literature search illustrated in **Table 1** showed that this was only done in a few of the references. Le Coupannec et al. (*36*) also observed a slight alternation of the retention time in the chromatograms when using $NaNO_3$ and CH_3COONH_4 as eluents for HPSEC. This could be due to the influence of the nature of the salt on the matrix pore and the compound size.

Once the interference from the sample solution was minimized, the area of a chromatographic profile could in principle be used for quantification, because there was a linear correlation between the area and the concentration of specific standards (**Figure 3**). This linear correlation has been widely used in high-performance anion exchange chromatography (HPAEC) for quantification, because most samples can be separated into distinct peaks when concentrations of specific molecules are estimated on the basis of a standard curve of known standards (*35*). However, HPSEC has mostly been used for measuring the molecular weight distribution, and therefore one peak could represent molecules with different molecular weights (*8*). HPSEC has therefore until now mainly been used as a qualitative method, for monitoring the molecular weight distribution.

With this new method, it has now been possible to quantify how much a molecular weight distribution has changed and also to calculate parameters such as standard deviation to tentatively estimate the reproducibility of the experiment and the method. Furthermore, the contribution from the substrate itself to the molecular weight distribution can now be subtracted, so only the enzyme activity can be estimated. This feature is particularly relevant when one is dealing with substrates such as wheat bran, which tend to autohydrolyze; at least, wheat bran autohydrolyzes to a higher degree than, for example, birchwood xylan.

Despite our literature search on HPSEC, we found that only Kabel et al. (*22*) used a similar semiquantitative method based on division of the HPSEC profiles into retention time intervals. In their work, the HPSEC profile was divided into three retention time intervals of different lengths based on standards representing molecules of high (DP > 25), medium (DP 9−25), and low (DP < 9) molecular weights (*22*). For the three parts, the percentage of the total area in the HPSEC diagram was calculated. By using this method, the internal molecular weight distribution was depicted after a certain treatment. However, when using this method, it would most likely not be possible to depict if the total area increased, because the area in each interval also would increase. Therefore, an increase or decrease in the total concentration would most likely not be detected.

In the present study, birchwood xylan and wheat bran were enzymatically catalyzed by treatment with the xylanase BsX (**Figures 6** and **7**). On the basis of the HPSEC profiles, fragments with a molecular mass below 6×10^3 g mol^{-1} were produced from both substrates, mainly during the first 4 h of incubation. When fragments after 4 h were compared, higher concentrations were released from birchwood xylan than from wheat bran. This corresponds to earlier results when the same enzymatic hydrolysis was analyzed by HPAEC, yielding a total amount of xylo-oligosaccharides (DP 2−6) of 6.5 mM released from birchwood xylan and of 3 mM from wheat bran (*37*). The observed differences in HPAEC and HPSEC of the two substrates may be related to differences in the complexity of the substrates. Because birchwood xylan was composed of approximately 97% xylose (*37*) and therefore only had relatively few arabinose substituents, this substrate was more accessible than wheat bran, where lower levels of fragments were produced. This was probably due to the more complex composition, where physical access to the β-1,4 linkages in xylan was restricted by the various substituents on the xylan backbone (*38*).

In any case, the appropriate selection of the mobile phase composition is important, because it has a large influence on the chromatographic profile of xylo-oligosaccharides. When using the same mobile phase as the sample matrix solution, this study showed that the interference from the sample solution was reduced to an acceptable minimum in the one-column HPSEC separation profile. A new method suitable for quantifying the molecular weight distribution of enzymatic hydrolysis allowed a more precise and detailed quantification of the product. The HPSEC can be used not only as a qualitative method but also as a quantitative method where more information can be gained from

HPSEC chromatograms. It is our belief that the quantitative approach reported here may be applied to other types of carbohydrate substrates and hydrolysates.

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Received for review September 10, 2009. Revised manuscript received November 9, 2009. Accepted November 17, 2009. This study was partly supported by the Innovative Bioprocess Technology Research Consortium financed by the Danish Research Council for Technology and Production Sciences, Chr. Hansen A/S, Danisco A/S, Novozymes A/S. Financial support from the FOOD Denmark Graduate School, Center for Advanced Food Studies, Denmark, is also acknowledged.