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Comparative Study of Xylanase Kinetics Using Dinitrosalicylic, Arsenomolybdate, and Ion Chromatographic Assays

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ABSTRACT

Xylanases are commonly assayed by the dinitrosalicylic acid (DNS) or the arsenomolybdate (ARS) method. However, specific activities are many times higher with DNS than with ARS. This is because the DNS assay is more reactive and the ARS assay is less reactive with xylooligosaccharides than with xylose. Xylose is often used as a standard, even though oligosaccharides are prevalent, so the DNS method overestimates and the ARS method underestimates specific activity. Ion chromatography, with pulsed amperometric detection, separates and measures all products and intermediates, but quantitation on a molar basis is difficult, because few xylooligosaccharide response factors are known. This report directly compares these three assay methods for the assay of xylanase activities.

Index Entries: Oligosaccharides; degradation; pulsed amperometric detection; xylanase; chromatography.

INTRODUCTION

Many xylanases have been isolated and characterized in recent years to determine their potential usefulness in bleaching kraft pulps *(1–5)*. High productivity is essential for commercial manufacture, so accurate measures of activities are important. However, xylanase activity, as measured by reducing-sugar release, does not always correlate well with increased pulp brightness or reduced chemical demand following enzyme treatment. The question remains whether some xylanases have inherent characteristics that make them better than others. The more critical fundamental question

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is, how does one measure the molar turnover number of an enzyme if reducing-sugar assays give such radically different values?

The first step in assessing relative efficacy is to determine how much enzyme is required to obtain a desired result. From a practical perspective, this is done on a volumetric or cost basis, but fundamental studies are needed that can relate the effect to specific enzymatic activities or molar turnover numbers. Despite interlaboratory studies *(6)*, there is no well-defined, reliable means of comparing the activity of one xylanase to another. The dinitrosalicylic acid (DNS) method has been recommended *(7)*, but several researchers have shown that this assay does not give a consistent response when used with various substrates *(8,9)*. Moreover, activities obtained by DNS do not correlate with those obtained with other reducing-sugar assays, such as the arsenomolybdate method (ARS) of Nelson and Somogyi *(10)*. This problem was first noted in trying to use the DNS assay for starch hydrolysis *(11)*. It is apparently a result of the partial hydrolysis of oligosaccharides by the DNS reagent.

The objective of the present research was to reassess the relative merits of the DNS and ARS methods in light of more contemporary ion chromatograph (IC) methods that separate product sugars. Reducing sugar assays can be used as broad indicators of enzymatic activity, but DNS tends to overestimate and ARS tends to underestimate oligosaccharide production, and HPLC-based IC, combined with pulsed amperometric detection (PAD), is a better means to determine enzyme kinetics.

MATERIALS AND METHODS

Xylanase Assay

Xylanase activity was determined by measuring the release of reducing sugars and oligosaccharides from an approx 1% (w/v) solution of water-soluble birch wood xylan, using ARS *(10)*, DNS *(12)* and IC/PAD *(13)* methods. Aliquots for ARS, DNS, and IC/PAD assays were removed from a single reaction mixture, and either transferred directly into the ARS or DNS reagents, or heat-inactivated for subsequent IC/PAD analysis. The ARS assays used 1.0 mL aliquots; the DNS assay used 0.5 mL; IC/PAD used 10 μL. Substrate preparation and assay conditions have been previously described *(14)*. SP342* xylanase was obtained from Novo Nordisk (Franklinton, NC, Ecozyme was obtained from Zeneca Bio Products (Mississauga, ON, Can). Each stock enzyme preparation was diluted as necessary to obtain appropriate levels of product formation for the assay employed (*see* Fig. 2 legend below).

* The use of trade or firm names in this publication is for reader information and does not imply endorsement by the US Department of Agriculture of any product or service.

Sugar Standards

Analytical grade xylose (Aldrich, Milwaukee, WI) and xylooligosaccharides from Megazyme (North Rocks, Australia) were used as standards for ARS, DNS, and IC/PAD.

Chromatographic Conditions

Because of artifacts created by the presence of phosphate buffer in the reaction mixtures, samples for IC were diluted to less than 10 m*M* PO_4 prior to analysis. The chromatography system consisted of a 738 autosampler (Alcott Chromatography, Norcross, GA), a GP40 quaternary gradient high-pressure pump (Dionex, Sunnyvale, CA) and a pulsed amperometric detector (PAD, Dionex). Product separation was performed with two Dionex Carbo Pac PA1 guard columns and a single analytical column connected in series. These were eluted at a flow rate of 1.0 mL/min, with the following sodium acetate gradient, in 100 m*M* NaOH: 50 m*M* for 7 min; linear ramp to 200 m*M* at 25 min. Following each 10-μL injection, column cleanup was performed by elution (2 min) with 800 m*M* acetate in 100 m*M* NaOH, and column equilibration was performed by elution (11 min) with 50 m*M* acetate in 100 m*M* NaOH. To remove hydrophobic components that have the potential to foul these columns, on-line solid-phase extraction with a Dionex IonPac NG1 guard column was employed. The NG1 guard column and the autosampler were removed from the flow path 1.2 min after each sample injection. Detector settings were as follows: E1 = 0.1 V (300 ms), E2 = 0.6 (120 ms), E3 = −9 V (300 ms).

RESULTS AND DISCUSSION

Although both the ARS and DNS assays are based on reducing group formation, they do not give similar responses with different xylose oligosaccharide standards (Table 1). The ARS method is almost 12× more sensitive than the DNS method when assaying the presence of xylose (X). With xylobiose (X2) as a standard, reactivity of ARS is less, but the reactivity of DNS is greater, so the ratio of sensitivities drops to 4.9. With xylotriose (X3), the reactivity of the DNS assay is still greater, and ARS method is only 4.4× as sensitive. Thus, for the ARS assay, the molar response factor decreases, and, for the DNS assay, the molar response factor increases, with increasing degree of polymerization (DP), for the X, X2, and X3 series.

This means that the apparent enzyme activity depends on the sugar assay method and the prevailing oligosaccharide present in the hydrolysis mixture, and that the DPs of the product sugars affect the assays in inverse ways. Moreover, because xylose is commonly used as a standard (even though most xylanases produce little xylose), the ARS assay tends to underestimate, and the DNS assay to overestimate, enzyme activity when it is expressed as international units (i.e., μ/min) of reducing sugars released (as xylose equivalents).

Table 1
Molar Response Factors for Xylose, Xylobiose, and Xylotriose as Determined by ARS and DNS Methods

	Molar response factor		
Analyte	ARS	DNS	Ratio
Xylose	1.038	0.087	12
Xylobiose	0.675	0.130	4.9
Xylotriose	0.642	0.152	4.4

The difficulty in determining relative efficacy of various enzyme preparations is compounded further when capacity for reducing-sugar production is compared at slightly different activities. The rate of reducing-sugar release is linear only when enzyme activities are very low, or when reaction times are short. If the reaction is allowed to proceed, the reaction rate falls off rapidly (data not shown).

Because most researchers like to report the highest possible activities for enzyme production by their isolates, the usual practice is to compare enzymes based on apparent activities following only limited hydrolysis, i.e., while the reaction is in the linear region. However, this approach does not reveal differences in action patterns or substrate specificities, which can only be discerned by separating and quantifying the oligosaccharides.

Figure 1A,B illustrates the product profiles formed from birch glucuronoxylan by SP342 and Ecozyme. The oligosaccharide products, ranging from X1 to X11, were separated into well-resolved peaks between 4 and 19 min. The linear xylan series is resolved to even higher DP, but the products are masked by a second series of oligosaccharides that begin to elute after 19 min. Their longer retention on the IC column suggests that these consist of 4-*O*-methylglucuronosides of xylan oligomers. A 4-*O*-methylglucuronic acid standard elutes at or immediately prior to the first member of this series of peaks, depending on the chromatographic conditions employed. Compositional analysis of the soluble xylan used as substrate indicated a minimal concentration of 11% (w/w) 4-*O*-methylglucuronic anhydride. Following prolonged incubations, late-eluting products disappeared and xylooligosaccharides accumulated, along with other earlier eluting members of the presumed acidic series (data not shown). But no standards are available, so the identities of the late-eluting products as acidic xylan oligosaccharides cannot be confirmed.

In examining these neutral and acidic products, it is first notable that almost no xylose is present in either reaction mixture at the 30-min time-point (when the reaction is still linear), so an estimate of enzyme activity based on a xylose standard is meaningless. Second, there is no apparent transition from an accumulation of higher DP oligosaccharides to an accumulation of lower DP oligosaccharides during the time frame examined.

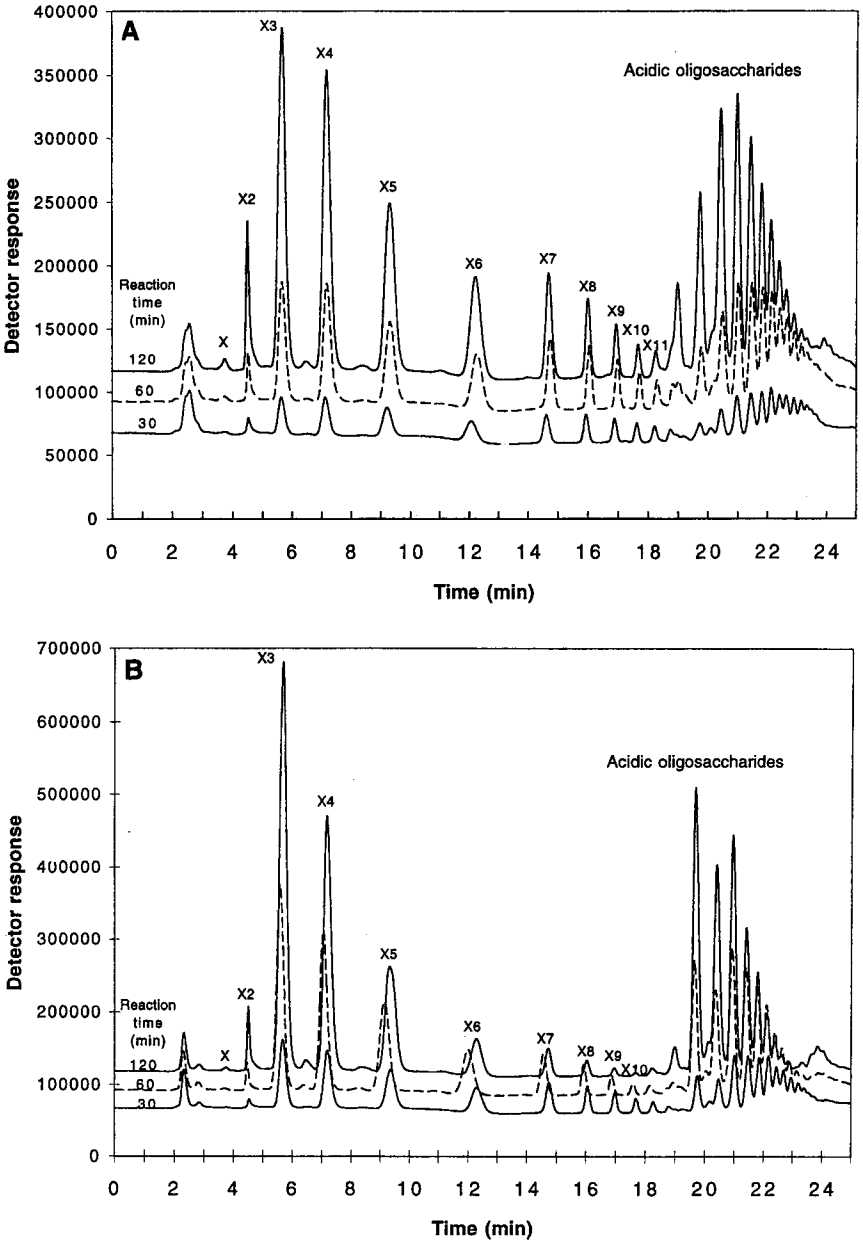

Fig. 1. Xylan oligosaccharide and acidic oligosaccharide products of **(A)** SP342 and **(B)** Ecozyme reaction mixtures at 30, 60, and 120 min. Reaction mixtures received 1.0 and 2.0 μL/mL of stock enzyme solution for SP342 and Ecozyme, respectively.

This is to say, the xylanases do not form intermediate DP (X5 to X11) products in a transient manner. Rather, what one observes is the progressive accumulation of X2, X3, and X4, plus acidic oligosaccharides, in the case of Ecozyme, and the accumulation of a wide range of oligosaccharides, in the case of SP342. These results suggest that the greatest affinity remains

with the high-mol-wt xylan, because the ratios of the DP × to X11 and acidic xylooligosaccharides do not change much in the course of this reaction.

It is informative to estimate sugar release based on the total carbohydrate present in the solubilized oligosaccharides. This can be done only if response factors are available for each of the products. Quantitative estimates of PAD responses, with standard mixtures of xylan oligosaccharides having a degree of polymerization of up to 5, show that the detector response is more closely related to the oligosaccharide mass than to its molarity (Table 2), but since oxidation at the electrode is incomplete and varies with the product, no definitive quantitation of higher DP products can be obtained without proper standards. This is particularly true with respect to the analysis of acidic oligosaccharides. The molar reactivities of uronic acids are known to be less than those of xylose, and the reactivities of xylan oligosaccharides with uronic acid moieties attached are probably lower than the reactivities of xylooligosaccharides, so the PAD response probably underestimates the accumulation of acidic oligo saccharides.

On a molar basis, the PAD response is similar for xylose and X2, but, on a weight basis, the PAD response drops dramatically. For X3 and X5, the molar PAD response factor increases significantly, but the change in the weight-response factor is less dramatic. A similar PAD response was previously reported for amylodextrins up to DP 7 *(15)*, so our observation is consistent with previous results.

After the sharp drop from xylose to xylobiose, the PAD mass-response factor seems to change only slightly (Table 2). If one assumes that the areas under the peaks are representative of the masses of these products, it is possible to obtain a rough estimate of the amounts of sugars present in the higher oligosaccharide series. To make the results comparable to ARS and DNS assays, these products have been expressed as xylose equivalents. For example, the amounts of xylotriose present in the reactions are reported as three xylose equivalents.

Using actual molar response factors for DP 2, 3, and 5, linear regression was used to estimate molar response factors for DP 4 and DP 6 to 11 xylooligomers (Response factor = [966563 * DP] + 948743; r^2 = 0.993 (3 pts).

The total products detected by IC/PAD on a mass basis were compared to reducing sugars measured by the DNS and ARS methods, and the DP 1 to X11 products, as calculated from their molar response factors (Fig. 2). By this comparison on a mass basis, the IC/PAD analysis detects almost twice as much product as the DNS method. The IC/PAD data in the mass analysis include materials eluted during the column wash that are not shown in Fig. 1. The total molar concentration of DP 1 to 11 xylooligosaccharides approximates, or is slightly less than, the molar xylose equivalents assayed by the ARS method. Because the molar response factors are unknown for the higher acidic oligosaccharide series, they are not

Table 2
Molar- and Mass-Based Response Factors for Xylooligosaccharides Relative to that Observed for Xylose when Analyzed by the IC/PAD Method

	Relative response factor	
Analyte	Molar	Mass
Xylose	1	1
Xylobiose	0.92	0.49
Xylotriose	1.31	0.47
Xylopentaose	1.88	0.42

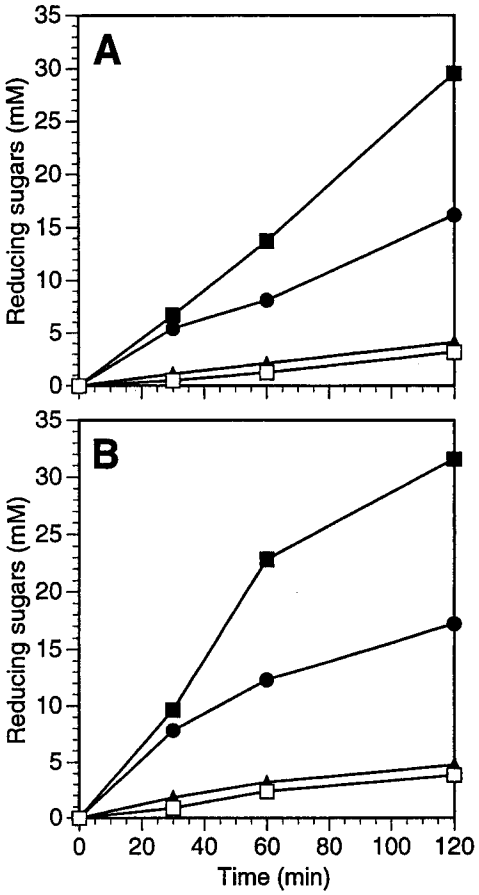

Fig. 2. Sugar production from birch xylan by **(A)** SP342 and **(B)** Ecozyme as assayed by ARS, DNS, and IC/PAD. ARS (●) and DNS (▲) reducing-sugar concentrations were calculated using xylose as a standard. For the IC/PAD assays, total oligosaccharide concentrations were estimated either by using a mass response factor and converting into xylose equivalents (■), or by using molar response factors and expressing the X1 to X11 series as total m*M* of reducing equivalents (□). SP342 and Ecozyme reaction mixtures received 0.1 μL/mL and 0.2 μL/mL of stock enzyme solution, respectively.

included in this latter calculation. This could account for the smaller amount of product observed by IC/PAD.

Under the conditions employed, kinetic assays based on the ARS method can be represented as international units (i.e., μmoles of xylooligosaccharide product/min), but kinetic assays based on the DNS method would be better represented as mg (or μg) of xylan solubilized. However, the extent to which the ARS or DNS methods measure the amounts of acidic oligosaccharides cannot be determined from these data.

CONCLUSIONS

In comparing these data, it is reassuring to note that the relative activities of the two enzyme preparations are similar by each of the three assay methods. However, DNS greatly overestimates reducing-group formation. The actual value for μmol of oligosaccharide products formed is better approximated by the ARS assay, but until more accurate response factors can be determined for xylooligosaccharides, and particularly for those in the acidic series, meaningful analysis of the kinetics, such as determination of specific molar turnover numbers, cannot be carried out, even by the IC/PAD method. Given the large difference in reactivities between xylose and X2, the declining difference in reactivities of higher oligosaccharides, and the prevalence of X2 or X3 as ultimate reaction products for most xylanases, it is better to employ X2 or X3, rather than xylose as a standard, when estimating activities and molar turnover numbers. Product profiles from pulp samples have many more complexities than reported here, so a sound basis for understanding xylanase bleaching in terms of enzyme action requires further study.

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