



Rapid 2,2'-bicinchoninic-based xylanase assay compatible with high throughput screening**

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Abstract

High-throughput screening requires simple assays that give reliable quantitative results. A microplate assay was developed for reducing sugar analysis that uses a 2,2'-bicinchoninic-based protein reagent. Endo-1,4- β -D-xylanase activity against oat spelt xylan was detected at activities of 0.002 to 0.011 IU ml⁻¹. The assay is linear for sugar concentrations from 0 to 86 μ g ml⁻¹ and can also be used to assay protein concentrations (0 to 143 μ g ml⁻¹) on the same plate. A variety of temperatures and pH conditions can be used and, after incubation, the assay requires only one detection reagent and one heating step.

Introduction

Xylanase (EC 3.2.1.8) catalyzes the hydrolysis of 1,4- β -D-xylosidic linkages. It has industrial uses in food products (Beg *et al.* 2001) and in enhancing kraft pulp bleaching (Dence & Reeve 1996, Kulkarni *et al.* 1999, Suurnakki *et al.* 1997). In the pulp and paper industry xylanases are applied just after the pulp washing. Residual alkali from the kraft cook often leaches slowly out of the fibers (Tolan & Guenette 1997). To avoid corrosion problems arising from the addition of acid, the application of this enzyme in pulp bleaching ideally requires enzymes with activity at pH values greater than 10.5. This need has stimulated research into identification and characterization of many new xylanases (Kenealy & Jeffries 2003). Enzymes that are active at more alkaline pH or at higher temperatures have been described and formulated into industrial products (Suurnakki *et al.* 1997). The ideal enzyme for application in the pulp and paper industry has not been discovered in nature, but there is potential to develop it via biotechnological means (Kenealy & Jeffries 2003).

Site-specific (Ebanks *et al.* 2000, Moreau *et al.* 1994) and random mutagenesis, gene or domain shuffling (Kulkarni *et al.* 1999), rational design and directed evolution (Kolkman & Stemmer 2001) are approaches to obtain a xylanase that has activity under the desired process conditions (Kenealy & Jeffries 2003). Recombinant approaches generate large libraries and require assaying of many variants to identify clones producing active enzyme.

Many xylanase assays have been described (Bailey *et al.* 1992, Biely *et al.* 1985, Doner & Irwin 1992, Fox & Robyt 1991, Jeffries *et al.* 1998, McCleary 1992, McFeeters 1980, Waffenschmidt & Jaenicke 1987). Most determine the amount of reducing sugars generated following hydrolysis of xylan and often require addition of multiple reagents, removal of insoluble substrate, or extensive heating steps. These manipulations are incompatible with, difficult, or expensive to accommodate in high throughput assays. The reagent 2,2'-bicinchoninate (BCA) can be used to rapidly detect and quantify from 0 to 2000 μ g ml⁻¹ of reducing sugar in solution (Fox & Robyt 1991, Garcia *et al.* 1993, McFeeters 1980) depending on the configuration of the assay. We have formulated a BCA xylanase assay that is suitable for high throughput screening

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in 96-well plates and have demonstrated its utility in screening production clones and testing temperature and pH requirements for xylanase activity.

Methods

Materials

Pulpzyme HA and Pulpzyme HC samples were obtained from Novozymes (Denmark). The enzyme solutions were stable at 0 to 5 °C. Oat spelt xylan, xylose, and a 2,2'-bichinchonic acid kit for protein determination were acquired from Sigma.

Substrate preparation

Oat spelt xylan (4% w/v in deionized water) was mixed and stored at 0 to 5 °C for at least 72 h. The supernatant (4000 × *g* for 30 min) of this solution is approx. 2% soluble xylan and is stored frozen when not in use. Fresh buffered xylan was made with 1 ml soluble xylan + 3.75 ml water and 0.25 ml 1 M buffer. Buffer solutions, 1 M, sodium acetate pH 5, Tris/HCl pH 8, and glycine/NaOH pH 10 were made, filter sterilized, and stored at room temperature.

Enzyme (10 µl) was dispensed into the wells of a microtiter plate (Uniwell, United Laboratory Plastics, St. Louis, MO). Buffered xylan (25 µl) was added to the wells of the plate using a multichannel dispenser. Plates were sealed (Polyolefin film, Nunc-232701, Fisher Scientific, Pittsburgh, PA) and immersed in 50 °C (or other temperature) water bath (supported underneath the dish) for 40 min for the screening assay. Plates were removed and placed on ice for 5 min. The sealing tape was removed and 150 µl fresh BCA reagent (50 ml part A carbonate/tartrate/BCA + 1 ml part B copper sulfate solution) was added. Another sealing tape was attached and the plate was incubated at 80 °C for 30 min. (About a 10% absorbance increase is observed by increasing the time to 45 min.) The plate was cooled on ice; the tape was then removed and read at 562 nm on a Molecular Devices Spectra Max Plus microtiter plate reader (Sunnyvale, CA). A control plate was included for screening assays that had the added enzyme source and no xylan to correct for protein contribution to the color. Appropriate blanks using deionized water in place of enzyme were included on each plate. Protein (0–5 µg BSA) and xylose (0–20 nmol) standards may be added to some wells at an initial volume of 35 µl.

The arsenomolybdate assay (Nelson–Somogyi) for reducing sugars described for the Institute of Biotechnology, Jülich, FRG (Ghose & Bisaria 1987) was conducted in test tubes using the above-described diluted xylan substrates. Buffered xylan (0.25 ml) and enzyme source (0.1 ml) were incubated at 50 °C for 30 min, removed from the water bath, and mixed with an equal volume of solution C. This mixture was then heated 15 min in boiling water and cooled. Solution D (2× volume solution C) and 10 ml water were added and absorbance at 520 nm was measured. The reaction with 3,5-dinitrosalicylic (DNS) acid was performed by mixing two volumes of DNS solution (1% DNS, 30% NaK tartarate, and 1.6% NaOH) with one volume of buffered xylan and enzyme source after incubation (as described above), heating in boiling water for 15 min, cooling, and measuring absorbance at 575 nm. Xylose standards were included in both assays through all the steps to determine enzymatic activity. For both DNS and the arsenomolybdate assays, the final reacted solution was dispensed (200 µl) into wells of a microplate to simultaneously determine the absorbance of all the solutions and blanks.

Results and discussion

BCA reacts with both protein and reducing sugars. The substrate xylan also contains reducing ends. The limitations of a xylanase assay depend upon the development of color with all reactants and the useful absorbance limits of the microplate reader ($A \approx 3$).

Table 1 shows the results using xylan and BCA and the additive effect of reactants in the assay. Soluble xylan (~100 µg) provided an average absorbance of 0.28 and a higher absorbance of 0.684 at pH 10 as a result of buffer interactions with BCA. Buffer also made a small contribution to color development at pH 8. Using this amount of xylan allows for up to 2.5 absorbance units to be formed by the generation of reducing sugars plus any contribution from protein. The final absorbance of the xylan, added xylose, and protein approximates the sum of the absorbance measurements of the individual components. The addition of 2 µg BSA to the xylan increased the absorbance by an average of 0.475. The addition of 5 nmol xylose increased the absorbance by an average of 0.565. These values agree well with those determined for BSA and xylose standards (Figure 1).

The protein contribution to the absorbance can be very small if active xylanase is used, but it can be

Table 1. Contribution of xylan and additions to final absorbance in reactions with BCA.

Xylan source	Absorbance 562 nm with blank subtracted ^a			
	No addition	+ BSA	+ Xylose	+ BSA and xylose
Xylan, pH 5	0.277 ± 0.051	0.829 ± 0.069	0.905 ± 0.137	1.332 ± 0.074
Xylan, pH 8	0.281 ± 0.037	0.731 ± 0.047	0.853 ± 0.063	1.242 ± 0.062
Xylan, pH 10	0.684 ± 0.038	1.106 ± 0.047	1.178 ± 0.036	1.551 ± 0.08

^aValues represent average ± standard deviation of at least 20 determinations with a reagent blank subtracted in total reaction volume of 35 μ l + 150 μ l BCA reagent.

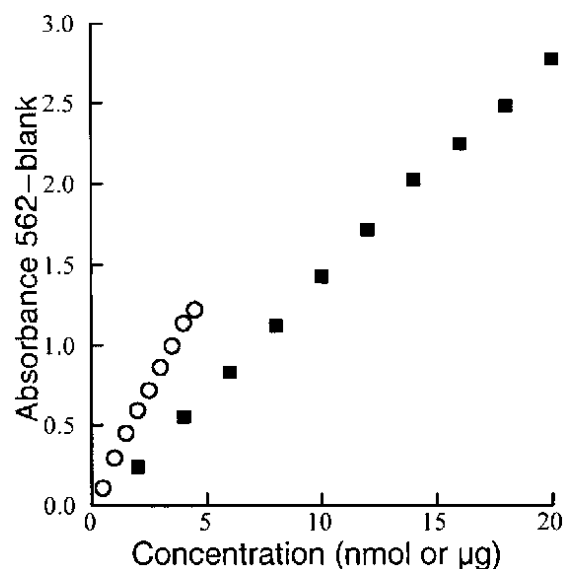


Fig. 1. Xylose and bovine serum albumin reactivity with BCA. Total volume of reaction was 35 μ l sample + 150 μ l BCA reagent. Data represent average of 8 samples minus absorbance of water blank. Units of xylose = nmol; units of bovine serum albumin = μ g. BSA, $\circ$; xylose, $\blacksquare$.

significant if protein from cell lysates and medium components are present. A control that measures the amount of absorbance resulting from the addition of enzyme is critical in determining xylanase activity in crude samples. Too much protein can also limit the sensitivity of the assay. In that case, the sample can be diluted and the incubation time of the assay extended to allow color development from generated reducing ends.

We used this assay for screening relative xylanase activities at different pH values. We found mutant enzymes with altered pH activity profiles produced in recombinant *Escherichia coli*. Table 2 shows the controls that were included on microplates during a screening of 1100 xylanase producing clones. These control enzymes from Novozymes have different pH activity profiles and served as a control for testing

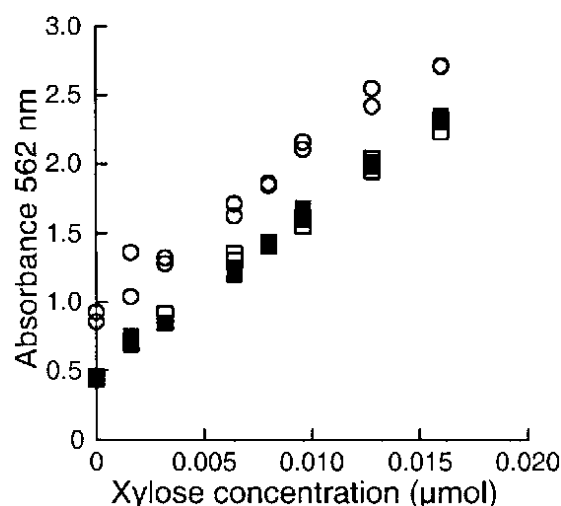


Fig. 2. Xylose reactivity with BCA in presence of xylan. Absorbance 562 nm measured for standards included with buffered xylan. Buffered xylan (25 μ l) at pH 5 $\square$, pH 8 $\blacksquare$, and pH 10 $\circ$ was dispensed with xylose standards (in 10 μ l) incubated at same time and conditions as enzyme samples and then reacted with 150 μ l BCA.

activity at altered pH. Pulpzyme HA was included in this assay at a dilution that provided some activity at pH 8 but would not be accurately determined since the absorbance of the samples at pH 5 was routinely at the maximum of the plate reader. For the purpose of screening, the background absorbance of wells containing the protein contribution can be subtracted from the final absorbance and the activity estimated at each pH by comparing the absorbance to a control containing only the buffered xylan. Pulpzyme HC was active at all the pH conditions, but was less active at pH 10.

The activity of xylanase can be measured directly when xylose standards are included on the plate in the presence of xylan. The results from such an assay is shown in Table 3 along with the results of other methods of analyzing reducing sugars while using the same buffered xylan. Xylose standards were included in wells with xylan at the different pH values (Figure 2). If the background absorbance of the xylan were

Table 2. Xylanase screening activity measurements using Pulpzymes HA and HC.

Enzyme	Absorbance at 562 nm (corrected for protein content) ^a		
	pH 5	pH 8	pH 10
HA	2.699 ± 0.223	1.409 ± 0.726	0.027 ± 0.26
HC	1.36 ± 0.448	1.321 ± 0.419	1.128 ± 0.493

^aData represent average ± standard deviation (from 9 separate plates) for absorbances corrected for water blank and protein contributions. Protein absorbances for HA and HC = 0.674 ± 0.136 and 1.571 ± 0.414, respectively. Samples incubated at 50 °C for 40 min.

Table 3. Xylanase activity measurements using Pulpzymes HA and HC.

Enzyme	pH	Activity ^a ($\mu\text{mol min}^{-1} \text{ml}^{-1}$ enzyme)		
		DNS	N-S	BCA
HA	5	218	261	210
	8	37	0	47
	10	0	48	4
HC	5	215	238	281
	8	210	265	332
	10	184	270	246

^aData obtained using enzymes at 1:1000 dilution for DNS, 1:5000 for Nelson–Somogyi (N–S), and 1:30 000 for BCA. Samples incubated at 50 °C for 30 min. Assays run in duplicate except for BCA assay (8 replicates).

subtracted from the values in Figure 2, the lines can be superimposed and correlate with the absorbencies shown in Figure 1.

The activity measured using the BCA reagent was similar to that of the other two reagents. The enzyme in the BCA assay (1:30 000) was sufficiently diluted so that the protein did not contribute to the color. The activity of Pulpzyme HA was highest at pH 5 and that of Pulpzyme HC was highest at pH 8 for the BCA assay. This is in agreement with the reported values of the manufacturer where Pulpzyme HA was acidic and Pulpzyme HC had a broad pH range.

The arsenomolybdate and DNS assays required the addition of a reagent and boiling to generate the color. The use of boiling baths required glass tubes since the plastic microplates warp in the boiling water. The BCA assay will develop color at 80 °C with reducing sugars and thus can be used with plastic microplates (Fox & Robyt 1991). The sealing tape used in our study prevented evaporation during extended incubations with the plate and was also used during the heating step.

The xylan present in this assay would be capable of being cleaved many times, creating multiple reducing ends. The reactivity of the reducing ends can be estimated by the use of xylose as the reducing sugar. Figure 1 shows linear results for xylose up to 20 nmol and protein (BSA) up to 5 μg . An increase of 20 nmol reducing ends would represent cleavage of less than 3% of the available 1,4- β -D-xylosidic linkages for the xylan present in the assay. The assay is not limited by the amount of xylan and accurate results can be obtained following proper dilutions of the enzyme.

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