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Abstract

The Oak Ridge strains of wild type *N. crassa* grow well with high molecular wight xylan from oat spelts (Sigma X-0376) as the sole carbon source. Xylan, a substituted ß-1,4 linked polymer of xylose, induces high levels of xylanase, xylosidase and ß-galactosidase activities in both culture medium and mycelium. To assay for xylanase activity, chromogenic and fluorogenic substrates have been prepared by procedures based on those of Biely et al. 1985. Anal. Biochem. 144:142-146; Biely et al. 1985. Anal. Biochem. 144:147-151; Rinderknecht et al. 1967. Experientia 23:805; and De Belder and Granath 1973. Carb. Res. 30:375-378. Batches of xylan that have a tan color and granular texture should be dissolved in boiling water, precipitated with two volumes of absolute ethanol, and dried as described below before use. These techniques may also be used to prepare chromogenic and fluorogenic substrates from dextrans, glucans or other high molecular weight polysaccharides to assay endoglycosidases for which suitable assay reagents are not readily available.

Chromogenic and fluorogenic substrates for assaying xylanases of Neurospora

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1. **Preparation of Blue Xylan (BX)** Disperse 2 g xylan in 60 ml deionized water with a magnetic stirrer at room temperature. Ad 0.5 g Remazol Brilliant Blue (RBB) (Sigma R-8001) and stir until dissolved. Add 20 ml of a 1 mg/ml solution of Na_2SO_4 dropwise over a period of 2 min. Then add 5 ml deionized water and 15 ml of a 10% (w/v) solution of NaOH to initiate the coupling reaction. At the end of 90 min of stirring at room temper-ature, add two volumes of absolute ethanol (200 ml) to precipitate the conjugated xylan. Let stand without stirring for 15 min at -20°C to complete the precipitation. The dyed xylan is collected on a vacuum filter and washed repeatedly with buffered 67% ethanol (1 volume of 0.05 M sodium acetate buffer, pH 5.4 and two volumes of absolute ethanol) until the filtrate is free from all trace of blue color. Break up clumps of precipitate on the filter if necessary. This washing step is crucial as any trapped dye will give an unacceptably high blank. When all traces of blue color are gone from the filtrate, rinse the BX on the filter with 80% ethanol in deionized water. Continue to dehydrate the xylan by washing with 95% ethanol, absolute ethanol and then acetone. Dry under vacuum overnight, pulverize the BX in a mortar, and store at room temperature. Blue xylan made by this procedure has a degree of substitution between 0.08 and 0.10 moles of dye per mole of xylose.

2. **Preparation of Fluorescein labelled Xylan (FX)** This procedure is nearly the same as that for BX except that it has been scaled down due to the higher cost of the labelling reagent, DTAF, 5-(4,6-dichloro triazin-2-yl) amino fluorescein hydrochloride (Sigma D-0531). The 6-DTAF isomer may also be used. Deviations from the BX procedure are noted. Disperse 1 g xylan in 30 ml deionized water. Alternatively, the xylan may be dissolved by heating the water to at least 70°C and cooling to room temperature before proceeding. Add 0.10 g DTAF and 10 ml of Na2SO4 as above. Bring to pH 10 with 10% NaOH and add NaOH as needed to keep reaction mixture near pH 10 over the next 2 h at room temperature. At the end, add sufficient deionized water to bring the final volume to 50 ml. Precipitate the labelled xylan with two volumes of absolute ethanol (100 ml) as before and wash on a filter until all of the yellow color is gone from the filtrate. The final traces of free label may be visualized by alkalizing aliquots of the filtrate

with NaOH and viewing the fluorescence. Dehydrate the FX and dry in vacuo as above. Fluorescent xylan made by this procedure has a degree of substitution of about 0.02.

3. **Xylanase Assays**. These assays directly measure the depolymerization of the high molecular weight substrate and are thus relatively insensitive to the presence of exoxylanases and xylosidases. The assays are run in a 1 ml volume consisting of 0.1 ml of sample and 0.9 ml of a reaction mix containing 5 mg/ml of BX or 2 mg/ml of FX in 0.05 M sodium phosphate buffer at pH 7.5. After incubation at 37°C, the undigested labelled xylan is removed by precipitation with 2 volumes of cold ethanol, chilling for 15 min at -20o C and centrifugation.

The solubilized dyed oligoxylosides are measured spectrophometrically at 590 or 660 nm or fluorometrically for fluorescein (max 493 nm, Emax nm). Reagent blanks consisting of sample and assay buffer are essential. Other buffers and condition may be used. These assays are quite sensitive to the ionic strength of the assay buffer since the alcohol solubility of the BX and FX fragments varies with buffer concentration. The absorbance of RBB is relatively unaffected by pH, but the fluorescence of DTAF should be measure above pH 7. Alcohol quenches the fluorescence somewhat so fluorescein standards should be prepared in 67% EtOH.

4. **Localizing Xylanase in gels**. BX may also be used to localize xylanase activities in non-denaturing PAGE slab gels. Separating gels are rinsed with 0.01 M sodium phosphate buffer, pH 7.5 and placed in contact with gels consisting of 2% purified agar (i.e. Ionagar #2) and 10 mg/ml of BX in 0.01 M sodium phosphate buffer, pH 7.5. The BX/agar gels are wrapped with Saran wrap to prevent drying and incubated at 34°-37° for several hours. The zymograms are then developed by soaking the BX/agar gels in buffered 67% ethanol (one volume 0.05 M sodium acetate buffer, pH 5.4 and two volumes of absolute ethanol). Enzyme bands show as clear zones against a blue background. Acknowledgement: I thank Dr. Patricia St. Lawrence for providing space and support for this work.