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Short Communication

# Apparent cellulase activity of purified xylanase is due to contamination of assay substrate with xylan

J.C. Royer[1], J.S. Novak and J.P. Nakas

*State University of New York, College of Environmental Science and Forestry, Syracuse, NY, USA*

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## SUMMARY

Purified xylanase A of *Trichoderma longibrachiatum* was active on one of two carboxymethyl cellulose (CMC) preparations used as cellulase assay substrates. The pattern of enzyme activity, and analysis of the substrate by acid hydrolysis and thin-layer chromatography (TLC) suggested that the enzyme had acted on xylan present in the CMC.

## INTRODUCTION

Considerable variability in the specificity of purified fungal xylanases has been reported, particularly with regard to activity on cellulose [1–5,13,14,16]. Endocellulase and xylanase activity are frequently estimated by measuring the quantity of reducing end groups formed from a solution of 1% (w/v) CMC or xylan, respectively [7,13].

*Trichoderma longibrachiatum* generates high levels of extracellular xylanase activity when cultured under the appropriate conditions [9,10]. Two endoxylanases were recently purified and characterized with respect to physiochemical and enzymatic properties [11]. In this paper, we provide evidence that xylanase A of *T. longibrachiatum* lacks cellulase activity, and that apparent cellulase activity is a result of activity on contaminating xylan in the CMC assay substrate.

## MATERIALS AND METHODS

### *Source of enzyme*

*T. longibrachiatum* Rifai was cultured on 1% (w/v) Solka Floc BW200 (James River Corp., Berlin, NH) in Vogel salts (pH 7.0) in shake flasks according to Royer and Nakas [9]. Xylanase A was purified from centrifuged culture medium according to Royer and Nakas [11]. Contaminating protein was precipitated at −20 °C by the addition of 2.5 vol. of ethanol to the crude enzyme. After centrifugation (6000 × *g*, −20 °C), the xylanase fraction was precipitated with an additional 7.5 vol. of ethanol. The precipitation mixture was then centrifuged (6000 × *g*, 5 °C) and the resulting pellet was air-dried, solubilized in sodium acetate (100 mM, pH 5.0), and subjected to gel filtration of BioGel P-60. Fractions containing the major peak of xylanase activity were pooled, dialyzed against sodium acetate (20 mM, pH 5.0) and applied to a column of carboxymethyl BioGel equilibrated in sodium acetate (20 mM, pH 5.0). Xylanase A was eluted from the column with a NaCl gradient of 0 to 500 mM.

### *Assays*

Xylan substrate was the soluble fraction from a 1.0% (w/v) suspension of larch xylan (lot No. 97109, Koch-Light, Haverhill, UK) in sodium citrate-HCl (0.05 M, pH 4.8) after centrifugation at 5000 × *g* for 20 min. A suitable dilution of enzyme, in 500 μl of sodium citrate buffer, was incubated with 500 μl of substrate at 50 °C for 30 min. (Enzyme was diluted to produce between 0.1 and 0.2 mg reducing sugar in the assay.) The reaction was terminated by boiling (5 min) and the reducing sugar concentration estimated by the procedure of Somogyi [12], with xylose as standard. Activity was expressed as micromoles reducing sugar released per min per ml undiluted enzyme (units/ml).

The cellulase assay was identical to the xylanase assay

Correspondence to: J.P. Nakas, State University of New York, College of Environmental Science and Forestry, Syracuse, NY 13210, USA.

[1] Present address: Biology Department, Stanford University, Palo Alto, CA 94305, USA.

except that 1% (w/v) carboxymethyl cellulose was used as substrate, assay tubes were centrifuged before reading, and activity was expressed as micromoles reducing sugar as glucose released per min per ml. Carboxymethyl cellulose preparation 1 was from Hercules (Wilmington, DE, type 4M65, lot No. 50535), while preparation 2 was obtained from Sigma (type 4888, lot No. 58c-0156). Protein was assayed by the procedure of Lowry et al. [6] with bovine serum albumin as standard.

*Thin-layer chromatography*

Acid hydrolysis of CMC was accomplished by incubation of 5 mg of material in 1 ml of HCl (1 M) for 6 h at 100 °C. Hydrolysis products were identified by TLC on Whatman 5K TLC silica gel glass plates. The solvent system was *n*-butanol/pyridine/water (6:4:3) and visualization was with 3% (w/v) *p*-anisidine-hydrochloride in *n*-butanol [8,15].

## RESULTS

Xylanase A was active on the carboxymethyl cellulose preparation used in initial studies (CMC preparation 1). However, the relationship between enzyme concentration and product formation during a 30-min assay was not linear. As a result, the calculated specific activity was highly dependent upon the concentration of enzyme used in the assay. Furthermore, while reducing sugar formation from xylan was linear during a 30-min assay, the rate of product formation from CMC preparation 1 decreased significantly during the assay period (Fig. 1B). Xylanase A did not generate detectable levels of reducing sugar from CMC preparation 2, even at more than 100-times the concentration of enzyme used in xylanase assays. In addition, HPLC analysis revealed that the enzyme was inactive on cellopentaose. Thin-layer chromatography of acid hydrolyzed material revealed only glucose residues ($R_F = 0.573$) in preparation 2, and confirmed the presence of both glucose and xylose ($R_F = 0.701$) residues in CMC preparation 1.

## DISCUSSION

The pattern of activity of xylanase A on CMC preparation 1 suggested that only a small fraction of the substrate was available to the enzyme. Unfortunately, the high viscosity of the CMC substrate, even after hydrolysis by xylanase A hindered direct identification of enzymatic end-products. However, the presence of xylan in CMC preparation 1 was confirmed by TLC. These results, combined with a lack of activity on cellopentaose and CMC preparation 2, suggest that xylanase A is inactive on cellulose and that apparent cellulase activity on CMC preparation 1 is a result of hydrolysis of contaminating xylan in the substrate.

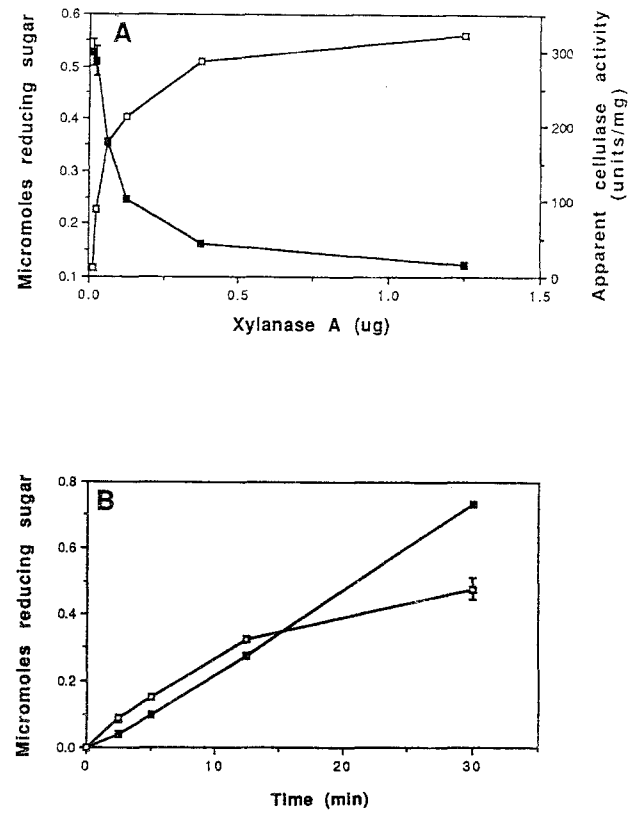


Fig. 1. Activity of xylanase A on CMC preparation 1 and xylan. (A) Effect of enzyme concentration on activity of xylanase A on CMC preparation 1. Activity (units/mg) is indicated by the closed squares (■) and was derived from the quantity of product (μmol glucose) generated at the corresponding enzyme concentration as denoted by the open squares (□). (B) Time-course of activity of xylanase A on CMC preparation 1 and oat spelt xylan. Assays contained 0.08 μg enzyme. Closed squares (■) indicate reducing sugar formed from xylan, while open squares (□) represent reducing sugar formed from carboxymethyl cellulose preparation 1. Results in A and B are the mean ± standard error of three replicates.

It is tempting to speculate that contamination of cellulase substrates with xylan could explain reported non-specificity in many purified xylanases. However, two classes of xylanolytic enzymes; one type possessing activity and one type lacking activity on the same cellulose assay substrate have been reported for *T. reesei* [3]. Steric factors suggest that cellulases could more likely possess xylanase activity than vice versa [16].

It was also noteworthy that higher apparent cellulase activity in the crude enzyme was consistently obtained with CMC preparation 1. Most likely, cellulase activity was overestimated due to the activity of xylanases on contaminating xylan in the substrate. Clearly, the purity of

assay substrates must be confirmed before meaningful enzyme assays can be performed. Qualitative analysis of the substrate is particularly critical when enzyme activity is estimated in a nonspecific manner (i.e., by measuring the generation of reducing end groups), rather than by quantifying the specific product(s) of the enzyme. In addition, these results emphasize the importance of establishing the linearity of product formation in fixed time assays.

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