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# A zymogram method to detect endoglucanases from *Bacillus subtilis*, *Myrothecium verrucaria* and *Trichoderma reesei*

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## SUMMARY

A purified endoglucanase (Eg) from *Bacillus subtilis* and crude Eg preparations from *Myrothecium verrucaria* and *Trichoderma reesei* were denatured by heat in the presence of SDS and separated by using SDS polyacrylamide gel electrophoresis. Eg zymogram activity bands were detected directly in carboxymethylcellulose-laden separating gels after enzyme renaturation. Denaturing zymogram analysis was a simple method for the separation and detection of Egs.

## INTRODUCTION

Cellulose is an abundant renewable resource and its bioconversion has many industrial applications. However, the characterization of cellulose-degrading enzymes (e.g. Egs) has been hampered by the complexity of the cellulase system [2]. Egs produced by some cellulolytic microorganisms can exist in forms that are difficult to resolve using nondenaturing separation methods [1,5,9].

Several investigators have used denaturing SDS polyacrylamide gel electrophoresis (SDS-PAGE), combined with zymogram analysis, to identify Egs from the thermophilic anaerobe *Clostridium thermocellum* [5,8]. Denaturing zymogram analysis was a simple method of characterizing *C. thermocellum* Egs, but further evaluation of its compatibility with cellulase systems from other sources is needed [8]. In this paper, a denaturing zymogram method was evaluated for its ability to detect Egs from *Bacillus subtilis, Myrothecium verrucaria,* and *Trichoderma reesei.*

## MATERIALS AND METHODS

### *Protein and endoglucanase measurements*

Protein was measured by a Coomassie Brilliant Blue G-250 dye-binding assay (Bio-Rad, Richmond, CA, USA) with bovine serum albumin as the standard (Sigma Chem. Co., St Louis, MO, USA).

Eg activity was measured as carboxymethylcellulase (CMCase) activity. Substrate solution for the CMCase assays consisted of 0.5% CMC (Fluka Chem. Corp., Ronkonkoma, NY, USA) in sodium acetate buffer (0.05 M, pH 5.0). The reaction mixture, which consisted of 0.2 ml of enzyme preparation and 4.8 ml of substrate solution, was incubated for 20 min at 50 °C. Samples (0.3 ml) were removed at 2-min intervals and the reducing sugar concentration was estimated by a copper-bicinchoninate method [3]. Glucose was used as a standard. One unit of enzyme activity was the amount of enzyme liberating 1 μmol of reducing sugars in 1 min.

### *Source of enzyme*

Purified endo-β-1,4-glucanase (30 kDa) from *B. subtilis* PAP115 was obtained from Dr Gordon Willick (Institute for Biological Sciences, Ottawa, Canada).

*M. verrucaria* ATCC 9095 and *T. reesei* ATCC 26921 were cultured on media described by Whitaker [10], and Mandels and Andreotti [6], except that cotton linters and Solka Floc were replaced with purified cellulose from Sigma. Fernbach flasks (2.8 L) containing 600 ml of culture media were inoculated with a 5% mycelial preinoculum and incubated on a reciprocating shaker for 7–9 days at 30 °C. Spent culture fluid (500 ml) was filtered through glass wool and freeze-dried. Portions (200 mg) of the dried culture fluid were dissolved in 1 ml of Tris-HCl buffer (0.03 M, pH 6.8) and desalted on a Bio-Gel P-2 column, using the same buffer.

### *Electrophoresis*

SDS-PAGE was performed as described by Laemmli [4]. Molecular mass markers (kDa) used were phosphorylase B (97), bovine serum albumin (66), egg albumin (45), carbonic anhydrase (29), and trypsin inhibitor (20). Protein bands

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were detected with a silver-stain kit (Sigma). Enzyme samples were denatured with 3% SDS in Tris-HCl buffer (0.03 M, pH 6.8) and heated according to various temperature–time protocols (60 °C – 30 min, 70 °C – 10 min, 80 °C – 10 min, and 95 °C – 5 min). No difference in the protein banding or Eg zymogram activity patterns were observed when enzyme samples were denatured using the various heat treatments (data not shown). To avoid the possibility of incomplete denaturation, the 95 °C – 5 min sample treatment was used for all subsequent studies. Separating gels contained 0.1% CMC. After separation of the enzyme samples by means of SDS-PAGE, CMCase activity was detected in the separating gels as described by Schwarz et al. [8]. After SDS-PAGE, the gels were washed for 30 min at room temperature with five changes of 0.1 M succinate (pH 5.8) containing 10 mM dithiothreitol. The wash step allowed for renaturation of enzyme components. The gels were then incubated for 30 min at 60 °C in 0.1 M succinate and stained in a 0.1% congo red solution for another 30 min. Pale red hydrolysis zones emerged against a red background after destaining with 1 M NaCl.

## RESULTS

Zymogram analysis of the 30 kDa *B. subtilis* Eg is shown in Fig. 1. The prominent Eg activity band was assigned a molecular mass estimate of 30 kDa.

Zymogram analysis of an enzyme sample from *M. verrucaria* is shown in Fig. 2. The prominent Eg bands were assigned mass values of 64, 49, 43, and 26 kDa. At least two faint Eg bands were evident near the 97 kDa marker.

Zymogram analysis of an enzyme sample from *T. reesei* is shown in Fig. 3. A large Eg band was observed in the 45–58 kDa mass region in lane 3. However, a distinct 56-kDa Eg band was evident in lanes 5–7 as the enzyme sample was diluted. Other distinct Eg bands evident in lane 3 were assigned mass values of 44, 36, 34, 27 and 25 kDa, respectively.

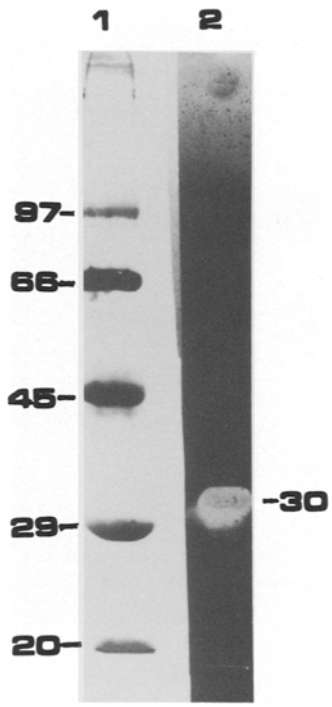


Fig. 1. Zymogram analysis of a purified Eg (30 kDa) sample from *B. subtilis* PAP115. The sample was subjected to 7.0% SDS-PAGE. After electrophoresis, the gel was divided into two sections that consisted of lane 1 (markers) and lane 2 (Eg, 1 $\mu$g). Lane 1 was silver-stained for protein. Values (kDa) of protein markers in lane 1 are on the left. Lane 2 was stained for Eg activity with congo red. The molecular mass estimate of the Eg band (lane 2) is on the right.

## DISCUSSION

A purified *B. subtilis* Eg with a mass of 30 kDa was subjected to zymogram analysis to determine the reliability and compatibility of the method (Fig. 1). The molecular mass zymogram estimate (30 kDa) assigned to the prominent Eg band (Fig. 1, lane 2) was consistent with the reported value of the native enzyme (30 kDa).

Crude Eg preparations from *M. verrucaria* and *T. reesei* were each subjected to zymogram analysis to determine if Eg components from these fungi could be detected by using this method. Four prominent Eg activity bands (64, 49, 43, and 26 kDa) from *M. verrucaria* and six Eg bands from *T. reesei* (56, 44, 36, 34, 27 and 25 kDa) were identified by using denaturing zymogram analysis (Figs 2 and 3).

Denaturing zymogram analysis was a simple method for the separation and detection of Egs from *B. subtilis, M. verrucaria*, and *T. reesei*. To our knowledge, this is the first report of a denaturing zymogram method used to detect endoglucanases from fungi and *B. subtilis*. The zymogram method would aid studies involving Eg induction, processing, preliminary characterization of new Eg systems, and confirming homogeneity of purified Eg samples. The zymogram method also will complement other techniques (e.g. immunostaining, [7]) in analyzing Eg multiple forms. Future research should address whether specific Eg forms (e.g. enzymatically active proteolytic fragments) will be detected by this method.

As discussed by Schwarz et al. [8], enzymes whose activity requires participation of subunits with different molecular masses will not renature in the gels. In addition, denaturation and renaturation conditions (e.g. heat treatments, wash conditions, buffer components) for different enzymes may vary so specific protocols must be determined empirically.

## ACKNOWLEDGEMENTS

We thank Dr Gordon Willick for providing us with the *B. subtilis* endoglucanase.

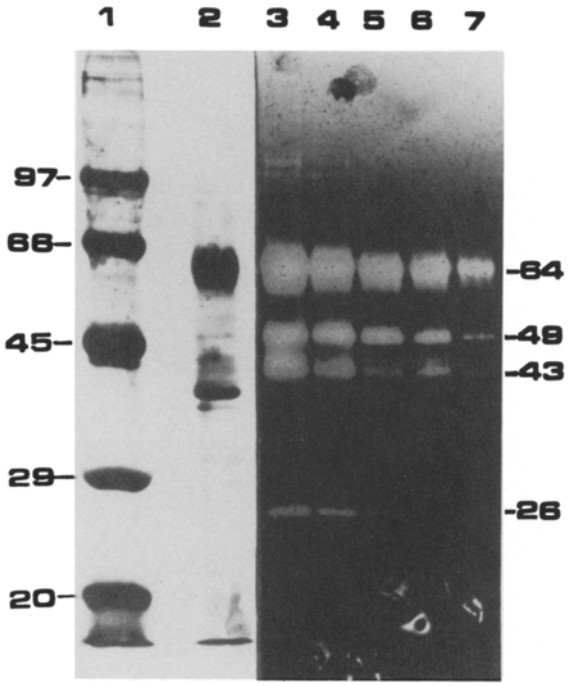


Fig. 2. Zymogram analysis of enzyme samples from *M. verrucaria*. Samples were subjected to 7.5% SDS-PAGE. After electrophoresis, the gel was divided into two sections consisting of lanes 1 (markers) and 2 (enzyme), and lanes 3–7 (enzyme). Lanes 1 and 2 were silver-stained for protein. Values (kDa) of protein markers in lane 1 are on the left. Lanes 3–7 were stained for Eg activity with congo red. The following amounts of enzyme sample were used: lanes 2 and 3, 8 μg (0.2 U); lane 4, 4 μg (0.1 U); lane 5, 2 μg (0.05 U); Lane 6, 1 μg (0.02 U); lane 7, 0.5 μg (0.01 U). Molecular mass estimates of Eg bands are on the right.

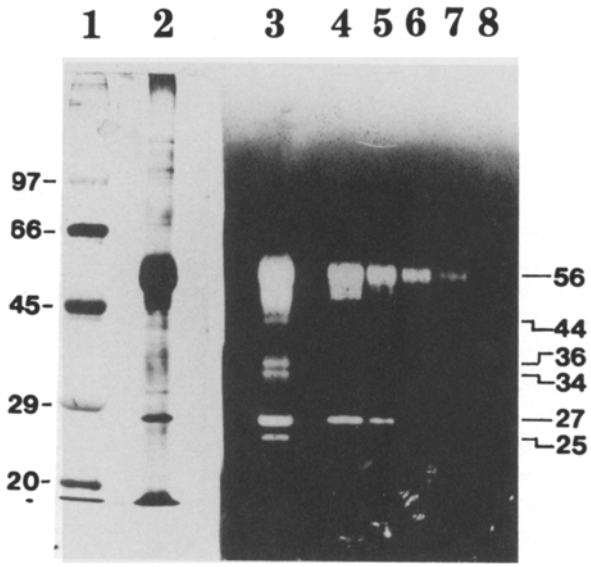


Fig. 3. Zymogram analysis of enzyme samples from *T. reesei*. The samples were subjected to 7.5% SDS-PAGE. After electrophoresis, the gel was divided into two sections that consisted of lanes 1 (markers) and 2 (enzyme), and lanes 3–8 (enzyme). Lanes 1 and 2 were silver-stained for protein. Values of markers in lane 1 are on the left. Lanes 3–8 were stained for Eg activity with congo red. The following amounts of enzyme sample were used: lane 2, 15 μg (1.5 U); lane 3, 4 μg (0.4 U); lane 4, 2 μg (0.2 U); lane 5, 1 μg (0.1 U); lane 6, 0.5 μg (0.05 U); lane 7, 0.2 μg (0.02 U); lane 8, 0.1 μg (0.01 U). Molecular mass estimates of Eg bands are on the right.

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