Xylanase Activity of *Phanerochaete chrysosporium*

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Xylan-degrading enzymes were induced when *Phanerochaete chrysosporium* was grown at 30°C in shake flask media containing xylan, Avicel PH 102, or ground corn stalks. The highest xylanase activity was produced in the corn stalk medium, while the xylan-based fermentation resulted in the lowest induction. Analytical and preparative isoelectric focusing were used to characterize xylanase multienzyme components. Preparative focusing was performed only with the cultures grown on Avicel and corn stalk. Of over 30 protein bands separated by analytical focusing from the Avicel and corn stalk media, three main groups (I, II, and III) of about five iso-enzymes each showed xylanase activity when a zymogram technique with a xylan overlay was used. Enzyme assays revealed the presence of 1,4-β-endoxylanase and arabinofuranosidase activities in all three isoenzyme groups separated by preparative isoelectric focusing. β-Xylosidase activity appeared in the first peak and also as an independent peak between peaks II and III. Denatured molecular masses for the three isoenzyme groups were found to be between 18 and 90 kDa, and pI values were in the range of 4.2 to 6.0. β-Xylosidase has an apparent molecular mass of 20, 30, and 90 kDa (peak I) and 18 and 45 kDa (independent peak), indicating a trimmer and dimer structure, respectively, with pI values of 4.2 and 5.78, respectively. Three more minor xylanase groups were produced on corn stalk medium: a double peak in the acidic range (pl 6.25 to 6.65 and 6.65 to 7.12) and two minor peaks in the alkaline range (pl 8.09 to 8.29 and 9.28 to 9.48, respectively). The profile of xylanases separated by isoelectric focusing (zymogram) of culture filtrate from cells grown on corn stalk media was more complex than that of culture supernatants from cells grown on cellulose. The pH optima of the three major xylanase groups are in the range of pH 4 to 5.5.

*Phanerochaete chrysosporium* is the most extensively studied lignocellulose-degrading white rot fungus. This basidiomycete secretes lignin-decomposing peroxidases (3, 7, 15), cellulases (4, 17), and hemicellulases (5). Xylanase activity has been detected in *P. chrysosporium* cultures (3, 13, 20), but to date no characterization of this enzyme has been reported. This paper describes the separation and characterization of the xylanase enzyme system of this fungus.

MATERIALS AND METHODS

**Microorganisms.** The fungal strain *P. chrysosporium* VKM F-1767 (ATCC 24725) was used in this study. It was routinely maintained on potato dextrose agar at 30°C.

**Culture conditions.** The fungus was cultured in shake flask medium which contained the following (per liter): 15 g of xylan from oat spelt (Sigma Chemical Co., St. Louis, Mo.) (medium A), 15 g of ground and sieved (<100 μm) corn stalk (medium B), or 15 g of Avicel PH 102 (medium C); 2 g of KH₂PO₄, 1.5 g of [NH₄]₂HPO₄; 1 g of corn steep liquor (100% [dry weight]); 1 g of Tween 80; 1 g of Struktol (antifoam); 0.5 g of NaCl; 0.3 g of urea; 0.3 g of MgSO₄·7H₂O; 0.3 g of CaCl₂; and 1 mL of trace element solution.

The pH value (before sterilization) was adjusted to 5.5 by sulfuric acid. Trace element solution contained (per liter) 5 g of FeSO₄·7H₂O; 3.45 g of ZnSO₄·7H₂O; 2 g of CoCl₂·6H₂O, and 1.6 g of MnSO₄. Main carbohydrates of corn stalk used in the experiments were as follows: cellulose, 33%; xylan, 27%.

Conidiospores (10⁶/ml, final concentration) were used for inoculation.

Cultures (50 ml) were incubated in 200-ml Erlenmeyer flasks at 30°C with shaking at 250 rpm for 144 h. Triplicate cultures were used for xylanase production, and the averages of titers are reported here.

**Enzyme assays.** All enzyme assays were performed with the extracellular fluid, which was collected by centrifugation at 10,000 × g for 15 min.

The buffer used in enzyme assays was sodium citrate-citric acid (0.05 M, pH 4.8). Xylanase assays were modified carboxymethyl cellulase assays (6). A 1-ml reaction mixture contained 0.5 ml of appropriately diluted supernatant and 0.5 ml of 2% oat spelt xylan (Sigma) in pH 4.8 buffer. The mixture was incubated at 50°C for 30 min, and the reducing sugars were assayed by the dinitrosalicylic acid procedure (9), with xylose as the standard. One unit of xylanase activity was defined as the amount of enzyme that released 1 μmol of reducing sugar (expressed as xylose equivalent) per min at 50°C and pH 4.8.

Arabinofuranosidase and β-xylosidase activities were measured by the methods described earlier (1, 2), with p-nitrophenyl-α-L-arabinofuranoside (Sigma) and xylobiotase (Sigma), respectively, as substrates.

Cellulolytic activity was determined according to the standards of the International Union of Pure and Applied Chemistry (IUPAC) by using Whatman no. 1 filter paper strips (50 mg) as a substrate (6). Protein content was determined by the method of Lowry et al. (8), with bovine serum albumin as a standard.

**Effect of pH and temperature on stability.** The enzyme was exposed to various conditions without the substrate. For pH
stability determination, purified samples were incubated in buffers (0.05 M acetate, 0.1 M citric acid–Na2HPO4; McIlvaine) and borate from pH 3 to 9 at room temperature for 30 min. For thermal stability characterization, various temperatures (4 to 80°C) were used at pH 4.8 for 10 min. The remaining xylanase activity was assayed under standard conditions described earlier.

**Protein separation methods.** Flat-bed analytical and preparative isoelectric focusing were performed as instructed (10) by using Ampholine (pH 3.5 to 10)–Agarose IEF for the analytical and Sephadex IEF for the preparative gel. All chemicals were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. To remove the salts, the supernatants were passed through a 1,000-Da-cutoff ultrafiltration membrane prior to the separations.

After analytical focusing, the gel was cut into two pieces, each containing identical samples. One half was stained for proteins by using Coomassie brilliant blue. For the other part, activity stain was used (11). Briefly, a substrate layer consisting of 200 mg of xylan from oat spels, 13 ml of deionized water, and 26 ml of 2% agarose in 0.2 M pH 4.8 sodium citrate buffer was cast onto a sheet (12 by 15 cm) of Agarose GelBond (FMC Corporation, Philadelphia, Pa.) and overlaid with the protein gel. After 20 min of incubation at 55°C, the protein gel was fixed and stained for proteins. The substrate gel was immersed in 75% ethanol for 30 to 60 min and was photographed above a dark background. After preparative focusing, the gel was cut into 38 strips. The fractions were eluted with distilled water, and the pH of each fraction was determined. The Ampholine content was removed by using a 1,000-Da-cutoff Millipore ultrafiltration membrane. Proteins were resolved by denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 5.0% stacking and 10% resolving gels) by the method of Sambrook et al. (12). Gels were silver stained by the method of Wray et al. (19).

Molecular weight standards were from Sigma.

**RESULTS AND DISCUSSION**

**Xylanase production.** Table 1 summarizes the results of shake flask fermentations. The cultures were grown on xylan from oat spels (medium A), corn stalk (medium B), or Avicel PH 102 (medium C) as the main carbon source.

The highest xylanase activity was produced in the corn stalk-containing medium, while the xylan-based fermentation resulted, contrary to expectation, in the lowest induction (Table 1). Similarly, the highest arabinofuranosidase and β-xylanosidase activities were found in the culture grown on corn stalk media.

The relatively low induction of xylanase activity on 1.5% xylan substrate (medium A) can be explained by the end product inhibitory effect of xylose and xylobiose. Reducing sugar (5.8 mg/ml, expressed as xylose) was detected in 6-day-old culture by the dinitrosalicylic acid method. Similar results for media B and C were only 0.2 and 0.05 mg/ml (expressed as glucose), respectively. Differences in the time of appearance and pattern of reducing sugar accumulation during growth of *P. chrysosporium* suggest that initial degradation of xylan was rapid whereas the corn stalk and cellulose were degraded more slowly. We assume that poor accessibility of xylan in the corn stalk and slow enzymatic release of xylose resulted in the best xylanase production on corn stalk media. No arabinofuranosidase activity was induced when xylan was used as the substrate.

Only low values of β-xylanosidase were determined. No cellulase activity was detected with medium A, while the highest values were found in the cultures grown on corn stalks and Avicel.

In other studies, *Trichoderma reesei* QM6a, the parent strain of the best existing cellulase-producing mutants, secreted 0.5 to 0.9 IU of filter paper activity (FPase) per ml on media B and C in our laboratory (unpublished results). These data indicate that *P. chrysosporium* is a relatively good producer of extracellular cellulase enzymes too.

**Separation of xylanases by isoelectric focusing.** Analytical focusing indicated the presence of numerous isoenzymes, mainly in the acidic pH range (Fig. 1). The culture supernatant of medium A contained relatively few bands in comparison to the two other cultures.

The profile of xylanases separated by isoelectric focusing (zymogram) of culture filtrate from cells grown on corn stalk
media was more complex than that of culture filtrate from cells grown on Avicel or xylan. Our observations are similar to the results of Smith and Forsberg (14), who used *Fibrobacter succinogenes* grown on barley straw or crystalline cellulose.

Preparative focusing was performed only with the cultures grown in media B and C (Fig. 2 and 3, respectively).

Of the 38 fractions separated in the Sephadex isoelectric focusing gel, 16 fractions in the pH range of 4.20 to 6.0 (medium B) and 9 fractions in the pH range of 3.91 to 5.53 (medium C) showed xylanase activities (Fig. 2 and 3, respectively). Both separations resulted in three isoenzyme groups: an acidic peak (IB and IC) of lower activity and a double peak (IIB and IIB and IIC and IIC, respectively) of higher activity in the higher pH range. Moreover, the culture grown on corn stalk (medium B) contained a xylanase peak in the range of pH 6.25 to 7.12 and two alkaline groups in the range of pH 8.09 to 8.28 and 9.28 to 9.48, respectively.

β-Xylosidase activity appeared in both cultures in the small peak of acidic pH and also between the two peaks in the higher pH range. These findings are similar to the multicomponent xylanase system found for *Thermomonosporum*.

FIG. 2. Preparative isoelectric focusing separation of *P. chrysosporium* culture supernatant grown on corn stalk (medium B). Xylanase activity was determined by the modified carboxymethyl cellulase assay with xylan from oat spels (6); arabinofuranosidase and β-xylosidase activities were assayed on p-nitrophenyl-α-L-arabinofuranoside and xylobiose, respectively, as substrates (1, 2); cellulase (FPase) activity was measured by using the IUPAC standards (6) with Whatman no. 1 filter paper strips as the substrate. Protein content was detected at 280 nm.
spora fusca (2). Xylanase recovery values are relatively high (68.8 and 88.5%, respectively [Table 2]). FPase was detected only in the fractions of the supernatant grown on Avicel (medium C) and was found to be identical to the β-xilosidase peak. Similarly, the culture grown on corn stalk (medium B) also showed FPase activity before the separation. However, as the protein recovery was high after the focusing (88 and 92%, respectively), it is very likely that the separated cellulase components act synergistically, as suggested by Wood and McCrae (18).

**SDS-PAGE separation of the fractions.** The molecular masses of the fractions separated by preparative focusing were found to cover the range of 18 to 90 kDa. The fractions of peak IB were found to be trimers, consisting of high (90 kDa)- and low-molecular-mass subunits (20 and 30 kDa). Peak IIB displayed dimers (18 and 45 kDa, respectively). The fractions of the IIIB group contained only a low-molecular-mass band of 18 kDa. These fractions show only β-xilosidase activity. The molecular weights of the three minor groups in the pH ranges of 6.25 (IVB), 8.09 to 8.29

### TABLE 2. Recovery of activities after preparative isoelectric focusing

<table>
<thead>
<tr>
<th>Medium</th>
<th>Xylanase</th>
<th>Arabinofuranosidase</th>
<th>β-Xilosidase</th>
<th>Cellulase A</th>
<th>Protein content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>68.8</td>
<td>62.8</td>
<td>57.3</td>
<td>0</td>
<td>88</td>
</tr>
<tr>
<td>C</td>
<td>88.5</td>
<td>83.3</td>
<td>81.4</td>
<td>69.4</td>
<td>92</td>
</tr>
</tbody>
</table>
(VB), and 9.28 to 9.48 (VIB) were not determined by SDS-PAGE. The separation of the culture grown on Avicel PH 102 (medium C) gave similar results. IC forms trimers (45, 47, and 60 kDa). The fractions of IIC were found to be different, showing an additional low fragment at 38 kDa. The β-xylosidase peak of pH 5.1 has a trimer structure of 38, 41, and 60 kDa. Group IIIC, as did group IC, displays three bands at 38, 46, and 60 kDa.

Ujiie et al. (16) also reported low-molecular-mass (20 kDa) xylanase isolated from Trichoderma viride.

The relatively small proteins may be secreted to render it possible for the enzyme to have a better access to the three-dimensional structure of the hemicellulose-lignin complex.

The effect of pH and temperature on the stability. The pH stability results show that the xylanase isoenzymes are stable in the acidic pH range, exhibiting almost 100% of total activity between pH 4 and 5.5 (Fig. 4).

The enzyme groups IIB, IIC, and IIIC showed better stability above pH 7.0 than the other separated fractions (e.g., IB and IC). However, according to the temperature stability curves, IA and IB are more stable, while the activities of the more alkaline enzyme groups (IIB, IIC, IIC, and IIIC) rapidly decrease above 37°C (Fig. 5).

Conclusion. P. chrysosporium produces several active xylanases. All three isoenzyme groups separated by isoelectric focusing showed β-xylanase and arabinofuranosidase activities. β-Xylosidase activity was also detected. Interestingly, xylanase isoenzymes in the highly alkaline range (between pI values of 8.0 and 9.5) were separated in the

FIG. 4. Effects of pH on the stability of xylanases separated by preparative isoelectric focusing.

FIG. 5. Effects of temperature on the stability of xylanases separated by preparative isoelectric focusing.
culture medium grown on a natural lignocellulose source (corn stalk). This phenomenon has not been detected in xylan- or Avicel-containing media. Xylanase production on highly purified microcrystalline cellulose (Avicel) suggests that at least part of the xylanase enzymes of P. chrysosporium is constitutive. Growth of the fungus on xylan resulted in accumulation of reducing sugars in the culture supernatants, whereas practically no reducing sugars were detected during growth on Avicel or corn stalk.

REFERENCES