

Purification and Some Properties of a Xylanase from *Aspergillus sydowii* MG49

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***Aspergillus sydowii* MG49 produces a 30-kDa exosplitting xylobiohydrolase during growth on xylan. A specific chemical modification and substrate protection analysis of purified xylanase provided evidence that tryptophan and carboxy and amino groups are present at the catalytic site of this enzyme. Thermal inactivation of the xylanase occurs because of irreversible polymolecular aggregation, which is slower in the presence of glycerol.**

Xylanase, the key enzyme which initiates the degradation of xylan to xylooligosaccharides, is produced by many microorganisms (23). The specific activities of *Aspergillus sydowii* MG49 xylanolytic enzymes are high during growth on xylan, xylose, or lignocellulosic residues (4). Recently, thermostable xylanases have been used in xylan digestion processes at elevated temperatures (16, 18) because of significant commercial interest in industrial applications of these processes. In this paper we describe the biochemical characteristics and thermostability of a xylanase produced by *A. sydowii* MG49 during growth on oat spelt xylan.

Xylanase was purified to homogeneity by a three-step procedure consisting of ultrafiltration of the culture filtrate through 50,000- and 20,000-molecular-weight cutoff membranes followed by gel filtration through a Sephadex G-75 column and cation-exchange (carboxymethyl cellulose; Sigma) chromatography (Table 1). The overall level of recovery of the enzyme was 60%, and 2.9-fold purification was sufficient to achieve homogeneity, suggesting that the xylanase is a major secreted protein in *A. sydowii* culture filtrates when oat spelt xylan is used as the sole carbon source. Using densitometric scans of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels containing crude culture filtrates, we estimated that approximately 45% of the total protein secreted into the culture medium was xylanase. A zymogram of a concentrated culture filtrate on a 10% polyacrylamide gel containing 0.1% oat spelt xylan and visualized by staining with cupric acetate (2) revealed the presence of only one active xylanase (Fig. 1). The apparent lack of multiple xylanases in *A. sydowii* is similar to the lack of such enzymes in *Penicillium chrysogenum* (7). Purified xylanase was detected as a single band at an M_r of 30,000 when the preparation was subjected to SDS-12.5% PAGE as described by Laemmli (13) and visualized by Coomassie brilliant blue R-250 staining (Fig. 2). This enzyme exhibits optimum activity at a temperature of 60°C and a pH of 5.5 (4). The purified enzyme was assayed for hydrolytic activity against various substrates, including oat spelt xylan, cellulose, carboxymethyl cellulose, cellobiose, and *p*-nitrophenyl- β -D-xylopyranoside, but no detectable activity was observed with any of the substrates except xylan. The hydrolysis product released by puri-

fied xylanase from oat spelt xylan after incubation for 15 min at 60°C and pH 5.5 was studied by performing paper chromatography with a solvent system consisting of ethylacetate, acetic acid and water (3:1:3, vol/vol/vol) and developing the preparation with silver nitrate (21). Xylobiose was found to be the only end product (Fig. 3), indicating that the enzyme which we studied is an exosplitting xylobiohydrolase equivalent to a cellobiohydrolase of cellulase rather than an endoxylanase.

Table 2 shows the effects of different functional group-specific chemical modifiers on xylanase activity. To investigate the inhibitory activities of different chemical modifiers various concentrations (1 to 10 mM) of the modifiers were incubated with 100- μ l portions of xylanase (10 μ g \cdot ml⁻¹) in 100 mM citrate buffer (pH 5.5) at 30°C for 1 h. Samples (10 μ l) of the reaction mixtures were removed and added to 90- μ l portions of the same buffer to quench the residual reagent. A control containing only xylanase was also examined under identical conditions. The remaining activities of the diluted enzyme derivatives were determined by measuring the amounts of reducing sugar released from 1% xylan by the method of Somogyi (17), as described previously (4); the results were expressed as percentages of the control value. *N*-Bomosuccinimide (1 mM) completely inhibited enzyme activity, suggesting that tryptophan is involved in the catalytic site of the enzyme. The xylanases of different microbial strains have been reported to have tryptophan residues at their active sites (12, 15, 22). Treatment of xylanase with 5 mM carbodiimide (cyanamide) reduced enzyme activity by 80%. Since carbodiimide very specifically modifies carboxy groups in proteins, inhibition of enzyme activity by this compound provided evidence that essential carboxy groups (aspartic and/or glutamic acid) are present in the enzyme which we studied. Involvement of carboxy groups at the active site has been observed in xylanases of other microorganisms, including *Streptomyces* spp. (10), *Schizophyllum commune* (1), *Thermomonospora fusca* (9), and *Thermoanaerobacterium saccharolyticum* B6A-R1 (14). Chemical modification of the *A. sydowii* xylanase by acylation with 5 mM succinic anhydride or 10 mM acetic anhydride resulted in 50 and 100% reductions in the enzyme specific activity, respectively, indicating that positively charged amino groups are critical for enzyme activity. However, addition of xylan before the xylanase was treated with these chemical modifiers prevented the loss of activity. This protection by the substrate confirmed the presence of reactive tryptophan and carboxy and amino

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TABLE 1. Purification of *A. sydowii* MG49 β -xylanase

Purification step	Vol (ml)	Total protein (mg)	Total activity (U)	Sp act (U mg ⁻¹) ^a	Yield (%)	Purification (fold)
Crude enzyme (culture filtrate)	1,000	50	3,500	70	100	
Ultrafiltered concentrate (after filtration through a 50,000-molecular-weight cutoff membrane and then through a 20,000-molecular-weight cutoff membrane)	50	30	3,300	110	94	1.6
Xylanase after gel filtration (Sephadex G-75-fractionated concentrate)	50	12	2,280	190	65	2.7
Xylanase after cation-exchange chromatography (negative adsorption) (elute from a carboxymethyl cellulose column concentrated with polyethylene glycol 6000)	35	10.3	2,100	204	60	2.9

^a We defined 1 U of enzyme specific activity as the amount of enzyme which produced 1 μ mol of reducing sugar as a xylose equivalent per min per mg of protein at 60°C and pH 5.5.

groups at the substrate binding site of the *A. sydowii* xylanase. Treatment with specific thiol inhibitors, including *p*-hydroxymercuribenzoate (1 mM), *N*-ethylmaleimide (5 mM), and iodoacetamide (5 mM), did not result in decreases in activity of more than 5, 15, and 20%, respectively. This indicated that no reactive cysteine residues were present at the active site of the enzyme. This observation is in contrast to the cysteine requirement of the *Streptomyces* sp. xylanase (11) but is similar to observations made with the *Aspergillus niger* xylanase (6). Alkylation of free amino groups may explain the small loss of enzyme activity that was observed in the presence of *N*-ethylmaleimide or iodoacetamide. Cystine residues apparently do not contribute to the xylanase reaction, as shown by the fact that no change in enzyme activity was observed in the presence of dithiothreitol or β -mercaptoethanol.

Experiments to study thermoinactivation of β -xylanase were performed by incubating the enzyme at different temperatures (60 to 90°C) and pH values (pH 3.0 to 7.0) in 100 mM citrate buffer with and without various additives. The time course of enzyme inactivation was monitored by periodically removing samples, which were then rapidly cooled in

ice for 1 h and assayed for the level of residual activity. The enzyme exhibited thermal half-lives of 165, 70, 22, and 16 min at pH 5.5 and temperatures of 60, 70, 80, and 90°C, respectively. The xylanase decay profile exhibited a slight deviation from first-order denaturation kinetics, which was also observed with the endoglucanase I of *Trichoderma reesei* QM 9414 (3) and the β -xylosidase of *A. sydowii* MG49 (5). Although thermoinactivation of the *A. sydowii* xylanase is strongly pH dependent, maximum thermostability occurred at pH 5.5, the optimum pH for enzyme activity. Heating of the enzyme at 60°C at pH 5.5 resulted in a gradual time-dependent loss of activity which was not restored by cooling (rapid freezing), indicating that denaturation was apparently irreversible. This inactivation was dependent on the initial enzyme concentration, and either dilution of the protein or cross-linking in the presence of a 2.5% glutaraldehyde solution reduced the rate of enzyme denaturation. This suggests that enzyme inactivation at 60°C is due to polymolecular processes, such as aggregation. The UV absorption spectra of heated enzyme (60°C, pH 5.5, 3 h) and native enzyme revealed that there was a very slight increase in the absorption of the partially denatured enzyme at 280 nm

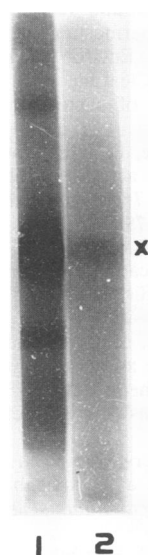


FIG. 1. Zymogram analysis of crude enzyme indicating the absence of any xylanase multiplicity. Lane 1, Coomassie blue-stained protein profile of a concentrated, culture filtrate; lane 2, activity gel. X, xylanase.

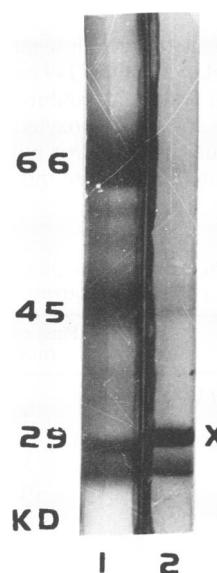


FIG. 2. SDS-PAGE of purified xylanase. Lane 1, molecular mass standards (Sigma) (the numbers on the left indicate the sizes of the standards, in kilodaltons [KD]); lane 2, purified xylanase (X).

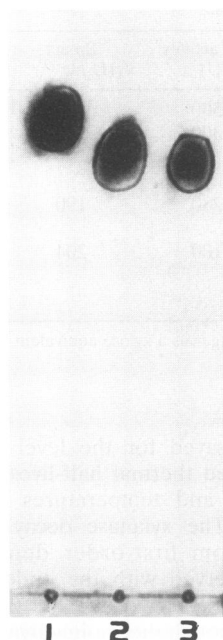


FIG. 3. Analysis of xylan hydrolysis products by paper chromatography. Lane 1, xylose; lane 2, xylobiose; lane 3, xylan hydrolysis product.

compared with the native enzyme, suggesting that no major alteration in the primary structure of the protein occurred. Incubation of the xylanase in the presence of 10% (vol/vol) glycerol at 60°C and pH 5.5 increased enzyme stability two-fold. Glycerol probably thermostabilizes the enzyme by decreasing the water activity because of strong hydrogen bonding with water. This results in a low water environment and a subsequent decrease in the extent of protein unfolding, as suggested by Timasheff and Kunihiro (19). However, a similar effect on xylanase stability was not observed in the presence of other polyhydric alcohols, including sorbitol, xylitol, and mannitol.

In conclusion, the fact that the low-molecular-weight, thermostable xylanase of *A. sydowii* MG49 exhibits specific activity only in the presence of xylan and exhibits no detectable activity in the presence of cellulose or carboxymethyl cellulose and the fact that reactive aspartic acid and/or glutamic acid is present at the xylanase substrate binding site indicate that this enzyme

might belong to family 11 in the Henrissat classification of glycosyl hydrolases (8, 20).

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TABLE 2. Effects of various chemical modifiers on xylanase activity

Addition	Final concn (mM)	Residual activity (%) ^a
None (native enzyme control)		100
<i>N</i> -Bromosuccinimide	1	0
Carbodiimide (cyanamide)	5	20
Succinic anhydride	5	50
Acetic anhydride	10	0
<i>para</i> -Hydroxymercuribenzoate	1	95
<i>N</i> -Ethylmaleimide	5	85
Iodoacetamide	5	80
Dithiothreitol	1	100
β -Mercaptoethanol	1	100

^a A xylanase specific activity value of 100% was equivalent to 200 U mg⁻¹.

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