A Thermostable Endo-Xylanase (Xyn10A) from *Acidothermus cellulolyticus* 11B

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We cloned and purified the major family 10 xylanase (Xyn10A) from *Acidothermus cellulolyticus* 11B. Xyn10A was active on oat spelt and birchwood xylans between 60°C and 100°C, and between pH 4-8. Optimal activity was at 90°C, pH 6; specific activity and $K_m$ for oat spelt xylan were 350 μmoles xylose produced min$^{-1}$ mg-protein$^{-1}$ and 0.53 mg ml$^{-1}$. Based on xylan cleavage patterns, Xyn10A is an endoxylanase, and its half-life at 90°C was approximately 1.5 h in the presence of xylan.
Xylanase enzymes are important in a wide variety of biotechnological and industrial applications (reviewed in (5, 7, 14, 20, 29, 31)). Thermostable xylanases from diverse mesophilic and thermophilic microbes have been described (5, 9, 24, 28, 38). An area of intensifying industrial application for xylanases is in the deconstruction of plant cell walls to facilitate biofuels production from lignocellulose (8). With the current dependence on acid and heat pretreatment of lignocellulosic feedstocks, bioconversion enzymes from thermoacidophilic microbes are of particular value (25). Here we report the characterization of a thermostable glycoside hydrolase (GH10) xylanase (designated Xyn10A) from *Acidothermus cellulolyticus* 11B, a Gram-positive actinomycete that was isolated from acidic hot springs in Yellowstone National Park (4, 18).

Transcriptional analyses of *A. cellulolyticus* 11B (ATCC 43068) grown in LPBM medium (4, 18) supplemented with 0.5% of either oat spelt xylan, cellulose, cellobiose, or glucose revealed that the *xyn10A* gene was more highly expressed in xylan-grown cultures (Fig. 1). Its expression was also detected in cellulose medium, but was almost undetectable in cellobiose or glucose media. Zymogram analysis (12) of oat spelt xylan- and cellulose-grown *A. cellulolyticus* culture supernatants showed a prominent clearing zone (using birchwood xylan as substrate and congo red staining) corresponding to the predicted molecular size of the Xyn10A protein; tandem mass spectrometry confirmed the presence of Xyn10A in the xylan medium (data not shown). These results suggested that Xyn10A hydrolyzes xylan and is the major xylanase produced on xylan by *A. cellulolyticus*.

The *xyn10A* gene was cloned by isolating the *A. cellulolyticus* chromosomal DNA as described (4) and using to PCR amplify the full-length *Acel_0372* gene. From the *A. cellulolyticus* genome sequence (4), PCR primers (5′-
GTGGTGGAGCTC and 5'-GCAATTCGTTCACGTTGAGG-3' and 5'-55
GTGGTGTCTAGAACCATCGAGTGGGAGTGACG-3') containing SacI and XbaI restriction
sites (underlined) were designed to facilitate cloning of the 1.4-kb PCR product into pK19 (21)
for expression in E. coli DH5α cells (26). Using hydroxyapatite column chromatography (19)
following heat treatment (65°C, 15 min) of the crude cell extract, Xyn10A was purified to > 90%
purity based on densitometry of SDS-PAGE gels (Fig. 2B; Table 1).

Specific activity of purified Xyn10A was quantified using a reducing sugars assay with
p-hydroxybenzoic acid hydrazide (16) and the Bradford assay (6). Xyn10A was active from 60°C
100°C and pH 4-8, with an optimum at 90°C and pH 6 under the conditions tested (Table 2).

Other polysaccharides (Sigmacell cellulose, carboxymethylcellulose [Fluka], and xanthan gum
[KELCO ZN 85192 A]) did not serve as substrates (data not shown). The specific activity and
Km of purified Xyn10A (at 90°C, pH 6) on oat spelt xylan were 350 ± 27 U mg-protein⁻¹, and
0.53 ± 0.18 mg ml⁻¹, respectively, values comparable to those of XynA from Thermatoga
maritima MSB8 (37). The optimal temperature for Xyn10A activity (T_opt = 90°C at pH 6) was
higher than that reported for the most thermostable cellulase (endoglucanase E1, T_opt = 81°C)
from Acidothermus (1, 3, 34), which has a growth optimum of 55°C (18). Relatively few
xylanases described to date have temperature optima ≥ 90°C; most of these were isolated from
hyperthermophiles (growth T_opt > 85°C) such as Thermatoga (27, 37), or from high temperature
environments where hyperthermophiles are found (32). Another exception is the high
temperature xylanase (T_opt 90°C) from a fungus (24).

Xylan hydrolysis products produced by Xyn10A were analyzed using thin layer
chromatography (TLC, Fig. 3) as described previously (15). The major products from oat spelt
and birchwood xylan had retention factor (Rf) values between those of xylose and xylopentaose
indicating that Xyn10A functions primarily as an endo-xylanase. The differences in the pattern of degradation products from the two xylans likely reflect the known differences in the structures of these xylans (13, 17). Oat spelt xylan consists of arabinoxylan with trace glucose substituents, while birchwood xylan is primarily an unsubstituted xylose polymer with traces of uronic acids as side groups (17, 22, 30).

Xylans protected Xyn10A against thermal inactivation. While Xyn10A had a half-life of 12 min at 90°C in buffer, negligible loss in activity occurred in 1 h in the presence of either oat spelt or birchwood xylans (Fig. 4A). Non-substrate polysaccharides (listed above) did not stabilize the enzyme (data not shown). In the presence of oat spelt xylan, approximately 15% of the activity was retained beyond 3 h and up to 24 h of 90°C heat treatment (Fig. 4B). These results indicate that xylans stabilize purified Xyn10A at high temperature; presumably, substrate-enzyme interactions prevent conformational changes at higher temperatures. It should be noted that Xyn10A is actively degrading xylan during the incubation at 90 degrees, and loss of activity after longer incubation periods may be due to the depletion of the stabilizing substrate. Certain other xylanases are known to be stabilized by substrate or by immobilization on glass beads at high temperature (24, 27), and in some cases, carbohydrate binding domains were shown to contribute to thermostability (2, 11, 33, 36). Xyn10A lacks predicted carbohydrate-binding domains, and therefore how Xyn10A interacts with xylans for stabilization is unknown.

The closest structurally characterized GH10 homolog of Xyn10A is from \textit{Clostridium thermocellum}, whose homolog shares only 39% identity over 86% of the length of Xyn10A. Therefore, structural deductions for understanding intrinsic and substrate-associated thermostability of Xyn10A are difficult and warrant the structural characterization of the enzyme. With its thermoacidic range for enzyme activity, the \textit{A. cellulolyticus} Xyn10A xylanase
should be highly suitable for use in the hydrolysis of acid- and heat-pretreated lignocellulose in bioenergy applications (10, 35), and would be particularly compatible for use in combination with thermoacidic cellulases such as the highly thermostable endoglucanase E1 from \textit{A. cellulolyticus} (3, 23). In addition, the broad effective temperature and pH ranges make it attractive for other emerging biotechnological processes.

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REFERENCES


FIG. 1. Expression of \textit{xyn10A} in \textit{A. cellulolyticus} at mid-exponential (open bars) and stationary (filled bars) growth phases. Densitometry-based relative intensity values of RT-PCR products are plotted along the y-axis. RNA was extracted using the RNeasy Plant Mini Kit (Qiagen). Primers specific to the \textit{xyn10A} gene (5’-CAAAGGAAGATCTGGCAATG-3’ and 5’-TGAGCATCCGTGGTAGTGAT-3’) were used to amplify a 485-bp product using the Qiagen OneStep RT-PCR kit. Expression of the housekeeping \textit{gyrB} (\textit{Acel}_0005) gene was used to normalize for the RNA (data not shown). Identical results were obtained in two independent experiments; data from one experiment are presented.

FIG. 2. Heterologous expression and purification of recombinant Xyn10A. (A) Zymogram assay showing xylanase activity following electrophoresis of crude cell extracts. Lanes ST, molecular weight standards, lanes 1 and 3, crude cell extracts from DH5\(\alpha\)(pK19) (vector control); lanes 2 and 4, crude cell extracts from DH5\(\alpha\)(pK19-xyl10A). Samples in lanes 3 and 4 were heated at 65°C for 15 min prior to loading. (B) SDS-PAGE (10% gel) showing purification of Xyn10A. Lane A, molecular weight standards; lane B, crude cell extract from DH5\(\alpha\)(pK19-xyl10A); lane C, heat treated extract from DH5\(\alpha\)(pK19-xyl10A); lane D, concentrated fractions from the hydroxyapatite column.

FIG. 3. Time course of Xyn10A product formation with birchwood (A) and oat spelt (B) xylans, using TLC. Xylan substrates (2% in 10 mM phosphate buffer, pH 6) were incubated with purified Xyn10A at 90°C. Lanes 1 and 8, standards: xylotetraose (X4) and xylotriose (X3)
(Megazyme, Wicklow, Ireland) and xylose (X1). Lane 2, unreacted xylan; lanes 3-7, increasing incubation time in the presence of purified Xyn10A (10, 20, 40, 60, 240 min, respectively).

FIG. 4. Stabilization of purified Xyn10A by xylans. (A) Purified Xyn10A was diluted 1:20 with 4% oat spelt xylan (white), 4% birchwood xylan (gray) or phosphate buffer (black), and incubated at 90°C for the times indicated. Rates of reducing sugar formation were determined and were linear for all samples. Activities relative to unheated Xyn10A (dark gray) are reported. Results are averages of three independent experiments; error bars indicate standard deviations.

(B) Long-term thermostability of Xyn10A at 90°C in the presence of 4% oat spelt xylan. Experiments were performed as in (A).
<table>
<thead>
<tr>
<th>Purification step</th>
<th>Amt of Protein (mg)</th>
<th>Activity&lt;sup&gt;a&lt;/sup&gt; Units</th>
<th>Units/ mg protein</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
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<tbody>
<tr>
<td>Crude cell extract</td>
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<td>14800</td>
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<tr>
<td>Heat treated extract</td>
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<td>13100</td>
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<td>Hydroxyapatite</td>
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<td>376</td>
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<sup>a</sup> Units = µmoles xylose reducing equivalents liberated from oat spelt xylan per min; activities measured at 90°C, pH 6.0.
Table 2. Specific activity of purified Xyn10A at different temperatures and pH.

<table>
<thead>
<tr>
<th>Temp</th>
<th>pH 4.0 (µmoles xylose reducing equivalents min⁻¹ mg protein⁻¹ ± standard deviation)</th>
<th>pH 5.0 (µmoles xylose reducing equivalents min⁻¹ mg protein⁻¹ ± standard deviation)</th>
<th>pH 6.0 (µmoles xylose reducing equivalents min⁻¹ mg protein⁻¹ ± standard deviation)</th>
<th>pH 7.0 (µmoles xylose reducing equivalents min⁻¹ mg protein⁻¹ ± standard deviation)</th>
<th>pH 8.0 (µmoles xylose reducing equivalents min⁻¹ mg protein⁻¹ ± standard deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100°C</td>
<td>37 ± 17 (12%)</td>
<td>206 ± 35 (65%)</td>
<td>91 ± 19 (28%)</td>
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<td>-</td>
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<tr>
<td>90°C</td>
<td>41 ± 9 (13%)</td>
<td>187 ± 15 (58%)</td>
<td>350 ± 27* (100%)</td>
<td>158 ± 7 (49%)</td>
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<tr>
<td>80°C</td>
<td>-</td>
<td>98 ± 44 (31%)</td>
<td>210 ± 4 (66%)</td>
<td>169 ± 9 (53%)</td>
<td>-</td>
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<tr>
<td>70°C</td>
<td>-</td>
<td>68 ± 5 (21%)</td>
<td>111 ± 7 (35%)</td>
<td>72 ± 11 (22%)</td>
<td>71 ± 1 (22%)</td>
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<tr>
<td>60°C</td>
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<td>63 ± 14 (20%)</td>
<td>67 ± 1 (21%)</td>
<td>68 ± 2 (21%)</td>
<td>48 ± 2 (15%)</td>
</tr>
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</table>

*Specific activity in µmoles xylose reducing equivalents min⁻¹ mg protein⁻¹ ± standard deviations, n ≥ 4. Values in parentheses are % activities relative to the maximum activity (at 90°C, pH 6.0). '-' indicates an activity value less than 10% of maximal; activities at 30-50°C, as well as at pH 3.0 and pH 9.0 were less than 10% of maximal (data not shown). The pH of the assay mixture was measured at the end of each assay and no significant change in pH was found in assays in the pH 3-8 range. The pH at the end of the pH 9 assays was consistently 8.6. No significant differences were seen between measurements of pH at room temperature or at 90°C.
Figure 1
Figure 2
Figure 3
Figure 4