Gene Cloning and Characterization of an Acidic Xylanase from *Acidobacterium capsulatum*

Kenji Inagaki, Ken Nakahira, Kazuisha Mukai, Takashi Tamura, and Hidehiko Tanaka

Department of Bioresources Chemistry, Faculty of Agriculture, Okayama University, Okayama 700-8530, Japan

Received October 23, 1997

The gene *xynA* encoding an acid endo-β-1,4-xylanase from an acidophilic bacterium, *Acidobacterium capsulatum* 161, was cloned and expressed in *Escherichia coli*. The nucleotide sequence of the 1.6-kb DNA fragment containing *xynA* was analyzed, revealing an open reading frame of 1,215 bp encoding a peptide of 405 amino acid residues. The deduced amino acid sequence of XynA was very similar to other xylanases that are from the glycosyl hydrolase family 10. XynA was purified to homogeneity by SDS-polyacrylamide gel electrophoresis from *E. coli* transformants. The molecular mass and isoelectric point of XynA were 41 kDa and 7.3, respectively. The xylanase activity of the cloned XynA shows optimal activity at pH 5.0 and 65°C, and is stable pH between 3.0 and 8.0. The *Km* and *Vmax* with oat spelt xylan as a substrate at pH 5.0 and 30°C are 35 mg/ml and 403 μmol/min/mg.

Key words: xylanase; glycosyl hydrolase family 10; *Acidobacterium capsulatum*; *xynA* cloning; nucleotide sequence

Introduction

Many microorganisms that grow under conditions of extreme pH, temperature, salinity, and pressure have been discovered. These extremophiles produce many unique and useful enzymes. For instance, numerous thermostable enzymes from thermophilic bacteria, and alkaline enzymes, that are characterized by pH optima and stabilities in the alkaline region, from alkaliophilic bacteria, have been found.

From acidic environments, a group of acidophilic, mesophilic, and chemoorganotrophic bacteria, classified in the genera *Acidiphilium*, *Acidobacterium*, and *Acidocella* have been isolated. We have purified and characterized several useful enzymes, such as restriction endonucleases and glycerol 3-phosphate dehydrogenase from the genus *Acidiphilium*.

*Acidobacterium* has been proposed as the name for a new genus of acidophilic, chemoorganotrophic bacteria containing menaquinone, isolated from an acidic mineral environment (Yañahara mine, Okayama, Japan). At present this genus consists of a single species, *Acidobacterium capsulatum*, which is a Gram-negative, aerobic, mesophilic, non-sporforming, capsulated, saccharolytic, and rod-shaped bacterium. *A. capsulatum* can grow between pH 3.0 and 6.0, but not at pH 6.5, and is able to use glucose, cellobiose, starch, maltose, or β-gentiobiose as a sole carbon source. In concurrence with that, this bacterium was found to produce various hydrolases such as α- and β-glucosidase, α- and β-galactosidase, β-glucuronidase, α-fucosidase, and N-acetyl-β-glucosaminidase. We found acidic β-glucosidase activity in *A. capsulatum*, and reported the purification and characterization of the enzyme. *A. capsulatum* β-glucosidase has an pH optimum of 3.0, and is stable from pH 1.5 to 6.0. No activity was detected above pH 6.5. The β-glucosidase in *A. capsulatum* was assumed to be localized in the periplasmic space. This assumption is supported by the observation that the enzyme demonstrated maximum activity at pH 3.0 and no activity around pH 7.0 (probably the intercellular pH).

Recently we found that *A. capsulatum* has cellulase and xylanase activities in the periplasmic space of the cell. Therefore, we expected that these novel endoglucanases show maximum activities under acidic conditions. In this paper, the cloning and nucleotide sequencing of a novel endo-β-1,4-xylanase gene is reported. We also describe some properties of the enzyme purified from the *Escherichia coli* transformants.

Materials and Methods

Bacterial strains and plasmid. *A. capsulatum* 161 was used as the donor strain of the xylanase gene. *E. coli* JM109 [RecA1 supE44 endA1 hsdR17 gyrA96 relA1 thi-Δ(lac-proAB)(F’traD36 proAB+ lacF’ lacZ ΔM15) λ-] was used as the recipient strain for transformation experiments. Plasmid pUC19 was used as the cloning vector.

Culture media and growth conditions. *A. capsulatum* 161 was grown aerobically at 30°C in a basal medium [0.2% (NH₄)₂SO₄, 0.01% KCl, 0.025% MgSO₄, 7H₂O, 0.25% glucose, 0.05% yeast extract, (pH 4.0)]. *E. coli* transformant was grown aerobically at 37°C in 2×YT medium containing 50 μg/ml ampicillin.

Cloning of xylanase gene. Chromosomal DNA of *A. capsulatum* 161 was isolated by the method of Saito and Miura, and digested with restriction endonuclease EcoRI. The EcoRI fragments were ligated into the EcoRI site of plasmid pUC19 with T4 DNA ligase (Takara Shuzo Co., Ltd., Kyoto, Japan). Plasmid DNA was prepared by the alkaline extraction procedure. The transformants were cultivated overnight on YT plates containing ampicillin at 37°C. All colonies on the

* Corresponding author.
plates were covered with 1% agar containing 1 mM 4-methylumbelliferyl-β-d-cellobiopyranoside (MUC) (Sigma Chemical Co., St. Louis, USA), and incubated for several hours at 37°C. Hydrolysis of MUC released 4-methylumbelliferone, a fluorescent compound that can be detected by a UV transilluminator (Atto Corp., Tokyo, Japan). Hybridization of DNA on a nylon membrane with the DNA probe was done as described by Southern.17 Plasmid DNA was labeled with [α-32P]dCTP (~ 220 TBq/mmol, Amersham International plc, Little Chalfont, UK) using a random primer DNA labeling kit (Takara Shuzo).18 The DNA from an agarose gel was transferred to a nylon membrane (Hybond-N+, Amersham).

**Nucleotide sequencing.** The nucleotide sequence of DNA was analyzed by the dideoxychain termination method19 with a model 373A automated DNA sequencer system (Applied Biosystems, Foster City, USA). The nucleotide and deduced amino acid sequences were analyzed using the GENETYX-Mac version 7.0.9 software (Software Development Co., Ltd., Tokyo, Japan).

**Purification of xylanase.** E. coli JM109 (pACC204) cells were harvested from 6 L of 2 × YT medium, suspended in 50 mM CP-buffer (citrate-phosphate buffer containing 5 mM 2-mercaptoethanol and 3 mM NaN3, pH 5.5), and then disrupted by ultrasonication on ice. After the removal of cell debris by centrifugation at 4,000 × g for 60 min, the supernatant solution was put on a CM-Toyopearl 650 M (Tosoh Corp., Tokyo, Japan) column equilibrated with 50 mM CP buffer (pH 5.5). The proteins were eluted with a linear gradient of 0 to 0.6 M sodium chloride at a flow rate of 4 ml/min. The active fractions concentrated with an ultrafiltration membrane (Diaflo YM-10, Amicon Inc., Beverly, USA), were then put on a phenyl-Toyopearl 650 M (Tosoh) column equilibrated with 50 mM CP buffer (pH 5.5) containing 20% ammonium sulfate. The proteins were eluted with a linear gradient of 20 to 0% saturation of ammonium sulfate at a flow rate of 4 ml/min. Protein in the samples was measured by the absorbance at 280 nm.

**Amino-terminal amino acid sequencing.** The xylanase was separated by SDS-polyacrylamide gel electrophoresis, blotted onto a PVDF-membrane (ProBlott membrane, Applied Biosystems), and the amino terminal amino acid sequences were analyzed with an Applied Biosystems 477A protein sequencer.

**Enzyme assays.** Xylanase activity was measured by the increase in reducing groups during the enzymatic hydrolysis of xylan by the dinitsroalicylic acid method of Miller et al.20 The soluble fraction of oat spellet xylan (Nacalai Tesque) was used as the substrate for the assay of the xylanase activity. The oat spellet xylan was shaken in distilled water for 24 h at room temperature. Insoluble and soluble fractions were separated by centrifugation (6,000 × g, 30 min.). Standard assay mixtures (500 μl) contained 1% soluble oat spellet xylan, 100 mM CP buffer (pH 5.0), and an appropriate amount of enzyme.

Unless mentioned otherwise, incubation conditions were 10 min at 30°C and after incubation the reaction was stopped by the addition of 1 ml dinitsroalicylic acid solution. After color development for 5 min at 100°C and centrifugation of the samples (15,000 × g, 5 min), the A525 was measured. One unit of xylanase activity is defined as the amount of enzyme which liberates 1 μmol of reducing group (xylose equivalents) per min. Carboxymethylcellulase (CMCase) activity was also measured by the dinitsroalicylic acid method20 with carboxymethylcellulose (CMC, Nacalai Tesque Inc., Kyoto, Japan). β-Xylosidase, β-celllobiosidase, and β-glucosidase activities were assayed with p-nitrophenyl-β-d-xylanopyranoside (Sigma), p-nitrophenyl-β-d-celllobioside (Sigma), and p-nitrophenyl-β-d-glucopyranoside (Sigma) as substrates, respectively.

**Effects of pH and temperature on xylanase activity.** To find the optimum pH, the following buffers were used: KCl- HCl buffer (pH 1.0 to 2.0), glycine-HCl buffer (pH 2.5 to 3.5), sodium acetate- HCl buffer (pH 3.5 to 4.0), citrate phosphate buffer (pH 4.0 to 6.5), potassium phosphate buffer (pH 6.5 to 7.5), and Tris-HCl buffer (pH 7.5 to 9.0). To measure the pH stability, the purified enzyme was incubated for 30 min at 30°C in a suitable buffer, and later, the substrate was added and the reaction allowed to proceed in 100 mM CP buffer (pH 5.0) for 10 min at 30°C. Optimal temperature and thermostability were measured in the range of 20 and 80°C in 100 mM CP buffer (pH 5.0). To measure the thermostability, the enzyme was incubated for 10 min at the corresponding temperature, and later, the substrate was added and the reaction allowed to proceed in 100 mM CP buffer (pH 5.0) for 10 min at 30°C.

**Analysis of the hydrolysis products.** Enzymatic hydrolysis products were obtained by incubating (30°C) 1% oat spellet xylan in 100 mM CP buffer (pH 5.0) and 1 U (μl) of enzyme. Five-μl samples were spotted on a silica gel thin-layer chromatography plate (Silica Gel 60

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Insert Size (kb)</th>
<th>Xylanase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>pACC1</td>
<td>8.8</td>
<td>+</td>
</tr>
<tr>
<td>pACC2</td>
<td>8.8</td>
<td>+</td>
</tr>
<tr>
<td>pACC201</td>
<td>7.3</td>
<td>+</td>
</tr>
<tr>
<td>pACC202</td>
<td>4.0</td>
<td>+</td>
</tr>
<tr>
<td>pACC203</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>pACC204</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>pACC205</td>
<td>1.1</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 1.** Restriction Map of pACC1 and Its Derivatives.

Restriction sites are denoted by BglII (B), EcoRI (E), HindIII (H), KpnI (K), PstI (P), SstI (S), XhoI (X). Plac, lac promoter. Oat spellet xylan was used as the substrate.
F. E. Merck, Darmstadt, Germany). The plate was developed with a solvent system of n-butanol-methanol-water (5:5:2, vol/vol/vol). The resolved sugars were detected by heating the plate to over 100°C after spraying them with diphenylamine reagent (1 g of diphenylamine, 1 μl of aniline, 5 μl of 85% phosphoric acid, and 50 μl of acetone).

**Nucleotide sequence accession number.** The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under the accession number, AB004452.

**Results**

**Cloning of xylanase gene from A. capsulatum 161**

Positive clones with fluorescence excited by UV irradiation were identified from approximately 15,000 colonies. All positive clones were found to harbor the same recombinant plasmid DNA, designated as pACC1, containing an 8.8 kb insert. α-32P-Labeled EcoRI fragments were hybridized to the EcoRI fragment of chromosomal DNA from A. capsulatum 161, indicating that the inserts in pACC1 originated from A. capsulatum 161. A restriction map of pACC1 and results of deletion ana-

---

**Fig. 2.** Nucleotide Sequence of Xylanase Gene and Deduced Amino Acid Sequence. The deduced amino acid sequence is indicated under the nucleotide sequence. The N-terminal (10 residues) sequence from the purified enzyme is underlined. In the 5′-flanking region of nucleotide sequence upstream from the ATG codon, the −35 and −10 regions of the putative promoter and the proposed Shine-Dalgarno sequence (SD) for the ribosome binding site are indicated.
Fig. 3. Comparison of the Amino Acid Sequence of the Catalytic Domains of Family 10 Xylanases.
Aca XynA, Acidobacterium capsulatum xylanase; Cst XynB, Clostridium sporogenes F-9 cellobiohydrolase; Csa XynA, Caldocellum saccharolyticum xylanase; Csa ORF4, C. saccharolyticum xylanase; Csa CelB, C. saccharolyticum exoglucanase; Cst XynZ, Clostridium thermocellum xylanase; Bsp XynA, Bacillus sp. strain C-125 xylanase; Cfi Cex, Cellulosimonas fimi exoglucanase; Pfl XynA, Pseudomonas fluorescens subsp. cellulosa xylanase; Bfi XynA, Butyribrio fibrisolvens xylanase. Conserved regions are indicated by numbers; (I)-(VIII). Shaded boxes indicate cases when more 7 out of 10 amino acids are conserved. The numbers in front of each sequence denote the first amino acids of individual lines.
lyses are shown in Fig. 1. Results with the recombinant plasmid pACC2, with an inverted insert of an EcoRI fragment, indicate that the promoter of the cloned A. capsulatum xylanase gene is functioning in E. coli. pACC201 harboring a 7.3-kb EcoRI-KpnI fragment, pACC202 harboring a 4.0-kb EcoRI fragment, and pACC204 harboring a 1.6-kb EcoRI-StyI fragment, in pUC19 expressed xylanase activity. However pACC203 harboring a 4.8-kb BglII fragment, and pACC205 harboring a 1.1-kb EcoRI-PstI fragment in pUC19 did not have any activity. From these results, it was concluded that the 1.6-kb EcoRI-StyI fragment was essential for xylanase activity.

**Nucleotide sequence of xynA**

Figure 2 shows the complete nucleotide sequence of the xynA structural gene along with its flanking regions. The open reading frame of xynA consists of 1,215 nucleotides encoding a protein of 405 amino acids with a deduced molecular weight of 44,100. The putative initiation codon ATG was preceded 6 bp upstream by a potential Shine-Dalgarno (SD) sequence, 5’-GAG-GAAG-3’, which allows base pairing between mRNA and the 3’-end of bacterial 16S rRNA.21 Upstream of the coding region, possible promoter sequences, TAGAGA for the −35 region and TTGGAT for the −10 region with a distance of 17 bp between them were observed. The open reading frame is terminated by the amber stop codon TAG at position 1489. The GC content of the xynA gene was calculated to be 65.2%, slightly higher than the reported GC content (60.8%) of genes from Acidobacterium species.60 No potential terminator sequence was found downstream from the stop codon.

**Amino acid sequence homology between XynA and other xylanases**

Comparison between the deduced amino acid sequence of XynA with those of other reported cellulases found a consensus sequence of the xylanases belonging to the glycosyl hydrolase family 102,239 (Fig. 3). XynA from A. capsulatum had much sequence similarity with XynB from Clostridium stercorarium F-9,245 XynA from Caldoccilum saccharolyticum,235 ORF4 from C. saccharolyticum,235 CelB from C. saccharolyticum,235 XynZ from Clostridium thermocellum,27,280 XynA from Bacillus sp. strain C-125,290 Cex from Cellulomonas fimii,290 XynA from Pseudomonas fluorescens subsp. cellulosa,31 and XynA from Butyrivibrio fibrisolvens.32 As shown in Fig. 3, the alignment of the amino acid sequences of the catalytic domains classified in family 10 showed that the highly conserved stretches are localized in 8 regions (I–VIII). Two glutamic acids (Glu167 and Glu282) in the proposed active center were also conserved in A. capsulatum XynA (region III and VI in Fig. 3). Moieties other than the catalytic domain of A. capsulatum XynA do not show any sequence similarity with other functional domains such as the cellulose-binding domain.

**Purification of XynA from a recombinant E. coli**

E. coli harboring pACC204 was grown for 12 h in 6 l of 2× YT medium containing ampicillin (50 μg/ml). XynA was purified from the cellular fraction of E. coli JM109 (pACC204) by CM-Toyopearl 650 M, phenyl-Toyopearl 650 M, and Sephacryl S-200 column chromatographies. The result of the purification of XynA is summarized in Table 1. The final preparation gave a single band on SDS-polyacrylamide gel and the molecular mass of the enzyme was estimated to be 41 kDa (Fig. 4). From gel filtration chromatography on a Sephacryl S-200 HR column, the corresponding native molecular mass of XynA was estimated to be about 40 kDa. These results indicate that the A. capsulatum XynA has a monomeric structure. This value was slightly smaller than that deduced from the nucleotide sequence (44,100 Da). The N-terminal amino acid sequence of XynA was identified as Ala-Pro-His-Leu-His-Glu-Val-Ser-Gly-Pro, which was found in the deduced amino acid sequence of XynA at amino acid positions 30 to 39 (Fig. 2).

**Characterization of XynA**

**Effects of pH and temperature on the enzyme activity.**

The effect of pH on XynA activity was measured in five different buffers covering the pH range of 1.5 to 8.5 (Fig. 5). The enzyme was most active at a pH of around 5.0 at 30°C and retained more than 80% of its activity at a pH of 3.0 to 8.0. The optimum temperature for enzyme activity was 65°C. According to the thermostabil-
Fig. 5. Effects of pH (A) and Temperature (B) on XynA Activity and Stability.

(A) ○, optimum pH; ■, pH stability (B) ○, thermal stability; ●, optimum temperature. The conditions are described in Materials and Methods.

Table II. Substrate Specificity of A. capsulatum XynA

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymeric substrate</td>
<td></td>
</tr>
<tr>
<td>Xylan (oat spellet)</td>
<td>100</td>
</tr>
<tr>
<td>Lichenan</td>
<td>9.7</td>
</tr>
<tr>
<td>CM-cellulose</td>
<td>5.4</td>
</tr>
<tr>
<td>Avicel</td>
<td>0</td>
</tr>
<tr>
<td>Filter paper</td>
<td>0</td>
</tr>
<tr>
<td>Laminarin</td>
<td>0</td>
</tr>
<tr>
<td>Soluble starch</td>
<td>0</td>
</tr>
<tr>
<td>Nitrophenyl glycosides</td>
<td></td>
</tr>
<tr>
<td>p-NP-β-D-celllobioside</td>
<td>100</td>
</tr>
<tr>
<td>p-NP-β-D-xylpyranoside</td>
<td>0</td>
</tr>
<tr>
<td>p-NP-β-D-glucopyranoside</td>
<td>0</td>
</tr>
</tbody>
</table>

The enzyme activities were measured by the standard assay containing 1% (w/v) of various substrates. In the case of nitrophenyl glycosides, the amount of p-nitrophenol released was measured at 410 nm.

Activity at a temperature of 20 to 50°C. The activity decreased greatly above 50°C, reaching 0 after incubation at 70°C for 10 min (Fig. 5).

Isoelectric point. The isoelectric point of the enzyme was 7.3 as estimated from the relative mobility of various standard proteins (data not shown).

Kinetic parameters. The initial reaction rates measured at 30°C in various concentrations of xylan and CMC for Lineweaver-Burk plots showed that the $V_{max}$ was estimated to be 403 ($\mu$mol/min/mg) for xylan, 95 ($\mu$mol/min/mg) for CMC, and the $K_m$ to be 3.5 (mg/ml) for xylan, and 4.4 (mg/ml) for CMC.

Substrate specificity. Purified XynA was tested on different substrates (Table II). The liberation of reducing groups were detected when both xylose and glucose polysaccharides were used. The enzyme was about 18-fold more active on oat spellet xylan (100%) than on CMC (5.4%), and it had about 1/10 activity (9.7%) when lichenan was used as the substrate. The enzyme was completely inactive on Avicel, filter paper, laminarin, soluble starch, p-nitrophenyl-β-D-xylpyranoside, and p-nitrophenyl-β-D-glucopyranoside.

Analysis of xylan hydrolysis products. The hydrolysis patterns of xylan by XynA was studied by thin-layer chromatography. The result showed an identical endo mode of action. Thus, as xylan degradation occurred, a large amount of intermediate products (xylotetraose, xylotetraose, xylotriose, and xylobiose) was obtained, but a very small quantity of xylose appeared. Even after long incubation periods, xylobiose was the most abundant product (Fig. 6).

Discussion

Xylan, a major component of hemicellulose in plant cell walls, is now regarded as a natural resource that is convertible to biofuels, chemicals, and value-added compounds by either microbial fermentation or enzymatic processes. Many xylan-degrading enzymes have been purified from various microorganisms. In this paper we report the cloning, nucleotide sequence, and expression of the gene encoding a novel xylanase from an acidophilic bacterium, A. capsulatum. To our knowledge, this is the first report on the gene cloning of an acidic xylanase from an acidophilic bacterium.

XynA was isolated from a recombinant E. coli clone (plasmid pACC204 containing the xynA gene). The enzyme was purified to approximately 91-fold with a recovery of 64%. The apparent purity of the enzyme was demonstrated by SDS-polyacrylamide gel electrophoresis, and native polyacrylamide gel electrophoresis. The notable characteristics of this enzyme were its acidic pH optimum (pH 5.0) and stability in the acidic region. The molecular mass of XynA was 41 kDa with SDS-polyacrylamide gel electrophoresis, and 40 kDa with gel
filtration. As such, this enzyme consists of a single polypeptide. The calculated molecular mass of the total \( xynA \) coding region was 44,100 Da (405 amino acids), and the molecular mass without the proposed signal peptide was 41,464 Da. These results suggest the existence of a signal peptide which consists of 29 amino acid residues. However this signal peptide does not have the characteristics of signal peptides and does not contain (+)-charged amino acids which are known to be related to the stability of ionic bonds with cell membranes. The clarification of the function of the signal peptide of the XynA will be useful for the analysis of the expression system of \( A. \text{capsulatum} \).

\( A. \text{capsulatum} \) XynA has hydrolytic activities toward CMC and \( p \)-nitrophenyl-\( \beta \)-d-cellobioside in addition to xylan (Table II). Xylanases in family 11 hydrolyze only xylan, but some of the xylanases in family 10 hydrolyze both xylan and cellulose. As shown in Fig. 3, the catalytic domains in family 10, which consist of about 350 amino acids, have 8 well-conserved regions. The three-dimensional structures of catalytic domains in family 10 closely resemble each other. Catalysts of the family 10 xylanase have eight-fold \( \alpha/\beta \)-barrels (TIM barrels) with two active-site glutamates, one of which is the proton donor and the other is the nucleophile. These two glutamates are conserved as Glu167 and Glu282 in \( A. \text{capsulatum} \) XynA.

\( A. \text{capsulatum} \) is surrounded by a capsule composed of polysaccharides. As mentioned above, this bacterium produces various carbohydrate degrading enzymes (\( \alpha \)- and \( \beta \)-glucosidase, \( \alpha \)- and \( \beta \)-galactosidase, \( \beta \)-glucuronidase, \( \alpha \)-fucosidase, and \( N \)-acetyl-\( \beta \)-glucosaminidase). These enzymes and synthesis of the capsule appear to be related. We have previously reported that \( \beta \)-glucosidase from this bacterium catalyzes not only the hydrolysis reaction but also the glycosyl transfer reaction. Therefore, further studies on XynA, particularly the analysis of the glycosyl transfer reaction of this enzyme, will yield significant and interesting results.

References