Cloning of a Family 11 Xylanase Gene from *Bacillus amyloliquefaciens* CH51 Isolated from Cheonggukjang

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Abstract *Bacillus amyloliquefaciens* CH51, an isolate from *cheonggukjang*, Korean fermented soyfood, secretes several enzymes into culture medium. A gene encoding 19 kDa xylanase was cloned by PCR. Sequencing showed that the gene encoded a glycohydrolase family 11 xylanase and named *xynA*. *xynA*His, *xynA* with additional codons for his-tag, was overexpressed in *Escherichia coli* BL21(DE3) using pET-26b(+). XynAHis was purified using HisTrap affinity column. K_m and V_{max} of XynAHis were 0.363 mg/ml and 701.1 μ mol/min/mg, respectively with birchwood xylan as a substrate. The optimum pH and temperature were pH 4 and 25 °C, respectively. When *xynA* was introduced into *Bacillus subtilis* WB600, active XynA was secreted into culture medium.

Keywords Family 11 xylanase · *Bacillus amyloliquefaciens* · Overexpression · Purification

Introduction

Xylan is a structural polymer consisting of D-xyloses connected via β -1,4-xylosidic bonds and a major constituent of hemi-cellulose in the plant cell walls. Xylan is present in plants in large quantities with varying contents [1]. Hemicellulose is a heteropolymer consisting of 5 and 6 carbon sugars including xylose, arabinose, glucose,

mannose, and galactose. Xylan backbone structure can be substituted with groups such as α -L-arabinofuranosyl, glucuronopyranosyl, acetyl, feruloyl, or p-coumaroyl group as side chains [1]. Although different types of hydrolases are required for complete breakdown of hemicelluloses because of the structural complexities, xylanases (endo-1,4- β -xylanases, EC 3.2.1.8) are the most important enzymes randomly cutting the β -1,4-xylosidic bonds in xylan [2]. Various microorganisms secrete xylanases into culture media and glycohydrolase family 10 or 11 xylanases are the most common [3]. Family 5, 7, 8, or 43 xylanases are also known but not much informations are available [1]. High crude oil prices drive development of alternative energy sources including bioenergy [4]. Isolation of microorganisms utilizing xylan efficiently is important since xylan occupies a significant portion of utilizable polymers in plants [4]. Isolation of novel xylanases [5] and improvement of their properties [6–8] are actively being studied. Here we report cloning of a family 11 xylanase gene from *Bacillus amyloliquefaciens* CH51.

Materials and Methods

Bacterial Strains and Culture Conditions

Bacillus amyloliquefaciens CH51 was isolated from *cheonggukjang* prepared at Sunchang county, Jellabukdo, Korea [9]. Bacterial strains and plasmids used are shown in Table 1. *E. coli* and bacilli were grown in Luria–Bertani broth (LB) at 37 °C with shaking. pET26b(+) (Novagen, Madison, WI, USA) was used to overexpress a xylanase gene in *E. coli* BL21(DE3). pHY300PLK (Takara, Shiga, Japan) was used to express a xylanase gene in *B. subtilis* WB600 [10].

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Table 1 Bacterial strains and plasmids used in this study

Bacterial strain and plasmid	Relevant characteristics	Reference
Strains		
<i>Escherichia coli</i>	ϕ 80dlacZ Δ M15, <i>recA1</i> , <i>endA1</i> , <i>gyrA96</i> , <i>thi-1</i> , <i>hdr17</i>	Gibco BRL
DH5 α	(r_k^- , m_k^+), <i>sup E44</i> , <i>relA1</i> , <i>deoR</i> , Δ (<i>lacZYA-argF</i>) U169	
BL21(DE3)	hsdS gal (<i>λclts857ind1 Sam7 nin5 lacUV5-T7 gene1</i>)	Novagen
<i>B. amyloliquefaciens</i> CH51	Isolated from cheonggukjang	[9]
<i>B. subtilis</i> WB600	Em ^r , Lm ^r , <i>npr</i> , <i>aprA</i> , <i>epr</i> , <i>bpf</i> , <i>mpr</i> , <i>mprB</i>	[10]
Plasmids		
pHY300PLK	<i>Bacillus</i> and <i>E. coli</i> shuttle vector, 4.87 kb, Ap ^r , Tc ^r	Takara
pET-26b(+)	expression vector, 5.36 kb, Km ^r	Novagen
pHxynA	pHY300PLK with <i>xynA</i> , 5.85 kb	This study
pExynA	pET-26b(+) with <i>xynA</i> without its own promoter, 6.15 kb	This study
pExynHis	pET-26b(+) with <i>xynA</i> without its own promoter, with 8 additional codons at the 3' end, 6.15 kb	This study

Cloning of *xynA*

Bacillus amyloliquefaciens CH51 was grown overnight in LB and chromosomal DNA was prepared [9]. PCR was done using a MJ mini gradient thermal cycler (BioRAD, Hercules, CA, USA). Reaction mixture (50 μ l) contained 1 μ l of template DNA, 1 μ l of each primer (10 μ M), 1 μ l of deoxynucleoside triphosphates (0.25 mM), and 0.5 μ l of ExTaq DNA polymerase (Takara). A primer pair was designed based on a presumptive xylanase gene (RBAM_033790) from *B. amyloliquefaciens* FZB42 where the whole genome sequences determined (GenBank: CP000560.1) : pHY-F (5'-AGAA TTCCATGGCG GAACAAGACCAC-3', *Eco*RI site underlined) and pHY-R (5'-GGGATCCGTCAGCTGTGAGC AAG TTAAACA-3', *Bam*HI site underlined). Amplification conditions were as follows: 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 2 min; and final extension at 72 °C for 4 min. Amplified fragment was ligated with plasmid vectors (5:1 molar ratio) for 16 h at 16 °C using T4 DNA ligase (Promega, Madison, WI, USA) after digested with *Eco*RI and *Bam*HI.

Transformation of *E. coli* and *B. subtilis*

Transformation of *E. coli* and *Bacillus* cells was done by electroporation method. *E. coli* competent cells were mixed with ligation mixture and transferred into an electroporation cuvette (0.1 mm). GenePulser II (BioRad) was used to deliver a single pulse (25 μ F capacitance, 600 Ω resistance, and 18 kV/cm field strength). Cells were diluted with 1 ml of LB, incubated for 1 h at 37 °C with shaking, and spreaded on LB plates with ampicillin (Ap, 50 μ g/ml). Electroporation of *B. subtilis* WB600 cells was done as described previously [11]. LB plates containing tetracycline (Tc, 15 μ g/ml) were used to select transformants (TFs).

Overexpression of *xynA* in *E. coli* and Purification of XynAHis

A *xynA* without promoter sequences was amplified using a primer pair: pET-F (5'-AGGATCCTGCCTCATGTCAA AGTC-3', *Bam*HI site underlined) and pET-R (5'-CGAAT TCGTCAGCTGTGAGCAAGTT-3', *Eco*RI site underlined). Amplified fragment was ligated with pET26b(+). *xynA* with additional codons for His-tag (*xynAHis*) was amplified by using a primer pair: pETHis-F (5'-AGGA TCCTGCCTCATGTCAAAGTC-3', *Bam*HI site underlined) and pETHis-R (5'-CCTCGAGCCACACTGTTACA TTAG-3', *Xho*I site underlined). *E. coli* BL21(DE3) harboring pExynAHis (pET26b(+)) with *xynAHis* was grown in LB (2 l) with kanamycin (Km, 50 μ g/ml). When the OD₆₀₀ was 0.7, IPTG was added (1 mM) and growth continued for 3 h. Cells were resuspended in 10 ml of 20 mM imidazole containing 8 M urea and 500 mM NaCl. Cells were disrupted by sonication (Bandelin electronic, HD2070, Berlin, Germany) and centrifuged at 12,000 $\times$ g for 10 min. XynAHis was purified using HisTrap HP column (GE Healthcare, Piscataway, NJ, USA). Fractions containing XynAHis were pooled and dialyzed against 20 mM sodium phosphate buffer (pH 7.0) for 24 h at 4 °C. Dialyzate was lyophilized and resuspended in 2 ml of sodium phosphate buffer.

Enzyme Assay

Escherichia coli cells were disrupted by ultrasonication. Cell pellet (insoluble fraction), obtained by centrifugation, and supernatant (soluble fraction) were examined for xylanase activity. Xylanase activity was determined by measuring the amount of reducing sugars released from birchwood xylan (Sigma, St. Louis, MO, USA) using 3,5-dinitrosalicylic acid

(DNS) reagent [12]. Two percent birchwood xylan in 50 mM sodium acetate buffer (pH 5.5) was the substrate. 0.3 ml of substrate solution was mixed with 0.1 ml of xylanase sample and incubated for 10 min at 37 °C. 1 ml of DNS reagent was added, boiled for 15 min, and OD₆₀₀ was read. One unit (U) of enzyme activity was defined as the amount of enzyme that released 1 µmol of reducing sugar equivalent to xylose per minute. Specific activity was expressed as unit per milligram protein and protein concentration was determined by the Bradford method [13] using bovine serum albumin (BSA) as the standard.

SDS-PAGE and Zymography

After cultivation in LB, cells were resuspended in PBS (phosphate buffered saline, pH 7.0) and disrupted by ultrasonication. Insoluble and soluble fractions were analyzed by SDS-PAGE and zymography. SDS-PAGE was done by Laemmli's method [14] using a 15 % polyacrylamide gel. Zymogram was obtained using a 15 % polyacrylamide gel containing 1 % birchwood xylan [15]. After electrophoresis, the gel was washed in 25 % isopropanol for 1 h followed by three times washing with 0.1 M phosphate buffer (pH 7.0) for 10 min each. After 30 min incubation at 40 °C, the gel was stained with 0.1 % Congo red and washed with 1 M NaCl and then 0.5 % acetic acid.

Properties of XynAHis

XynAHis was incubated at pH 3–10 (pH 3–4, 0.1 M citrate buffer; pH 5–6, 0.1 M sodium phosphate buffer; pH 7–8, 0.1 M Tris–HCl buffer; pH 9–10, 0.1 M glycine–NaOH buffer) for 30 min at 37 °C. Then the activity was measured. Stability at different pHs was determined by measuring the remaining activity after 3 h incubation. The optimum temperature was examined by measuring the activity after 30 min incubation at 25–80 °C in 0.1 M citrate buffer (pH 4.0). Thermal stability was determined by measuring the activity for 1 h incubation at 37, 45, and 50 °C. XynAHis was incubated in 0.1 M citrate buffer (pH 4.0) containing 5 mM metal ions or 1 mM enzyme inhibitors for 30 min at 25 °C and the remaining activity was measured. K_m and V_{max} were determined from the Lineweaver–Burk plot of reactions done with birchwood xylan as a substrate.

Results and Discussion

Cloning of *xynA* from *B. amyloliquefaciens* CH51

Bacillus amyloliquefaciens CH51 was isolated from *cheonggukjang*, a Korean fermented soyfood and the strain

secretes several proteins into culture medium [16]. Some secreted proteins were identified by tandem mass spectrometry after each band excised from a gel. Identified proteins included a 27 kDa fibrinolytic protease (AprE51), a 23 kDa endoglucanase, and a 19 kDa xylanase [16]. In this work, the gene encoding the 19 kDa xylanase was cloned by PCR. A primer pair was designed based on *xynA* of *B. amyloliquefaciens* FZB42 where genome sequences are available. Amplified 985 bp fragment was first ligated with pHY300PLK and then introduced into *E. coli* DH5 α by electroporation. TFs with the expected plasmid, pHxynA, were obtained. BLAST (Basic Local Alignment Search Tool) search confirmed that it was a xylanase gene encoding a family 11 xylanase. The gene was named *xynA* and the sequence was deposited in Genbank (HM060310). The ORF is 642 nt (nucleotide) in size and starts at 172 nt (AUG) and stops at 813 nt (UAA), encoding a protein of 213 amino acids. Calculated pI and molecular mass are 9.5 and 23,276.55 Da, respectively. The first 28 amino acids seem to be a signal peptide as predicted by SignalP 3.0 program (<http://www.cbs.dtu.dk/services/SignalP/>). Mature enzyme, 185 amino acids in length, has molecular weight of 20,232.92 Da and pI of 9.03. The calculated size matched well with the 19 kDa band on a SDS-gel. Putative –35 and –10 promoter sequences were located upstream of the first codon, TTAATA (47–52 nt) and TATTAT (72–77 nt) with 9 nt space.

XynA was similar to other family 11 xylanases: 100 % with endo-1,4-beta-xylanase from *Bacillus* sp. (AAD10834.1), 99 % with XynA from *B. amyloliquefaciens* FZB42 (ABS75708.1), *B. subtilis* (AAZ17392.1), and *Paenibacillus macerans* (AAZ17386.1), 96 % with endo-1,4-beta-xylanase from *B. licheniformis* (AAZ17387.1), *Brevibacillus brevis* (AAZ17389.1), and *B. megaterium* (ACT21830.1). Family 11 xylanases are known to have high pI values and low molecular weights in common, considered useful for industrial applications [1]. In contrast, family 10 xylanases are larger enzymes.

Expression of *xynA* in *E. coli* DH5 α

Escherichia coli DH5 α harboring pHxynA (Table 1) produced the 19 kDa band but control (cells harboring pHY300PLK) did not (data not shown). The same result was obtained by zymography, an active 19 kDa band observed only from cells harboring pHxynA (data not shown). The band was observed from both soluble (cytoplasmic fraction) and insoluble fractions.

Overexpression of *xynA* in *E. coli* BL21 (DE3)

A 791 bp *xynA* without promoter sequences was amplified and cloned into pET-26b(+). *E. coli* BL21(DE3) harboring

Fig. 1 SDS-PAGE and zymogram of purified XynAHis. M, Dok-Do-MARK proteins (Elpisbio, Daejeon, Korea); 1, coomassie blue stained XynAHis; 2, zymogram. XynAHis was purified by HisTrap affinity column and 5 μ g was analyzed on 15 % acrylamide gel

the recombinant plasmid (pExynA) was grown in LB until OD_{600} reached 0.7. IPTG (1 mM) was added and growth continued for 3 h. When cell extracts were analyzed by SDS-PAGE, a thick 19 kDa band was observed from insoluble fraction (results not shown) and the band with much less intensity was detected from soluble fraction, indicating that most XynA molecules accumulated as inactive inclusion bodies. No xylanase activity was detected from supernatant of cells harboring pET26b(+) and uninduced cells harboring pExynA.

To facilitate purification, *xynAHis* was amplified. *xynAHis* contains 8 extra codons encoding LE (*Xho*I site) and 6H at 3' end. After induction by IPTG, XynAHis was purified by using a HisTrap affinity column (Fig. 1). XynAHis molecules, accumulated as inclusion bodies inside *E. coli*, were recovered from insoluble fraction of cells and loaded into a HisTrap column. They were eluted with 20 ml of 100 mM imidazole containing 8 M urea and 500 mM NaCl and regained activity after dialysis as shown by enzyme assay and zymography. Many foreign proteins form inclusion bodies when overproduced in *E. coli* [17]. XynAHis as inclusion bodies has advantages over soluble enzyme if renaturation steps are not difficult or time consuming because inclusion bodies are easily recovered by centrifugation and less prone to degradation by proteases of *E. coli* [18].

Properties of XynAHis

K_m of XynAHis for birchwood xylan was calculated to be 0.363 mg/ml and V_{max} was 701.1 μ mol/min/mg protein. K_m value was smaller than that of a family 11 xylanase

Fig. 2 Optimum pH (a) and pH stability (b) of XynAHis. **a** Purified XynAHis (10 μ g) was incubated in different pHs for 30 min at 37 $^{\circ}$ C **b**. XynAHis was incubated in different pHs for 3 h. Then the remaining activity was measured in each case

from *Bacillus licheniformis*, which had 6.7 mg/ml as K_m and 379 μ mol/min/mg as V_{max} , respectively [19]. XynAHis hydrolyzed birchwood and beechwood xylans efficiently but did not hydrolyze CMC and pectin (results not shown).

XynAHis is active and stable at pH 4–5 (Fig. 2) but rapidly inactivated at pH 3.0 or less and alkaline pHs above pH 8.0. After 3 h at pH 10, XynAHis maintained 65 % of activity observed at pH 4.0. XynAHis was active and stable below 40 $^{\circ}$ C with 25 $^{\circ}$ C as optimum temperature, rapidly inactivated above 45 $^{\circ}$ C (Fig. 3). After 30 min at 50 $^{\circ}$ C (pH 4.0), <10 % of activity remained. At 45 $^{\circ}$ C, 29.5 % of activity remained after 1 h. Thermostability of native enzyme was checked. *B. amyloliquefaciens* CH51 was grown in LB for 12 h and culture supernatant was concentrated by 80 % ammonium sulfate precipitation. Ammonium sulfate pellet was dialyzed, freeze-dried, and resuspended in small volume of 0.1 M citrate buffer (pH

Fig. 3 Optimum temperature (a) and temperature stability (b) of XynAHis. **a** XynAHis (1 μ g) in 0.1 M citrate buffer (pH 4.0) was incubated for 30 min at different temperatures. Then the activity was measured. **b** XynAHis was incubated for 1 h at different temperatures. Sample was taken every 10 min and the activity was measured. Filled circle, 37 °C; filled square, 45 °C; filled triangle, 50 °C

4). When thermostability of native XynA was measured, 91.2 and 45.7 % activity remained after 30 min at 45 and 50 °C, respectively (results not shown). Although native XynA was more thermostable than XynAHis, XynA was not stable above 45 °C. Reduced thermostability of XynAHis might be due to the additional 8 amino acids or incomplete renaturation. Thermostability is important if a xylanase is used for industrial applications such as bio-bleaching of pulp [2] since bio-bleaching proceeds at high temperature and alkaline pHs. Thermostability might be improved by protein engineering. Zhang et al. [8] improved thermostability of a mesophilic xylanase from *Streptomyces olivaceovirdis* by site-specific mutagenesis. Changes as few as five amino acids in the N-terminus improved thermostability significantly. Shibuya et al. [20] improved the thermostability of a mesophilic xylanase from *Streptomyces lividans* by random gene shuffling technique.

Table 2 Effect of metal ions and inhibitors on the xylanase activity

Metal ions or inhibitors	Concentration	Relative activity (%)
None		100
KCl	5 mM	80 $\pm$ 0.7
MgCl ₂	5 mM	85 $\pm$ 0.6
CaCl ₂	5 mM	79 $\pm$ 0.3
CuSO ₄	5 mM	103 $\pm$ 1.8
MnCl ₂	5 mM	50 $\pm$ 2.6
ZnCl ₂	5 mM	78 $\pm$ 1.0
HgCl ₂	5 mM	77 $\pm$ 0.2
FeSO ₄	5 mM	80 $\pm$ 0.5
SDS	1 mM	72 $\pm$ 5.7
EDTA	1 mM	85 $\pm$ 5.1
PMSF	1 mM	93 $\pm$ 0.1
Cantharidic acid	1 mM	94 $\pm$ 0.1
Pepstatin A	1 mM	94 $\pm$ 0.6
Bestatin hydrochloride	1 mM	99 $\pm$ 0.6
E-64	1 mM	95 $\pm$ 0.9
Triton-100	1 %	116 $\pm$ 0.1
Ethanol	1 %	94 $\pm$ 3.3
Methanol	1 %	96 $\pm$ 2.1
Isopropylalcohol	1 %	100 $\pm$ 0.2
Glycerol	1 %	84 $\pm$ 0.6

Among the metal ions, Cu²⁺ slightly increased the activity but all other ions inhibited (Table 2). Mn²⁺ reduced the activity by 50 % but Hg²⁺ reduced by 23 %. For a family 10 xylanase from an alkalophilic *Bacillus* sp., Hg²⁺ and Mn²⁺ inhibited the activity by 81 and 73 %, respectively [2]. Among the inhibitors, SDS reduced the activity by 28 % followed by glycerol (16 %). Similar effects of SDS and glycerol were reported [2].

Expression of *xynA* in *B. subtilis*

pHxynA was introduced into *B. subtilis* WB600, a strain lacking six proteases [10]. The 19 kDa xylanase was observed from cells harboring pHxynA but not from cells harboring pHY300PLK (Fig. 4). XynA was detected from culture supernatant but not from cell extract (results not shown), indicating XynA secreted into culture medium. Xylanase activity was 1.68 unit/mg protein for culture supernatant and 0.16 unit/mg protein for cell extract. The band intensity of XynA increased for concentrated culture supernatant (Fig. 4, lane 4). *B. subtilis* WB600 may be used as a host to produce active XynA. Secretion of xylanase has an advantage, obtaining unmodified enzyme in addition to simple recovery. Optimization of XynA production in *B. subtilis* WB600 deserves further studies.

Fig. 4 SDS-PAGE and zymogram of *B. subtilis* strains. M, Dok-Do-MARK proteins (Elpisbio); 1, culture supernatant from *B. subtilis* WB600 [pHY300PLK]; 2, culture supernatant from *B. subtilis* WB600 [pHxyna]; 3, concentrated culture supernatant from *B. subtilis* WB600 [pHY300PLK]; 4, concentrated culture supernatant from *B. subtilis* WB600 [pHxyna]. Culture supernatant was concentrated by ammonium sulfate precipitation (80 %). Each 10 µg sample was analyzed on 15 % acrylamide gel

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