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Research article

Improving the thermostability of *Trichoderma reesei* xylanase 2 by introducing disulfide bonds

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Background: Xylanases are considered one of the most important enzymes in many industries. However, their low thermostability hampers their applications in feed pelleting, pulp bleaching, and so on. The main aim of this work was to improve the thermostability of *Trichoderma ressei* xylanase 2 (Xyn2) by introducing disulfide bonds between the N-terminal and α-helix and the β-sheet core.
Results: In this work, two disulfide bonds were separately introduced in the Xyn2 to connect the N-terminal and α-helix to the β-sheet core of Xyn2. The two disulfide bonds were introduced by site-directed mutagenesis of the corresponding residues. The half-life of the mutants $Xyn2^{C14–52}$ (disulfide bond between β-sheets B2 and B3) and $Xyn2^{C59–149}$ (disulfide bond between β-sheets A5 and A6) at 60°C was improved by approximately 2.5- and 1.8-fold compared to that of the wild type Xyn2. In addition, the enzyme's resistance to alkali and acid was enhanced.
Conclusion: Our results indicated that the connection of the N-terminal and α-helix to the β-sheet core is due to the stable structure of the entire protein.

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1. Introduction

Xylan mainly consists of D-xyloses connected by β-1,4-D-linkage and is the major constituent of hemicellulose in cells and the second-most abundant polysaccharide in nature [1]. There are several xylanolytic enzymes involved in the complete degradation procedure of xylan because of its complex structure. Endo-β-1,4-D-xylanases play a key role in the hydrolysis of xylan because of their ability to cleave the internal β-1,4-D-xylosedic linkages, which are the main linkages in xylan [2]. In the last few decades, xylanase has attracted much attention because of its wide industrial applications in food technology, feed technology, and pulp industry, and it has even been used in biofuel production [3,4,5,6]. Xylanases have been primarily classified into glycoside hydrolase (GH) family 5, 8, 10, 11, 30, and 43 according to the hydrophobic cluster analysis of the catalytic domains and similarities in the amino acid sequences [7]. Among these, GH family 11 xylanases have gained much attention because of their high substrate selectivity, high catalytic efficiency, and a relatively simple structure that mainly consists of an α-helix and two β-sheets packed against each other [8].

However, most naturally isolated enzymes are not competent for industrial applications because of their low thermostability, acid (or alkali) resistance, and so on [2,9,10]. Although thermostable xylanase can be isolated from natural thermophiles such as *Thermotoga maritime* (producing XynA and XynB), their low specific activity and contamination of other side activities limits their use [11,12].

Improving the thermal properties of mesophilic xylanases through genetic engineering is considered an effective approach to obtaining thermostable xylanases [10,13]. Introduction of a disulfide bond is the most efficient method for improving the stability of a protein and has been widely used in many other proteins [14,15,16]. Previous studies on xyalnases indicated that the β-sheet core is a stable structure of GH family 11 xylanases. Moreover, the N-terminal, C-terminal, and α-helix are the most notable differences between thermophilic and mesophilic xylanases [17,18,19,20]. In addition, many studies have successfully improved the thermostability of mesophilic xylanases by connecting the N-terminal, C-terminal, or α-helix [17,21,22,23,24,25]. However, the connection between the N-terminal, C-terminal, and α-helix and the β-sheet core was barely mentioned in previous studies.

The endo-1,4-β-xylanase 2 (Xyn2) of *Trichoderma reesei* is one of the most important xylanases used in industry [26]. It is a

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low-molecular-mass (20 kDa) enzyme with an alkaline isoelectric point (pI 9.0) and activity optimum at pH 4–6. However, the temperature optimum of *T. reesei* Xyn2 is approximately 50–55°C, and it cannot withstand temperatures above 65°C, the most common temperature range in industrial applications [18,27]. Therefore, in this work, we aimed to improve the thermostability of Xyn2 through protein engineering. Previous studies have implicated the role of disulfide bond in the determination of thermostability; we accordingly designed amino acid mutations in the N-terminal and C-terminal of *T. reesei* Xyn2. Our aim was to increase the thermostability of Xyn2 by introducing disulfide bonds between the unstable regions (N-terminal and α-helix) and the stable core (β-sheet core).

2. Materials and methods

2.1. Vector, microbial strains, and culture media

T. reesei Rut C-30 (Mutated type, ATCC56765) preserved in laboratory was used as the Xyn2 gene donor (GenBank Accession No. EU532196.1). *Escherichia coli* (TianGen CB101) and vector pMD19-T (Takara D104A) were used for gene cloning, and *E. coli* Origami (DE3) and vector pET32a were used for expression. *T. reesei* Rut C-30 was cultured in a basal medium (61% tryptone, 0.3% yeast extract) for the expression of the Xyn2 gene. *E. coli* DH5α and Origami (DE3) were cultured at 37°C in Luria–Bertani medium containing 100 μg/mL Amp for selection.

2.2. Cloning of Xyn2 from T. ressei

Spores of *T. ressei* Rut C-30 (100 μL) were inoculated in 100 mL of basal medium and cultured at 28°C for 48 h. Then the fungal mycelia were harvested for total RNA isolation using RNAiso Plus (Takara D312) as described previously [28]. The first strand cDNA was synthesized with 500 ng of total RNA using Prime Script II 1st Strand cDNA Synthesis Kit (Takara) according to the manufacturer's instructions. The protein-coding sequence of *T. ressei* Xyn2 (GenBank Accession No. EU532196.1) was amplified by a pair of primers, F1 (5′-GCTGAATTCCAGACGATTCAGCCCGGCA-3′) and R1 (5′-ATGCGGCCGCTTAGCTGACGGTGATGGAA-3′), supplied with the restriction sites *Eco*RI and *Not*I, respectively. The PCR fragments were ligated to pMD19-T (Simple) vector (Takara) by TA cloning, and the recombinant cloning vector was named pMD19T-Xyn2. The *T. reesei* Xyn2 genes were sequenced by Sangon Biotech Co. and corrected by DNAman 6.0 (http://www.lynnon.com) with the template from GenBank (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/nuccore/EU532196.1).

Table 1
Primers for mutants.

Enzyme[a]	Mutant site[b]	Primers[c]
Xyn2$^{C14–52}$	F14C	5′-CGTTCCAGTACGAGTAGCAGTAGCCGTTGTTG-3′
		5′-GCTACTCGTACTGGAACGATGGCCACGGC-3′
	Q52C	5′-TCTTGGTGCCGGGACACCATCCCTTGCCG-3′
		5′-CGGCAAGGGATGGTGTCCCGGCACCAAGA-3′
Xyn2$^{C59–149}$	V59C	5′-CTGCCCGAGAAGTTGATGCACTTGTTCTTGGTGCCGG-3′
		5′-TGCATCAACTTCTCGGGCAGCTACAACCCCAACGG-3′
	S149C	5′-GTTCGCCGTGTTGACGCAGCCGCTCGAGC-3′
		5′-CGTCAACACGGCGAACCACTTCAACGCGTGGG-3′

[a] The mutant enzymes Xyn2$^{C14–52}$ and Xyn2$^{C59–149}$ contain two mutations F14C, Q52C and V59C, S149C respectively.
[b] The introduced mutations in this study are underlined.
[c] All the mutation sites in the primers are underlined.

2.3. Mutagenesis of Xyn2 gene

Each mutant of Xyn2$^{C14–52}$ and Xyn2$^{C59–149}$ were separately constructed with a pair of primers (Table 1) using the *Fast* Mutagenesis System Kit (TransGen Biotech Co, FM111-01) according to the manufacturer's instructions. The nucleic acid sequence of the mutants was identified by sequencing (Sangon Co, Shanghai) and confirmed by DNAMAN6.0.

2.4. Prediction of the crystal structure of Xyn2 and its mutants

A homologous crystal structure (PDB code: 3akq) that was fully identical to the amino acid sequence of the wild type *T. ressei* Rut C-30 Xyn2 was chosen as the template model for mutants. The three-dimensional crystal structure of the mutants was modeled using Swiss PDB Viewer 4.1 (http://spdbv.vital-it.ch) with Swiss Model programs based on the coordinates of the PDB code 3akq. The final coordinates of the mutants were calculated using the energy minimization program.

2.5. Expression and purification of Xyn2 and its mutants

All the Xylanase genes Xyn2, Xyn2$^{C14–52}$, and Xyn2$^{C59–149}$ located in the recombinant cloning vector were digested by *Eco*RI (Takara Code: 1611) and *Not*I (TaKaRa Code: 1623) and ligated to pET32a(+) having the corresponding sticky ends. Recombinant vectors with the wild and mutant genes were then transformed into *E. coli* Origami (DE3) cells and induced by 0.1% IPTG. The cells were then harvested by centrifugation at 10,000 × *g* at 4°C for 5 min. The cells were lysed by 0.1 mg/mL lysozyme, and the supernatant (soluble protein) was harvested by centrifugation at 10,000 × *g* at 4°C for 10 min. The supernatant (crude enzymes) was removed and directly loaded onto a 2-mL Ni^{2+}-chelating chromatography column according to the instruction manual (Bio-Rad, USA) and subsequently eluted with an elution buffer (300 mM NaCl, 50 mM sodium phosphate, and 500 mM imidazole, pH 8.0).

2.6. Mass spectrometry

The samples were analyzed by matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF–TOF-MS) performed by BGI Tech., China (http://www.bgitechsolutions.com). Spectra were acquired using the enhanced scanning mode covering a mass range of m/z 500–3500 with a distinguishability of 50,000. Database searches using the peak list files of the processed mass spectra were performed using Mascot 2.3.02 (http://www.matrixscience.com) and UniProt *T. reesei* database (http://www.uniprot.org/taxonomy/?query=Trichoderma_reesei&sort=score).

2.7. Enzyme activity and protein assays

The activity of the endo-1,4-β-xylanases was determined colorimetrically by a standard procedure using 3,5-dinitrosalicylic acid (DNS), as described by Zhang [29]. In brief, 40 μL of enzyme diluted with McIlvaine buffer (0.2 M sodium phosphate, 0.1 M citric acid, pH 6.0) was added to 360 μL of 1% (*w*/*v*) beech wood xylan, which was suspended in the same buffer, and incubated at 50°C for 10 min. The reducing sugars hydrolyzed by xylanase were developed by the developer 600 μL of DNS after boiling at 100°C for 10 min. The absorbance of the solution was then measured at 540 nm using a visible-spectrophotometer. In the control experimental group, xylanase was added in the last step. One unit (IU) of xylanase activity was defined as the amount of enzyme liberating 1 μmol of reducing sugar equivalent per minute.

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