International Journal of Biological Macromolecules 101 (2017) 366–372

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Engineering a xylanase from *Streptomyce rochei* L10904 by mutation to improve its catalytic characteristics

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ARTICLE INFO

Article history:
Received 8 February 2017
Received in revised form 21 March 2017
Accepted 24 March 2017

Keywords:
Xylanase
N-terminal
Cord
Catalytic characteristics

ABSTRACT

Protein engineering was performed by N-terminal region replacement and site-directed mutagenesis in the cord of a xylanase (Srxyn) from *Streptomyce rochei* L10904 to improve its catalytic characteristics. Three mutants SrxynF, SrxynM and SrxynFM displayed 2.1-fold, 3.2-fold and 5.3-fold higher specific activities than that of Srxyn, respectively. Moreover, all of the mutants showed greater substrate affinity and k_{cat}/K_m than the native Srxyn. In addition, the enzymes showed improved hydrolysis characteristics, of which the most noteworthy is the enhanced ability of producing xylobiose (X2) and xylotriose (X3) from polymeric substrates. The engineered xylanases have greater potential for applications in oligosaccharide preparation industry.

1. Introduction

Xylanases are used in several different biotechnological applications, alone or in combination with other enzymes: processing aid of bakery products [1], starch separation, clarification of juices, animal feed biotechnology and production of functional food ingredients, especially for those have special properties [2,3]. Currently, an application of xylanases is the production of emerging prebiotics xylooligosaccharides due to the high specificity and little side product generation of the enzymatic preparation [4–8]. During the production of xylooligosaccharides, xylose (X1) is a undesirable component that requires additional costs to be removed [9]. Therefore, it's necessary to explore appropriate xylanase by either traditional screening from special environment or the application of protein engineering. To date, many xylanases were reported to possess special hydrolysis characteristics in producing xylooligosaccharides. Xylanases from *Bacillus Methylotrophicus* CSB40, *Streptomyces thermovulgaris* TISTR1948, *Streptomyces rameus* L2001, *Paenibacillus campinasensis* BL11, *Streptomyces matensis* and xylanase from *Aspergillus niveus* expressed in *Aspergillus nidulans*, can produce xylooligosaccharides when using birchwood xylan, oat-spelt xylan, beechwood xylan or others as substrates [10–15].

Xylanase (β-1,4-endoxylanase, EC3.2.1.8) is a category enzyme which cleave the β-D-xylopyranose bond between two D-xylopyranosyl residues linked by β-(1,4) bond, and the ones form the glycoside hydrolase family 11 (GH11) of the CAZy database are considered true xylanase [16–18]. GH11 xylanase is the overall conservation of the β-jelly-roll domain [19]. It resembles the shape of a partially closed right hand and consists of two twisted antiparallel β-sheets and a single α-helix. The β-sheet A is composed of a maximum of six β-sheets named A1-A6, whereas β-sheet B is composed of nine β-sheets denoted B1-B9 [20]. Both β-sheet A and B constitute the "fingers", while the loop between β-sheets B7 and B8 makes the "thumb". The twisted part of β-sheet B and the α-helix form the "palm" of the hand. As more than 60% of the residues are embedded in β-strands and α-helix of xylanase structure, the loops that collect all these elements are consequently short, about 5 residues on average. However, there are two exceptions: the "cord", part of a long irregular loop (10 residue-long) joining the β-strands B6-B9, which connects the fingers with the base of the thumb and partially closes the active site on the aglycon side; the loop joining the β-strands B8-B7, about 12 residue-long [19].

Xylanases possessing special hydrolysis characteristics in producing xylooligosaccharides with a possible lowest X1 units have

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http://dx.doi.org/10.1016/j.ijbiomac.2017.03.135
0141-8130/

great industrial application value [12,21]. In our previous work, a xylanase gene (*Srxyn*) from *Streptomyce rochei* L10904 [22] was cloned and expressed in *Escherichia coli* BL21(DE3). The recombinant enzyme showed attractive hydrolysis characteristic, with negligible X1 in hydrolysis products. However, the specific activity is too low to meet the requirement for broad industrial applications. In the present study, protein engineering was conducted by N-terminal region replacement and site-directed mutagenesis in the cord of the xylanase (Srxyn) from *Streptomyce rochei* L10904 to improve the catalytic characteristics.

2. Materials and method

2.1. Bacterial strains, vectors and substrates

Streptomyce rochei L10904 was newly isolated from the soil in our previous study and identified using 16S rDNA PCR-RFLP [22]. *E. coli* DH5α was used for propagation and manipulation of plasmids, and *E. coli* BL21(DE3) was used for protein expression. Plasmids pMD18-T and pET28a (+) were used for gene cloning and expression in *E.coli*, respectively. Taq polymerases were from Takara (Japan). Restricition endonucleases, T4 DNA ligase and Q5® High-Fidelity DNA polymerase were from NEB Inc. (USA). Bovine serum albumin was from Roche (738328). Beechwood, birchwood and oat spelt xylan were from Sigama(X4252, X0502 and X0627). Xylobiose (X2), xylotriose (X3), xylotetraose (X4) and xylopentaose (X5) were from Megazyme (6860-47-5, 47592-59-6, 22416-58-6, 49694-20-4).

2.2. Gene cloning and expression of xylanase

Streptomyce rochei L10904 was cultivated in LB medium. *Srxyn* (Genbank code: X81045) without the native secretion signal was cloned by polymerase chain reaction (PCR) from the chromosomal DNA with primers StreX11NcoF and StreX11XhoR (Table S1. in supplementary data). The amplified DNA fragment was subcloned into pMD18-T and transformed into *E. coli* DH5α cells. The gene was subcloned into the pET28a(+) at *Nco*I and *Xho*I restriction sits and transformed into *E. coli* BL21(DE3) for expression. The expressed recombinant xylanase was denoted as Srxyn.

2.3. Engineering of xylanase

Amino acid sequence homology analysis showed that Srxyn has a similarity of 91.36% (full length) to *Streptomyces* sp. JHA19 xylanase (Genbank: WP_055619964). Interestingly, six amino acids in Srxyn differed from *Streptomyces* sp. JHA19 xylanase (Fig. 1B). Four of the six are replaced by Thr in amino acid sequence of *Streptomyces* sp. JHA19 xylanase. Thus the four sites were selected according to the distinction of amino acid residue, excepting amino acids with similar characteristics, to be replaced with corresponding amino acids of *Streptomyces* sp. JHA19 xylanase (Fig. 1B). Furthermore, in an attempt to gain some insight into the functional role of the N-terminal of Srxyn (Fig. 1A), it has been replaced with a β-strand A1 "KFTVGNGQ" from *Neocallimastix patriciarum* xylanase [23,24], to create a hybrid enzyme named SrxynF. Excision method has usually been used to investigate the function of N-terminal, but we only obtained some inactivity mutants, whether long or short sequences have been deleted (data not shown). The primers containing mutated codons were used to introduce mutation (Table S1 in Supplementary data).

2.4. Production and purification of xylanases

All the mutants were cloned and expressed in *E.coli* BL21(DE3). The cells bearing the recombinant plasmids were cultured and expressed in LB medium at 37 °C by shaking at 200 rpm. 1.0 mM IPTG was used to induce xylanase expression. The enzymes were collected after centrifugation at 5000 rpm for 5 min at 4 °C. The cells were re-suspended with 50 mM Tris-HCl buffer (pH 7.0), and subjected to cell wall-breaking with ultrasonic. The disrupted cells were centrifuged at 10000 rpm for 10 min at 4 °C to obtain the supernatant with the enzymes ready for the following purification. Srxyn and the mutants were purified by Ni sepharose HP column (1 × 10 cm) with 50 mM phosphate buffer (pH 7.8) including 300 mM NaCl and different concentrations of imidazole, working on an ÄKTA FPLC purification system (GE Healthcare, Uppsala, Sweden). The purified enzymes were adjudged homogeneous after examination with SDS-PAGE. Protein concentrations were determined by Coomassie brilliant blue method with bovine serum albumin (BSA) as the standard.

2.5. Xylanase activity assay

Xylanase activity was measured according to the procedure reported by Bailey [25]. The reaction mixture containing 0.9 mL of 1% (w/v) beechwood xylan and 0.1 mL of a suitable diluted enzyme solution was incubated (50 mM acetate buffer, pH 5.5) at 55 °C for 5 min. The amount of reducing sugar liberated was determined by the 3,5-dinitrosalicylic acid (DNS) method, using X1 as the standard [26]. One unit (U) of xylanase activity was defined as the amount of enzyme releasing 1 μmol of X1 equivalent per min under the assay condition.

2.6. Characterization of pH and temperature properties

The effect of pH on the activity of xylanase was studied at 55 °C and pH 4.0–8.5 (50 mM): citrate buffer for pH 4.0–6.5; Tris-HCl buffer for pH 7.0–8.5. To determine the pH stability of the enzyme, the xylanase was incubated in the above mentioned appropriate buffers of different pH at 50 °C for 12 h, the residual xylanase activities were measured by the standard assay procedure. Relative activity calculated with percentage of residual activity to initial activity was used to evaluate the pH and temperature stability, respectively. The optimum temperature for xylanase activity was determined by incubating the enzyme at different temperature (40–85 °C) in 50 mM citrate buffer (pH 6.0). To determine the temperature stability of the enzyme, the xylanase was incubated at different temperatures (50 °C, 60 °C and 70 °C) at pH 6.0 for 60 min followed by cooling on ice for 30 min, and the residual xylanase activity was measured following the standard assay procedure.

2.7. Substrate specific activity and kinetic parameters

To determine the substrate specificity of the enzyme, the purified xylanase was incubated with 1% (w/v) of each substrate in 50 mM citrate buffer (pH 6.0) at 55 °C for 10 min. The amount of reducing sugars produced was estimated using the dinitrosalicylic acid method as described above. For the kinetic experiments, six different concentrations of each substrate were dissolved in 50 mM citrate buffer (pH 6.0), and incubated with the purified xylanase at 40 °C for 5 min [27]. The K_m and k_{cat} values were calculated from the kinetic data using the GraphPad Prism software.

2.8. Hydrolysis characteristics for oligosaccharide

To evaluate hydrolysis characteristics of xylanases, X3, X4 and X5 were used as substrates. Reaction systems (500 μl) containing 0.5 mg ml^{-1} substrate were incubated at 50 °C for 8 h in 50 mM citrate buffer (pH 6.0) with 1 U xylanase. The mixtures were then heated in boiling water for 5 min. Reaction mixtures were filtered with 0.22 μm membrane filter and injected (20 μl) onto a COSMOSIL sugar-D packed column (4.6ID × 500 mm,