Available online at www.sciencedirect.com
SCIENCE DIRECT®
Protein Expression and Purification 42 (2005) 122–130
Protein Expression & Purification
www.elsevier.com/locate/yprep

Improvement of the thermostability and catalytic activity of a mesophilic family 11 xylanase by N-terminus replacement

Jian-Yi Sun *, Ming-Qi Liu, Ying-Lei Xu, Zi-Rong Xu, Lin Pan, Hui Gao

Microbiology Division, Feed Science Institute, College of Animal Science, Zhejiang University, Hangzhou, 310029, China

Received 20 January 2005, and in revised form 3 March 2005
Available online 30 March 2005

Abstract

To improve the thermostability and catalytic activity of *Aspergillus niger* xylanase A (AnxA), its N-terminus was substituted with the corresponding region of *Thermomonospora fusca* xylanase A (TfxA). The constructed hybrid xylanase, named ATx, was overexpressed in *Pichia pastoris* and secreted into the medium. After 96-h 0.25% methanol induction, the activity of the ATx in the culture supernatant reached its peak, 633 U/mg, which was 3.6 and 5.4 times as high as those of recombinant AnxA (reAnxA) and recombinant TfxA (reTfxA), respectively. Studies on enzymatic properties showed that the temperature and pH optimum of the ATx were 60 °C and 5.0, respectively. The ATx was more thermostable, when it was treated at 70 °C, pH 5.0, for 2 min, the residual activity was 72% which was higher than that of reAnxA and similar to that of reTfxA. The ATx was very stable over a broader pH range (3.0–10.0) and much less affected by acid/base conditions. After incubation at pH 3.0–10.0, 25 °C for 1 h, all the residual activities of the ATx were over 80%. These results revealed that the thermostability and catalytic activity of the AnxA were enhanced. The N-terminus of TfxA contributed to the observed thermostability of itself and the ATx, and to the high activity of the ATx. Replacement of N-terminus between mesophilic eukaryotic and thermostable prokaryotic enzymes may be a useful method for constructing the new and improved versions of biologically active enzymes.

Keywords: Xylanase; Thermostability; Catalytic activity; N-terminus; *Pichia pastoris*

Xylanases (EC 3.2.1.8) are glycoside hydrolases that catalyze the hydrolysis of internal β-1, four bonds of xylan, the major hemicellulose component of the plant cell wall [1]. According to sequence similarities and hydrophobic cluster analysis, xylanases have been classified into families 10 (or F) and 11 (or G) of glycosyl hydrolases [2]. Xylanases were useful for several different biotechnological applications, such as paper, food, and feed industries [3]. The processes of different industries need various xylanases with their corresponding biochemical properties.

The TfxA[1] is known as one of the most thermostable family 11 xylanases [4]. The AnxA has been produced at an industrial scale and its crucial properties are the high activity and low pH optimum, which are essential for releasing important nutrients in such acidic environments as the chicken stomach [5]. The amino acid sequences of the catalytic domain of the TfxA showed that it belongs to family 11 and has 40% identity with the catalytic domain of AnxA. However, the TfxA is much more thermostable than the AnxA. Further study

* Corresponding author.
E-mail address: jysun@zju.edu.cn (J.-Y. Sun).

[1] *Abbreviations used:* TfxA, *Thermomonospora fusca* xylanase A; AnxA, *Aspergillus niger* xylanase A; atx, the constructed hybrid xylanase gene; ATx, the hybrid xylanase encoded by atx; reAnxA, recombinant AnxA expressed in *P. pastoris*; reTfxA, recombinant TfxA expressed in *P. pastoris*; LB, Luria–Bertani.

1046-5928/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.pep.2005.03.009

showed that the homology of their N-terminuses was relatively low. It was supposed that the N-terminus of TfxA contributed to its significant thermostability [6].Therefore, both the enzymes are suitable for studying the mechanism of thermostability.

During the past years, several approaches have been studied to obtain thermostable proteins [7,8]. In feed application, xylanases have to be stable in high temperature during feed preparation, but the catalytic activity is needed at physical conditions of domestic animals. Although all existing wild-type xylanases have their own advantages, they still cannot satisfy the demand of being applied in feed industries. Hence, attentions are focused on discovery of new xylanases or improvement of existing ones to meet the requirements of industries. However, as we know, no work of improving the thermostability and activity of AnxA has been done. In this study, we constructed a hybrid xylanase using AnxA and TfxA as parents, and expressed it in *Pichia pastoris*. The biochemical properties of the hybrid xylanase, particularly in catalytic activity and thermostability, were investigated.

Materials and methods

Materials

The *P. pastoris* expression kit including the pPIC9K vector, *P. pastoris* strain GS115, and *Escherichia coli* strain TOP10F′ were purchased from Invitrogen. Medium components were from Difco. Birchwood xylan was from Sigma Chemical. Restriction endonucleases were from TaKaRa. T_4 DNA ligase and PCR kit were from Promega. Primers were synthesized by Sangon. The recombinant pBS-T anx plasmids containing the AnxA gene and pBS-T tfx plasmids containing TfxA gene were stored at −20 °C in our laboratory. All other chemicals used were of reagent grade obtained from standard sources.

Constructions of hybrid gene and expression plasmid

Hybrid xylanase gene, atx, was constructed by substituting 5′ end segment (129 bases) of the TfxA for its corresponding region (108 bases) of the AnxA using the method of splicing by overlap extension (SOE).

Fragment A was amplified from recombinant pBS-T tfx plasmid, by using the primers of A1 (5′-CG^AATTC GCTGTTACATCCAACGAGACCG-3′) with the *Eco*RI recognition site (underlined) and A2 (5′-TCCAGTATTA CGCCAAGAGGTGCTGTAGTTTCC-3′). Fragment B was amplified from the recombinant pBS-T anx plasmid using the following PCR primers: B1 (5′-ACCTCTTG GCGTAATACTGGAGATTTTGTCGTTGGTCTG-3′) and B2 (5′-AGC^GGCCGCAGAGGAAATCGTG AC ACTG-3′) with the *Not*I recognition site (underlined). The primary PCR parameters for fragments A and B were: denaturation at 94 °C for 2 min first; 30 cycles of (1 min at 94 °C, 1 min at 57 °C, and 1 min at 72 °C). The resulting PCR products were separated by electrophoresis in a 1% (w/v) agarose gel. Fragments A and B were recovered and served as templates for subsequent amplification to produce the complete hybrid xylanase gene using the A1 and B2 as primers. The second PCR program for atx was: 2 min at 94 °C; 30 cycles of (1 min at 94 °C, 1 min at 54 °C, and 1 min at 72 °C); followed by 10 min at 72 °C. The atx was also recovered by electrophoresis in a 1%(w/v) agarose gel.

The amplified atx was double digested with *Eco*RI and *Not*I, and ligated into pPIC9K vector. The recombinant pPIC9K-atx plasmid was obtained. Correct insertion nucleotide sequences of the atx were confirmed using the dideoxy nucleotide chain-termination method. Competent *E. coli* strain TOP10F′ was transformed with pPIC9K-atx plasmid according to the procedures described in Sambrook et al. [9]. Transformants were screened on LB plates with 100 μg/ml kanamycin. A large amount of pPIC9K-atx plasmids were extracted by using Maxicolumns (Promega, Wizard Plus Maxipreps DNA Purification Kit).

Transformation of Pichia pastoris and expression of ATx

The pPIC9K-atx plasmid was linearized with *Bgl*II and transformed into *P. pastoris* strain GS115 with electroporator (Eppendorf, 2510, USA) using the protocol provided by the manufacturer. Transformants were selected for their ability to grow on plate without histidine (His^+). His^+ transformants were recovered on MD (minimal dextrose) agar plates (1.34% YNB, 2% glucose, 0.4 μg/ml biotin, and 1.7% agar). After 3-day incubation at 30 °C, single colonies were streaked onto MD and MM (minimal methanol, the same as MD but with 0.5% methanol instead of glucose) plates. After 2-day incubation at 30 °C, transformants were scored as mut^s (methanol utilization slow) phenotype based on slow growth on the MM plates. 125 His^+ Mut^s phenotype transformants were tested for expression of xylanase in 3-ml YPM medium (1% yeast extract, 2% peptone, and 0.5% methanol). The transformant with the best expression performance was used for further studies.

Scale-up expression was achieved in 2-L baffled shake flask containing 0.5 L BMGY medium (2% tryptone, 1.34% YNB, 0.4 μg/ml biotin, 1% glycerol, and 0.1 M K-phosphate, pH 6.0) at 30 °C for 24 h. Cells grown in BMGY were harvested and resuspended in 0.5 L BMMY medium (the same as BMGY but with 0.5% methanol instead of glycerol), and then incubated with shaking (250 r/min) at 30 °C for 96 h. To maintain induction, 100% methanol was added to the culture to a final concentration of 0.25% every 24 h. The ATx secreted into the culture was determined by activity.