Protein Expression and Purification xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Protein Expression and Purification

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

High-level expression and characterization of a thermostable xylanase mutant from *Trichoderma reesei* in *Pichia pastoris*

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ARTICLE INFO

Article history:
Received 17 January 2014
and in revised form 18 November 2014
Available online xxxx

Keywords:
Trichoderma reesei
Xylanase
Pichia pastoris
Fermentation

ABSTRACT

A gene encoding xylanase 2 mutant from *Trichoderma reesei* (T2C/T28C, named mxyn2) was cloned into the *Pichia pastoris* X33 strain using the vector pPICZαA. Recombinant Mxyn2p was functionally expressed in *P. pastoris* X33 and secreted into the supernatant. Real time qPCR demonstrated that an increase in gene copy number correlated with higher levels of expression. Supernatant from methanol induced cells was concentrated by ultrafiltration with a 10 kDa cut off membrane, and purified with ion exchange chromatography using SP Sepharose Fast Flow chromatography. Recombinant Mxyn2p protein had the highest activity at 75 °C, while recombinant protein encoded by the "wild type" xylanase gene xyn2, also expressed in *Pichia*, was 20 °C lower. The Mxyn2p enzyme retained more than 70% of its activity after incubation at 80 °C for 10 min. The effects of the optimal pH and temperature for higher expression levels in *P. pastoris* were also determined, 6.0 and 22 °C, respectively. The maximum xylanase activity of Mxyn2p was 13,000 nkat/mg (9.88 g/l) in fed-batch cultivation after 168 h induction with methanol in a 50 l bioreactor.

Introduction

Xylan is a heteroglycan with a backbone made up of β-1,4-linked D-xylopyranose residues with substitutions of L-arabinofuranose, D-glucuronic acid, and 4-O-methyl-D-glucuronic acid at 2′ and 3′ positions [1]. Complete degradation of xylans requires the concerted and synergistic action of a variety of enzymes. Endo-1,4-β-xylanase (EC 3.2.1.8) catalyzes the hydrolysis of the main polysaccharide chain presented in xylan. It is a crucial enzyme for depolymerization to produce xylooligosaccharides and xylose [2]. Based on amino acid sequence homologies and hydrophobic cluster analysis, xylanase has been grouped mainly into two families of glycosyl hydrolases: the GH10 and GH11 families [3,4]. Most widely industrially applied xylanases belong to the glycoside hydrolase family GH11 [5]. The structure of GH11 xylanase consists of two large pleated sheets and a single alpha-helix that form a structure resembling a partially closed right hand [6].

Most xylanases used at an industrial scale are produced by *Aspergillus* and *Trichoderma* [7]. Industrial processes include pulp bleaching, baking, and animal feeding [8–10]. Importantly, Xylanase activity at extreme conditions, such as high temperature, is required in some of these applications. Xylanase 2 (Xyn2p) secreted by *Trichoderma reesei* belongs to the glycosyl hydrolase family 11 and has a right hand β-sandwich structure [6]. It represents more than 50% of the total xylanolytic activity of *T. reesei* cultivated on xylan: Xyn1p and Xyn2p activity account for more than 90% of the xylan-degrading ability of this fungus [11]. However, prior work indicated that native Xyn2p is not stable at high temperature and loses activity rapidly when incubated at temperatures over 50 °C [11]. Thus, improving the performance of the Xyn2p to resist inactivation at high temperature has been a goal of many studies [12–15]. Researchers reported that a engineered disulfide bridge within the N-terminal region of Xyn2p is effective at resisting thermal inactivation (T2C and T28C) [16,17]. Furthermore, Mxyn2p made in *Escherichia coli* [16,17], had elevated thermal stability suggesting potential industrial applications [16,17].

The methylotrophic yeast *Pichia pastoris* has been widely reported as a suitable expression system for heterologous protein and many recombinant proteins have been expressed successfully in this system [18,19]. *P. pastoris* has particular industrial interest due to its powerful and tightly regulated methanol-inducible, alcohol oxidase 1 promoter (pAOX1), a capacity for foreign protein secretion and the ability to perform post-translational modifica-

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http://dx.doi.org/10.1016/j.pep.2014.11.014
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tions including glycosylation and disulfide-bond formation [20,21]. The objective of this work is to develop an efficient expression system for Mxyn2p using *P. pastoris* and characterize the recombinant protein.

Materials and methods

Strains, plasmids, culture medium and reagents

Plasmid pPICZαA, *P. pastoris* X33 strain and Zeocin™ were purchased from Invitrogen (USA). *E. coli* DH5α was used for all plasmid constructions. *P. pastoris* was cultured in shake flask in BMGY (1% yeast extract, 2% peptone, 1% glycerol, 1.34% YNB, 4×10^{-5}% biotin and 100 mM potassium phosphate, pH 6.0) before induction, or BMMY for induction (BMGY with 0.5% methanol instead of 1% glycerol). YPDS plates (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, 2% agar) containing 1 mg/ml Zeocin™ or 100 μg/ml Zeocin™ were used for selecting transformants with multiple copies of the expression vector. Other molecular biological reagents were purchased from Takara (Japan) or Invitrogen (USA). Birchwood xylan was purchased from Sigma (USA).

Construction of recombinant expression vectors and transformation of P. pastoris

DNA encoding the xyn2 open reading frame (ORF, Genbank Accession No: U24191.1) and the mature mxyn2 ORF derived from *T. reesei* were synthesized by Genscript (Suzhou, China) and subsequently cloned into pPICZαA vector using *Eco*RI and *Not*I restriction sites (Figs. 1 and 2). *Sac*I linearized recombinant plasmids were transformed into *P. pastoris* strain X33 by electroporation using a MicroPulse (Bio-Rad, Hercules, CA, USA). The transformed cells were cultured on YPDS plates containing 100 μg/ml and 1 mg/ml Zeocin™ respectively. After incubation at 30 °C for 3 days, ten of the larger colonies from each Zeocin concentration were selected for protein expression.

Expression of recombinant Mxyn2p in P. pastoris

Selected colonies were cultured in 25 ml BMGY in 250 ml shake flask at 28 °C, 250-rpm, for 18 h. Cells were collected by centrifugation at 3000*g* for 5 min at 4 °C, and re-suspended in 25 ml BMMY for continued growth under the same conditions. Methanol was added to a final concentration of 0.5% every 24 h to maintain induction. After 72 h with methanol, the culture supernatant was separated from the cells by centrifugation as above and used for protein purification in further experiment. The collect cells were analyzed for determination of gene copy number by real time qPCR.

Enzyme activity analysis

Xylanase activity was assayed with 1% soluble birchwood xylan as the substrate at 50 °C for 10 min. Dilutions of the recombinant protein in the culture supernatant were in 50 mM sodium citrate buffer with pH 5.0. A 500 μl reaction mixture contained 100 μl of diluted enzyme and 400 μl of a 1% soluble xylan and was incubated at 50 °C for 10 min. The amount of released sugar was determined by the dinitrosalicylic acid (DNS)[2] method [22]. One unit (nkat) of xylanase activity was defined as the quantity of enzyme that liberated reducing sugar at the rate of 1 nanomole per second.

[2] *Abbreviations used:* DNS, dinitrosalicylic acid; BSA, bovine serum albumin.

Determination of gene copy number by real time qPCR

To determine the copy number of the expression vectors in the select recombinant strains, genome DNA was extracted using a Yeast DNA Extracting Kit (Sangong, China). The quantity of DNA was measured by NanoDrop 2000 (Thermo Fisher, USA). Real time qPCR was carried out following the method described in previous report [23] with the LightCycler 480 instrument (Roche Diagnostics, Switzerland). Since the AOX1 promoter (pAOX1) exists both the pPICZaA plasmid DNA and the *P. pastoris* genome, the pAOX1 copy number, minus one, equals the copy number of the mxyn2 gene. The results were normalized using the GAPDH gene as a single-copy gene, and the untransformed *P. pastoris* X33 strain to normalize the data.

Protein purification and determination of protein concentration

After methanol induction for 72 h, 100 ml supernatant was concentrated by ultrafiltration using a Amicon® Ultra Centrifugal Filter (Millipore, 10 kDa cut off) according to the manufacturer's instructions and subsequent dialysis against 20 mM phosphate buffer solution (pH 6.0) at 4 °C overnight. For further purification, the enzyme solution was loaded onto an SP Sepharose Fast Flow column (GE Healthcare, Sweden) (2.0 cm × 20 cm) equilibrated with 20 mM phosphate buffer solution. Elution was performed with a linear gradient of 0–0.5 M NaCl at a rate of 2 ml/min. The active fractions were pooled and the protein concentration determined by the Bradford method (Bio-Rad, USA) with bovine serum albumin (BSA) as a standard. To determine protein purity, protein samples were analyzed by electrophoresis on a 15% (w/v) SDS–PAGE as described by Laemmli [24] and the proteins in the gel were visualized by staining with Coomassie brilliant blue R-250. The protein bands on the SDS–PAGE gel were quantified with the software Gene Tools from SynGene (Bio-Rad, USA).

Biochemical characterization of the recombinant protein

The temperature optimum was measured by performing the xylanase activity assay at temperatures ranging from 45 °C to 80 °C. Assays at different pH values were performed at the optimum temperature over a pH range of 3.0–8.0. The buffers used were 50 mM citrate buffer (pH 3.0–3.5), 50 mM citrate phosphate buffer (pH 4.0–7.0), and 50 mM phosphate buffer (pH 7.5–8.0), respectively. Thermostability was tested by preincubating the enzyme samples for 10 min at various temperatures (45 °C–80 °C). The residual activity was then measured at 50 °C as described above.

Optimization of expression in shake flask

The strain with the highest productivity of Mxyn2p was chosen for testing to determine the optimal pH and induction temperature for Mxyn2p expression. The selected strain was cultured in BMGY, as above, and similarly shifted to BMMY for expression induction 28 °C, 18 h at 250 rpm. Cells were harvested and re-suspended in BMMY medium for expression induction. For the purpose of determining the optimum pH value for expression, BMMY medium was adjusted to different initial pH value (4.5, 5.0, 5.5, 6.0, and 6.5). Methanol was added to a final concentration of 0.5% every 24 h to maintain induction. The culture was collected and centrifugated after induction for 72 h. The resulting supernatant from culture of different pH value was tested for enzyme activity. To optimize induction temperature, induction was performed under three temperature (28 °C, 25 °C, and 22 °C), respectively. After induction for 72 h, enzyme activity of recombinant Mxyn2p in the three resulting supernatant was measured with the method mentioned above.

Please cite this article in press as: Y.-y. Li et al., High-level expression and characterization of a thermostable xylanase mutant from *Trichoderma reesei* in *Pichia pastoris*, Protein Expr. Purif. (2014), http://dx.doi.org/10.1016/j.pep.2014.11.014