Protein Expression and Purification 124 (2016) 55–61

Contents lists available at ScienceDirect

Protein Expression and Purification

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

Cloning and high-level expression of β-xylosidase from *Selenomonas ruminantium* in *Pichia pastoris* by optimizing of pH, methanol concentration and temperature conditions

Ehsan Dehnavi [a], Seyed Omid Ranaei Siadat [b, c, **], Mehrnoosh Fathi Roudsari [d], Khosro Khajeh [a, *]

[a] Department of Biochemistry, Faculty of Biological Science, Tarbiat Modares University, Tehran, Iran
[b] Nanobiotechnology Engineering Laboratory, Faculty of Engineering and New Technologies, Shahid Beheshti University, GC, Tehran, Iran
[c] Protein Engineering Laboratory, Protein Research Center (PRC), Shahid Beheshti University, GC, Tehran, Iran
[d] National Institute of Genetic Engineering and Biotechnology [NIGEB], Tehran, Iran

ARTICLE INFO

Article history:
Received 27 May 2014
Received in revised form
28 April 2016
Accepted 2 May 2016
Available online 3 May 2016

Keywords:
β-xylosidase
Pichia pastoris
Heterologous expression
Optimization

ABSTRACT

β-xylosidase and several other glycoside hydrolase family members, including xylanase, cooperate together to degrade hemicelluloses, a commonly found xylan polymer of plant-cell wall. β-D-xylosidase/α-L-arabinofuranosidase from the ruminal anaerobic bacterium *Selenomonas ruminantium* (SXA) has potential utility in industrial processes such as production of fuel ethanol and other bioproducts. The optimized synthetic SXA gene was overexpressed in methylotrophic *Pichia pastoris* under the control of alcohol oxidase I (AOX1) promoter and secreted into the medium. Recombinant protein showed an optimum pH 4.8 and optimum temperature 50 °C. Furthermore, optimization of growth and induction conditions in shake flask was carried out. Using the optimum expression condition (pH 6, temperature 20 °C and 1% methanol induction), protein production was increased by about three times in comparison to the control. The recombinant SXA we have expressed here showed higher turnover frequency using ρ-nitrophenyl β-xylopyranoside (PNPX) substrate, in contrast to most xylosidase experiments reported previously. This is the first report on the cloning and expression of a β-xylosidase gene from glycoside hydrolase (GH) family 43 in *Pichia pastoris*. Our results confirm that *P. pastoris* is an appropriate host for high level expression and production of SXA for industrial applications.

1. Introduction

Hemicellulose, a heterogeneous polysaccharide found in plants and cell wall of some microorganisms, is the second most abundant organic biopolymer in nature [1]. Biodegradation of xylan polymers as lignocellulosic components of cell wall needs a cocktail of hydrolytic enzymes. Cooperation of the glycoside hydrolase family, including endo-xylanase (EC.3.2.1.8) and β-xylosidase (EC.3.2.1.37), with accessory enzymes like α-L-arabinofuranosidase, α-glucoronidase and other enzymes results in biodegradation of such polymers [2]. According to Carbohydrate-Active enZYmes (CAZY) database, β-xylosidases have been classified into glycoside hydrolase (GH) families 3, 30, 39, 43, 51, 52 and 54 based on their protein sequences (http://www.cazy.org). Among these families, GH 43 have a distinct feature. The active site of the β-xylosidases and arabinofuranosidases are highly conserved, consistent with the observation that β-xylosidases and some arabinofuranosidases are capable of also cleaving α-arabinofuranosyl and β-xylopyranosyl linkages, respectively [3].

β-D-xylosidase/α-L-arabinofuranosidase from the ruminal anaerobic bacterium *Selenomonas ruminantium* (SXA) is a bifunctional glycoside hydrolase enzyme which simultaneously shows β-D-xylosidase and α-L-arabinofuranosidase activities. With respect to protein sequence similarity, SAX is assumed as a member of glycoside hydrolase family 43 (GH43) [4]. This enzyme has potential utility in industrial processes such as the production of ethanol fuel and other bioproducts [5].

* Corresponding author. Department of Biochemistry, Faculty of Biological Science, Tarbiat Modares University, Tehran, Iran.
** Corresponding author. Nanobiotechnology Engineering Laboratory, Faculty of Engineering and New Technologies, Shahid Beheshti University, GC, Tehran, Iran.
E-mail addresses: o_ranaei@sbu.ac.ir (S.O. Ranaei Siadat), khajeh@modares.ac.ir (K. Khajeh).

http://dx.doi.org/10.1016/j.pep.2016.05.001
1046-5928/

Nowadays, yeasts have been developed as host systems for the industrial production of heterologous proteins. Significant advantages of yeasts, such as easy genetic manipulation, fast growth rate and post-translational modification, has ranked them among the most popular expression hosts [6]. One of the most commonly used yeast hosts is *Pichia pastoris*. This methylotrophic yeast contains a copy of alcohol oxidase I (AOX1) promoter, which is highly controllable for inducible expression of recombinant proteins. The strong AOX promoter is induced by methanol and repressed in the presence of glucose. Another important feature of *Pichia pastoris* is its ability to achieve high cell densities, enabling efficient protein production [7].

Here, we describe cloning, expression and characterization of recombinant β-xylosidase from *Selenomonas ruminantium* in *Pichia pastoris* host. In this study we have tried to optimize the expression of SXA gene in *P. pastoris.* The extracellular secretion and biochemical characterization of recombinant enzymes was also investigated.

2. Materials and methods

2.1. Strains, medium, chemicals and plasmids

Escherichia coli strain DH5α was used for cloning and propagation of plasmids. *Pichia pastoris* strain GS115 and pPinkα-HC expression vector were obtained from Invitrogen (USA). PCR reagents were prepared from Sinaclon Bioscience (Iran). All restriction enzymes, DNA marker and T4 DNA ligase enzyme were purchased from Vivantis (Tehran, Iran). Protein markers prepared from Vivantis and Thermo scientific companies.

E. coli cells were grown in Luria and Bertani (LB) medium at 37 °C with 200 rpm shaking. Yeast extract peptone dextrose (YPD) medium and buffered glycerol-complex (BMGY) medium were used for culture of *Pichia pastoris.* Gene expression was induced in Buffered Methanol-complex (BMMY) medium. All yeast mediums were prepared from Invitrogen. Temperature of 30 °C and 300 rpm shaking was used as the optimum condition for growth of *Pichia*. Unless stated, standard DNA methodologies were carried out according to the Sambrook manual of Molecular Biology [8].

2.2. Codon optimization and gene synthesis

The protein sequence of SXA gene from *Selenomonas ruminantium* (Accession No. ABA97967.1) was used for synthetic gene preparation. The codon usage was adapted to the codon preference of *P. pastoris* genes using NCBI-related database at http://www.kazusa.or.jp/codon, according to 30 native genes known to be highly expressed in *P. pastoris* [Table S1 from supplementary material]. Furthermore, mRNA secondary structures which might reduce translation efficiency, AT-rich and GC-rich regions and cryptic splice sites were removed and finally the GC content was adjusted to 46%. The optimized sequence was synthesized by GeneArt Company (Germany) (GenBank Accession No. JF193553.1) and cloned into pMA plasmid named pMA-SXA.

2.3. Construction of expression vector and transformation into *Pichia pastoris*

The synthetic SXA gene was flanked by *Xho*I and *Kpn*I restriction sites at the 5′ and 3′ ends, respectively. The gene was subcloned into the *Xho*I/*Kpn*I cleavage sites of pPinkα-HC expression vector, placing under the control of the AOX1 promoter. The desired gene was cloned downstream of α-mating factor secretion signal originating from the yeast *Saccharomyces cerevisiae* [9]. The ATG start codon of the SXA gene was inserted immediately after the last codon of signal peptide. The TAG stop codon was used at the end of SXA gene.

Recombinant pPinkα-SXA was linearized by *Bsp*T1 restriction enzyme to increase the efficiency of integration into the TRP2 locus of *P. pastoris*. The linearized pPinkα-SXA was transformed into *P. pastoris* GS115 by electroporation (BTX, ECM630 at 1800 V, 200 Ω and 25 μF) following Invitrogen protocol. After transformation, the cells were spread on PAD plates (*Pichia* Adenine Dropout) and incubated at 30 °C for 3–7 days. Grown *P. pastoris* colonies on selection plates were used for colony PCR using specific SXA gene primers (Forward: 5′-TCTCTCGAGTCAAAGGCAACAAACTGT-3′ and Reverse: 5′-GGTACCGCATGCCTACTACTTTCTT-3′). The following program was used for PCR: 5 min at 95 °C, 30 cycles of 1 min at 95 °C, 1 min at 60 °C, and 90 Sec at 72 °C; and 1 cycle of 5 min at 72 °C.

2.4. Expression of recombinant SXA and deglycosylation analysis

Single colonies of the positive transformants were inoculated in 10 ml BMGY medium and the cultures were incubated at 30 °C for 48 h with constant shaking (300 rpm) until OD600 was reached to 2–5 [10]. For induction, the cells were collected by centrifugation and resuspended in 1 mL BMMY induction medium and allowed to grow at 30 °C. For continuous expression, methanol was added to the flasks every 24 h in order to constantly keep the final methanol concentration at 0.5%. The supernatant was collected by centrifugation and analyzed on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The amount of total protein was determined by Bradford method using bovine serum albumin (BSA) as standard.

Expressed SXA was deglycosylated by Endo H enzyme according to New England Biolabs manual. 1 mg of protein dissolved in citrate buffer (pH 4.8) was denatured by boiling for 10 min. Then it was treated with 0.1 U Endo H for 1 h at 37 °C and analyzed on 5% stacking and 10% resolving SDS-PAGE.

2.5. Purification of recombinant SXA protein

After four days expression of SXA in shake flask, the 50 ml crude supernatant was obtained by centrifugation of the culture at 3000 g for 5 min at 4 °C. Then recombinant protein was concentrated 10-fold by ammonium sulfate precipitation (ammonium sulfate required for 80% saturation was slowly added to the supernatant at 4 °C). The saturated medium with ammonium sulfate was stirred overnight in cold room and pelleted by centrifugation at maximum speed for 10 min at 4 °C. The resulting pellet was resuspended in 5 ml citrate buffer pH 4.8 (xylosidase assay buffer) and dialyzed against the same buffer at 4 °C. The purification was carried out using size exclusion chromatography on a Sephadex S-200 column (Amersham Pharmacia Biotech, Sweden) previously equilibrated with 50 mM potassium phosphate buffer pH 4.8 containing 150 mM sodium chloride. The fractions were collected at a flow rate of 0.8 ml min^{-1}. Active fractions were combined and used as the purified SXA in the following experiments.

2.6. Characterization of recombinant SXA protein, enzymatic assay and determination of kinetic parameters

Xylosidase activity was determined using artificial choromophoric 4-Nitrophenyl β-D-xylopyranoside (PNPX) substrate (Sigma) based on the release of ρ-nitrophenyl. β-xylosidase activity was assayed using the standard procedure described in the *Pichia* expression kit (Invitrogen, USA). According the standard assay method, β-xylosidase activity was assayed using 1 mM of PNPX in sodium acetate buffer 50 mM (pH 4.8) at 50 °C for 10 min and the