Journal of Molecular Catalysis B: Enzymatic 111 (2015) 29–35

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

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

Screening and characterization of a highly active chitosanase based on metagenomic technology

Heng Li[a,1], Zhong Fei[a,1], Jinsong Gong[a], Tao Yang[a], Zhenghong Xu[a,b], Jinsong Shi[a,*]

[a] School of Pharmaceutical Science, Jiangnan University, Wuxi 214122, PR China
[b] The Key Laboratory of Industrial Biotechnology, Ministry of Education, Jiangnan University, Wuxi 214122, PR China

ARTICLE INFO

Article history:
Received 4 June 2014
Received in revised form 3 November 2014
Accepted 14 November 2014
Available online 24 November 2014

Keywords:
Metagenomics
Fosmid
Chitosanase
Expression
E. coli Rosetta-gami (DE3)

ABSTRACT

A metagenomic fosmid library containing 8×10^4 clones was constructed from a soil sample taken from the stacking place of shrimp and crab shells. 12 clones with chitosanase activity were screened using LB agar plates containing 0.12 wt.% chitosan. Through subsequent construction of a subclone library, 3 chitosanase positive clones were selected. The gene *csn*A, which was expressed in *Escherichia coli* Rosetta-gami, consisted of 834 base pairs encoding 277 amino acid residues. The corresponding recombinant chitosanase CsnA was overexpressed and showed a molecular mass of 31 kDa. The specific activity of CsnA reached 2373 U/mg toward chitosan, with the optimal pH and temperature of 6.0 and 55 °C, respectively. CsnA performed stable activity at a wide pH range from 4.5 to 6.5 and was thermostable at 50 °C and 55 °C. The enzyme is significantly inhibited by various heavy metal ions and SDS, while enhanced by Mn^{2+}, Sr^{2+}, Tweens and Tritons. The values of K_m, V_{max} and k_{cat} for CsnA were 7.29 mg mL^{-1}, 7.48 μmol min^{-1}, and 1.33×10^4 s^{-1}, respectively. This recombinant chitosanase could hydrolyze chitosan efficiently into chitosan dimer, trimer, tetramer, pentamer and hexamer. This study demonstrates that functional screening based on metagenomics is a useful approach for isolating useful enzymes.

1. Introduction

Chitosanase (EC 3.2.1.132), which are generally endo-splitting enzymes, could catalyze the hydrolysis of the β-1,4-glycosidic bond of chitosan [1]. As the degradation products of chitosan, chitooligosaccharides (COSs) have been reported to have various biological activities and applications in gene delivery, drugs, antibacterial and antifungal agents, ingredients for wound dressing and bone strengthening, as well as substances for lowering serum glucose in diabetics [2,3]. Compared with the chemical/physical hydrolytic pathway, the enzymatic hydrolysis was performed under mild conditions without any production of monosaccharides [4,5].

Chitosanase has been found in a wide variety of microorganisms, including bacteria, fungi and yeast [6]. Generally, these chitosanases have all been isolated through the cultivated cultures. However, more than 99% microorganisms are not amenable to cultivation, which limited the exploration of novel enzymes. Metagenomics, a culture-independent approach, may be an appropriate solution to circumvent the problem of utilizing untapped gene resources from uncultured microorganisms [7,8]. Many biocatalysts with either novel sequences or high activities have been screened out and identified with this method, such as chitinolytic enzyme [9], protease [10], oxygenase [11], amylase [12], esterase [13], alginate lyase [14], *etc.* The traditional method of metagenomic library construction involves insertion of small sequences of less than 10 kb into a standard sequencing vector. However, this method was subject to both the omission of large gene clusters or operons and heavy workload for screening [15]. Therefore, it is gradually being replaced by large-insert libraries constructions which employ large cloning vectors of approximately 40–200 kb, like cosmid, fosmids or bacterial artificial chromosomes (BACs). The use of fosmids has found a balance between the requirements for inserts large enough to include complete gene clusters and the relative simplicity of library construction [16].

In this study, a fosmid metagenomic library for chitosanase screening was constructed from soil samples taken from the stacking place of shrimp and crab shells. The soil was rich in chitin and corresponding degraded matters, which was just like a natural medium beneficial to microorganisms with the ability of hydrolyzing chitin and its derivatives enrichment. It then could

* Corresponding author at: School of Pharmaceutical Science, Jiangnan University, 1800 Lihu Avenue, Wuxi 214122, PR China. Tel.: +86 510 85328177; fax: +86 510 85918206.
E-mail address: shijs@163.com (J. Shi).
[1] These authors contributed equally to this article.

http://dx.doi.org/10.1016/j.molcatb.2014.11.005
1381-1177/

be prospected that chitosanases with special properties might be screened out. Through functional screening and subcloning, a gene encoding chitosanse with high activity was isolated. After being overexpressed in *Escherichia coli*, the biochemical properties of the purified enzyme were characterized.

2. Materials and methods

2.1. Bacterial strains, vectors, reagents and enzymes

E. coli JM109 (Novagen, Madison, WI), *E. coli* Rosetta-gami (Novagen, Madison, WI), and *E. coli* EPI300 (Epicentre, Madison, WI) were used as host strains for cloning and expression. Moreover, the pMD 19-T simple vector (Takara, Dalian, China), pET-28a(+) (Novagen, USA), pCC2FOS (Epicentre, Madison, WI) were used as vectors. Restriction enzymes were obtained from Takara Bio Inc. (Dalian, China). Chitosan (deacetylated degree of 80–95%) and other reagents were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Metagenomic DNA extraction and DNA quality examination

The soil samples were taken from the stacking place of shrimp and crab shells. The whole DNA was extracted using Meta-G-Nome DNA Isolation Kit (Epicentre) following the manufacturer's protocols with minor modifications [17]. 10 g soil sample was first dissolved in an extraction buffer (Epicentre) with Tween 20, and centrifuged at 1500 × *g* to remove large debris. The soil pellet was further dissolved in the extraction buffer and centrifuged at 900 × *g*. The supernatant was combined with the previously collected supernatant. After being briefly centrifuged at 900 × *g* for 2 min, the collected supernatant was filtered through eight layers of gauze, 1.2 μm filter membrane and 0.45 μm filter membrane in succession to dissociate particles and collect the microbials. Further purification of the DNA was performed by gel electrophoresis in 1.0% agarose gel with low melting temperature (Takara). Gel electrophoresis was performed at 30 V for 12 h and the DNA fragments of approximately 40–50 kb were then isolated. Metagenomic DNA quality was assessed using agarose gel electrophoresis. The DNA concentration and purity was further analyzed by spectrophotometry with the ratio of A_{260}/A_{280} and A_{260}/A_{230}, respectively.

2.3. Construction of a metagenomic library and screening of chitosanase gene

The metagenomic library was constructed in the following manner using a fosmid vector of pCC2FOS (Epicentre). The purified DNA was end-repaired with END-Repair Enzyme Mix (Epicentre) which caused the DNA to be blunt-ended and 5′-phosphorylated. The blunt-ended DNA was ligated into a pCC2FOS vector (Epicentre). Lambda packaging extracts were then added for ligations, and the infection of *E. coli* EPI300 was performed. *E. coli* transformants were transferred into 96-well microtiter plates and stored at −80 °C. For colonies with chitosanase activity screening, the transformants were plated on LB agar medium with 12.5 μg/mL chloramphenicol addition and 0.12 wt.% chitosan as the substrate. With transparent circle method, colonies were selected as candidates. The positive clones were reconfirmed and subcloned.

The positive clones were cultured at 37 °C in LB media supplemented with 12.5 μg/mL chloramphenicol and autoinduction buffer (Epicentre). The fosmid DNA was purified using the modified alkaline lysis method as previously described [18] and partially digested with the restriction enzyme HindIII and EcoRI to collect 2–6 kb DNA fragments, which were isolated from a 1.0% low melting temperature agarose (Takara). The digested DNA fragments of 2–6 kb were subcloned in pUC18 digested with the corresponding enzymes and introduced into *E. coli* JM109. The transformants were plated onto LB agar plates containing ampicillin (100 μg/mL) and 0.12 wt.% chitosan. After incubation at 37 °C for 24 h, colonies surrounded by a clear halo were selected. The desired clone was picked out and the plasmid it carried was extracted and sequenced at Sangon Biotech Co., Ltd. (Shanghai, China).

2.4. Sequence analysis and the phylogenetic tree built

The signal peptide was identified by SignalP 3.0 server [19] and the open reading frame (ORF) analysis was performed by ORF Finder online tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Analysis of nucleotide and protein sequence homology was conducted with BLAST program at NCBI (http://www.blast.ncbi.nlm.nih.gov/) and DNAMAM. The protein sequences were aligned using CLUSTAL X. Phylogenetic tree indicating relationship of CsnA and other known chitosanases was constructed using neighbor-joining method with MEGA 3.1 [20].

2.5. Gene cloning and expression

The *csn*A gene was amplified without its signal sequence using the plasmids carrying the *csn*A gene from a subclone transformant as the template. The primers used were as following: 5′-GGATCCGCGGGACTGAATAAAGATCAAAA-3′ and 5′-AAGCTTTTATTTGATTACAAAATTACCG-3′ (BamHI and HindIII restriction enzyme sites are underlined). The thermal cycles of denaturation (94 °C, 1 min), annealing (55 °C, 1 min), and polymerization (72 °C, 1 min) were performed for 30 times. The amplified DNA fragment was then ligated with pMD19-T simple vector, and the resultant recombinant plasmid was used to transform *E. coli* JM109. Once the sequence had been confirmed by sequencing, the recombinant plasmid was digested with BamHI and HindIII and inserted into pET-28a(+) which was also digested with the corresponding two enzymes, and the recombinant plasmid was transformed into *E. coli* Rosetta-gami. For overexpression of the recombinant CsnA in *E. coli*, a single colony which harboring the plasmid pET28a-*csn*A was picked after overnight incubation on selection plate with kanamycin (50 μg/mL) and chloramphenicol (25 μg/mL) at 37 °C, and then inoculated into liquid LB medium for amplification culture. When the optical density (OD) at 600 nm reached 0.6, isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added into the culture to a final concentration of 0.5 mM. After an additional 8 h incubation at 28 °C with agitation, the cell mass was harvested by centrifugation at 6000 × *g* for 20 min and washed twice with phosphate buffered saline (pH 7.4).

2.6. Protein purification

The washed cells were resuspended in 20 mL buffer (50 mM PBS, pH 7.4, 0.5 M NaCl) and then disrupted by sonicated on ice and centrifuged (12,000 × *g* for 20 min, 4 °C). The supernatant was filtered with a 0.22 μm filter to remove small particles. A Ni-NTA superflow column (10 mL) and elution buffer containing imidazole (200 mM) were employed for purification. The protein concentration was determined using the method of Bradford protein assay [21].

2.7. Chitosanase activity assay

Standard chitosanase activity was determined using the dinitrosalicylic acid (DNS) method [22]. The reaction mixture consisted of 30 μL of dilute enzyme and 970 μL of 1.0 wt.% chitosan (dissolved in 0.2 M sodium acetate buffer, pH 6.0, which was pre-incubated at 55 °C for 30 min). The reaction was incubated at 55 °C for 10 min.