Heterologous Expression and Efficient Secretion of Chitosanase from *Microbacterium* sp. in *Escherichia coli*

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Abstract A recombinant expression vector, pCT7-CHISP6H, was constructed for the secretory expression of mature peptide of chitosanase (mMschito) from *Microbacterium* sp. OU01. The vector contains several elements, including T7 promoter, signal peptide sequence of *mschito*, 6 × His-tag sequence and *PmaCI* restriction enzyme cloning site. In pCT7-CHISP6H, *mMschito* was fused into signal peptide sequence of *mschito* gene to construct recombinant plasmid pCT7-CHISP6H-mMschito. The recombinant plasmid was transformed into *Escherichia coli* BL21(DE3) and then expressed. The recombinant protein was secreted into the Luria–Bertani broth and the chitosanase activity in supernatant of the culture could reach up to 67.56 U/mL. The rmMschito in the broth supernatant was purified using HisTrapTM FF Crude column and the purified rmMschito was shown to be apparent homogeneity by 12 % SDS–PAGE analysis. Detected by 4700 MALDI-TOF–TOF-MS, the molecular weight of the purified rmMschito was 26,758.1875 and it was consistent

with the predicted molecular weight. Chitosan (degree of deacetylation of 99 %) was mostly hydrolyzed into chitopentaose, chitotriose, and chitobiose by the purified rmMschito.

Keywords Heterologous · Secretion · *Microbacterium* sp. OU01 · Recombinant expression vector · Chitosanase

Introduction

Chitooligosaccharides (COSs) have various applications in physiological, pharmaceutical, agricultural and food fields [1]. One of the most important applications is bioconversion of chitosan into COSs. However, chitosanase activity of most wild type is too low to meet the requirements of industrial production. In addition, purification of chitosanase from the culture broth of wild type is complex and expensive. In previous reports, chitosanases were mostly expressed in the periplasmic space or inclusion bodies in the intracellular space of *Escherichia coli* [2–6]. If recombinant chitosanase can be secreted into culture broth, it can reduce the processing cost. Therefore, it is necessary and significant to obtain an efficient secretory expression vector for production of recombinant chitosanase.

In 2006 and 2007, a strain of *Microbacterium* sp. OU01, was isolated in our lab which could secrete chitosanase and β-D-glucosaminidase at a high level, and identified a chitosanase gene (*mschito*) (GenBank accession no. EF159153) [7, 8]. It has been verified both the *E. coli* OmpA and native signal peptide could be used to direct the secretion of *Bacillus* extracellular enzymes in *E. coli* expression systems [9, 10]. A signal peptide was predicted by online software (SignalP 4.1), in the deduced amino acid sequence of *mschito* gene at the position 1–25. In the

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Fig. 1 Secretory expression plasmid pCT7-CHISP6H

present study, we reported the secretory expression of mMschito under the control of signal peptide of *mschito*.

Materials and Methods

Strains, Vector, Reagents and Enzymes

Escherichia coli host strain TOP10 used for routine plasmid amplification and recombinant expression vector pCR-T7/NT-TOPO-mMschito was maintained in our laboratory [8]. *E. coli* host strain BL21(DE3), ampicillin and IPTG were purchased from Invitrogen (San Diego, CA, USA). Chitosan from shrimp shells was purchased from Sigma-Aldrich. The molecular weight and degree of deacetylation (DDA) were 41×10^4 Da and 95 %, respectively [7]. Various chitosan-oligosaccharides (GlcN)₂₋₆ were purchased from Seikagaku (Tokyo, Japan). In-Fusion HD Cloning Kit, restriction enzymes (*PmaCI*, *NdeI* and *EcoRI*) and Ex Taq DNA polymerase were purchased from Takara (Japan). HisTrapTM FF Crude column (5 mL), HiPrep Desalting column (HiPrep 26/10 Desalting Sephadex G-25

Fine) was purchased from GE Healthcare (Sweden). Other reagents were from Sangon (Shanghai, China).

Construction of Secretory Expression Plasmid pCT7-CHISP6H

As shown in Fig. 1, an expression vector (pCT7-CHISP6H), containing several elements, including T7 promoter, signal peptide sequence of *mschito*, 6 × His-tag sequence and *PmaCI* restriction enzyme cloning site, was constructed. The native signal peptide of *mschito* was inserted in between the restriction enzyme sites for *NdeI* and *EcoRI*. In addition, a restriction enzyme site for *PmaCI* and the 6 × His-tag sequence was also introduced into the expression vector.

Construction of Expression Plasmid pCT7-CHISP6H-mMschito

Based on vector pCT7-CHISP6H and sequence information of *mschito*, two oligonucleotide primers were designed (pCT7-CHISP6H-*mschito*F: CATCACCATCACCCTCCGCCGA AACGGCCGGGA; pCT7-CHISP6H-*mschito*R: AAGCTTC GAATTCACCTAGTTCAGGGAGAACTGG. The underlined 15 bases in both primers are in homology with the ends of the linearized vector pCT7-CHISP6H, pre-cut using restriction enzyme *PmaCI*) to amplify the mature peptide sequence of *mschito*. The plasmid pCR-T7/NT-TOPO-mMschito constructed previously was used as the template [8].

The PCR amplification was performed as follows: 1 circle of 94 °C for 3 min; 35 circles of 94 °C for 30 s, 57 °C for 30 s and 72 °C for 30 s; 1 circle of 72 °C for 10 min. The PCR product was isolated on a 1.2 % agarose gel and purified by PCR purification kit (BBI, USA). According to the In-fusion[®] HD Cloning kit User Manual, the purified PCR product was ligated into the linearized plasmid pCT7-CHISP6H pre-cut with restriction enzyme *PmaCI*. The constructed recombinant plasmid, named pCT7-CHISP6H-mMschito, was then transformed into TOP10 *E. coli* strain and verified by sequencing in BGI (Beijing, China).

Expression, Purification and Electrophoresis of Chitosanase and Protein Assay

The recombinant plasmid pCT7-CHISP6H-mMschito was transformed into *E. coli* BL21(DE3) and the transformants were cultured by incubation overnight at 37 °C with shaking at 250 rpm in 5 mL of Luria–Bertani (LB) broth supplemented with 100 µg/mL ampicillin (LB-Amp). On the subsequent day, 4 mL overnight culture was inoculated into 200 mL fresh LB-Amp and grown until an OD₆₀₀ nm reached 0.6. Then, 0.5 mM IPTG was added to induce

expression at about 24 h. The supernatant was collected by centrifugation at $10,000\times g$ for 30 min at 4 °C. Preparation of cell lysate was done as previously described [8].

One-ninth volume of Buffer A (500 mM $\text{Na}_2\text{HPO}_4\text{--NaH}_2\text{PO}_4$, 3.0 M NaCl, and 100 mM imidazole, pH 7.2) was added the supernatant and mixed. Then, the sample was loaded onto HisTrapTM FF Crude column preequilibrated with 10 column volumes (CVs) Buffer B (50 mM $\text{Na}_2\text{HPO}_4\text{--NaH}_2\text{PO}_4$, 300 mM NaCl, and 10 mM imidazole, pH 7.2). The rmMschito was eluted using a flow rate of 3 mL/min with Buffer C (50 mM $\text{Na}_2\text{HPO}_4\text{--NaH}_2\text{PO}_4$, 300 mM NaCl, and 20 mM imidazole, pH 7.2) and Buffer D (50 mM $\text{Na}_2\text{HPO}_4\text{--NaH}_2\text{PO}_4$, 300 mM NaCl, and 250 mM imidazole, pH 7.2) for 20 CVs.

The purified rmMschito was desalted with HiPrep Desalting column, lyophilized. Protein concentration was estimated by Lowry's method, using bovine serum albumin (BSA) as the standard. The purity of rmMschito was estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using 12 % separating gel and 5 % stacking gel. The molecular weight of the purified rmMschito was detected by 4700 MALDI-TOF–TOF-MS (ABI, USA).

Chitosanase Activity Assay

Chitosan with 95 % DDA was used as the substrate in chitosanase assay. Chitosanase activity was determined by quantitative estimation of reducing sugars produced from chitosan [7]. One unit of chitosanase was defined as the amount of enzyme that could liberate 1 μmol of reducing sugar as GlcN per min under conditions described above.

Characterization of rmMschito and Analysis of Hydrolyzed Products

The end products of enzymatic hydrolysis of chitosan (99 % DDA) with the rmMschito and its oligosaccharides were qualitatively analyzed by thin layer chromatography (TLC) on silica gel 60 aluminium plates according to the method described by Choi et al. [11].

Results

Construction of the Recombinant Plasmid, Expression and Secretion of rmMschito

The presence of signal peptide in the deduced amino acid sequence means that the protein is synthesized as a pre-protein of *Microbacterium* sp. OU01. Based on the secretory expression plasmid pCT7-CHISP6H and the sequence information of mMschito, an expression plasmid pCT7-CHISP6H-mMschito was constructed (Fig. 2). To analyze

Fig. 2 Recombinant expression plasmid pCT7-CHISP6H-mMschito

Fig. 3 Efficiency of different signal peptides directing the secretion of target protein in *E. coli*. a No signal peptide; b Enx-endoxylanase from *Bacillus* sp.; c PelB signal peptide from *Erwinia carotovora*; d OmpA signal peptide from *E. coli*; e Native signal peptide of mschito

the expression and distribution of the mMschito produced in *E. coli* culture, the chitosanase activity in extracellular, cytoplasmic extract and periplasm extract was measured. In the extracellular supernatant, the chitosanase activity could reach up to 67.56 U/mL; however, the chitosanase activity in cytoplasmic extract and periplasm extract was only 10.31 and 22.39 U/mL. The result showed that the secretion of rmMschito by *E. coli* was directed by the infused signal peptide (Sample e in Fig. 3).

To determine the efficiency of different signal peptides directing the secretion of mMschito by *E. coli*, three different signal peptide sequences (Enx-endoxylanase of *Bacillus* sp., PelB of *Erwinia carotovora*, OmpA of *E. coli*) were inserted in between the restriction enzyme sites for *NdeI* and *EcoRI* of the plasmid pCT7-CHISP6H-mMschito,

Fig. 4 SDS-PAGE analysis of the purified rmMschito. Lane M represents protein MW markers, Lane 1 and 2 represent elution of rmMschito purified by affinity chromatography on Ni-NTA agarose with 20 and 250 mM imidazole

separately. The previously constructed plasmid pCR-T7/NT-TOPO-mMschito that no signal peptide was infused was used as the negative control. These four recombinant plasmids were transformed into *E. coli* BL21(DE3) and the transformants were cultured and induced expression as described over. As shown in Fig. 3, under the same conditions, the native signal peptide of *mschito* was more efficient for directing rmMschito into extracellular supernatant than others. In addition, no chitosanase activity was detected in the extracellular supernatant of transformant containing plasmid pCR-T7/NT-TOPO-mMschito (Fig. 3). Therefore, it is necessary to combine a signal peptide when construct a secretory expression plasmid and the signal peptide sequence of *mschito* may be a good candidate.

Purification of rmMschito and Measurement of Molecular Weight

During the purification process using HisTrapTM FF Crude (5 mL) column (GE Healthcare), pH and ionic strength of extracellular supernatant was adjusted by adding Buffer A (500 mM Na₂HPO₄–NaH₂PO₄, 3.0 M NaCl, and 100 mM imidazole, pH 7.2) to make them consistent with the pre-equilibrated Buffer B. The purified rmMschito was run on 12 % SDS–PAGE gel (Fig. 4). Only one band was shown on the gel and its size was estimated about 27.0 kDa by SDS–PAGE referring to a size marker, which was consistent with the predicted molecular weight of mMschito. Furthermore, the molecular weight of the purified rmMschito was detected by 4700 MALDI-TOF–TOF-MS

(ABI, USA) and a peak was produced at mass (*m/z*) of 26,758.1875 which is consistent with peptide mass MH⁺ (26,757.9974) of mature chitosanase and six infused histidines calculated by Ion Fragmentation Calculator of Date ExplorerTM Software (Fig. 5).

Characterization of rmMschito and Analysis of Hydrolyzed Products

The rmMschito in this study was similar to the characterization of the rmMschito from inclusion body of our previous report [8]. The products of enzymatic hydrolysis of chitosan (degree of deacetylation of 99 %) were analyzed by TLC after incubation with rmMschito at 40 °C (Fig. 6). After complete hydrolysis, the products produced were predominantly chitopentaose, chitotriose and chitobiose.

Discussion

Several enzymes, including cellulase, pepsin, lipase A, and chitosanase, have the ability to hydrolyze chitosan into COSs, however, chitosanase is probably the most efficient because of its specific activity and because the nature of the hydrolysates can be controlled [12]. At present, there were many reports about heterologous expression of chitosanases in *E. coli* [2–4, 6, 8, 13, 14]. In these reports, most chitosanases were from *Bacillus* except our previous report about chitosanase gene from *Microbacterium* sp. OU01 [8]. It has been verified that both *E. coli* OmpA and native signal peptide could be used to direct the secretion of *Bacillus* extracellular enzymes in *E. coli* expression system [9, 10]. It is the first report that the signal peptide of chitosanase from *Microbacterium* sp. OU01 can be recognized by *E. coli* secretion machinery and be a guideline for recombinant chitosanase secreted into the culture supernatant. The absence of the signal peptide was confirmed by analyzing the data MALDI-TOF–TOF-MS and the deduced amino acid sequence of mMschito. With native signal peptide of chitosanase from *Microbacterium* sp. OU01, the yield from culture supernatant is much higher than that with *E. coli* OmpA, *Bacillus* Enx-endoxylanase, *E. carotovora* PelB. In previous reports, chitosanases were mostly expressed in the periplasmic space or inclusion bodies in the intracellular space of *E. coli* [2–6, 8]. Chitosanase gene from *Bacillus subtilis* 168 was recombinantly expressed in the secretion mode with *E. coli* OmpA signal peptide in *E. coli* [13]. Chitosanase gene from *B. subtilis* V26 was cloned into the pBAD vector and expressed in *E. coli* in the secretory mode [2]. Our result showed that the expression yield of rmMschito in extracellular supernatant is the highest among in extracellular supernatant, cytoplasmic extract and periplasm extract.

Fig. 5 Detection of the purified rmMschito by 4700 MALDI-TOF-TOF-MS

Fig. 6 TLC analysis of hydrolysis products of chitosan by rmMschito. Lane 0 standard mixture of (GlcN)₂₋₆; Lane 1, 2, 3, 4, 5 0.1 mL chitosan incubated with rmMschito (1.2 U) for 5, 15, 30, 45 min and 1 h

Since *Bacillus* chitosanase is an extracellular bacterial enzyme, the gene encoding mature enzyme from *B. subtilis* 168 was fused with OmpA signal peptide for efficient secretion using *E. coli* expression system by Pechsrichuang et al. [13]. Their research showed that the OmpA signal peptide for secretion expression was much better than the native signal peptide, which was different from our result. Therefore, it is important to select an efficient signal peptide for secretion expression in *E. coli* expression system. Kilani-Fek et al. constructed the recombinant expression plasmid pBAD-csnV26 containing a chitosanase gene from *B. subtilis* V26 for its heterologous expression in *E. coli* and found the recombinant chitosanase has a significant effect on the mycelium structure of two *Rhizopus* species [2]; however, they did not report the composition of the hydrolyzed product. In this work, the purified rmMschito could hydrolyze colloidal chitosan into a biofunctional oligomer predominantly chitopentaose, chitotriose and chitobiose. Therefore, the secretory expression of mMchito makes the enzyme as a good candidate for the

biotechnological applications in the industrial production of functional COSs.

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