Carbohydrate Research 343 (2008) 3001–3004

Contents lists available at ScienceDirect

Carbohydrate Research

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

Note

Expression, purification and characterization of *endo*-type chitosanase of *Aspergillus* sp. CJ22-326 from *Escherichia coli*

Songlin Li [a], Liang Chen [b], Chen Wang [b], Wenshui Xia [a,*]

[a] State Key Laboratory of Food Science and Technology, School of Food Science and Technology, Jiangnan University, Lihu Road 1800, Wuxi, 214122 Jiangsu, PR China
[b] Laboratory of Molecular Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Yue Yang Road 320, Shanghai 200031, PR China

ARTICLE INFO

Article history:
Received 11 July 2008
Received in revised form 26 August 2008
Accepted 31 August 2008
Available online 9 September 2008

Keywords:
Aspergillus
endo-Type chitosanase
High chitosanolytic activity
Gene cloning
His-Tag purification

ABSTRACT

An *endo*-chitosanase gene was cloned from *Aspergillus* sp. CJ22-326 and expressed in *Escherichia coli*. The purified protein showed an *endo*-chitosanase activity during viscosimetric assay and TLC analysis. The enzyme had higher chitosanolytic activity than previously reported fungal chitosanases.

Chitosanases (EC 3.2.1.132) are glycosyl hydrolases that catalyze the hydrolysis of β-1,4-glycosidic bonds of chitosan, which is the partly acetylated or nonacetylated counterpart of chitin.[1] Chitosan and its partially hydrolyzed form, chitooligosaccharides, have received much attention because of their many applications in function foods, medical aids, and agricultural agents.[1–4] Until now, chitosanolytic enzymes, which are divided into *endo*- and *exo*-chitosanases, have been reported from many microorganisms.[5–11]

We previously reported that a chitosanase was produced by *Aspergillus* sp. CJ22-326, which was found to secrete an *exo*-β-D-glucosaminidase and an *endo*-type chitosanase (csn).[11] Here, we report the gene cloning, expression, purification, and characterization of the csn from CJ22-326.

According to 18s rDNA partial base sequencing, CJ22 is mostly closely related to the species of *Aspergillus* with a similarity of more than 98%. We identified the strain as a member of the genus *Aspergillus*.[12] And it is not same with *Aspergillus fumigatus* according to the shape of the spores.[11] Therefore, CJ22 belongs to a new species of *Aspergillus*. With the purpose of obtaining a chitosanase hyperproducing mutant, parent strain (CJ22) was subjected to mutagenesis with UV and ^{60}Co. Then CJ22-326 was isolated, and its ability to produce chitosanase increased 1.54 times compared with the parent strain. The mycelia of CJ22-326 were used as a source for gene cloning.

In order to clone csn gene, the PCR primers (degenerated primers) were made according to NCBI by using BLAST search of the GenBank Database. Both 5′- and 3′-rapid amplification of cDNA Ends (RACE) and end-to-end PCR were employed to isolate a full-length cDNA from the PCR fragment. Its open reading frame (ORF) consisted of 717 bp sequences. And the deduced amino acid sequence was 95% identical to that of *Neosartorya fischeri* NRRL 181 (GenBank Accession No. EAW25580), however, only 40% identical to that of *A. fumigatus* A1163 (GenBank Accession No. EDP47847). The encoding sequence of csn of *Aspergillus* CJ22-326 was registered in the GenBank, and the GeneBank Accession number is EU302818.

As shown in Figure 1A, a protein with a molecular weight of 30 kDa was expressed upon induction. The identity of this band as the recombinant protein was demonstrated by Western blot assay using anti-His antibody to the His-Tag (Fig. 1B). There were both cytoplasmic proteins and heterologous His-Tag-csn expressed protein in the lysate. However, the major recombinant protein remained located in the insoluble fraction as inclusion bodies after cellular disruption (data not shown).

After a treatment with a highly denaturing buffer (regeneration buffer I), for dissolution of the aggregated recombinant protein, the protein could be applied to the Ni^{2+}–NTA column[13] (Fig. 2, lane 1). Then the targeted protein was eluted from the column under a

* Corresponding author. Tel./fax: +86 510 85329057.
E-mail address: Xiaws@jiangnan.edu.cn (W. Xia).

0008-6215/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2008.08.032

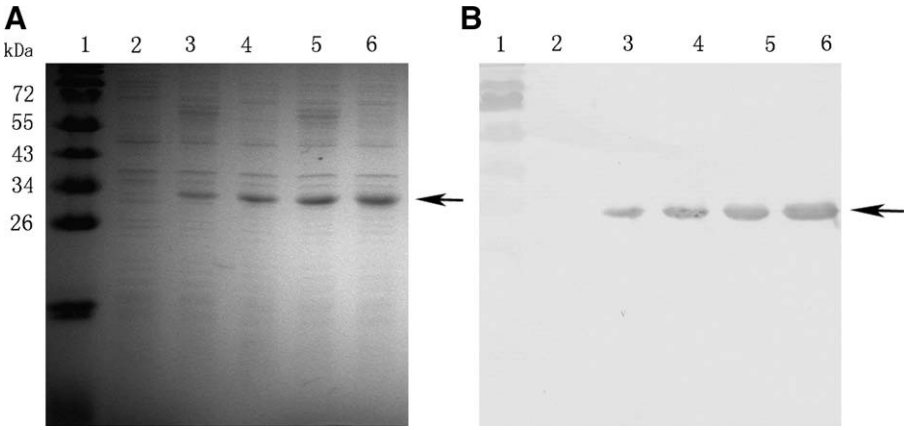

Figure 1. Expression and localization of the His-Tag csn. (A) 15% SDS–PAGE and (B) Western blot of the whole cell lysate before induction (lane 2) and after 1, 2, 3, and 4 h induction (lanes 3–6, respectively). Lane 1, protein molecular weight marker.

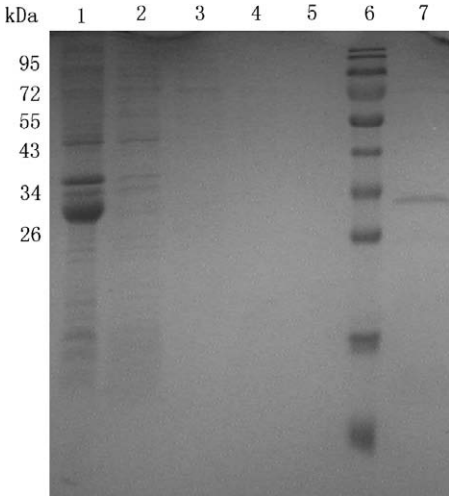

Figure 2. Purification of the recombinant csn. 15% SDS–PAGE corresponds to fractions obtained by Ni–NTA column: lane 1, crude soluble recombinant csn; lane 2, flow-through; lanes 3–5, sequential column washings; lane 6, protein molecular weight marker; lane 7, elution of recombinant csn from the column.

linear gradient of 20–300 mM imidazole in buffer III. A summary of the purification steps and protein yield is presented in Table 1.

Chitosanolytic activity of CJ22-326 (1.18 U/mL) was higher than that of previously reported chitosanases from *Mucor rouxii, Fusarium solani* and three different species (*oryzae*, *sojae*, and *flavus*) of the genus *Aspergillus* (Table 2).[14] As shown in Table 3, His_6-csn showed activities toward chitosan with different degrees of deacetylation (DDA), but no activity toward colloidal chitin and carboxymethyl cellulose (CMC).

Table 1
Summary of purification of His_6-csn from *E. coli*

Purification step	Total protein[a] (mg)	Step yield (%)	Overall yield (%)	Total activity[b] (U)
Crude extract	81	100	100	–
Ni–NTA	7.5	9.3	9.3	–
Ultra-filtration	2.3	30.7	2.8	1.18

[a] Total protein was isolated from a 1 L culture.
[b] One unit of enzyme was defined as the amount of enzyme required to produce 1 μmol of reducing sugar as glucosamine per minute.

His_6-csn reduced the viscosity of chitosan solution drastically at an early stage of the reaction (Fig. 3). The hydrolysates of chitosan by His_6-csn were analyzed by thin layer chromatography (TLC). Chitosan was hydrolyzed to chitotriose and chitotetraose predominantly (Fig. 4). The results above suggest that His_6-csn is an endo-type enzyme, which catalyzes an endo-type cleavage of chitosan.

The optimum pH value of His_6-csn (pH 6) was different from that of csn of *A. oryzae* IAM2660[14] (pH 5.5), *A. fumigatus* KH-94[15] (pH 5.5), and *Aspergillus* sp. Y2K[16] (pH 6.5). His_6-csn showed an optimum temperature of 65 °C, comparable to that of csn of *A. fumigatus* KH-94 (70–80 °C), and *A. oryzae* IAM2660 (50 °C). The kinetics of recombinant chitosanase displayed typical Michaelis–Menten behavior. The reaction rate was calculated, and the K_m and V_{max} values were 0.826 mg/mL and 0.094 mg/mL min, respectively.

In conclusion, the cDNA encoding csn from *Aspergillus* mutant CJ22-326 was cloned, expressed, and its product was purified from E. coli. Chitosanolytic activity of the recombinant protein was much higher than that of previously reported fungal recombinant chitosanases.

Table 2
Activity of various fungal chitosanase towards different chitosan substrates

Strains	CJ22-326	*M. rouxii*	*F. solani*	*A. oryzae* IAM 2660	*A. sojae* 703	*A. flavus* IFO 6343
Maximum activity (U/mL)	1.18	0.0002	0.0015	0.05	0.012	0.026
Substrate	Chitosan (100% DDA)	Chitosan (100% DDA)	Chitosan (70% DDA)	Chitosan (70% DDA)	Chitosan (100% DDA)	Chitosan (100% DDA)

Table 3
Substrate specificity of His_6-csn

Substrate	Chitosan (95% DDA)	Chitosan (83% DDA)	Chitosan (70% DDA)	Chitosan (60% DDA)	Colloidal chitin	CMC
Relative activity (%)	100.0	76	58	37	0	0

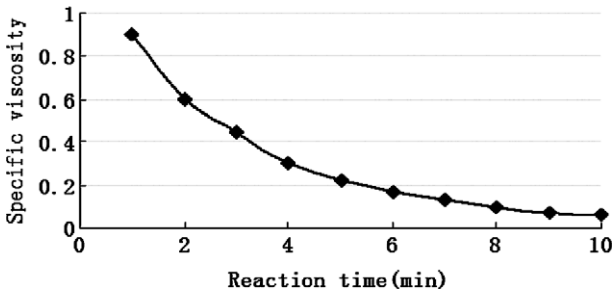

Figure 3. Reduction in viscosity of chitosan solution by His_6-csn. The reaction mixture contained 0.1% chitosan as the substrate. The flow time of the mixture was measured at interval times. Specific viscosity = the flow time of the reaction mixture/the flow time of distilled water.

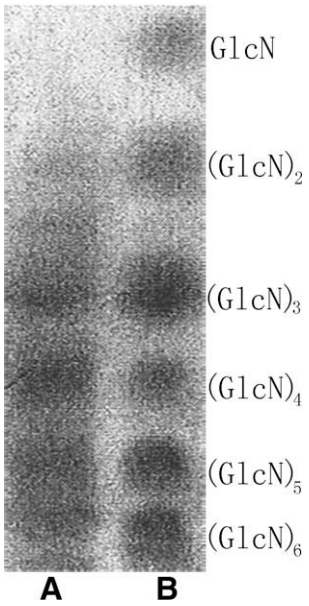

Figure 4. Analysis of enzymatic hydrolysates by TLC. The reaction was done at 37 °C overnight. Lane A, reaction products from the recombinant csn using 95% deacetylated chitiosan as the substrate. Lane B, a mixture of chitooligomers ranging from GlcN to chitohexaose $(GlcN)_6$.

1. Experimental

1.1. Microorganism, plasmid and culture conditions

The strain (CJ22) used in this study was isolated from marine soil in China. The *E. coli* strains DH5a and BL21 (DE3)pLysS were purchased from Invitrogen. Plasmid pET-28a(+) was used for expression. *E. coli* cells were cultivated overnight with vigorous shaking (200 rpm) at 37 °C in Luria-Bertani (LB) medium (1% tryptone,0.5% yeast extract, 1% sodium chloride, with 50 μg/ml kanamycin added when required).

1.2. Analytical techniques

Total RNA was isolated using Trizol reagent (Invitrogen, USA) with a modified Trizol/phenol/chloroform method according to Chomczynski.[17] Chitosanase activity was assayed by estimating the amount of the reducing ends of sugars using the dinitrosalicylic acid (DNS) method.[18] The induced cells were sonicated with at least 10 times short burst of 10 s pulses by intervals of 30 s for cooling, using an ultrasonic processor (Rongshun Instrument Factory, China). Protein concentration was quantified by the method of Bradford.[19] Viscosimetric assay was performed by the method of A. Krikstaponis.[20] The reaction products were analyzed by TLC and the products were detected by spraying on the plate with ninhydrin. Western blotting was performed with primary antibodies anti-His (Santa Cruz Biotechnology) and alkaline phosphatase (AP)-conjugated (Promega) anti-mouse IgG. And the bound antibodies were visualized by using nitroblue tetrazolium (NBT)/BCIP. SDS–PAGE[21] was performed with a 5% stocking gel and a 15% separating gel at 160 V for 1 h. K_m and V_{max} values were determined by Lineweaver–Burk plot.

1.3. Gene cloning

First strand cDNA was synthesized by RT-PCR with $Oligo(dT)_{15}$ using mRNA isolated from CJ22-326. 5′- and 3′-RACE were carried out using the 5′-Full RACE kit and the 3′-Full RACE Core Set Ver. 2.0 kit (TaKaRa, Japan) to obtain the full-length sequence. 3′-RACE reaction was performed using degenerate primers 1 (DP1): TAYGGHATHTGGGGHGAY and 3′-RACE Outer Primer: TACCGTCGTTCCACTAGTGATTT. 5′-RACE Outer PCR was performed using degenerate primers 2 (DP2): YTTYAARTTRTTXGGYAARTCRTA and 5′-RACE Outer Primer: CAT GGCTACATGCTGACAGCCTA. Then 5′-RACE Inner PCR was performed using degenerate primers 3 (DP3): RTCRCARTCDATRTCCATRTT and 5′-RACE Inner Primer: CGCGGATCCACAGCCTACTGATGATCAGTCGATG. And using the sequence of 5′- and 3′-RACE products, end-to-end PCR was performed. A pair of primers: csn-ORF(+), GCGGGATCCATGCGTCTCTCCGAAATT (the underlined sequence is the position of a *Bam*HI site) and csn-ORF(-), GCGCTCGAGCTATGCTTTCAAACCAGC (the underlined sequence is the position of an *Xho*I site) was designed to amplify cDNA encoding fragment. The PCR program was 95 °C for 5 min followed by 36 cycles of 94 °C for 30 s, 58 °C for 45 s, and 72 °C for 1 min, followed by an elongation at 72 °C for 10 min.

1.4. Inducing expression

His_6-csn fusion protein expression plasmid pET28a-His_6-csn was transformed into *E. coli* BL21(DE3)pLysS. Transformants were cultured in LB medium and shaken overnight at 37 °C. Expression of the fused protein was induced by the addition of isopropyl-β-D-thiogalactoside (IPTG). The induced cells were harvested by centrifugation at 6000 rpm for 15 min at 4 °C and analyzed by SDS–PAGE.

1.5. His-Tag purification

The recombinant protein was subjected to His-Tag purification using a gravity column which could be packed with nickle–NTA agarose (Qiagen). After the mixture of csn solution and Ni^{2+}–NTA slurry flowed through it, the column was washed with buffer I (0.5 M NaCl, 8 M urea, 0.02 M NaH_2PO_4, pH 7.5) until the A_{280nm} <0.01. The weakly bound protein was washed away from the column using buffer II (20 mM imidazole, 0.5 M NaCl, 2 M urea, 0.02 M NaH_2PO_4, pH 7.6). Then a linear urea gradient from 8 M to 0 M of refolding buffer (20 mM imidazole, 0.5 M NaCl, 0.02 M NaH_2PO_4, pH 7.6) was used to wash the column for refolding of the bound protein at flow rate of 0.5 mL/min. Finally, the recombinant fusion protein was eluted by a linear gradient of 20–300 mM imidazole in buffer III (0.5 M NaCl, 0.02 M NaH_2PO_4, pH 7.6) at 1 mL/min. Both the flow-through and the eluted fractions were collected and analyzed by SDS–PAGE. The purified His_6-csn was ultrafiltered by Amicon Ultra-4 (10 K) centrifugal devices for removing imidazole.

Acknowledgment

This research was financially supported by NSFC (No. 20876068), PCSIRT0627, 111 project (No. B07029), and State Key Laboratory of Food Science and Technology, Jiangnan University (No.SKLF-MB-200805).

References

1. Arvanitoyannis, I. S.; Nakayama, A.; Abia, S. *Carbohydr. Polym.* **1998**, *37*, 535–541.
2. Smoum, R.; Rubinstein, A.; Srebnik, M. *Bioconjugate Chem.* **2006**, *17*, 1000–1007.
3. Wang, Y.; Zhou, P.; Yu, J.; Pan, X.; Wang, P.; Lan, W.; Tao, S. *Asia-Pac. J. Clin. Nutr.* **2007**, *16*, 174–177.
4. Shahidi, F.; Arachchi, J. K. V.; Jeon, Y. J. *Trends Food Sci. Technol.* **1999**, *10*, 37–51.
5. Zhang, J.; Sun, Y. *Biotechnol. Lett.* **2007**, *29*, 1221–1225.
6. Zhou, W.; Yuan, H.; Wang, J.; Yao, J. *Lett. Appl. Microbiol.* **2008**, *46*, 49–54.
7. Wang, S. L.; Peng, J. H.; Liang, T. W.; Liu, K. C. *Carbohydr. Res.* **2008**, *343*, 1316–1323.
8. Masakazu, I.; Yohane, K.; Kengo, Y.; Jun-Ichi, S.; Takashi, K.; Wataru, O.; Hirofumi, O.; Yasushi, M. *J. Mol. Catal. B: Enzym.* **2007**, *47*, 159–163.
9. Kittur, F. S.; Kumar, A. B.; Gowda, L. R.; Tharanathan, R. N. *Carbohydr. Polym.* **2003**, *53*, 191–196.
10. Alfonso, C.; Martinez, M. J.; Reyes, F. *FEMS Microbiol. Lett.* **1992**, *95*, 187–194.
11. Chen, X. E.; Xia, W. S.; Yu, X. B. *Food Res. Int.* **2005**, *38*, 315–322.
12. *The Genus Aspergillus*, Raper, K. B., Fennel, D. I., Eds.; The Williams and Wilkins Co.: Baltimore, 1965.
13. Abdullah, N.; Chase, H. A. *Biotechnol. Bioeng.* **2005**, *92*, 501–513.
14. Kim, S. Y.; Shon, D. H.; Lee, K. H. *J. Microbiol. Biotechnol.* **1998**, *8*, 568–574.
15. Zhang, X. Y.; Dai, A. L.; Zhang, X. K.; Kuroiwa, K.; Kodaira, R.; Shimosaka, M. *Biosci., Biotechnol., Biochem.* **2000**, *64*, 1896–1902.
16. Cheng, C. Y.; Li, Y. K. *Biotechnol. Appl. Biochem.* **2000**, *32*, 197–203.
17. Chomczynski, P.; Sacchi, N. *Nat. Protoc.* **2006**, *1*, 581–585.
18. Miller, L. *Anal. Chem.* **1959**, *31*, 1321–1326.
19. Bradford, M. M. *Anal. Chem.* **1976**, *72*, 248–254.
20. Krikstaponis, A.; Lugauskas, A.; Krysinska-Traczyk, E.; Prazmo, Z.; Dutkiewicz, J. *Ann. Agric. Environ. Med.* **2001**, *8*, 227–234.
21. Laemmli, U. K. *Nature* **1970**, *227*, 680–685.