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Note

Expression, purification, and characterization of *exo*-β-D-glucosaminidase of *Aspergillus* sp. CJ22-326 from *Escherichia coli*

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ABSTRACT

An *exo*-β-D-glucosaminidase gene was cloned from *Aspergillus* sp. CJ22-326 and expressed in *Escherichia coli*. The purified protein showed an *exo*-chitosanase activity in a viscosimetric assay and TLC analysis. This is the first report on cloning of a gene encoding an *Aspergillus* sp. *exo*-β-D-glucosaminidase.

Chitosanase (EC 3.2.1.132) catalyzes the hydrolysis of the glycosidic bonds of chitosan, and has been found in a variety of microorganisms.[1–7] Chitosan and its partially hydrolyzed form, chitooligosaccharides, as well as glucosamine (GlcN), have received much attention because of their many applications in physiological, pharmaceutical, agricultural, and food fields.[8–12] We previously reported that a chitosanase was produced by *Aspergillus* sp. CJ22-326, which was also found to secrete an *exo*-β-D-glucosaminidase (csx) and an *endo*-type chitosanase.[7] To broaden our understanding of csx, we cloned and characterized the recombinant *exo*-chitosanase.

Aspergillus sp. CJ22-326 is a chitosanase hyperproducing mutant and its mycelia were used as a source for gene cloning.[13] A full-length cDNA was obtained using RT-PCR with Oligo(dT)$_{15}$ and 3′-RACE (rapid amplification of cDNA ends). Its open reading frame (ORF) consisted of 2652 bp and the sequence was deposited in GenBank under position number FJ449570. The nucleotide sequence of CJ22-326 is shown in Figure 1. The deduced amino acid sequence was 93% identical to that of *Neosartorya fischeri* NRRL 181 (GenBank Accession No. XP_001267603), but only 62% and 60% identical to that of *Aspergillus flavus* NRRL3357 (GenBank Accession No. EED55155) and *Aspergillus oryzae* (GenBank Accession No. BAE58464), respectively. To our knowledge, this is the first report on the cloning of a gene encoding *Aspergillus* sp. *exo*-β-D-glucosaminidase.

As shown in Figure 2A, a protein with a molecular weight of 100 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. 2B). The recombinant protein was aggregated mostly as inclusion bodies although these could be disrupted by sonication (data not shown). After purification under denaturing conditions using an Ni^{2+}–NTA column[14] (Fig. 3), the denatured recombinant proteins were refolded by stepwise dialysis through 8, 6, 4, 2, 1, and 0 M urea solutions. A summary of the purification steps and protein yield is presented in Table 1.

When compared to other reported specific activity of *exo*-enzymes, such as *Mucor rouxii*[6] ChA (42.7 U/mg), *A. oryzae* IAM2660[15] ChA (38.8 U/mg), and *Trichoderma reesei* PC-3-7[16] ChA (27.3 U/mg), chitosanase activity of His_6–csx is only 5.8 U/mg. The reasons for this are possibly due to the recombinant protein formed inclusion bodies.

The relative activities of His_6–csx with various substrates are summarized in Table 2. His_6–csx degraded chitosan with a DDA of 83–95%, respectively, but exhibited low activity with chitosan having DDA of 60%. There was no activity toward colloidal chitin and carboxymethyl cellulose (CMC).

His_6–csx reduced the viscosity of a chitosan solution to a small extent (Fig. 4). The hydrolysates of chitosan by His_6–csx were analyzed by thin layer chromatography (TLC). His_6–csx gave only GlcN as a final product (Fig. 5), suggesting an *exo*-type cleavage manner to release a single GlcN residue from the substrate.

The optimum pH value of His_6–csx (pH 4.1) was different from the optimum pH value of csx of *A. oryaze* IAM2660[15] (pH 5.5),

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1 ATGGATTGGATCATCTTGCTGTATGGTCTATATGTGGCGGCCTTTGGCGTGACCGTTGTCGACGGGATCGGCGAGACGGCA
1  M  D  W  I  I  L  L  Y  G  L  Y  V  A  A  F  G  V  T  V  V  D  G  I  G  E  T  A
82 GGGGATACGACCACGCTCAGTCAATGGCGACTGCAGTCTTCCCTTGCGGTGTCCAACGTCTCTGCATGGTCGCTTCCAGGG
28  G  D  T  T  T  L  S  Q  W  R  L  Q  S  S  L  A  V  S  N  V  S  A  W  S  L  P  G
163 AATGACGACTCTTCCTGGTATCAGGTTGGGGCGCGGGCGACCGTCATGGCGGGTTTACTTGAGAACGGCGTCTACGACGAC
55  N  D  D  S  S  W  Y  Q  V  G  A  R  A  T  V  M  A  G  L  L  E  N  G  V  Y  D  D
244 ACGACCCTGTTCTACTCCAACAACATGGAAACCAAAATCGGCGACGCTGCCAAGTTCGACGCGCCTTGGCTATACAGACAT
82  T  T  L  F  Y  S  N  N  M  E  T  K  T  G  D  A  A  K  F  D  A  P  W  L  Y  R  H
325 GAGCTGTTGATCAAGAACAAGATCCGCCAAGGAGAGCATTATCTGCTTCACACGCATGGCATCACCTCCAAGGCGGATATA
109  E  L  L  I  K  N  K  I  R  Q  G  E  H  Y  L  L  H  T  H  G  I  T  S  K  A  D  I
406 TACGTCAACGGGGTGCTGGTTGCACCGAGCTCGGTTCAGCAGGGATCGTACGGCGGTCACACGTACGACATCACAAAATAC
136  Y  V  N  G  V  L  V  A  P  S  S  V  Q  Q  G  S  Y  G  G  H  T  Y  D  I  T  K  Y
487 CTGCGTGTTGACGCAAACGCGCTGCTCATCCATGCGTATCCGACGAACTATCTCCGGGATTTCGCGCAGGGGTTTGTGGAT
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568 TGGAATCCGTACCCAGCGGACAATGGCACCGGTCTATGGCGGGATGTCATGATCTCAAAGACGGGGCCGGTGTCGATCTCG
190  W  N  P  Y  P  A  D  N  G  T  G  L  W  R  D  V  M  I  S  K  T  G  P  V  S  I  S
649 TCGCCGCGGGTACTCACCGACTTCAGAAAGCCTGATGGAAAGCCCGTTACGGTGAATGCTCTCGCGGATGTGACGAACCAT
217  S  P  R  V  L  T  D  F  R  K  P  D  G  K  P  V  T  V  N  V  L  V  D  V  T  N  H
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244  E  D  E  T  V  T  G  T  V  Q  G  S  I  Q  S  D  T  E  S  L  V  L  S  Q  R  F  S
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271  L  Q  P  H  E  T  K  T  I  P  M  T  V  R  I  E  K  P  K  V  W  W  P  A  Q  W  G
892 TCCCAGCCCTTGTACACGGTCAATGTCAATGCGACAGTGGGCAAGGATCAGGTCTCGGATGTTGCGCTTCCAAGGCGGTTT
298  S  Q  P  L  Y  T  V  N  V  N  A  T  V  G  K  D  Q  V  S  D  V  A  L  P  R  R  F
973 GGCATTCGCCACGTAGACTCTCATGTAAATTCTCATAACGACACAGCGTTTACCGTGAACGGCTCGCCATTCCTAGTTCTC
325  G  I  R  H  V  D  S  H  V  N  S  H  N  D  T  A  F  T  V  N  G  S  P  F  L  V  L
1054 GGGGGAGGGTACTCGCCTGATTTGTTTCTCCGGTTCGATGCCAATCGTGTGCGGAATATCTTCCAGTATATGCTCGACATG
352  G  G  G  Y  S  P  D  L  F  L  R  F  D  A  T  R  V  R  N  I  F  Q  Y  M  L  D  M
1135 GGGCTGAACACTGTCCGACTGGAGGGCAAGCAGGAGCATCCAGAGTTATACGATCTGGCAGATGAAATGGGATTGATGGTC
379  G  L  N  T  V  R  L  E  G  K  Q  E  H  P  E  L  Y  D  L  A  D  E  M  G  L  M  V
1216 ATTGCCGGATGGGAGTGTTGCGACAAATGGGAGGGATGGGATGTAAGTTCACTTGCCCGTGCTTCAACAACGCGGAATAC
406  I  A  G  W  E  C  C  D  K  W  E  G  W  D  •  •  •  •  •  •  •  •  •  •  •  •  •
1296 TGACAGGCACAGTACAATGATGAAGCCAGCGGCGAAAAATGGACCGAGAAGGACTACCCCATTGCCAATGCGTCCATGCTC
420  •  •  •  Y  N  D  E  A  S  G  E  K  W  T  E  K  D  Y  P  I  A  N  A  S  M  L
1377 CACGAAGCTGCCATGATGCAGACCCATCCGTCCATGCTAGCCTTTCTAATTGGATCCGATTTTTGGCCCAACGACCGTGCA
444  H  E  A  A  M  M  Q  T  H  P  S  M  L  A  F  L  I  G  S  D  F  W  P  N  D  R  A
1458 ACCAAGATCTACCTCGATGCGCTGCATCGCATGGACTGGCCGGTGCCGATTATCGCGTCCGCCAGCAAACGGGGATACCCT
471  T  K  I  Y  L  D  A  L  H  R  M  D  W  P  V  P  I  I  A  S  A  S  K  R  G  Y  P
1539 AAAGTTCTCGGACCGTCTGGTATGAAGATGGACGGCCCGTATGACTGGGTCCCCCCGAATTATTTCTATGGCGATCAGCTG
498  K  V  L  G  P  S  G  M  K  M  D  G  P  Y  D  W  V  P  P  N  Y  F  Y  G  D  Q  L
1620 GGTGCCGCATTTGGATTCGGGTCAGAAGAGGGTGCTGGGGTGGGAACGCCCGAGTTGACCAGTCTGCGAAAGTTTCTGTCG
525  G  A  A  F  G  F  G  S  E  E  G  A  G  V  G  T  P  E  L  T  S  L  R  K  F  L  S
1701 CCAAAGGACTTGGATTCCTTGTGGAAGAACCCCGATCAGAACCAGTACCATATGTCGCGGTATGACTCGTCCTTCTATAAC
552  P  K  D  L  D  S  L  W  K  N  P  D  Q  N  Q  Y  H  M  S  R  Y  D  S  S  F  Y  N
1782 CGCGCTCTGTATAACAAAGCTCTCTTTGCGCGGTACGGCGCCCCAGGCAGCCTGGAGGATTATCTCCTCAAGGTGCAGATG
579  R  A  L  Y  N  K  A  L  F  A  R  Y  G  A  P  G  S  L  E  D  Y  L  L  K  V  Q  M
1863 ATGGACTATGAGGCCACGCGTGCAGAATTCGAAGCGTTCTCTGTGCGCCAGAATGCCTCCCGTCCGGCCACCGGAGTCATA
606  M  D  Y  E  A  T  R  A  E  F  E  A  F  S  V  R  Q  N  A  S  R  P  A  T  G  V  I
1944 TACTGGATGTTGAATAGCGCATGGCCGAATCTGCACTGGCAGCTGTTTGACTATTATTTGAGTCCTGCAGGGGCATATTTC
633  Y  W  M  L  N  S  A  W  P  N  L  H  W  Q  L  F  D  Y  Y  L  S  P  A  G  A  Y  F
2025 GGAACCAAGATCGGTGGAAGAATGGAACACGTGGCGTATGACTACGAGGAGCGTGCAGTCTACCTGATCAATCGCTCCCTT
660  G  T  K  I  G  G  R  M  E  H  V  A  Y  D  Y  E  E  R  A  V  Y  L  I  N  R  S  L
2106 GAGAAGAAAGGGCCTCGCATCGTCTCTGTTGACCTTGTTGATCGGCATGGCAAATCGCTCTTCAACCAGGAGGTCACGATT
687  E  K  K  G  P  R  I  V  S  V  D  L  V  D  R  H  G  K  S  L  F  N  Q  E  V  T  I
2187 GATACCACACCTACCGCCTCCAAGAGGGTCATCCCAGTCAAGGCCGTTACTCAGATCAAGGACGTCGCATTCCTCCGGCTG
714  D  T  T  P  T  A  S  K  R  V  I  P  V  K  A  V  T  Q  I  K  D  V  A  F  L  R  L
2268 GTGCTCAAGAACCCCAGTACAGGAGCCATCCTCAGCCGCAATGTATACTGGTTGTCCGCGAAGAACGATGTCCTGGACTGG
741  V  L  K  N  P  S  T  G  A  I  L  S  R  N  V  Y  W  L  S  A  K  N  D  V  L  D  W
2349 GAGAATTCAGACTGGTATTACACTCCCGTTACAAAGTACGTCGACTACAAGGCACTGATATCGATGCCGACTGCTGCTGTG
768  E  N  S  D  W  Y  Y  T  P  V  T  K  Y  V  D  Y  K  A  L  I  S  M  P  T  A  A  V
2430 ACTGCATCGCTGAAGCGGCTGCCCGCCAAGGACGGCCTGTCCCAGGTGCAGGTAGTCTTGAAGAATCCTTCAGCCACCCCT
795  T  A  S  L  K  R  L  P  A  K  D  G  L  S  Q  V  Q  V  V  L  K  N  P  S  A  T  P
2511 GCCGTGTTCATGCACCTGTCAGCCATCAACAAGGACACGCAGGAGGAGATCACCCCGGTATTCTGGTCTGATAATTATGTG
822  A  V  F  M  H  L  S  A  I  N  K  D  T  Q  E  E  I  T  P  V  F  W  S  D  N  Y  V
2592 ACTGTCTTCAAGGGCGAGAGTGTGACCCTGACAGCGGCATTCCCGGGAGGTCGAAGTAATTGGGAGGTCATTCTGTCGGGT
849  T  V  F  K  G  E  S  V  T  L  T  A  A  F  P  G  G  R  S  N  W  E  V  I  L  S  G
2673 GCAAATGTGAAGAGAACTCTCTTGTCGTAA
876  A  N  V  K  R  T  L  L  S  *
```

Figure 1. Nucleotide sequence of the csx gene and deduced amino acid sequence of the gene product from *Aspergillus* sp. CJ22-326. The intron is marked with dots.

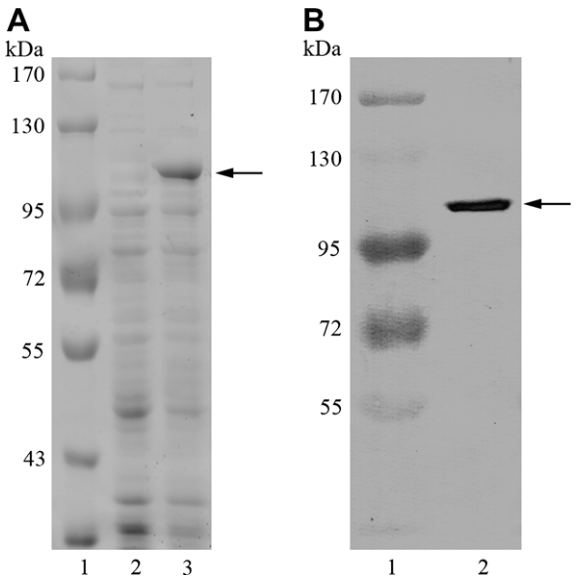

Figure 2. Expression and localization of the His-Tag csx. (A) 7.5% SDS–PAGE: lane 2, before induction; lane 3, after 4 h induction. The amount of protein loaded in lane 1 and lane 2 was both 50 μg. (B) Western blot of the whole cell lysate after induction (lane 2). Lane 1, protein molecular weight marker. The amount of protein loaded in lane 1 was 18 μg.

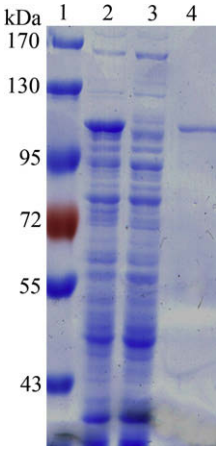

Figure 3. Purification of the recombinant csx. 7.5% SDS–PAGE corresponds to fractions obtained by Ni–NTA column: lane 1, protein molecular weight marker; lane 2, crude soluble recombinant csx; lane 3, flow-through; lane 4, elution of recombinant csx from the column. The amount of protein loaded in lane 2, lane 3, and lane 4 was 60 μg, 60 μg, and 0.5 μg, respectively.

Table 1
Summary of purification of His_6–csx from *E. coli*

Steps	Total activity (U)[a]	Total protein (mg)[b]	specific activity (U/mg)	Overall yield (%)
Crude extract	58.8	49	1.2	100
Ni–NTA	15.7	3.8	4.13	7.8
Ultra-filtration	6.96	1.2	5.8	2.4

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

Aspergillus fumigatus KH-94[17] (pH 4.5–5.5), and *Aspergillus* sp. Y2 K[18] (pH 5.5). His_6–csx showed an optimum temperature of 50 °C, comparable to the optimum temperature of csx of *A. fumigatus* KH-94 (50–60 °C) and *A. oryaze* IAM2660 (50 °C). The kinetics of recombinant chitosanase displayed typical Michaelis–Menten behavior. The reaction rate was calculated and the V_{max} and K_m values were 7.758 mg/mL and 0.146 mg/mL min, respectively. In the case of *A. fumigatus* S-26,[19] V_{max} and K_m values were 7.7×10^{-8} mol/s/mg protein and 1.0 mg chitosan/mL. K_m of *M. rouxii*[6] ChA was 8.13 mg/mL. *Penicillium funiculosum* KY616[20] GlcNase showed V_{max} of 0.81 mM/min and K_m of 1.40 mM for chitobiose, and the values decreased in accordance with an increase in the size of chitooligosaccharides.

Table 2
Substrate specificity of His_6–csx

Substrate	Chitosan (95% DDA)	Chitosan (83% DDA)	Chitosan (70% DDA)	Chitosan (60% DDA)	Colloidal chitin	CMC
Relative activity (%)	100.0	88	80	68	0	0

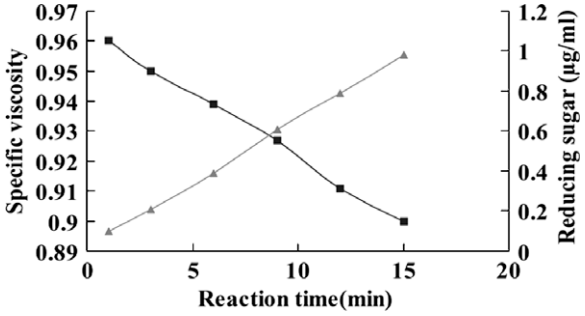

Figure 4. Profiles of viscosity change (■) and reducing sugar production (▲) during incubation of chitosan with His_6–csx. Specific viscosity = (the flow time of the reaction mixture/the flow time of distilled water).

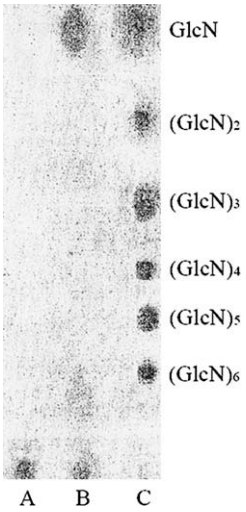

Figure 5. Analysis of enzymatic hydrolysates by TLC. The substrate used was 95% deacetylated chitosan. Lane A , the substrate before hydrolysis; lane B, csx hydrolysates after 24 h at 37 °C; lane C, standard GlcN to $(GlcN)_6$.

In conclusion, we reported the cloning and characterization of a csx gene from *Aspergillus* mutant CJ22-326.

1. Experimental

1.1. Microorganisms and culture conditions

The strain (CJ22) used in this study was isolated from marine soil in China. *Escherichia 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).

1.2. Isolation and screening of chitosanase-producing strains

The basic medium consists of 1.0% soluble chitosan, 2.0% wheat bran, 0.2% $(NH_4)_2SO_4$, 0.2% KH_2PO_4, 0.05% $MgSO_4$, and 2% agar per liter (pH 5.6). The plates were seeded with the irradiated spores and were incubated at 28 °C for 2–3 days, after which clear zones could be observed only around colonies of the mutant strains. This is due to the more immediate hydrolysis of the chitosan in the vicinity of the colonies of the mutant strains, as a result of the greater chitosanase production by these colonies. Those organisms obtained from the screening were subcultured in liquid media and the supernatants were collected for the measurement of chitosanase activity using the procedure described below. The strain CJ22-326 that showed the highest chitosanase activity was isolated, maintained on nutrient agar, and used throughout the study.

1.3. Analytical techniques

Total RNA was isolated with a modified Trizol–phenol–chloroform method according to Chomczynski.[21] Chitosanase activity was assayed using the dinitrosalicylic acid (DNS) method.[22] Protein concentration was quantified by the method of Bradford.[23] Viscosimetric assays were performed by the method of Krikstaponis.[24] 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. Bound antibodies were visualized by using nitroblue tetrazolium (NBT)–BCIP. SDS–PAGE[25] was performed with a 5% stocking gel and a 7.5% separating gel at 160 V for 1 h. To determine the K_m of chitosanase toward the substrate chitosan, 0.2 u/mL His_6–chitosanase was added to 0.45, 0.5, 0.56, 0.63, 0.71, 0.83, 1 mg chitosan (95% DDA), respectively. V_{max} and K_m values were determined by Lineweaver–Burk plot.

1.4. Gene cloning

The first strand of cDNA was synthesized by RT-PCR with Oligo$(dT)_{15}$ using mRNA isolated from CJ22-326 according to manufacturer's protocol (Clotech, USA). The cDNA of CJ22-326 encoding csx was amplified by the 3′-RACE method with the primers 5′-atg gat tgg atc atc ttg-3′ and Oligo$(dT)_{15}$, which were designed based on the sequences of NH_2-terminus and the cDNA encoding csx reported on the National Center for Biotechnology Information (NCBI) and the structure of mRNA, respectively. The first strand cDNAs synthesized above were used as the template. The PCR program was 95 °C for 5 min followed by 30 cycles of 94 °C for 30 s, 57 °C for 45 s, and 72 °C for 3 min, followed by an elongation at 72 °C for 10 min. The resulting PCR fragment was ligated with pMD18-T (Takara, Japan) by using the T/A cloning procedure. The constructed vector was transferred to the component cell *E. coli* JM109. Subsequently a positive clone was obtained. After sequencing, a pair of specific primers: csx-ORF(+), 5′-gcg aag ctt gc atg gat tgg atc atc ttg-3′ (the underlined sequence is the position of an *Hind*III site) and csx-ORF(−), 5′-gcg ctc gag tta cga caa gag agt tct-3′ (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 30 cycles of 94 °C for 30 s, 58 °C for 45 s, and 72 °C for 3 min, followed by an elongation at 72 °C for 10 min.

1.5. Inducing expression and His-Tag purification

His_6–csx fusion protein expression plasmid pET28a–His_6–csx was transformed into the host *E. coli* BL21(DE3) and the positive clone was induced with isopropyl-β-D-thiogalactoside (IPTG) at 16 °C. The induced cells were harvested by centrifugation at 6000 rpm for 15 min at 4 °C and were analyzed by SDS–PAGE. The recombinant csx was purified using Ni^{2+}–NTA column at the denatured conditions and refolded according to our previous method.

Acknowledgments

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