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Characterization of a novel fungal chitosanase Csn2 from *Gongronella* sp. JG

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ABSTRACT

A 28 kDa chitosanase designated as Csn2 was purified from the culture broth of the fungus *Gongronella* sp. JG through three chromatography steps: CM-Sepharose FF, Superdex 200 and SP-Sepharose FF. Its optimal reaction pH and temperature were pH 5.6 and between 55 °C and 60 °C. The half-lives of Csn2 at 50 °C and 55 °C were estimated to be 30 min and 11 min, respectively. The K_m value of Csn2 in sodium acetate buffer (pH 5.6) at 55 °C was 8.86 mg/mL. Mn^{2+}, Ca^{2+} and Sr^{2+} were activators of Csn2; ETDA was an inhibitor. Cu^{2+} stimulated Csn2 at 1 mM, but inhibited Csn2 activity at 10 mM. Csn2 displayed strong activity on colloidal chitosan, but did not hydrolyze colloidal chitin and carboxylmethyl cellulose. Thin layer chromatography analysis showed the end products of colloidal chitosan hydrolyzed by Csn2 were chitobiose, chitotriose and chitotetraose with chitotriose as the major product. The N terminus of Csn2 was determined to be YQLPANLKKIYDSHKSGTC. Part of the genomic DNA sequence corresponding to Csn2 was cloned. Sequence alignment showed DNA sequence of Csn2 was partly identical to chitosanase genes from *Metarhizium anisopliae* var. *acridum*, *Hypocrea lixii* and *Aspergillus fumigatus*. Based on sequence similarity, Csn2 was classified as a GH-75 chitosanase.

1. Introduction

Chitosan is a polysaccharide of β-(1→4)-linked glucosamine (GlcN) residues, partially substituted with *N*-acetyl groups. In recent years, chitosan oligosaccharides have received much attention because they have various biological activities, such as inhibiting the growth of microorganisms, activating immune responses, exerting antitumor activity and preventing negative mineral balance.[1–4] Traditional chemical hydrolysis method for preparing chitosan oligosaccharides is performed at high temperatures and under acidic conditions. This method results in low yields of oligosaccharides because the hydrolysis reaction is difficult to control.[5] Enzymatic processes are an ideal alternative to chemical degradation. Chitosanases (EC 3.2.1.132) catalyze the cleavage of the β-(1→4)-linked glycosidic linkages of chitosan. These enzymes have been found in microorganisms including viruses, bacteria and fungi.[6–19] Compared to chemical hydrolysis, preparation of chitosan oligosaccharides with chitosanases can be preformed under mild reaction conditions and the yield is higher.[5]

Chitosanases have been classified into five glycoside hydrolase families: GH-5, GH-8, GH-46, GH-75 and GH-80 (http://www.cazy.org/fam/acc_GH.html). Among these families, GH-46 chitosanases, especially those from *Bacillus* and *Streptomyces* have been studied extensively in terms of their catalytic features, enzymatic mechanisms and protein structures.[20–23] GH-75 chitosanase is another major chitosanase family, and members belonging to this family are mainly fungal in origin. Cheng et al. explored a GH-75 chitosanase, CSN, produced by *Aspergillus fumigatus*. They concluded that CSN was an inverting enzyme and cleaved linkages of GlcNAc-GlcN and GlcN-GlcN in its substrates. They also found Asp160 and Glu169 are the essential residues for the hydrolysis.[24] However, compared to bacterial chitosanases, catalytic mechanisms of fungal chitosanases remained poorly studied. Therefore, characterization of chitosanases from novel chitosanase-producing fungus is of value in understanding the mechanisms.

Gongronella sp. JG is a novel chitosanase-producing fungus isolated by this group.[25] In our previous study, we found that strain JG produced at least two chitosanase isoenzymes and the major one, Csn1, with molecular weight of 90 kDa, had been purified and characterized.[26] In this work, we purified another chitosanase, Csn2, from stain JG. Its biochemical features were characterized and the corresponding genomic DNA sequence was determined.

2. Materials and methods

2.1. Fungus and chemicals

Gongronella JG was kept on PDA plates at 4 °C. Column chromatography media were purchased from Pharmacia. pMD18-T Vector, restriction endonucleases, LA-Taq and T4 ligase were purchased from Takara (Dalian). Commercial chitosan (85% deacetylated) was used for the culture medium and measurement of chitosanase activity. All other chemicals were of reagent grade. Colloidal

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chitosan was prepared according to the method described by Kurakake et al.[27] with a little modification. Commercial chitosan (1 g, 85% deacetylated) was dissolved in water (10 mL) by adding acetic acid (0.285 mL). Then, the pH was adjusted to 5.5 and water was added to a total volume of 100 mL. Chitosan oligosaccharides mixture was purchased from Seikagaku (Tokyo, Japan).

2.2. Determination of chitosanase activity

Chitosanase activity was assayed using colloidal chitosan as substrate. The reaction mixture consisted of 1.8 mL 1% colloidal chitosan in 50 mM sodium acetate buffer (pH 5.5) and 0.2 mL enzyme solution. The hydrolysis reaction was performed at 55 °C for 30 min and then was terminated by adding 2 mL of dinitrosalicylic acid reagent. After that, the mixture was boiled for 10 min and cooled to room temperature. Insoluble chitosan in the reaction mixture was removed by centrifugation and the resulted adducts of reduced sugars were measured at 540 nm. The amount of chitosanase that produced 1 μmol of reduced sugars per min was taken as one unit; D-glucosamine was used as the calibration standard.

2.3. Production of Csn2

The production medium per liter contained glucose (1 g), glutamic acid (1 g), KH_2PO_4 (1.5 g), $MgSO_4$ (0.2 g), $CaCl_2$ (0.11 g), chitosan (5 g) at pH 6.0. This medium was autoclaved at 118 °C for 30 min. For production of chitosanase, mycelia of strain JG were inoculated into 100 mL production medium in a 250 flask and cultivated at 30 °C and 180 rpm for 72 h.

2.4. Purification of Csn2

Ten liters of culture broth were collected and centrifuged at 8000*g* and 4 °C for 30 min to remove cell debris. The supernatant was concentrated to 200 mL by a Millipore Pellicon ultrafiltration apparatus with membranes of a nominal molecular size cut-off of 3 kDa. The concentrated supernatant was dialyzed against buffer A (20 mM sodium acetate buffer, pH 5.0) and then was subjected to the following chromatographic purification steps: (1) The crude sample was loaded on CM-Sepharose Fast Flow column pre-equilibrated with buffer A. A linear gradient sodium acetate buffer (pH 5.0, from 20 mM to 1 M) was used to elute the chitosanase containing fraction. (2) This fraction was concentrated in an Amicon ultrafiltration stirred cell equipped with a 3 kDa weight cut-off membrane and then was loaded on Superdex 200 gel filtration column pre-equilibrated with buffer B (200 mM sodium acetate buffer, pH 5.0). The same buffer was used to elute the chitosanase containing fraction. (3) The fraction obtained in step 2 was dialyzed against buffer A and further separated by SP-Sepharose Fast Flow column pre-equilibrated with buffer A in the same running condition described in step 1. The purified Csn2 was pooled, dialyzed against buffer A and kept at 4 °C.

2.5. Enzyme characterization

Protein concentration was estimated by the method of Lowry et al.[28] The molecular weight of the denatured Csn2 was determined by SDS–PAGE on 12% polyacrylamide gels according to standard protocols.[29] The optimal pH was tested in sodium acetate buffer with various pH values. The optimal temperature was measured at the range of 30–70 °C. The thermal stability was tested by incubating Csn2 at 50 °C and 55 °C and measuring the residual activity at different time intervals. The effect of additives on Csn2 activity was performed by adding the additives to the reaction mixture to a final concentration of 1 mM or 10 mM and then measuring the enzymatic activity according to the standard enzyme assay. Kinetic studies were performed in sodium acetate buffer (pH 5.6) at 55 °C with colloidal chitosan as substrate.

2.6. Substrate specificity and hydrolysis product

Chitosan or chitosan analogues were used as hydrolysis substrates and the corresponding enzymatic activities were measured using the standard enzyme assay. The activity with colloidal chitosan as substrate was set as 100%. For analysis of the hydrolysis products, a 0.5 mL reaction mixture containing 100 μg chitosan and 10 μg Csn2 was incubated at 45 °C overnight. Hydrolysis products were analyzed by thin layer chromatography (TLC) on Silica G plate. The developing solvent was *n*-butanol–isopropanol–25% aqueous ammonia (3:6:4). To visualize the product, the plate was sprayed with ninhydrin solution (0.2% in ethanol) and heated at 150 °C for 15 min.

2.7. Protein blotting and sequencing

Purified Csn2 was blotted directly from the SDS–PAGE gel onto a polyvinylidene difluoride (PVDF) membrane with a Trans-Blot Semi-Dry Transfer Cell (Bio-Rad). The blotting buffer contained Tris (3.02 g), glycine (14.4 g) and methanol (200 mL/L). The band on the PVDF membrane corresponding to Csn2 was visualized by staining with Coomassie Brilliant Blue R-250. Then the protein band was excised from the PVDF membrane and subjected to Edman degradation. The N-terminal amino acid sequence was determined in Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences.

2.8. Molecular cloning of the genomic sequence

Genomic DNA of strain JG was extracted using the Universal Genomic DNA Extraction Kit (Takara). Genomic DNA (5 μg) was digested with *EcoR* I overnight, then cleaned by alcohol precipitation and ligated into pUC19 plasmid, which was predigested with *EcoR* I. This ligation mixture was used as template for PCR amplification with M13 sequencing primer (CAGGAAACAGCTATGAC) and a degenerated primer [TA(C/T)GA(A/G)(C/T)T(C/G)CC(C/T)GC(C/T)AA(C/T)(C/T)T(C/G)AAGAAGAT(C/T)TA(C/T)G] designed based on the N-terminus sequence of Csn2. PCR was performed with LA-Taq according to the following parameters: denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2 min. The PCR was run for 30 cycles. PCR products were separated by 1% agarose electrophoresis. Each DNA band was excised from the agarose gel and cleaned up. The purified PCR products were ligated into pMD18-T vector and transformed into *Escherichia coli* strain DH5α, respectively. DNA sequence was determined by bidirectional sequencing.

2.9. DNA sequence accession number

The GenBank accession number of Csn2 reported in this article was EU377605.

3. Results

3.1. Purification of Csn2

Strain JG produces at least two chitosanases. Both were inducible enzymes because no chitosanase activity was detected in media that did not contain the inducer, chitosan. Among the isoenzymes, Csn1 contributed much more in total chitosanase activity and it had been purified and characterized in our previous work.[26]

Csn1 and Csn2 were co-eluted in the CM-Sepharose Fast Flow chromatography step (Fig. 1). Remarkable differences in their molecular sizes (90 kDa for Csn1 and 28 kDa for Csn2) resulted in separation of the two proteins in the gel filtration chromatography step. After the SP-Sepharose Fast Flow chromatography step (Fig. 2), about 0.2 mg Csn2 was obtained and it appeared as a single band by SDS–PAGE (Fig. 3). Its apparent molecular weight assessed based on SDS–PAGE was 28 kDa. Because there were at least two chitosanase isoenzymes in the liquid culture of strain JG and they both hydrolyzed chitosan, the yield of Csn2 in liquid culture and after CM-Sepharose Fast Flow purification step was difficult to determine exactly. Also, it was difficult to calculate the purification fold throughout the purification steps.

3.2. Enzymatic properties of Csn2

The optimal pH for Csn2 was tested in sodium acetate buffers with pH values between 4.2 and 5.8. Result showed Csn2 displayed its highest activity at pH around 5.6 (Fig. 4). The optimal reaction temperature was between 55 °C and 60 °C (Fig. 5). Almost no activity was detected at temperatures below 30 °C or above 70 °C. The thermal stability of Csn2 at 50 °C and 55 °C was further investigated. Csn2 was incubated at 50 °C or 55 °C, the residual activity was measured every 5 min and the logarithm of the relative residual activity was plotted against incubation time. The half-lives of Csn2 at 50 °C and 55 °C were estimated to be 30 min and 11 min, respectively. The K_m value of Csn2 in sodium acetate buffer (pH

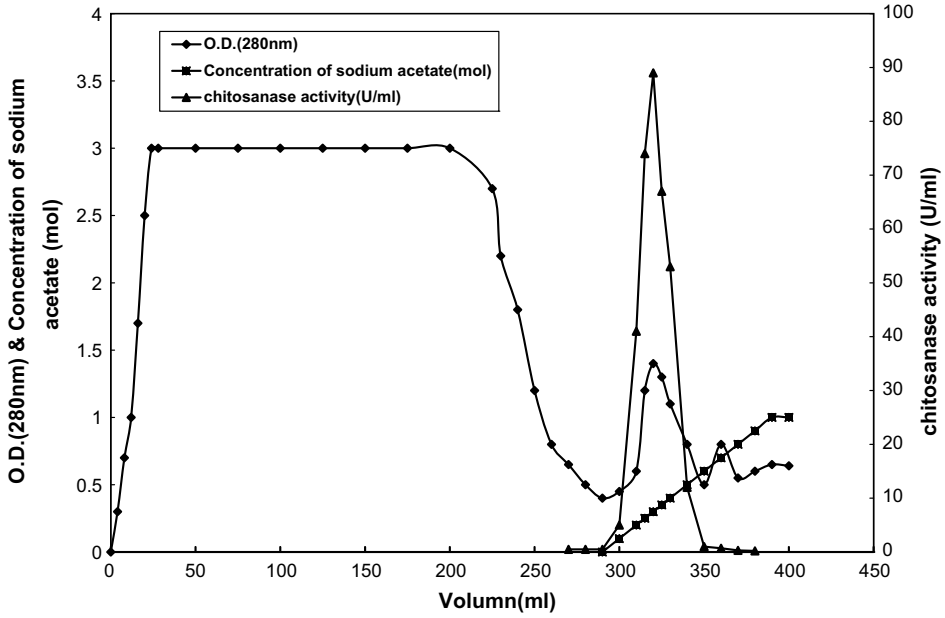

Figure 1. Elution profile of CM-Sepharose FF column.

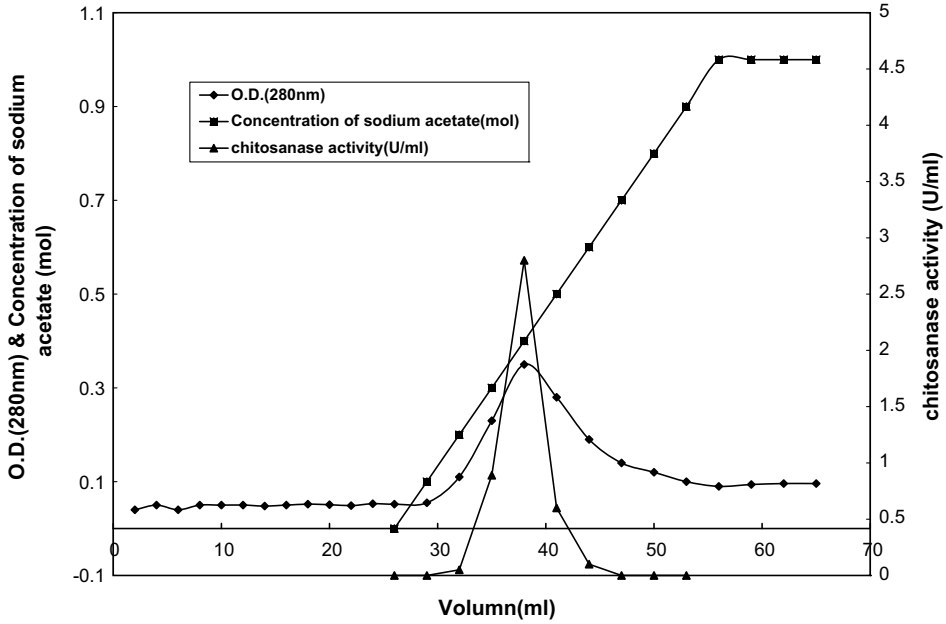

Figure 2. Elution profile of SP-Sepharose FF column.

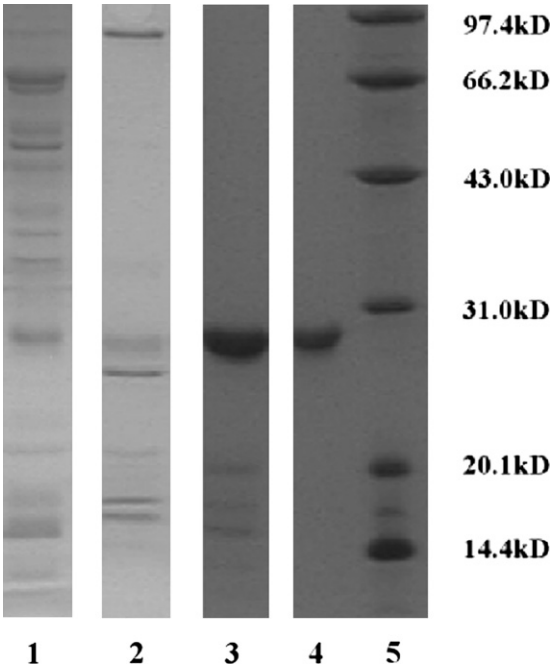

Figure 3. SDS–PAGE analysis. Samples were analyzed on 12% polyacrylamide gels and stained with Coomassie Brilliant Blue R-250. Lane 1: crude extract; lane 2: sample collected from CM-Sepharose FF chromatography; lane 3: sample collected from Superdex 200 chromatography; lane 4: sample collected from SP-Sepharose FF chromatography; lane 5: protein marker.

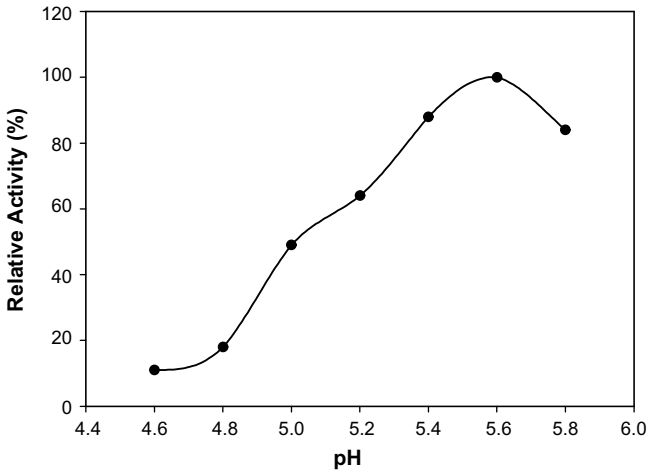

Figure 4. Effect of reaction temperature on Csn2 activity.

5.6) at 55 °C was 8.86 mg/mL. Mn^{2+}, Ca^{2+} and Sr^{2+} were activators of Csn2, and ETDA was an inhibitor. Cu^{2+} stimulated Csn2 at 1 mM, but inhibited Csn2 activity at 10 mM.

3.3. Substrate specificity

The activities of Csn2 upon colloidal chitosan, soluble chitosan, glycol chitosan, colloidal chitin and carboxylmethyl cellulose are presented in Table 1. Csn2 displayed stronger hydrolysis capability against colloidal chitosan than soluble chitosan. No obvious activity was detected when colloidal chitin or carboxylmethyl cellulose was used as the substrate. Csn2 showed weak activity against glycol chitosan, only 4% of the activity for colloidal chitosan.

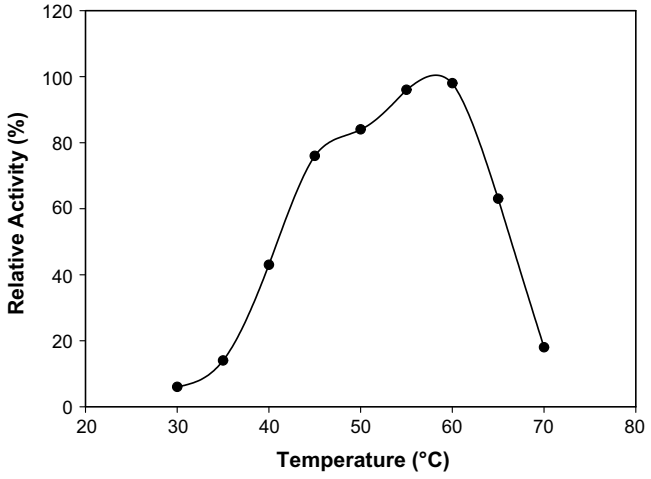

Figure 5. Effect of reaction pH on Csn2 activity.

Table 1
Substrate specificity of Csn2[a]

Substrate	Relative activity (%)
Colloidal chitosan (85% deacetylated)	100
Soluble chitosan (85% deacetylated)	14
Glycol chitosan	4
Carboxylmethyl cellulose	Not detected
Colloidal chitin	Not detected

[a] The activity with colloidal chitosan as substrate was set as 100%.

3.4. Hydrolysis product

The end product of 85% deacetylated chitosan hydrolyzed by Csn2 was analyzed by TLC (Fig. 6). After overnight incubation, $(GlcN)_2$, $(GlcN)_3$ and $(GlcN)_4$ were detected in the hydrolysates with $(GlcN)_3$ as the major product. No GlcN was detected in the hydrolysates, indicating Csn2 was an endo-type chitosanase.

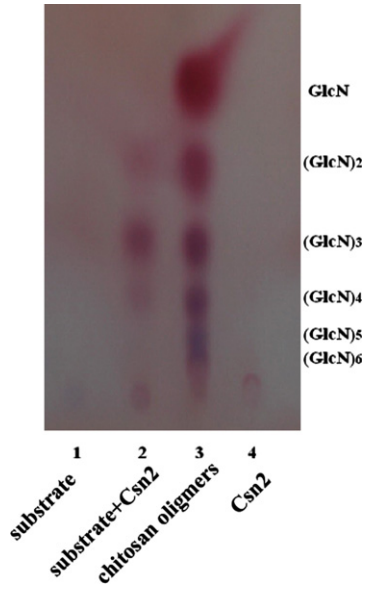

Figure 6. TLC analysis of the end products of 85% deacetylated chitosan hydrolyzed by Csn2. Hydrolysis products were analyzed on Silica G plate with *n*-butanol–isopropanol–25% aqueous ammonia (3:6:4) as the developing solvent. The plate was then sprayed with ninhydrin solution (0.2% in ethanol) and heated at 150 °C for 15 min for visualization.

3.5. N-Terminus sequence of Csn2

The first 19 amino acids of the N-terminus of Csn2 were determined and they were YQLPANLKKIYDSHKSGTC. A BLAST search revealed that this peptide sequence was 89% identical to a putative chitosanase, CSN1, of *Metarhizium anisopliae* var. *acridum* (CAC07218), 88% identical to *Hypocrea lixii* chitosanase (AAS77607) and 68% identical to a chitosanase from *Aspergillus oryzae* (BAA92250).

3.6. Partial genomic DNA sequence of Csn2

A DNA library was constructed by ligating the genomic DNA fragments digested by *EcoR* I into pUC19 plasmid, which was predigested with the same restriction enzyme. Based on the peptide sequence, a degenerate primer was designed corresponding to the peptide sequence YQLPANLKKIYD. Using this primer and a M13 general sequencing primer, we cloned the genomic sequence of Csn2 from the DNA library by PCR. This sequence was submitted to Genbank, and was assigned an accession number EU377605. The first 45bp of the DNA sequence was consistent with the peptide sequence YQLPANLKKIYDSHK. A BLASTN was performed with Csn2 DNA sequence. Part of the Csn2 DNA sequence showed 75% identity to the cDNA sequence of a putative chitosanase gene from *M. anisopliae* var. *acridum* (AJ293219), 73% identity to a *H. lixii* chitosanase gene (AY571342), 70% identity to a *A. fumigatus* endo-chitosanase gene (AY190324), 69% identity to the csnB gene from *A. oryzae* (AB090327) and 67% identity to the *Beauveria bassiana* chitosanase 1 gene (AY008269). No significant similarity was found between the Csn2 gene and reported *Bacillus* and *Streptomyces* chitosanase genes.

4. Discussion

The microorganism *Gongronella* JG is a novel chitosanase producing fungus isolated by this group.[25] To our knowledge, strain JG is the first chitosanase producing fungus of *Gongronella* species that had been reported. Co-existence of isoenzymes is normal for microbial chitosanases.[30] In our production condition, two chitosanases at least were secreted by strain JG.

Chitosanases from various fungi differed in their enzymatic properties. A brief summary is presented in Table 2. The molecular weight, optimal pH and temperature of Csn2 were in the range of reported values, but differed from those of Csn1 produced by the same strain. Metal ions had different impacts on fungal chitosanases. Cu^{2+} and Mn^{2+} could be activator or inhibitor to different chitosanases. As to Csn2, Mn^{2+} was activator. 1 mM Cu^{2+} stimulated Csn2, while 10 mM Cu^{2+} had negative effect on Csn2 activity. This finding is consistent with that of Csn1. Ca^{2+} and Sr^{2+} were activators of Csn2, which was not found with Csn1. Cu^{2+} and Mn^{2+} were activators of another GH-75 chitosanase from *A. fumigatus* Y2K at low concentration, but strongly inhibited the activity at high concentration.[8] Ca^{2+} did not affect chitosanase from *A. fumigatus* Y2K, but it stimulated Csn2. It was also reported that Ca^{2+} triggered the refolding of chitosanase from *Bacillus subtilis* GM9804.[31] Therefore, we hypothesized that different metal ions and metal ions at different concentrations might affect Csn2 activity through influencing the structure of the protein. EDTA was an activator of *Bacillus* R-4 chitosanase.[30] But to Csn2, it was an inhibitor. The optimal reaction temperature of Csn2 was slightly higher than that of Csn1, 55 °C for Csn2 and 50 °C for Csn1. At 55 °C, the half-life of Csn2 was 11 min. However, almost no hydrolysis activity was detected with Csn1 after 10 min treatment at the same temperature. These results indicated that Csn2 was more stable than Csn1 at 55 °C. The K_m value of Csn2 at its optimal reaction condition was 8.86 mg/mL, while it was 4.5 mg/mL for Csn1 at the optimal reaction conditions.

Several chitosanases can hydrolyze polysaccharides besides chitosan. For example, chitosanase from *Bacillus* sp. MET 1299 showed activity on β-glucan,[11] chitosanases from *Streptomyces griseus* HUT 6037 hydrolyzed carboxymethylcellulose,[32] chitosanase of *Paenibacillus fukuinensis* D2 hydrolyzed carboxymethylcellulose and glycol chitosan,[33] chitosanase II of *Acinetobacter* sp. Strain CHB101 degraded colloidal chitin and glycol chitin.[34] As for Csn2, besides soluble chitosan and colloidal chitosan, it displayed a weak hydrolysis capability on glycol chitosan. No obvious activity was detected with colloidal chitin and carboxymethylcellulose as substrates. This type of substrate specificity was similar to that of Csn1, which is also produced by strain JG. Csn2 also showed great discrepancy in hydrolysis capability with soluble or colloidal chitosan as the substrate. Substrate preference of chitosanases might be a kind of adaptation to evolutionary pressure as suggested by Fenton and Eveleigh[10] Endo-chitosanases are needed for producing chitooligosaccharides. The hydrolysis product analysis showed Csn2 was an endo-type chitosanase, which could be considered for preparing chitobiose, chitotriose and chitotetraose from colloidal chitosan.

Based on the N-terminus peptide sequence of Csn2, part of the genomic DNA corresponding to Csn2 was cloned. This is the first *Gongronella* chitosanase gene determined to date. Based on sequence similarity, Csn2 was classified as a GH-75 chitosanase (http://www.cazy.org/fam/GH75.html). A BLASTN analysis showed that the gene sequence of Csn2 was significantly different from those of other bacterial chitosanases which have determined to date. This might reflect the evolutionary divergence of chitosanases from different origins. Part of the Csn2 DNA sequence showed more than 70% identity to the DNA sequences of a putative chitosanase gene from *M. anisopliae* var. *acridum* (AJ293219) and a chitosanase gene from *H. lixii* (AY571342). However, to our knowledge, no detailed characterizations of these two chitosanases have been reported. Csn2 DNA sequence was 70% identical to an *A. fumigatus* endo-chitosanase gene (AY190324), a 25 kDa enzyme,[8] which is similar in size to Csn2. Its optimal reaction pH and

Table 2
Some properties of fungal chitosanases

Microorganism	Molecular weight (kDa)	Optimal pH	Optimal temperature (°C)	Metal ion activators	Metal ion inhibitors	Ref.
Gongronella JG	90	4.6–4.8	50	Mn^{2+}, Cu^{2+} (1 mM)	Cu^{2+} (10 mM)	26
	28	5.6	55	Mn^{2+}, Cu^{2+} (1 mM), Ca^{2+}, Sr^{2+}	Cu^{2+} (10 mM)	This work
Aspergillus fumigatus Y2K	25	6.5	65–70	Mn^{2+}, Cu^{2+}, Cu^{+}	Hg^{2+}, Cd^{2+}	8
Aspergillus oryzae IAM 2660	40	5.5	50			19
	135	5.5	50			
Aspergillus CJ22-326	29	6.0	60–65	Mn^{2+}	Hg^{2+}, Cu^{2+}	7
	109	4.0	50–55	Mn^{2+}, Cu^{2+}	Hg^{2+}	
Trichoderma reesei PC-3-7	93	4.0	50			14
Mucor rouxii	76	5.0	55	Ca^{2+}	Cu^{2+}, Mn^{2+}	6
	58	5.0	50		Cu^{2+}, Mn^{2+}	
Penicillium islandicum	30	4.5–6.0	45	Ca^{2+}	Cu^{2+}	10

temperature were 6.5 and 65–70 °C, and its major hydrolysis products were chitotriose, chitotetraose and chitopentaose. These properties were totally different from those of Csn2.

In conclusion, an endo-chitosanase, Csn2, was purified from *Gongronella* strain JG. Biochemical and molecular characterizations showed Csn2 was a new chitosanase, which can be considered as a potential candidate for producing chitooligosaccharides from colloidal chitosan.

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