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Cellobiohydrolase I (Cel7A) from *Trichoderma reesei* has chitosanase activity

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Abstract

Cellobiohydrolase CBH I (Cel7A) from the filamentous fungus *Trichoderma reesei* (*Tr*CBHI), which is a member of glycoside hydrolase family (GHF) 7, was expressed in *Aspergillus oryzae*. We found that the recombinant enzyme showed significant chitosanase activity, as well as cellulase activity, and acted in an endo-type manner on soluble polymeric substrate. Furthermore, another GHF7 CBH I from *Aspergillus aculeatus* (*Aa*CBHI) expressed in *A. oryzae* also had chitosanase activity, while endoglucanase EG I (Cel7B) from *T. reesei* had no activity towards chitosan. To our knowledge, this is the first report of GHF7 enzymes possessing chitosanase activity.

Keywords: *Trichoderma reesei* (*Hypocrea jecorina*); *Aspergillus aculeatus*; Cellobiohydrolase; Chitosanase; Glycoside hydrolase family (GHF) 7

1. Introduction

Cellulose and chitosan are biopolymers consisting of β-1,4-linked glycopyranoses, and they differ in the functional groups at the C-2 positions of their constituent sugars, i.e., hydroxyl and amino groups, respectively. Enzymes that hydrolyze the β-1,4-glycosylic bonds of these biopolymers (cellulase and chitosanase, respectively) have been found in many microorganisms, such as bacteria and fungi. Cellulases are endoglucanases (EGs, EC 3.2.1.4) that cleave internal bonds in amorphous cellulose chains to cello-oligosaccharides, and cellobiohydrolases (CBHs, EC 3.2.1.91), which release cellobiose from the ends of the cellulose chain. Cellulases have been, to date, classified into 12 glycoside hydrolase families (GHFs), based on amino acid sequence similarity [1]. Chitosanase (EC 3.2.1.132) breaks the internal bonds in the chitosan chain. Chitosanase activity has been confirmed in five GHFs; GHF 5, 8, 46, 75 and 80.

The filamentous fungus *Trichoderma reesei*, an anamorph of *Hypocrea jecorina*, is known to be one of the most powerful producers of cellulase. This fungus secretes two CBHs, CBH I and CBH II (Cel7A and Cel6A, respectively), and at least six EGs, EG I–VI (Cel7B, Cel5A, Cel12A, Cel61A, Cel45A and Cel74A, respectively). These enzymes have been extensively studied at the biochemical and genetic levels [2–8]. Among these cellulases, CBH I, the most secreted protein under cellulase-induced conditions [5], is considered to be a highly processive strict exoglucanase, although some controversial results have been reported [9–11]. This enzyme belongs to GHF7, whose members are almost all CBHs and EGs from ascomycotal and basidiomycotal fungi, along with EG I.

We previously found that the crude enzyme from cellulose-induced *T. reesei* possesses a chitosan-degrading activity and that this activity could not be separated from CBH I (*Tr*CBHI)-containing fractions after several column chromatography steps (unpublished data). However, it is unclear whether the chitosanase activity was caused by *Tr*CBHI, as there is a possibility that trace amounts of chitosan-degrading enzyme were not separated. The aim of this study was to clarify this issue, and we

Abbreviations: GHF, glycoside hydrolase family; GlcN, glucosamine; CMC, carboxymethyl cellulose; PSC, phosphoric acid-swollen cellulose; *p*NP, *p*-nitrophenol; *p*NPG2, *p*-nitrophenyl-β-D-cellobioside; CBH, cellobiohydrolase; *Tr*CBHI, CBH I from *Trichoderma reesei*; *Aa*CBHI, CBH I from *Aspergillus aculeatus*; EG, endoglucanase; *Tr*EGI, EG I from *T. reesei*

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showed that *Tr*CBHI and CBH I from *Aspergillus aculeatus* (*Aa*CBHI), which also belongs to GHF7, have chitosanase activity, in addition to cellulase activity, using the recombinant CBH Is in *A. oryzae*. To our knowledge, this is the first report to confirm that GHF7 enzymes possess chitosanase activity.

2. Experimental

2.1. Strains and plasmids

T. reesei QM9414 was used as the source of the *cbh1* gene. *Aspergillus oryzae niaD300* [12], which lacks nitrate reductase (*niaD*) activity for nitrate assimilation, and the plasmid pNEN142 [13] were used as the recipient strain and high-level expression vector for the heterologous expression of the *Trcbh1* gene, respectively. The transformant *A. oryzae* with the *cbh1* gene from *A. aculeatus* no. F-50 was constructed previously [14].

2.2. Materials

Taq DNA polymerase was obtained from Takara Shuzo Co. Ltd. (Kyoto, Japan). Glycol chitosan and chitosan oligomer (dimer, trimer, tetramer and pentamer; $GlcN_{2–5}$) were purchased from Wako Junyaku Co. Ltd. (Osaka, Japan) and Seikagaku Co. (Tokyo, Japan), respectively. Chitosan 10B (degree of deacetylation (DDA): >98%) and microcrystalline cellulose (Avicel) were obtained from Funakoshi Co. Ltd. (Tokyo, Japan). Carboxymethyl cellulose (CMC) and *p*-nitrophenyl-β-D-cellobioside (*p*NPG2) were purchased from Sigma Chemical Co. (MO, USA). Phosphoric-acid-swollen cellulose (PSC) was prepared as described by Walseth [15].

2.3. DNA manipulations

All DNA manipulations were performed by standard techniques [16] or according to manufacturer's recommendations. Dideoxy DNA sequencing reactions were performed with a "CEQ^{TM}DTCS Quick Start Kit" (Beckman Coulter, CA, USA) as specified by the manufacturer, and DNA fragments were analyzed on a CEQ2000XL (Beckman Coulter).

2.4. Construction of Trcbh1 expression plasmid and expression in A. oryzae

Trcbh1 genomic DNA was isolated from a *T. reesei* QM9414 genomic DNA library constructed previously [3] using a [γ-^{32}P]-labeled synthetic oligonucleotide, 5′-GCCAAGTACGGCACGGGGTACTGTGACAGC-3′, as a probe. The *Trcbh1* chromosomal fragment was amplified by polymerase chain reaction (PCR) using the primers 5′-TTGGTACCATGTATCGGAAGTTGGCCGTCA-3′ (forward) and 5′-TTTCTAGATTACAGGCACTGAGAGTAGTAA-3′ (reverse), which were tagged with *Kpn*I and *Xba*I restriction sites (underlined), respectively. The amplified product was digested with *Kpn*I and *Xba*I, and cloned into pUC119 treated with the same restriction enzymes to give pUCBH. The removal of introns by site-directed mutagenesis was carried out using pUCBH as a template and two oligonucleotide primers, 5′-TGTTTCGCAGCTGCCGTGCGGCTTGAACGG-3′ and 5′-AGTCTGTGGGATGATTACTACGCCAACATG-3′, with the "U.S.E. Mutagenesis Kit" (Pharmacia), according to the manufacturer's instructions. After confirmation by sequencing, the *Trcbh1* coding region was excised from the resulting plasmid with *Kpn*I treatment followed by blunting and *Xba*I digestion, and was then inserted to pNEN142 vector, which was blunted at the *Sal*I site and treated with *Xba*I, to give pNENTcbh1. pNENTcbh1 was linearized with *Mfe*I and then introduced into *A. oryzae niaD300* strain by the method of Gomi et al. [17]. Stable transformants were obtained as described by Honda et al. [13].

2.5. Production and purification of recombinant CBHIs

For recombinant *Tr*CBHI purification, spores (10^7 per flask) of *A. oryzae* with pNENTcbh1 were inoculated into glucose–peptone medium containing 4% glucose, 1% polypeptone, 0.5% KH_2PO_4, 0.1% NH_4Cl, 0.05% $MgSO_4$ and 0.1% Tween 80, and were incubated for 6 days at 30 °C with vigorous shaking (180 rpm). After cultivation, all procedures were carried out at temperatures below 4 °C and 10 mM sodium citrate buffer (pH 5.5) was used. Crude enzyme from the culture filtrate was precipitated with ammonium sulfate (80% saturation). The precipitate was collected and dissolved in buffer. After desalting, the *Tr*CBHI-fractions having *p*NPG2 degrading activity were subjected to DEAE-Sepharose FF (Pharmacia) column chromatography with a linear gradient of buffer containing 0–0.3 M NaCl. The *Tr*CBHI-fractions were desalted and concentrated by ultrafiltration, and were used as purified *Tr*CBHI. For recombinant *Aa*CBHI purification, 20 mM sodium acetate buffer (pH 5.0) was used. *A. oryzae* with the *cbh1* gene from *A. aculeatus* no. F-50 [14] was cultivated for 4 days at 30 °C in minimum medium [18] containing 5% glucose as a carbon source, with supplementation of glucose (final 5%) at 50 h. The culture filtrate was treated with ammonium sulfate at 20% saturation, subjected to Butyl-Toyopearl 650 M (Tosoh, Tokyo, Japan) column chromatography and separated with a linear gradient of buffer containing ammonium sulfate at 20–0% saturation. The *Aa*CBHI-fractions were collected, desalted and concentrated by ultrafiltration, and used as purified *Aa*CBHI.

2.6. Enzyme assay and analytical methods

All enzyme reactions were carried out at 50 °C in 50 mM sodium acetate buffer (pH 5.0), unless specified otherwise. Cellulase and chitosanase activities were usually determined by measuring the concentration of reducing sugar liberated during the hydrolysis of substrates using the Somogyi-Nelson method [19] and a modified version of the Schales method [20], respectively. One unit of activity was defined as the amount of enzyme that liberated 1 μmol of reducing sugar from substrate per minute with glucose or GlcN as the standard. *p*NPG2-cleaving activity was measured by monitoring the release of *p*-nitrophenol (*p*NP) from *p*NPG2 at 410 nm. The reaction mixture (500 μl) contain-

ing 1 mM *p*NPG2 and enzyme was incubated for an appropriate time, and the reaction was then terminated by the addition of 500 μl of 1 M sodium carbonate. One unit of activity was defined as the amount of enzyme that librated 1 μmol of *p*NP from the substrate per min.

Protein concentration was determined by the Lowry method with bovine serum albumin as a standard. The hydrolysates from chitosan or its oligomer were analyzed by thin-layer chromatography (TLC). A reaction mixture containing 1% GlcN oligomer or 0.5% Chitosan 10B was incubated with 2 mg/ml or 0.5 mg/ml *Tr*CBHI, respectively. The reaction mixture (8 μl) was spotted onto silica gel plates (Wako) and developed twice in a solvent system composed of *n*-butanol–methanol–28% ammonia water–water (5:4:2:1, v/v). Sugars on the TLC plate were visualized by spraying with Ninhydrin Spray (Wako) followed by heating. Viscosimetric assay was carried out as described previously [21]. Reaction mixtures containing 0.05% chitosan 10B or 0.1% CMC and 8 μg/ml or 34 μg/ml of *Tr*CBHI, respectively, were used. Reactions were performed in an Ubbelohde viscosimeter (Shibata Kagaku, Tokyo, Japan) kept at 50 °C.

3. Results and discussion

3.1. Overexpression of Trcbh1 in A. oryzae

When the pNENTcbh1 transformant was grown on glucose, *p*NPG2-cleaving activity was detected in culture filtrates and this activity was increased by prolonged cultivation. On the other hand, no significant activity could be observed in culture filtrates of pNEN142 transformants cultivated under the same conditions (Fig. 1). Furthermore, in culture filtrates of the pNENTcbh1 transformant, which was grown for 6 days, chitosan 10B-degrading activity was also detected, but this activity was not detected for pNEN142 transformants. These data clearly indicate heterologous expression of *Tr*CBHI in *A. oryzae* and imply that *Tr*CBHI has both cellulase activity and chitosanase activity. The specific activity of *Tr*CBHI, which was purified to apparent homogeneity in SDS-PAGE (data not shown), for *p*NPG2 was 27.0 mU/mg, which is similar to that of native enzyme, indicating that approximately 0.23 mg/ml-culture *Tr*CBHI was secreted during the 6-day cultivation.

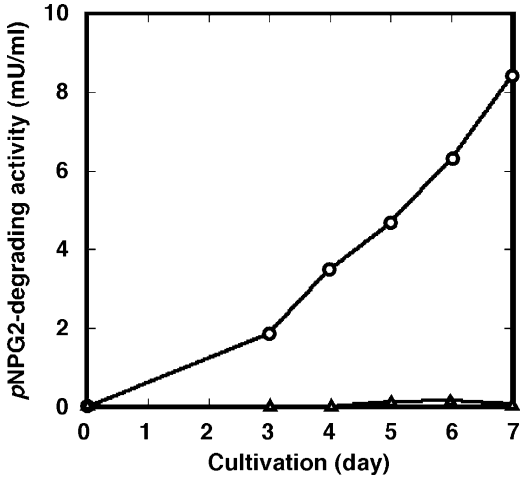

Fig. 1. *p*NPG2-degrading activity of culture filtrate of transformants. pNENTcbh1 and pNEN142 transformants are indicated by circles and triangles, respectively.

3.2. Substrate specificity of enzymes

Using the purified recombinant *Tr*CBHI, we measured cellulase and chitosanase activities against several substrates. As shown in Table 1, *Tr*CBHI clearly showed Chitosan 10B-degrading activity comparable to cellulase activity, *p*NPG2-cleaving and PSCase activities, in addition to slight hydrolytic activity for glycol chitosan. We further examined the chitosanase activities of other GHF7 enzymes, CBHI from *A. aculeatus* (*Aa*CBHI) and EGI from *T. reesei* (*Tr*EGI), both of which were individually expressed in *A. oryzae* [14 and unpublished data, respectively] and were purified to apparent homogeneity in SDS-PAGE (data not shown), in order to investigate whether this broad substrate specificity to cellulose and chitosan is restricted to *Tr*CBHI among GHF7 enzymes. Similarly to *Tr*CBHI, recombinant *Aa*CBHI also had chitosanase activity, although this activity was lower than that of *Tr*CBHI (Table 1), while the recombinant *Tr*EGI having full specific activity for CMC (unpublished data) did not show any chitosanase activity (detection limit, 1 mU/mg). The finding that two CBHIs have chitosanase activity is a novel property among GHF7 enzymes, and may imply that the cellobiohydrolases of GHF7 appear to be able to degrade chitosan. The three-dimensional structures of *Tr*CBHI and *Tr*EGI have been determined [22–24]. Both enzymes exhibit similar overall folding, but differ in the shape of their active sites; *Tr*CBHI has a 50 Å tunnel-shaped active site whose roof is created by four surface loops, whereas *Tr*EGI possesses an expanded, open substrate-binding cleft. This difference could explain the discrepancy in the type of degradation exhibited by these enzymes. However, it could be difficult to clarify the differences in substrate specificity due to the structural similarities between cellulose and chitosan; they differ only in having hydroxyl and amino groups, respectively, at the C-2 position. Furthermore, *Tr*EGI also degrades xylan, which is a β-1,4-linked polysaccharide of xylose [4], while *Tr*CBHI does not. With regard to the substrate specificity of GHF7 enzymes, it remains to be clarified how GHF7 enzymes can distinguish similar substrates, such as cellulose, chitosan

Table 1
Specific activity of recombinant enzymes

Substrate	Specific activity (mU/mg)	
	*Tr*CBHI	*Aa*CBHI
*p*NPG2	27.0	28.2
Avicel[a]	34.4	32.3
CMC	34.9	44.1
PSC	257	346
Chitosan 10B	350	128
Glycol chitosan	2.9	1.5

[a] Reaction mixture containing 0.5% Avicel and 100 μg/ml enzyme was incubated for 20 min.

and xylan. To resolve this issue question, further studies into the structure of both enzymes and substrates are required.

3.3. Cleavage specificity of chitosan by TrCBHI

In order to determine the mode of cleavage mediated by recombinant *Tr*CBHI, we analyzed the reaction products from chitosanous material by TLC. The hydrolysis products from $GlcN_4$ and $GlcN_5$ were a mixture of $GlcN_{1–3}$ and $GlcN_{1–4}$, respectively, and the amount of each oligomer increased with reaction time (Fig. 2). However, no products could be detected from $GlcN_2$ or $GlcN_3$ (data not shown). These results indicate that *Tr*CBHI has no or very low hydrolytic activity towards $GlcN_2$ and $GlcN_3$. When Chitosan 10B was used as a substrate, $GlcN_{2–5}$ could be seen at 4 h, where $GlcN_2$ was the main product, and detectable levels of GlcN were also produced at later stages (Fig. 2), thus suggesting that *Tr*CBHI has endo-type activity on Chitosan 10B. To confirm whether *Tr*CBHI has endo-type activity, we further carried out viscosimetric assay of the enzyme reaction using Chitosan 10B and CMC as substrates. As shown in Fig. 3, the viscosity of the reaction mixture using recombinant *Tr*CBHI decreased in the early stages of the reaction, but viscosity reductions were not observed when the exo-type chitosanase exo-β-D-glucosaminidase [21] was used as an enzyme. These data clearly indicate that *Tr*CBHI acts on Chitosan 10B and CMC in an endo-type manner. In contrast to our results, however, it has also been considered to act strictly as an exoglucanase with high processing activity on crystalline cellulose degradation [10,11], while another report suggests that *Tr*CBHI has endo activity [9]. Based on these differing reports, we concluded that the manner by which *Tr*CBHI cleaves cellulosic and chitosanous materials varies depending on substrate state (soluble or insoluble), regardless of substrate (cellulose or chitosan), and that it acts with endo-type activity or lower processivity on soluble substrates and with exo-type showing higher processivity on solid insoluble cellulose containing crystalline substrates.

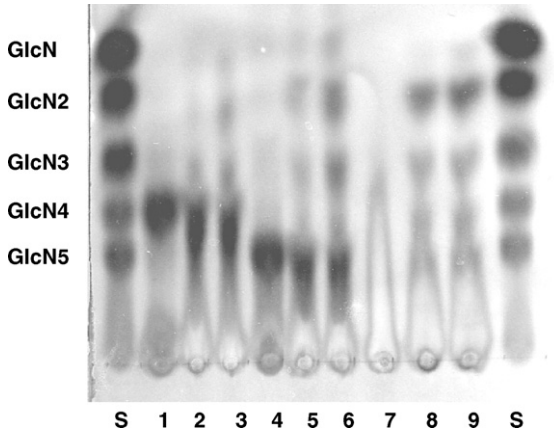

Fig. 2. TLC analysis of degradation products from $GlcN_4$, $GlcN_5$ and Chitosan 10B by *Tr*CBHI. Lane S, standard mixture ($GlcN_{1–5}$); lanes 1–3, hydrolysates from $GlcN_4$ obtained after 0, 4 and 20 h, respectively; lanes 4–6, hydrolysates from $GlcN_5$ obtained after 0, 4 and 20 h, respectively; lanes 7–9, hydrolysates from Chitosan 10B obtained after 0, 4 and 20 h, respectively.

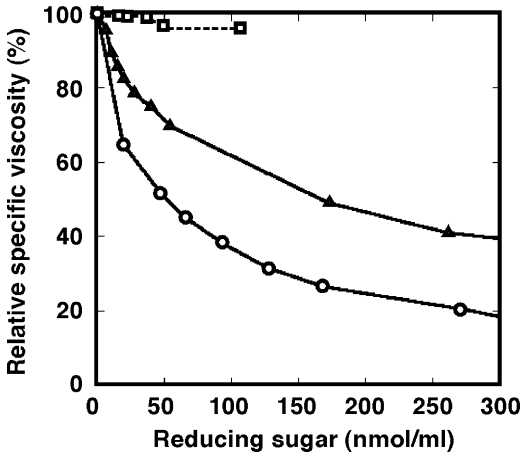

Fig. 3. Relationship between reduction in viscosity and liberation of reducing sugars from substrate solution by *Tr*CBHI. Chitosan 10B (circles) and CMC (triangles) were used as substrates. The results for Chitosan 10B treated with exo-β-D-glucosaminidase (squares) are shown as representative of an exo-type enzyme [21].

4. Conclusions

In this article, we demonstrated that the two GHF7 cellobiohydrolases, *Tr*CBHI and *Aa*CBHI, have both cellulase activity and chitosanase activity, whereas a GHF7 endoglucanase, *Tr*EGI, does not possess chitosanase activity. Thus, the cellobiohydrolases of GHF7 appear to be able to degrade chitosan. To our knowledge, this is the first report on enzymes that have both cellulase and chitosanase activities among GHF7.

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