Cloning and characterization of a small family 19 chitinase from moss (*Bryum coronatum*)

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Chitinase-A (BeChi-A) was purified from a moss, *Bryum coronatum*, by several steps of column chromatography. The purified BeChi-A was found to be a molecular mass of 25 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and an isoelectric point of 3.5. A cDNA encoding BeChi-A was cloned by rapid amplification of cDNA ends and polymerase chain reaction. It consisted of 1012 nucleotides and encoded an open reading frame of 228 amino acid residues. The predicted mature BeChi-A consists of 205 amino acid residues and has a molecular weight of 22,654. Sequence analysis indicated that BeChi-A is glycoside hydrolase family-19 (GH19) chitinase lacking loops I, II, IV and V, and a C-terminal loop, which are present in the catalytic domain of plant class I and II chitinases. BeChi-A is a compact chitinase that has the fewest loop regions of the GH19 chitinases. Enzymatic experiments using chitooligosaccharides showed that BeChi-A has higher activity toward shorter substrates than class II enzymes. This characteristic is likely due to the loss of the loop regions that are located at the end of the substrate-binding cleft and would be involved in substrate binding of class II enzymes. This is the first report of a chitinase from mosses, nonvascular plants.

Keywords: *Bryum coronatum* / chitin oligosaccharide / GH19 chitinase / moss / plant chitinase

Introduction

Chitinases (EC 3.2.1.14) catalyze the hydrolysis of chitin, which is a β-1,4-linked homopolymer or oligomer of N-acetylglucosamine (GlcNAc). These enzymes have been divided into two families, the glycoside hydrolase families 18 (GH18) and 19 (GH19) based on their catalytic modules according to the CAZy database (http://www.cazy.org/). The enzymes of the individual families differ in their primary sequences, three-dimensional structures and catalytic mechanisms (Beintema 1994; Iseli et al. 1996). GH18 chitinases are widely distributed in a variety of organisms, such as bacteria, fungi, animals and higher plants. However, GH19 chitinases have been found mainly in seed plants (Collinge et al. 1993; Graham and Sticklen 1994) and rarely in bacteria (Kawase et al. 2004). There are several sequences of GH19 chitinases in other organisms in the database, but their experimental characterizations have not been done. Independent of the CAZy classification, plant chitinases are divided into five classes and two subclasses based on their amino acid sequences and domain architectures (Taira 2010). Classes III, IIIb and V have GH18 catalytic domains. The classes containing enzymes with GH19 catalytic domains are as follows: class I chitinases, consist of an N-terminal chitin-binding domain and a GH19 catalytic domain; class II chitinases consist of only a GH19 domain; class IV enzymes resemble class I enzymes but are smaller due to the lack of several loops; class II-L enzymes are variants of the class II enzymes lacking several loops.

Plant GH19 chitinases have been well studied. However, an endogenous substrate for plant chitinases has not yet been found. One of the physiological roles of these chitinases is to protect plants against fungal pathogens by degrading chitin, a major component of the cell wall of many fungi (Schlumbaum et al. 1986). However, some chitinases do not show any antifungal activity (Taira, Toma, et al. 2005; Taira, Ohdomari, et al. 2005). Plant chitinases are induced not only by pathogenesis but also by abiotic stress. Several plant chitinases are constitutive, developmentally regulated, and tissue- and organ-specific. Thus, it appears that the role of plant chitinases does not consist solely of defense against pathogen attacks (Kasprzewska 2003). In addition, their roles seem to differ among different plants as far as the results of various experiments using various plants have shown. If their functions differ among different plants, we should examine many chitinases from many plant species to understand the physiological roles of chitinases in the plant kingdom.

This diversity in the structures and roles of plant chitinases has developed during the course of plant evolution. To study the fundamental role of plant chitinases, we have examined chitinases derived from evolutionarily older plants. We
previously researched chitinases from fern (Onaga and Taira 2008) and cycad (Taira et al. 2009) plants, which are evolutionarily older than angiosperms. We are currently interested in chitinases derived from mosses. Mosses are nonvascular plants and evolutionarily the oldest of the land plants. We think that structural and physiological research on chitinases in mosses will probably lead to a better understanding of the fundamental aspects of chitinases in the plant kingdom. However, to date, there has been no report of chitinases from mosses.

We screened various moss species for chitinase activity. *Bryum coronatum* extracts showed high chitinase activity relative to the other mosses assayed. Here, we describe the successful isolation, cloning and characterization of a chitinase from *B. coronatum*. This is the first report of a plant chitinase from a moss. Sequence analysis showed that this chitinase is the smallest GH19 chitinase, lacking the largest number of the loop regions that exist in class I and II chitinases. In this study, we show that the smallest chitinase is more adapted to shorter oligomeric substrates than a class II chitinase. The phylogenetic relationship between moss chitinase and other GH19 chitinases and the roles of loop regions of GH19 chitinases is discussed.

**Results**

**Physicochemical properties of purified *B. coronatum* chitinase-A**

*Bryum coronatum* chitinase A (BeChi-A) was purified from *B. coronatum* using gel filtration, hydrophobic interaction and ion-exchange column chromatographies (Supplementary data, Figure S1). A 100 g sample of *B. coronatum* yielded 0.05 mg of BeChi-A. The purified BeChi-A was found to be a 25 kDa (Figure 1) by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) with an isoelectric point (pI) of 3.5 by pI-PAGE (data not shown).

**Cloning and sequence analysis of BeChi-A cDNA**

To obtain the N-terminal amino acid sequences, BeChi-A was applied to a protein sequencer. After Edman degradation, the N-terminus appeared to be blocked and no sequence information was obtained. However, internal amino acid sequences were obtained from tryptic peptides of BeChi-A. Employing an reverse transcription-polymerase chain reaction (RT-PCR) method using degenerate primers designed with internal amino acid sequences (VTYYTFCQTLGVDPTDLR and GPIQLSWNYNYGEAG, underlines indicate the sequences used for primer design), we obtained internal sequences of the cDNA encoding BeChi-A. The strategy used for the cloning of BeChi-A cDNA is given in Supplementary data, Figure S2A. cDNA fragments of ~500 and 350 bp were amplified by the 5′-rapid amplification of cDNA end (RACE) and 3′-RACE reactions, respectively, using a gene-specific primer based on the sequence of the cDNA fragment obtained from RT-PCR. The resulting full-length BeChi-A cDNA consisted of 1012 nucleotides and encoded an open reading frame (ORF) of 228 amino acid residues (Supplementary data, Figure S2B). Analysis using the computer program SignalP (http://www.cbs.dtu.dk/services/SignalP; Bendtsen et al. 2004) indicated that the presequence of 23 amino acids is a putative signal peptide for transportation to the endoplasmic reticulum. The remaining 205 amino acids (Gln1 to Arg205) were considered to constitute the mature BeChi-A protein.

Structure-based sequence alignments of BeChi-A, plant class I, II, II-L and IV chitinases and bacterial GH19 chitinases are shown in Figure 2. There are some numberings of loops of GH19 chitinase. In this paper, we represent loop numbers I to V in sequence from N-terminus according to Ubyhayasekera et al. (2007, 2009). BeChi-A has no chitin-binding domain and lacks loops I, II, IV and V and a C-terminal loop, which are present in plant class I and II chitinases. This is similar to the catalytic domains of class IV, class II-L [lacking loops II, IV and V and a C-terminal loop, hereafter pokeweed leaf chitinase-A (PLC-A) type class II-L] and bacterial GH19 chitinases (lacking loops I, II and V and a C-terminal loop) but is more compact than these chitinases because BeChi-A lacks one more loop region compared with plant class II-L and bacterial GH19 chitinases (Figure 2, inset). The pathogenesis-related protein-P and -Q (PR-P and PR-Q) from tobacco (Payne et al. 1990), one of the class II-L chitinases lacking only loop III (represented by “II-L*”), hereafter PR-P type class II-L), differ from that of BeChi-A.

To study the phylogenetic relationships of BeChi-A and other GH19 chitinases, a multiple alignment of the amino acid sequences corresponding to the sequence regions shown in Figure 2 of various GH19 chitinases available from CAZy (http://www.cazy.org/) was prepared. The corresponding regions do not have all of the loop regions and the highly variable regions contain many gaps. As shown in Figure 3, three main clusters are apparent on the phylogenetic tree. The first cluster consists of plant class I, class II-L and the PR-P type class II-L (represented by “II-L*”) chitinases; the second cluster consists of plant class IV and PLC-A type class II-L chitinases and the third consists of bacterial GH19 chitinases and BeChi-A. Phylogenetically, BeChi-A is closely related to GH19 chitinases from bacteria.

**Preparation and characterization of recombinant BeChi-A**

To clarify detailed enzymatic properties of BeChi-A, we prepared a recombinant BeChi-A as described in the Materials and methods section. The isolation of recombinant BeChi-A
was successful, and remarkably, the yield of recombinant BeChi-A was ~100 mg/L of induced culture. The molecular mass of the recombinant BeChi-A was the same as that of the native BeChi-A (Figure 1, lane 2). The specific activities of the native and recombinant BeChi-A were 1.8 × 10^5 and 2.5 × 10^5 U/mol enzyme, respectively, using glycolchitin as the substrate. As shown in Figure 4, the optimum pH and temperature for both the native and recombinant BeChi-A were 5.0–6.0 and 60–70°C, respectively. The effects of pH and temperature for chitinase activity and pH stability of recombinant enzyme were almost the same as the native chitinase. BeChi-A was stable between pH 6 and 11, but unstable below pH 5 and above pH 12. It was stable to 70°C and unstable above 80°C. These results indicate that the recombinant enzyme was correctly folded in the cells.

Comparison of chitin-hydrolytic activity of BeChi-A with those of class II chitinases

Because BeChi-A is a plant GH19 chitinase that lacks the largest number of loops, we were interested in the roles of the loop regions in the chitin-hydrolytic abilities of plant GH19 chitinases. To clarify the roles of loop regions, the chitin-hydrolytic activity of BeChi-A and that of rye seed chitinase-c (RSC-c), typical class II enzyme from rye seeds, were compared. As shown in Table I, using glycolchitin as a polymer substrate, the chitin-degrading activity of BeChi-A was about three times higher than that of RSC-c. Using (GlcNAc)_6, (GlcNAc)_2 and (GlcNAc)_3 oligosaccharides, the chitin-degrading activity of BeChi-A was ~10 times, 100 times and 1000 times higher than that of RSC-c, respectively. BeChi-A was equally active on tetra- and hexamers, in contrast to RSC-c. These results indicate that BeChi-A is optimized for shorter substrates than class II enzymes.

Stereochemical course of hydrolysis reaction catalyzed by BeChi-A

To investigate the substrate bond-splitting modes of BeChi-A, we analyzed the anomer form of the degradation products of (GlcNAc)_4 of the enzyme using high-performance liquid chromatography (HPLC). The time-dependent HPLC profiles are shown in Figure 5. At the initial stage of (GlcNAc)_6 hydrolysis, BeChi-A produced (GlcNAc)_2, (GlcNAc)_3 and (GlcNAc)_4 that were a mixture of the α- and β-anomers. In all cases, the α-anomers were predominant, indicating that BeChi-A catalyzes an anomer inversion reaction. In addition, the α/β ratio of (GlcNAc)_4 was slightly higher than that of (GlcNAc)_2, indicating that the splitting to (GlcNAc)_2 + (GlcNAc)_3 was more frequent than that to (GlcNAc)_2 + (GlcNAc)_4. The middle linkage of (GlcNAc)_6 was also cleaved, producing (GlcNAc)_4. In the case of (GlcNAc)_5 hydrolysis, BeChi-A produced (GlcNAc)_2 and (GlcNAc)_3 that were a mixture of the α- and β-anomers. In this case, the α-anomer products were also predominant and the α/β ratio of (GlcNAc)_4 was slightly higher than that of (GlcNAc)_2. Starting with (GlcNAc)_4, the enzyme produced only (GlcNAc)_2 that were rich in α-anomer. These results indicate that BeChi-A hydrolyzed mainly the second glycosidic linkage from the reducing end of (GlcNAc)_4-6.

Antifungal activity of BeChi-A

Antifungal activity was determined by using the hyphal extension inhibition assay on agar plates with Trichoderma viride as the test fungus. A small amount (20 pmol) of RSC-c inhibited hyphal extension sufficiently, whereas a large amount (1000 pmol) of BeChi-A did not exhibit any antifungal activity (Figure 6).

Mutagenesis of catalytic center

Figure 2 shows that glutamate (67th in barley enzyme) thought to be a catalytic residue in 26 kDa barley class II chitinase (Andersen et al. 1997) is conserved in BeChi-A. The role of the glutamate was demonstrated by site-directed mutagenesis. The mutant E61Q was prepared in the same procedure as wild-type BeChi-A (Figure 1, lane 3). The mutation of Glu61 reduced activity to below the level that could be detected with our assay (data not shown).
In this study, we demonstrated the existence of a low-molecular-weight GH19 chitinase, BeChi-A, found in a moss. There are several low-molecular-weight chitinases that consist of a GH19 catalytic domain with some deletion(s). The deletion(s) are restricted to loop regions and the C-terminal loop region of class II enzymes. To date, mainly two types of low-molecular-weight class II chitinases, class II-L, in plants have been reported. One is the loop III lacking type chitinases such as PR-P and PR-Q that are the prototypes of the class II chitinases from tobacco (Payne et al. 1990). The other lacks loops II, IV and V and a C-terminal loop such as PLC-A which is first of this type of chitinase characterized (Ohta et al. 1995; Yamagami et al. 1998). BeChi-A is a class II-L chitinase, which lacks one more loop (loop I) than the PLC-A type. The catalytic domain of bacterial GH19 chitinases has no loops I, II and V and a C-terminal loop [loop V in the numbering of this paper indicates “loop III” and “loop C” described in Kezuka et al. (2006) and Hoell et al. (2006), respectively]. Because the number of missing loops is the highest in GH19 chitinases, BeChi-A is the smallest chitinase. Glu67 and Glu89 in barley class II chitinase are the catalytic acid and base, respectively (Andersen et al. 1997). In spite of the loop lacking regions, the positions of the corresponding residues, Glu61 and Glu70 in BeChi-A, are conserved. In this study, by site-directed mutagenesis confirmed that Glu61 is essential for the catalytic activity of BeChi-A. The positions of cysteine residues are almost completely conserved in plant GH19 chitinases, and two disulfide bonds (Cys23–Cys85 and Cys97–Cys105 in RSC-c) in the N-terminal end of the catalytic domains are conserved, but there are two variations of disulfide bonds in their C-terminal end (Yamagami and Funatsu 1993b; Hart et al. 1995; Araki et al. 1996; Yamagami et al. 1998). At least, BeChi-A lacks the first disulfide bridge due to the deletion of loop I, which contains the cysteine residue involved in the bridge, like the bacterial GH19 chitinases.

The phylogenetic analysis in this study suggests that BeChi-A is closely evolutionally related to the GH19 Chitinases from bacteria. Despite the exclusion of loop regions from the analysis, the phylogenetic relationships among GH19 chitinases agree well with their manner of loop deletions. Loop loss is likely to be involved with evolutionary events. In fact, the catalytic domain of a putative GH19 chitinase (EDQ60220) from Physcomitrella patens, a model moss plant, has a similar primary structure as BeChi-A. How moss plants obtained the GH19 chitinases would be interesting to investigate.

Roles of loop regions of GH19 chitinases
BeChi-A seems to be optimized for degradation of shorter substrates than class II enzymes. This characteristic is likely due to the loss of the loop regions that are located at the end of the substrate-binding cleft and would be involved in substrate binding in class II enzymes. One role of the loop regions of GH19 chitinases has been reported. Using chemical modification with N-bromosuccinimide, Yamagami and Funatsu (1995) showed that Trp72, located in loop II of RSC-c (a rye seed class II chitinase), is involved in substrate binding. Loop II is located at the end of the substrate-binding cleft (Hart et al. 1995). PLC-A (lacking loop II) easily hydrolyzes (GlcNAc)₄ into two moles of (GlcNAc)₂, whereas RSC-c dose (GlcNAc)₄ so little if at all, although both are active toward glycolchitin, a high-molecular-weight substrate (Ohta et al. 1995). Based on these experimental findings and sequence comparisons, they suggested that the deletion of the region containing Trp72 causes PLC-A to have high hydrolytic activity toward (GlcNAc)₄ by removal of the interference of this region with the binding of (GlcNAc)₂ to the substrate-binding site (Yamagami et al. 1998). This suggestion has been confirmed by several groups using several GH19 chitinases, including RSC-c, ChiC from Streptomyces griseus; ChiG from S. coelicolor; and OsChi1b (class I), OsChi1c (class I) and OsChi2e (class II-L) from rice (Ohnuma et al. 2002; Truong et al. 2003; Hoell et al. 2006; Kezuka et al. 2006; Mizuno et al. 2008). Fukamizo et al. (2009) showed that loop II is localized to the +3/4 subsite of the substrate-binding cleft of class II enzyme from barley seeds and concluded that Trp72 interacts with sugar residues but that Trp82 modulates loop flexibility, which controls the protein stability and enzymatic properties. Kezuka et al. (2006) hypothesized that ChiC (lacking loops I, II and V) has six subsites, +4 to +2, as in hen egg-white lysozyme. Hoell et al. (2006) showed by experimental analysis that ChiG (lacking loops I, II and V) has only four subsites (−2 to +2). These findings indicate that loop II comprises at least the +3/4 subsite in GH19 chitinases. In the case of BeChi-A, it is likely to lack the +3/4 subsite due to lack of the loop II. Because BeChi-A easily hydrolyzes (GlcNAc)₄ into two moles of (GlcNAc)₂, BeChi-A has at least four subsites (−2 to +2). However, although BeChi-A can bind to (GlcNAc)₆ and (GlcNAc)₅ in the all productive binding modes, the subsite would extend to a minus site (nonreduced end side) beyond −2. In fact, the α/β-anomer ratio of the tetramer product from hemexamer substrates was slightly higher than that of the dimer by BeChi-A. Similarly, the α/β-anomer ratio of the trimer product from pentamer substrates was slightly higher than that of the dimer. Although there would be subsites having little binding affinity at a minus site beyond −2, it is striking that BeChi-A hydrolyzed tetramer, pentamer and hexamer substrates at similar rates. In contrast, the efficiency of RSC-c (class II) is strongly dependent on substrate length. This ascertains that class II-L enzyme and class II have different catalytic properties, in accordance with the existence of loop region(s).

BeChi-A exhibits higher thermostability (stable up to 70°C) than RSC-c (stable up to 50°C; Yamagami and Funatsu 1993a). BeChi-A has smaller loop regions than RSC-c. A number of thermostable proteins, e.g. ferredoxin from Thermotoga maritima (Macedo-Ribeiro et al. 1996) and a thermostable endocellulase (Sakon et al. 1996), do show smaller loop regions, indicating a possible role in protein thermostability.

Physiological roles of chitinase in a moss
One of the physiological roles of plant chitinases is to protect plants against fungal pathogens. BeChi-A did not exhibit any antifungal activity, whereas the basic class II enzyme strongly did. ChiC from S. griseus inhibited hyphal growth of
Fig. 3. Phylogenetic relationships of GH19 chitinases from plants and bacteria. An unrooted phylogenetic tree was constructed based on an alignment of partial amino acid sequences of all GH19 chitinases by using the neighbor-joining method (Saitou and Nei 1987) implemented in the Clustal W program. The sequence regions used for the alignment are indicated in Figure 2. The optimal tree with the sum of branch lengths = 11.98392927 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling 1965) and are in units of the number of amino acid substitutions per site. There were a total of 123 positions in the final data set. Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007). Classes I, II and IV indicate the plant chitinase classes I, II and IV, respectively. Class II-L indicates the chitinases lacking loops II, IV, V and the C-terminal loop. “Class II-L*” indicates the chitinases lacking only loop III. Organisms with accession numbers are placed at the tips of branches. I, II, II-L, IV and Bac in parentheses at the ends of the explanations at the tips of branches indicate the chitinases of plant class I, II, II-L, IV and bacterial chitinases, respectively.
This activity was significantly reduced by the deletion of an N-terminal chitin-binding domain (Itoh et al. 2002). Taira et al. (2002), Taira, Ohdomari, et al. (2005), and Taira et al. (2010) reported that the chitin-binding domain and basicity of chitinase strongly contribute to their antifungal ability. BcChi-A has a low pI and no chitin-binding domain. Yamada et al. (1993) showed that GlcNAc oligomers smaller than trimers had almost no activity to induce phytoalexin formation in suspension-cultured rice cells. Because BcChi-A rapidly hydrolyzed tetramers, it may be not adequate to produce elicitors from fungal cell walls. A possible role of chitinase is in symbiosis. Some chitinases in Medicago truncatula have been reported to be specifically transcribed during interactions with mycorrhizal fungi (Salzer et al. 2000). Moss plants can also establish symbiosis with mycorrhizal fungi as well as vascular plants (Zhang and Guo 2007). Salzer et al. (1997) showed that the cleavage of chitinous elicitors (larger than tetramer) from the mycorrhizal fungus to monomeric and dimeric fragments by host chitinases prevents induction of a defense reaction in Picea abies cells. From these results, they suggested the following: first, only some of the fungal chitin elicitors reach their receptors at the plant plasma membrane, initiating reactions of the hypersensitive response in the host cells; and then, the remaining fungal elicitors will be degraded to varying extents by host chitinases, reducing the defense reactions of the plant and allowing symbiotic interactions of both organisms. BcChi-A may contribute to a symbiosis system by trimming or abolishing chitin-related signal molecules. The physiological functions of class IV chitinases, having a similar catalytic domain as BcChi-A, seem to be the most diverse among plant chitinases. They have been demonstrated to be involved in many biological functions and processes including pathogenesis, embryogenesis and programmed cell death (Kasprzewska 2003). Although seed plants express a large number of chitinase isozymes, we have not found other chitinase isozymes in B. coronatum. There are 10 candidate genes coding for chitinases which have enough length of polypeptide and conserved catalytic residues. Eight

### Table I. Comparison of molar-specific activity between BcChi-A and RSC-c

<table>
<thead>
<tr>
<th>Glycolchitin</th>
<th>(GlcNAc)_6</th>
<th>(GlcNAc)_5</th>
<th>(GlcNAc)_4</th>
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<tr>
<td>BcChi-A</td>
<td>2.54 × 10^9</td>
<td>3.44 × 10^6</td>
<td>2.12 × 10^6</td>
</tr>
<tr>
<td>RSC-c</td>
<td>8.31 × 10^8</td>
<td>3.55 × 10^5</td>
<td>1.74 × 10^4</td>
</tr>
</tbody>
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**Fig. 4.** Effect of pH and temperature on chitinase activity and stability of native and recombinant BcChi-A. (A) The effect of pH on activity was examined after incubation at 37°C for 15 min. (B) The effect of pH on stability was examined by measuring residual activity after incubation with various pH at 37°C for 24 h. (C) The effect of temperature on activity was examined after incubation for 15 min. (D) The effect of temperature on stability was examined by measuring residual activity after incubation in 0.1 M sodium phosphate buffer, pH 7.0, for 1 h. Details are described in the Materials and methods section. Closed and open circles indicate native and recombinant BcChi-A, respectively.

**Fig. 5.** Time-dependent HPLC profile showing the hydrolysis of chitin oligosaccharides by BcChi-A. Enzyme and substrate concentrations were 0.04 μM and 4.75 mM, respectively. The enzyme reaction was conducted in 50 mM sodium acetate buffer, pH 5.0, at 25°C. HPLC separations were conducted.
of them are estimated to be GH19 chitinases and others are done to be GH18 chitinases. Now, we examine their enzymatic properties and expression patterns. The physiological roles of chitinases in mosses, which are nonvascular and the oldest land plants, should be clarified by future structural and physiological studies on chitinases.

**Advantage of BeChi-A in structure-function analysis of GH19 chitinases**

Because BeChi-A is a thermostable protein, consisting of a small number of amino acid residues, and large amounts of recombinant BeChi-A can be obtained in E. coli overexpression systems, the enzyme should have advantages in detailed structure-function analysis using NMR and thermal analyses such as differential scanning calorimetry and isothermal titration calorimetry. NMR and thermal analyses of BeChi-A are now in progress by our research group. These analyses will probably lead to a better understanding of the catalytic mechanism of the GH19 chitinases.

**Materials and methods**

**Materials**

*Bryum coronatum* was collected on the campus of University of the Ryukyus. Chitin was purchased from Seikagaku Kogyo Co. (Tokyo, Japan). TPCK-treated trypsin was purchased from Sigma-Aldrich Co. (St. Louis, MO). *Escherichia coli* BL21(DE3) cells and expression vector pET-22b were purchased from Novagen (Madison, WI). RSC-c was provided by Dr. Yamagami of Kyushu University. All other reagents were of analytic grade.

**Protein measurement**

Protein concentrations were measured by the bicinchoninic acid method (Smith et al. 1985), using bovine serum albumin as the standard.

**Assay of chitinase activity**

Chitinase activity was assayed colorimetrically using glycolchitin. Ten microliters of the sample solution was added to 250 µL of 0.2% (w/v) glycolchitin in 0.1 M buffer. After incubation at 37°C for 15 min, the reducing power of the reaction mixture was measured using the ferri-ferrocyanide reagent by the method of Imoto and Yagishita (1971). The chitinase activity of each chromatographic fraction was expressed by the absorbance of the reaction mixture at 420 nm (ΔA420). One unit of enzyme activity was defined as the amount of enzyme releasing 1 µmol of GlcNAc per minute at 37°C.

**Purification of chitinase from B. coronatum**

All procedures were performed in a cold room. Absorbance was measured at 280 nm to monitor the proteins, and chitinase activity was measured at pH 5 during chromatographic separation. *B. coronatum* (100 g) was homogenized with 500 mL of deionized water and then left for 4 h at 4°C. After centrifugation, the supernatant was collected as crude chitinase. The crude chitinase was mixed with an equal volume of 2 M ammonium sulfate solution, and the mixture was blended with 10 mL of butyl-Toyopearl 650M (Tosoh, Tokyo, Japan) resin. After 4 h, the resin with adsorbed proteins was collected with a glass filter, and then packed into a column (1.6 × 5 cm). The adsorbed proteins were eluted with 50 mM sodium acetate buffer, pH 5.0. The fractions containing chitinase activity were pooled and applied to a Sephadex G-75 (GE Healthcare, Little Chalfont, Bucks, UK) column (2 × 140 cm) previously washed with 50 mM sodium acetate buffer, pH 5.0, and developed using the same buffer. The fractions containing chitinase activity were pooled and mixed with an equal volume of 2 M ammonium sulfate solution. The mixture was applied to a phenyl Superose column (Pharmacia, Milton Keynes, UK, 0.5 × 5 cm) previously equilibrated with 25 mM sodium acetate buffer, pH 5.0, containing 1 M ammonium sulfate. The column was washed with the same buffer, and then the adsorbed proteins were eluted with a linear gradient of ammonium sulfate from 1 to 0 M in the same buffer. A peak containing chitinase activity was obtained, and the peak was collected. The active fraction obtained by phenyl Superose column chromatography was dialyzed against 10 mM sodium acetate buffer, pH 5.0. The dialysate was applied to a Mono-Q column (GE Healthcare, 0.5 × 5 cm) equilibrated with 1 M NaCl from 0 to 0.3 M in the same buffer. The active fraction was collected as BeChi-A. The purified BeChi-A gave a single band having a molecular mass of 25 kDa on SDS–PAGE (Figure 1, lane 1). One hundred grams of *B. coronatum* yielded 0.05 mg of BeChi-A.

**Electrophoresis**

SDS–PAGE was done by the method of Laemmli (1970) using a 15% acrylamide gel. Proteins on the gel were stained with Coomassie brilliant blue R250 (CBB). The molecular mass was measured in the presence of 2-mercaptoethanol using a molecular weight marker kit (Fermentas, Glen Burnie, MD). The pI of chitinase was measured by electrofocusing on a PhastGel IEF 3–9 with the PhastSystem electrophoresis system (GE Healthcare). Proteins on the gel were detected by silver staining. The pI was determined with a pI calibration kit (GE Healthcare).
To determine internal amino acid sequences, the purified protein was digested with in-gel trypsin digestion as follows. Purified protein was separated by SDS-PAGE, and then the gel was stained with CBB. The protein band was cut out of the gel and sliced into ~1-mm pieces. The pieces were washed with water, and then dehydrated with acetonitrile, before being dried in a speed vac. Dried gel pieces were rehydrated with 50 μL of 100 mM dithiothreitol in 100 mM NH₄HCO₃ solution, and then the protein in the gel was reduced at 56°C for 30 min. After removing the reducing solution, 100 μL of 100 mM iodoacetamide in 100 mM NH₄HCO₃ solution was added to the gel pieces, and then the protein in the gel was alkylated at 37°C for 30 min. After removing the alkylating solution, the pieces were washed with water, and then dehydrated with acetonitrile before being dried in a speed vac. Dried gel pieces were rehydrated with 10 μL of protease solution, trypsin (100 ng in 100 mM NH₄HCO₃) and then covered with 100 mM NH₄HCO₃. Digestion was carried out overnight at 37°C. After reaction, the supernatant was removed and stored. And then, to extract the remaining peptides in gels, the same volume of acetonitrile/water/formic acid (30:70, v/v/v) was added to the gel. The resulting peptide fragments. The extract was mixed with the previous supernatant, and then dehydrated in a speed vac. The protein in the gel was alkylated at 37°C for 30 min. After removing the alkylating solution, the pieces were washed with water, and then dehydrated with acetonitrile before being dried in a speed vac. Dried gel pieces were rehydrated with 10 μL of trypsin (100 ng in 100 mM NH₄HCO₃) and then covered with 100 mM NH₄HCO₃. Digestion was carried out overnight at 37°C. After reaction, the supernatant was removed and stored. And then, to extract the remaining peptides in gels, the same volume of acetonitrile/water/formic acid (30:70, v/v/v) was added to the gel pieces. The extract was mixed with the previous supernatant, and the mixture dried in a speed vac and dissolved with formic acid/water (30:70, v/v). The resulting peptide fragments were fractionated by reverse-phase HPLC on a C4 column (3.9 × 150 mm, Waters, Milford, MA). The amino acid sequences of tryptic peptides were determined by automated sequential Edman degradations using a PPSQ-23A protein sequencing system (Shimadzu, Kyoto, Japan).

**cDNA cloning**

The sequences of all primers are presented in Table II. Total RNA was isolated from *B. coronatum* using an RNeasy kit (Qiagen, Valencia, CA). First-strand cDNA synthesis was performed on 5 μg of total RNA using a GeneRacer kit (Invitrogen, Carlsbad, CA) with oligo(dT) primer adapter. The resulting cDNA was used as a template for PCR amplification with degenerate primers. The PCR was performed with primers P1 (a forward primer designed on the basis of the tryptic peptide sequence, GPIQLSWNYYNGAG) and P2 (a reverse primer designed on the basis of other tryptic peptide sequence, VTVYTQFCQTLGVDGPTDLR). The resulting PCR products were cloned into a pGEM-T vector (Promega, Madison, WI) and sequenced using the ABI Prism system (Model 310; ABI). To obtain the full-length cDNA of BeChi-A, both 5'- and 3'-RACE were performed using a GeneRacer kit according to the manufacturer’s instructions. The gene-specific primers P3 (first PCR) and P4 (nested PCR) were used for the 5'-RACE and the gene-specific primers P5 (first PCR) and P6 (nested PCR) for the 3'-RACE. Finally, a cDNA fragment containing the entire coding region of BeChi-A cDNA was amplified using the forward primer P7 (designed from the 5'-RACE products) and reverse primer P8 (designed from the 3'-RACE products). The sequences of the resulting PCR products were analyzed using the above-mentioned procedure.

**Phylogenetic analysis**

Phylogenetic analysis was restricted to the proteins exhibiting enzyme activity, which was confirmed by experimental characterization. All the sequences in the data set possess the glutamate residue that acts as a acid catalyst and another residue capable of acting as a base is expected to be present in ~22 (in the case of chitinases having loop II) or 9 (in those lacking loop II) residues downstream of the glutamate residue. Sequences that did not meet the above criteria were removed from the final data set. In addition, if there were several sequences with high homology to each other in the same species, only one of them was used for the analysis. Multiple sequence alignment was performed using the program ClustalW (Larkin et al. 2007). The alignment was performed using default parameters. Then, all loop regions and the highly variable regions containing many gaps were manually removed from the alignment, and phylogenetic tree was constructed. Phylogenetic analysis using the neighbor-joining method was performed with MEGA, version 4 (Tamura et al. 2007). Complete deletion of gaps and missing data was performed, and 1000 bootstrap replicates were employed for the test of the inferred phylogenetic tree.

**Expression and purification of recombinant BeChi-A and its mutants in *E. coli***

We expressed recombinant BeChi-A [Met(–1)-Cys205, an ORF without a putative signal sequence] in *E. coli*. The corresponding cDNA region was amplified by PCR using BeChi-A cDNA as the template, with the primers P9 and P10 (P9 is a forward primer containing the Ndel recognition site, and P10 is a reverse primer containing the BamHI recognition site). The amplified DNA fragment was digested with Ndel and BamHI and ligated onto an expression vector, pET 22b (Novagen), previously digested with the same enzymes. The resulting PCR products were analyzed using the above-mentioned procedure.

**Table II. Primers for PCR, RACE, expression and mutation**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>5'-TGGAAATTAYAATGNGAGA-3'</td>
</tr>
<tr>
<td>P2</td>
<td>5'-CAGAATGATGACATCTGTC-3'</td>
</tr>
<tr>
<td>P3</td>
<td>5'-TGGCGTGACTGCGCTCCCG-3'</td>
</tr>
<tr>
<td>P4</td>
<td>5'-GGCGGAGATTGAAATGCA-3'</td>
</tr>
<tr>
<td>P5</td>
<td>5'-GGGATACGAGATGGAATGCTG-3'</td>
</tr>
<tr>
<td>P6</td>
<td>5'-TTGGGACGCCGTTTCTAGA-3'</td>
</tr>
<tr>
<td>P7</td>
<td>5'-TTGGGACGCCGTTTCTAGA-3'</td>
</tr>
<tr>
<td>P8</td>
<td>5'-CCGGTCTCTAATCTAGGAT-3'</td>
</tr>
<tr>
<td>P9</td>
<td>5'-GGGATACGAGATGGAATGCTG-3'</td>
</tr>
<tr>
<td>P10</td>
<td>5'-GGGATACGAGATGGAATGCTG-3'</td>
</tr>
<tr>
<td>P11</td>
<td>5'-GGGATACGAGATGGAATGCTG-3'</td>
</tr>
</tbody>
</table>

Single and double underlines indicate Ndel and BamHI recognition sites, respectively. A broken line indicates a codon encoding an amino acid change.
dialyzed against 10 mM sodium acetate buffer, pH 4.0. After dialysis, the resulting insolubilized proteins were eliminated by centrifugation at 10,000 × g for 15 min. The supernatant was dialyzed against 10 mM sodium acetate buffer, pH 5.0. The dialysate was applied to a Mono-Q column (0.5 × 5 cm) equilibrated with the dialysis buffer. The column was washed with the same buffer and adsorbed proteins were eluted with a linear gradient of NaCl from 0 to 0.3 M in the same buffer. After confirmation of the purity of the corresponding fraction by SDS-PAGE, the fraction was collected as purified recombinant protein. Site-directed mutagenesis was performed using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The primer used for site-directed mutagenesis was P11 for the Glu61→Gln mutation (E61Q). A mutation was introduced into pET-BcChi-A and the resulting plasmid designated pET-E61Q. The pET-E61Q was introduced into E. coli BL21(DE3), and then the mutant E61Q was prepared from the resulting E. coli cells by the same procedure as that for recombinant BcChi-A (wild type).

**HPLC analysis of hydrolysis products of chitin oligomers by chitinases**

The anomic form of the enzymatic products was determined by HPLC. The enzymatic hydrolysis of chitin oligosaccharides [(GlcNAc)n, n = 4, 5 or 6] was carried out in 50 mM sodium acetate buffer, pH 5.0, at 25°C. Concentrations of BcChi-A and the substrate were 0.04 mM and 4.75 mM, respectively. After incubation for a given period of time, a portion of the reaction mixture was directly injected into a TSK-GEL Amide 80 column (Tosoh), and the elution was performed with acetonitrile:H2O (7:3) at a flow rate of 0.7 mL/min. The substrate and enzymatic products were detected by ultraviolet absorption at 220 nm. The splitting mode of the oligosaccharide substrates was qualitatively estimated from the α/β ratio of each oligosaccharide product in the HPLC profiles (Koga et al. 1998).

**Antifungal assay**

An antifungal assay was done according to the method of Schlumbaum et al. (1986) with modification. An agar disk (6 mm in diameter) containing the fungus T. viride, prepared from actively growing fungus that had previously been cultured on potato dextrose broth with 1.5% (w/v) agar (PDA), was placed in the center of a Petri dish containing PDA. The plates were incubated at room temperature for 12 h. Wells were subsequently punched into the agar at a distance of 15 mm from the center of the Petri dish. The samples to be tested were placed into the wells containing 10 µL of sterile water. The plates were incubated for 24 h at room temperature and then photographed.

The nucleotide sequence for the BcChi-A gene has been deposited in the GenBank database under GenBank accession number AB368988. The amino acid sequence of this product can be accessed through NCBI protein database under NCBI accession number BAB90002.

**Supplementary data**

Supplementary data for this article is available online at [http://glycob.oxfordjournals.org/](http://glycob.oxfordjournals.org/).

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**Conflict of interest**

None declared.

**Abbreviations**

BcChi, *Bryum coronatum* chitinase; CBB, Coomassie brilliant blue R250; GH19, glycoside hydrolase family-19; GlcNAc, N-acetylglucosamine; HPLC, high-performance liquid chromatography; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PDA, potato dextrose broth with agar; PR-P, pathogenesis-related protein P; PR-Q, pathogenesis-related protein Q; pf, isoelectric point; RACE, rapid amplification of cDNA ends; RSC-c, rye seed chitinase-c.

**References**


Itoh Y, Kawase T, Nakaidou N, Fukada H, Mitsutomi M, Watanabe T, Itoh Y. 2002. Functional analysis of the chitin-binding domain of a family 19 chitinase from *Streptomyces griseus* HUT6057: Substrate-binding affinity and...


