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Note

Insertion of a Loop Structure into the "Loopless" GH19 Chitinase from *Bryum coronatum*

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Shoko Takenaka,[1] Takayuki Ohnuma,[1] and Tamo Fukamizo[1,†]

[1]*Department of Advanced Bioscience, Kindai University*
(*3327−204 Nakamachi, Nara 631−8505, Japan*)

Abstract: Chitinases belonging to the GH19 family have diverse loop structure arrangements. A GH19 chitinase from rye seeds (RSC-c) has a full set of (six) loop structures that form an extended binding cleft from -4 to +4 ("loopful"), while that from moss (BcChi-A) lacks several loops and forms a shortened binding cleft from -2 to +2 ("loopless"). We herein inserted a loop involved in sugar residue binding at subsites +3 and +4 of RSC-c (Loop-II) into BcChi-A (BcChi-A+L-II), and the thermal stability and enzymatic activity of BcChi-A+L-II were then characterized and compared with those of BcChi-A. The transition temperature of thermal unfolding decreased from 77.2 ˚C (BcChi-A) to 63.3 ˚C (BcChi-A+L-II) by insertion of Loop-II. Enzymatic activities toward the chitin tetramer $(GlcNAc)_4$ and the polymeric substrate glycol chitin were also suppressed by the Loop-II insertion to 12 and 9 %, respectively. The Loop-II inserted into BcChi-A was found to be markedly flexible and disadvantageous for protein stability and enzymatic activity.

Key words: *Bryum coronatum*, GH19 chitinase, loop structure, thermal stability, chitin hydrolysis

Chitinases (EC 3.2.1.14) are chitin-degrading enzymes that are capable of hydrolyzing the β-1,4-linkages of the polysaccharide, and have been divided into two major families, GH18 and GH19 (http://www.cazy.org) based on the amino acid sequences of their catalytic domains.[1)] GH18 chitinases are widely distributed in living organisms, while GH19 enzymes are mainly found in plants and some bacteria.[2)] GH19 enzymes have been further subdivided into classes I, II, and IV, based on loop structure arrangements and domain organization.[3)] The X-ray crystal structures of various types of GH19 chitinases have been examined in several studies,[4)5)6)7)8)] and the findings obtained revealed that the loop structure arrangement defines the length of the substrate-binding cleft. As shown in Fig. 1 (the right panel; PDB code, 4J0L), a GH19 chitinase from rye seeds (RSC-c) has a full set of (six) loop structures ("loopful"), referred to as Loop-I, Loop-II, Loop-III, Loop-IV, Loop-V, and the C-terminal Loop from the N-terminus (shaded in grey). The binding cleft of RSC-c is composed of the following eight subsites, −4, −3, −2, −1, +1 +2, +3, and +4, accommodating two molecules of the chitin tetramer $(GlcNAc)_4$. On the other hand, a GH19 chitinase from moss (BcChi-A) only has one loop structure, Loop-III, and lacks the loop structures involved in sugar residue binding at subsites −4, −3, +3, and +4 ("loopless"; the left panel of Fig. 1; PDB code, 3WH1). Thus, BcChi-A accommodates only one $(GlcNAc)_4$, and the binding cleft consists of only four subsites from −2 to +2. In the core regions (unshaded regions of the two structures in Fig. 1), the main chain structure of BcChi-A is almost completely overlapped with that of RSC-s, and is unlikely affected by the change in loop structure arrangement. These structural data suggest that engineering of the loop structure of GH19 chitinases may control the length of the substrate-binding cleft; and, hence, the substrate-binding mode and chain length of enzymatic products. In the present study, we inserted Loop-II derived from RSC-c into "loopless" BcChi-A (BcChi-A+L-II), and the protein stability and enzymatic activity of BcChi-A+L-II were compared with those of BcChi-A.

In order to insert the Loop-II of RSC-c (GGWATAPDGAFAW) into BcChi-A, we carried out the following two steps. In the first step, 21 nucleotides encoding the heptapeptide (GGWATAP), the N-terminal half of the Loop-II structure of RSC-c (NterL-II), were inserted into pET-BcChi-A, an expression vector of wild-type BcChi-A,[9)] using the QuickChange method[10)] with primers P1 and P2 (Table 1). The resulting plasmid was referred to as pET-BcChi-A-NterL-II. In the second step, 18 nucleotides encoding the hexapeptide (DGAFAW), the C-terminal half of Loop-II, were inserted into pET-BcChi-A-NterL-II with primers P3 and P4 (Table 1), creating the BcChi-A+L-II expression vector pET-BcChi-A+L-II. Primers P1, P2, P3, and P4 were designed to insert Loop-II into the position between Gly63 and Gly64 in BcChi-A, which corresponds to the position of Loop-II in RSC-c. Sequences of primers P1, P2, P3, and P4 are listed in Table 1. After nucleotide se-

†Corresponding author (Tel. +81−742−43−8237, Fax. +81−742−43−8976, E-mail: fukamizo@nara.kindai.ac.jp).
Abbreviations: BcChi-A, a family GH19 chitinase from *Bryum coronatum*; RSC-c, a family GH19 chitinase from rye (*Secale cereal*) seeds; BcChi-A+L-II, an engineered BcChi-A to which Loop-II derived from RSC-c is inserted; GlcNAc, 2-acetamido-2-deoxy-D-glucopyranose; $(GlcNAc)_n$, β-1,4-linked oligosaccharide of GlcNAc with a polymerization degree of *n*; HPLC, high performance liquid chromatography.

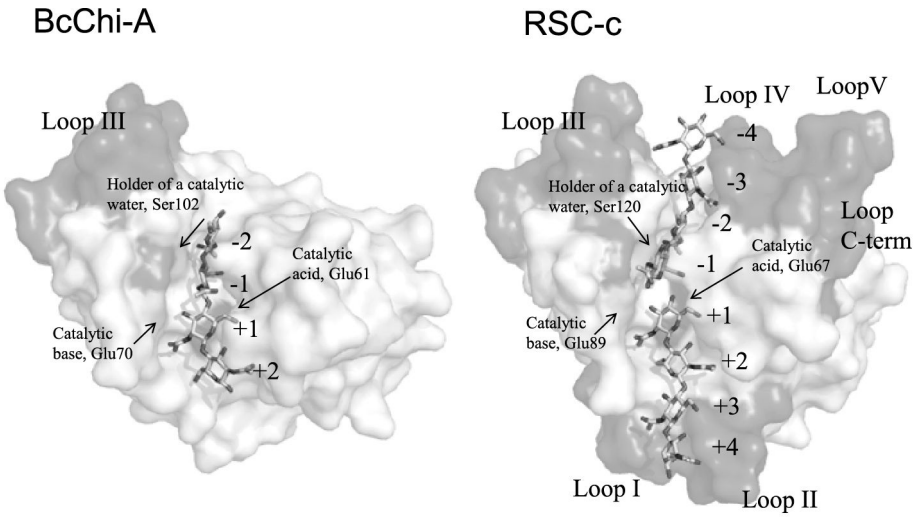

Fig. 1. Structural comparison between "loopless" BcChi-A and "loopful" RSC-c.

Surface models of crystal structures of BcChi-A in complex with $(GlcNAc)_4$ (Left panel; PDB code, 3WH1) and RSC-c in complex with two molecules of $(GlcNAc)_4$ (Right panel; PDB code, 4J0L). Regions shaded in grey are the loop structures for individual GH19 chitinases. Loop structures are numbered from the N-terminus with roman numerals. Subsites are numbered according to the nomenclature proposed by Davies *et al*.[17] The positions of the catalytically important residues are indicated by arrows.

Table 1. Sequences of oligonucleotide primers used for the Loop II insertion.

Primers	Seqeunces
P1	5′-AACATCAACCAGGAATCCGGAGGCGGGTGGGCGACCGCACCCGGGTTGCAGTTTATCCAAGAG-3′
P2	5′-CTCTTGGATAAACTGCAACCCGGGTGCGGTCGCCCACCCGCCTCCGGATTCCTGGTTGATGTT-3′
P3	5′- GGCGGGTGGGCGACCGCACCCGACGGGGCCTTCGCATGGGGGTTGCAGTTTATCCAAGAG -3′
P4	5′- CTCTTGGATAAACTGCAACCCCCATGCGAAGGCCCCGTCGGGTGCGGTCGCCCACCCGCC -3′

The underlined regions correspond to the inserted sequence (Loop II from RSC-c).

quencing, we confirmed that the resulting plasmid had the complete sequence for BcChi-A+L-II.

BcChi-A was produced and purified using previously described methods.[9] The Loop-II-inserted BcChi-A mutant, BcChi-A+L-II, was expressed in *E. coli* using essentially identical procedures to those for BcChi-A. Briefly, *E. coli* BL21(DE3) cells harboring the expression vector pET-BcChi-A+L-II were grown to $A_{600\ nm}$ = 0.6 before induction with 1 mM isopropyl β-D-1-thiogalactoside. Growth was then continued at 15 ˚C for 24 h. Cells were harvested by centrifugation, suspended in 20 mM Tris–HCl buffer (pH 7.5), and disrupted with a sonicator. After cell debris had been removed by centrifugation (10,000 × G, 10 min), the supernatant dialyzed against 10 mM sodium acetate buffer, pH 5.0. was then applied onto a Q-Sepharose Fast Flow column (1.6 × 2.5 cm) equilibrated with dialysis buffer. The adsorbed proteins were eluted with a linear gradient of NaCl from 0 to 0.3 M in the same buffer. The fractions eluted were pooled and further separated using a Sephacryl S-100 HR column (1.6 × 60 cm) equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl. Fractions exhibiting a single protein band on SDS-PAGE were collected as purified BcChi-A+L-II.

We successfully produced 27 mg of the purified BcChi-A+L-II from 1 L of culture medium. Since the yield of BcChi-A from 1 L of culture medium was 48 mg protein, the expression level of BcChi-A+L-II was reduced by the insertion of Loop-II. Far-ultraviolet CD spectra of BcChi-A and BcChi-A+L-II were obtained using a Jasco J-720 spectropolarimeter (JASCO Corporation, Tokyo, Japan) (cell length 0.1 cm), and are shown in Fig. 2A. Both spectra were essentially identical, indicating that the insertion of Loop-II did not affect the global conformation of the enzyme. In order to obtain the thermal unfolding curves of BcChi-A and BcChi-A+L-II, the CD value at 222 nm was monitored while the solution temperature increased at a rate of 1 ˚C/min using a temperature controller (PTC-423L, Jasco). As shown in Fig. 2B, individual unfolding curves were highly cooperative. BcChi-A unfolded at 77.2 ˚C, while the transition temperature of BcChi-A+L-II was 63.3 ˚C. The insertion of loop structure appears to be disadvantageous for the protein stability. As described above, the structure of core region (unshaded region of BcChi-A in Fig. 1) is unlikely affected by the insertion of loop structure. Thus, the strong reduction in protein stability may be due to the flexibility of inserted Loop-II, which is unlikely to form an interaction with the other structural elements in BcChi-A +L-II, but is likely to fluctuate at the +3/+4 region. The data are consistent with our findings reported in the previous paper,[11] in which the mutations of tryptophan residues in Loop-II of "loopful" chitinase from barley seeds enhanced the flexibility of Loop-II and destabilized the protein structure.

Using a gel-filtration HPLC (column, TSK-GEL G2000PW, 7.5 × 600 mm, TOSOH Corporation, Tokyo, Japan; eluent, distilled water), the enzymatic products from the substrate chitin tetramer $(GlcNAc)_4$ were determined, and the time-courses of enzymatic hydrolysis were ob-

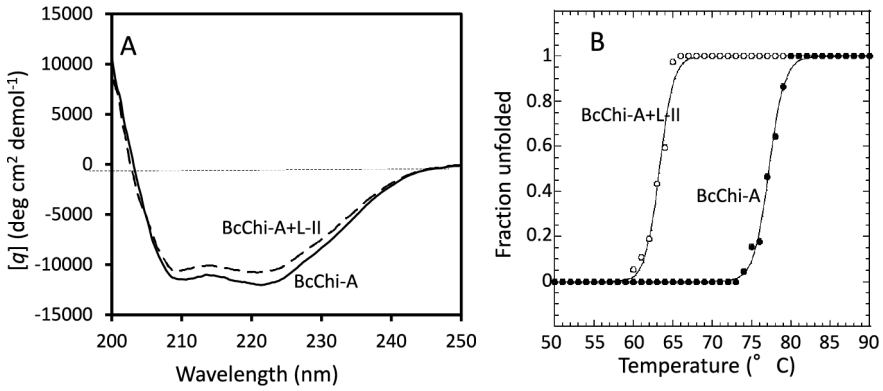

Fig. 2. Far-ultraviolet CD spectra (**A**) and thermal unfolding curves of BcChi-A and BcChi-A+L-II (**B**).

Measurements were conducted in 20 mM sodium acetate buffer pH 5.0 at 25 ˚C, using a Jasco J-720 spectropolarimeter. The protein concentrations were 4 μM. The solid and broken lines in CD spectra correspond to BcChi-A and BcChi-A+L-II, respectively. In the thermal unfolding experiments (**B**), the solution temperature increased at a rate of 1 ˚C/min using a temperature controller (PTC-423L, Jasco Corporation, Tokyo, Japan). T_m values obtained from the unfolding curves in (**B**) were 77.2 (the line with closed circles, BcChi-A) and 63.3 ˚C (the line with open circles, BcChi-A+L-II).

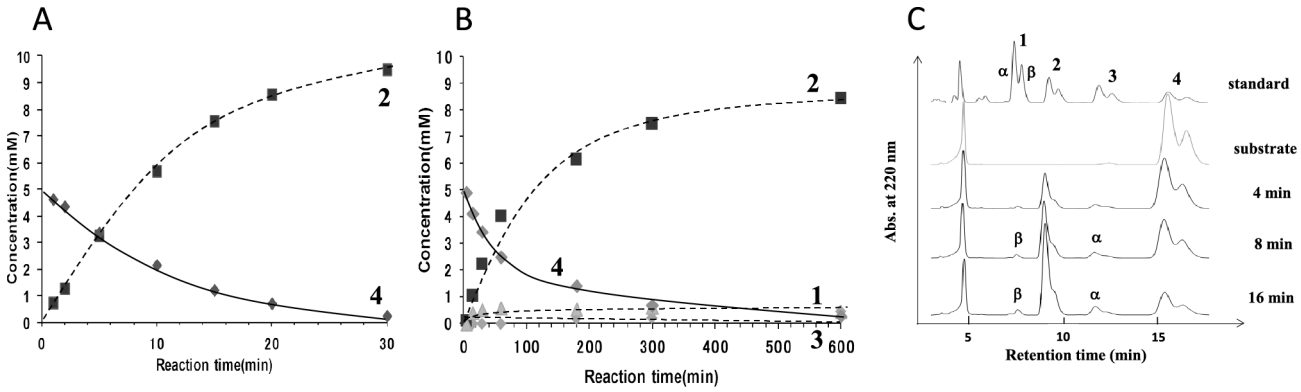

Fig. 3. Time-courses of $(GlcNAc)_4$ hydrolysis catalyzed by BcChi-A (**A**) and BcChi-A+L-II (**B**). Time-dependent HPLC profiles of the reaction products from incubation of $(GlcNAc)_4$ with BcChi-A+L-II (**C**).

A and B: The enzymatic reaction was conducted in 20 mM sodium acetate buffer pH 5.0 at 37 ˚C. Enzyme and substrate concentrations were 0.1 μM and 5.0 mM, respectively. Substrate and product concentrations at a given reaction time were determined by gel-filtration HPLC using a column of TSK-GEL G2000PW (Tosoh Corporation, Tokyo, Japan). **C:** The enzymatic reaction was conducted in 20 mM sodium acetate buffer pH 5.0 at 25 ˚C. Enzyme and substrate concentrations were 0.4 μM and 5.0 mM, respectively. HPLC conditions: column, TSK-GEL Amide-80 column (Tosoh); elution solvent, 70 % acetonitrile; flow rate, 0.7 mL/min. Numerals in the figures represent the polymerization degree of $(GlcNAc)_n$. α and β are anomeric forms of the corresponding oligosaccharides.

tained by the method previously described.[12] The results are shown in Figs. 3A and 3B. BcChi-A rapidly hydrolyzed the substrate, which was completely consumed within 30 min (Fig. 3A). The product was only $(GlcNAc)_2$, indicating symmetrical hydrolysis of the initial substrate $(GlcNAc)_4$. The rate of $(GlcNAc)_4$ degradation by BcChi-A+L-II was markedly lower than that of BcChi-A (Fig. 3B). Specific activities evaluated from the rate of $(GlcNAc)_4$ degradation were 167.4 μmol/min/mg for BcChi-A and 19.9 μmol/min/mg for BcChi-A+L-II (12 %). It is important to note that small amounts of GlcNAc and $(GlcNAc)_3$ were produced from $(GlcNAc)_4$ by BcChi-A+L-II (Fig. 3B). The $(GlcNAc)_4$-binding mode was affected by the insertion of Loop-II. In order to examine the mode of hydrolysis by BcChi-A+L-II, the anomeric forms of the individual reaction products were quantitatively determined using HPLC (column, TSK-GEL Amide 80, 0.46 × 25 cm; eluent, 70 % CH_3CN)[13]. As shown in Fig. 3C, the product GlcNAc was predominantly β-anomer, while the product $(GlcNAc)_3$ was rich in α-anomer. Since BcChi-A is an inverting enzyme producing α-anomer, the products GlcNAc and $(GlcNAc)_3$ were derived from the reducing-end side and another side of the substrate $(GlcNAc)_4$, respectively. Although most $(GlcNAc)_4$ was symmetrically hydrolyzed, a fraction of $(GlcNAc)_4$ was hydrolyzed at the first glycosidic linkage from the reducing end. The predominant production of β-GlcNAc over α-GlcNAc suggested that subsite +1 specifically recognizes β-anomer of the substrate $(GlcNAc)_4$. When Loop-II was inserted into BcChi-A, the large flexibility of inserted Loop-II may disturb the binding mode of $(GlcNAc)_4$. Although a major fraction of $(GlcNAc)_4$ may bind to subsites from −2 to +2 for symmetrical hydrolysis, some fractions may bind to subsites from −4 to −1 without spanning the catalytic site, resulting in the lower enzymatic activity. Another small fraction may bind to subsites from −3 to +1, producing $(GlcNAc)_3$ and GlcNAc.

Chitinase activity toward glycol chitin[14] was evaluated from the production rate of reducing sugars by the method of Imoto and Yagishita.[15] The enzymatic reaction was conducted in 20 mM sodium acetate buffer pH 5.0 at 37 ˚C.

One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of GlcNAc per minute at 37 ˚C. The specific activity of BcChi-A toward glycol chitin was 2.0×10^5 U/mg, while the activity of BcChi-A+L-II was markedly reduced (1.9×10^4 U/mg, 9 %), as compared with that of BcChi-A. Since the core structure is unlikely affected by the loop insertion (Fig. 1), the reduction in the activity may be due to loss of the binding ability. The flexibility of inserted Loop-II in BcChi-A+L-II may have acted as an obstacle to binding of the polymeric substrate; and, thus, resulted in the lower activity (9 %). The relationship between enzyme activity and loop arrangement can be confirmed by the data reported by Mizuno *et al*.[16] They determined the activities of Loop-II-truncated mutant of a "loopful" GH19 chitinase from rice and reported that the enzyme activities toward a reduced form of $(GlcNAc)_6$ and polymeric chitin substrate were enhanced by the truncation of Loop-II.

In conclusion, Loop-II inserted into BcChi-A was found to be disadvantageous for protein stability and enzymatic activity, possibly due to the loop flexibility. The isolated situation of the inserted loop at the +3/+4 region did not form any intramolecular interactions, resulting in the highly flexible movement of the loop. This movement destabilized the protein structure and perturbed the binding mode of the substrate, lowering the enzymatic activity of BcChi-A+L-II.

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