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Role of the Loop Structure of the Catalytic Domain in Rice Class I Chitinase

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In the three-dimensional structure of a rice class I chitinase (OsChia1b) determined recently, a loop structure (loop II) is located at the end of the substrate-binding cleft, and is thus suggested to be involved in substrate binding. In order to test this assumption, deletion of the loop II region from the catalytic domain of OsChia1b and replacement of Trp159 in loop II with Ala were carried out. The loop II deletion and the W159A mutation increased hydrolytic activity not only towards $(GlcNAc)_6$ but also towards polysaccharide substrates. Similar results were obtained for k_{cat}/K_m values determined for substrate reduced-$(GlcNAc)_5$. The two mutations shifted the splitting positions in $(GlcNAc)_6$ to the reducing end side, but the shift was less intensive in the Trp mutant. Theoretical analysis of the reaction time course indicated that sugar residue affinity at the +3 subsite was reduced from –2 kcal/mol to +0.5 kcal/mol by loop II deletion. Reduced affinity at the +3 subsite might enhance the release of product fragments, resulting in higher turnover and higher enzymatic activities. Thus, we concluded that loop II is involved in sugar residue binding at the +3 subsite, but that Trp159 itself appears to contribute only partly to sugar residue interaction at the subsite.

Key words: family 19 chitinase, loop structure, *Oryza sativa*.

Abbreviations: OsChia1a, OsChia1b and OsChia1c, *Oryza sativa* L., var Nipponbare class I chitinase, respectively; GH, glycoside hydrolases; CBM, carbohydrate-binding modules; ChBD, chitin binding domain; CatD, catalytic domain; IPTG, isopropyl-β-D-thiogalactopyranoside; GlcNAc, 2-deoxy-2-acetamido-D-glucose; Red-(GlcNAc)5, reduced *N*-acetylchitopentaose; GC, glycol chitin; CC, colloidal chitin.

Chitinases (EC 3.2.1.14) catalyse the degradation of chitin, a linear β-1,4-linked homopolymer of *N*-acetylglucosamine, and are distributed in a wide range of organisms. In spite of the wide distribution and variety of roles of chitinases, they are classified into only two families of glycoside hydrolases (GH), families 18 and 19, according to the CAZy database (http://afmb.cnrs-mrs.fr/CAZY/), in which glycoside hydrolases are divided into more than 110 families based on the amino acid sequence similarity of their catalytic domains (*1*, *2*). The members of two different families differ in their amino acid sequences, three-dimensional (3D) structures (*3–6*), and molecular mechanisms of catalytic reactions (*7–10*), and are thus considered to have different evolutionary origins. GH family 18 chitinases are widely distributed in a variety of organisms, such as bacteria, fungi, viruses, animals and higher plants (classes III and V). The distribution of GH family 19 enzymes is more restricted and they are mainly found in higher plants (classes I, II and IV) and some bacteria (*11*, *12*).

Plant family 19 chitinases are thought to be part of the defence mechanism against fungal pathogens. This role has been deduced from the following observations: (i) chitin is the major component of the cell wall of plant pathogens, (ii) chitinase is a pathogenesis-related protein (*13*) and (iii) some plant chitinases exhibit anti-fungal activity *in vitro* (*14*, *15*). This hypothesis has been supported by the observation that transgenic plants constructed by introducing the plant chitinase gene enhanced resistance against fungal diseases (*16*, *17*). Independent of the CAZy classification, plant chitinases are divided into five classes on the basis of their primary structures (*14*). Classes I, II and IV chitinases are members of GH family 19, whereas classes III and V chitinases are members of GH family 18. Class II chitinases consist of only a catalytic domain, whereas classes I and IV chitinases have an N-terminal cysteine-rich chitin-binding domain (ChBD) and a C-terminal catalytic domain (CatD) connected by a linker peptide of 10 to 20 residues. Class IV chitinases have characteristic deletions in the catalytic domain as compared with those of class I chitinases.

The 3D structure of family 19 chitinase was first reported with class II chitinase (a single domain chitinase) from barley (*5*), and then from jack bean (*18*). For barley chitinase, Hart *et al*. (*5*) showed the resemblance of its secondary structure at the active site region with lysozyme and predicted two acidic residues

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as catalytic residues, Glu67 as a general acid (proton donor) and Glu89 as a general base. The candidates were later confirmed to be essential for catalytic activity by site-directed mutagenesis (*19*). On the other hand, the crystal structure of bacterial family 19 chitinases has been reported recently with ChiC, a two-domain chitinase from *Streptomyces griseus* HUT6037, by Kezuka *et al*. (*6*), and ChiG, a single-domain chitinase from *Steptomyces coelicolor* A3(*2*), by Hoell *et al*. (*20*). ChiC consists of the N-terminal ChBD of carbohydrate-binding module (CBM) family 5 and family 19 CatD. Overall, structures of the catalytic domains of the two bacterial chitinases are similar to that of barley and jack bean chitinases, however, the characteristic deletion of three loop structures was observed in the catalytic domains of bacterial chtinases. These structural differences are also described by Ubhayasekera *et al*. (*21*), who reported the 3D-structure of CatD of BjCHI1 (consisting of two ChBDs and CatD) from *Brassica juncea*.

Recently, the crystal structure of full-length rice class I chitinase (OsChia1b, also referred to as Cht-2) containing N-terminal ChBD of CBM family 18 has been determined by Kezuka *et al*. (PDB accession code: 2DKV). This is the first structure of two-domain chitinase from plants. The catalytic domain of OsChia1b is very similar to class II chitinases from barley and jack bean. The catalytic domain of OsChia1b has three loop structures, loop I, II and III, which are absent from the catalytic domains of ChiC and ChiG. In this study, we focused on the function of loop II and the effect of a mutation introduced into the loop structure was studied.

MATERIALS AND METHODS

Strains, Plasmids, and Medium—Escherichia coli DH5α was used as a host strain in plasmid construction. The *RCC2* gene encoding a rice class I chitinase, OsChia1b, has been isolated from *Oryza sativa* L. ssp. *japonica* cv. Nipponbare and characterized by Nishizawa *et al*. (*22*) previously. OsChia1b and its mutants were produced in *E. coli* Origami B(DE3) (Novagen, Madison, WI, U.S.A.) cells harbouring plasmid p22-cht2 and its derivatives. The plasmid p22-cht2 carries *RCC2* without a region corresponding to the signal sequence in the expression vector pET22b(+) (Novagen). Plasmids p22-ΔlooII and p22-W159A carry DNA regions encoding mutated OsChia1b, OsChia1b$_{\Delta loopII}$ and OsChia1b$_{W159A}$, respectively. *Escherichia coli* cells carrying recombinant plasmids were grown in Luria–Bertani (LB) medium containing 100 μg/ml ampicillin.

Construction of OsChia1b$_{\Delta loopII}$ and OsChia1b$_{W159A}$—The gene encoding mature portion of OsChia1b was subcloned into pUC119 (p119-cht2) and the plasmid was used to construct mutated OsChia1b. OsChia1b$_{\Delta loopII}$ was constructed by PCR using p119-cht2 as a template, and amplified fragments were phosphorylated and self-ligated. The forward primer (5′-GGCTACTGCTTCAA GCAGGAGC-3′) and the reverse primer (5′-GCCGGTCT CGTGGGAGGTCTGGC-3′) used for PCR are identical to the sense strand and anti-sense strand, respectively, of the 3′-flanking region of loop II. The primer set was designed to delete the loop II-encoding region, based on careful comparison of the 3D-structures of ChiC and OsChia1b as described in RESULTS section. Site-directed mutagenesis to replace Trp159 with Ala in OsChia1b was performed using the Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The primers used for mutagenesis were 5′–AGACCACCGG CGGGGCGCCGACCGCGCCCG-3′ and 5′-CGGGCGCGG TCGGCGCCCCGCCGGTGGTCT-3′. The mutated gene was sequenced to confirm that only the desired mutation had occurred, by using an automated laser fluorescence DNA sequencer (Model 4,000L; LI-COR) and a ThermoSequenase fluorescent-labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for the sequencing reaction.

Production and Purification of OsChia1b and Its Mutants—Escherichia coli Origami B(DE3) cells carrying plasmid p22-cht2 or its derivatives were grown at 33°C in LB medium containing 100 μg/ml ampicillin. IPTG was added at $OD_{600} = 0.6$ and cultivation was continued for a further 48 h. *Escherichia coli* cells were harvested by centrifugation at 8 000 *g* for 10 min at 4°C. The cells were disrupted by sonication and the soluble protein fraction was prepared by centrifugation at 12 000 *g* for 15 min at 4°C. Proteins in the fraction were collected by ammonium sulphate precipitation (40% saturation), dissolved in 20 mM sodium phosphate buffer, and dialysed against the same buffer. Dialysed protein solution was applied onto a chitin affinity column (1.5 × 30 cm), filled with powdered crab shell chitin (Funakoshi Chemical Co.), previously equilibrated with 20 mM sodium phosphate buffer (pH 6.0) containing 0.5 M NaCl. The chitin affinity column was then washed with a five-column volume of 20 mM sodium phosphate buffer (pH 6.0) containing 0.5 M NaCl and a five-column volume of 20 mM sodium acetate buffer (pH4.0) at a flow rate of 1.5 ml/min. Then, absorbed proteins were eluted with 20 mM acetic acid and peak fractions containing OsChia1b or its mutants were collected, dialysed against 1 mM sodium phosphate buffer (pH 6.0), and lyophilized.

SDS–PAGE analysis of purified chitinases was conducted by the method of Laemmli (*23*).

Enzyme and Protein Assay—The reaction mixture for the chitinase assay contained purified chitinase and either 0.1% (w/v) reduced *N*-acetylchitopentaose [Red-$(GlcNAc)_5$], 0.2% (w/v) glycol chitin or 0.2% (w/v) colloidal chitin in 0.1 M sodium citrate buffer (pH 4.0). The reaction was performed at 37°C. The amount of reducing sugar generated was measured by a modified version of Schales' procedure (*24*) and 1 U of chitinase activity was defined as the amount of enzyme that produces 1 μmol of reducing sugar per minute.

Kinetic studies of wild-type and mutant chitinases were carried out using Red-$(GlcNAc)_5$ as the substrate. Reaction mixtures containing purified chitinase and various concentrations of the substrate in sodium citrate buffer (pH 4.0), were incubated at 37°C for 2 min. The reaction velocity was determined from the amount of reducing sugar produced, and plotted against the substrate concentration.

Protein concentration was estimated by measuring UV absorbance at 280 nm. The molar absorption coefficients used for the UV method were ε(OsChia1b) = 61,265, ε(OsChia1b$_{\Delta loopII}$) = 50,265, and ε(OsChia1b$_{W159A}$) = 55,765, which were calculated based on the deduced amino acid compositions of these proteins (*25*).

HPLC Analysis of Hydrolysis Products from (GlcNAc)$_6$—The hydrolysis of (GlcNAc)$_6$ by wild-type and mutant chitinases was carried out in 50 mM sodium acetate buffer pH 6.0 at 25°C. The concentration of chitinases was either 100 nM or 180 nM and that of the substrate was 2.5 mM. After incubation for a given period, the reaction mixture was filtered through a cellulose acetate membrane and quickly frozen in ethanol-dry ice bath. HPLC analysis of the hydrolysis products to monitor the time course of hydrolysis was carried out with a TSK amide 80 column using 70% acetonitrile as an elution solvent. The flow rate was 0.7 ml/min. The initial substrate and the oligosaccharide products were detected by ultraviolet absorption at 220 nm. The cleavage mode of (GlcNAc)$_6$ was qualitatively estimated from the α/β ratio of each oligosaccharide product in HPLC profiles.

Estimation of Sugar Residue Affinities of Individual Subsites—Theoretical analysis of reaction time courses obtained by OsChia1b and OsChia1b$_{\Delta loopII}$ was carried out using the reaction model reported for barley class II chitinase (*26*). Based on the splitting mode of the substrate estimated from HPLC profiles (Fig. 5), OsChia1b was assumed to possess subsites from −3 to +3. In the practical calculation, all of the possible binding modes were taken into consideration. Details of the calculation method are described in the previous report (*26*).

To estimate sugar residue affinities at individual subsites, an optimization technique based on the modified Powell method (*27*) was employed using the cost function,

$$F = \sum_i \sum_n [(\mathrm{GlcNAc})^c_{n,i} - (\mathrm{GlcNAc})^e_{n,i}]^2$$

where e and c represent experimental and calculated values, respectively, n is the size of oligosaccharides and i is the reaction time. Optimization was conducted by changing the binding free energy values of the individual subsites while fixing the estimated rate constant values. The k_{cat} values obtained for the substrate-reduced (GlcNAc)$_5$ (Table 1) were directly adopted for the rate constant value for cleavage of the glycosidic linkage (k_{+1}). At present, it is difficult to estimate the value of k_{+2} (rate constant for hydration) with sufficient accuracy. Thus, a higher value, 200.0 s^{-1}, was tentatively allocated to k_{+2}, because the bond cleavage process (k_{+1}) is a rate-limiting step in the entire course of enzymatic hydrolysis.

Chemicals—*N*-acetylchitooligosaccharides were purchased from Yaizu Suisan Chemical Co. (Shizuoka, Japan). Red-(GlcNAc)$_5$ was prepared from (GlcNAc)$_5$ as described by Yanase *et al*. (*28*). Colloidal chitin and ethylene glycol chitin were prepared from powdered crab shell chitin (Funakoshi Chemical Co., Tokyo, Japan) by the methods of Jeuniaux (*29*) and Yamada and Imoto (*30*).

Table 1. **Hydrolytic properties of OsChia1b and its mutants.**

OsChia1b	Specific activity (U/μmol of enzyme)		Kinetic parameter		
	GC	CC	Red-(GlcNAc)$_5$		
			K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M
Wild-type	232	191	0.33	4.32	13.2
Δloop II	479	265	0.72	10.6	14.8
W159A	946	399	0.69	20.9	30.3

GC, glycol chitin; CC, colloidal chitin; Red-(GlcNAc)$_5$, reduced *N*-acetylchitopentaose.

RESULTS

Structural Comparison of the Catalytic Domain of OsChia1b with that of Chitinase C from Streptomyces griseus and Designing Deletion Mutagenesis—OsChia1b consists of the N-terminal ChBD belonging to CBM family 18 and the catalytic domain belonging to GH family 19. The catalytic domain of OsChia1b has three loop structures, loop I, II and III, which are absent in the catalytic domain of *S. griseus* ChiC (*6*) and *S. coelicolor* ChiG (a single domain chitinase) (*20*). These loop structures may differentiate plant class I and class II chitinases from bacterial family 19 chitinases and plant class IV chitinases with respect to its enzymatic properties and biological function. Among these loop structures, we focused on loop II in the present study, because it is located at the reducing end side of the substrate-binding cleft and thus assumed to be involved in substrate binding. In order to test this assumption and to gain an insight into the function of class I and class II plant chitinases, construction of the deletion mutant of loop II region from the catalytic domain of OsChia1b was attempted.

Loop II corresponds to the amino acid sequence region from Thr156 to Trp169 (TGGWPTAPDGPFSW) and, thus, deletion of loop II was carried out by replacing the sequence region with Gly residue by referring to the structures (Fig. 1a) and amino acid sequence alignment (Fig. 1b) of ChiC and OsChia1b. In addition, replacement of Trp159 in loop II with Ala was carried out by site-directed mutagenesis, because this residue is in loop II and has been assumed to play an important role in the interaction of OsChia1b with a bound substrate.

Production and Purification of Mutant Chitinase—Wild-type OsChia1b and the two mutant chitinases, loop II-deletion mutant (OsChia1b$_{\Delta loopII}$) and W159A mutant (OsChia1b$_{W159A}$), were produced in *E. coli* cells harbouring a plasmid encoding either wild-type or mutated genes. The produced chitinases were extracted from cells, collected by ammonium sulphate precipitation and purified by chitin affinity column chromatography. The purified chitinases thus obtained were analysed by SDS–PAGE analysis as shown in Fig. 2. The protein band of OsChia1b$_{\Delta loopII}$ was detected at a position slightly smaller than those of wild-type OsChia1b and OsChia1b$_{W159A}$ as expected from the molecular sizes of these proteins. Circular dichroism (CD) spectra of mutant chitinases are similar to those of wild-type OsChia1b (data not shown). Mutant chitinases were

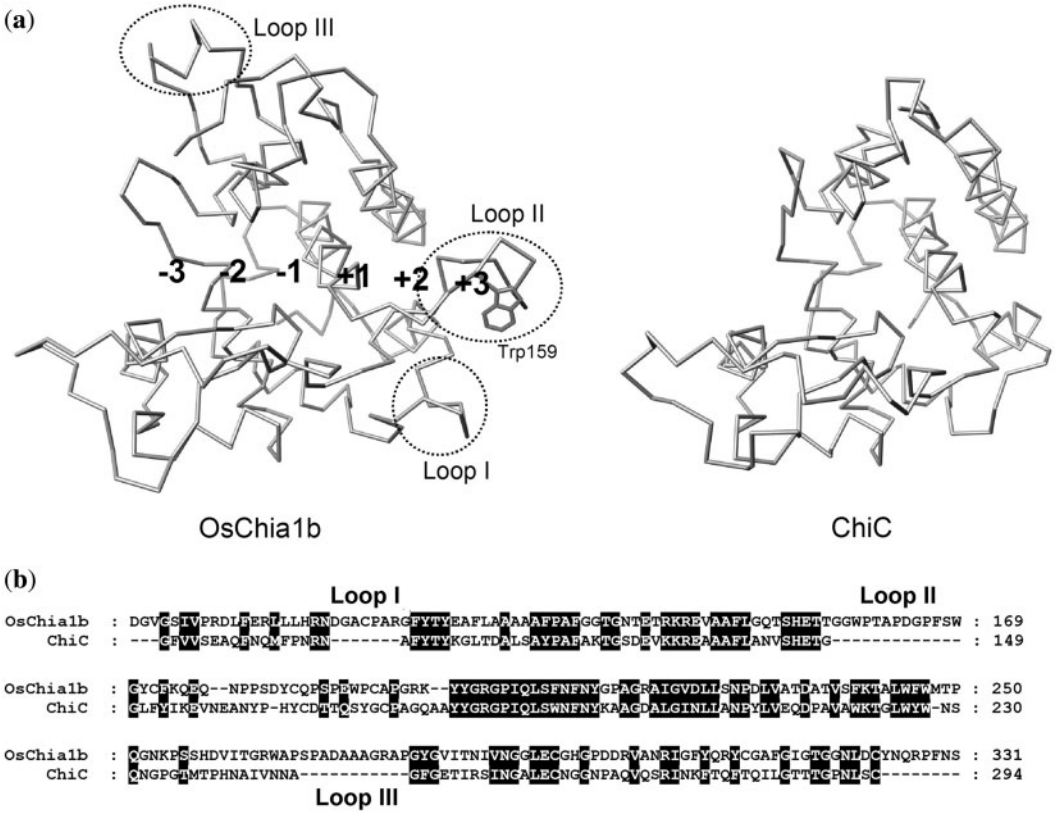

Fig. 1. **Comparison of three-dimensional structure (a) and amino acid sequences (b) between catalytic domains of OsChia1b (PDB accession code: 2DKV) and ChiC (PDB accession code: 1WVU).** Dotted circles indicate loop structures, which are absent in ChiC. Identical amino acids in the alignment are indicated by white type on a black background. Putative binding subsites are noted by the subsite number, –3, –2, –1, +1, +2 and +3.

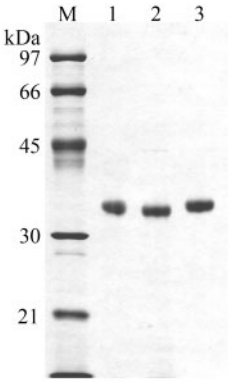

Fig. 2. **SDS–PAGE analysis of purified chitinases.** Purified chitinases (20 μg each) were applied and protein bands were visualized by Coomassie brilliant blue R-250 staining. Lane M, size markers; lane 1, OsChia1b; lane 2, OsChia1b$_{\Delta loopII}$; lane 3, OsChia1b$_{W159A}$.

produced at a level similar to that of wild-type OsChia1b. Approximately 0.6 mg of purified chitinases was obtained from 1 l culture of *E. coli* cells carrying each chitinase gene.

Effect of Mutations on the Hydrolytic of OsChia1b—The effect of mutations on hydrolytic activities towards an oligosaccharide substrate, Red-(GlcNAc)$_5$, and high Mr substrates, glycol chitin and colloidal chitin, were examined as shown in Table 1. Enzymatic hydrolysis was monitored by measuring the amount of reducing sugar released from these substrates. Interestingly, deletion of loop II and the W159A mutation enhanced hydrolytic activity towards the polysaccharide substrates, glycol chitin and colloidal chitin. Activity enhancement was more intensive in OsChia1b$_{W159A}$ than in OsChia1b$_{\Delta loopII}$.

Table 1 also shows kinetic parameters for the enzymatic hydrolysis of Red-(GlcNAc)$_5$. K_m values for mutant enzymes were about 2-fold higher than that of the wild-type, and k_{cat} values were also higher than that of the wild-type by 2.5–4.8-fold. Overall, the values of k_{cat}/K_m were found to be enhanced for both mutant enzymes. This is consistent with activity data for polysaccharide substrates. Activity enhancement induced by both mutations was also seen from the time course of enzymatic degradation of (GlcNAc)$_6$ (Fig. 3).

Effect of Mutations on the Splitting Specificity Towards (GlcNAc)$_6$ Substrate—Mutations on loop II, which is located at the end of the substrate-binding cleft, might alter the splitting specificity of the oligosaccharide substrate. To examine this effect, hydrolysis products generated from (GlcNAc)$_6$ were analysed by HPLC. The time courses of (GlcNAc)$_6$ hydrolysis by wild-type OsChia1b, and OsChia1b$_{\Delta loopII}$ were monitored by HPLC. As shown in Fig. 3a, wild-type OsChia1b produced

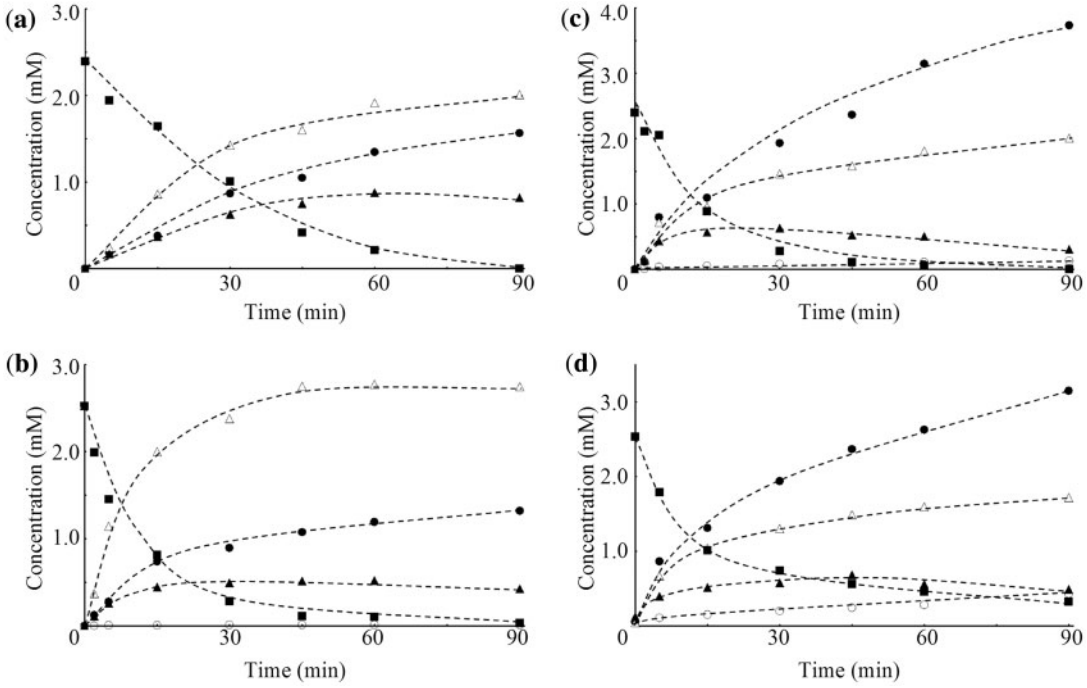

Fig. 3. **Time course of $(GlcNAc)_6$ hydrolysis by OsChia1b (a), OsChia1b$_{W159A}$ (b), OsChia1b$_{\Delta loopII}$ (c) and ChiC (d).** Chitinase concentration was either 180 nM (OsChia1b, OsChia1b$_{\Delta loopII}$), 100 nM (OsChia1b$_{W159A}$) or 72 nM (ChiC). Substrate concentration was 2.5 mM. The reaction was conducted in 50 mM sodium acetate buffer pH 6.0 at 27°C. Symbols: open circles, GlcNAc; filled circles, $(GlcNAc)_2$; open triangles, $(GlcNAc)_3$; filled triangles, $(GlcNAc)_4$; filled squares, $(GlcNAc)_6$. Broken lines were drawn by roughly following experimental data points.

$(GlcNAc)_3$ most abundantly, followed by $(GlcNAc)_2$ and $(GlcNAc)_4$ in this order. OsChia1b$_{W159A}$ also produced $(GlcNAc)_3$ predominantly and then $(GlcNAc)_2$ and $(GlcNAc)_4$ similarly (Fig. 3b). $(GlcNAc)_3$ production, however, was enhanced by W159A mutation when compared with the wild-type enzyme. On the other hand, OsChia1b$_{\Delta loopII}$ produced $(GlcNAc)_2$ predominantly, and then $(GlcNAc)_3$ and $(GlcNAc)_4$ (Fig. 3c). The amount of $(GlcNAc)_4$ produced by OsChia1b$_{\Delta loopII}$ was significantly smaller than that by wild-type OsChia1b, but increased up to 30 min and then gradually decreased. This observation suggested that the produced $(GlcNAc)_4$ was readily hydrolysed into two molecules of $(GlcNAc)_2$.

To compare the hydrolysis pattern of OsChia1b$_{\Delta loopII}$ with that of *S. griseus* ChiC that originally lacked loop II, hydrolysis of $(GlcNAc)_6$ by ChiC was also carried out. As shown in Fig. 3d, the pattern of hydrolysis products by ChiC was very similar to that by OsChia1b$_{\Delta loopII}$. In addition, a significant amount of GlcNAc monomer was detected as a hydrolysis product by ChiC. The monomer was also produced by OsChia1b$_{\Delta loopII}$ but was not detected in the reaction products by OsChia1b and OsChia1b$_{W159A}$.

Anomer formation from $(GlcNAc)_6$ by enzymatic hydrolysis was also analysed by HPLC using a partition column which can separate α- and β-anomers. HPLC profiles of hydrolysis products from $(GlcNAc)_6$ in the early reaction stage by individual chitinases are shown in Fig. 4. In the case of the wild-type enzyme, the α/β ratios of $(GlcNAc)_2$ and $(GlcNAc)_4$ were 26.8 and 2.95, respectively (Fig. 4a). Since GH family 19 enzymes produce only α-anomer through hydrolysing the β-glycosidic linkage (*11*, *20*, *31*), the almost exclusive generation of α-anomer of $(GlcNAc)_2$ and the low α/β ratio of $(GlcNAc)_4$ in the early reaction stage indicated that $(GlcNAc)_2$ and $(GlcNAc)_4$ are mainly generated by hydrolysis of the fourth linkage from the reducing end of $(GlcNAc)_6$. Similar cleavage frequency at the middle linkage of $(GlcNAc)_6$ was also found in the wild-type enzyme as deduced from Figs 3a and 4a. In OsChia1b$_{W159A}$, however, the α/β ratios of $(GlcNAc)_2$ and $(GlcNAc)_4$ were 8.62 and 10.4, respectively (Fig. 4b). The α/β values suggested that cleavage of the second linkage from the reducing end of $(GlcNAc)_6$ is relatively enhanced in the mutant enzyme. Deletion of loop II further increased the α/β ratio of $(GlcNAc)_4$ slightly and decreased the α/β ratio of $(GlcNAc)_2$ significantly as shown in Fig. 4c. Cleavage of the second linkage from the reducing end of $(GlcNAc)_6$ appears to be more enhanced in OsChia1b$_{\Delta loopII}$ than in OsChia1b$_{W159A}$. As in the case of the reaction time course (Fig. 3), the HPLC profile obtained with ChiC was similar to that obtained with OsChia1b$_{\Delta loopII}$ (Fig. 4d). Based on these HPLC analyses (Figs 3 and 4), we finally estimated the splitting mode of the wild-type and two mutant chitinases, as shown in Fig. 5.

Estimation of Binding Free Energy Changes of the Subsites—To examine the effect of mutation on sugar residue binding to individual subsites of the binding cleft, we tried to estimate the sugar residue affinity (binding free energy change) for individual subsites for

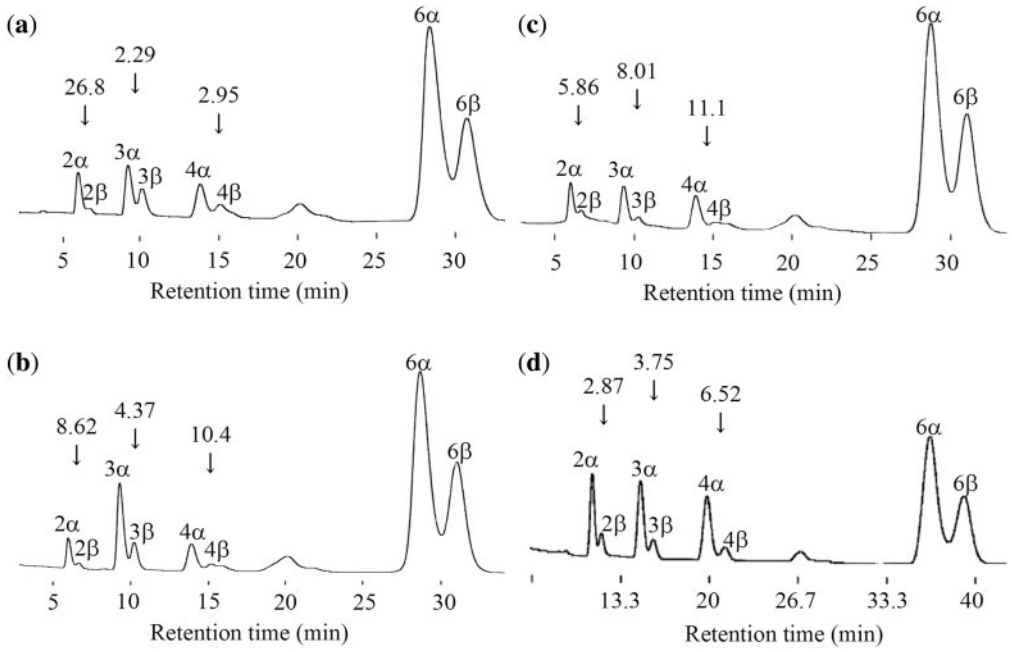

Fig. 4. **HPLC profile showing the hydrolysis of $(GlcNAc)_6$ by OsChia1b (a), OsChia1b$_{W159A}$ (b), OsChia1b$_{\Delta loopII}$ (c) and ChiC (d) in the early reaction stage.** The α- and β-anomers of $(GlcNAc)_2$, $(GlcNAc)_3$, $(GlcNAc)_4$ and $(GlcNAc)_6$ are labelled as 2α, 2β, 3α, 3β, 4α, 4β, 6α and 6β, respectively. Numerals on the vertical arrows indicate α/β ratios of produced oligosaccharides. The α/β ratio of $(GlcNAc)_6$ at equilibrium was ~1.8.

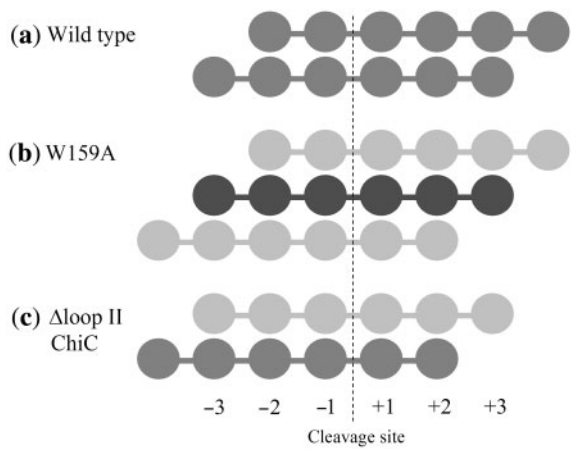

Fig. 5. **Binding mode of $(GlcNAc)_6$ to OsChia1b (a), OsChia1b$_{W159A}$ (b) and OsChia1b$_{\Delta loopII}$ (c).** Individual circles illustrate GlcNAc residues. The right end corresponds to the reducing end of the substrate. The density of the shadow of the circles reflects the population roughly estimated from the α/β ratio of each oligosaccharide product.

OsChia1b, OsChia1b$_{W159A}$ and OsChia1b$_{\Delta loopII}$. Based on the splitting mode of $(GlcNAc)_6$ (Fig. 5), the subsite structure of OsChia1b was assumed to be (–3) (–2) (–1) (+1) (+2) (+3) and was used for theoretical analysis of the reaction time course. From the 3D structure, the size of the substrate-binding cleft of OsChia1b was found to be consistent with the subsite model of (–3) (–2) (–1) (+1) (+2) (+3). To optimize binding free energy values for OsChia1b, OsChia1b$_{W159A}$ and OsChia1b$_{\Delta loopII}$, the initial estimated values were set at the free energy values for OsChia1c, as previously reported (*31*). Starting from these values, optimization was conducted by changing the free energy values to fit the experimental time course.

The calculated time courses best fitting the experimental time courses of OsChia1b and OsChia1b$_{\Delta loopII}$ are shown in Fig. 6 and the optimized values of binding free energy change finally obtained are listed in Table 2. The results indicate that loop II deletion decreases sugar residue affinity at subsite +3 by 2.5 kcal/mol. It is clear that loop II truly contributes to form this subsite, as estimated from the 3D structure of OsChia1b. Unfortunately, data fitting for the time course of $(GlcNAc)_6$ degradation by OsChia1b$_{W159A}$ was not successful because of its poor fitness, so a very high cost function (Eq. 1) was obtained for this mutant enzyme, thus, sugar residue affinities were not obtained for OsChia1b$_{W159A}$.

DISCUSSION

Three loop structures found in the crystal structure of GH family 19 chitinase from rice are missing in *Streptomyces* family 19 chitinases (ChiC and ChiG). Such flexible loop structures have been recognized to participate in the substrate binding in various glycoside hydrolases (*32–34*). Among the three loop structures, loop II is located at the right-endmost site (+3) of the putative substrate binding cleft and assumed to be involved in substrate binding (Fig. 1). To examine the role of loop II, we produced, purified and characterized the two mutant enzymes, one of which has deletion of the entire loop II

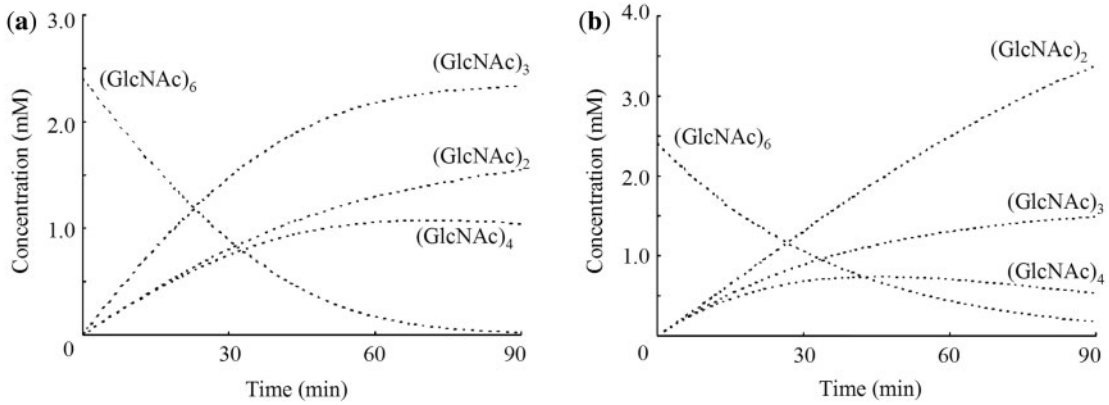

Fig. 6. **Theoretical time courses of OsChia1b (a) and OsChia1b$_{\Delta loopII}$ (b) obtained by data fitting to the experimental time courses.** The free energy changes of sugar residue binding obtained from data fitting are listed in Table 2.

Table 2. **Estimated free energy changes of subsites for OsChia1b and OsChia1b$_{\Delta loopII}$.**

OsChia1b	Binding free energy change (kcal/mol)					
	Subsite					
	(−3)	(−2)	(−1)	(+1)	(+2)	(+3)
Wild-type	0.0	−5.0	+4.1	−0.5	−3.8	−2.0
Δloop II	0.0	−5,0	+4.1	−0.5	−3.8	+0.5

region and the other which has substitution of Trp159 in loop II with alanine.

Loop II deletion and the W159A mutation significantly enhanced hydrolytic activities not only towards polysaccharide substrates, colloidal chitin and glycol chitin (Table 1), but also towards $(GlcNAc)_6$ (Fig. 3). Activity enhancement of both mutant enzymes was also seen from the values of k_{cat}/K_m towards reduced-$(GlcNAc)_5$. It should be noted that activity enhancement was derived from the higher values of turnover (k_{cat}), especially in OsChia1b$_{W159A}$. Structural changes at the endmost site of the substrate-binding cleft induced by the mutations might reduce the sugar residue affinity at this region, enhancing the release of hydrolysis products. This situation might increase the k_{cat} value. In fact, from theoretical analysis of the reaction time course, sugar residue affinity at the +3 subsite was found to be reduced by –2.5 kcal/mol through loop II deletion. Lower affinity at the +3 subsite might thus bring about activity enhancement of the mutant enzymes.

Anomer analysis of hydrolysis products from $(GlcNAc)_6$ by HPLC afforded information on the splitting mode of the substrate. Since the newly produced reducing end is exclusively α-anomer, α/β ratios of oligosaccharide products containing new reducing ends should be higher than those containing original reducing ends, in which mutarotation attains equilibrium. Based on this criterion, we found that the loop II deletion and W159A mutation shift the splitting positions of $(GlcNAc)_6$ to the reducing end side, but the shift is less intensive in the W159A mutant. From these results, $(GlcNAc)_6$ binding modes to the enzyme binding cleft were estimated as shown in Fig. 5. The lower affinity to the sugar residue at the +3 subsite would affect the binding mode of the substrate, shifting the substrate molecule to the non-reducing end side. Finally, we concluded that loop II is involved in sugar residue binding at the +3 subsite of OsChia1b.

As described in RESULTS section, data fitting was not successful for the W159A mutant. Unexpected binding modes such as 1:2 (enzyme:substrate) complexes, which can be neglected in reactions catalysed by the wild-type and Δloop II mutant, might occur in the course of $(GlcNAc)_6$ hydrolysis catalysed by OsChia1b$_{W159A}$. Nevertheless, from the substrate-binding modes estimated from HPLC data (Fig. 5), the +3 subsite of OsChia1b$_{W159A}$ was supposed to have a sugar residue affinity lower than that of the wild-type, but higher than that of OsChia1b$_{\Delta loopII}$.

The subsite structure from the –3 subsite to +3 subsite has been reported for class II chitinases from barley seeds (*26*) and a rice class I chitinase, OsChia1c (*31*). The present study indicated that OsChia1b also has a subsite structure similar to those of plant chitinases, and that loop II is required for sugar residue binding to subsite +3. Hoell *et al*. (*20*) reported that ChiG lacking loop II has only four subsites (–2 to +2), unlike plant chitinases. Their results are in very good agreement with the results obtained in this study; that is, OsChia1b$_{\Delta loopII}$ lacking loop II does not have significant sugar residue affinity at the +3 subsite (+0.5 kcal/mol). GH family 19 chitinase from *Aeromonas* sp. No.10S-24 was also examined with respect to binding subsites (*35*, *36*). *Aeromonas* chitinase was reported to possess the subsite structure from –2 subsite to +4 subsite. This is considerably different from the subsite structures estimated for GH family 19 chitinases from plants and *Streptomyces*. The *Aeromonas* enzyme has two ChBDs at the N-terminus and a unique catalytic domain (46.7 kDa), which is less homologous to other GH family 19 catalytic domains. This unique structural feature might form a subsite arrangement different from those of other GH family 19 chitinases.

Among the three loop structures, loop I is adjacently located to loop II on the same side of the domain as shown in Fig. 1a. Loop I contains Cys110 that forms

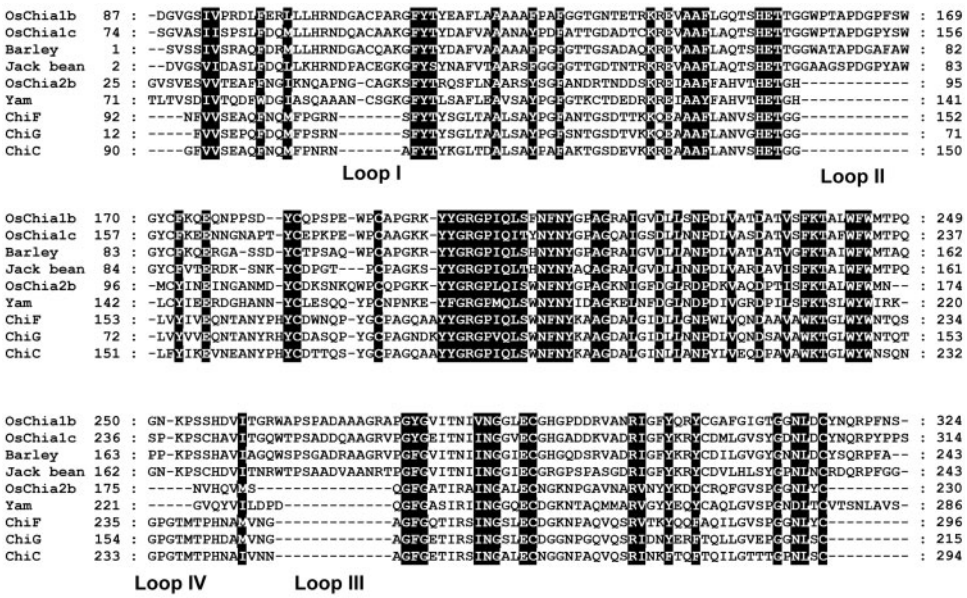

Fig. 7. **Amino acid sequence alignment of the catalytic domains of family 19 chitinases.** Sequence alignment was performed using the CLUSTAL X program. Amino acid residues conserved in all sequences are indicated by black backgrounds. The labels, Loop I, II and III indicate the loop regions, which are absent in *Streptomyces* chitinases, ChiC, ChiG and ChiF. The label, Loop IV, indicates the loop region, absent in class IV chitinases. OsChia1b, rice class I chitinase (accession number D16222); OsChia1c, rice class I chitinase (D16223); Barley, barley class II chitinase (M62904), Jack bean, jack bean class II chitinase (AJ006992); Oschia2b, rice class II chitinase with class IV type catalytic domain (AB003194); Yam, yam class IV chitinase (BAC56863); ChiC, chitinase C from *S. griseus* (AB009289); ChiF, chitinase F from *S. coelicolor* (AB017012); ChiG, chitinase G from *S. coelicolor* (AB017013).

a disulphide bond with Cys172 at the base of loop II and this loop has been considered to maintain the stability of loop II. On the other hand, loop III is located relatively distant to the catalytic site and it is thus difficult to predict its functional role. In addition to these three loops, there is one major structural difference between plant chitinases (class I and II) and *Streptomyces* family 19 chitinases as pointed out by Hoell *et al*. (*20*), and it is found in the structural region corresponding to '161–166 loop' (Ala161 to Pro166 in barley chitinase). The '161–166 loop' corresponds to the 152–158 loop of *S. coelicolor* ChiG, 231–237 loop of *S. griseus* ChiC and 248–253 loop of OsChia1b. To avoid confusion, these loops are temporarily named loop IV. Loop IV of barley chitinase contains Gln162 and Lys165, which has been suggested to be important for sugar binding to subsites –3 and –4. Loop IV of OsChia1b also contains corresponding residues Gln249 and Lys252.

As shown in Fig. 7, as well as *Streptomyces* chitinases, plant class IV chitinases have deletion of the region corresponding to loop II, however there is a considerable difference between *Streptomyces* chitinases and plant class IV chitinases. Plant class IV chitinases have a small deletion (the region corresponding to Pro248 to Pro253 of OsChia1b) in addition to a longer deletion of the loop III region, as shown. This small deletion corresponds to the region of loop IV in other chitinases, therefore, class IV chitinase most probably lacks loop IV and, therefore, has a structure considerably different from both *Streptomyces* chitinases and plant class I, II chitinases in this region. This means that the structures of the corresponding regions in the three types of family 19 chitinases are all different and these differences would affect the substrate-binding properties of each chitinase. In order to study the effect of such structural differences on enzymatic properties, construction of the expression system of rice class IV chitinase is now underway.

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