Rice chitinases: sugar recognition specificities of the individual subsites

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Sugar recognition specificities of class III (OsChib1a) and class I (OsChia1cChBD) chitinases from rice, Oryza sativa L., were investigated by analyzing 1H- and 13C-nuclear magnetic resonance spectra of the enzymatic products from partially N-acetylated chitosans. The reducing end residue of the enzymatic products obtained by the class III enzyme was found to be exclusively acetylated, whereas both acetylated and deacetylated units were found at the nearest neighbor to the reducing end residue. Both acetylated and deacetylated units were also found at the nonreducing end residue and its nearest neighbor of the class III enzyme products. Thus, only subsite (–1) among the contiguous subsites (–2) to (+2) of the class III enzyme was found to be specific to an acetylated residue. For the class I enzyme, the reducing end residue was preferentially acetylated, although the specificity was not absolute. The nearest neighbor to the acetylated reducing end residue was specifically acetylated. Moreover, the nonreducing end residue produced by the class I enzyme was exclusively acetylated, although there was a low but significant preference for deacetylated units at the nearest neighbor to the nonreducing end residue. These results suggest that the three contiguous subsites (–2), (–1), and (+1) of the class I enzyme are specific to three consecutive GlcNAc residues of the substrate. In rice plants, the target of the class I enzyme might be a consecutive GlcNAc sequence probably in the cell wall of fungal pathogen, whereas the class III enzyme might act toward an endogenous complex carbohydrate containing GlcNAc residue.

Key words: chitinase/Oryza sativa L./partially N-acetylated chitosan/specificity/subsites

Introduction

It has been recognized that plant chitinases are major components of pathogenesis-related proteins, which are induced in response to pathogenic attack and play an important role in self-defense against pathogen (Schlumbaum et al., 1986; Brogie et al., 1991; Collinge et al., 1993). Chitinases catalyze the hydrolysis of N-acetyl-β-D-glucosaminide 1,4-linkages in chitin and chitodextrins, which do not occur in plants themselves. Therefore, the chitinous components of the cell wall of fungal phytopathogen have been regarded as the chitinase targets. Recently, however, several reports indicated that plant chitinases are induced not only on the pathogenic attack but on various environmental stresses, such as osmotic pressure, drought, salinity, wounding, and frost (Hamel and Bellemare, 1995; Yun et al., 1996; Yeh et al., 2000; Pinheiro et al., 2001; Tateishi et al., 2001). Moreover, recent studies suggest that the enzymes regulate growth and developmental processes by generating or degrading signal molecules (van der Holst et al., 2001; Kasprzewska, 2003). In these cases, an endogenous complex carbohydrate possessing N-acetyl-β-D-glucosaminide linkages, such as glycoprotein or glycolipid, might be a possible chitinase target, which mediates the signal transduction in such a biological process. Thus, to correctly understand the physiological roles of plant chitinases, it is very important to identify the chitinase target.

According to Neuhaus and others (1996), plant chitinases are divided into several classes (I, II, III, IV, V, and VI) based on their sequence similarity. The classes I, II, and IV correspond to family GH-18 according to the CAZY database (Henrissat, 1997; Sasaki et al., 2002, 2003). The retaining chitinases have an N-terminal chitin-binding domain (ChBD), but the molecular sizes of class IV chitinases are smaller than those of class I chitinases due to deletions in both ChBD and the catalytic domain. Class II chitinases are homologous to those of class I and IV enzymes but do not have ChBD (Collinge et al., 1993). The individual classes of chitinases were found to have different substrate specificities and reaction mechanisms. For example, in tobacco plant, the class III chitinases exhibit a significant level of lysozyme activity together with the chitinase activity, whereas the class VI chitinases have only chitinase activity (Brunner et al., 1998). Class I and II chitinases hydrolyze β-glycosidic linkage through an inverting mechanism, whereas class III chitinases through a retaining mechanism (Fukamizo et al., 1995; Iseli et al., 1996; Hollis et al., 1997; Sasaki et al., 2002, 2003). The retaining chitinases require specifically an N-acetylglosamnine (GlcNAc or A) unit at subsite (–1), whereas the inverting chitinases do not. Chitinases may also differ with respect to the specificity for their mode of action, that is, endo- or exo-mode of action, both of which can be either processive or nonprocessive.

Several chitinases from rice were produced using the Pichia pastoris expression system, and the enzymatic properties were characterized (Park et al., 2002; Truong et al., 2006; Park et al., 1996).
Among the class III enzymes from rice, the chitinase activity of OsChib1b was found to be lower than that of OsChib1a. The former enzyme showed lytic activity toward Micrococcus lysodeikticus cells and antifungal activity toward Trichoderma reesei, whereas no lytic or antifungal activity was observed for the latter. pH optima of the class III enzymes were 8.4 for the former and 4.3 for the latter. The rice class I enzyme, OsChia1c, also exhibited antifungal activity as well as chitinase activity, and the pH optimum was 4.8. It appears that specificity and other enzymatic properties of the rice chitinases are more diverse than expected even among the identical class of the enzymes. To examine the mechanism of substrate recognition, we have estimated and refined the subsite structure of various types of chitinases from rice, Oryza sativa L., based on the time-course of enzymatic hydrolysis of hexasaccharide of GlcNAc. The two class III chitinases from rice (OsChib1a and OsChib1b) were found to have a subsite structure represented by \((-4)(-3)(-2)(-1)(+1)(+2)\) (Sasaki et al., 2002), and the class I chitinases (OsChia1c and OsChia1cChBD) from rice possess a subsite structure \((-3)(-2)(-1)(+1)(+2)(+3)\) (Sasaki et al., 2003). As a succeeding step for understanding more closely the substrate recognition mechanism, we have to determine sugar recognition specificity of the individual subites of the chitinases. Mitsutomi and his coworkers determined the sugar sequence of the products from the chitinase digestion of partially N-acetylated chitosans (Mitsutomi, Hata, et al., 1995; Mitsutomi, Kidoh, et al., 1995; Koga et al., 1999). The sequence data provided information on the sugar recognition specificity at subsites \((-1)\) and \((+1)\) of the enzymes. Recently, a more convenient technique has been reported by Vårum and others (1996). This method has enabled the determination of sugar recognition specificity of the contiguous subites from \((-2)\) to \((+2)\) of chitinolytic enzymes based on \(^{1}H\)- and \(^{13}C\)-nuclear magnetic resonance (\(^{13}C\)-NMR) spectra of the enzymatic products obtained from partially N-acetylated chitosans.

To get insight into the physiological role of plant chitinases, we analyzed the enzymatic products obtained from the substrate partially N-acetylated chitosans by \(^{1}H\)- and \(^{13}C\)-NMR spectroscopy using class III chitinase (family GH-18, OsChib1a) and class I chitinase without ChBD (family GH-19, OsChia1cChBD) from rice (O. sativa L.). Based on the identity of the sugar unit (N-acetylglucosamine [GlcNAc or A] or glucosamine [GlcN or D]) at the reducing and nonreducing ends as well as the identity of their nearest neighbors, the sugar recognition specificities at individual subites from \((-2)\) to \((+2)\) in the substrate-binding cleft were estimated and compared between the two rice chitinases.

Results

Mode of action of rice chitinases toward partially acetylated chitosans

Information on sugar recognition specificity of individual subites of chitinases can be obtained from the sugar residue identity of the reducing and nonreducing ends as well as their nearest neighbors of the enzymatic products. This analysis is suitable for chitinases operating to a nonprocessive endo-mode of action, because many of the products from the other mode of actions (monomer or dimer) would complicate the interpretation of the NMR spectra with respect to the identification of reducing and nonreducing ends and their nearest neighbors. Thus, we at first examined the mode of action of rice chitinases (endo or exo; processive or nonprocessive); that is, the size distribution of the enzymatic products produced on the cleavage of the chitosan (degree of acetylation \(F_{A} = 0.64\)) was characterized to reveal information regarding their mode of action. As can be seen from the chromatograms (Figure 1), rice chitinases seem to operate according to a nonprocessive endo-mode of action as the size distribution shows a continuum of the size of the oligomers without any preference for the formation of even- or odd-numbered oligomers, as previously found for other chitinases (Sorbotten et al., 2005; Horn et al., 2006). Thus, we concluded that the rice chitinases tested are suitable for determining sugar recognition specificity of the individual subites from sugar residue identity of the reducing and nonreducing ends as well as their nearest neighbors by NMR spectroscopy of the enzymatic products.

Class III chitinase

At first, the class III chitinase from rice was used to hydrolyze partially N-acetylated chitosan \(F_{A} = 0.64\). The \(^{1}H\)-NMR spectra of the enzymatic products from the chitosan are shown in Figure 2A. The number-average degree of polymerization \((DP_{n})\) was reduced to 14 by 20 min incubation with the enzyme. The \(\alpha\)-anomer signal of the reducing end GlcNAc is located at 5.19 ppm, whereas the signal for the reducing end GlcN (\(\alpha\)-form) could not be detected at all (5.43 ppm). The \(\beta\)-anomer signal of the reducing end GlcNAc (appearing as a doublet) was found to split into two doublets depending on the neighboring unit to the...
reducing end: \(-\text{AA} (4.70 \text{ ppm})\) and \(-\text{DA} (4.75 \text{ ppm})\), as previously described (Sørbotten et al., 2005). The signal intensity of \(-\text{AA}\) was higher than that of \(-\text{DA}\) in the case of this chitosan substrate (\(F_A = 0.64\)). A similar experiment was conducted with the chitosan substrate (\(F_A = 0.32\)), and the result is shown in Figure 2B. As to the reducing end \(\alpha\)-anomer signal, we found exclusively an acetylated unit. With this substrate, the doublet signal intensity of \(-\text{AA} (4.70 \text{ ppm})\) was lower than that of \(-\text{DA} (4.75 \text{ ppm})\), reflecting the relative abundance of \(A\)- and \(D\)-units in the substrates. The ratio between the two signal intensities was similar to the ratio between the \(A\)- and \(D\)-units in the substrate. This would be the expected result when the enzyme is absolutely specific to acetylated units at subsite (–1) but with no specificity at subsite (–2).

Further analysis was performed using \(^{13}\text{C}\)-NMR spectra of the enzymatic products as previously assigned (Vårum et al., 1996). The results are shown in Figures 3 and 4. The C5 signals of acetylated and deacetylated units at nonreducing ends were detected at 78.4 and 79.0 ppm, respectively. When the chitosan with \(F_A = 0.64\) was used as the substrate (Figure 3), both signal intensities increased with the reaction time. The ratio between the signal intensities of \(D\)- and \(A\)-units was approximately 2:3 (0.67), which is similar to the \(F_A\)-value of the chitosan substrate (0.64). The nonreducing end C3 signal of the acetylated unit was found to split into two signals (76.0 and 76.1 ppm) depending on the neighboring residue, \(\text{AA}\) or \(\text{AD}\), indicating that both acetylated and deacetylated units were found at the nearest neighbor to the nonreducing end \(A\)-unit. The \(^{13}\text{C}\)-NMR analysis was also conducted using the hydrolytic products from the chitosan substrate with \(F_A = 0.32\) (Figure 4). The newly produced nonreducing ends of both acetylated and deacetylated units were also found at C5 region (78.4 and 79.0 ppm), and the ratio of the signal intensities was 3:10 (0.3), which is comparable with the \(F_A\)-value of the substrate. The nonreducing end C3 signal (76.0 and 76.1 ppm) was not clearly detected in this case. Nevertheless, it is evident that both the nonreducing end residue and its nearest neighbor of the enzymatic products were both acetylated and deacetylated units and consequently that the (+1) and (+2) sites are not specific to \(A\)- or \(D\)-unit. These results clearly demonstrated that the class III chitinase is specific to acetylated units only at subsite (–1) but not specific at subsites (–2), (+1), and (+2).

Class I chitinase without ChBD

Next, the class I chitinase without ChBD was employed for the chitosan hydrolysis experiments. In the previous article (Sasaki et al., 2003), the kinetic properties of class I chitinase (OsChia1c) and the ChBD-truncated enzyme (OsChia1cΔChBD) toward GlcNAc hexasaccharide were examined, but no significant change in the kinetic properties was observed by the ChBD truncation. ChBD of the class I enzyme is unlikely to participate in the oligosaccharide binding to the enzyme. In this study, we examined the sugar recognition specificity only in the active site region...
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from (–2) to (+2). Thus, the ChBD truncation would not affect the specificity data of the class I enzyme.

1H-NMR spectra of the enzymatic products obtained by the rice class I enzyme are shown in Figure 5. When the chitosan with \( F_A = 0.64 \) was hydrolyzed by the enzyme for 5 min, the newly produced reducing end was found to be exclusively acetylated units (Figure 5A, 5.19 ppm). The chitosan substrate with lower \( F_A \) (0.32) was not efficiently hydrolyzed by the enzyme. Ten days of incubation was needed for obtaining detectable signals of the newly produced reducing ends (Figure 5B). Both the acetylated and deacetylated signals were found at the reducing end of the enzymatic products from the lower \( F_A \) chitosan substrate. However, despite the lower \( F_A \) of the substrate, the acetylated residue still predominated at the reducing end residue of the enzymatic products. Thus, the reducing end residue was preferentially acetylated but not absolutely. As in the case of class III enzyme, the class I enzyme preferentially binds acetylated units at subsite (–1), even though subsite (–1) of the class I enzyme is somewhat less specific to an acetylated unit than that of class III enzyme. The doublet signal of \( \beta \)-anomer of the reducing end GlcNAc was found only at 4.70 ppm and did not split at all for any of the chitosan substrates (\( F_A = 0.32 \) and \( F_A = 0.64 \)). These results indicate that the nearest neighbor to the reducing end A-unit was exclusively acetylated. Thus, the class I enzyme is highly specific to acetylated units at subsite (–2) and also specific to acetylated units at subsite (–1).

Then, the reaction products were also analyzed by 13C-NMR spectroscopy to obtain information on the nonreducing ends of the reaction products. Figure 6 shows the 13C-NMR spectra of the enzymatic products from the chitosan with \( F_A = 0.64 \). As shown in this figure, the C5 signal of the nonreducing end GlcNAc was found at 78.5 ppm, whereas the signal of the nonreducing end GlcN was not detected at the corresponding position (79.0 ppm). Thus, the nonreducing ends of the products are exclusively acetylated. In the nonreducing end C3 region (76.0 and 76.1 ppm), the intensity of \( \Delta_D^- \) was higher than that of \( \Delta_A^- \) (Figure 6B). Because the fraction of acetylated units is higher in the substrate chitosan, the class I enzyme has a low but significant preference toward deacetylated units at subsite (+2). Then, the sequence -AAAD- in the chitosan chain preferentially binds...
Fig. 4. C5 and C3 regions of 13C-NMR spectra of the class III chitinase products from chitosan with $F_A = 0.32$. The chitosan with $F_A = 0.32$ (12.5 mg/mL) was hydrolyzed by 2 μM class III chitinase for 15 min (A), 50 min (B), and 52 h (C) at 40°C. $D_P$-values were calculated to be 37 (A), 17 (B), and 6 (C) from the anomeric proton signals of the 1H-NMR spectra. The symbols used for the signal assignments are the same as in Figure 2.

Discussion

To analyze sugar recognition specificity of plant chitinases, we produced and employed class III and I chitinases without ChBD for chitosan digestion experiments. Because ChBD does not affect oligosaccharide-binding ability to the active site, the data obtained for class I enzyme without ChBD would be true for the mature class I enzyme. Figure 8 shows the sugar recognition specificities of the individual subsites of the substrate-binding cleft of the rice chitinases. The specificity of the class III chitinase is a great contrast to that of the class I enzyme. The class III enzyme has a specificity to GlcNAc only at subsite (–1), whereas the class I enzyme is specific to GlcNAc at the three contiguous subsites (–2) to (+1).

Mitsutomi and coworkers reported the sugar recognition specificities at subsites (–1) and (+1) of various chitinases (Mitsutomi, Hata, et al., 1995; Mitsutomi, Kidoh, et al., 1995; Koga et al., 1999). They purified the oligosaccharide fragments produced by the chitinase digestion of partially N-acetylated chitosan and analyzed the sequences of the fragments to determine the sugar recognition specificity. As a result, they found that GlcNAc is absolutely specific to subsite (–1) of family GH-18 chitinases but not specific to (+1), whereas in family GH-19 chitinases GlcNAc is absolutely specific to (+1) but not to (–1). The data presented in this article for class III chitinase from rice (family GH-18) are completely consistent with the Mitsutomi’s findings. Family GH-18 chitinases have been recognized to catalyze the hydrolytic reaction through substrate-assisted mechanism (Tews et al., 1997), in which the oxocarbonium ion intermediate is stabilized by an anchimeric assistance of N-acetyl group of the (–1) sugar. Thus, the specificity data for the class III enzyme are consistent with the catalytic mechanism generally accepted. On the contrary, for class I chitinase without ChBD (family GH-19), the data contain some inconsistency with the Mitsutomi’s findings, especially for the specificity at subsite (–1). As shown in Figure 5B, however, both the acetylated and deacetylated signals were found at the reducing end of the enzymatic products from the lower $F_A$ chitosan substrate ($F_A = 0.32$). Subsite (–1) of the class I chitinase from rice is less specific to GlcNAc when compared with class III chitinase from rice. However, despite the lower $F_A$ of the substrate, the acetylated residue still predominated at the reducing end residue of the enzymatic products, and from the chitosan substrate with $F_A = 0.64$, the reducing end residues of the products are absolutely GlcNAc. Thus, we conclude that the subsite (–1) of the class I chitinase from rice binds preferentially GlcNAc but that the specificity is not absolute. This is consistent with the mechanism of the family GH-19 enzymes (single-displacement mechanism), which do not require an acetylated unit at subsite (–1) (Fukamizo, 2000).

The findings in this study provide information on the chitin targets in rice plants. The class I enzyme is likely to act toward a consecutive GlcNAc sequence. In the early stage of pathogenesis, the apoplastic chitinases (class IV, family GH-19; de A Gerhardt et al., 1997) appear to release elicitor molecules from the cell wall of fungal pathogen, and the released compounds, probably GlcNAc oligosaccharides, elicit the defense system. After eliciting the system, vacuolar chitinases (class I, family GH-19; Collinge et al., 1993) degrade the newly synthesized chitin chain of the fungal pathogen, inhibiting the fungal growth. In this defensive action, plant chitinases should hydrolyze a consecutive GlcNAc sequence of the chitinous components of the fungal cell wall. The class I chitinase examined in this study is most likely to function in the defense against fungal pathogens. On the contrary, for the class III chitinase, GlcNAc specificity was found only at subsite (–1), indicating that the target of the class III enzyme is not necessarily a consecutive GlcNAc sequence. It is possible that the class III rice chitinase examined in this study might act toward GlcNAc-containing glycolipid or glycoprotein, producing or degrading the signal molecules to control important biological processes other than pathogenesis. However, it is also known that chitin that is present in the cell wall of mature hyphae is subjected to intensive modifications including deacetylation (Ruiz-Herrera and Martinez-Espinoza, 1999). Such a partially deacetylated chitin in the cell wall might be recognized and hydrolyzed by the class III
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Thus, the possibility that the class III enzyme acts in the defensive action against pathogens cannot be ruled out at present. Although class III chitinase from *Arabidopsis thaliana* was reported to act in cell wall remodeling or developmental regulation (Samac and Shah, 1991; Kwon *et al.*, 2005), these physiological functions cannot be directly connected with the specificity data obtained in this study. To clarify this problem, it is highly desirable to make clear the localization of these chitinases and to find endogenous substrates in plants themselves.

Using the chitinase genes (class I gene [accession number M38240] and class III gene [accession number M34107]) obtained from *A. thaliana*, we also analyzed the expression levels of the genes in each organ at the various developmental stages of the *Arabidopsis* plant and under various stress conditions by RT–PCR and quantitative real-time PCR (Tamoi *et al.*, unpublished data). As a result, the class I enzyme gene was found to be constitutively expressed in the entire developmental stages, whereas the class III gene was not expressed at any developmental stages and in any plant organs. On the contrary, the class III enzyme gene was found to be expressed when the *Arabidopsis* plants were exposed to various environmental stresses, such as cold, drought, high light, salt, and chemical treatment with paraquat and ethephon. Obviously, the class III enzyme has a different physiological function from that of the class I enzyme and is likely responsible for the transduction of environmental stress signals. These physiological data appear to be consistent with the sugar recognition specificities presented in this article and will be reported elsewhere.

**Materials and methods**

**Materials**

*Pichia* expression kit was the product of Invitrogen (Tokyo, Japan). A Ni$^{2+}$-chelating affinity column (His-Trap, GE Healthcare, Piscataway, NJ) was purchased from Pharmacia Biotech (Tokyo, Japan). The pepton for culture medium was purchased from Sigma (Tokyo, Japan), and another chemicals used for culture medium were purchased from Difco Laboratories (Tokyo, Japan). Deuterated products used for NMR measurements were purchased from Cambridge Isotope Laboratories (Andover, MA). Other reagents were of analytical grade commercially available.

**Production of chitosans**

Chitin was isolated from shrimp shells. Two chitosan samples with different degrees of N-acetylation were prepared by the homogeneous deacetylation of chitin (Sannan *et al.*, 1976). $F_A$-values were determined to be 64% ($F_A = 0.64$) and 32% ($F_A = 0.32$) from $^1$H-NMR spectra (Vårum *et al.*, 1991). $DP_n$-values could not be obtained, because the NMR signals for reducing end residues were hardly detected for these chitosan preparations. Instead, the average molecular weights of the chitosans were determined to be 320,000 ($F_A = 0.64$) and 220,000 ($F_A = 0.32$) from intrinsic
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viscosity measurements. Thus, $DP_n$-values of the chitosans were estimated to be $>1000$.

**Enzyme expression and purification**

Details of the strain construction were described in the articles published previously (Park et al., 2002; Truong et al., 2003). Briefly, chitinase genes encoding the mature part of class III chitinase (OsChib1a) and class I chitinase without ChBD (Oschia1cΔChBD) were kindly donated by Dr. Yoko Nishizawa, National Institute of Agrobiological Resources, Japan, and fused to the His-tag coding sequence at the 3’ end. The chitinase genes containing His-tag were expressed in yeast *P. pastoris* as follows: *P. pastoris* strains harboring the recombinant chitinase genes were grown in YPD (1% yeast extract, 2% pepton, 2% dextrose) for general growth and BMGY (1% yeast extract, 2% pepton, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, $4 \times 10^{-5}$% biotin, 1% glycerol) or BMMY (1% glycerol in BMGY is replaced with 1% methanol) medium to induce chitinase gene expression. After the induction of the chitinase gene, the culture supernatant was applied onto a Ni$^{2+}$-chelating affinity column, using 20 mM phosphate buffer, pH 7.6, containing 10 mM imidazole and 0.5 M NaCl. The protein adsorbed was eluted with the same buffer containing 0.5 M imidazole and 0.5 M NaCl.

**Fig. 6.** C5 and C3 regions of $^{13}$C-NMR spectra of the class I chitinase products from chitosan with $F_A = 0.64$. The chitosan with $F_A = 0.64$ (8.85 mg/mL) was hydrolyzed by 0.002 μM class I chitinase without ChBD for 5 min (A) and by the same enzyme (0.02 μM) for 10 min (B) at 40°C. $DP_n$-values were calculated to be 17 (A) and 11 (B) from the anomeric proton signals of the $^1H$-NMR spectra. The symbols used for the signal assignments are the same as in Figure 2.

**Fig. 7.** C5 and C3 regions of $^{13}$C-NMR spectra of the class I chitinase products from chitosan with $F_A = 0.32$. The chitosan with $F_A = 0.32$ (12.5 mg/mL) was hydrolyzed by 0.2 μM class I chitinase without ChBD for 76 h (A) and 10 days (B) at 40°C. $DP_n$-values were calculated to be 26 (A) and 18 (B) from the anomeric proton signals of the $^1H$-NMR spectra. The symbols used for the signal assignments are the same as in Figure 2.

**Fig. 8.** Sugar recognition specificity of class I and III chitinases from rice, *Oryza sativa* L. (A) class III; (B) class I.

Fractions containing chitinase activity were pooled and dialyzed against 20 mM phosphate buffer, pH 7.6. The purity was confirmed by SDS–PAGE.


**Protein concentration**

Protein concentration was determined from the ultraviolet absorption at 280 nm using the extinction coefficient calculated from the equation reported by Pace and others (1995).

**Hydrolysis of chitosan by chitinases from rice**

Individual chitans ($F_A = 0.64$ and 0.32, 20–30 mg) were dissolved in 0.05 M KCl solution to obtain 8.85 mg/mL and 12.5 mg/mL of chitosan solutions (20 mM as GlcN-ac unit concentration), respectively, and the solution pH was adjusted to 4.3 for the class III enzyme and 4.8 for the class I enzyme without ChBD. Each enzyme was added to the chitosan solution, and the enzymatic reaction was conducted at 40°C. After a given incubation period, we terminated the reaction by boiling for 3 min. The resultant solutions containing enzymatic products were employed for size-exclusion chromatography and NMR measurements.

**Size-exclusion chromatography**

Products obtained by the enzymatic degradation of chitosan were separated on three columns in series, packed with Superdex 30, from GE Healthcare (overall dimension 2.6 × 180 cm), as previously described (Sorbona et al., 2005). The column was eluted with 0.15 M ammonium acetate, pH 4.5, at a flow rate of 0.8 mL/min. The effluent was monitored with an online refractive index (RI) detector (Shimadzu RID 6A) coupled with a datalogger. Fully acetylated and fully deacetylated oligosaccharides were used as standards (monomer to hexamer, Seikagaku Kogyo, Tokyo, Japan).

**NMR spectroscopy**

For NMR determination of the enzymatic products, the reaction mixtures were repeatedly lyophilized from D_2O and finally dissolved with 0.6 mL of D_2O. The solution pH was adjusted to 5 for $^{13}$C-NMR experiments and pH 3–4 for $^1$H-NMR experiments by adding a small volume of concentrated DCl or NaOD solution. NMR measurements were conducted using a Bruker Avance DPX spectrometer. NMR spectra of the enzymatic products were obtained at 90°C. $D_{Rn}$-value of the enzymatic products was determined from signal intensities of the reducing end H1 resonance relative to that of the internal H1. The chemical shifts were obtained relative to internal sodium 3-(trimethylsilyl)-propionate-$d_4$ (0.00 ppm). Signal assignments of the spectra were performed according to the chemical shift data previously reported (Boyd et al., 1985; Domard and Cartier, 1989; Vårum et al., 1996; Sorbona et al., 2005).

**Conflict of interest statement**

None declared.

**Abbreviations**

ChBD, chitin-binding domain; $D_{Rn}$, number-average degree of polymerization; $F_A$, fraction of N-acetylated residue (degree of N-acetylation); GlcN or D, 2-amino-2-deoxy-D-glucopyranose; GlcNAc or A, 2-acetamido-2-deoxy- D-glucopyranose; NMR, nuclear magnetic resonance.

**References**


