The cell wall of *Fusarium oxysporum* was digested with commercial *Bacillus pumilus* chitosanase. The chitosanase produced low molecular weight heterooligosaccharides consisting of GlcN and GlcNac from the cell wall. A main component of the digestion products was identified as 2-amino-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranose. The chitosanase appeared to be more effective than *Streptomyces griseus* chitinase for cell wall digestion. Moreover, maltose was unexpectedly found in the digestion products, indicating that the cell wall contains α-1,4-linked glucan chain as a polysaccharide component.

**Key words:** *Fusarium oxysporum*; cell wall structure; chitosanase; chito-oligosaccharide; 13C-NMR

Chitinolytic enzymes have been applied to biological control of plant disease, because many fungal pathogens have chitinous components in their cell wall.1–3) For effective use of chitinolytic enzymes for plant protection from fungal disease, we have been trying to delineate a precise cell wall structure of a fungal pathogen. Analysis of chemical structure of *Fusarium oxysporum* cell wall by CP/MAS 13C-NMR spectroscopy indicated that the cell wall contains chitinous components of which the acetylation degree is 25–35%.4) Chitinase digestion experiments of the cell wall indicated that N-acetylated residues in the chitinous components of the cell wall are most likely to be clustered.5) Chitosanase digestion of the cell wall may also provide additional information on the chemical structure of chitinous component of the cell wall. In this study, the cell wall was digested with commercial *Bacillus pumilus* chitosanase, and structures of the products were identified by NMR spectroscopy.

The cell wall fraction (350 mg) prepared from *Fusarium oxysporum* f. sp. *lycopersici* (race 1) as described previously5) was suspended in 60 ml of 0.1 M sodium acetate buffer, pH 5.3. Chitosanase from *Bacillus pumilus* BN-262 (50 units) purchased from Wako Pure Chemical Co. and 0.5 ml of 2% NaNO3 were added to the cell wall suspension, and then the reaction mixture was incubated for 48 h at 37°C. We have confirmed that the chitosanase preparation does not contain any chitinate activity nor β-N-acetylglucosaminidase activity. The enzymatic reaction was terminated by 5 min of incubation in boiling water. After centrifugation of the reaction mixture, the soluble products were adsorbed on a column of charcoal (3.5 × 10cm). The salts and unadsorbed fractions were removed by eluting with distilled water, and adsorbed oligosaccharides were recovered by eluting with 60% ethanol and evaporated under reduced pressure. The amount of the oligosaccharide mixture recovered from the charcoal column was 32 mg. The dried oligosaccharides were dissolved in 0.01 M sodium acetate buffer, pH 5.0 (buffer A), and were put on a column of CM-Sephadex C-25 previously equilibrated with buffer A. After elution with buffer A, oligosaccharides adsorbed to CM-Sephadex resin were eluted with a linear gradient of NaCl from 0 to 1.0 M in buffer A. The result is shown in Fig. 1. Several reducing sugar fractions were separated by eluting with buffer A, and the others were separated by gradient elution.

The reducing sugar fractions designated by I, II, and III in Fig. 1 were dialyzed against distilled water using an electric dialyzer, Micro Acilizer GI (Asahikasei Kogyo), and lyophilized. The amounts of the reducing sugar fractions obtained were 10 mg for I, 3 mg for II, and 1 mg for III. The recovery of reducing sugars in the ion exchange chromatography was 44%, suggesting that some oligosaccharide fractions are strongly adsorbed to CM-Sephadex resin and not eluted with the gradient elution. The dried sample was dissolved in 0.6 ml of D2O, and used for NMR measurement.13C-NMR spectrum of fraction I is shown in Fig. 2B. The main signals in the spectrum were completely consistent with those detected in the spectrum of maltose (Fig. 2A). Thus, the main component of fraction I was identified to be maltose. Fraction II eluted at 0.2–0.3 M of NaCl concentration (Fig. 1) was analyzed by HPLC, and the elution profile is shown in Fig. 3A. Although fraction II contained several peaks derived from impurities, the retention time of the main peak was identical to that of 2-amino-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranose (GlcN-GlcNac, Fig. 3B), which was

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Abbreviations: GlcN, 2-amino-2-deoxy-α-D-glucopyranose; GlcNac, 2-acetamido-2-deoxy-α-D-glucopyranose; (GlcN)n, β-1,4-linked oligosaccharide of GlcN with a polymerization degree of n; (GlcNac)n, β-1,4-linked oligosaccharide of GlcNac with a polymerization degree of n; GlcN-GlcNac, 2-amino-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranose; NMR, nuclear magnetic resonance; HPLC, high performance liquid chromatography.
obtained by digestion of 25–35% acetylated chitosan with chitosanase from *Streptomyces* sp. N174. Fraction II was also analyzed by $^{13}$C-NMR spectroscopy, and the spectrum is shown in Fig. 4A. For methyl and carbonyl carbon regions (24–25 ppm and 176.5–177.5 ppm, respectively), the profiles were identical to those of the spectrum of GlcN-GlcNAc (Fig. 4B). For C-1-C-6 carbon region (55–100 ppm), although the spectrum contained several signals derived from impurities (designated by asterisks), the main signals were consistent with those detected in the spectrum of GlcN-GlcNAc. Thus, the main component of fraction II was identified as GlcN-GlcNAc. Because of the small quantity, a clear $^{13}$C-spectrum could not be obtained from fraction III. From the $^1$H-spectrum of fraction III, fraction III was found to be a mixture of heterooligosaccharides consisting of GlcN and GlcNAc. In addition, the reducing end residues of the heterooligosaccharides were found to be GlcNAc from their anomeric proton signals. However, further characterization was impossible from the $^1$H-spectrum because of its heterogeneity. From the retention time of HPLC, the main component of fraction III appeared to be a partially N-acetylated chitotrisaccharide.

*B. pumilus* chitosanase hydrolyzes the glycosidic linkage of GlcNAc-GlcN in partially acetylated chitosan as well as that of GlcN-GlcN, producing (GlcN)$_n$ (n = 2 and 3) and heterooligosaccharides in which the reducing and nonreducing end residues are GlcNAc and GlcN, respectively. From the cell wall, the chitosanase produced low molecular weight heterooligosaccharides of which the structures are similar to those of the products from 25–35% acetylated chitosan. (GlcN)$_n$ (n = 2 and 3) produced from the cell wall should have been removed by charcoal column fractionation. On the other hand, *Streptomyces griseus* chitinase produced only (GlcNAc)$_2$ as a low molecular weight product from chitosan component of the cell wall. The amount of the insoluble cell wall fraction obtained after the chitosanase digestion was 69% of the amount before digestion, while, in the case of *S. griseus* chitinase digestion, it was 83%. From these results, we concluded that *B. pumilus* chitosanase is more effective for digestion of cell wall of this species than *S. griseus* chitinase.

In our previous paper, we proposed a probable structure for the chitosin component in the *F. oxysporum* cell wall on the basis of the product composition from *S. griseus* chitinase digestion (Fig. 5). As shown in the proposed structure, GlcNAc residues form a cluster at some region in the chitosin component of the cell wall. Considering the specificity of *B. pumilus* chitosanase, the chitosan should not hydrolyze the GlcNAc cluster region, but the GlcN cluster region. Thus, we estimated that the chitosanase digestion product obtained in this study, GlcN-GlcNAc, is derived from the GlcN cluster region and that the GlcN cluster region is studded with a small number of GlcNAc residues as shown in the figure. The acetylation degree of chitosin component of the cell wall was estimated to be 25–35%. This indicates that the chitinase-accessible region is restricted to a small part of the chitosin component. Thus, we confirmed that *B. pumilus* chitosanase is more accessible to the chitosin component of the cell wall than *S. griseus* chitinase.
Chitinous Component of *Fusarium* Cell Wall

Fig. 4. 67.8 MHz $^{13}$C-NMR Spectra of Fraction II (A) and GlcN-GlcNAc (B). The signals designated by asterisks are derived from impurities. pH of the samples were adjusted to 3.0-4.0 by addition of concentrated DC1 or NaOD.

![Chemical shift (ppm)](image)

**F. oxysporum** cell wall

- \(\text{\textcircled{C}}\) glucosamine residue
- \(\text{\textcircled{O}}\) N-acetylglicosamine residue
- \(\text{\textuparrow} B. pumilus \text{ chitosanase cleavage site}\)
- \(\text{\textuparrow} S. griseus \text{ chitosanase cleavage site}\)

Fig. 5. Proposed Structure of the Chitinous Component in *F. oxysporum* Cell Wall. The arrows indicate the cleavage sites for *B. pumilus* chitosanase and *S. griseus* chitosanase.

In the ion-exchange chromatography of the chitosanase digest from 25-35% acetylated chitosan, no reducing sugar was eluted before NaCl gradient elution. From the cell wall, however, reducing sugar fractions were eluted before the gradient elution. A main component of the fractions was identified to be maltose, an \(\alpha\)-1,4-linked disaccharide of \(\alpha\)-glucose. The disaccharide should be a structural unit of the cell wall. The enzyme preparation commercially available might have contained other carbohydrate enzymes, such as \(\alpha\)-1,4-glucanase, giving rise to the additional disaccharide in the digest. A small amount of laminariobiose, a \(\beta\)-1,3-linked disaccharide of \(\beta\)-glucose, was found in the cell wall digest with *S. griseus* chitosanase commercially available. From these results, we assumed that \(\alpha\)-1,4- and \(\beta\)-1,3-linked glucan chains are also structural components of the cell wall. A glucanase
digestion study should be done for delineating the fine structure of the cell wall.

References