Chitosanase activity of the enzyme previously reported as β-1,3-1,4-glucanase from *Bacillus circulans* WL-12

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Received April 28, 1998; Accepted July 17, 1998

Chitosanases 33 kDa and 40 kDa in size were detected in the culture supernatant of *Bacillus circulans* WL-12. One of the two chitosanases, chitosanase 40 (40-kDa chitosanase) has been shown to be identical to the enzyme which has been reported previously as a β-1,3-1,4-glucanase by Bueno *et al.*† The enzyme has been classified into family 8 glycosyl hydrolases together with the enzymes formally known as cellulase family D. This enzyme named chitosanase 40/β-1,3-1,4-glucanase hydrolyzed both chitosan and β-1,3-1,4-glucan with similar efficiency. However, the production of the enzyme was induced with chitosan but not by β-1,3-1,4-glucan. Therefore, it seems possible that the major substrate of this enzyme is chitosan rather than β-1,3-1,4-glucan. Analysis of degradation products generated from partially N-acetylated chitosan showed that chitosanase 40/β-1,3-1,4-glucanase hydrolyzes GlcN-GlcN and GlcN-GlcNAc linkages but not GlcNac-GlcNac nor GlcNac-GlcN. The specificity for hydrolyzing linkages of this enzyme is similar to that of the chitosanase from *S. griseus* HUT6037.

Key words: chitosanase; β-1,3-1,4-glucanase; *Bacillus circulans* WL-12; family 8

*Bacillus circulans* WL-12, originally isolated as a yeast and fungal cell wall lytic bacterium, produces a number of extracellular enzymes including chitinases. More than 10 chitinases are detected in the culture supernatant of this bacterium when grown in the presence of chitin. These chitinases are shown to be derived from only three genes, *chiA*, *chiC*, and *chiD* encoding chitinases A1, C1, and D1, respectively, and proteolytic cleavage occurs at the inter domain regions of initial chitinases give rise to so many chitinases with different sizes. In addition to chitinases, we recently found that this bacterium also produces chitosanases into culture medium.

Chitosanase is the enzyme that catalyzes the hydrolysis of glycosidic bonds in chitosan, deacetylated derivative of chitin. Chitosanase and chitinase both degrade partially N-acetylated chitosan quite efficiently as a common substrate. The overlap of the range of degradable substrate sometimes cause confusion in the distinction of the two enzymes. Recently we proposed a new definition of chitinase and chitosanase based on the hydrolyzing linkages of the two enzymes as follows. Chitinase is the enzyme that requires a GlcNac residue at least at one side of hydrolyzing linkages and chitosanase requires a GlcN residue at least at one side of hydrolyzing linkages. Consequently, chitinase does not hydrolyze the linkages between GlcN and GlcN. On the other hand, chitosanase does not hydrolyze the linkages between GlcNac and GlcNac.

Primary structures of the chitosanases so far as we know do not have significant similarity to those of chitinases. However, the chitinases and chitosanases compared are mostly derived from different organisms. Therefore, we decided to clone a chitosanase gene from *Bacillus circulans* WL-12 in order to compare the primary structure with those of chitinases from the same organism. Comparison between chitosanase 40 and the other proteins showed that this enzyme is identical to the enzyme previously reported by Bueno *et al.* as a β-1,3-1,4-glucanase which belongs to the family 8 glycosyl hydrolases. We found that production of this enzyme was induced with chitosan but not by β-1,3-1,4-glucan. Analysis of chitooligosaccharides produced in the hydrolysis of partially N-acetylated chitosan showed that the enzyme splits the β-glucosaminidic linkages in chitosan molecules with a mode of action similar to that of the chitosanase from *Streptomyces griseus* HUT 6037.

Materials and Methods

Bacterial strains, plasmids, and culture media. *Bacillus circulans* WL-12 was grown at 30°C with shaking in modified yeast nitrogen base (YNB) medium containing 0.2% (w/v) chitosan and 0.5% yeast extract for chitosanase production and in Luria-Bertani (LB) medium with 0.5% glucose for chromosomal DNA extraction. *Escherichia coli* JM109 was used as a host and pUC19 was used as a vector for gene cloning. For subcloning of restriction fragments to sequence the nucleotides of the cloned gene, pUC118 was used as a vector for plasmid. *E. coli* cells carrying either plasmid pUC19, pUC118, or their derivatives were grown in LB medium containing 100 µg/ml ampicillin.

DNA sequencing. Various restriction fragments of pCS01 were subcloned into pUC118, and the resulting

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plasmids were sequenced with an automated laser fluorescence sequencer (Model 4000L; LI-COR). Sequencing reactions were done by using the Thermosequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham) according to the supplier’s instructions with a double-stranded template. Nucleotide sequence data were analyzed using the Gene tyx system (Software Kaibatsu Co., Tokyo). The amino acid sequence was compared with those available in the SWISS-PROT protein data bank by using the Lipman-Pearson algorithm.9

**SDS-PAGE and zymogram.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% slabs was done as described by Laemmli.10 After electrophoresis was completed, renaturation of the enzymes and detection of either chitosanase or chitinase activity in the gel were done as described previously,11 except that 0.05% colloidal chitosan or chitin, respectively, was included in the agar sheet instead of pachymen, and that 0.1 M sodium phosphate buffer (pH 6.0) was used throughout the procedure.

**N-terminal amino acid sequence.** N-terminal amino acid sequences of the proteins separated by SDS-PAGE were determined as described in our previous paper.6

**Extraction of chitosanase from E. coli.** The product of the cloned BGC gene was extracted from the periplasmic space of E. coli harboring the plasmid pCS01 by a cold osmotic shock procedure described by Manoil and Beckwith.12 The extracted periplasmic proteins were precipitated with ammonium sulfate (80% saturation) and the precipitate was dissolved in a small volume of 20 mM sodium phosphate buffer, pH 6.0, dialyzed against the same buffer, and lyophilized.

**Enzymes and protein assay.** Chitosanase activity was measured using colloidal chitosan prepared from chito san 10B (less than 1% acetylated chitosan) by measuring the amount of reducing sugar formed after the enzyme reaction according to the method described by Imoto and Yagishita.13 One unit of chitosanase activity was defined as the amount of enzymes that liberate reducing sugar corresponding to one μmole of glucosamine per minute. β-N-Acetylglicosaminidase (β-GlcNAcase) and exo-β-glicosaminidase (β-GlcNase) activities were assayed by the procedures described in our previous paper.14

Protein concentration was measured by the method of Lowry et al.15 using bovine serum albumin as a standard.

**Reducing sugar measurement.** The amount of reducing sugar during column chromatography was measured by a modification of Schales method,13 using GlcN as a reference compound.

**Isoelectric focusing.** The isoelectric focusing analysis of chitosanase was done using Ampholine electrofocusing equipment (LKB 8100-10; LKB Instruments AB, Sweden) according to the manufacturer's instruction.

**HPLC.** The HPLC system consisted of an 880-PU pump, an 830-R1 RI detector (Japan Spectroscopic Co. Ltd., Tokyo), and a D-2500 Chromato-Integrator (Hitachi Ltd., Tokyo). Sugars were separated on a Radi al-PK μBondapak NH2 column (8.0 × 100 mm, Millipore Co., Milford, MA, USA) using acetonitrile-water (6:35) mixture as the mobile phase, at a flow rate of 2.0 ml per min. Chitooligosaccharides were detected by monitoring the refractive index.

**Mass spectrometry.** The mass analysis of oligosaccharides was done by a Voyager Elite matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) (PerSeptive Biosystems, Framingham, MA, USA). The acceleration voltage was 20 kV and 2,5-dihydroxybenzoic acid was used as the matrix.

**Exoglycosidase digestion.** Partially N-acetylated chitooligosaccharides were hydrolyzed by successive action of β-GlcNCase and β-GlcNase, and the hydrolysis products were analyzed by HPLC as described previously.9

**Hydrolysis of chitosan by chitosanase.** A reaction mixture consisting of 500 ml of 0.6% chitosan (27% N-acetylated chitosan) in 0.1 M acetic buffer (pH 5.0), 5 ml of 2% NaN3, and 1 ml of chitosanase (12 units) was incubated for 24 hr at 37°C. After the addition of 1 ml of chitosanase (12 units), the mixture was further incubated for 24 hr at 37°C. The reaction was stopped by boiling for 5 min. The extent of the hydrolysis of chitosan after 48 hr was 66%. The reaction mixture was concentrated in a rotary evaporator under reduced pressure at a temperature below 35°C and dialyzed through an electric dialyzer (Micro Aclyzer G1, Asahikasei Kogyo Co. Ltd., Kanagawa). The dialyzed solution was adjusted to pH 4.6 by adding 1 N acetic acid.

**Chemicals.** Chitosan 7B (approximately 30% N-acetylated chitosan) and 10B (less than 1% N-acetylated chitosan) were purchased from Funakoshi Chemical Co. (Tokyo). Chitosan with 27% N-acetylation, which was prepared by homogeneous alkaline hydrolysis of chitin, was the product of Katakura Chikkarin Co., Ltd. (Tokyo).16 Colloidal chitosan was prepared from chitosan 7B and 10B according to the method described by Price and Storck.17 Glycol chitin and colloidal chitin were prepared from powdered chitin purchased from Funakoshi Chemical Co. by the method described by Yamada and Imoto18 and, Jeuniaux19 respectively. A series of (GlcN)n, (n = 2–6) was purchased from Sekagaku Kogyo Co., Ltd. (Tokyo). Restriction enzymes were purchased from Toyobo Biochemicals (Osaka) and New England Biolabs (Beverly, MA, USA). β-GlcNA case was prepared from Pycnoporus cinnabarinus IFO 6139 as described previously.20 β-GlcNase from Penicillium sp. AF9-P-128 was a gift from Prof. Y. Uchida of Saga University.21
Results

Chitosanase produced by Bacillus circulans WL-12

Bacillus circulans WL-12 was grown in the medium containing either powdered chitin, 30% acetylated chitosan (chitosan 7B), fully decacetylated chitosan (chitosan 10B) or glucose, and chitosanase activity was measured by using colloidal chitosan as an assay substrate. The highest chitosanase activity was detected in the medium containing 30% N-acetylated chitosan (Fig. 1). The activity reached its maximum level at day 2 of cultivation and remained constant during further cultivation. Chitin and fully decacetylated chitosan also induced significant levels of chitosanase activity but it was much lower than that observed in the medium containing 30% N-acetylated chitosan. When B. circulans WL-12 was grown in the medium containing glucose, essentially no chitosanase activity was observed.

Crude enzyme was prepared by ammonium sulfate precipitation (80% saturation) from the day-2 culture supernatant of B. circulans WL-12 grown in the medium containing 30% N-acetylated chitosan, which supported highest chitosanase production. As shown in Fig. 2(A), the crude enzyme preparation contained a major chitosanase of 40 kDa (chitosanase 40) and a minor chitosanase of 33 kDa (chitosanase 33). The major chitosanase of 40 kDa was partially purified by isoelectric focusing electrophoresis and the N-terminal amino acid sequence was analyzed by automated Edman degradation. The major chitosanase was recovered in the peak fraction at pH at around 9.5 (data not shown). The analyzed N-terminal sequence, APNKFPQHTTYXSGXIKPNHVTQXAM-, was compared with the sequences in the SWISS-PROT protein data bank. It was found to be identical to the sequence from Ala-32 in the deduced amino acid sequence of the gene (BGC) encoding ß-1,3,1,4 glucanase of B. circulans WL-12 previously reported by Bueno et al. Due to the perfect agreement of N-terminal amino acid sequence of chitosanase 40 to Bueno’s ß-1,3,1,4 glucanase, we suspected that the two enzymes might be the same. Therefore, we examined whether chitosanase 40 had ß-1,3,1,4 glucanase activity. As demonstrated by zymogram analysis after running SDS-PAGE (Fig. 2(B)), chitosanase 40 had lichenan (ß-1,3,1,4 glucan) degrading activity.

Cloned ß-1,3,1,4 glucanase had chitosanase activity

To discover whether our chitosanase 40 is identical to Bueno’s ß-1,3,1,4 glucanase, we decided to clone the gene (BGC) encoding ß-1,3,1,4 glucanase and compare the properties of the gene product with chitosanase 40. Bueno et al. cloned the BGC gene from Sau3A1

Fig. 1. Course of Chitosanase Production by B. circulans WL-12.

B. circulans WL-12 was grown in the medium containing either chitosan 10B (.), chitosan 7B (○), powdered chitin (○), or glucose (△) as a carbon source. Chitosanase activity in the culture supernatant was measured using colloidal chitosan prepared from chitosan 10B (less than 1% acetylated chitosan) as the assay substrate.

Fig. 2. Chitosanases Detected in the Culture Supernatant of B. circulans WL-12.

(A) SDS-PAGE analysis of the crude enzyme prepared from culture supernatant of B. circulans WL-12 grown in the presence of chitosan 7B. a, protein staining; b, chitosanase activity detected on an agar replica of the polyacrylamide gel. lane 1, size marker; lane 2, crude enzyme. The arrow indicates the position of chitosanase 40. (B) Partially purified chitosanase 40 by isoelectric focusing. a, protein staining; b, chitosanase activity; c, lichenan activity. lane 1, size marker; lane 2, partially purified chitosanase 40.
digested genomic library of *B. circulans* WL-12. They described how the cloned DNA fragment containing the *BGC* gene hybridized with the 2.2-kb fragment in the *HindIII* digested *B. circulans* genomic DNA. Therefore, the *BGC* gene was cloned as the 2.2-kb *HindIII* fragment instead of using *Sau3A1* partial digestion. After digestion of chromosomal DNA of *B. circulans* WL-12 with *HindIII*, fragments around 2.2 kb were collected, ligated with *HindIII* cut-pUC19, and *E. coli* JM109 cells were transformed with it. The transformants carrying inserted DNA were tested for the formation of clearing zones around colonies on the agar plate containing lichenan, a β-1,3-1,4-glucan. Among 1,600 transformants tested, four formed clearing zones and all of them contained the same plasmid (designated as pCS01) carrying the 2.2-kb inserted DNA fragment in the same orientation.

To confirm that the gene we have cloned is the gene that Bueno *et al.* reported, the nucleotide sequence of inserted DNA in pCS01 was analyzed. The sequence found was identical to the nucleotide sequence of the *BGC* gene (GenBank X52880) except for the upstream region from the *Sau3A1* site 38 bp upstream from the initiation codon. This disagreement can be explained by misincorporation of a short *Sau3A1* fragment together with the fragment carrying the entire coding region of the *BGC* gene in the course of their cloning experiment. Thus, we considered that the gene we have cloned is the *BGC* gene reported by Bueno *et al.* The gene encodes a polypeptide of 409 amino acids with a calculated size of 44,815 Da. The enzyme produced by this gene has been classified into glycosyl hydrolase family 8 together with several endoglucanases formerly known as cellulase family D.2123

Total proteins of *E. coli* JM109 cells carrying either only the vector plasmid pUC19 or pCS01 carrying the *BGC* gene were analyzed by SDS-PAGE as shown in Fig. 3. *E. coli* cells carrying pCS01 contained a 40-kDa protein which was not observed in the *E. coli* cells carrying only pUC19. The migration position of this protein was indistinguishable to that of purified chitosanase 40 prepared from the culture supernatant of *B. circulans* WL-12. The N-terminal amino acid sequence of the 40-kDa protein produced by *E. coli* JM109 carrying pCS01 was analyzed and found to be APNKFPQHTTTYTSG-. This sequence perfectly matched the N-terminal amino acid sequence of chitosanase 40, and, therefore, matched the sequence from Ala-32 in the deduced amino acid sequence of the *BGC* gene. In addition, this protein had chitosanase activity as well as β-1,3-1,4-glucanase as demonstrated by zymogram analysis of an SDS-PAGE gel (Fig. 3B).

All of these results clearly indicate that the 40-kDa protein detected here is the product of the *BGC* gene and is identical to chitosanase 40.

**Substrate specificity and induction of chitosanase 40 production**

As shown in Table 1, chitosanase 40/β-1,3-1,4-glucanase had its highest hydrolyzing activity on lichenan and colloidal chitosan. Partially N-acetylated (30%) chitosan was hydrolyzed faster than fully deacetylated chitosan. CM-cellulose was hydrolyzed significantly but the rate of hydrolysis was approximately 1/10 of those of lichenan and chitosan. Low but significant activities were detected on glycol chitin and colloidal chitin, probably due to the deacetylation occurred during the process of preparation of these substrates. Pachyman, laminarin, and lutean were not hydrolyzed at all.

The substrate specificity showed that chitosanase 40/β-1,3,1,4-glucanase hydrolyzes chitosan and lichenan with similar efficiency. Now the question became which is the major substrate for this enzyme. Thus, production of this enzyme in media containing either chitosan or

![Fig. 3. Detection of Chitosanase and Lichenase Activities of the Gene Product of the Cloned BGC (con40). E. coli JM109 cells harboring either pUC19 or pCS01 were grown in LB medium containing 100 µg/ml ampicillin at 37°C for 18 hr. Cells were collected and treated at 100°C for 5 min. with SDS/sample buffer and analyzed by SDS-PAGE. lane 1, size marker; lane 2, total proteins of E. coli JM109 cells harboring vector plasmid pUC19; lane 3, total proteins of E. coli JM109 cells harboring recombinant plasmid pCS01 carrying the BGC (or con40) gene; lane 4, partially purified chitosanase 40 prepared from culture supernatant of B. circulans WL-12. A, protein staining; B, chitosanase activity; C, lichenase activity.](image)

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<th>Table 1. Substrate Specificity of Chitosanase 40</th>
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<td>Substrate</td>
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<td>Colloidal chitosan 10B*</td>
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* Colloidal chitosan prepared from chitosan 10B (more than 99% deacetylated chitosan).

** Colloidal chitosan prepared from chitosan 7B (approximately 70% deacetylated chitosan).
Chitosanase 40 from Bacillus circulans WL-12

Fig. 4. SDS-PAGE Analysis of the Proteins Produced in the Medium Containing either Chitosan or Lichenan. B. circulans WL-12 was grown in the presence of either chitosan 7B (lane 2) or lichenan (lane 3) as a carbon source, proteins in the culture supernatants were collected by ammonium sulfate precipitation and analyzed by SDS-PAGE. Lane 1, size marker. A, protein staining; B, chitosanase activity. The arrow indicates the position of chitosanase 40.

lichenan was compared. B. circulans WL-12 was grown in the medium containing either 0.2% lichenan or 0.2% chitosan 7B (30% N-acetylated chitosan) for 2 days. Proteins in the culture supernatant was collected by ammonium sulfate precipitation and analyzed by SDS-PAGE. As shown in Fig. 4, chitosanase 40 was detected in the medium containing chitosan but not in the medium containing lichenan. Production of chitosanase 40 was induced with chitosan but not with β-1,3-1,4-glucan. From these observation, we suspected that the major substrate for this enzyme may be chitosan, although the enzyme hydrolyze both chitosan and β-1,3-1,4-glucan with similar efficiency.

Products of enzymatic hydrolysis of partially N-acetylated chitosan by chitosanase 40/β-1,3-1,4-glucanase

To clarify the specificity for hydrolyzing linkages of chitosanase 40/β-1,3-1,4-glucanase, structures of oligosaccharides generated from partially N-acetylated chitosan by the action of the enzyme were studied. Chitosan with 27% N-acetylation was treated with the enzyme and the hydrolyzate was separated by CM-Sephadex C-25 column chromatography (Fig. 5). Oligosaccharides consisting of only GICNac are always eluted with 0.02 M acetate buffer (pH 4.6) in this chromatography. However, no reducing sugar was eluted with the same buffer indicating that the hydrolyzate does not contain (GICNac)4. Fractions from F-1 to 11 were collected, dialyzed through an electric dialyzor, and lyophilized. Each fraction was further purified by gel filtration on Bio-Gel P-4 column (2.6 × 180 cm) with the elution of 0.15 M acetate buffer (pH 4.2). Fractions F-3, 5, 6, 7, 8, 9, 10, and 11 were homogeneous based on the HPLC and, therefore, used to study sugar compositions and sequences of oligosaccharides.

The structures of chito-oligosaccharides were studied by exoglycosidase digestion and MALDI-TOF MS. In MALDI-TOF MS, chito-oligosaccharides were detected in the positive ion mode as sodium and potassium adducts. In the mass spectra of F-5, 8, 9, and 11, the ion peaks at m/z 363.0, 524.3, 524.3, and 685.3 were observed as a sodium adduct, respectively. These oligosaccharides were completely decomposed by β-GlcNacase to only GlcN. Thus, F-5, 8, 9, and 11 were identified to be (GlcN)2, (GlcN)3, (GlcN)4, and (GlcN)5, respectively.

In the MALDI-TOF MS analyses of F-3, 6, 7, and 10, the [M + Na]+ ions were detected at m/z 769.1, 930.3, 727.3, and 888.3, indicating that these are diacetylchito-oligosaccharides, each sample and an example of the MALDI-TOF MS spectra of the oligosaccharides obtained by exo-splitting of F-3 by successive action of both enzymes is shown in Fig. 7. F-3 was hydrolyzed by β-GlcNacase to produce GlcNac and F-3-1, which was further split by β-GlcNase to produce GlcN and F-3-2. In the MALDI-TOF MS spectra of F-3-1 and F-3-2, the peaks of [M + Na]+ ion of tri- and disaccharides containing two and one GlcN residue(s) were detected at m/z 566.1 and 405.1, respectively (Fig. 7B and 7C). F-3-2 was hydrolyzed to GlcNAc and GlcN by β-GlcNacase (data not shown). From these results, F-3 was identified as GlcNAc-GlcN-GlcNac-GlcN.
Fig. 6. HPLC of the Hydrolysis Products of F-3 (A), F-6 (B), F-7 (C), and F-10 (D) by Successive Action of \( \beta \)-GlcNAcase and \( \beta \)-GlcNase. The experimental details are described in the text. Hydrolysis products were eluted with acetonitrile-water (65:35) and monitored by the refractive index. Chitooligosaccharide (a) was digested with \( \beta \)-GlcNAcase (b) followed by \( \beta \)-GlcNase (c).

F-6 was hydrolyzed by \( \beta \)-GlcNAcase to GlcNac, F-6-1, and F-6-2. In the mass spectrum of F-6-1, the peak of [M+Na]+ ion of (GlcN) was observed at \( m/z \) 524.3. The [M+Na]+ ion was observed at \( m/z \) 727.4 corresponding to monoacetylchitotetraose, in the mass spectrum of F-6-2. Furthermore, \( \beta \)-GlcNase split F-6-2 into GlcN and F-6-3. In the MALDI-TOF MS spectrum of F-6-3, the peak at \( m/z \) 405.1 corresponding to [M+Na]+ ion of monoacetylchitobiase was observed. These results indicate that the structure of F-6-2 is (GlcN)\(_2\)-GlcNac-GlcN. Thus, we concluded that F-6 is a mixture of (GlcNac)\(_2\)-(GlcN) and GlcNac-(GlcN)\(_2\)-GlcNac-GlcN.

F-7, which is monoacetylchitotetraose, was hydrolyzed by \( \beta \)-GlcNacase to GlcNac and F-7-1 which gave the [M+Na]+ ion corresponding to (GlcN) at \( m/z \) 524.2. Therefore, F-7 was identified to be GlcNac-(GlcN).

In the incubation of F-10 with \( \beta \)-GlcNAcase, GlcNac, F-10-1, and F-10-2 were formed as hydrolysis products. In the mass spectra of F-10-1 and F-10-2, the peaks of [M+Na]+ ions of (GlcN)\(_4\) (\( m/z \) 685.4) and monoacetylchitopentaose (\( m/z \) 888.5) were observed, respectively. F-10-1 was completely hydrolyzed by \( \beta \)-GlcNase to form GlcN. F-10-2 was also hydrolyzed by \( \beta \)-GlcNase to GlcN and F-10-3. In the mass analysis of F-10-3, the mass number of [M+Na]+ ion was 727.3, which agreed with the mass number calculated for GlcNac-(GlcN)\(_3\). These results indicate that F-10 is a mixture of two pentasaccharides with sugar sequence of GlcNac-(GlcN)\(_3\) and GlcN-GlcNac-(GlcN)\(_3\).

From the results described above, it was concluded that chitosanase 40 hydrolyze GlcN-GlcN and GlcN-GlcNac linkages but not GlcNac-GlcNac nor GlcNAc-GlcN linkage in partially N-acetylated chitosan.

Fig. 7. MALDI-TOF MS Spectra of F-3 (A), F-3-1 (B), and F-3-2 (C).
Discussion

Bueno et al. described in their report how the $\beta-1,3,1,4$-glucanase of \textit{B. circulans} WL-12 was not produced until stationary phase of growth.\textsuperscript{3} Although the reason why the $\beta-1,3,1,4$-glucanase was produced at stationary phase is not clear, their observation is consistent with ours, that chitosanase 40 was not induced with lichenan. Although the enzyme had a slightly higher hydrolyzing activity on lichenan than chitosan, it is probably inadequate to judge that $\beta-1,3,1,4$-glucan is preferred to this enzyme over chitosan because the solubility of lichenan and chitosan in aqueous solution are different. Therefore, it seems possible that the major substrate of this enzyme is chitosan rather than $\beta-1,3,1,4$-glucan. However, this does not necessarily mean that the $\beta-1,3,1,4$-glucan hydrolyzing activity of this enzyme is an unimportant side activity. Since $\beta-1,3,1,4$-glucan is a common component of fungal cell walls,\textsuperscript{20} the wide substrate specificity of chitosanase 40/$\beta-1,3,1,4$-glucanase must be advantageous for \textit{B. circulans} WL-12 when degrading and using components of fungal cell walls. This bacterium produces two $\beta-1,3$-glucanases, A1 and B, and $\beta-1,3$-glucanase B efficiently degrades $\beta-1,3,1,4$-glucan while $\beta-1,3$-glucanase A1 does not.\textsuperscript{25-27} $\beta-1,3$-glucanase B is assumed to degrade $\beta-1,3,1,4$-glucan by hydrolyzing $\beta-1,3$-linkages. On the other hand, chitosanase 40/$\beta-1,3,1,4$-glucanase had not high but significant hydrolyzing activity on CM cellulose, indicating that this enzyme degrades $\beta-1,3,1,4$-glucan by hydrolyzing $\beta-1,4$-linkages. Therefore, cooperative action of chitosanase 40/$\beta-1,3,1,4$-glucanase and $\beta-1,3$-glucanase B will result in efficient and complete degradation of $\beta-1,3,1,4$-glucan.

Chitosanases can be divided into three classes according to their specificity for the hydrolysis of the $\beta$-glycosidic linkages in partially $\text{N}$-acytlylated chitosan.\textsuperscript{20} Chitosanases from \textit{Bacillus} sp. No. 7-M\textsuperscript{16} and \textit{Streptomyces griseus} HUT 6037\textsuperscript{7} have the specificity toward the $\beta$-d-glucosaminidic linkages but not N-acetyl-$\beta$-d-glucosaminidic linkages in chitosan molecules, and the former splits only GlcN-GlcN linkages, while the latter splits both GlcN-GlcN and GlcN-GlcNAc linkages. On the other hand, chitosanases from \textit{Bacillus pumilus} BN-26\textsuperscript{20} and \textit{Streptomyces} sp. N174\textsuperscript{20} split the linkages of GlcNAc-GlcN as well as GlcN-GlcN in partially $\text{N}$-acytlylated chitosan molecules. In the hydrolysis of partially $\text{N}$-acytlylated chitosan by chitosanase 40/$\beta-1,3,1,4$-glucanase, hetero-chitooligosaccharides with GlcN at reducing end residues and GlcN or GlcNAc at non-reducing end residues together with (GlcN)$_{n}$, (GlcN)$_{n}$, and (GlcN)$_{n}$ were isolated. These results indicate that chitosanase 40 hydrolyzes GlcN-GlcN and GlcN-GlcNAc bonds but not GlcNAc-GlcNAc nor GlcNAc-GlcN in chitosan molecules. Thus, the specificity of this enzyme was similar to that of chitosanase from \textit{S. griseus} HUT 6037.\textsuperscript{8} Chitosanases from \textit{S. griseus} HUT 6037\textsuperscript{8} and \textit{Bacillus} sp. No. 7-M\textsuperscript{16} also hydrolyze CM-cellulose. Therefore, it seems likely that chitosanase with $\beta-1,4$-glucanase activity splits only the $\beta$-d-glucosaminidic linkage but not the N-acetyl-$\beta$-d-glucosaminidic linkage.

The nucleotide sequences and deduced amino acid sequences of three bacterial chitosanases and one fungal chitosanase have been reported so far. The three bacterial chitosanases from \textit{Bacillus circulans} MH-K1.\textsuperscript{32} \textit{Streptomyces} sp. N174,\textsuperscript{34} and \textit{Nocardiooides} sp. N106\textsuperscript{36} have been classified into family 46 according to the glycosyl hydrolases classification based on the amino acid sequence similarity proposed by Henrissat \textit{et al.}\textsuperscript{22,23} Recently, two different proteins coded by a \textit{Chlorella} virus have shown to be similar to chitosanases in family 46 and chitosanase activity of these proteins have been experimentally demonstrated.\textsuperscript{35} The fungal chitosanase from \textit{Fusarium solani}\textsuperscript{36} does not have significant sequence similarity to family 46 bacterial and viral chitosanases and has not been classified yet into any family. On the other hand, $\beta-1,3,1,4$-glucanase (chitosanase 40) of \textit{B. circulans} WL-12 has been classified into family 8 and no significant sequence similarity was observed with the family 46 chitosanases nor the fungal chitosanase. In addition, chitosanase 40/$\beta-1,3,1,4$-glucanases did not show significant sequence similarity with any other $\beta-1,3,1,4$-glucanases (lichenases) which were mostly classified as family 16 glycosyl hydrolases. All of the other members of family 8 are endo-$\beta-1,4$-glucanases formally known as cellulase family D with one exception of a hypothetical protein of \textit{E. coli}. It is probably not surprising that chitosanase 40/$\beta-1,3,1,4$-glucanases is in the same family with cellulases, since chitosan and cellulose are structurally very similar. In fact, chitosanase 40/$\beta-1,3,1,4$-glucanases had significant activity on CM-cellulose, although it was not high compared with chitosanase or lichenase activity. It will be very interesting to examine whether the other member of family 8 have chitosanase activity or not.

Chitosanases with hydrolyzing activity on CM cellulose from \textit{Myxobacter} AL-1,\textsuperscript{37} \textit{S. griseus} HUT 6037,\textsuperscript{39} \textit{Bacillus} sp. No. 7-M,\textsuperscript{31} and \textit{B. megaterium} P1\textsuperscript{38} have been reported. However, the primary structures of these bacterial chitosanases have not yet been clarified. Recently, Pedraza-Reyes and Gutierrez-Corona\textsuperscript{39} reported that the N-terminal acid sequence of a chitosanase-cellulase produced by \textit{Myxobacter} sp. AL-1 had 76% identity to \textit{B. subtilis} endocellulases belong to family 5. Further studies on the properties, structure, and function of the enzymes that are able to cleave $\beta-1,4$-glycosidic linkages in different substrates should be done to understand the real role of the enzymes in the degradation of $\beta-1,4$-glucan in nature.

Acknowledgments

We are grateful to Prof. Yasushi Uchida of Saga University for his generous gift of $\beta$-GlcNase and to Dr. Yuto Kamei and Mr. Hideki Harada in Marine and Highland Bioscience Center, Saga University for help in mass-spectrometry analysis.

References


