ENZYMATIC PREPARATION OF MONO-*N*-ACETYLATED CHITO-OLIGOSACCHARIDES

Masaru Mitsutomi[a]*, *Mitsuhiro Ueda*[b], *Masami Nakazawa*[b], *Kazutaka Miyatake*[b], *and Kiyohiko Seki*[a].

[a]Department of Applied Biochemistry and Food Science, Saga University, 1-Honjo, Saga 840-8502, Japan; [b]Department of Applied Biological Chemistry, Osaka Prefecture University, Sakai, Osaka 599-8531, Japan.

Abstract

A series of mono-*N*-acetylated chitooligosaccharides [$(GlcN)_n$-GlcNAc, n=1-10] was prepared by enzymatic hydrolysis of partially *N*-acetylated chitosan. *Aeromonas* sp. No. 10S-24 chitinase II hydrolyze highly deacetylated chitosan very well. The enzyme hydrolyzed *N,N*^{2}-diacetylchitotetraose [$(GlcN)_2$-$(GlcNAc)_2$] and *N,N*^{2}-diacetylchitopentaose [$(GlcN)_3$-$(GlcNAc)_2$] to produce $(GlcN)_2$-GlcNAc and $(GlcN)_3$-GlcNAc, respectively. In order to obtain chitooligosaccharides with high degree of polymerization, the hydrolyzate of 25% *N*-acetylated chitosan by *Aeromonas* sp. No. 10S-24 chitinase II and *Pycnoporus cinnabarinus* β-*N*-acetylglucosaminidase was separated by CM-Sephadex C-25 and Bio-Gel P-6. The purified mono-*N*-acetylated chitooligosaccharides had GlcNAc as the reducing end residues. By this method, mono-*N*-acetylated chitooligosaccharides with a DP of 2 to 11 were obtained in high yield.

Introduction

Chitooligosaccaharides produced by the hydrolysis of chitin and chitosan are known to have many bioactivities, such as elicitor, antifungal, antibacterial, and antitumor activities [1]. However, it is difficult to obtain the functional chitooligosaccharides with degree of polymerization (DP) higher than 6 by acid degradation of chitinous compound. Chitinolytic enzymes, such as chitosanase (EC 3.2.1.132) and chitinase (EC 3.2.1.14), are useful for preparing chitooligosaccharides. Chitosanase is used to prepare D-glucosamine (GlcN) oligomers with DP 2 to 6 from chitosan [2], whereas chitinase hydrolyze partially *N*-acetylated chitosan to produce a mixture of hetero-chitooligosaccharides consisting of *N*-acetyl-D-glucosamine (GlcNAc) and GlcN with various degree of acetylation [3,4]. However, separation of hetero-chitooligosaccarides with different degree of acetylation is difficult. On the other hand, chitinases are classified into two families (18 and 19) of glycosyl hydrolases based on the amino acid sequence similarity of the catalytic domains [5]. Chitinases of the two families have different specificities for the hydrolysis of the β-glycosidic linkages in partially *N*-acetylated chitosan [4, 6, 7]. Family 18 chitinases exclusively cleave the GlcNAc-GlcNAc and GlcNAc-GlcN linkages, whereas family 19 chitinases exclusively cleave the GlcNAc-GlcNAc and GlcN-GlcNAc linkages. Among the microbial chitinases tested, *Aeromonas* sp. No. 10S-24 chitinase II (family 18) was able to hydrolyze highly deacetylated chitosan [4, 8, 9]. In this study, we found that chitinase II hydrolyzed *N,N*^{2}-diacetylchitotetraose [$(GlcN)_2$-$(GlcNAc)_2$] and *N,N*^{2}-diacetylchitopentaose [$(GlcN)_3$-$(GlcNAc)_2$] to produce $(GlcN)_2$-GlcNAc and $(GlcN)_3$-GlcNAc, respectively. These results suggest that chitinasae II is suitable for preparing mono-*N*-acetylated chitooligosaccharides with GlcNAc at the reducing end (Fig. 1). It is expected that a series of the mono-*N*-acetylated chitooligosaccharides can be separated each other by cation exchange chromatography. Therefore, we attempted to convert hetero-chitooligosaccharides to the

mono-*N*-acetylated chitooligosaccharides using chitinase II and β-*N*-acetylglucosaminidase (β-GlcNAcase). The mono-*N*-acetylated chitooligosaccharides with a DP of 2 to 11 were purified by CM-Sephadex C-25 column chromatography. This paper deals with the new method for preparing the biofunctional chitooligosaccharides with high DP by enzymatic reaction.

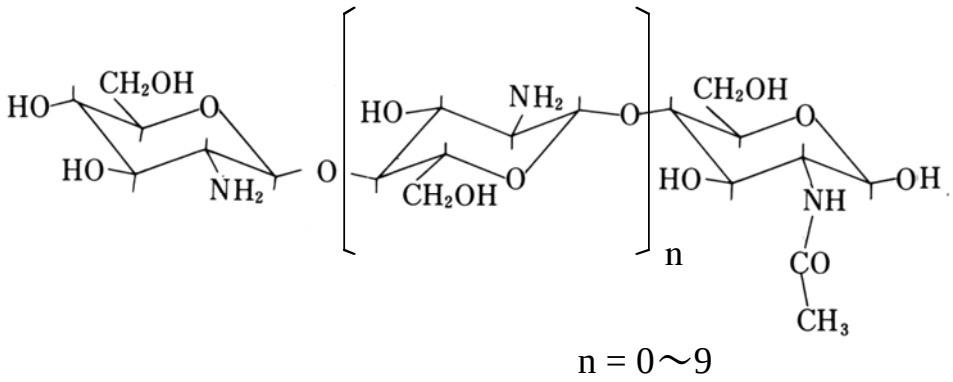

Figure 1: Structure of mono-*N*-acetylated chitooligosaccharides.

Material and Methods

Materials

The 25% *N*-acetylated chitosan was prepared according to the method of Hirano et al. [10]. $(GlcNAc)_{1\text{-}6}$ and $(GlcN)_{1\text{-}6}$ were supplied by Yaizu Suisan Kagaku Industry Co. Ltd., Shizuoka. $(GlcN)_2$-$(GlcNAc)_2$ and $(GlcN)_3$-$(GlcNAc)_2$ were obtained by the method of Mitsutomi et al. [11]. Chitinase II from *Aeromonas* sp. No. 10S-24 was purified according to the procedure described in the previous paper [8]. Chitinase A from *Ralstonia* sp. A-471 was purified as described previously [12]. β-GlcNAcase and Exo-β-glucosaminidase (β-GlcNase) were purified from the culture filtrate of *Pycnoporus cinnabarinus* IFO6139 [13] and *Penicillium* sp. AF9-P-128 [14], respectively. CM-Sephadex C-25 was product of Pharmacia Biotech. Bio-Gel P-6 was purchased from Bio-Rad Laboratories.

Enzyme assay

Chitinase activity was assayed in a reaction mixture containing 0.5 ml of 0.5% colloidal chitin, 1.0 ml of McIlvaine buffer (pH 4.0), and 0.5 ml of enzyme solution. After incubation with shaking for 10 min at 37 ºC, the reaction was stopped by boiling, then centrifuged. The amount of reducing sugar liberated was determined by modified Schales' method described by Imoto and Yagishita [15]. One unit of chitinase activity was defined as the amount of enzyme which produced 1 μmol of reducing sugar per min under the standard conditions. β-GlcNAcase and β-GlcNase activities were assayed by the procedures described previously [16].

Reducing sugar measurement

The reducing sugar in each fraction separated by column chromatography was measured by Schales' method with modification [15], using GlcN as a standard.

TLC of chitooligosaccharides

The products of the enzymatic hydrolysis of partially *N*-acetylated chitooligosaccharide were analyzed by TLC. The reaction mixture (50 μl) consisted of 0.2% oligosaccharide, 0.04 M phosphate buffer (pH 5.5), and 0.0125 units of β-GlcNase, incubated for 20 h at 37ºC. After the mixtures were boiled for 3 min to stop the reaction, 2 μl of the samples were applied to TLC. Sugars were developed on a silica gel plate (Silica gel 60, Merck) with a *n*-propanol-water-30% ammonia (70:15:15) mixture as the developing solvent. The spots were made visible with by diphenylamine-aniline reagent.

High performance liquid chromatography (HPLC)

The HPLC system consisted of an 880-PU pump, an 875-UV/VIS detector (Japan Spectroscopic Co. Ltd., Tokyo), and D-2500 Chromato-Integrator (Hitachi Ltd., Tokyo). Sugars were separated on a Radial-PAK μBondapak NH_2 column (8.0×100 mm, Millipore Co., Milford, MA, USA) using acetonitrile-water (70:30) mixture as the mobile phase, at a flow rate of 2.0 ml per min. *N*-Acetylchitooligosaccharides were detected by monitoring the absorbance at 210 nm.

Mass spectrometry

The molecular weights of chitooligosaccharides were measured by a Voyager Elite matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS, PerSeptive Biosystems, Framingham, MA, USA) [17].

Hydrolysis of 25% N-acetylated chitosan by chitinase II and β-GlcNAcase

A reaction mixture consisting of 500 ml of 0.5% chitosan with 25% *N*-acetylation in 0.1 M acetate buffer (pH 4.0), 2 ml of 2% NaN_3, and 2 ml of chitinase II (4.0 units) was incubated for 96 h at 37˚C. After the addition of 0.8 ml of β-GlcNAcase (5.0 units), the mixture was further incubated for 24 h at 37 ºC. The reaction was stopped by boiling for 5 min. The reaction mixture was centrifuged for 15 min at 13,000 rpm. The supernatant was concentrated in a rotary evaporator under reduced pressure at a temperature below 35 ºC, and dialyzed through an electric dialyzer (Micro Acilyzer G1, Asahikasei Kogyo Co. Ltd., Kanagawa).

Results and Discussion

Hydrolysis of partially N-acetyated chitosan

The hydrolysis products of 25% *N*-acetylated chitosan with *Aeromonas* sp. No. 10S-24 chitinase II were analyzed by MALDI-TOF MS. In the mass spectrum of the hydrolyzate, the peaks corresponding to the mass number of $[M + Na]^+$ ions of mono-*N*-acetylated chitooligosaccharides with DP 4 to 17 were observed (Fig. 2). Diacetylated chitooligosaccharides with DP 4 to 6 were also detected in the reaction mixture. In our previous paper [4], the diacetylated oligosaccharides (DP 3 to 5) possessing GlcNAc both at the reducing end and the non-reducing end have been isolated from the hydrolyzate of 54% *N*-acetylated chitosan with chitinase II.

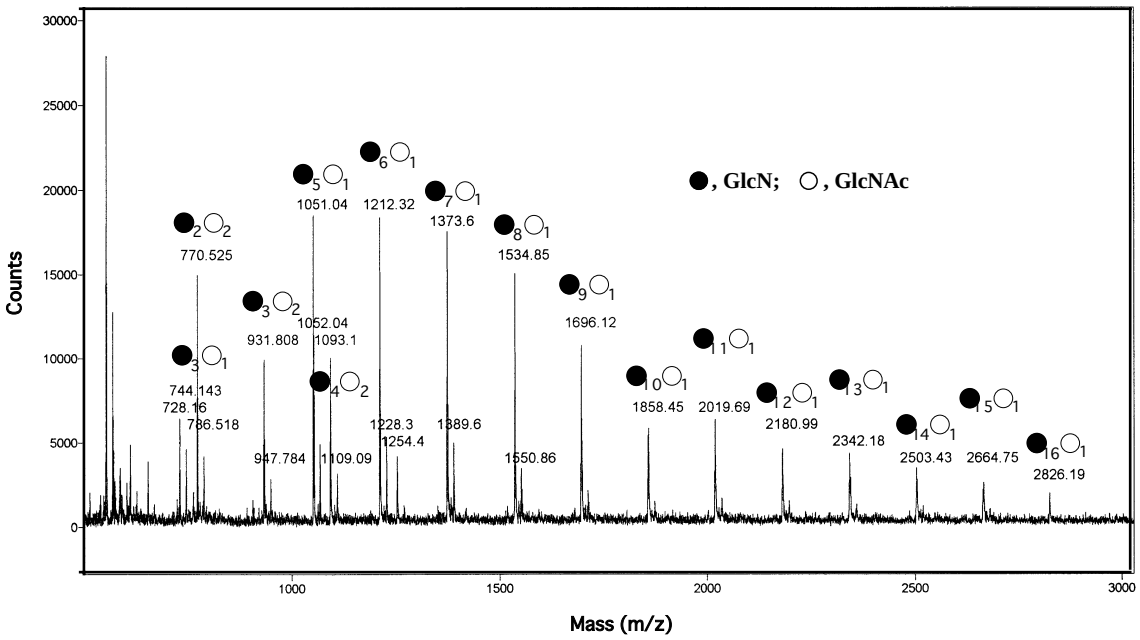

Figure 2: MALDI-TOF MS of chitooligosaccharides produced in the hydrolysis of 25% *N*-acetylated chitosan with *Aeromonas* sp. No. 10S-24 chitinase II.

Table 1: Hydrolysis products of diacetylated chitooligosaccharides by chitinases

Substrate	Products		
	Bacillus circulans WL-12 chitinase A1	*Ralstonia* sp. A-471 chitinase A	*Aeromonas* sp. No.10S-24 chitinase II
●●○∅	Not attacked	Not attacked	○ + ●●∅
●●●○∅	Not attacked	Not attacked	○ + ●●●∅

○ , GlcNAc ; ● , GlcN ; ∅ , ◐ , reducing end residue

As shown in Table 1, chitinase II hydrolyzed [$(GlcN)_2$-$(GlcNAc)_2$] and [$(GlcN)_3$-$(GlcNAc)_2$] to produce GlcNAc and $(GlcN)_2$-GlcNAc or $(GlcN)_3$-GlcNAc, respectively. Recently, we have purified chitinase A (family 18) from *Ralstonia* sp. A-471, which is thermostable and highly active against various partially *N*-acetylated chitosan [12]. However, chitinase A could not attack β-1,4-*N*-acedtylglucosaminidic linkages existing at the reducing end of the diacetyl chitooligosaccharides. These results suggest that chitinasae II is suitable for preparing mono-*N*-acetylated chitooligosaccharides with GlcNAc at the reducing end. Furthermore, we decided to use β-GlcNAcase from *P. cinnabarinus* to remove GlcNAc at the non-reducing end of the diacetylated chitooligosaccharides.

Separation and characterization of chitooligosaccharides

Chitosan with 25% *N*-acetylation was treated with chitinase II and β-GlcNAcase, and the hydrolyzate was separated by CM-Sephadex C-25 column chromatography (Fig. 3). The dialyzed hydrolyzate was put onto a column (2.6 x 68 cm) of CM-Sephadex C-25 previously equilibrated with 0.02 M acetate buffer (pH 5.0). After the column was washed with the same buffer, oligosaccharides were eluted with a linear gradient from 0 to 2.0 M NaCl in the same buffer, at a flow rate of 42 ml per h. Fractions from F-1 to F-13 were collected, dialyzed through an electric

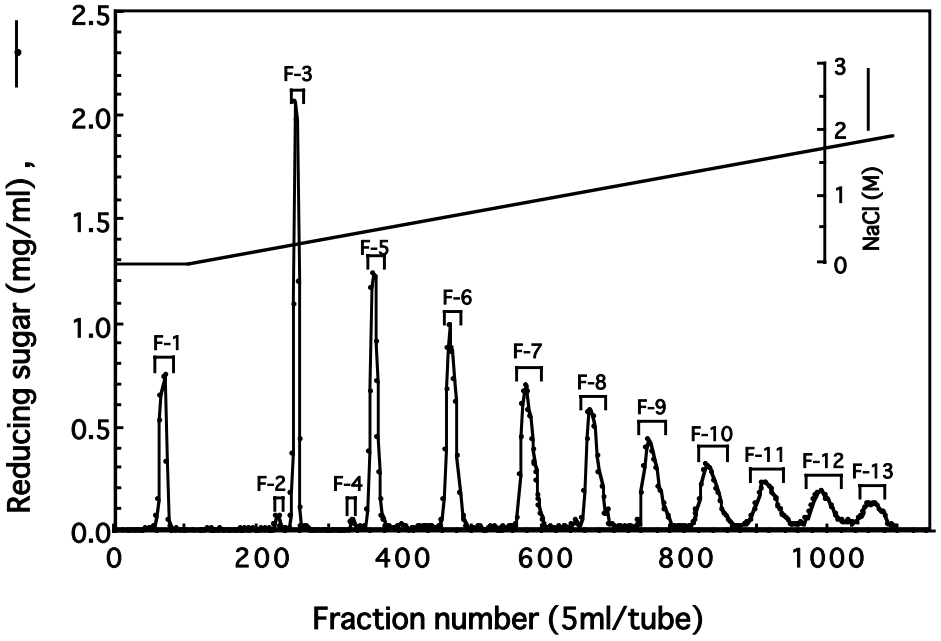

Figure 3: Chromatography of hydrolyzates of 25% *N*-acetylatyed chitosan with chitinase II and β-GlcNAcase on CM-Sephadex C-25 column.

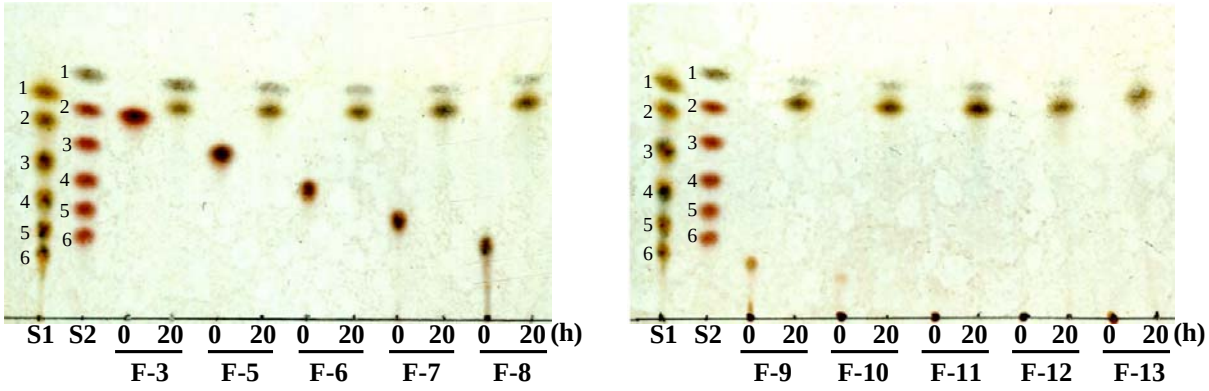

Figure 4: TLC of the hydrolysis products of F-3, F-5, F-6, F-7, F-8, F-9, F-10, F-11, F-12, and F-13 by β-GlcNase. Lanes S1, $(GlcN)_{1-6}$; lanes S2, $(GlcNAc)_{1-6}$.

dialyzer, and lyophilized. Fractions F-8 to F-11 were further purified by gel filtration on Bio-Gel P-6 column (2.6 x 195 cm) with the elution of 0.15 M ammonium acetate buffer (pH 5.0). The yields of fractions F-1, F-3, F-5, F-6, F-7, F-8, F-9, F-10, F-11, F-12, and F-13 were 72, 121, 146, 176, 162, 120, 97, 92, 90, 148, and 110 mg, respectively. F-1 contained GlcNAc. The hetero-chitooligosaccharides were characterized by MALDI-TOF MS analysis combined with exo-glycosidase digestion. In MALDI-TOF MS, chitooligosaccharides were detected in the positive ion mode as sodium and potassium adducts. In the MALDI-TOF MS analyses of F-3, F-5, F-6, F-7, F-8, F-9, F-10, F-11, F-12, and F-13, the $[M+Na]^+$ ions were detected at *m/z* 405.3, 566.4, 727.4, 888.3, 1049.8, 1211.1, 1372.2, 1533.4, 1694.1, and 1854.9, indicating that these are monoacetylated chitooligosaccharides with DP from 2 to 11, respectively. These oligosaccharides were completely decomposed by β-GlcNase to GlcN and GlcNAc (Fig. 4). From these results, F-3, F-5, F-6, F-7, F-8, F-9, F-10, F-11, F-12, and F-13 were identified to be GlcN-GlcNAc, $(GlcN)_2$-GlcNAc, $(GlcN)_3$-GlcNAc, $(GlcN)_4$-GlcNAc, $(GlcN)_5$-GlcNAc, $(GlcN)_6$-GlcNAc, $(GlcN)_7$-GlcNAc, $(GlcN)_8$-GlcNAc, $(GlcN)_9$-GlcNAc, and $(GlcN)_{10}$-GlcNAc, respectively.

It is very difficult to prepare hetero-chitooligosaccharides with a defined distribution of acetyl group. In this study, we succeeded in preparation of a series of mono-*N*-acetylated chitooligosaccharides (DP 2 to 11) with GlcNAc at the reducing end. Family 75 chitosanase from *Aspergillus fumigatus* also hydrolyzed partially *N*-acetylated chitosan into $(GlcN)_{2-5}$ and $(GlcN)_{1-3}$-GlcNAc [18]. However, the preparation of the mono-*N*-acetylated chitooligosaccharides with DP higher than 6 using the chitosanase is difficult, because the chitosanse cleave GlcN-GlcN and GlcNAc-GlcN linkages in chitosan molecule. The mono-*N*-acetylated chitooligosaccharides can be converted to corresponding fully *N*-acetylated chitooligosaccharides. Recently, Côté et al. [19] employed the mono-*N*-acetylated chitooligosaccharides as a useful substrate to examine the catalytic mechanism of chitosanolytic enzyme. Study of the physiological activities of the mono-*N*-acetylated chitooligosaccharides would be also expected for their useful applications.

Acknowledgements
We thank Dr. Yuto Kamei and Mr. Tatsuya Ida (Coastal Bioenvironment Center, Saga University) for their help with the mass-spectrometric analysis. We also thank Miss Saori Takahara, Miss Shoko Kobayashi for their technical assistance during this study.

References

[1] F. Shahidi, J. K. V. Arachchi, Y.-J. Jeon, Trends Food Sci. Technol., 10 (1999) 37-51.

[2] M. Izume, A. Ohtakara, Agric. Biol. Chem., 51 (1987) 1189-1191.

[3] S. Aiba, Carbohydr. Res., 265 (1994) 323-328.

[4] M. Mitsutomi, M. Ueda, M. Arai, A. Ando, T. Watanabe, Chitin Enzymology, 2 (1996) 273-284.

[5] B. Henrissat, Biochem. J., 280 (1991) 309-316.

[6] T. Ohno, S. Armand, T. Hata, N. Nikaidou, B. Henrissat, M. Mitsutomi, T. Watanabe, J. Bacteriol., 178 (1996) 5065-5070.

[7] M. Mitsutomi, A. Uchiyama, T. Yamagami, T. Watanabe, Advances in Chitin Science, 2 (1997) 250-255.

[8] M. Ueda, M. Arai, Biosci. Biotechnol. Biochem., 56 (1992) 460-464.

[9] M. Ueda, T. Kawaguchi, M. Arai, J. Ferment. Bioeng., 78 (1994) 205-211.

[10] S. Hirano, S.Tsuneyasu, Y. Kondo, Agric. Biol. Chem., 45 (1981) 1335-1338.

[11] M. Mitsutomi, H. Kidoh, H. Tomita, T. Watanabe, Biosci. Biotechnol. Biochem., 59 (1995) 529-531.

[12] A. Sutrisno, M. Ueda, Y. Abe, M. Nakazawa, K. Miyatake, Appl. Microbiol. Biotechnol., 63 (2004) 398-406.

[13] A. Ohtakara, M. Yoshida, M. Murakami, T. Izumi, Agric. Biol. Chem., 45 (1981) 239-247.

[14] Y. Uchida, H. Takeda, A. Ohkuma, K. Seki, Chitin and Chitosan Research (in Japanese), 1(1995) 138-139.

[15] T. Imoto, K. Yagishita, Agric. Biol. Chem., 35 (1971) 1154-1156.

[16] M. Mitsutomi, A. Ohtakara, T. Fukamizo, S. Goto, Agric. Biol. Chem., 54 (1990) 871-877.

[17] M. Mitsutomi, M. Isono, A. Uchiyama, N. Nikaidou, T. Ikegami, T. Watanabe, Biosci. Biotechnol. Biochem., 62 (1998) 2107-2114.

[18] C.-Y. Cheng, C.-H. Chang, Y.-J. Wu, Y.-K. Li, J. Biol. Chem., 281 (2006) 3137-3144.

[19] N. Côté, A. Fleury, E. Dumont-Blanchette, T. Fukamizo, M. Mitsutomi, R. Brzezinski, Biochem. J., 394 (2006) 675-686.