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A novel method for chemo-enzymatic synthesis of elicitor-active chitosan oligomers and partially *N*-deacetylated chitin oligomers using *N*-acylated chitotrioses as substrates in a lysozyme-catalyzed transglycosylation reaction system

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Abstract

N,N′,N″-Tri(monochloro)acetylchitotriose prepared by *N*-monochloroacetylation of chitotriose trihydrochloride was successfully polymerized into higher-molecular-weight oligomers by a lysozyme-catalyzed transglycosylation reaction, and a following base-catalyzed *N*-demonochloroacetylation gave a chitosan oligomer mixture mainly composed of oligomers with dp > 6. Partially *N*-deacetylated chitin oligomers (DAC oligomers) with dp 4–12 were synthesized by the enzyme reaction using *N,N′,N″*-tri(monochloro)acetylchitotriose and *N,N′,N″*-triacetylchitotriose (chitin trimer) as initial substrates followed by *N*-demonochloroacetylation. The structures of synthetic oligomers were analyzed by ^{1}H NMR spectroscopy, enzymatic hydrolysis and nitrous acid deamination–$NaBH_4$ reduction treatment. The dp of synthetic oligomers was measured by MALDI TOF MS (matrix-assisted laser desorption ionization time-of-flight mass spectrometry) using per-*N*-acetylated derivatives. The synthetic chitosan and DAC oligomers were strong elicitors for phytoalexin induction in *Pisum sativum* and *Phaseolus vulgaris*. This chemo-enzymatic method utilizing *N*-acylated chitotrioses as substrates is a novel approach to the synthesis of high-molecular-weight chitosan oligomers and DAC oligomers of biological importance.

Abbreviations: DAC, partially *N*-deacetylated chitin; GlcNAc, 2-acetamido-2-deoxy-D-glucopyranose; GlcN, 2-amino-2-deoxy-D-glucopyranose; $(GlcNAc)_n$ ($n = 2, 3$), β-(1 → 4)-linked *n*-mer of GlcNAc; dp, degree of polymerization; da, degree of *N*-acetylation

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1. Introduction

Oligosaccharide fragments derived from fungal cell walls have been shown to induce defense responses in plants [1]. These oligosaccharides, called elicitors, have also been employed to increase the yield of secondary products in various plant cell cultures [2]. Chitin [(1 → 4)-linked 2-acetamido-2-deoxy-β-D-glucan], chitosan (*N*-deacetylated form of chitin) and their oligosaccharide fragments have been reported to exhibit elicitor activities towards several plants, and have been widely used as elicitors for the induction of secondary products in plant cell cultures [1,2].

During our research work on the biological potentiality of natural polysaccharides including chitin and algal β-glucan, laminaran [3,4], we have found that the oligosaccharide fragments of both chitosan and DAC derived from chitin are potent inducers of a phytoalexin, (+)-pisatin, and antimicrobial flavonoids in pea epicotyls [5,6], and recently showed that chitosan hexamer and DAC heptamer is the smallest unit for elicitor activity in the two series of oligomers [7]. MALDI TOF MS analysis of the DAC heptamer, which was prepared by partial *N*-acetylation of chitosan heptamer, revealed that mono-, di-, and tri-*N*-acetylated oligomers are major constituents in the DAC heptamer preparation, and that there was no detectable amount of chitosan heptamer in the analyte. These facts suggest the possible presence of different recognition mechanisms for chitosan oligomer elicitors and DAC oligomer elicitors, prompting us to develop an effective method for the preparation of the two series of higher oligomers for further investigation on the induction mechanism of chemical defenses in *Pisum sativum* as well as other plants potentially elicited by chitin-derived oligosaccharides.

Conventional methods for preparing chitosan oligomers by partial hydrolysis of chitosan using acids or enzymes predominantly yield lower oligomers. The chemical synthesis of chitosan oligomers up to a dodecamer with a 4-methoxyphenyl group at the reducing end has been carried out [8] and their elicitor activities towards *Pisum sativum* have been evaluated [9]. However, chemical syntheses of oligosaccharides require complicated processes for multiple protection and deprotection. DAC oligomers can be prepared by selective *N*-acetylation of the chitosan oligomers.

We report here a novel method for the synthesis of chitosan oligomers and DAC oligomers utilizing a transglycosylation reaction catalyzed by hen egg-white lysozyme with the use of *N,N′,N″*-tri(monochloro)acetylchitotriose (**1**) and *N,N′,N″*-triacetylchitotriose (**2**) as glycosyl donors (Fig. 1).

2. Results and discussion

Synthetic strategy for chemo-enzymatic synthesis of chitosan oligomers and DAC oligomers (Fig. 1).—Hen egg-white lysozyme (EC 3.2.1.17) has been shown to catalyze

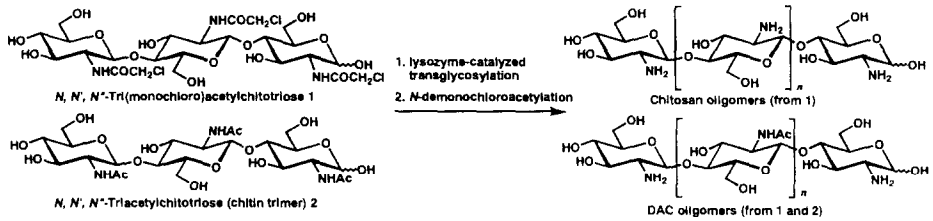

Fig. 1. Chemo-enzymatic synthesis of chitosan oligomers and DAC oligomers utilizing a lysozyme-catalyzed transglycosylation reaction followed by *N*-demonochloroacetylation.

a transglycosylation reaction. Usui et al. reported a method for the enzymatic synthesis of hexa-*N*-acetylchitohexaose and hepta-*N*-acetylchitoheptaose by the lysozyme-catalyzed transglycosylation reaction using di-*N*-acetylchitobiose as an initial substrate [10]. An X-ray crystallographic study of lysozyme-hexa-*N*-acetylchitohexaose complex clearly showed that the presence of *N*-acetyl groups in chitin oligomers was essential for binding of the oligomers to the active site of lysozyme [11]. This result suggests that structural analogs of chitin oligomers, such as *N*-acylated chitosan oligomers, could bind to the active site of the enzyme, and act as glycosyl donors in a lysozyme-catalyzed transglycosylation reaction. According to this hypothesis, we tried to use a *N,N',N''*-tri(monochloro)acetylated chitosan oligomer for the enzyme reaction as an analog of chitin oligomer because of its structural similarity. Another reason for choosing this analog is that monochloroacetamides are more easily cleaved by base than unsubstituted acetamides, thus enabling *N*-demonochloroacetylation in the presence of both *N*-acetyl and *N*-monochloroacetyl groups. We therefore expected that higher chitosan oligomers could be synthesized by the transglycosylation reaction followed by complete *N*-demonochloroacetylation using **1** as an initial substrate, and similarly, higher DAC oligomers with different da could be obtained using a mixture of **1** and **2**. Results from a preliminary experiment dictated the use of trimer as a substrate for this purpose.

Chemo-enzymatic synthesis of chitosan oligomers using N,N',N''*-tri(monochloro)acetylchitotriose* (**1**) *as a substrate (Table 1).*—*N,N',N''*-tri(monochloro)acetylchitotriose (**1**) was polymerized by hen egg-white lysozyme in 50% acetone–0.1 M acetate buffer (pH 4.0) or in 0.85% $(NH_4)_2SO_4$–0.1 M acetate buffer (pH 4.0). Reaction conditions are listed in Table 1. Only a trace amount of a transglycosylation product was formed at 40°C, and most of the substrate still remained even after 112 h incubation. However, incubation at 60°C accelerated the transglycosylation reaction. As the polymerization reaction proceeded, high-molecular-weight products precipitated as white solids. Addition of $(NH_4)_2SO_4$ to the buffer resulted in a 1.1-fold increase in product (Table 1, entry 5), while the formation of precipitate was not observed at a higher concentration of $(NH_4)_2SO_4$ (1.7%) (data not shown). A preliminary experiment examining the effect of organic solvent on the reaction showed that a comparable amount of white precipitate was formed in the buffer system containing 25% Me_2SO, 10% DMF, 10% 1,4-dioxane, and 5% MeCN at 60°C.

Table 1
Chemo-enzymatic synthesis of chitosan oligomers using *N,N′,N″*-tri(monochloro)acetylchitotriose, **1** [a], in various conditions

Entry	Solvent	Temp. (°C)	Time (h)	Yield (%) [b]	Dp [c]
1	buffer [d]	40	112	4.3	5–10
2	50% acetone–buffer	40	112	3.5	5–10
3	0.85% $(NH_4)_2SO_4$–buffer	40	112	8.4	5–10
4	buffer	60	52	29.8	4–10
5	0.85% $(NH_4)_2SO_4$–buffer	60	52	33.2	4– 9

[a] 34×10^{-3} mol/L; lysozyme (hen egg-white) 0.17% (w/v).
[b] Solvent insoluble part in a transglycosylation reaction.
[c] Dp of the oligomers was measured by MALDI TOF MS as per-*N*-acetylated derivatives.
[d] 0.1 M acetate buffer (pH 4.0).

The ^{1}H NMR spectra of synthetic chitosan oligomers were superimposable on those of a chitosan oligomer mixture obtained by HCl hydrolysis of chitosan. The signals at δ 5.43 and δ 4.85–4.90 were assigned to the α- and β-anomeric protons of the GlcN units respectively, suggesting that the oligomers are β-(1 → 4)-linked (Fig. 2a). In order to obtain more detailed information on regio- and stereo-chemistry of the linkage, the mixtures were converted to the corresponding per-*N*-acetylated derivatives by *N*-acetylation, and were subjected to lysozyme digestion in 0.1 M acetate buffer (pH 5.6) at 40°C [10]. HPLC analysis showed the occurrence of GlcNAc, $(GlcNAc)_2$, and $(GlcNAc)_3$ in the hydrolysis products. These results clearly indicate that glycosidic bond formation occurred in a regio- and stereo-selective manner to afford chitosan oligomers containing β-(1 → 4)-linkages.

The MALDI TOF MS analysis of the *N*-acetylated derivatives revealed that the products consist of tetramer through decamer. Although the amount of each component in the products was not determined in detail, TLC and HPLC analysis indicated that the major constituents in the mixtures were oligomers with dp > 6.

Chemo-enzymatic synthesis of DAC oligomers using N,N′,N″*-tri(monochloro)acetylchitotriose* (**1**) *and* N,N′,N″*-triacetylchitotriose* (**2**) *as substrates (Table 2).*—Lysozyme-catalyzed transglycosylation using both *N,N′,N″*-tri(monochloro)acetylchitotriose (**1**) and *N,N′,N″*-triacetylchitotriose (**2**) as substrates was carried out in the same way as in the chitosan oligomer synthesis described above. Reaction temperatures for a 50% acetone–buffer system and a 0.85% $(NH_4)_2SO_4$–buffer system were 40 and 60°C, respectively. In contrast to the reaction using **1** as a single substrate in a 50% acetone–buffer system (Table 1, entry 2), the transglycosylation reaction using both **1** and **2** proceeded very smoothly, eventually resulting in precipitation of high-molecular-weight oligomers in all experiments performed. After 112 h of incubation when the initial substrates were almost consumed, the reaction mixture was processed as described above to give a DAC oligomer mixture. The reaction in a buffer containing $(NH_4)_2SO_4$ also proceeded smoothly to give a white precipitate of high molecular weight oligomers. A series of work-up processes described above afforded a DAC oligomer mixture. Although the enzymatic polymerization of **1** and **2** also occurred in the buffer system containing 50% MeCN, 50% EtOH, 50% 1,4-dioxane, and 50% DMF at 40°C, these

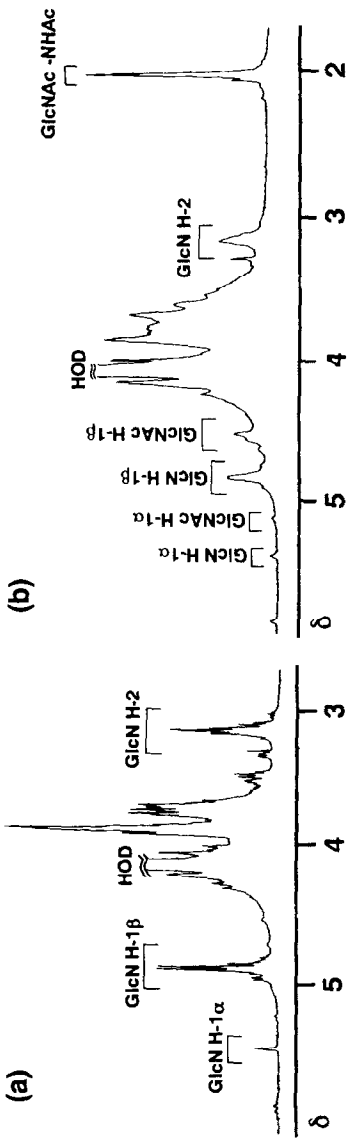

Fig. 2. 500 MHz ^1H NMR spectra of synthetic chitosan oligomers (a, Table 1, entry 5) and DAC oligomers (b, Table 2, entry 5).

Table 2
Chemo-enzymatic synthesis of DAC oligomers using *N,N',N''*-tri(monochloro)acetylchitotriose (**1**) and *N,N',N''*-triacetylchitotriose (**2**)

Entry	Substrate concn ($\times 10^{-3}$ mol/L)		Molar ratio	Yield (%) [a]	Da (%) [b]	Dp [c]
	1	2				
1	25	8.2	3:1	16.6	23	4–11
2	17	17	1:1	15.8	53	4–12
3	8.4	25	1:3	14.1	76	4–12
4	25	8.2	3:1	21.3	13	4–10
5	17	17	1:1	10.1	36	4–11
6	8.4	25	1:3	3.4	73	4–12

Entry 1–3; synthesized in 50% acetone–0.1 M acetate buffer (pH 4.0) at 40°C for 112 h; Entry 4–6; synthesized in 0.85% $(NH_4)_2SO_4$–0.1 M acetate buffer (pH 4.0) at 60°C for 52 h; lysozyme (hen egg-white) 0.17% (w/v).

[a] Solvent insoluble part in a transglycosylation reaction.

[b] Da was determined by comparison of the area under the H-1 proton signals of the GlcNAc units with that of the GlcN units in 500 MHz ^{1}H NMR spectrum (D_2O, 90°C).

[c] Dp of the oligomers was measured by MALDI TOF MS as per-*N*-acetylated derivatives.

gave low yields compared to the acetone- or $(NH_4)_2SO_4$-containing buffer systems (data not shown). No reaction was observed in 50% MeOH- and 50% Me_2SO-containing buffer systems at 40°C.

The ^{1}H NMR spectra of the synthetic DAC oligomers showed signals due to the H-1 protons of the GlcN units (δ 4.85–4.90 for H-1β, δ 5.43 for H-1α) and those of the GlcNAc units (δ 4.55–4.65 for H-1β, δ 5.19 for H-1α) (Fig. 2b). The spectra also revealed that the removal of *N*-monochloroacetate residues with base proceeded without any detectable epimerization of reducing GlcNAc to ManNAc, whose anomeric protons were observed at δ 5.11 (H-1β) and δ 5.01 (H-1α) in the same analytical conditions. Lysozyme digestion of the per-*N*-acetylated derivatives of DAC oligomers afforded hydrolysis products of GlcNAc, $(GlcNAc)_2$, and $(GlcNAc)_3$. These results confirmed the β-(1 → 4)-linkage of the synthetic oligosaccharides.

Based on the ^{1}H NMR spectrum, the degree of acetylation was determined by comparison of the area under the H-1 proton signals of the GlcN units with that of the GlcNAc units. The degree of acetylation of DAC oligomers synthesized in a 50% acetone–buffer was in close agreement with the molar ratio of **1** and **2**, while that obtained in a 0.85% $(NH_4)_2SO_4$–buffer was lower than that expected from the molar ratio.

MALDI TOF MS analysis of the per-*N*-acetylated derivatives of DAC oligomers showed that the highest dp of the oligomers in each experiment increased as the molar ratio of **2** to **1** increased, probably because **2** has better solubility than **1**. Fig. 3 shows a MALDI TOF MS spectrum of the per-*N*-acetylated derivatives of DAC oligomers synthesized in entry 5 of Table 2. Under the conditions described in the Experimental section, chito-oligomers were detected as monosodium and monopotassium carbohydrate adduct ions. All signals in the low-mass range (below 600 Da) exclusively represented ions of the matrix and were observed also in the absence of the analyte. In the MALDI

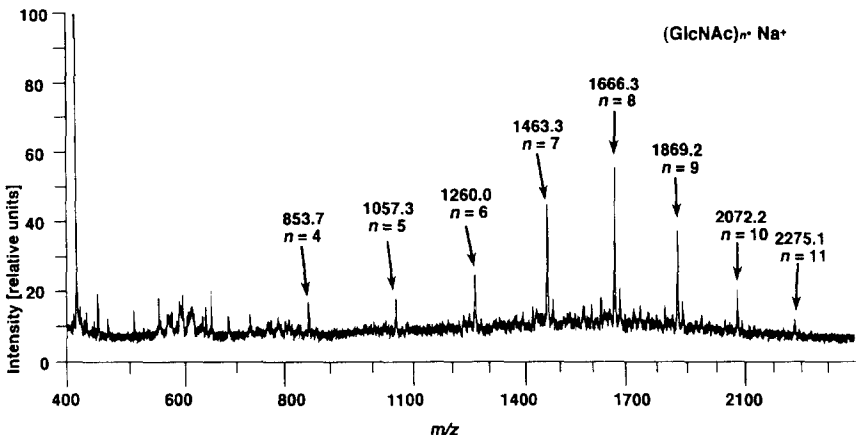

Fig. 3. MALDI TOF MS spectrum of per-*N*-acetylated derivative of synthetic DAC oligomers (Table 2, entry 5).

TOF MS, the octamer was observed as the most prominent peak. Nevertheless, the relative amount of each oligomer product could not be determined from relative intensity in the spectrum since a linear correlation between the relative ion-intensity and the quantification of the product has not yet been established. However, a preliminary analysis by TLC and HPLC of the synthetic products indicated that the oligomers with dp > 6 were major constituents.

In order to obtain information about the distribution of GlcNAc units in the DAC oligomers, the oligomers synthesized in entry 2 (Table 2) were subjected to nitrous acid deamination–$NaBH_4$ reduction in which the D-glucosamine units of DAC oligomers are converted into 2,5-anhydro-D-mannitol. ESI-MS and HPLC analyses revealed the occurrence of oligomers, $(GlcNAc)_n$-2,5-anhydro-D-mannitol (n = 1, 2, 3, and 4), in the deamination–reduction product. This result showed that GlcNAc, $(GlcNAc)_2$, $(GlcNAc)_3$, and $(GlcNAc)_4$ sequences are present in the DAC oligomers.

Elicitor activities of the synthetic chitosan oligomers and DAC oligomers towards Pisum sativum *and* Phaseolus vulgaris.—The chitosan and DAC oligomers synthesized in a $(NH_4)_2SO_4$–buffer system at 60°C were examined for phytoalexin-inducing activity in pea epicotyl assay and bean cotyledon assay at concentrations ranging from 6.25 to 100 μg/mL (Fig. 4a,b). The results showed that two of the synthetic DAC oligomers with a degree of acetylation of 13% and 36% being the most effective elicitors for phytoalexin induction in both assay systems. These oligomers significantly induced (+)-pisatin in the pea assay and (±)-kievitone in the bean assay at all concentrations tested. The two compounds are known to be major phytoalexins in plants [12,13]. The synthetic chitosan oligomers also exhibited strong activity at relatively high concentrations (50, 100 μg/mL) in the pea epicotyl assay, while the oligomers showed little activity in the bean cotyledon assay. The DAC oligomer with a degree of acetylation of 73% was a weak elicitor towards both plants.

Chitin- and chitosan-derived oligosaccharides with dp > 5 have been shown to induce plant defensive responses, and to exert antitumor [14] and antimicrobial activities [15].

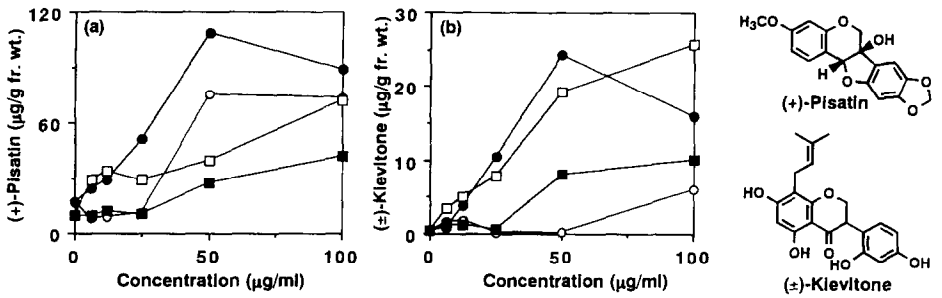

Fig. 4. Phytoalexin-inducing activities of the synthetic chito-oligomers in (a) pea epicotyl assay and (b) bean cotyledon assay, and the structures of (+)-pisatin and (±)-kievitone. Chito-oligomers synthesized in Table 1, entry 5 (○, dp 4–9, degree of acetylation 0%), in Table 2, entry 4 (●, dp 4–10, degree of acetylation 13%), entry 5 (□, dp 4–11, degree of acetylation 36%), and entry 6 (■, dp 4–12, degree of acetylation 73%).

The method described here is effective for preparing high-molecular-weight chitosan oligomers and DAC oligomers with β-(1 → 4)-linkages. The transglycosylation reaction using **1** and **2** as substrates in a 50% acetone–buffer system is especially useful for the synthesis of high molecular weight DAC oligomers with a desired degree of acetylation. Chitotriose trihydrochloride, which was used as a starting compound for the preparation of *N,N',N''*-tri(monochloro)acetylchitotriose and *N,N',N''*-triacetylchitotriose, is easily prepared in gram quantities by ion-exchange chromatography of an HCl-catalyzed hydrolysate of chitosan. *N*-Acylation of the trimers is straightforward, and hen egg-white lysozyme is readily available from commercial sources at a low price.

3. Experimental

Materials.—Chitosan was purchased from Tokyo Chemical Industry Co., Ltd. Hen egg-white lysozyme was provided by Wako Pure Chemical Industries, Ltd. Chitotriose trihydrochloride (chitosan trimer) was purified from an HCl-catalyzed hydrolysis product of chitosan by Dowex 50W-X8 (H^+) column chromatography [16].

Measurements.—^{1}H NMR spectra were recorded on a Varian VXR-500 spectrometer. The values of δ (in D_2O) are expressed in ppm downfield from the reference, internal sodium 3-(trimethylsilyl)propionate-d_4. ESI-MS (electrospray ionization mass spectrometry) was performed in the positive ion mode on a Perkin–Elmer–Sciex API-III spectrometer. MALDI TOF MS spectra were measured on a Finnigan MAT Vision 2000 with 10 mg/mL 2,5-dihydroxybenzoic acid in 30% MeCN–H_2O (0.1% CF_3COOH) as a matrix solution. Ions were accelerated to an energy of 6 keV before entering the TOF mass spectrometer. Under these conditions, chito-oligomers were detected as monosodium and monopotassium carbohydrate adduct ions. HPLC analysis was carried out using a column of TSKgel Amide-80 (4.6 mm i.d. × 250 mm, TOSOH Co. Ltd.). The eluent for synthetic chitosan oligomers was 35% MeCN–H_2O (0.2% phosphoric acid, 0.2% triethylamine), and that for chitin oligomers and the oligomers from nitrous acid deamination–$NaBH_4$ reduction was 65% MeCN–H_2O. The flow rate was 0.5

mL/min at 40°C and peaks were detected with an RI monitor. TLC analysis was conducted using a DC-Alufolien kieselgel 60 F_{254} plate (Merck). The solvent system for chitosan oligomers was 4:3:3 *n*-BuOH–MeOH–28% NH_4OH, and that for chitin oligomers and the oligomers from deamination–reduction was 5:5:2 *n*-BuOH–MeOH–H_2O. Three times development was performed for the TLC analysis, and the carbohydrates were detected with diphenylamine–aniline–phosphoric acid.

N,N′,N″-*tri(monochloro)acetylchitotriose* (**1**).—To a solution of chitotriose trihydrochloride (2.6 g, 4.2 mmol) in H_2O (100 mL) and triethylamine (30.3 mL, 215 mmol) was added dropwise a solution of monochloroacetyl anhydride (17.3 g, 101 mmol) in 1,4-dioxane (50 mL) at 2–5°C. After stirring for 15 min, the mixture was diluted to 2 L with H_2O, and the solution was chromatographed on an activated charcoal column. The column was washed with H_2O (3 L) and eluted with acetone (2 L). The eluate was concentrated to give **1** (2.17 g, 71%). MALDI TOF MS *m*/*z* 752.4 (MNa^+). ^1H NMR data (500 MHz, D_2O, 60°C): δ 4.16–4.22 (m, 6 H, NHCOCH_2Cl), 4.65–4.68 (2 H, H-1β), 4.75 (d, *J* 8.1 Hz, H-1β), 5.21 (d, *J* 2.7 Hz, H-1α).

N,N′,N″-*triacetylchitotriose* (**2**).—*N,N′,N″*-triacetylchitotriose (**2**) was prepared from chitotriose trihydrochloride by the method for selective *N*-acetylation [17].

Chemo-enzymatic synthesis of chitosan oligomers.—*N,N′,N″*-tri(monochloro)acetylchitotriose (**1**) (150 mg, 205 μmol) was dissolved in acetone (3.0 mL)–0.1 M acetate buffer (pH 4.0, 2.5 mL) or in 0.1 M acetate buffer (pH 4.0, 5.5 mL) containing $(NH_4)_2SO_4$ (528 mg). To this solution was added a 0.1 M acetate buffer (0.5 mL) solution containing hen egg-white lysozyme (10 mg, final concn 0.17%). Final concentrations for acetone and $(NH_4)_2SO_4$ were 50% and 0.85%, respectively. The reaction conditions are listed in Table 1. As the polymerization reaction proceeded, high molecular weight products precipitated as white solids. After incubation, the precipitate was collected by centrifugation, washed 3 times with aq 50% acetone, and suspended in H_2O (2 mL). The suspension was heated at 100°C for 10 min, and lyophilized. The resulting products (e.g. 49.8 mg, entry 1, Table 1) were treated with aq 5 M NaOH for 20 min at 40°C, neutralized with 1 M HCl, desalted using a Micro Acilyzer (Asahikasei Kogyo Co. Ltd.), and lyophilized to give chitosan oligomers with high molecular weights (32.3 mg, entry 1, Table 1).

Chemo-enzymatic synthesis of DAC oligomers.—Chemo-enzymatic synthesis of DAC oligomers was carried out in the same way as in the chitosan oligomer synthesis described above. In entries 2 and 5 of Table 2, *N,N′,N″*-tri(monochloro)acetylchitotriose (**1**) (73 mg, 100 μmol) and *N,N′,N″*-triacetylchitotriose (**2**) (63 mg, 100 μmol) were dissolved in acetone (3.0 mL)–0.1 M acetate buffer (pH 4.0, 2.5 mL) or in 0.1 M acetate buffer (pH 4.0, 5.5 mL) containing $(NH_4)_2SO_4$ (528 mg). To this solution was added a 0.1 M acetate buffer (0.5 mL) solution containing hen egg-white lysozyme (10 mg, final concn 0.17%). Reaction conditions are listed in Table 2. After incubation, the resulting precipitates were collected by centrifugation, washed, and suspended in H_2O (2 mL). The suspension was heated at 100°C for 10 min, and lyophilized. The resulting products (21.5 and 13.8 mg, respectively) were demonochloroacetylated by base, neutralized, and desalted. Lyophilization of the desalted products gave DAC oligomers (17.9 and 12.2 mg, respectively).

Nitrous acid deamination–$NaBH_4$ reduction of DAC oligomers.—The nitrous acid

deamination–$NaBH_4$ reduction of DAC oligomers was performed according to the method of Shively et al. [18]. The products obtained were analyzed by ESI-MS, HPLC, and TLC.

According to the method by Sashiwa et al. [19], authentic samples of chitin oligomers with 2,5-anhydro-D-mannitol at the reducing end were obtained from the deamination–reduction product of DAC with a degree of acetylation of 49% (390 mg) prepared by the method previously reported [5]. The deamination–reduction product was separated by activated charcoal column chromatography employing gradient elution from H_2O to 90% MeOH–H_2O to afford oligomers [$(GlcNAc)_n$-2,5-anhydro-D-mannitol, $n = 1$, 2, 3, and 4]. The structures of the oligomers were assigned by ESI-MS analysis, which gave m/z 368.2 (MH^+), 571.2 (MH^+), 774.2 (MH^+), and 977.5 (MH^+), respectively.

Elicitor assay.—Pea epicotyl elicitor and bean cotyledon elicitor assays were performed as described previously [5,20].

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