Enzymatic Polymerization to Novel Polysaccharides Having a Glucose-*N*-acetylglucosamine Repeating Unit, a Cellulose−Chitin Hybrid Polysaccharide

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A cellulose−chitin hybrid polysaccharide having alternatingly $\beta(1\rightarrow4)$-linked D-glucose (Glc) and *N*-acetyl-D-glucosamine (GlcNAc) was synthesized via two modes of enzymatic polymerization. First, a sugar oxazoline monomer of Glc$\beta(1\rightarrow4)$GlcNAc (**1**) was designed as a transition-state analogue substrate (TSAS) monomer for chitinase catalysis. Monomer **1** was recognized by chitinase from *Bacillus* sp., giving rise to a cellulose−chitin hybrid polysaccharide (**2**) via ring-opening polyaddition with perfect regioselectivity and stereochemistry. Molecular weight (M_n) of **2** reached 4030, which corresponds to 22 saccharide units. Second, a sugar fluoride monomer of GlcNAc$\beta(1\rightarrow4)$Glc (**3**) was synthesized for the catalysis of cellulase from *Trichoderma viride*. The enzyme catalyzed polycondensation of **3**, providing a cellulose−chitin hybrid polysaccharide (**4**) in regio- and stereoselective manner. M_n of **4** reached 2840, which corresponds to 16 saccharide units. X-ray diffraction measurements revealed that these hybrid polysaccharides did not form any characteristic crystalline structures. Furthermore, these unnatural hybrids of **2** and **4** were successfully digested by lysozyme from human neutrophils.

Introduction

Cellulose is one of the most abundant organic materials on the Earth and is produced by plants, algae, bacterial species, and tunicates.[1] It is biologically synthesized by the catalysis of cellulose synthase (EC 2.4.1.12), sequentially transferring D-glucose (Glc) using uridine-5′-diphospho-glucose (UDP-Glc) as substrate to a growing chain end in a $\beta(1\rightarrow4)$ fashion.[2] Human beings have utilized cellulose for housing, clothing, and also industrial products such as papers and fibers, because it is a renewable material that can be easily obtained and fabricated.[3] It is chemically functionalized to cellulose derivatives such as nitrocellulose as a component of coatings and films,[4] cellulose acetate as materials for hollow fibers and filters,[5] methyl and carboxymethyl cellulose as additives for cosmetics and foods,[6] and hydroxyethyl cellulose as thickener and colloid for various substances.[7] Because of the excellent characteristics of the biological inactivity, biocompatibility, and chirality, cellulose is applied for medical and pharmaceutical purposes[8] and separation of enantiomers.[9]

Chitin is the most abundant in the animal world. It is a linear polysaccharide of *N*-acetyl-D-glucosamine (GlcNAc) connecting through a $\beta(1\rightarrow4)$ glycosidic linkage. Chitin is found as a structural biopolymer in animals such as insects and invertebrates,[10] which is biologically produced by the action of chitin synthase (EC 2.4.1.16).[11] The enzyme catalyzes sequential $\beta(1\rightarrow4)$ glycosidic bond formation via transferring a GlcNAc unit from UDP-GlcNAc as substrate to a growing chain end. In contrast to cellulose, chitin and its derivatives in themselves are biologically active, and they are utilized as immuno-adjuvant substances,[12] inhibitor of metastases of tumor cells,[13] wound-healing materials,[14] additives for cosmetics,[15] and drug carriers.[16]

The structural difference between chitin and cellulose is the C2 substituent of a pyranoside unit: an acetamido group on the chitin molecule and a hydroxy group on the cellulose molecule. Thus, the C2 acetamido groups provide various bioactivities for chitin. However, the acetamido groups participate in the formation of inter- and intramolecular hydrogen bonding with the C6 hydroxy groups,[17] leading to a stiff chitin crystal insoluble in almost all solvents. From these two natural polysaccharides, a blend polymer was prepared by spinning an aqueous alkaline solution of their sodium xanthates.[18] The blend showed good mechanical properties as materials for wound healing[19] and improving the quality of soil and water.[20]

Enzymatic polymerization catalyzed by glycoside hydrolases has been demonstrated as an effective method for synthesis of natural and unnatural oligo- and polysaccharides.[21] For example, synthetic cellulose was produced from β-cellobiosyl fluoride by cellulase,[22] synthetic chitin from an *N*,*N*′-diacetylchitobiose oxazoline derivative by chitinase,[23] and synthetic hyaluronan, chondroitin, and their derivatives from oxazoline derivatives of hyalobiuronate and chondrosine by hyaluronidase.[24] Furthermore, a cellulose−xylan hybrid polysaccharide consisting of alternating $\beta(1\rightarrow4)$-linked Glc and D-xylose was successfully synthesized from β-D-xylosyl-$(1\rightarrow4)$-β-D-glucosyl fluoride by xylanase.[25] Thus, this methodology enables a single-step

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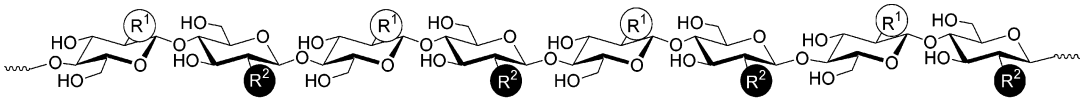
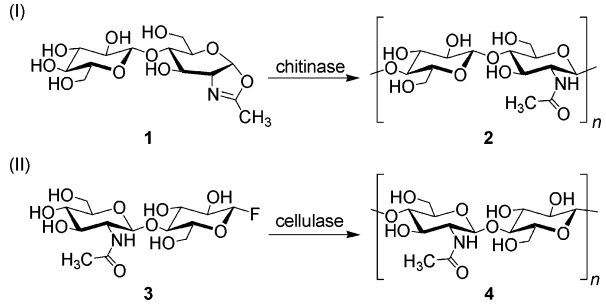


		R^1	R^2
(a)	a cellulose-chitin hybrid polysaccharide	OH	$NHCOCH_3$
(b)	cellulose	OH	OH
(c)	chitin	$NHCOCH_3$	$NHCOCH_3$

Figure 1. Chemical structures of (a) a cellulose−chitin hybrid polysaccharide, (b) cellulose, and (c) chitin.

Scheme 1. Enzymatic Polymerizations to a Cellulose−Chitin Hybrid Polysaccharide



production of natural and unnatural polysaccharides with well-defined structure via regioselective and stereocontrolled polymerization of designed carbohydrate monomers.

The present paper reports the first synthesis of a cellulose−chitin hybrid polysaccharide via two modes of enzymatic polymerization, i.e., chitinase-catalyzed ring-opening polyaddition of a sugar oxazoline monomer (**1**) and cellulase-catalyzed polycondensation of a sugar fluoride monomer (**3**) (Scheme 1). These polysaccharides have a disaccharide repeating unit composed of β(1→4)-linked Glc and GlcNAc, which are a constituent of cellulose and chitin, respectively. Therefore, these unnatural polysaccharides are a hybrid polysaccharide of cellulose and chitin at a molecular level and are expected to exhibit biological functions resulting from both natures (Figure 1).

Experimental Section

Materials. Dichloromethane and 1,2-dichloroethane were purified by distillation from P_2O_5 and stored over activated molecular sieves (4 Å) prior to use. Molecular sieves AW-300 (MSAW300) purchased from Aldrich (lot no. 04024CI) were ground and activated by heating over 100 °C under reduced pressure. Other chemicals were used without further purification. Cellulase from *Trichoderma viride* "ONOZUKA R-10" was purchased from Yakult Honsha Co., Ltd. (lot no. 201093). Chitinase from *Bacillus* sp. was purchased from Wako Pure Chemicals Inc. (lot no. LDH7046). Lysozyme from human neutrophils was purchased from Sigma-Aldrich Co. (lot no. 123K0837). All enzymes were used without further purification. Di-*N*-acetylchitobiose, tri-*N*-acetylchitotriose, tetra-*N*-acetylchitotetraose, penta-*N*-acetylchitopentaose, and hexa-*N*-acetylchitohexaose were purchased from Seikagaku Corp. (lot nos. 9612020, 9809100, 9812110, 9906280, and 9812110, respectively) and were used as chitooligosaccharide standards for SEC measurements. Cellobiose was purchased from ICN Biochemicals, Inc. (lot no. 9643F), and cellotriose, cellotetraose, cellopentaose, and cellohexaose were purchased from Seikagaku Corp. (lot nos. 400400, 400402, 400404, and 400406, respectively). These were used as cellooligosaccharide standards for SEC measurements. Pullulan standards (lot no. 80301) were purchased from Showa Denko K.K.

Measurements. NMR spectra were recorded on a Bruker DPX-400 spectrometer with tetramethylsilane or acetone as a reference in $CDCl_3$ or in D_2O, respectively. All assignments were based on correlation spectroscopy (COSY) experiments. High-resolution fast atom bombardment (HRFAB) mass spectra were obtained on a JEOL HX-110 spectrometer using 2,4-dinitrobenzyl alcohol or dithiothreitol/thioglycerol (1:1, v/v) as a matrix. Optical rotations were measured with a Jasco P-1010 polarimeter. Melting points were determined with a Yamato MP-21. High-performance liquid chromatography (HPLC) measurements were carried out by using a Tosoh LC-8020 system with Chemco Chemcobond ODS-W column (4.6 × 250 mm) eluting with a water−acetonitrile mixture (98:2, v/v; flow rate, 1.0 mL/min; 30 °C for monomer **1** or 50 °C for monomer **2**). An aliquot of the reaction mixture (5 μL) was sampled at the respective period and injected to the column to monitor reaction progress. Molecular weight values (M_n) of the product given after the reactions were determined by size exclusion chromatography (SEC) measurements using a Tosoh GPC-8020 system with Tosoh α-M column (7.8 × 300 mm) eluting with 0.6 M LiCl in an NMP−DMAc mixture (2:1, v/v; flow rate, 0.6 mL/min; 40 °C) calibrated with pullulan standards (M_n = 5900, 11 800, and 22 800), chitooligosaccharides (M_w = 424, 628, 831, 1034, and 1237), and cellooligosaccharides (M_w = 342, 504, 667, 829, and 991). Yields of a water-soluble part were determined by using a Tosoh LC-8020 system with Shodex Sugar KS-802 column (8.0 × 300 mm) eluting with water (flow rate, 0.5 mL/min; 80 °C), and this system was also used for purification of a water-soluble part and for monitoring the reaction of enzymatic digestibility. Matrix-assisted laser desorption ionization time-of-flight mass spectroscopy (MALDI-TOF/MS) analysis was performed on JEOL JMS-ELITE spectrometer using 2,5-dihydroxybenzoic acid as a matrix containing 0.1% trifluoroacetic acid under positive ion mode. Cross-polarization magic angle spinning (CP/MAS) ^{13}C NMR was recorded with a Bruker MSL-400 spectrometer. X-ray diffraction (XRD) measurements were carried out on Rigaku Miniflex with Cu radiation at 30 kV/15 mA. All diffractograms were scanned in a 2θ range of 7.5−32.5° at a rate of 1° min^{-1}.

2-Methyl-4,5-dihydro-[4-*O*-(2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl)-3,6-di-*O*-acetyl-1,2-dideoxy-α-D-glucopyranoso][2,1-*d*]-1,3-oxazole (7). To a solution of compound **6**[26] (1.0 g, 1.48 mmol) in anhydrous 1,2-dichloroethane (14 mL) was added trimethylsilyl triflate (TMSOTf; 0.31 mL, 0.38 g, 1.70 mmol) at 65 °C under argon atmosphere. The reaction mixture was kept stirring for 13 h followed by addition of triethylamine (0.70 mL) at 0 °C under argon atmosphere. The mixture was concentrated under reduced pressure, and the residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate, 1:1, v/v, containing 0.3% (v/v) triethylamine) to afford **7** (0.83 g, 1.34 mmol, 92%) as a colorless syrup: R_f 0.50 ($CHCl_3$/methanol, 9:1, v/v); $[\alpha]_D^{24}$ +5.8° (*c* 1.0, $CHCl_3$); ^1H NMR ($CDCl_3$): δ 5.92 (1H, d, $J_{1,2}$ = 7.52 Hz, H-1), 5.66 (1H, d, $J_{2,3}$ = 2.48 Hz, H-3), 5.19

(1H, t, $J_{2',3'} = J_{3',4'} = 9.52$ Hz, H-3′), 5.10 (1H, t, $J_{3',4'} = J_{4',5'} = 10.04$ Hz, H-4′), 5.00 (1H, dd, $J_{1',2'} = 8.04$ Hz, $J_{2',3'} = 9.56$ Hz, H-2′), 4.72 (1H, d, $J_{1',2'} = 8.04$ Hz, H-1′), 4.29 (1H, dd, $J_{5',6a'} = 4.52$ Hz, $J_{6a',6b'} = 12.04$ Hz, H-6a′), 4.20 (1H, dd, $J_{5,6a} = 2.00$ Hz, $J_{6a,6b} = 12.04$ Hz, H-6a), 4.14 (1H, dd, $J_{5',6b'} = 2.52$ Hz, $J_{6a',6b'} = 12.52$ Hz, H-6b′), 4.13 (1H, m, H-2), 4.04 (1H, dd, $J_{5,6b} = 5.48$ Hz, $J_{6a,6b} = 12.04$ Hz, H-6b), 3.78 (1H, ddd, $J_{5',6b'} = 2.48$ Hz, $J_{5',6a'} = 4.52$ Hz, $J_{4',5'} = 9.52$ Hz, H-5′), 3.64 (1H, d, $J_{4,5} = 9.52$ Hz, H-4), 3.48 (1H, ddd, $J_{5,6b} = 2.00$ Hz, $J_{5,6a} = 5.52$ Hz, $J_{4,5} = 9.52$ Hz H-5), 2.11, 2.10, 2.10, 2.08, 2.04, 2.02 (18H, s, COCH_3), 1.99 (3H, s, CH_3 of oxazoline). HRMS (FAB) *m*/*z*: calcd for $C_{26}H_{36}NO_{16}$ $[M + H]^+$ 618.2034, found 618.2035.

2-Methyl-4,5-dihydro-[4-*O*-(β-D-glucopyranosyl)-1,2-dideoxy-α-D-glucopyranoso][2,1-*d*]-1,3-oxazole (1). To a solution of compound **7** (0.10 g, 0.15 mmol) in anhydrous methanol (8.0 mL) was added sodium methoxide (28% methanol solution, 1.4 mg, 0.0075 mmol) in anhydrous methanol (1.0 mL) dropwise. The reaction mixture was kept stirring at 0 °C for 4 h under dry atmosphere. Then, the mixture was concentrated to dryness under reduced pressure to afford **1** (0.055 g, 0.15 mmol, purity 99%) as a white amorphous powder; $[\alpha]_D^{22}$ −55.3° (*c* 0.1, H_2O); ^{1}H NMR (D_2O): δ 6.04 (1H, d, $J_{1,2} = 7.04$ Hz, H-1), 4.44 (1H, d, $J_{1',2'} = 8.04$ Hz, H-1′), 4.24 (1H, dd, $J_{2,3} = 3.00$ Hz, $J_{3,4} = 2.00$ Hz, H-3), 4.14 (1H, m, H-2), 3.87 (1H, dd, $J_{5,6a} = 2.00$ Hz, $J_{6a,6b} = 12.56$ Hz, H-6a), 3.77 (1H, dd, $J_{5',6a'} = 2.00$ Hz, $J_{6a',6b'} = 12.04$ Hz, H-6a′), 3.69 (1H, dd, $J_{5,6b} = 5.52$ Hz, $J_{6a,6b} = 12.04$ Hz, H-6b), 3.67 (1H, dd, $J_{3,4} = 1.52$ Hz, $J_{4,5} = 9.04$ Hz, H-4), 3.62 (1H, dd, $J_{5',6b'} = 6.52$ Hz, $J_{6a',6b'} = 12.52$ Hz, H-6b′), 3.44−3.09 (4H, m, H-3′, H-5′, H-5, H-4′), 3.23 (1H, dd, $J_{1',2'} = 8.04$ Hz, $J_{2',3'} = 9.04$ Hz, H-2′), 2.01 (3H, s, CH_3 of oxazoline). HRMS (FAB) *m*/*z*: calcd for $C_{14}H_{24}NO_{10}$ $[M + H]^+$ 366.1400, found 366.1392.

2,3,6-Tri-*O*-acetyl-4-*O*-(3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-D-glucopyranosyl Acetate (12). To a mixture of compounds **10**[27] (4.58 g, 7.90 mmol) and **11**[28] (2.50 g, 7.52 mmol) with activated MSAW300 (5.0 g) was added anhydrous dichloromethane (80 mL) under argon atmosphere. After cooling to −20 °C, boron trifluoride diethyl ether complex (BF_3·OEt_2) (0.18 mL, 1.44 mmol) in anhydrous dichloromethane (1.0 mL) was added dropwise to the mixture. The reaction mixture was kept stirring at −20 °C for 6 h under argon atmosphere followed by addition of triethylamine (1.0 mL). After stirring for 1 h under argon atmosphere, the mixture was filtered through diatomaceous earth (Celite; no. 545), extracted with chloroform, and washed with saturated aqueous $NaHCO_3$ and brine. The organic layer was dried over $MgSO_4$ and then filtered through Celite and concentrated to dryness. The residue was purified by silica gel column chromatography (toluene/ethyl acetate, 3:1, v/v) to afford pure **12** (3.19 g, 4.17 mmol, 58%) as a white solid: R_f 0.63 ($CHCl_3$/diethyl ether/methanol, 10:10:1, v/v/v);^{1}H NMR for β-anomer compound ($CDCl_3$): δ 7.87−7.75 (4H, m, aromatic), 5.74 (1H, dd, $J_{3',4'} = 9.52$ Hz, $J_{2',3'} = 10.52$ Hz, H-3′), 5.59 (1H, d, $J_{1,2} = 8.52$ Hz, H-1), 5.42 (1H, d, $J_{1',2'} = 8.52$ Hz, H-1′), 5.24 (1H, t, $J_{2,3} = J_{3,4} = 9.28$ Hz, H-3), 5.14 (1H, t, $J_{3',4'} = J_{4',5'} = 9.78$ Hz, H-4′), 5.00 (1H, dd, $J_{1,2} = 8.04$ Hz, $J_{2,3} = 9.52$ Hz, H-2), 4.45 (1H, dd, $J_{5',6a'} = 4.52$ Hz, $J_{6a',6b'} = 12.04$ Hz, H-6a′), 4.31−4.23 (2H, m, H-2′, H-6a), 4.15−4.08 (1H, dd, $J_{5',6b'} = 2.00$ Hz, $J_{6a',6b'} = 12.04$ Hz, H-6b′), 3.94 (1H, t, $J_{3,4} = J_{4,5} = 9.28$ Hz, H-4), 3.86 (1H, ddd, $J_{5',6b'} = 2.00$ Hz, $J_{5',6a'} = 4.52$ Hz, $J_{4',5'} = 10.04$ Hz, H-5′), 3.67−3.61 (2H, m, H-5, H-6b), 2.13, 2.06, 2.05, 2.04, 2.02, 2.01, 2.00 (21H, s, COCH_3). HRMS (FAB) *m*/*z*: calcd for $C_{34}H_{39}NO_{19}$ $[M + Na]^+$ 788.2014, found 788.2005.

3,6-Di-*O*-acetyl-4-*O*-(3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-[1,2-*O*-(1-ethoxyethylidene)]-α-D-glucopyranose (13). To a solution of compound **12** (2.02 g, 2.64 mmol) in anhydrous dichloromethane (10 mL) was dropwise added 25 wt % HBr/AcOH (10.0 mL, 41.9 mmol) at 0 °C under dry atmosphere. The reaction mixture was kept stirring in the dark at room temperature for 6 h. Then, the mixture was extracted with $CHCl_3$ and washed with iced water, saturated aqueous $NaHCO_3$, and brine. The organic layer was dried over $MgSO_4$, filtered through Celite, and concentrated under reduced pressure. The residue was dissolved in pyridine (20 mL), followed by addition of tetrabutylammonium bromide (0.57 g, 1.77 mmol) and ethanol (0.54 mL, 9.23 mmol). The reaction mixture was kept stirring at 50 °C for 3 h under argon atmosphere and then extracted with $CHCl_3$ and washed with 4% aqueous $KHSO_4$, saturated aqueous $NaHCO_3$, and brine. The organic layer was dried over $MgSO_4$, filtered through Celite, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate, 3:2, v/v) to afford pure **13** (1.27 g, 1.69 mmol, 64%) as a white amorphous powder: R_f 0.23 (*n*-hexane/ethyl acetate, 1:1, v/v); ^{1}H NMR ($CDCl_3$): δ 7.85−7.74 (m, 4H, aromatic), 5.72 (dd, 1H, $J_{3',4'} = 9.52$ Hz, $J_{2',3'} = 10.54$ Hz, H-3′), 5.60−5.59 (2H, m, H-1, H-3), 5.55 (1H, d, $J_{1',2'} = 8.52$ Hz, H-1′) 5.22 (1H, t, $J_{3',4'} = J_{4',5'} = 10.24$ Hz, H-4′), 4.36 (1H, dd, $J_{1',2'} = 8.52$ Hz, $J_{2',3'} = 10.52$ Hz, H-2′), 4.33 (1H, dd, $J_{5',6a'} = 3.52$ Hz, $J_{6a',6b'} = 12.04$ Hz, H-6a′), 4.28 (1H, dd, $J_{2,3} = 3.00$ Hz, $J_{1,2} = 5.00$ Hz, H-2), 4.18 (1H, dd, $J_{5',6b'} = 2.52$ Hz, $J_{6a',6b'} = 12.52$ Hz, H-6b′), 3.94 (1H, ddd, $J_{5',6b'} = 2.52$ Hz, $J_{5',6a'} = 3.52$ Hz, $J_{4',5'} = 10.52$ Hz, H-5′), 3.90 (1H, d, $J_{6a,6b} = 11.52$ Hz, H-6a), 3.72−3.67 (2H, m, H-4, H-5), 3.64 (1H, dd, $J_{5,6b} = 4.00$ Hz, $J_{6a,6b} = 12.04$ Hz, H-6b), 3.52 (2H, q, $J = 7.02$ Hz, OCH_2CH$_3$), 2.12, 2.10, 2.02, 1.93, 1.85 (15H, s, COCH_3), 1.64 (3H, s, CH_3 of ortho ester), 1.16 (3H, t, $J = 7.03$ Hz, OCH$_2$CH_3). HRMS (FAB) calcd for HRMS (FAB) *m*/*z*: calcd for $C_{34}H_{41}NO_{18}$ $[M + H]^+$ 752.2402, found 752.2404.

4-*O*-(2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-3,6-di-*O*-acetyl-[1,2-*O*-(1-ethoxyethylidene)]-α-D-glucopyranose (14). To a solution of compound **13** (1.06 g, 1.41 mmol) in ethanol (15 mL) was added hydrazine monohydrate (1.85 mL, 38.1 mmol). The reaction mixture was kept stirring at 80 °C under dry atmosphere for 48 h and then concentrated under reduced pressure. The residue was dissolved in dry pyridine (20 mL), and then, acetic anhydride (10 mL) was added at room temperature under dry atmosphere. The reaction mixture was kept stirring at room temperature under dry atmosphere overnight, followed by addition of methanol to quench excess reagents. The mixture was concentrated under reduced pressure and coevaporated with toluene (20 mL) three times. The residue was diluted with $CHCl_3$ and washed with 4% aqueous $KHSO_4$, saturated $NaHCO_3$, and brine. The organic layer was dried over $MgSO_4$, filtered through Celite, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate, 1:2, 1:4, 0:1, v/v, stepwise) to afford pure **14** (0.77 g, 1.17 mmol, 83%) as a white solid: R_f 0.28 (ethyl acetate); ^{1}H NMR ($CDCl_3$): δ 5.89 (1H, d, $J_{2',NH'} = 8.52$ Hz, N*H*′), 5.66 (1H, d, $J_{1,2} = 5.00$ Hz, H-1), 5.57 (1H, d, $J_{3,4} = 2.00$ Hz, H-3), 5.21 (1H, t, $J_{2',3'} = J_{3',4'} = 9.80$ Hz, H-3′), 5.10 (1H, t, $J_{3',4'} = J_{4',5'} = 9.52$ Hz, H-4′), 4.70 (1H, d, $J_{1',2'} = 8.56$ Hz, H-1′), 4.42 (1H, dd, $J_{5,6a} = 4.04$ Hz, $J_{6a,6b} = 12.04$ Hz, H-6a), 4.31 (1H, m, H-2), 4.26 (1H, dd, $J_{6a',6b'} = 4.52$ Hz, $J_{5',6a'} = 12.56$ Hz, H-6a′), 4.14 (1H, dd, $J_{5,6b} = 2.00$ Hz, $J_{6a,6b} = 12.04$ Hz, H-6b), 4.11 (1H, dd, $J_{5',6b'} = 2.48$ Hz, $J_{6a',6b'} = 12.04$ Hz, H-6b′), 3.95 (1H, dt, $J_{1',2'} = J_{2',NH'} = 9.04$ Hz, $J_{2',3'} = 10.04$ Hz, H-2′), 3.80 (1H, ddd, $J_{5,6b} = 2.00$ Hz, $J_{5,6a} = 4.00$ Hz, $J_{4,5} = 10.04$ Hz, H-5), 3.75 (1H,ddd, $J_{5',6b'} = 2.48$ Hz, $J_{5',6a'} = 4.00$ Hz, $J_{4',5'} = 10.00$ Hz, H-5′), 3.55 (2H, q, $J = 7.04$ Hz, OCH_2-CH$_3$), 3.50 (1H, d, $J_{4,5} = 9.52$ Hz, H-4), 2.14, 2.09, 2.08, 2.03, 2.01, 1.95 (18H, s, COCH_3), 1.72 (3H, s, CH_3 of ortho ester), 1.18 (3H, t, $J = 7.04$ Hz, OCH$_2$CH_3). HRMS (FAB) *m*/*z*: calcd for $C_{28}H_{41}NO_{17}$ $[M + H]^+$ 664.2453, found 664.2463.

4-*O*-(2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl Fluoride (15). To a solution of compound **14** (0.57 g, 0.86 mmol) in dichloromethane (5 mL) was added 25 wt % HBr/AcOH (5.0 mL, 20.9 mmol) at 0 °C under dry atmosphere. The reaction mixture was kept stirring in the dark at room temperature under dry atmosphere for 3 h. The mixture was extracted with $CHCl_3$ and washed with iced water, saturated aqueous $NaHCO_3$,

and brine. The organic layer was dried over $MgSO_4$, filtered through Celite, and concentrated under reduced pressure. A solution of the residue dissolved in acetonitrile (25 mL) was added to silver fluoride (0.68 g, 6.23 mmol), and the reaction mixture was vigorously stirred under argon atmosphere for 18 h in the dark. The mixture was filtered through Celite, extracted with $CHCl_3$, and washed successively with water, aqueous saturated $NaHCO_3$, and brine. The organic layer was dried over $MgSO_4$, filtered through Celite, and evaporated to dryness. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate, 1:3 then 1:5, v/v) to afford pure **15** (0.44 g, 0.69 mmol, 80%) as a white solid: R_f 0.60 (chloroform/methanol, 1:1, v/v); $[\alpha]_D^{27}$ +3.80° (*c* 0.1, $CHCl_3$); mp 117−119 °C; ^{1}H NMR ($CDCl_3$): δ 5.82 (1H, d, $J_{NH',2'} = 8.52$ Hz, N*H*′), 5.36 (1H,dd, $J_{1,2} = 7.00$ Hz, $J_{1,F} = 52.70$ Hz, H-1), 5.26 (1H, t, $J_{2',3'} = J_{3',4'} = 10.56$ Hz, H-3′), 5.18 (1H, t, $J_{2,3} = J_{3,4} = 7.52$ Hz, H-3), 5.04 (1H, t, $J_{3',4'} = J_{4',5'} = 10.04$ Hz, H-4′), 5.00 (1H, m, H-2), 4.69 (H, d, $J_{1',2'} = 8.00$ Hz, H-1′), 4.42 (1H, dd, $J_{5,6a} = 2.00$ Hz, $J_{6a,6b} = 12.04$ Hz, H-6a), 4.36 (1H, dd, $J_{6a',6b'} = 4.04$ Hz, $J_{5',6a'} = 12.04$ Hz, H-6a′), 4.27 (1H, dd, $J_{5,6b} = 4.00$ Hz, $J_{6a,6b} = 12.04$ Hz, H-6b), 4.02 (1H, dd, $J_{5',6b'} = 2.00$ Hz, $J_{6a',6b'} = 12.04$ Hz, H-6b′), 3.91 (1H, t, $J_{3,4} = J_{4,5} = 9.02$ Hz, H-4), 3.85 (1H, m, H-5), 3.78 (1H, dt, $J_{1',2'} = J_{2',NH'} = 8.52$ Hz, $J_{2',3'} = 10.56$ Hz, H-2′), 3.66 (1H, ddd, $J_{5',6b'} = 2.04$ Hz, $J_{5',6a'} = 4.04$ Hz, $J_{4',5'} = 10.04$ Hz, H-5′), 2.15, 2.09, 2.08, 2.05, 2.01, 2.00, 1.94 (21H, s, COC*H*$_3$). HRMS (FAB) calcd for $C_{26}H_{36}O_6NF$ $[M + H]^+$ 638.2096, found 638.2096.

4-*O*-(2-Acetamido-2-deoxy-*β*-D-glucopyranosyl)-*β*-D-glucopyranosyl Fluoride (3). To a solution of compound **15** (40 mg, 0.063 mmol) in anhydrous methanol (1.0 mL) was dropwise added sodium methoxide (28% methanol solution, 3.6 mg, 0.019 mmol) in anhydrous methanol (0.1 mL) under dry atmosphere. The reaction mixture was kept stirring at 0 °C for 50 min under dry atmosphere and then neutralized by addition of ion-exchange resin (Amberlite MB-3). After filtration through cotton, the mixture was concentrated to dryness under reduced pressure to afford pure **3** (24 mg, 0.063 mmol, purity 96%) as a white solid; ^{1}H NMR (D_2O): δ 5.23 (1H, dd, $J_{1,2} = 7.00$ Hz, $J_{1,F}$ 53.18 Hz, H-1), 4.55 (1H, d, $J_{1',2'} = 8.56$ Hz, H-1′), 3.91 (d, 1H, $J_{6a,6b} = 12.52$ Hz, H-6a), 3.83 (d, 1H, $J_{6a',6b'} = 11.52$ Hz, H-6a′), 3.73 (m, 2H, H-3, H-2′), 3.68 (m, 2H, H-2′, H-5′), 3.64 (m, 4H, H-3′, H-4′, H-6b, H-6b′), 3.54 (dd, 1H, $J_{3,4} = 7.04$ Hz, $J_{4,5} = 8.52$ Hz, H-4), 3.48 (m, 2H, H-2, H-5), 2.04 (s, 1H, COC*H*$_3$). HRMS (FAB) *m*/*z*: calcd for $C_{14}H_{25}NO_{10}F$ $[M + H]^+$ 386.1462, found 386.1459.

A Typical Polymerization Procedure for 1 Using Chitinase from *Bacillus* sp. Monomer **1** (15 mg, 40 mmol) was dissolved in carbonate buffer (10 mM, pH 10.5, 150 μL), and the mixture was equally divided into two sample tubes. To a solution in one sample tube was added 10 mM carbonate buffer (25 μL) without enzyme, and to a solution in the other sample tube was added 10 mM carbonate buffer (25 μL) with chitinase from *Bacillus* sp. (0.75 mg). The reaction mixtures in these two sample tubes were incubated at 30 °C. The concentration change of **1** was monitored by means of HPLC with a Chemco Chemcobond ODS-W column eluting with distilled water−acetonitrile mixture. After the reaction was completed, the mixture was heated at 90 °C for 10 min so as to inactivate the enzyme. A water-insoluble part of **2** was separated by centrifugation, washed with distilled water (200 μL) three times, and dried under reduced pressure (2.5 mg, 33%). A water-soluble part of **2** was subjected to SEC with Shodex Sugar KS-802 to remove inorganic salts and the disaccharide component. Fractions containing product **2** were collected and combined, followed by lyophilization to give the lower-molecular-weight polysaccharide of **2** (more than tetrasaccharides; 3.0 mg, 40%).

A Typical Polymerization Procedure for 3 with Using Cellulase from *Trichoderma viride*. Monomer **3** (23.1 mg, 60 mmol) dissolved in a mixture of acetonitrile−acetate buffer (50 mM, pH 5.0, 5:1, v/v, 0.80 mL) was added to a solution of cellulase from *Trichoderma viride* (EC 3.2.1.4; 1.16 mg) in a mixture of acetonitrile−acetate buffer (50 mM, pH 5.0, 5:1, v/v, 0.40 mL). In parallel, monomer **3** (5.78 mg, 15 mmol) dissolved in a mixture of acetonitrile−acetate buffer (50 mM, pH 5.0, 5:1, v/v, 0.30 mL), was prepared as reference. Both reaction mixtures were kept standing at 30 °C, and the concentration change of **3** was monitored by HPLC with a Chemco Chemcobond ODS-W column eluting with distilled water−acetonitrile mixture. After 8 h, the mixture was heated at 90 °C for 10 min in order to inactivate the enzyme. The white precipitate of **4** formed during the reaction was separated as a water-insoluble part by centrifugation, washed three times by distilled water (200 μL), and dried under reduced pressure (12.0 mg, 52%). The water-soluble part was subjected to SEC with Shodex Sugar KS-802, and fractions containing **4** were collected and combined. The combined fraction was lyophilized to give lower molecular weight of **4** (5.78 mg, 25%).

Enzymatic Digestibility of a Cellulose−Chitin Hybrid Polysaccharide Using Lysozyme from Human Neutrophils. A typical procedure for enzymatic digestion is given as follows: A water-soluble part of **2** containing more than octasaccharide was separated by SEC with Shodex Sugar KS-802. The collected fractions were combined and lyophilized. To a solution of lower-molecular-weight polysaccharides of **2** containing more than octasaccharide (1.0 mg) in a phosphate buffer (100 mM, pH 7.5, 80 μL) was added lysozyme from human neutrophils (0.05 mg) dissolved in a phosphate buffer (100 mM, pH 7.5, 20 μL), and the mixture was kept standing at 37 °C. Concentration changes of **2** and its hydrolysis products were monitored by HPLC with Shodex Sugar KS-802. Chitohexaose and cellohexaose were used for positive and negative control experiments, respectively (data not shown). After the reaction was completed, the produced oligosaccharides were analyzed by MALDI-TOF/MS.

Results and Discussion

Monomer Design. To synthesize a cellulose−chitin hybrid polysaccharide via enzymatic polymerization, there are two possibilities for monomer design (Figure 2). The target polysaccharide has two kinds of glycosidic linkages, i.e., (1→4)-*β*-*N*-acetyl-D-glucosaminide linkage and (1→4)-*β*-D-glucoside linkage. Chitinase from *Bacillus* sp. (EC 3.2.1.14) is capable of producing polysaccharide **2** via ring-opening polyaddition through the formation of the (1→4)-*β*-*N*-acetyl-D-glucosaminide linkage[23] with using Glc*β*(1→4)GlcNAc oxazoline (**1**) as monomer. On the other hand, cellulase from *Trichoderma viride* (EC 3.2.1.4) responsible for (1→4)-*β*-D-glucosidic bond formation[22] is capable of providing polysaccharide **4** via polycondensation of GlcNAc*β*(1→4)Glc-*β*-fluoride (**3**). These two enzymes are easy to obtain; therefore, we used monomers **1** and **3** for the production of polymers **2** and **4** via enzymatic polymerization. The differences between polysaccharides **2** and **4** are their polymer chain ends: **2** has GlcNAc and Glc units at reducing and nonreducing terminals, respectively, whereas **4** has Glc and GlcNAc units, respectively.

Synthesis of Monomer 1. Monomer **1** was synthesized starting from cellobiose (**5**) via nine step reactions (Scheme 2). A Glc*β*(1→4)GlcNAc disaccharide derivative (**6**) was prepared via azidonitration as previously reported.[26] Compound **6** was converted to the oxazoline derivative (**7**) by treatment with trimethylsilyl triflate (TMSOTf). Finally, all *O*-acetyl groups of **7** were removed by sodium methoxide in methanol to afford monomer **1**.

Synthesis of Monomer 3. Monomer **3** was synthesized starting from D-glucosamine hydrochloride (**8**) and D-glucose (**9**) according to the reactions outlined in Scheme 3. Compound **8** was converted to a glycosyl donor having an *N*-phthaloyl

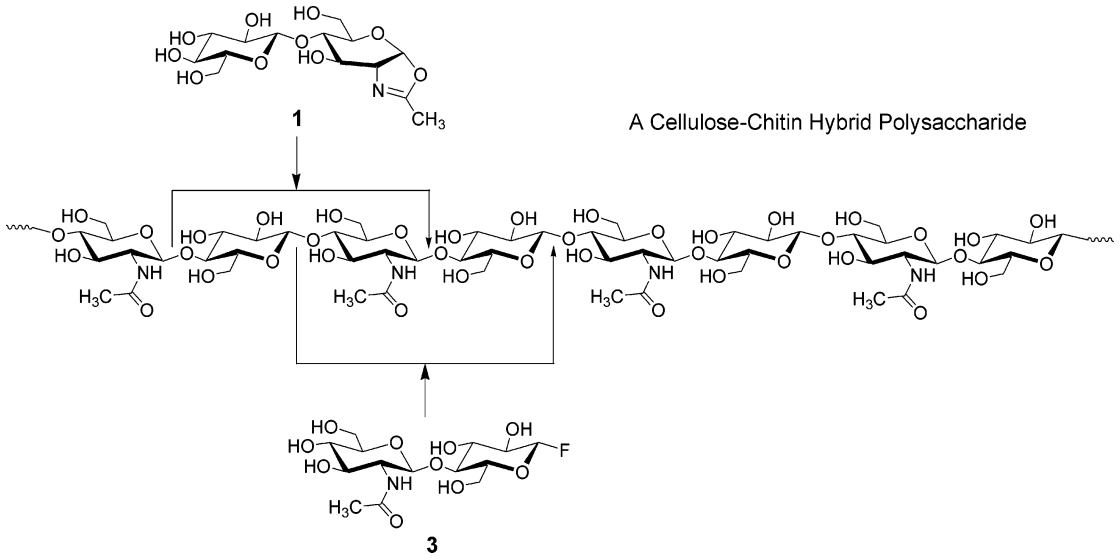
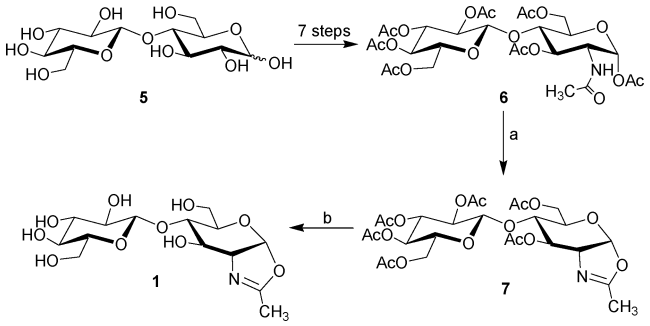


Figure 2. Possible monomer designs for the synthesis of a cellulose−chitin hybrid polysaccharide.

Scheme 2



(a) TMSOTf/1,2-dichloroethane, 75%; (b) CH_3ONa/CH_3OH, purity 99%.

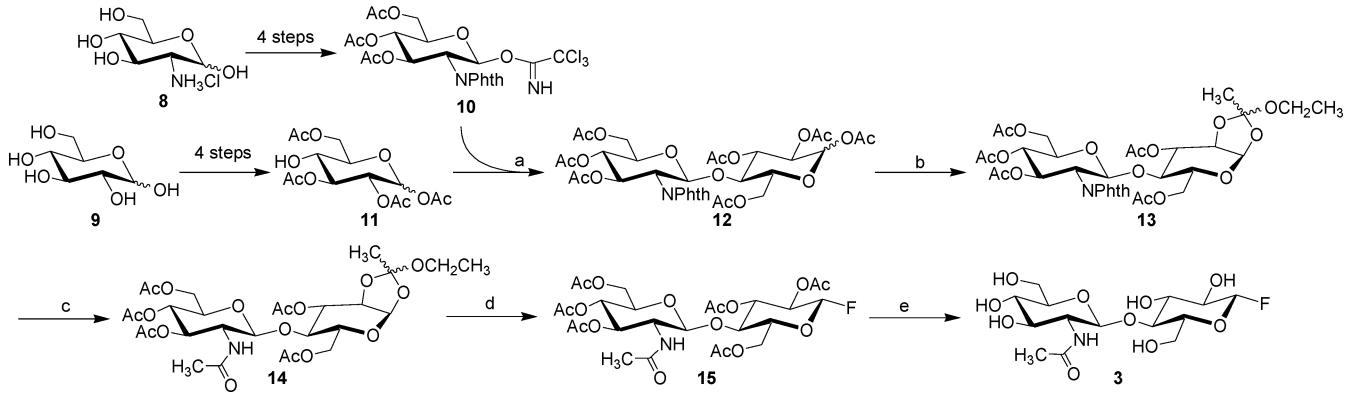
group (**10**) via four step reactions.[27] A glycosyl acceptor (**11**) was synthesized from compound **9** via four step reactions as previously reported.[28] Compounds **10** and **11** were coupled by glycosylation using boron trifluoride diethyl ether complex (BF_3·OEt_2) as a promoter to afford a disaccharide derivative (**12**) in a 68% yield. After conversion of compound **12** to the corresponding ortho ester derivative (**13**), the *N*-phthaloyl group was removed with hydrazine monohydrate in hot ethanol, followed by acetylation by acetic anhydride in pyridine to afford compound **14**. The ortho ester was directly converted the corresponding α-bromide by treatment with 25% hydrogen bromide−acetic acid solution, and then fluorinated by silver fluoride in acetonitrile to give compound **15**. Finally, all *O*-acetyl groups of **15** were deprotected by sodium methoxide in methanol to afford monomer **3**.

Enzymatic Polymerization of Monomer 1 Catalyzed by Chitinase from *Bacillus* sp. With the enzyme catalysis, two kinds of reactions are possible, i.e., enzymatic polymerization of **1** to provide corresponding polymer **2** and hydrolysis of **1** enzymatically and nonenzymatically to afford hydrolysate **15** via the oxazoline ring-opening (Figure 3). Monomer **1** bearing an oxazoline structure is an activated, high-energy form of GlcNAc; therefore, it hydrolyzes gradually in aqueous media without enzyme.

Figure 4 shows the reaction time courses of **1** with chitinase from *Bacillus* sp. and without enzyme. With the enzyme catalysis, monomer **1** disappeared completely within 3 h, and a white precipitate was formed during the reaction. Without enzyme, **1** was gradually decomposed, and it remained at 95% after 3 h without formation of a white precipitate. These results indicate that monomer **1** was definitely recognized and catalyzed by chitinase from *Bacillus* sp., leading to the oxazoline ring-opening reaction.

After termination of the reaction by heating at 90 °C, a white precipitate was separated by centrifugation. The supernatant was subjected to MALDI-TOF/MS measurement (Figure 5). On the

Scheme 3



(a) BF_3·OEt_2, MSAW300/CH_2Cl_2, 68%; (b) (i) HBr/AcOH, CH_2Cl_2; (ii) EtOH, $(n\text{-Bu})_4NBr$/pyridine, 70%; (c) (i) $H_2NNH_2-H_2O$/EtOH; (ii) Ac_2O/pyridine, 83%; (d) (i) HBr/AcOH, CH_2Cl_2; (ii) AgF/CH_3CN, 80% (2 steps); (e) CH_3ONa/CH_3OH, purity 96%.

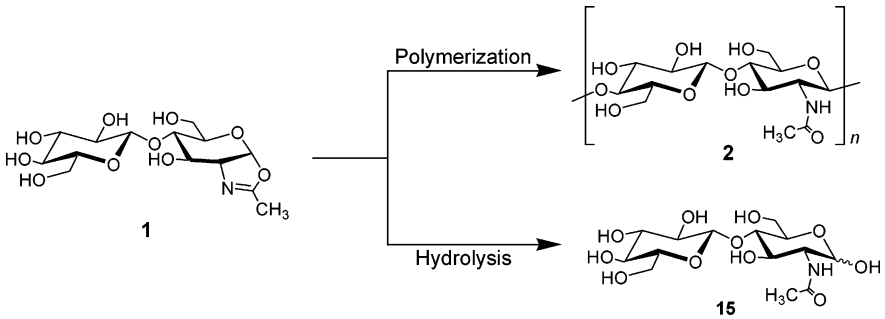


Figure 3. Two kinds of reactions are possible during enzymatic reaction of monomer **1**.

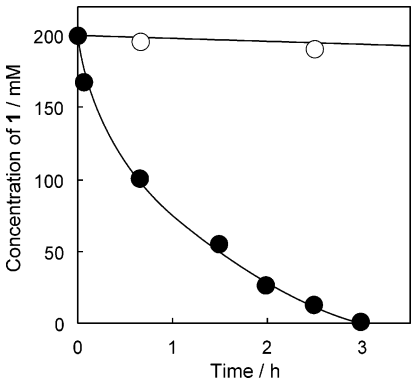


Figure 4. Reaction time courses of **1** with chitinase (●) and without enzyme (○). Reaction conditions: in a carbonate buffer (10 mM, pH 10.5); amount of enzyme, 5 wt % for **1**; reaction temperature, 30 °C; initial concentration of **1**, 200 mM.

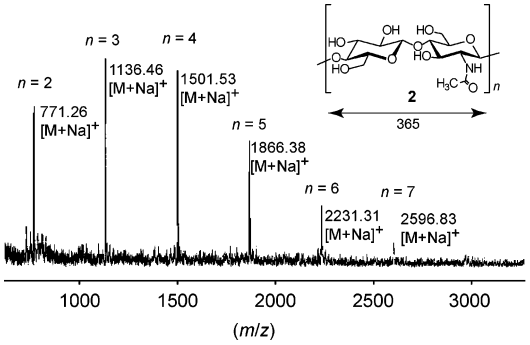


Figure 5. MALDI-TOF mass spectrum of **2** (a water-soluble part).

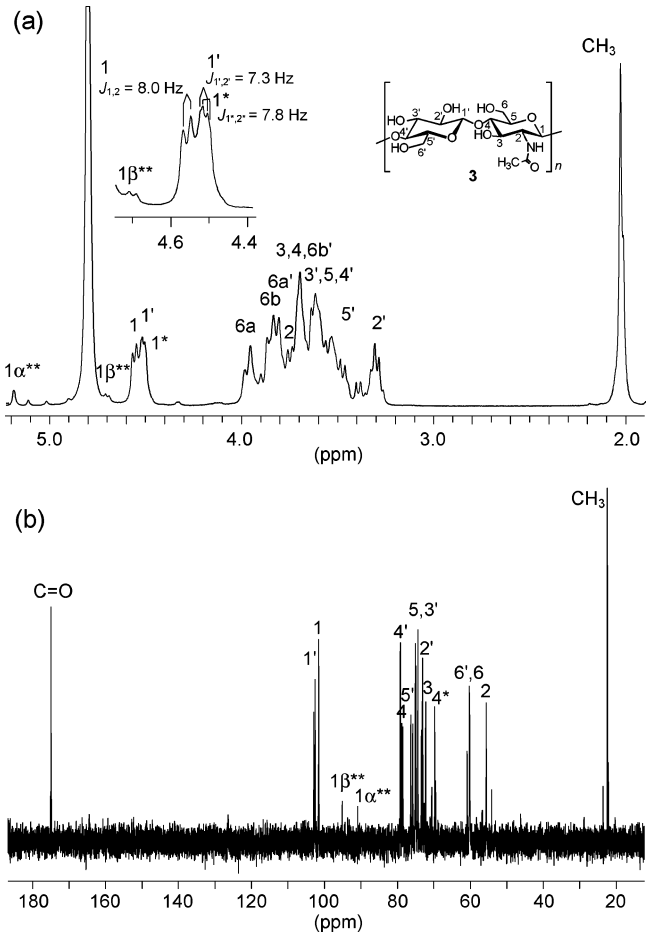


Figure 6. (a) ^{1}H and (b) ^{13}C NMR spectra of **2**. Single and double asterisks indicate the nonreducing and reducing ends of **2**.

spectrum, significant peaks were observed at every *m*/*z* of 365, which corresponds to the molecular mass of **1**, the same as that of the repeating disaccharide unit of **2**. Therefore, the water-soluble part consisted of lower-molecular-weight polysaccharides of **2**.

The water-soluble part of **2** was further purified by size exclusion chromatography (SEC) to remove the hydrolysate **15** and subjected to ^{1}H and ^{13}C NMR measurements (Figure 6). On the ^{1}H NMR spectrum, two significant signals derived from the anomeric protons of internal GlcNAc and Glc units were observed at δ 4.56 and δ 4.51, respectively. Coupling constants of the respective signals were 8.0 and 7.3 Hz, indicating that the lower-molecular-weight polysaccharides **2** were connected through β-glycosidic linkages. Furthermore, on the ^{13}C NMR spectrum, two signals at δ 80 and δ 79 were assigned to the C4 peaks of GlcNAc and Glc units, respectively. It is generally accepted that the signal derived from the C1 carbon atom connecting to the glycosidic oxygen is found in lower magnetic field (at higher δ values of δ 102 and δ 103) than that from any other saccharide ring carbon. Therefore, these results indicate that lower-molecular-weight polysaccharides of **2** were connected through (1→4)-glycoside. Thus, the water-soluble part of **2** was composed of alternating β(1→4)-linked Glc and GlcNAc.

A white precipitate was then analyzed by cross-polarization/magic angle spinning (CP/MAS) ^{13}C NMR measurement (Figure 7a). Assignment of the signals was performed by comparing with those of synthetic chitin (Figure 7b) and synthetic cellulose (Figure 7c).[22] Two significant signals derived from the acetamido group of the GlcNAc unit were found at δ 20 (methyl) and δ 175 (carbonyl) in Figure 7a as well as in Figure 7b. Furthermore, the C2 carbon of GlcNAc was observed at δ 55 in Figure 7a,b. Signals derived from the Glc unit in Figure 7a

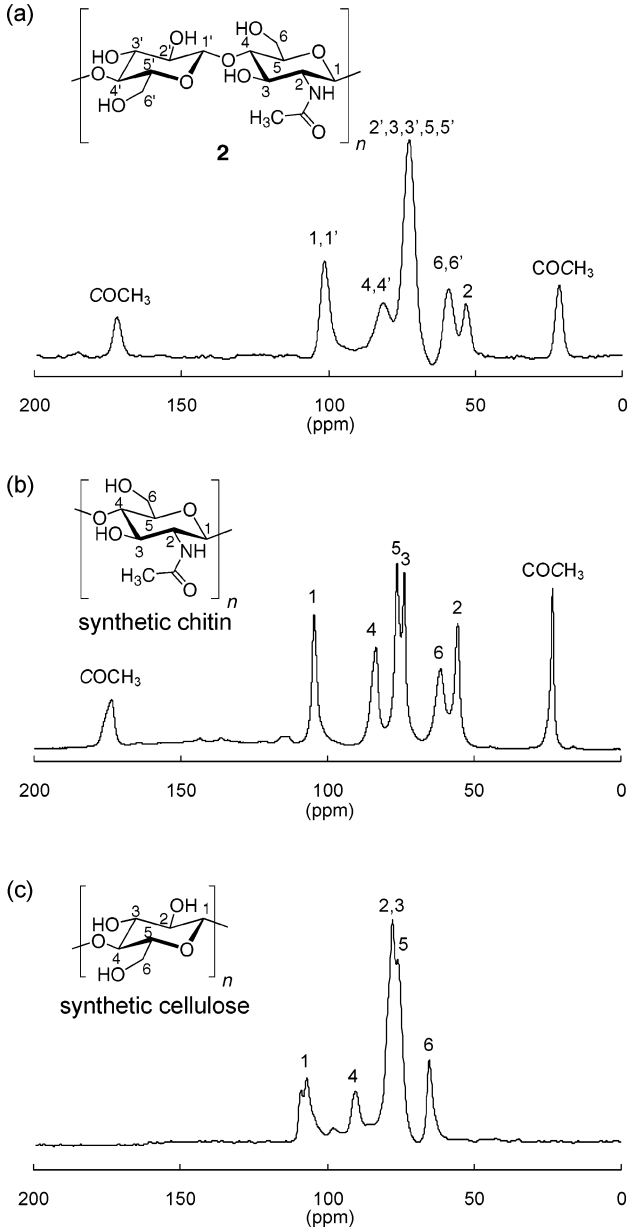


Figure 7. CP/MAS ^{13}C NMR spectra of (a) **2**, (b) synthetic chitin, and (c) synthetic cellulose.

were also detected at similar δ values from synthetic cellulose depicted in Figure 7c. Note that signals from the C4 and C4′ carbons were observed at around δ 81, showing that the product has a (1→4)-linked glycosidic bond exclusively.[29] Thus, the precipitate of the water-insoluble part is a cellulose−chitin hybrid polysaccharide of **2**.

X-ray diffraction (XRD) measurement of **2** provided an interesting result (Figure 8a), i.e., no significant peaks were observed on the spectrum of **2**. XRD spectrum of synthetic chitin (Figure 8b) shows significant, sharp peaks at 2θ = 9.3, 19.2, 23.4, and 26.1, which forms antiparallel, α-chitin-type molecular organization.[23,30] Furthermore, synthetic cellulose normally assembles in antiparallel molecular chain packing (cellulose II),[22,31] which provides an XRD spectrum exhibiting sharp peaks at 2θ = 12.2, 19.9, and 22.0 (Figure 8c). Therefore, these results clearly indicate that no crystals were formed from polysaccharide **2** produced via enzymatic polymerization of **1**.

Both α-chitin and cellulose II have antiparallel molecular chain organizations. However, the crystal structure of α-chitin

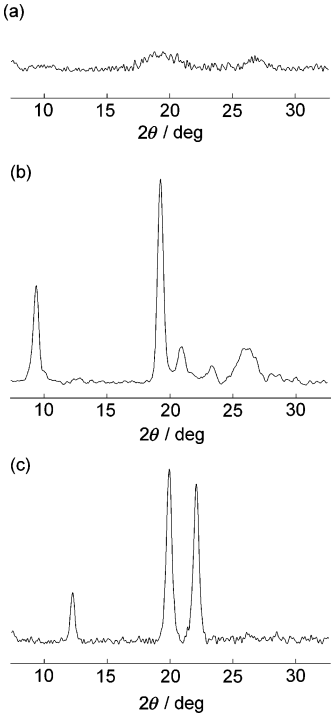


Figure 8. X-ray diffractograms of (a) **2**, (b) synthetic chitin, and (c) synthetic cellulose.

Table 1. Enzymatic Polymerization of **1** under Various Conditions

	polymerization[a]					product			
						insoluble polymer **2**			soluble polymer **2**
entry	pH	chitinase wt % for **1**	temp °C	concn of **1** mM	time[b] h	yield[c] %	M_n[d]	M_w[d]	yield[e] %
1	6.0	5.0	30	200	0.4	5	3880	4230	14
2	7.0	5.0	30	200	0.8	12	3880	4300	48
3	8.0	5.0	30	200	1	12	3900	4330	47
4	9.0	5.0	30	200	2	20	3780	4420	40
5	10.0	5.0	30	200	2	28	3790	4480	48
6	10.5	5.0	30	200	3	34	3750	4170	45
7	11.0	5.0	30	200	10	46	4030	4660	33
8	11.5	5.0	30	200	78	0			4
9	11.0	1.0	30	200	53	20	3990	4410	47
10	11.0	3.0	30	200	21	42	3800	4130	35
11	11.0	7.0	30	200	10	34	3860	4230	40
12	11.0	10	30	200	4	14	3920	4310	50
13	11.0	5.0	5	200	24	28	3220	3480	51
14	11.0	5.0	10	200	11	34	3390	3800	41
15	11.0	5.0	20	200	11	38	3510	3940	37
16	11.0	5.0	40	200	25	30	3620	4000	41
17	11.0	5.0	30	50	30	6	3630	3880	58
18	11.0	5.0	30	100	13	30	3750	4220	47
19	11.0	5.0	30	300	3	46	3740	4260	32

[a] In a phosphate buffer (10 mM; entries 1−4) or in a carbonate buffer (10 mM; entries 5−19). [b] Indicating the time for disappearance of **1**. [c] Isolated yields. [d] Determined by SEC using pullulan, cellooligosaccharide, and chitooligosaccharide standards. [e] Determined by HPLC (more than tetrasaccharides, RI detector, calibrated by cello- and chito-oligosaccharides).

is an orthorhombic cell unit having dimensions a = 0.474 nm, b = 1.886 nm, and c (chain axis) = 1.032 nm,[30] whereas cellulose II is monoclinic cell unit having dimensions a = 0.801 nm, b = 0.904 nm, c (chain axis) = 1.036 nm, and γ = 117°.[31] Therefore, the present cellulose−chitin hybrid polysaccharide is unlikely to form either a crystalline structure or a higher-ordered assembly.

Enzymatic Polymerization of 1 under Various Reaction Conditions. Table 1 shows the results of chitinase-catalyzed

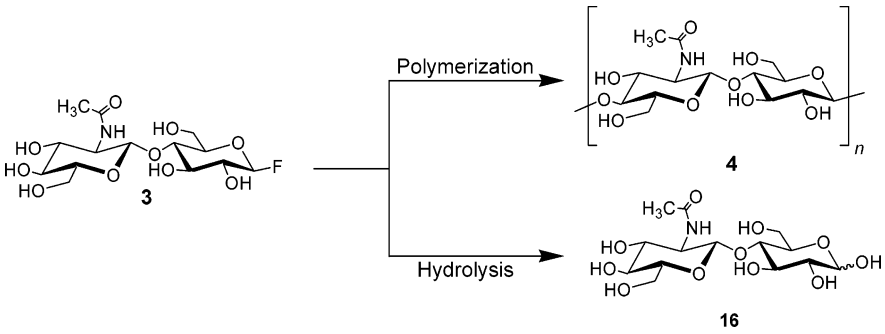


Figure 9. Two kinds of reactions conceivable for the enzymatic reaction of monomer **3**.

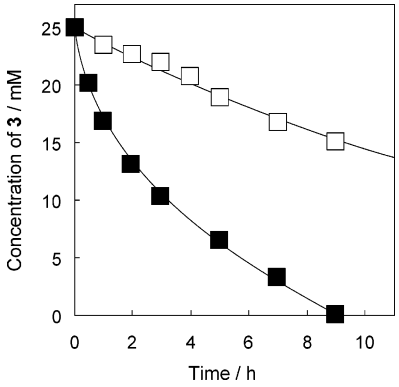


Figure 10. Reaction time courses of **3** with cellulase (■) and without enzyme (□). Reaction conditions: in acetonitrile−acetate buffer (50 mM, pH 5.0) mixture; amount of enzyme, 5 wt % for **3**; reaction temperature, 30 °C; initial concentration of **3**, 25 mM.

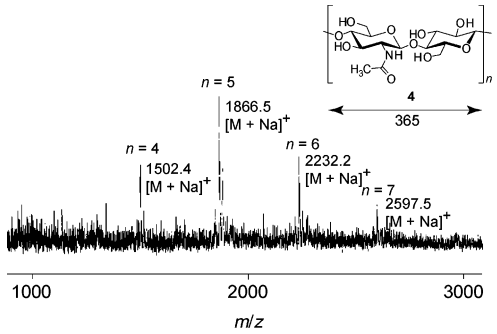


Figure 11. MALDI-TOF/MS spectrum of **4** (a water-soluble part).

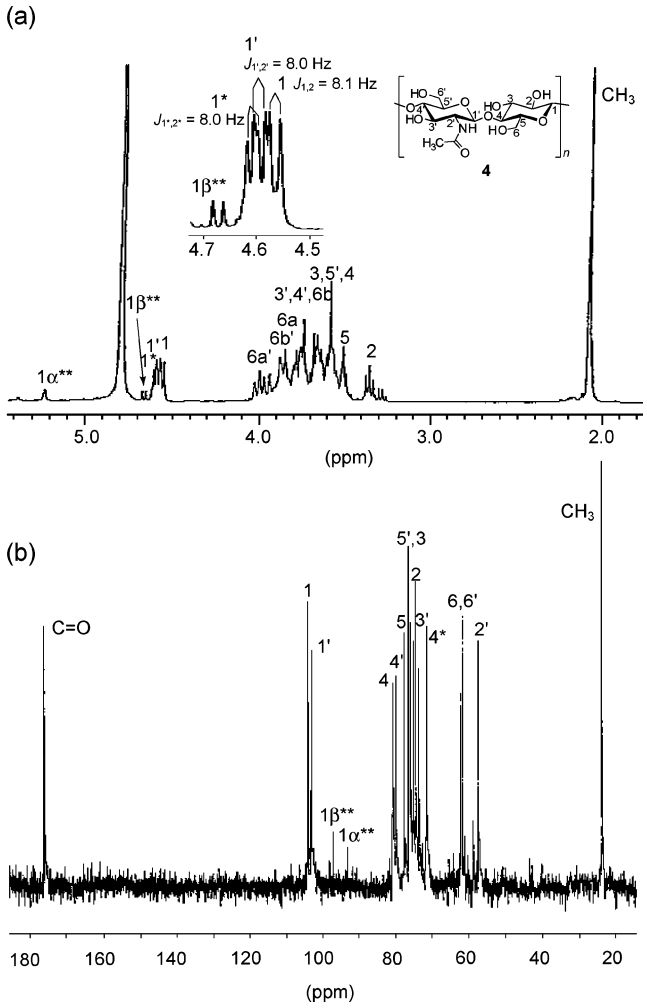


Figure 12. (a) ^{1}H and (b) ^{13}C NMR spectra of oligomers of **4**. Single and double asterisks indicate the nonreducing and reducing ends of **4**, respectively.

polymerization of **1** under various conditions. Yield and M_n of a water-insoluble part of **2** became higher with increase of pH value (entries 1−7) and reached the maxima of 46% and M_n of 4030 within 10 h at pH 11.0 (entry 7). Note that monomer **1** disappeared rapidly at pH 6.0 within 0.4 h to provide a water-insoluble part of **2** in a very low yield (5%; entry 1). Furthermore, a water-soluble part of **2** was obtained in a small amount (14%), indicating that almost all of **1** was hydrolyzed to compound **15**. This is due to the unstable nature of the oxazoline under acidic conditions, leading to nonenzymatic hydrolysis in aqueous media prior to enzymatic reaction. At pH 11.5, the polymerization did not occur at all (entry 8). A water-insoluble part of **2** contains the polymer with higher molecular weights (M_n), whereas a water-soluble part is a mixture of its oligomers lower than dodecasaccharide detected by HPLC. For example, it contained tetra- (7%), hexa- (8%), and octa- to dodecasaccharide (18%) in the case of entry 7. Therefore, yield and M_n of a water-insoluble part of **2** are discussed in the following. The amount of chitinase affected the yield of **2** (entries 7, 9−12); 5 wt % chitinase for monomer **1** was most effective to produce **2** (entry 7). Yield of **2** was reduced to 14% by using 10 wt % chitinase (entry 12). In contrast to variation of the yield, polymer **2** was obtained with M_n ranging narrowly from 3800 (mainly decasaccharide; entry 10) to 4030 (mainly dodecasaccharide; entry 7). Reaction temperature exerted influence on the polymer yield and M_n; the higher reaction temperature gave higher yield and M_n of **2** (entries 7, 13−15). In entry 16, however, yield and M_n of **2** decreased to 30% and 3620 at 40 °C. In contrast to almost constant M_n ranging from 3630 (mainly decasaccharide; entry

Table 2. Enzymatic Polymerization of **3** under Various Conditions[a]

	polymerization[a]					product			
						insoluble polymer **4**			soluble polymer **4**
entry	pH	cellulase wt % for **3**	temp °C	concn of **3** mM	time[b] h	yield[c] %	M_n[d]	M_w[d]	yield[e] %[e]
20	3.0	5.0	30	25	7	40	2580	2750	39
21	4.0	5.0	30	25	7	44	2600	2800	35
22	4.5	5.0	30	25	9	52	2620	2800	32
23	5.0	5.0	30	25	9	55	2820	3070	28
24	5.5	5.0	30	25	10	51	2760	3030	30
25	6.0	5.0	30	25	12	49	2680	2930	31
26	7.0	5.0	30	25	24	48	2640	2940	34
27	8.0	5.0	30	25	30	0			19
28	5.0	1.0	30	25	120	0			2
29	5.0	3.0	30	25	24	32	2390	2560	24
30	5.0	7.0	30	25	4	58	2300	2620	23
31	5.0	10	30	25	2	30	2200	2510	36
32	5.0	5.0	20	25	15	31	2350	2620	46
33	5.0	5.0	40	25	7	28	2130	2460	33
34	5.0	5.0	30	10	24	13	2820	3070	23
35	5.0	5.0	30	50	8	63	2840	3220	25
36	5.0	5.0	30	100	6	26	2320	2650	47

[a] In CH_3CN−acetate buffer (50 mM) mixture (5:1, v/v; entries 20−26, 28−36) or in CH_3CN−phosphate buffer (50 mM) mixture (5:1, v/v; entry 27). [b] Indicating the time for disappearance of **3**. [c] Isolated yields. [d] Determined by SEC calibrated with pullulan standards, cellooligosaccharides, and chitooligosaccharides. [e] Determined by HPLC (more than tetrasaccharides, RI detector, calibrated with cellooligosaccharides and chitooligosaccharides).

17) to 4030 (entry 7), the initial concentration of **1** drastically affected the polymer yield; polymer **2** was produced in a 6% yield during the reaction starting from 50 mM of **1**, whereas the yield increased to 46% starting from 200 or 300 mM of **1**.

Enzymatic Polycondensation of Monomer 3 Catalyzed by Cellulase from *Trichoderma viride*. Monomer **3** is a high-energy substrate monomer activated at the anomeric carbon by a fluorine atom, which acts as a good leaving group, leading to a corresponding oxocarbenium intermediate.[21] Two kinds of reactions are possible during enzymatic reaction, i.e., enzymatic polycondensation of **3** to polymer **4** and enzymatic and nonenzymatic hydrolysis of **3** to **16** (Figure 9).

Figure 10 illustrates the time dependence of monomer concentration with cellulase from *Trichoderma viride* and without enzyme. With the enzyme catalysis, the consumption rate of **3** was significantly accelerated, and it disappeared within 9 h with formation of a white precipitate of **4**. In contrast, monomer **3** was gradually decomposed nonenzymatically without formation of any precipitates, and it remained at 73% at 9 h. After completion of the enzymatic reaction, a white precipitate was separated by centrifugation, and the supernatant was analyzed by MALDI-TOF/MS measurement (Figure 11). The spectrum shows the peaks at *m*/*z* of 1502.4, 1866.5, 2232.2, and 2597.5, which appeared at every *m*/*z* of 365, corresponding to the molecular mass of the repeating disaccharide unit of **4**.

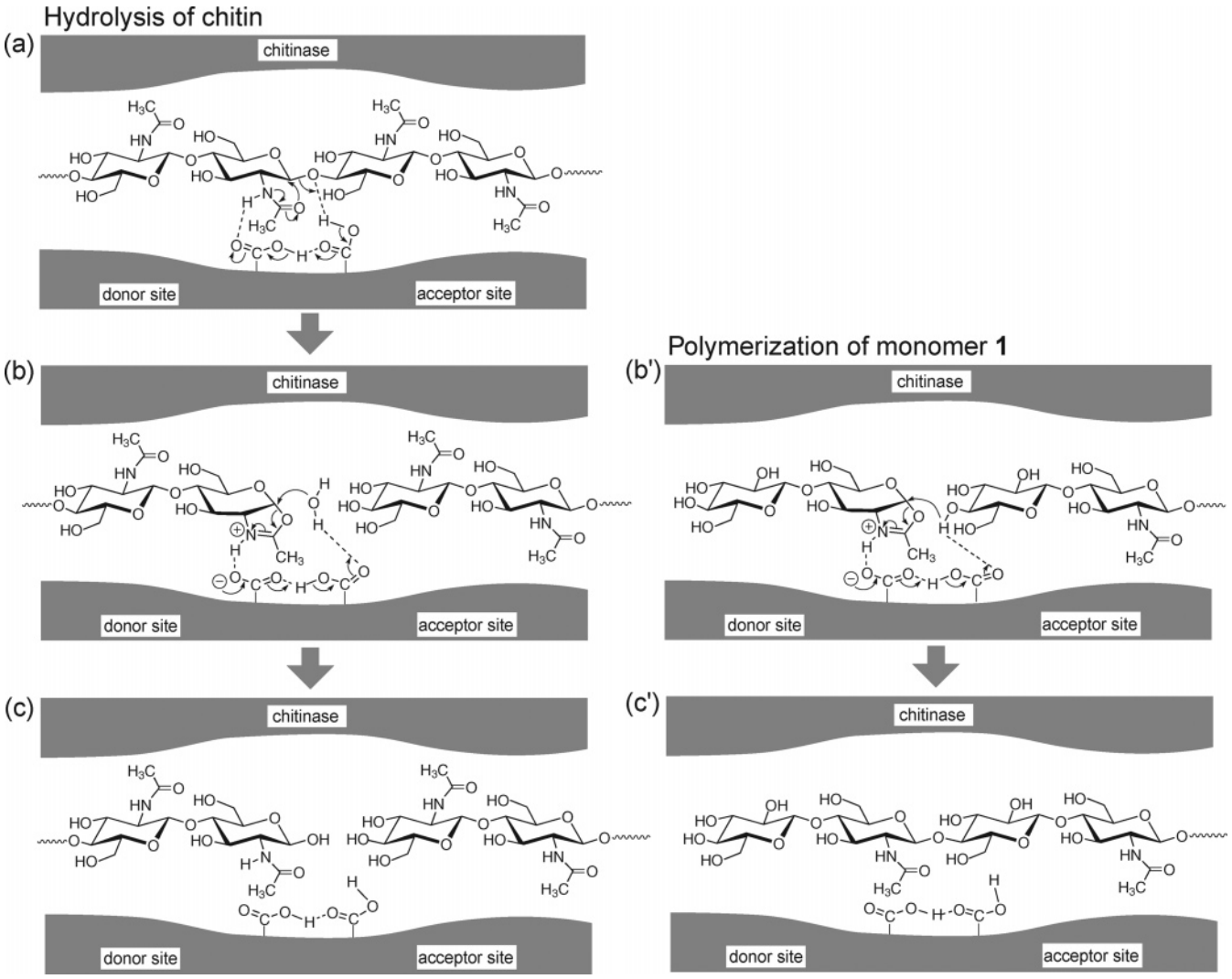


Figure 13. Posulated reaction mechanism of chitinase from *Bacillus* sp. for (a) hydrolysis of chitin and for (b) polymerization of monomer **1**.

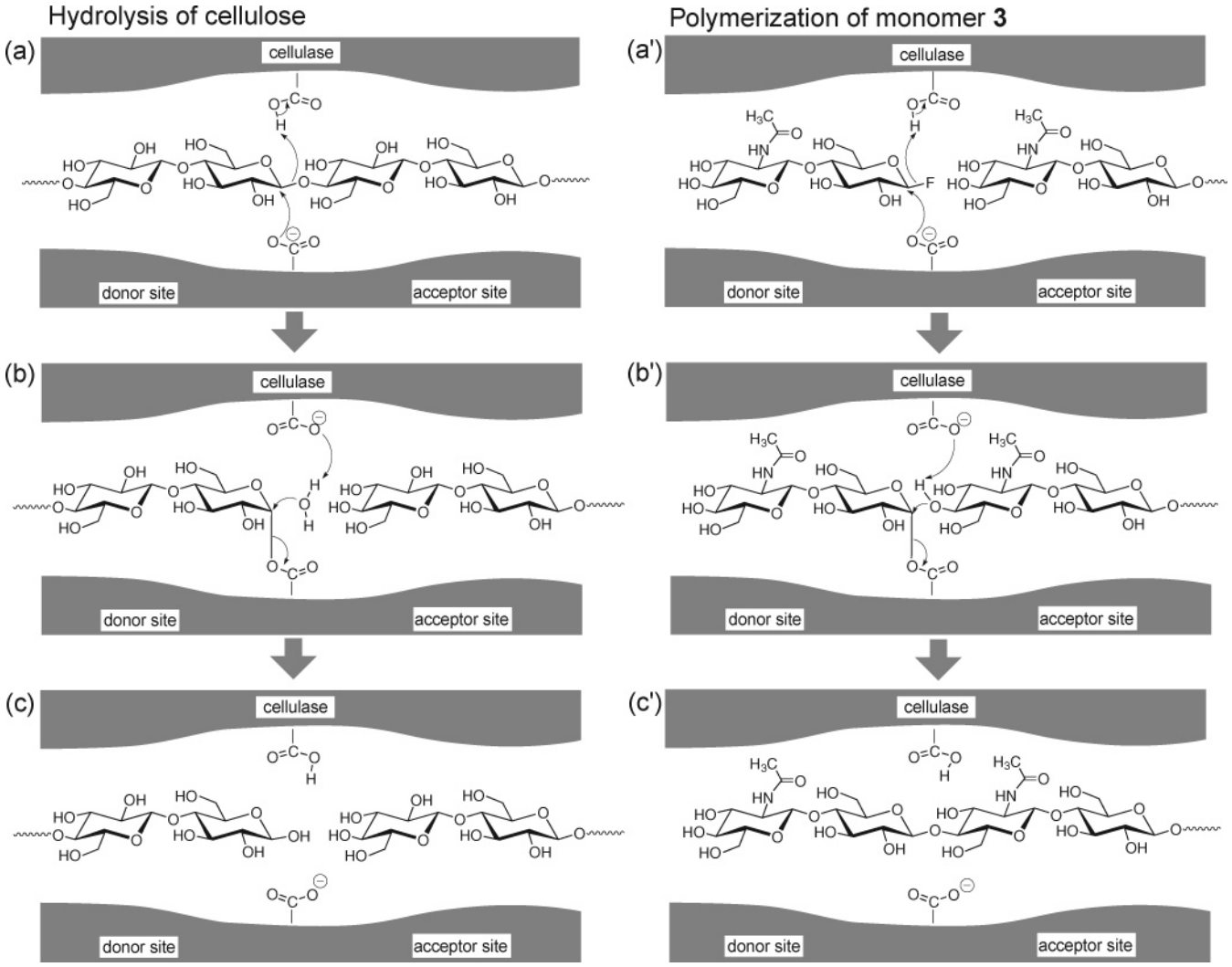


Figure 14. Posulated reaction mechanisms of cellulase for (a) hydrolysis of cellulose and (b) polymerization of monomer **3**.

Therefore, the water-soluble part is the lower-molecular-weight polysaccharides of **4**.

The water-soluble part of **4** was subjected to SEC to separate the fractions including those of higher molecular weight than hexasaccharide. From ^{1}H NMR spectrum of the separated lower-molecular-weight polysaccharide **4** (Figure 12a), two significant signals derived from the anomeric protons of internal GlcNAc and Glc were observed at δ 4.60 and δ 4.56, respectively. Their coupling constants were 8.0 and 8.1 Hz, respectively, indicating that these saccharide units are connected through the β-glycosidic linkage. Furthermore, two characteristic signals around δ 80 on the ^{13}C NMR spectrum (Figure 12b) were assigned to those from the C4 of internal GlcNAc and Glc units. These results indicate the formation of a (1→4)-glycoside linkage by cellulase-catalyzed reaction. Thus, cellulase from *Trichoderma viride* catalyzed the regioselective and stereo-controlled polycondensation of **3**, providing cellulose−chitin hybrid polysaccharide **4** with the alternating structure of β(1→4)-linked Glc and GlcNAc.

Enzymatic Polymerization of 3 under Various Reaction Conditions. The reaction parameters of pH, amount of enzyme, reaction temperature, and initial concentration of **3** were varied in order to optimize the polymerization (Table 2). Similarly to polymerization of **1** to polymer **2**, a water-insoluble part of **4** contains the polymer with higher-molecular-weight segments (M_n), whereas the water-soluble part is a mixture of its oligomers smaller than dodecasaccharide detected by HPLC. For example, it contained tetra- (5%), hexa- (5%), and octa- to dodecasaccharide (18%) in the case of entry 23. Therefore, yield and M_n of a water-insoluble part of **4** are discussed here. With increase of pH value (entries 20−27), yield and M_n of **4** gradually increased to 55% and 2820 (mainly octasaccharide; entry 23), respectively, then decreased to 48% and 2640 (mainly hexa- and octasaccharides; entry 26), respectively. At pH 8.0, the polymerization no longer proceeded, only affording a small amount of water-soluble part of **4** in a 19% yield (entry 27). The optimal pH of cellulase from *Trichoderma viride* for hydrolysis of cellulose was reported to be 5.0.[32] Similarly, the enzyme catalyzed the polymerization of **3** most effectively at around pH 5. The amount of cellulase drastically influenced the polymer yield (entries 23, 28−31); 7.0 wt % cellulase produced **4** in a higher yield of 58% (entry 30), whereas 1.0 wt % enzyme did not provide **4** (entry 28). M_n of **4** was not varied so much from 2820 (entry 23) to 2200 (mainly hexasaccharide; entry 31). Polymerization of **3** proceeded at a temperature ranging from 20 to 40 °C (entries 23, 32, and 33). The initial concentration of **3** exerted a critical influence on the polymer yield (entries 23, 34−36); polymerization of **3** in 50 mM produced polymer **4** in the highest yield of 63% (entry 35), whereas those in 10 mM (entry 34) and in 100 mM (entry 36) gave **4** in lower yields of 13% and 26%, respectively. Notably,

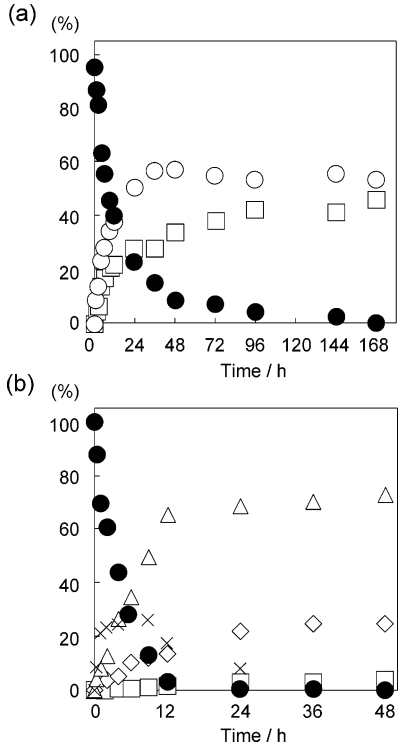


Figure 15. Lysozyme-catalyzed hydrolysis of (a) **2** and (b) **4** (starting substrates **2** or **4**; ●, more than octasaccharides; products: ×, hepta-; ○, hexa-; △, penta-; □, tetra-; ◇, trisaccharide). Reaction conditions: in a phosphate buffer (100 mM, pH 7.5); amount of enzyme, 11 450 units; reaction temperature, 37 °C; initial concentrations of **2** and **4**, 10 mg/mL.

M_n of **4** in entry 35 was 2840, which was the highest throughout all reactions in Table 2.

Reaction Mechanisms of Chitinase and Cellulase. Chitinase from *Bacillus* sp. belonging to the glycoside hydrolase family 18 primarily catalyzes hydrolysis of the (1→4)-*β*-*N*-acetylglucosaminide linkage in chitin.[33,34] The hydrolysis mechanism is postulated as follows: After recognition of a chitin molecule at the active site of the enzyme, the glycosidic oxygen was immediately protonated (Figure 13, stage a). Then, the carbonyl oxygen atom at the C-2 acetamido group placed at the donor site of the enzyme nucleophilically attacks the neighboring C1 carbon to form an oxazolinium ion intermediate, leading to the glycosidic bond cleavage (stage a to b). A water molecule attacks the anomeric carbon of the oxazolinium from the *β*-side (stage b) to terminate the hydrolysis, resulting in the formation of the hydrolyate having *β*-configuration (stage c).

In the polymerization, monomer **1**, which already has an oxazoline moiety, is recognized and protonated at the donor site of the enzyme to form the corresponding oxazolinium ion (stage b′). The anomeric carbon of the oxazolinium is attacked from the *β*-side by the C4 hydroxy group of Glc in another monomer or in the growing chain end placed at the acceptor site, resulting in the formation of a *β*(1→4)-glycosidic linkage (stage c′). Repetition of this glycosylation is a ring-opening polyaddition, providing a cellulose−chitin hybrid polysaccharide. Note that the oxazolinium structure of stage b′ is very close to that of stage b. Therefore, we named such an oxazoline-type monomer a transition-state analogue substrate (TSAS) monomer.[23]

Cellulase from *Trichoderma viride* belonging to the glycoside hydrolase family 5 normally catalyzes cleavage of the (1→4)-*β*-glucoside linkage in cellulose.[34,35] The hydrolysis mechanism of cellulose, which is generally accepted, proceeds as follows (Figure 14): After recognition of a cellulose molecule by the enzyme, the glycosidic oxygen is immediately protonated by one carboxyl group (catalytic proton donor; stage a). Then, another carboxylate (catalytic nucleophile) attacks nucleophilically to the anomeric carbon from the α-side to form a glycoside−enzyme intermediate (or transition state),[35] resulting in the glycosidic bond cleavage (stage b). A water molecule attacks the anomeric carbon in the intermediate from the *β*-side (stage b) to terminate the hydrolysis, leading to formation of the hydrolysate with *β*-configuration (stage c).

In the polymerization, monomer **3** placed at the donor site of the enzyme through recognition by cellulase is nucleophilically attacked by the carboxylate (catalytic nucleophile) from the α-side (stage a′), resulting in formation of a glycosyl−enzyme intermediate (or transition state) (stage b′)[21b] as well as in leaving the fluorine atom as hydrogen fluoride. Note that the structure of the intermediate in stage b′ is very similar to that in stage b. The 4-OH group of GlcNAc in the growing chain end or in another monomer molecule placed at the acceptor site attacks the anomeric carbon of the intermediate nucleophilically from the *β*-side (stage b′), leading to the formation of a *β*(1→4)-glycosidic linkage (stage c′). Repetition of this regio- and stereoselective glycosylation process is a polycondensation, giving rise to a cellulose−chitin hybrid polysaccharide.

To our surprise, both types of enzyme recognize the respective monomer despite their high substrate-specific character. Recent investigations revealed that chitinase from *Bacillus* sp. has a (−2)(−1)(+1)(+2)(+3)(+4)-type subsite structure in the active site, and the shape of catalytic domain looks like a cleft.[36] The catalytic domain of chitinase from *Bacillus* sp. is well superimposed on that of chitinase from *Serratia marcescens*. From the three-dimensional structural analysis of chitinase from *Serratia marcescens*, it is indicated that the C2 of the substrate at the −2 and +1 subsites face the outside of the cleft.[37] Judging from these situations, the C2 at the nonreducing end of the monomer does not seem to play an important role for the monomer recognition and the following glycosylation. On the other hand, XRD analysis on the catalytic domain of the cellulase not from *Trichoderma viride* but from *Bacillus agaradherans*,[38] *Thermoascus aurantiacus*,[39] and *Acidothermus cellulolyticus*[40] has recently been reported. The C2 substitutions of substrates located at −2 and +1 subsites seem not to be important for the cellulase catalysis.

Another surprising aspect is the behavior of monomers when changing the pH of the polymerization medium. Polymerization of both monomers took place to produce the polymer in the very wide range of pH values: monomer **1** between 6 and 11 and monomer **2** between 3 and 6. These in vitro behaviors are quite different from those of the in vivo reactions of chitinase and cellulase catalysis, which is normally very sensitive toward the pH change.

Emzymatic Digestion of Hybrid Polymers 2 and 4. The cellulose−chitin hybrid polysaccharide is a novel unnatural polymer. However, its components of Glc and GlcNAc are natural substances widely found in nature. Therefore, it is very important and worthwhile to examine the biodegradability of the hybrid, in particular, the in vivo digestibility in mammalian systems for the development of advanced biomaterial architecture. In the following sections are examined the enzymatic digestions of polymers **2** and **4** by using lysozyme from human

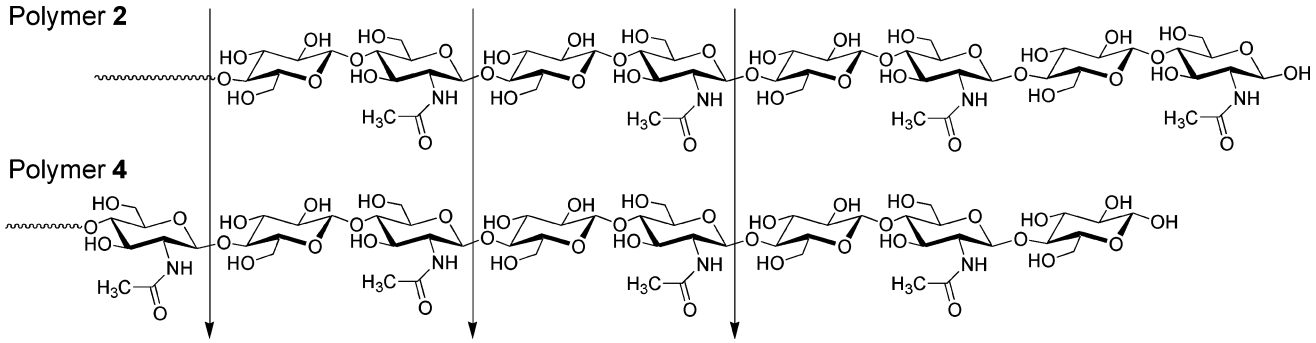


Figure 16. Possible bonds in polymers **2** and **4** cleaved by hydrolysis of lysozyme from human neutrophils. Arrows show the $\beta(1\rightarrow4)$-glycosidic bond in these polymers susceptible to the enzymatic hydrolysis. Each polymer is illustrated from the reducing end and is aligned with the same saccharide units.

neutrophils as model experiments of in vivo digestibility. Lysozyme (EC 3.2.1.17) is one of the glycoside hydrolases responsible for the cleavage of the $\beta(1\rightarrow4)$-glycosidic linkage between GlcNAc and GlcNAc and between GlcNAc and *N*-acetylmuramic acid (MurNAc).[41] The enzyme exists in cytoplasmic granules of the polymorphonuclear neutrophils (PMN) and is released through the mucosal secretions.[42]

Polymers **2** and **4** containing segments larger than octasaccharide were separated from the respective water-soluble part by using SEC. The M_n values of purified **2** and **4** were both 1620 as determined by GPC measurements, which corresponds to 8−10 saccharide units. First, control experiments were carried out using chitohexaose and cellohexaose by lysozyme catalysis. As expected, chitohexaose was effectively hydrolyzed to produce mono-, di-, and trisaccharides, whereas cellohexaose was not metabolized at all (data not shown).

Figure 15 shows the time dependence of concentrations of the purified **2** and **4** by the lysozyme-catalyzed hydrolysis. It is very interesting to note that both hybrid polymers were catalyzed by lysozyme. During the enzymatic reaction, polymer **2** was consumed effectively and disappeared within 168 h (Figure 15a). Hexa- (○) and tetrasaccharides (□) from **2** were selectively produced; the hexasaccharide was produced as the major component. Polysaccharide **4** was also hydrolyzed by the enzyme (Figure 15b). Hydrolysis products obtained were mainly odd-numbered saccharides, i.e., tri- (◇) and pentasaccharides (△) were the major products. Heptasaccharide (×) of **4** was initially formed within 10 h, then it disappeared within 36 h, and a very small amount of tetrasaccharide (□) was formed. Thus, it should be noted that both hybrid polymers **2** and **4** were effectively hydrolyzed by lysozyme from human neutrophils, suggesting that these are well-metabolized in vivo.

These results can be reasonably explained as in Figure 16, which illustrates possible cleaving bonds by the catalysis of lysozyme in polymers **2** and **4**. By the catalysis, poymer **2** selectively produced even-numbered hexa- and tetrasaccharides. On the other hand, polymer **4** has a structure of one GlcNAc unit shifted from that of polymer **2**; therefore, odd-numbered oligosaccharides were mainly formed.

Conclusion

This study demonstrated two new enzymatic polymerizations: the ring-opening polyaddition of Glc$\beta(1\rightarrow4)$GlcNAc oxazoline derivative **1** catalyzed by chitinase from *Bacillus* sp. and the polycodensation of GlcNAc$\beta(1\rightarrow4)$Glc fluoride **3** catalyzed by cellulase from *Trichoderma viride*. Monomers **1** and **3** were polymerized under total control of regioselectivity and stereochemistry, giving rise to corresponding cellulose−chitin hybrid polysaccharides **2** and **4** in good yields. These polysaccharides, having $\beta(1\rightarrow4)$-linked alternating aligned Glc and GlcNAc, are difficult to construct by a conventional chemical method or by a modification of cellulose and/or chitin. The M_n values of the resulting products **2** and **4** reached 4030 and 2840, which correspond to 22 and 15−16 saccharide units, respectively. The present cellulose−chitin hybrid polysaccharide did not exhibit a crystalline structure. They were hydrolyzed in vitro by the lysozyme catalysis. The M_n values of the resulting products are rather low compared with naturally occurring chitin and cellulose; however, these cellulose−chitin hybrid polysaccharides may be expected as possible candidates for biomedical materials.

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Supporting Information Available. ^{13}C and ^{19}F NMR spectra data of new compounds **1**, **3**, **7**, and **12**−**15**. This material is available free of charge via the Internet at http://pubs.acs.org.

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