Chemoenzymatic Syntheses of Amylose-Grafted Chitin and Chitosan

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Amylose-grafted chitin and chitosan were synthesized by chemoenzymatic methods according to the following reaction manners. First, maltoheptaose was introduced to chitosan by a reductive amination using sodium cyanotrihydroborate in a mixed solvent of 1.0 mol/L aqueous acetic acid and methanol at room temperature to produce a maltoheptaose-grafted chitosan (**1**). The functionality of maltoheptaose to chitosan in **1** depended on reaction time. The phosphorylase-catalyzed enzymatic polymerization of α-D-glucose 1-phosphate was then performed from **1** to obtain amylose-grafted chitosan (**2**). Maltoheptaose-grafted chitin (**3**) was synthesized by *N*-acetylation of **1** using acetic anhydride in a mixed solvent of aqueous acetic acid and methanol. Then, synthesis of amylose-grafted chitin (**4**) was performed by the phosphorylase-catalyzed enzymatic polymerization under conditions the same as those for **2**. The average DPs of amylose graft chains in **2** and **4** depended on the feed ratios of α-D-glucose 1-phosphate to maltoheptaose primers in **1** and **3**.

Introduction

Starch, cellulose, and chitin are the most abundant polysaccharides on earth and have widely been studied for their potential to become substitutes for petroleum-based materials. Because the polysaccharides are naturally recycled carbon resources and are considered to be eco-friendly because of their biodegradability,[1] it is expected that the use of polysaccharides will lead to the production of environmentally benign materials.

Besides the abundant materials, polysaccharides having branched structures are often found in nature. For example, arabinoxylan, gum arabic, and guaran play important roles in moisture maintenance and protection against bacteria.[2] These materials are composed of two or more different kinds of saccharide components, which contribute to their prominent functions. On the basis of the above, the development of an efficient method for the preparation of branched (grafted) artificial heteropolysaccharides using common polysaccharides is a promising topic in material research fields. For example, the synthesis of starch−chitosan hybrid materials by direct reaction of these two polysaccharides has been reported.[3,4] However, the reaction proved inefficient as a result of steric hindrance, and their detailed structures have not been elucidated because of their insolubility in any solvents.

The enzymatic polymerization is a useful tool for preparation of precisely regio- and stereocontrolled polysaccharides.[5] For example, phosphorylase-catalyzed enzymatic polymerization using α-D-glucose 1-phosphate (G-1-P) proceeds with the regio- and stereoselective construction of α-glycosidic bond under mild conditions, leading to the direct formation of (1→4)-α-glucan chain, i.e., amylose, in the aqueous media. This polymerization is initiated from a maltooligosaccharide primer like maltoheptaose. Then, the propagation proceeds through the following reversible reaction to produce amylose.

$$((\alpha, 1\rightarrow4)\text{-G})_n + \text{G-1-P} \rightleftharpoons ((\alpha, 1\rightarrow4)\text{-G})_{n+1} + \text{P}$$

In the reaction, a glucose unit is transferred from G-1-P to the nonreducing 4-OH terminus of a (1→4)-α-glucan chain, resulting in inorganic phosphate (P).[6]

Because the combined method of the above-mentioned enzymatic polymerization forming amylose with the chemical reaction, i.e., a chemoenzymatic approach, has fascinated us for obtaining grafted artificial heteropolysaccharides with well-defined structures, we briefly reported a chemoenzymatic synthesis of amylose-grafted chitosan, which had the structure of a chitosan main chain covalently linked to amylose graft chains.[7] This was achieved by the introduction of a maltooligosaccharide primer to chitosan by chemical reaction, followed by elongation of α-glucan chains by phosphorylase-catalyzed polymerization using G-1-P. In this approach, functionality and degree of polymerization (DP) of graft chain is possibly controlled by the conditions in both the chemical reaction and the enzymatic polymerization, leading to the further extension of this study.

In this paper, we describe the comprehensive study on the chemoenzymatic synthesis of amylose-grafted chitosan and the further application to formation of amylose-grafted chitin. Synthetic strategy for these materials is shown in Scheme 1. Introduction of maltoheptaose primer to chitosan by reductive amination was first carried out to produce primer-grafted chitosan having well-defined molecular structure. This was converted into a maltoheptaose-grafted chitin by *N*-acetylation. Phosphorylase-catalyzed enzymatic polymerization of G-1-P from the primers in the materials was then performed to obtain amylose-grafted chitin and chitosan, respectively. This approach consisting of two- or three-step reactions can be considered as more effective than direct reaction of two polysaccharides for production of covalently bonded polysaccharide hybrids having well-defined structures.

Experimental Section

Materials. Chitin powder from crab shells was purchased from Nakalai Tesque, Inc., Kyoto, Japan, and used as a source of chitosan. The general degrees of polymerization of chitin from the origins were

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Scheme 1. Schematic Image for Chemoenzymatic Syntheses of Amylose-Grafted Chitin and Chitosan

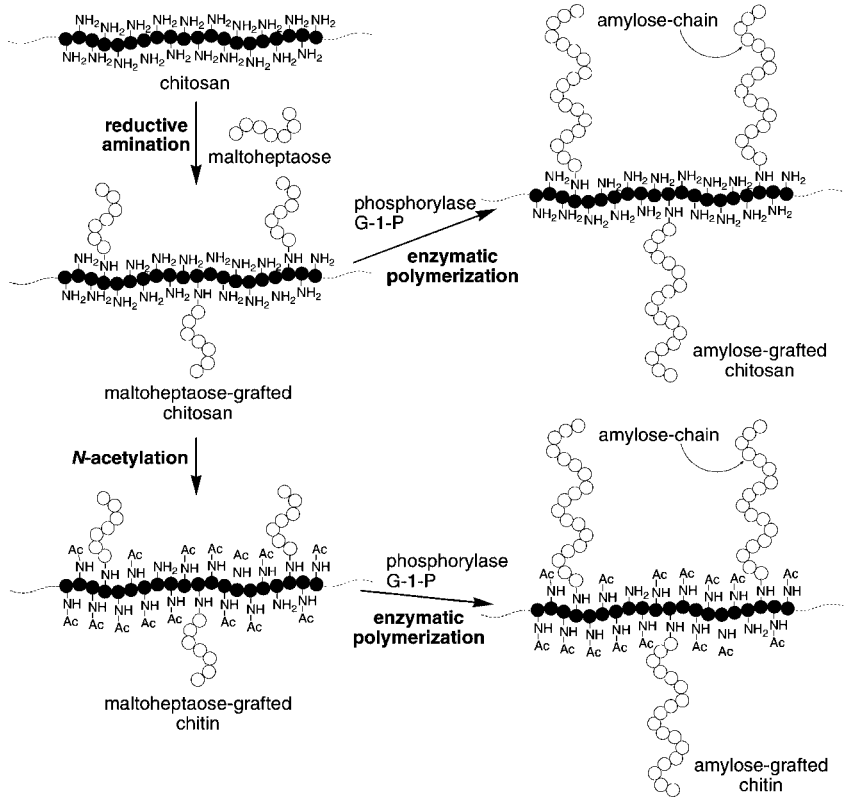


Table 1. Relationship between Functionality of Maltoheptaose to Chitosan in **1** and Reaction Time on the Reductive Amination[a]

	reaction time	functionality of maltoheptaose to chitosan in **1** (%)	
run	(day)	NMR	CHN elemental analysis
1	1	2.4	2.8
2	3	3.1	3.3
3	6	7.7	7.3
4	10	14.2	14.0

[a] Reaction conditions: chitosan = 0.5 mmol unit (0.081 g), maltoheptaose = 0.5 mmol (0.577 g), $NaBH_3CN$ = 2.5 mmol (0.175 g), mixed solvent of 1.0 mol/L aqueous acetic acid and methanol (1/1 (v/v), 18.5 mL), reaction temperature = rt.

Table 2. Results of Enzymatic Polymerization of G-1-P from Maltoheptaose in **1**[a]

run	feed molar ratio of G-1-P to maltoheptaose in **1**	C/N elemental ratio of **2**[b]	DP of amylose graft chain in **2**[b]
1	100	15.5	48
2	250	33.4	139
3	500	61.2	282

[a] Reaction conditions: **1** = 0.9 μmol based on maltoheptaose unit (0.0055 g), phosphorylase = ca. 16 unit, sodium acetate buffer (0.2 mol/L, pH 6.2) = ca. 4 mL, reaction time = 7 h, reaction temperature = 40–45 °C. [b] Determined by CHN elemental analyses.

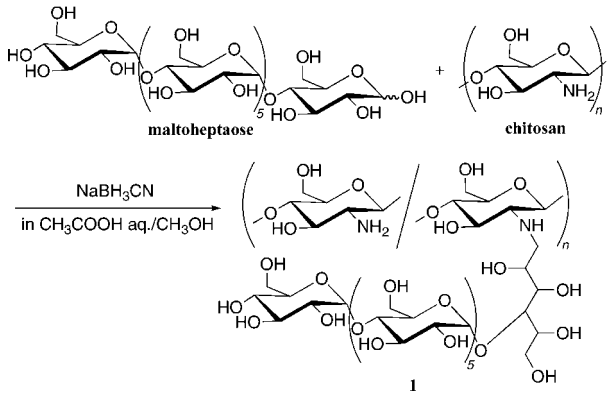
reported to be ca. 2000–4000.[8,9] Chitosan was prepared by deacetylation of chitin according to literature procedure.[10] The degree of deacetylation of chitosan was evaluated to be 99.4% by CHN elemental analysis, which was in good agreement with the literature value (97%).[10] Maltoheptaose was prepared by selective cleavage of one glycosidic bond of β-cyclodextrin under acidic condition.[11] Phosphorylase (300 unit/mL) was supplied from Ezaki Glico Co. Ltd.[12] Chitosanase (from *Bacillus pumilus*, 835 unit/g) and Chitinase (from *Bacillus* sp., 60 unit/g) were purchased from Wako Pure Chemical Industries, Ltd. Other reagents and solvents were used as received.

Synthesis of Maltoheptaose-Grafted Chitosan (1).[7] A typical experimental procedure for synthesis of **1** was as follows (run 2 in Table 1). To a solution of chitosan (0.081 g, 0.5 mmol unit) in a mixed solvent of 1.0 mol/L aqueous acetic acid and methanol (1/1 (v/v), 10 mL), a solution of maltoheptaose (0.577 g, 0.5 mmol) in the mixed solvent (7.5 mL) and a solution of sodium cyanotrihydroborate ($NaBH_3CN$) (0.175 g, 2.5 mmol) in the mixed solvent (1.0 mL) were successively added with vigorous stirring at room temperature. After the solution was stirred further for 3 days, the solution was poured into methanol (200 mL) to precipitate the powdered product, which was isolated by filtration. Dimethyl sulfoxide (DMSO) was added to the obtained product and the suspension was stirred to dissolve remaining unreacted maltoheptaose. The DMSO-insoluble product was isolated by filtration, successively washed with DMSO, acetone, saturated sodium hydrogencarbonate aqueous solution, and water, and then dried under reduced pressure at room temperature to yield 0.119 g of white powdered **1**. ^{1}H NMR (400 MHz, 1.0 mol/L CD_3COOD/D_2O mixed solvent): δ 5.38–5.31 (br, **H-1** of maltoheptaose), δ 4.96–4.78 (br, **H-1** of chitosan), δ 4.13–3.46 (br, **H-3-H-6** of chitosan, **H-2-H-6** of maltoheptaose), δ 3.25–3.10 (br, $-CH-N$, $-CH_2-N$).

Synthesis of Amylose-Grafted Chitosan (2).[7] A typical experimental procedure for synthesis of **2** was as follows (run 3 in Table 2). Maltoheptaose-grafted chitosan **1** (0.0055 g, 0.9 μmol based on maltoheptaose unit) was dissolved in aqueous acetic acid (0.2 mol/L, 0.5 mL), and then sodium acetate buffer (0.2 mol/L, pH 6.2, 4 mL) and sodium salt of G-1-P (0.137 g, 0.45 mmol) was added to this solution. After the pH value of the solution was adjusted to 6.2 by addition of 0.2 mol/L aqueous acetic acid, phosphorylase (ca. 16 unit) was added to this solution, and the solution was stirred further for 7 h at 40–45 °C. After lyophilization of the reaction mixture was performed to obtain powdered product, saturated sodium hydrogen carbonate aqueous solution was added to this powdered product. The insoluble product was isolated by filtration, washed with water and DMSO, and then dried under reduced pressure at room temperature to yield 0.049 g of white powdered **2**.

Hydrolysis of Amylose-Grafted Chitosan 2 by Chitosanase. Amylose-grafted chitosan **2** (0.049 g) was dispersed in sodium acetate buffer

Scheme 2. Synthesis of Maltoheptaose-Grafted Chitosan **1**



(0.3 mol/L, pH 5.6, 9.75 mL) with ultrasonic waves, and then a solution of chitosanase in sodium acetate buffer (0.3 mol/L, pH 5.6, 0.5 mL) was added to the mixture. After the suspension was stirred for 45 min at 50 °C, 0.05 mol/L aqueous sodium carbonate (6.25 mL) was added to the reaction mixture. The insoluble product was isolated by filtration, washed with water, and then lyophilized to yield 0.041 g of white powdered product.

Synthesis of Maltoheptaose-Grafted Chitin (3). To a solution of **1** (0.050 g) in aqueous acetic acid (10 w/v%, 3.0 mL), methanol (10 mL) and acetic anhydride (2.5 mL) were added with stirring at room temperature. After the solution was stirred further for 12 h, the mixture was poured into acetone (100 mL) to precipitate the powdered product, which was isolated by filtration, successively washed with acetone, and then dried under reduced pressure at 100 °C for 2 h to yield 0.044 g of white powdered **3**.

Synthesis of Amylose-Grafted Chitin (4). Maltoheptaose-grafted chitin **3** (0.0066 g, 0.9 μmol based on maltoheptaose unit) was dispersed in aqueous acetic acid (0.2 mol/L, 0.5 mL), and the following procedures were same as those for synthesis of **2**.

Hydrolysis of Amylose-Grafted Chitin 4 by Chitinase. Amylose-grafted chitin **4** (0.013 g) was dispersed in sodium acetate buffer (0.4 mol/L, pH 5.6, 3.0 mL) with ultrasonic waves, and then a solution of chitinase in sodium acetate buffer (0.4 mol/L, pH 5.6, 1.0 mL) was added to the mixture. After the suspension was stirred for 1 day at 37 °C, the insoluble product was isolated by filtration, washed with water, and then dried under reduced pressure at room temperature to yield 0.011 g of white powdered product.

Measurements. The ^{1}H NMR and the solid-state ^{13}C CP/MAS NMR spectra were recorded using a JEOL ECX-400 spectrometer (JEOL Ltd.). The IR spectra were recorded using a Shimadzu FTIR-8400 spectrometer. The CHN elemental analyses were performed using a Perkin-Elmer 2400 II CHN element analyzer. The X-ray diffraction (XRD) measurements were performed at a scanning speed of $2\theta = 2$ deg/min using a Geigerflex RAD-IIB diffractometer (Rigaku Co.) with Ni-filtered Cu Kα radiation (= 0.15418 nm).

Results and Discussion

Synthesis of Maltoheptaose-Grafted Chitosan 1. As previously reported,[7] introduction of maltoheptaose to amino groups of chitosan was carried out by reductive amination using $NaBH_3CN$ in a mixed solvent of 1.0 mol/L aqueous acetic acid and methanol at room temperature to produce a maltoheptaose-grafted chitosan **1** (Scheme 2).[13] The product **1** was soluble in aqueous acetic acid, but insoluble in typical organic solvents such as chloroform, methanol, *N,N*-dimethylformamide (DMF), and DMSO, as well as water. The structure of **1** was characterized by the ^{1}H NMR spectroscopy and the CHN elemental analysis.

The ^{1}H NMR spectrum of **1** in 1.0 mol/L CD_3COOD/D_2O mixed solvent showed not only signals arising from the **H-1** of the chitosan as a main chain but also a signal that is attributable to the **H-1** of maltoheptaose as a grafted side chain in spite of the washing with DMSO, which would dissolve any unbound maltoheptaose. This result indicates that the product has the structure **1**, in which maltoheptaose is connected to the chitosan by covalent bonds. To further prove the covalent bonding of maltoheptaose to the chitosan by the above reductive amination, a similar procedure without use of $NaBH_3CN$ was performed. Consequently, signals that correspond to maltoheptaose as a grafted side chain are not observed in the ^{1}H NMR spectrum, which strongly confirms that **1** is composed of maltoheptaose covalently linked to chitosan.

Table 1 shows the functionalities of maltoheptaose to chitosan in **1** obtained by various reaction times. The values were determined by both the ^{1}H NMR and CHN elemental analysis measurements. The functionalities increase with prolonged reaction time. For example, the values in the products calculated by the ^{1}H NMR spectra are 2.4, 3.1, 7.7, and 14.2% when the reaction times are 1, 3, 6, and 10 days, respectively. These values are relatively in good agreement with those estimated by the CHN elemental analysis.

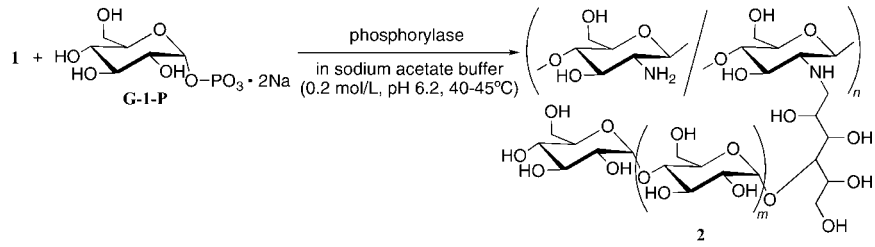
Synthesis of Amylose-Grafted Chitosan 2. Synthesis of amylose-grafted chitosan **2** was achieved by the phosphorylase-catalyzed enzymatic polymerization of G-1-P from maltoheptaose primer in **1** (feed molar ratio of G-1-P to maltoheptaose in **1** = 500) in sodium acetate buffer (0.2 mol/L, pH 6.2) at 40–45 °C (Scheme 3).[7] Here, we employed **1** with the functionality of maltoheptaose to chitosan = 3.3% (run 2 in Table 1). The products were insoluble in any solvents, e.g., aqueous acetic acid and DMSO, which were good solvents for chitosan and amylose, respectively.

The IR spectra of chitosan and **1** show absorptions at 1595 cm^{-1}, attributable to the primary amino groups of chitosan (Figure 1a,b),[14] while the IR spectrum of **2** does not show this absorption (Figure 1c). This indicates that the ratio of primary amino groups of chitosan main chain in **2** has relatively decreased in comparison with that of **1** because of the increase of amylose contents in **2** by progress of the phosphorylase-catalyzed polymerization of G-1-P from **1**.

The XRD pattern of the product (Figure 2b) shows the two diffraction peaks ($2\theta = 16.8$ and 22.4°) derived from the A-type crystalline structure as observed in the XRD pattern of amylose (Figure 2a), indicating the formation of amylose chain by phosphorylase-catalyzed enzymatic polymerization. Such a crystalline structure is generally attributed to the formation of a double helix.[15] Therefore, we assume that one of the reasons for the insolubility of **2** is probably caused by molecular aggregation as a result of this crystalline structure. The aggregation in the material would contribute its conversion into film and hydrogel forms. For example, the film of **2** was prepared by drying the reaction mixture on the flat plate at room temperature, followed by washing with water (Figure 3). It resisted treatment of any solvent. Although the hydrogel of **2** could be formed by drying the reaction mixture slowly in the vessel at 40–50 °C (Figure 4), it broke down easily under compression.

To further confirm the formation of amylose graft chain in **2** by enzymatic polymerization of G-1-P, the chitosan main chain of **2** was hydrolyzed by chitosanase as a catalyst to obtain soluble product.[16] This was achieved by reaction of **2** with chitosanase in sodium acetate buffer (0.3 mol/L, pH 5.6) at 50 °C for 45 min. After neutralization of the reaction mixture using 0.05 mol/L aqueous sodium carbonate, the water-insoluble product was isolated. The product was soluble in DMSO and

Scheme 3. Synthesis of Amylose-Grafted Chitosan **2** by Phosphorylase-Catalyzed Polymerization of G-1-P



thus characterized by ^{1}H NMR measurement in DMSO-d_6. As a result, the spectroscopic pattern was the same as that of amylose, indicating existence of amylose graft chain in **2**.

In the present study, we controlled the average DP of amylose graft chain by changing the feed ratios of G-1-P to **1**. The DPs of amylose graft chains in **2** were calculated by CHN elemental analyses. The C/N elemental ratios of **2**, which were prepared in feed molar ratios of G-1-P to maltoheptaose in **1** = 100, 250, and 500, were calculated to be 15.5, 33.4, and 61.2, respectively. From these data, the DPs of the amylose graft chains were calculated to be ca. 48, 139, and 282, respectively (Table 2), indicating that the DPs of amylose graft chains depended on the ratios of G-1-P to **1**. The DP values were lower than feed molar ratios of G-1-P to maltoheptaose in **1** in all cases, indicating the existence of unreacted G-1-P in the reaction mixture. This is because that the phosphorylase-catalyzed polymerization is in equilibrium with depolymerization of **3**.[17]

The one-step reaction of amylose with chitosan by reductive amination does not afford the amylose-grafted chitosan **2**. This is probably because of a heterogeneous reaction system as a result of the insolubility of the amylose in the solvent for reductive amination. The result indicates that the chemoenzymatic reaction procedure is quite an efficient tool for the production of amylose-grafted chitosan **2**.

Synthesis of Maltoheptaose-Grafted Chitin 3. The present chemoenzymatic approach described above is applied to preparation of amylose-grafted chitin **4**, which has a structure composed of two representative natural polysaccharides. Chitin is the *N*-acetylated derivative of chitosan. Therefore, maltoheptaose-grafted chitin **3** was first synthesized by *N*-acetylation of **1** with the functionality of maltoheptaose to chitosan = 3.3% (run 2 in Table 1) by using acetic anhydride in a mixed solvent of aqueous acetic acid (1.67 mol/L) and methanol (Scheme 4).[18] The obtained product was insoluble in any solvents. The IR spectrum of the product shows absorptions at 1659 and 1558 cm^{-1} due to C=O stretching vibration and NH bending vibration of the amido groups, respectively (Figure 1d), and the spectroscopic pattern is similar to that of commercial chitin (Figure 1e), indicating that **1** was converted into **3**. The functionalities of maltoheptaose and acetamido groups were calculated to be ca. 3.3 and 94.5%, respectively, based on the C/N elemental ratio of 9.26 by CHN elemental analysis of **3**.

Synthesis of Amylose-Grafted Chitin 4. Synthesis of amylose-grafted chitin **4** was performed by the phosphorylase-catalyzed enzymatic polymerization of G-1-P from maltoheptaose primer in **3** (feed molar ratio of G-1-P to maltoheptaose in **3** = 500) under the conditions same as those for **2** (Scheme 5). The obtained product was insoluble in any solvents and the structure was confirmed by the IR, solid-state ^{13}C CP/MAS NMR, XRD, and CHN elemental analysis measurements.

The IR spectrum of **3** shows an absorption at 1558 cm^{-1}, which is attributed to the NH bending vibration of amido group of chitin (Figure 1d), while that of **4** does not show this

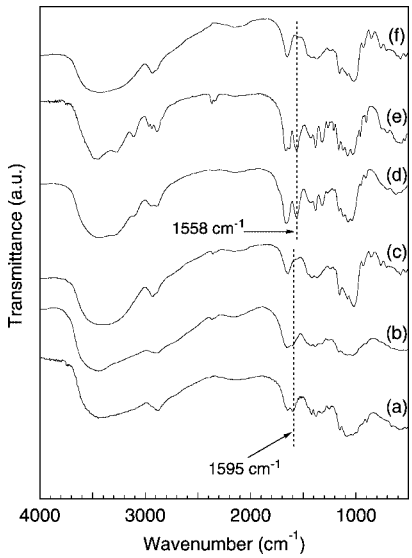


Figure 1. IR spectra of (a) chitosan, (b) maltoheptaose-grafted chitosan **1**, (c) amylose-grafted chitosan **2**, (d) maltoheptaose-grafted chitin **3**, (e) chitin, and (f) amylose-grafted chitin **4**.

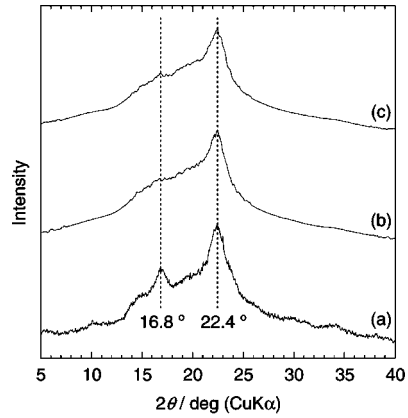


Figure 2. XRD Patterns of (a) amylose, (b) amylose-grafted chitosan **2**, and (c) amylose-grafted chitin **4**.

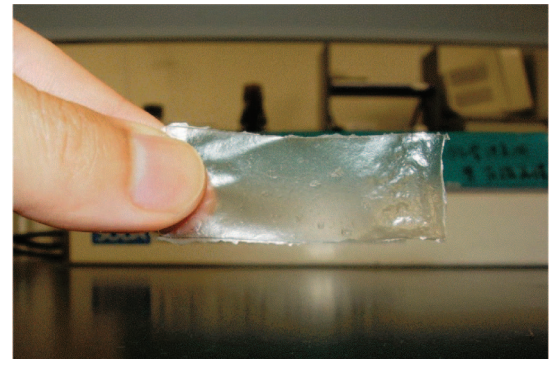


Figure 3. Photograph of a film obtained from **2**.

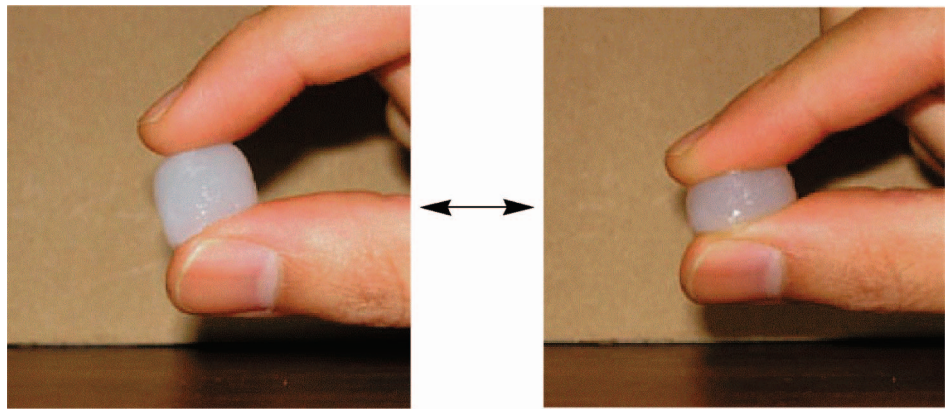
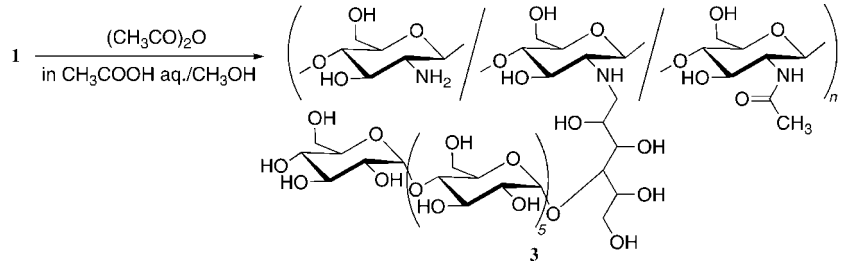
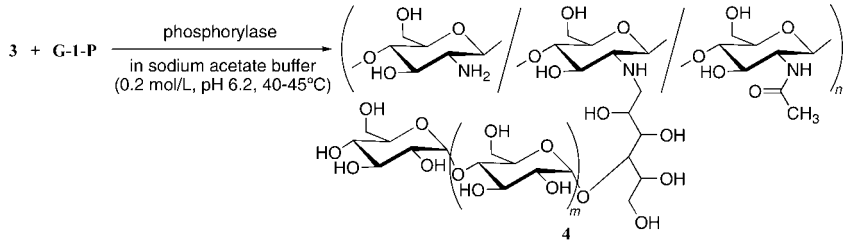


Figure 4. Photographs of a hydrogel obtained from **2**.

Scheme 4. Synthesis of Maltoheptaose-Grafted Chitin **3**



Scheme 5. Synthesis of Amylose-Grafted Chitin **4** by Phosphorylase-Catalyzed Polymerization of G-1-P



absorption (Figure 1f). This indicates that the ratio of amido group of the chitin main chain in **4** has relatively decreased in comparison with that of **3** because of the increase of amylose contents in **4** by progress of the enzymatic polymerization from **3**.

The solid-state ^{13}C CP/MAS NMR spectrum of **4** shows signals at δ 102.7, 82.8, 72.8, and 62.1 ppm corresponding to C-1, C-4, C-3, C-5, and C-6 of both amylose and chitin, respectively (Figure 5a). In addition, signals due to the carbonyl carbon, C-2, and the methyl carbon of chitin are observed at δ 174.8, 55.9, and 23.6 ppm, respectively. This spectroscopic pattern includes signals derived from amylose and chitin (Figure 5b,c), indicating that the product was composed of both the polysaccharide chains.

The XRD pattern of the product shows the two characteristic diffraction peaks of amylose, indicating the formation of amylose graft chain (Figure 2c).[15] Furthermore, to confirm the formation of amylose graft chain in **4** by enzymatic polymerization of G-1-P, the chitin main chain of **4** was hydrolyzed by chitinase as a catalyst to obtain soluble product,[19] which was carried out in acetate buffer (pH = 5.6) at 37 °C. The hydrolyzed product was characterized by ^1H NMR measurement in DMSO-d_6 and the spectroscopic pattern of the product was the same as that of amylose, indicating existence of amylose graft chain in **4**.

The average DPs of amylose graft chains in **4** were controlled by changing the feed ratios of G-1-P to **3**. When the enzymatic polymerizations were performed in feed molar ratios of G-1-P to maltoheptaose in **3** = 100, 250, and 500, the C/N elemental ratios of **4** were calculated to be 12.5, 20.3, and 50.6, respectively, corresponding to the DPs of amylose = 23, 63, and 208, respectively (Table 3). These DP values were lower than those of **2** (Table 2). This is probably because that the enzymatic polymerization of G-1-P using **3** as a primer proceeded heterogeneously due to insolubility of **3**. From all results described above, we concluded that the product had the structure **4**, in which amylose was connected to the chitin by covalent bonds.

Conclusions

In the present paper, we reported chemoenzymatic syntheses of amylose-grafted chitin **4** and amylose-grafted chitosan **2**. First, introduction of maltoheptaose to amino groups of chitosan was carried out by reductive amination using $NaBH_3CN$ in a mixed solvent of 1.0 mol/L aqueous acetic acid and methanol at room temperature to produce a maltoheptaose-grafted chitosan **1**. The functionalities of maltoheptaose to chitosan in **1** increased with prolonged reaction time. The phosphorylase-catalyzed enzymatic polymerization of G-1-P was then performed from **1** to obtain amylose-grafted chitosan **2**. Maltoheptaose-grafted chitin **3** was synthesized by *N*-acetylation of **1** using acetic anhydride in a mixed solvent of aqueous acetic acid and methanol. The

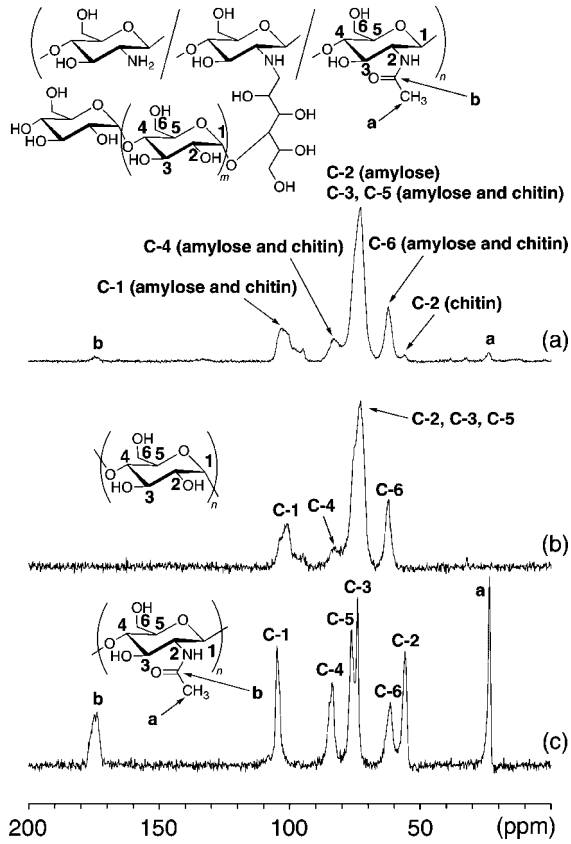


Figure 5. Solid-state ^{13}C CP/MAS NMR Spectra of (a) **4**, (b) amylose, and (c) chitin. Chemical shifts were referenced to hexamethylbenzene (δ 17.36 ppm).

Table 3. Results of Enzymatic Polymerization of G-1-P from Maltoheptaose in **3**[a]

run	feed molar ratio of G-1-P to maltoheptaose in **3**	C/N elemental ratio of **4**[b]	DP of amylose graft chain in **4**[b]
1	100	12.5	23
2	250	20.3	63
3	500	50.6	208

[a] Reaction conditions: **3** = 0.9 μmol based on maltoheptaose unit (0.0066 g), phosphorylase = ca. 16 unit, sodium acetate buffer (0.2 mol/L, pH 6.2) = ca. 4 mL, reaction time = 7 h, reaction temperature = 40–45 °C. [b] Determined by CHN elemental analyses.

synthesis of amylose-grafted chitin **4** was performed by the phosphorylase-catalyzed enzymatic polymerization under conditions the same as those for **2**. The average DPs of amylose graft chains in **2** and **4** depended on the feed ratios of G-1-P to maltoheptaose primers in **1** and **3**. Because the present chemoenzymatic approach is efficient procedure for the production of amylose-grafted polysaccharides, the development of various branched heteropolysaccharides can be expected by the present method.

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Note Added after ASAP Publication. This paper was published ASAP on November 16, 2007. The reaction equation in the Introduction was revised. The updated paper was reposted on November 27, 2007.

References and Notes

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