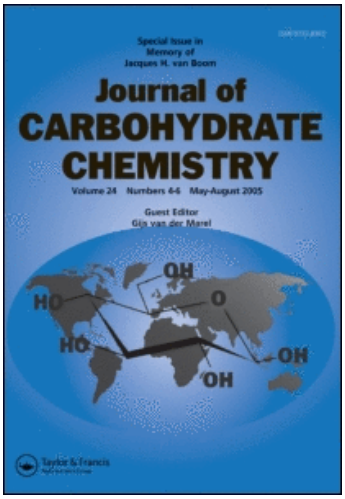

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Apparent Production of Enzymatically Synthesized Amylose in DMSO by Means of Calcium Alginate Hydrogel Beads/DMSO System

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In this paper, we describe the apparent production of enzymatically synthesized amylose in DMSO by means of the calcium alginate hydrogel beads/DMSO system as the reaction field of the phosphorylase-catalyzed enzymatic polymerization. When the calcium alginate hydrogel beads including glucose-1-phosphate, maltoheptaose, and phosphorylase were suspended in DMSO and the system was slowly stirred at 40°C for 12 h, the reaction proceeded to produce amylose, which eluted to the DMSO solution. The obtained amylose was purified by the treatment with ion-exchange resins, and its structure was confirmed by the ^{1}H NMR spectrum. The time-course experiment in the present system revealed that the phosphorylase-catalyzed enzymatic polymerization was carried out for 15 min on the inside of the calcium alginate hydrogel beads and the produced amylose was gradually eluted to the surrounding DMSO solution. The comparison of the present system with the general enzymatic polymerization in aqueous buffer solution suggested that the yield and the degree of polymerization of amylose in the present system were comparable to those in aqueous buffer solution.

Keywords Calcium algninate hydrogel beads, Phosphorylase, Enzymatic polymerization, Amylose

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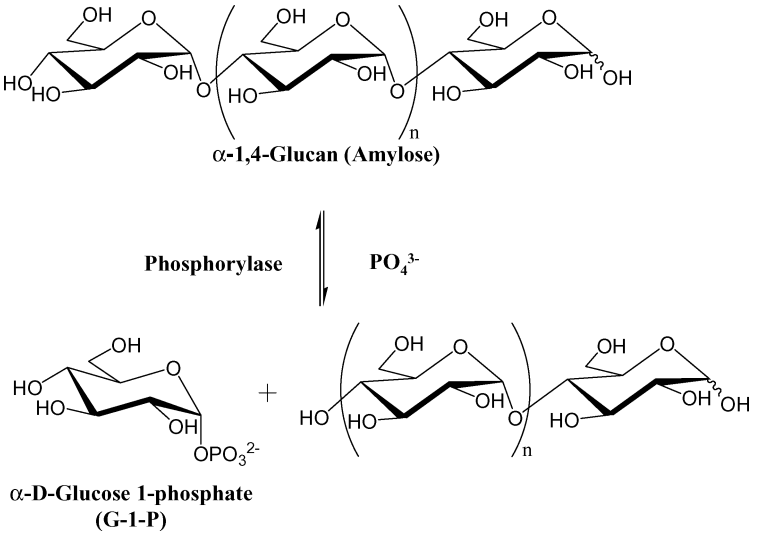
INTRODUCTION

Because the enzymatic polymerization is a useful tool for preparation of precisely regio- and stereocontrolled polysaccharides, this method has been demonstrated as the efficient approach for the synthesis of natural and unnatural polysaccharides.[1–5] For example, amylose can be prepared by the enzymatic polymerization of α-D-glucose 1-phosphate (G-1-P) as a monomer catalyzed by α-glucan phosphorylase (or simply called as "phosphorylase," EC 2.4.1.1).[6] Phosphorylase is basically the enzyme that catalyzes the reversible phosphorolysis of α-1,4-glucans at the nonreducing end in the presence of inorganic phosphate to give G-1-P (Sch. 1).[7,8] By means of the reversibility of the phosphorylase catalysis, the reaction proceeds to the way of the chain elongation with the regio- and stereoselective construction of α-glycosidic bond, leading to the direct formation of (1→4)-α-glucan, that is, amylose. At the beginning of the reaction system, a maltooligosaccharide as a primer is required to initiate the polymerization. Then, the propagation proceeds through the following reversible reaction to produce amylose: $((\alpha, 1\rightarrow4)\text{-G})_n$+ G-1-P $\leftrightarrows$ $((\alpha, 1\rightarrow4)\text{-G})_{n+1}$+ 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). In this reaction system, the produced amylose is gradually precipitated from the aqueous solvent due to the double-helix formation of the each product.



Scheme 1. Catalysis of phosphorylase.

The phosphorylase-catalyzed enzymatic polymerization has been used to prepare the amylosic materials such as the amylose-grafted materials.[9–15] By means of this enzymatic polymerization, we have developed a new method for

the preparation of the inclusion complexes composed of amylose and synthetic polymers,[16–23] since little had been reported regarding the formation of inclusion complexes composed of amylose and polymeric compounds due to the difficulty of including the polymer chains into the cavity of amylose.[24,25] When the phosphorylase-catalyzed polymerization was carried out in the presence of the appropriate guest polymers in our developed method, the propagation proceeded with the formation of the corresponding inclusion complexes. The representation of this reaction system is similar to the way that vines of plants grow twining around a rod. Accordingly, we have proposed that this polymerization method is named "vine-twining polymerization." Although the hydrophobic interaction has been considered as a driving force for the formation of the inclusion complexes, the guest polymers are necessarily dispersed in aqueous buffer solution of the polymerization solvent. In the previous study on vine-twining polymerization, therefore, only a limited number of the polymers having the appropriate hydrophobicity have successfully been used as the guest polymers. For example, the inclusion complexes were obtained from the polyethers having the appropriate hydrophobicity such as poly(trimethylene oxide) and poly(tetramethylene oxide), whereas no inclusion complex was formed from the more hydrophobic polyether of poly(hexamethylene oxide), attributed to its aggregation in the aqueous polymerization solvent.[18] If the phosphorylase-catalyzed enzymatic synthesis of amylose can be performed in organic solvents, a variety of hydrophobic guest polymers are possibly employed in the vine-twining polymerization to form the various kinds of the inclusion complexes. In addition to this point, because DMSO is a good solvent of amylose, the use of DMSO as the solvent will achieve the homogeneous system of the enzymatic polymerization, resulting in a possibility for construction of novel amylosic materials by this system. However, in our preliminary research, phosphorylase from potato did not catalyze the enzymatic polymerization of G-1-P in acetate buffer solution containing DMSO amounting to larger than 50% due to the rapid deactivation of the phosphorylase in such the solvent. Although we confirmed that the phosphorylase-catalyzed enzymatic polymerization proceeded in acetate buffer solution containing 25% v/v of DMSO, the reaction system became heterogeneous due to the precipitation of the product. As one of the common approaches achieving the enzymatic reaction in the organic media, we prepared the phosphorylase/surfactant complex.[26,27] However, the phosphorylase/surfactant complex did not exhibit the activity of the catalysis for the enzymatic polymerization in acetate buffer solution containing 50% v/v DMSO. On the basis of the aforementioned results, we needed to develop a new reaction system that provides the production of enzymatically synthesized amylose in DMSO solution even though the reaction is performed in aqueous media.

In this study, we newly designed the hydrogel beads/DMSO system for the above purpose. In the system, the hydrogel beads have an important role, to

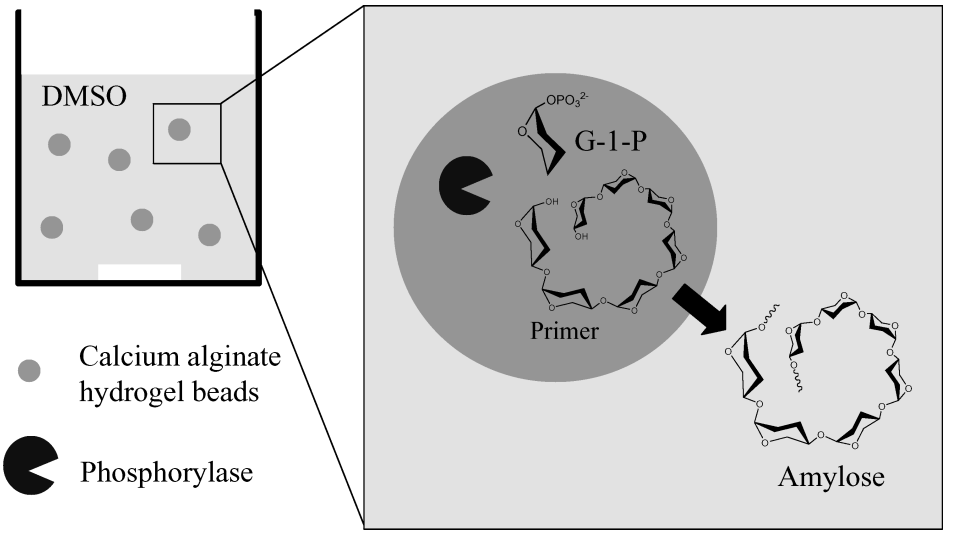


Figure 1. Image of the calcium alginate hydrogel beads/DMSO system.

provide the separated aqueous media from DMSO, in which the deactivation of the phosphorylase caused by contact with DMSO molecules is reduced. Consequently, amylose synthesized by the enzymatic polymerization in aqueous media included by the beads elutes to the surrounding DMSO solution. Therefore, the system can be considered as the apparent production of amylose in DMSO and, thus, will be possibly applicable to the preparation of the novel amylose-polymer inclusion complexes using the extensive kinds of the guest polymers by means of the vine-twining polymerization in the future study.

To achieve this system, in this paper, we selected the calcium alginate hydrogel as the material for the beads including the enzymatic reaction field, and examined the development of the calcium alginate hydrogel beads/DMSO system for the apparent synthesis of amylose in DMSO (Fig. 1).

RESULTS AND DISCUSSION

The phosphorylase-catalyzed enzymatic synthesis of amylose using G-1-P (185.9 μmol) and maltoheptaose (Glc_7) (3.4 μmol) was performed in the calcium alginate hydrogel beads/DMSO system according to the procedure as described in the Experimental section. The similar amount of phosphorylase was used for this system (52 units for 3.4 μmol of Glc_7) as that employed for the phosphorylase-catalyzed polymerization in our recent studies.[13,14] After the reaction, the resulting mixture was separated into the two parts, the DMSO filtrate and the calcium alginate beads, by filtration. The DMSO filtrate was poured into acetone to precipitate the crude product containing amylose. The ^{1}H NMR spectrum of the crude product in DMSO-d_6/D_2O (v/v = 20/1)

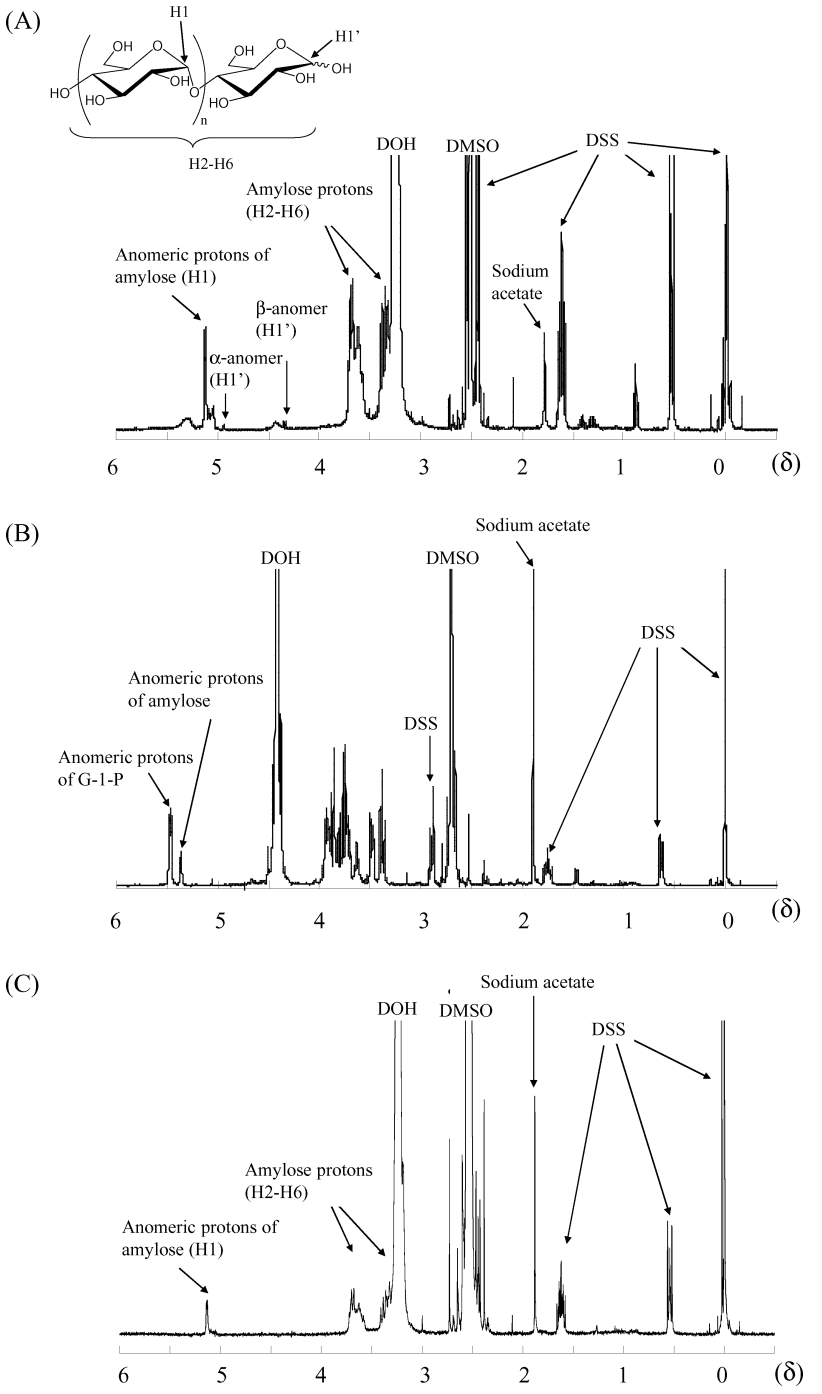


Figure 2. ^{1}H NMR spectra of (A) the crude product in DMSO solution (DMSO-d_6/D_2O ,v/v = 20:1), (B) the material extracted from the dried calcium alginate with D_2O (D_2O), and (C) the material extracted from the dried calcium alginate with DMSO-d_6/D_2O (v/v = 20:1) (DMSO-d_6/D_2O, v/v = 20:1).

(Fig. 2A) showed the signals at 3.31–3.87 ppm (H2-H6), 5.02–5.18 ppm (H1), and 4.94 and 4.35 ppm (H1′, α- and β-anomers of reducing end, respectively) assignable to amylose, whose degree of polymerization (DP) was estimated by the integrated ratio of the signals due to the H1 protons to the signals due to the protons of the reducing end (H1′) to be 32. This result suggested that the enzymatic polymerization proceeded in the beads suspended in the large amount of DMSO and the produced amylose eluted to DMSO solution from the inside of the beads. The yield of amylose eluting to DMSO was estimated by the ^{1}H NMR spectrum using a 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (DSS) standard of 56.7%. However, in the ^{1}H NMR spectrum, the signal due to sodium acetate was observed at 1.80 ppm, and further, the ^{31}P NMR spectrum of the crude product showed a signal at 2.52 ppm assignable to inorganic phosphate generated from G-1-P by the progress of the enzymatic polymerization. These results suggested that the crude product as the fraction insoluble in acetone included sodium acetate and inorganic phosphate as well as probably some impurities that were not detected by the NMR analysis. To isolate the pure amylose from the present enzymatic reaction system, the DMSO filtrate after separation from the reaction mixture was passed through the ion-exchange resins (Amberlite IR120(H), IRA400(OH)). The eluted DMSO solution was poured into acetone to precipitate the product, which was corrected by filtration, washed with a large amount of acetone, and dried under reduced pressure to obtain the isolated product. The ^{1}H NMR spectrum of the isolated material in DMSO-d_6/D_2O (v/v = 20/1) (Fig. 3) showed the signals due to only amylose and no signal was detected in the ^{31}P NMR

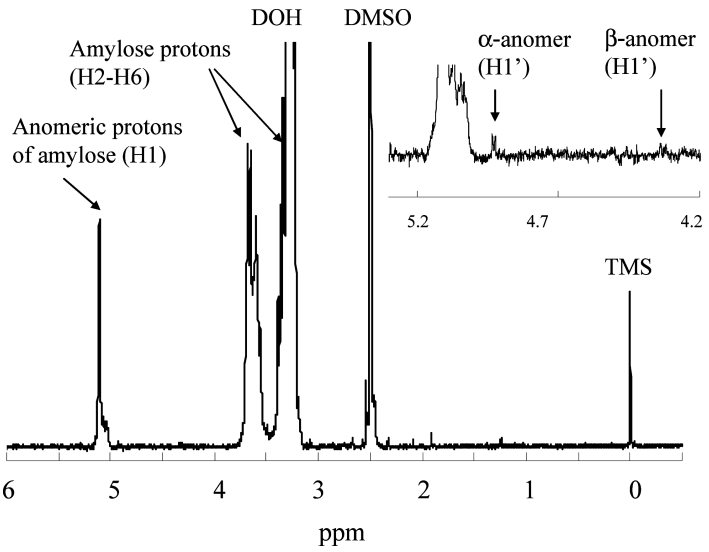


Figure 3. ^{1}H NMR spectrum of the purified amylose (DMSO-d_6/D_2O, v/v = 20:1).

spectrum of the same sample. These results indicated that the product was isolated as the pure amylose by the treatment with the ion-exchange resins, and the isolated yield was 36.6%.

On the other hand, the ^{1}H NMR spectra of the extracted materials from the dried calcium alginate residue with D_2O (Fig. 2B) and DMSO-d_6/D_2O (v/v = 20/1) (Fig. 2C) showed the existence of 29.0% of unreacted G-1-P and 11.7% of amylose, respectively. The amylose remaining in calcium alginate hydrogel beads was also purified using ion-exchange resins, and the yield was 9.7%.

The time-course experiment using G-1-P (164.0 μmol) and Glc_7 (0.6 μmol) was carried out to reveal the detailed behavior of the present reaction system (Fig. 4). The yields of amylose obtained in DMSO (Fig. 4A) were gradually increased with the time advances up to 35.6% after 720 min. However, the highest yield of amylose in gel was confirmed after 15 min, and then the yields were gradually decreased with the time advances. The total yields as the total values of the yields in DMSO plus those in gel were rapidly increased from 11.5% to 43.9% between the reaction times of 1 min and 15 min, and then the values of the yields were maintained during the later reaction times. These results indicated that the further reaction hardly proceeded for the prolonged reaction time after 15 min in spite of the remaining G-1-P as shown in Table 1. This was probably caused by the deactivation of phosphorylase in the present system during the reaction time of 15 min because the activity of phosphorylase decreased to be much less than the original value by incubating the calcium alginate hydrogel beads, including the enzyme in DMSO, for 15 min. The number-average DPs calculated from the yields and DPs of the amylose both in DMSO and in gel were also rapidly increased and reached the highest DP (120) after 15 min (Fig. 4B). The number-average DPs at the later reaction times were not calculated because of fewer amounts of amylose in the gel. The DPs of amylose eluting to DMSO were gradually increased during the reaction, probably caused by the difference in the eluting rate depending on the size of each amylose molecule. These results indicated that the enzymatic polymerization in the calcium alginate hydrogel beads/DMSO system was completed after 15 min and the products gradually eluted from the inside of the beads to DMSO.

Table 1: Phosphorylase-catalyzed enzymatic polymerization in the calcium alginate hydrogel beads/DMSO system.

		Amylose obtained in DMSO		Materials remaining in gel	
Entry	**G-1-P/Glc_7**	**Yield (%)[a]**	**DP[a]**	**Amylose (%)[a]**	**G-1-P (%)[a]**
1	55	56.7	32	11.7	29.0
2	165	40.7	83	8.2	50.2
3	293	35.6	108	8.5	40.7

[a]Estimated by ^{1}H NMR spectra.

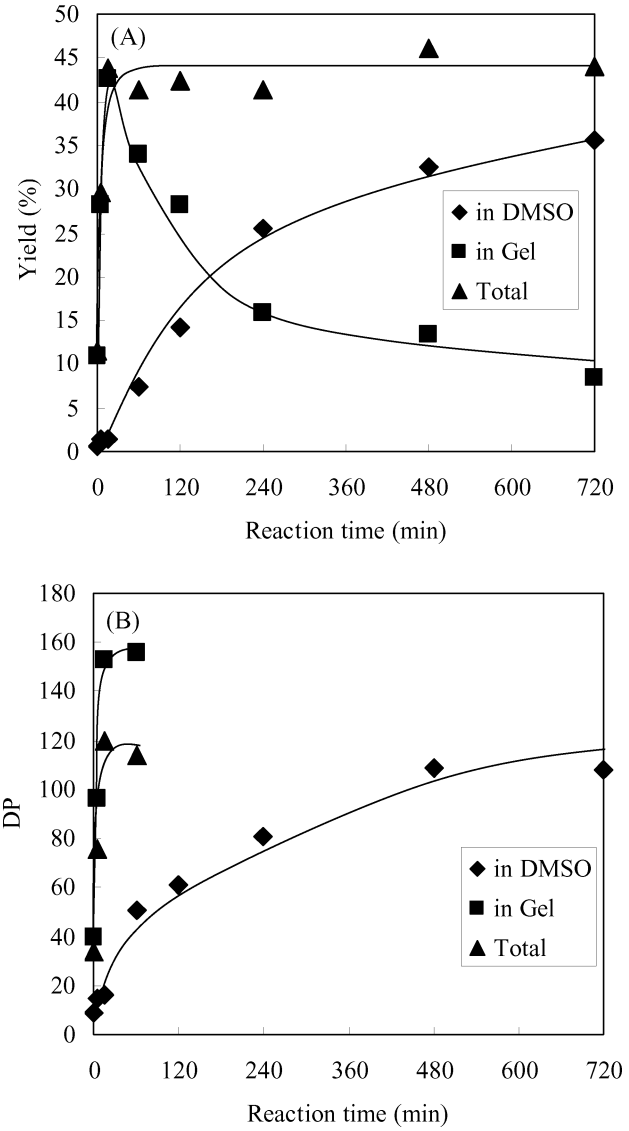


Figure 4. Time-course experiments (A) yield vs. reaction time and (B) DP vs. reaction time.

We performed the comparison of the enzymatic polymerization in the present system with that in acetate buffer solution (Table 1, in the calcium alginate hydrogel beads/DMSO system; Table 2, in acetate buffer solution). These experiments were carried out using the same feed ratios of G-1-P to the primer. In these experiments, the yields and DPs of amylose obtained in DMSO by the calcium alginate hydrogel beads/DMSO system were comparable to those obtained in the general enzymatic polymerization.

Table 2: General phosphorylase-catalyzed enzymatic polymerization in sodium acetate buffer.

Entry	G-1-P/Glc_7	Yield (%)[a]	DP[a]
4	55	54.0	32
5	165	43.5	80
6	293	36.8	109

[a]Estimated by ^1H NMR spectra.

In conclusion, in this study, the calcium alginate hydrogel beads/DMSO system was developed as the new reaction field for enzymatic synthesis of amylose. In the present system, the phosphorylase-catalyzed enzymatic polymerization proceeded in the inside of the gel beads and the product eluted to DMSO as the apparent production of the enzymatically synthesized amylose in DMSO. The results of the time-course experiment suggested that the enzymatic polymerization in the present system was completed after 15 min and the products gradually eluted from the inside of the beads to DMSO. The yield and DP of amylose in the present system were comparable to those in the general enzymatic polymerization in aqueous buffer solution. The preparation of the novel amylose-polymer inclusion complexes using a variety of the guest polymers by means of the present calcium alginate hydrogel beads/DMSO system is now in progress in our research group. Furthermore, the data of the time-course experiments suggested that the amylose molecules are possibly separated based on their size by means of the calcium alginate hydrogel beads/DMSO phase. This system will be applied to the construction of the practical separation method in the future.

EXPERIMENTAL

General Method

α-Glucan phosphorylase from potato was supplied from Ezaki Glico Co. Ltd. (Osaka, Japan).[28] Maltoheptaose (Glc_7) was prepared by selective cleavage of one glycosidic bond of β-cyclodextrin under acidic condition.[9] Sodium salt of G-1-P and sodium alginate were purchased from Wako Pure Chemical Industries, Ltd. Other reagents and solvents were used as received. NMR spectra were recorded on a JEOL ECX400 spectrometer.

Enzymatic preparation of amylose in the calcium alginate hydrogel beads/DMSO system

Sodium alginate (30.0 mg) was dissolved in 100 mM acetate buffer (pH = 6.2) (2.0 mL) and a sodium salt of G-1-P (56.5 mg, 185.9 μmol) and Glc_7 (3.9 mg, 3.4 μmol) were added to the solution. After the resulting solution was cooled

to 0°C, phosphorylase (0.2 mL, 52 units) was added. The obtained solution was added dropwise into 5wt% $CaCl_2$/*n*-butanol solution (4.0 mL) at 0°C to prepare the calcium alginate hydrogel beads including G-1-P, Glc_7, and phosphorylase. The calcium alginate hydrogel beads were expeditiously added to DMSO (6.0 mL) and then the obtained mixture was stirred slowly at 40°C for 12 h. After the calcium alginate beads were filtered off, the filtrate was poured into acetone to precipitate the polymeric material, which was collected by filtration, washed with acetone and ethanol, and dried under reduced pressure to obtain 19.3 mg of the crude product. The yield of amylose in crude product was estimated by ^{1}H NMR spectrum (JEOL ECA400 spectrometer) using DSS as standard in DMSO-d_6/D_2O (v/v = 20/1) to be 56.7%.

For the purification of amylose existing in DMSO, the reaction was conducted in the same scale as described above and the DMSO filtrate after separation from the reaction mixture was passed through the ion-exchange resins (Amberlite IR120(H), IRA400(OH)). The eluted DMSO solution was poured into acetone to precipitate the product, which was corrected by filtration, washed with a large amount of acetone, and dried under reduced pressure to obtain the purified amylose (12.4 mg) in 36.6% yield.

Analysis of the materials remaining in the calcium alginate hydrogel beads

The calcium alginate beads, obtained by the aforementioned work-up procedure, were mashed, washed with acetone, and dried under reduced pressure to give 462.0 mg of the dried material. The material (123.7 mg) was added to D_2O (1.0 mL) and the mixture stirred for 1 h at rt. After the insoluble fraction was filtered off, DSS was added to the filtrate, and the solution was subjected to ^{1}H NMR measurement to estimate the amount of the unreacted G-1-P to be 29.0%. The remaining amylose in gel was extracted from the dried material of the beads by the same procedure using DMSO-d_6/D_2O (v/v = 20/1) and the yield was estimated by ^{1}H NMR spectrum of the solution using DSS standard to be 11.7%.

General enzymatic synthesis of amylose in buffer solution

A solution of G-1-P (58.0 mg, 190.8 μmol), Glc_7 (4.0 mg, 3.5 μmol), and phosphorylase (0.2 mL, 52 unit) dissolved in 100 mM acetate buffer solution (pH = 6.2) (2.0 mL) was incubated for 12 h at 40°C. After the reaction solution was lyophilized, the yield of amylose was directly estimated by ^{1}H NMR spectrum of the lyophilized residue using DSS standard in DMSO-d_6/D_2O (v/v = 20/1) to be 54.0%.

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