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Novel carbonyl-group-containing dextran synthesis by pyranose-2-oxidase and dextransucrase

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ABSTRACT

The carbonyl polysaccharide, keto-dextran, was synthesized by the regioselective oxidation of sucrose and by the subsequent transfer reaction of the oxidized sucrose. The regioselective oxidation of sucrose was performed by bioconversion with pyranose-2-oxidase (EC 1.1.3.10). After 24 h, the conversion percentage of sucrose into keto-sucrose was 100% as determined by a colorimetric method with dinitrophenylhydrazine. Converted keto-sucrose was polymerized to keto-dextran by dextransucrase (EC 2.4.1.5). Polymerization of keto-dextran was confirmed by the increase in molecular weight and amount of keto-dextran produced. The amount of keto-dextran produced decreased to 80% of the amount of dextran produced owing to the substrate recognition of DSase. From a Lineweaver–Burk reciprocal plot, the Michaelis constants for sucrose and keto-sucrose were 4.6 mmol L^{-1} and 14.0 mmol L^{-1}, respectively. The keto-dextran had a carbonyl group in all glucose units.

1. Introduction

Modification of polysaccharides is an important process in preparing a functional, bioactive, and biodegradable carbohydrate.[1–3] In particular, dextran, consisting of glucose units linked mainly by α-(1,6) glycoside bonds, has been widely developed in analytical engineering and the pharmaceutical industry as well as in biochemical engineering owing to the hydrophilicity of plural hydroxyl groups and the flexibility of dextran backbone from random structure. For example, a solution of dextran with a molecular weight of 75–100 kDa is used in transfusion as a blood-plasma volume expander,[4] and cross-linked dextran is used as a packed bed in a column to separate and purify molecules with molecular weights in the range of 0.7–200 kDa.[5,6]

Traditionally, the modification of dextran has involved the direct introduction of a functional group into the dextran backbone via chemical reaction. Etherification of dextran enables us to control the solubility, hydrophilicity or hydrophobicity, ionic strength, and degradability using the properties of ether moieties.[7] For example, Dellacherie et al. prepared an amphiphilic dextran (Dex-P) by introducing aromatic groups,[8–10] and Jozefonvicz et al. prepared a cation-exchanging dextran (CMD) by carboxymethylation.[11,12] Dex-P and CMD are utilized as nano particles in drug delivery systems (DDS) and as anticoagulants. Many studies on the synthesis of dextran ester have also been reported.[7] Dextran maleate with unsaturated bonding is obtained by reaction with maleic anhydride in a medium of DMF/LiCl at 353 K, and forms hydrogel by cross-linking under irradiation. The hydrogel is applicable to DDS and protecting encapsulants. Inorganic dextran esters such as dextran phosphates and dextran sulfates are achieved by treatment in formamide with polyphosphonic acid and chlorosulfonic acid, respectively. These dextran esters are important polymers in the electrical and medical fields. In addition, thiol-dextran, a hydroxyl group converted to a thiol group, is obtained by nucleophilic displacement reactions via the tosylation of dextran. The thiolation of dextran leads to wide application, for example, metal adsorption and self-assembly.

However, using a chemical method, the functional group is randomly introduced to dextran at low density due to steric hindrance, and thus it is difficult for the functional group to be regioselectively introduced. A by-product of dextran during a chemical reaction is also obtained by hydrolysis.

Natural dextran is produced enzymatically from sucrose as a substrate by dextransucrase (DSase) extracted from species of *Leuconostoc* and *Streptococcus*.[13] The amount and molecular weight of dextran are controllable by the DSase reaction time, temperature, pH, and substrate concentration.[14,15] To produce modified dextran effectively, the functional group is readily introduced to the sucrose, and subsequently the modified sucrose is polymerized by DSase, resulting in a dextran derivative with high functional density. When modified sucrose is polymerized by DSase, the substrate recognition of DSase should be considered. Robyt et al. reported that hydroxyl groups of sucrose at C-3 and C-4 positions are important in the binding of sucrose to the active sites of DSase using sucrose analogs.[16] Enzymatic polymerization using DSase

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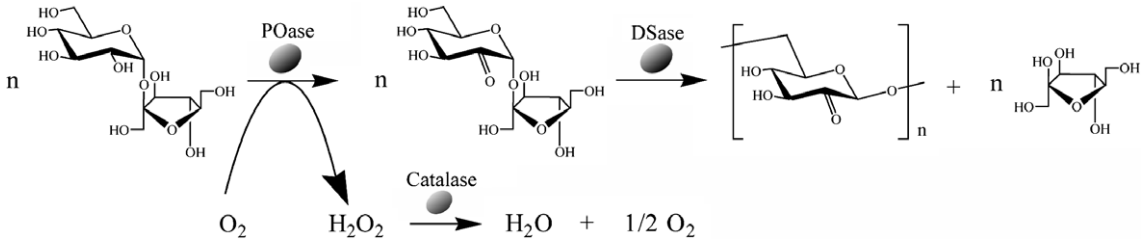


Figure 1. Preparation of keto-dextran using three enzymatic reactions; first regioselective oxidation of sucrose by POase, subsequently polymerization of carbonyl-group-containing sucrose at C-2 by DSase.

requires an appropriate molecular design of sucrose at the C-2 position. However, as it is difficult to chemically modify the particular hydroxyl group in sucrose in a chemical method, processes for protection and deprotection are required.[17]

The enzymatic specificity is also used, in this study, for regioselective modification of sucrose at C-2. Pyranose-2-oxidase (POase) catalyzes the regioselective oxidation at C-2 hydroxyl group of the glucose unit and the reduction of O_2 to H_2O_2.[18,19] The sugar is converted into the corresponding 2-keto-sugar, having a carbonyl group at C-2, by the POase reaction. POase is applied as a biosensor to determine the amount of carbohydrate,[20,21] and the keto-sugar is used as the intermediates for synthetic carbohydrates.[22]

In this paper, regioselectively introduced keto-dextran at a high introduction percentage of the carbonyl group was prepared by the reactions of three enzymes (POase, catalase, and DSase) as shown in Figure 1. First, sucrose was converted into keto-sucrose with carbonyl groups at the C-2 position by POase (Fig. 1 left side). Hydrogen peroxide as by-product was eliminated by catalase because it is an inhibitor of the POase reaction.[23] The conversion percentage into keto-sucrose was determined from the concentration of the carbonyl group from a colorimetric assay. The presence and modified position of the carbonyl group in sucrose from the POase reaction were confirmed by ^{13}C nuclear magnetic resonance (NMR) and ^{1}H NMR. Subsequently, keto-dextran is polymerized from keto-sucrose by DSase (Fig. 1 right side), and the concentration of keto-dextran produced is a function of the reaction time. The substrate recognition of DSase was quantitatively analyzed via a Lineweaver–Burk reciprocal plot to calculate the Michaelis constant and maximum velocity.

2. Experimental

2.1. Reagents and materials

Pyranose-2-oxidase (EC 1.1.3.10, Lot No. 056K1489), catalase (EC 1.11.1.6, Lot No. 096K7035), and dextransucrase (EC 2.4.1.5, Lot No. 067K4164) were purchased from Sigma Chemical Co. Sucrose (first grade, product code: 193-00025) and dinitrophenylhydrazine (special grade, product code: 049-03612) were purchased from Wako Chemical Ltd. Deuterium oxide (D_2O) and dimethyl sulfoxide-d_6 (D, 99.9% + 0.05% TMS) were purchased from Merck and Cambridge Isotope Laboratories Inc., respectively. An ultrafiltration membrane (molecular weight cut-off: 100,000) was obtained from Millipore Co. Other reagents were of analytical grade or higher.

2.2. Bioconversion of sucrose into keto-sucrose

Bioconversion of sucrose into keto-sucrose was performed by regioselective oxidation using POase. Sucrose (5 mg mL^{-1}) was dissolved in 200 mL distilled water as solvent, and POase (0.25 U mL^{-1}) and catalase (25 U mL^{-1}) were added to the solution with aeration (0.5 mL min^{-1}) at 303 K. After 24 h, enzymes were inactivated by heating and removed by filtering with the ultrafiltration membrane. The removal of enzymes was confirmed by the Bradford method.[24] The filtrate was dried in a vacuum and obtained as a hygroscopic white powder.

2.3. Polymerization of keto-sucrose into keto-dextran

To polymerize dextran or keto-dextran using DSase, the enzyme (0.1 U mL^{-1}) was added to 20 mL sucrose or keto-sucrose (100 mg mL^{-1}) solution buffered with acetate buffer distilled water solution at 303 K. The solution of acetate buffer and distilled water was prepared by mixing acetic acid and sodium acetate solution (10 mmol L^{-1}, pH 5.5).

To calculate the Michaelis constant and maximum velocity, the reaction rate was determined by changing the substrate concentration. DSase (0.1 U mL^{-1}) was added to 1 mL sucrose or keto-sucrose solution (3–150 mmol L^{-1}, 10 mmol L^{-1} acetate buffer distilled water solution, pH 5.5). The reaction rate was calculated from the concentration of fructose in the solution. The low concentration of fructose ranging from 0.01 to 0.5 g L^{-1} was measured by the Somogyi–Nelson method.[25,26] The values of the Michaelis constant and maximum velocity were estimated using the Lineweaver–Burk reciprocal plot[27]

$$\frac{1}{r} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}} \quad (1)$$

where K_m and V_{max} are the Michaelis constant (mol L^{-1}) and maximum velocity (mol L^{-1} min^{-1}), respectively. [S] and r are the substrate concentration (mol L^{-1}) and reaction rate (mol L^{-1} min^{-1}), respectively.

2.4. Confirmation of carbonyl groups in keto-sucrose and keto-dextran

The existence of carbonyl groups in keto-sucrose was verified by ^{13}C NMR. The position of carbonyl groups introduced to the hydroxyl group of keto-sucrose was estimated by ^{1}H NMR. ^{1}H NMR of keto-dextran was then determined to estimate the degree of branching. ^{13}C NMR and ^{1}H NMR spectra were determined using an NMR spectrometer (Jeol, JNM-AL300) at 300 MHz and 75 MHz, respectively. The measurements of sucrose and keto-sucrose were carried out in deuterated water (D_2O), and measurements of dextran and keto-dextran were carried out in 9:1 D_2O-deuterated dimethyl sulfoxide (DMSO-d_6) at room temperature (293 K).

The amount of carbonyl group in keto-sucrose and keto-dextran was determined by a colorimetric assay using dinitrophenylhydrazine.[28] The conversion percentage into keto-sucrose was calculated as

$$\text{Conversion } (\%) = \frac{C_C}{C_S} \times 100 \quad (2)$$

where C_C and C_S are the concentrations of carbonyl groups (mol L^{-1}) and sucrose (mol L^{-1}), respectively.

2.5. High performance liquid chromatography (HPLC) determination

To confirm the production of dextran and keto-dextran, the concentration of fructose produced by DSase and the molecular weight of the product were determined. The molecular weights of dextran and keto-dextran were determined by size exclusion chromatography (SEC) using a column (Tosoh Corp., TSKgel G4000PW_{XL}) connected to a pump (Waters Corp., 515 HPLC Pump), refractive index detector (Waters Corp., 2414 refractive index detector), and integrator (Shimadzu Corp., C-R8A Chromatopac). The mobile phase was distilled water permeating at a flow rate of 1.0 mL min^{-1} at a column temperature of 313 K. The concentration of fructose produced by DSase was quantified by HPLC using a column (Tosoh Corp., Amide-80). The mobile phase was a mixture of acetone and distilled water with a ratio of 8:2 and a flow rate of 1.0 mL min^{-1} at 303 K. The amount of dextran and keto-dextran produced was calculated from the concentration of produced fructose,

$$\text{Concentration of product during DSase reaction}(\text{mg mL}^{-1}) = C_F \times \frac{M_M}{M_F} \quad (3)$$

where C_F, M_F, and M_M are the fructose concentration (mg mL^{-1}), molecular mass of fructose (g mol^{-1}), and molecular mass of the monomer unit (g mol^{-1}), respectively.

3. Results and discussion

3.1. Bioconversion of sucrose into keto-sucrose

The use of POase leads to the regioselective oxidation of sucrose at the C-2 hydroxyl group. To achieve the appropriate introduction site at C-2, sucrose was converted into keto-sucrose using the specificity of POase. The conversion of sucrose into keto-sucrose during the POase reaction is shown in Figure 2. The conversion was gradually increased with increasing reaction time. After 24 h, the conversion reached 100%.

The presence of the carbonyl group and its position in keto-sucrose were determined by ^{13}C NMR and ^{1}H NMR. In the ^{13}C NMR spectrum (Fig. 3), a peak for carbon in carbonyl groups of keto-sucrose appeared at 210 ppm, confirming the existence of carbonyl groups in keto-sucrose. In the ^{1}H NMR spectrum for both sucrose and keto-sucrose (Fig. 4), a peak corresponding to anomeric protons was observed in the 5.3–5.5 ppm range. The peak for sucrose was a doublet, while that for keto-sucrose was a singlet. The singlet peak for keto-sucrose indicates that its neighboring carbon does not have a proton, demonstrating that the carbonyl group was introduced at C-2 of sucrose.

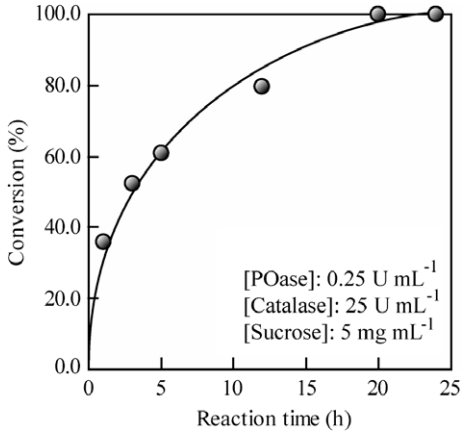


Figure 2. Conversion percentage of sucrose into keto-sucrose during POase reaction.

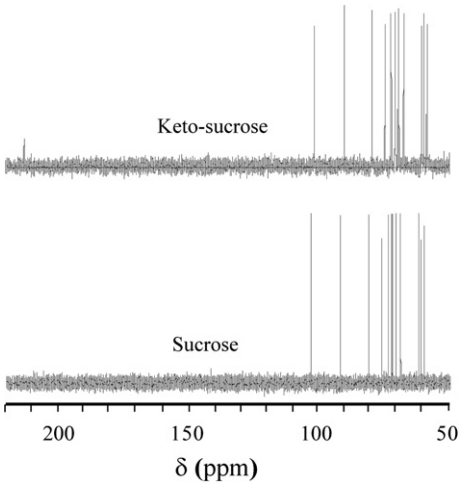


Figure 3. ^{13}C NMR spectra of sucrose and keto-sucrose.

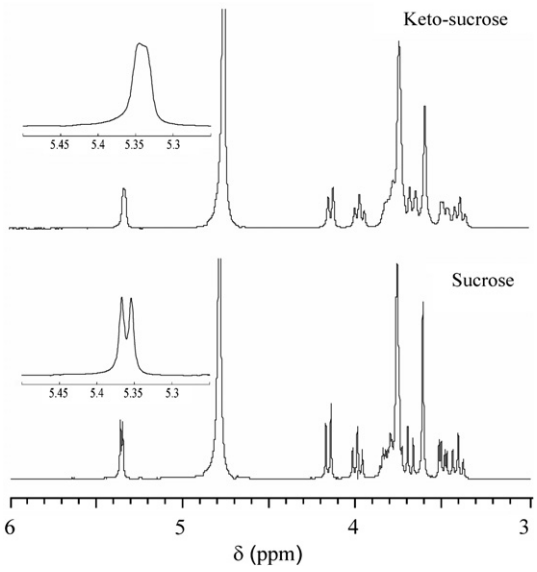


Figure 4. ^{1}H NMR spectra of sucrose and keto-sucrose.

3.2. Polymerization of keto-sucrose into keto-dextran

Sucrose and keto-sucrose were polymerized into dextran and keto-dextran, respectively, by DSase reaction. The amount of dextran and keto-dextran produced is shown in Figure 5. When keto-dextran was produced from keto-sucrose using DSase, the reactivity of keto-sucrose decreased to 80% of the reactivity of sucrose owing to the substrate recognition of DSase. The introduction percentage of the carbonyl group was 100% for enzymatic polymerization, that is, the carbonyl group was introduced to all glucose units of keto-dextran.

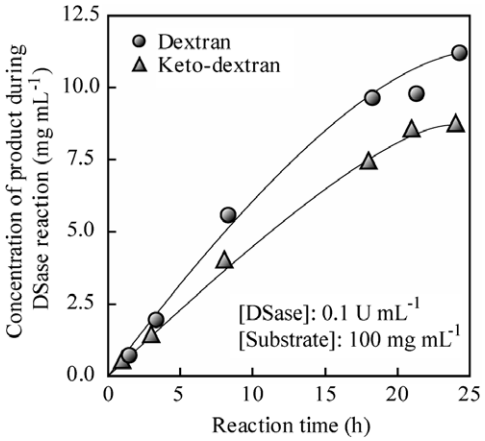


Figure 5. Concentration of dextran and keto-dextran produced by DSase reaction.

In contrast, Larm et al. reported the oxidation of dextran using bromine.[29] Dextran oxidized by bromine was present at 21.5% of the carbonyl groups at the C-2 position, at 4.0% of the carbonyl groups at the C-3 position, and at 25.0% of the carbonyl groups at the C-4 position. Moreover, 12% of glucose rings were cleaved by acid as a side reaction. Using the protection of C-2 and C-4 positions with phenylboronic acid, the percentage of regioselective introduction of the carbonyl group by oxidation with dimethyl sulfoxide acetic-anhydride was 19.5% (C-2 position: 8.0%, C-3 position: 11.5%). Therefore, the enzymatic method is more effective than the chemical method in preparing a dextran derivative with high selectivity and high-density introduction of functional groups.

To confirm the growth of dextran and keto-dextran chains, the resulting polymer was characterized by SEC (Fig. 6). The retention time of SEC for the obtained dextran and keto-dextran was 5.5 min. The retention time of commercially available dextran with a molecular mass of 2000 kDa was less than that of the obtained dextrans, indicating that the obtained dextrans had an apparent molecular mass of more than 2000 kDa.

The branched structure of dextran and keto-dextran was determined by ^{1}H NMR, as shown in Figure 7. For keto-dextran the anomeric proton from 1,6-glycoside bonding was observed, indicating that keto-dextran had a similar structure to that of dextran. The peak at 5.4 ppm was the C-1 proton of the dextran terminal. For keto-dextran, the peaks at 5.2–5.3 ppm assigned as α-(1,3) and α-(1,4) glycoside bonding were observed because the anomeric carbon of the C-1 position of sucrose attacked the C-3 and C-4 positions of keto-dextran instead of the C-6 position due to the misrecognition by DSase. The branch percentage was calculated to be 5.6% from the integral ratio of α-(1,6) glycoside bonding to α-(1,3) and α-(1,4) glycoside bonding. Doman et al. synthesized the branched structure of dextran synthesis by changing the sucrose concentration, pH, and temperature.[30] To regulate the branched structure of keto-dextran it is also possible to change the reaction condition of DSase from keto-sucrose. The assignment of dextran and keto-dextran in ^{1}H NMR is shown in Table 1.

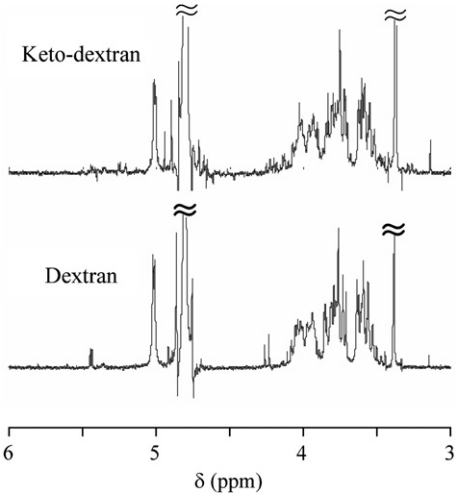


Figure 7. ^{1}H NMR spectra of dextran and keto-dextran.

Table 1
Assignments of ^{1}H chemical shifts for dextran and keto-dextran produced by DSase reaction

Dextran		Keto-dextran	
Chemical shift (ppm)	Assignment	Chemical shift (ppm)	Assignment
3.38	DMSO[a]	3.38	DMSO
3.52–4.05	H_2–H_6	3.52–4.07	H_3–H_6
4.80	Water	4.80	Water
5.01	H_1 of 1,6 bonding	5.01	H_1 of 1,6 bonding
5.44	Terminal H_1	5.21	H_1 of 1,3 bonding
		5.25	H_1 of 1,4 bonding
		5.44	Terminal H_1

[a] H_n denotes the ring proton of the glucose unit.

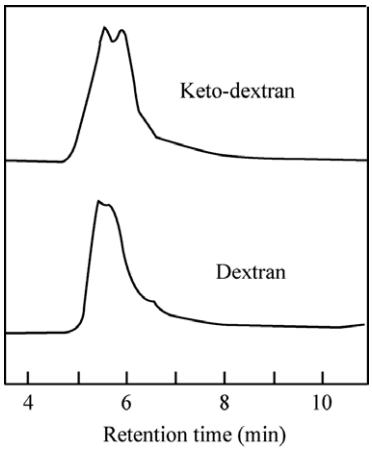


Figure 6. Gel permeation chromatogram of dextran and keto-dextran produced by DSase reaction for 24 h.

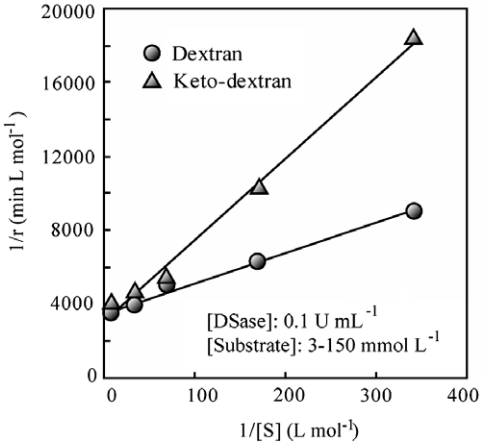
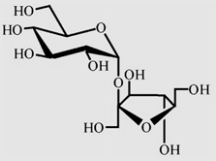
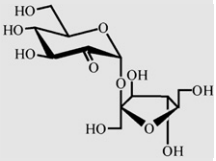


Figure 8. Lineweaver–Burk plots of DSase reaction using sucrose and keto-sucrose.

Table 2
The value of K_m and V_{max} using sucrose and keto-sucrose

Substrate	 Sucrose	 Keto-sucrose
K_m (mmol L^{-1})	4.6	14.0
V_{max} (mmol L min^{-1})	0.28	0.32

3.3. Kinetic analysis of dextran and keto-dextran polymerization by DSase

To investigate the difference in substrate recognition for sucrose and keto-sucrose, dextran and keto-dextran were produced from substrates at varying concentrations. A Lineweaver–Burk reciprocal plot is shown in Figure 8. $1/r$ linearly increased with increasing 1/[S]. The Michaelis constant and maximum velocity were estimated from the slope and intercept of the Lineweaver–Burk reciprocal plot. The Michaelis constant and maximum velocity of DSase for sucrose and keto-sucrose are summarized in Table 2. The value of the Michaelis constant for keto-sucrose was higher than that for sucrose, because it is more difficult for DSase to recognize keto-sucrose at the active site. The values of maximum velocity for keto-sucrose and sucrose were approximately equal. Therefore, although keto-sucrose is inhibited in taking in DSase, the turn-over activity of DSase in the production of keto-dextran is maintained.

4. Conclusion

Keto-dextran, dextran with carbonyl groups, was prepared by the enzymatic polymerization of keto-sucrose, which in turn was produced by the enzymatic oxidation of sucrose. Initially, sucrose was converted to keto-sucrose with a carbonyl group at the C-2 position due to the specificity of POase. The converted keto-sucrose was then polymerized to keto-dextran by the DSase reaction. Keto-dextran prepared using various enzymes had a higher introduction percentage of the carbonyl group than the chemical method. Therefore, the enzymatic method suggested in this study has a higher potential for synthesis of modified dextran than does the chemical method. The technique using various enzymatic reactions will enable us to functionalize other carbohydrate polymers.

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