Conformation and Cooperative Order-Disorder Transition in Aqueous Solutions of β-1,3-D-Glucan with Different Degree of Branching Varied by the Smith Degradation.

Kazuto Yoshiba[a], Toshihiko Saheki[a], Bjørn E. Christensen[b], and Toshiaki Dobashi[a]

a. Division of Molecular Science, Graduate School of Science and Technology, Gunma University, 1-5-1 Tenjin-cho, Kiryu, Gunma, 376-8515, Japan
b. NOBIPOL, Department of Biotechnology and Food Science, Norwegian University of Science and Technology (NTNU), Trondheim NO-7491, Norway

Corresponding Author
* Kazuto Yoshiba
Division of Molecular Science, Graduate School of Science and Technology, Gunma University, 1-5-1 Tenjin-cho, Kiryu, Gunma, 376-8515, Japan
Tel: +81 277 30 1486 Fax: +81 277 30 1409
e-mail: yoshiba@gunma-u.ac.jp

ABSTRACT

β-1,3-D-glucan with different degrees of branching were obtained by selectively and gradually removing side chains from schizophyllan, a water-soluble triple helical polysaccharide, using the Smith degradation. Size exclusion chromatography combined with a multi-angle light scattering detection was performed in aqueous 0.1 M NaCl. The degree of branching decreased after the Smith degradation, while the molar mass distributions were almost unchanged. The molecular conformation of the Smith-degraded β-1,3-D-glucan was analyzed on the basis of the molar mass dependency of the radius gyration, and found to be comparable to the original triple helix of schizophyllan. Differential scanning calorimetry in deuterium oxide – hexadeuterodimethylsulfoxide mixtures was performed to investigate the effects of the degree of branching on the cooperative order-disorder transition. Removal of side chains affects both the transition temperature and transition enthalpy. The ordered structure is formed by the residual side chains in the triplex unit, so that the linear cooperative system of the triplex is maintained after the Smith degradation.

Keywords: β-1,3-D-glucan, schizophyllan, Smith degradation, triple helix, order-disorder transition

1. Introduction

β-1,3-D-glucan is a family of polysaccharides consisting of the β-1,3-glycoside linkage in the main backbone. β-1,3-D-glucans are classified into sub-groups by the chemical architectures. The simplest linear β-1,3-D-glucan is curdlan, which has been widely used for an industrial gum as a gelator, a viscosity enhancer in food industry. Branched β-1,3-D-glucans, having a side chain of β-1,6-D-glucose linked with the main chain of β-1,3-D-glucose, exist in nature. There are some analogues with different degrees of branching, which are produced from fungi and yeast, in their cell walls and mycelia and so on. The native structure of these β-1,3-D-glucans is the triple helix, which is found not only in dried state but also in solution. The triple helical β-1,3-D-glucans have characteristic properties which have not been observed in other polysaccharides. For instance, these β-1,3-D-glucans stimulate the innate immune systems in human body recognized by the cell wall receptors on leucocytes, monocytes and so on, where the receptor proteins can selectively recognize the triple helical conformation of β-1,3-D-glucans and modulate the innate immunity.[1,2] Furthermore, the β-1,3-D-glucan chain can forms a helical complex with polynucleotide with the immunological activity.[3,4]

Schizophyllan is an extracellular water-soluble β-1,3-D-glucan produced by *Schizophyllum commune*, which has a repeating unit of three consecutive β-1,3-D-glucose residues, and one β-1,6-linked D-glucose as side chain.[5,6] Three chains are linked together through the intermolecular hydrogen bonds to form a triple helix, whereas the side chain of is located outwards from the triple helix core.[7,8] The triple helix of schizophyllan is quite stable in water, which is maintained even over 100°C without denaturation. The triple helical conformation of this polysaccharide forms a very rigid structure,[9,10] and leads to remarkable physical and biological properties in water, *i.e.* liquid crystal formation[11-13] and anti-tumor activity[14,15], respectively. A cooperative order-disorder transition (CODT) is also one of the characteristic properties of the schizophyllan triple helix.[16-24] This transition occurs between the side chains and the solvent molecules to form an ordered hydration structure below 7°C in H_2O, without dissociation of the triple helix. CODT of aqueous schizophyllan solution has mainly two characteristic properties. Firstly, the transition temperature strongly depends on the sample molar mass, reflecting a highly cooperative phenomenon, such as the helix-coil transition of synthetic polypeptides.[17,18,21] Secondly, the transition temperature and the transition enthalpy are affected by the solvent, for instance, adding small amounts of aprotic solvents in water.[25,26] In previous studies, experimental results have been obtained from optical rotation, heat capacity, and dielectric relaxation of the schizophyllan solutions, which can be analyzed quantitatively with the linear cooperative transition (LCT) theory.[27] Furthermore, other native β-1,3-D-glucans with different degrees of branching also show

CODT behavior in the solution.[28,29] Kitamura *et al.*[28] studied the effects of the side chain on CODT with seven analogues of β-1,3-D-glucans with different degrees of branching using differential scanning calorimetry, and showed that the degree of branching of the side chain affects the transition temperature (T_r) and the transition enthalpy (ΔH_r). However, no theoretical analysis was available for their data, so that the effects of the degree of branching on CODT have not been clarified yet.

The Smith degradation is an important method in carbohydrate chemistry normally used to determine the chemical structure of polysaccharides.[30] The degradation includes three successive steps: 1) periodate oxidation, 2) reduction with sodium borohydride, and 3) mild acid hydrolysis (selective for the oxidized/reduced residues). In the case of schizophyllan, the β-1,6-D-glucose of the side chains can be selectively oxidized by periodate into the corresponding dialdehyde side chain, the β-1,3-linked main chain being resistant. The dialdehydes are reduced with sodium borohydride into hydroxyl groups. Finally, the modified side chain can be selectively removed by acid hydrolysis with dilute H_2SO_4 (Figure 1). The degree of branching can thus be varied by controlling the degree of periodate oxidation. Such a modified β-1,3-D-glucan is useful to investigate the effects of the degree of branching on their physical and biological properties compared with native branched β-1,3-D-glucan. Koumoto *et al.*[31] studied the thermal stabilization of β-1,3-D-glucan/polynucleotide complex and the cytoxicity of the Smith degraded schizophyllan. Magree *et al.*[32] investigated the aggregation behavior in an aqueous solution of highly modified scleroglucan, chemical analogue of schizophyllan, by size exclusion chromatography equipped with a multi-angle light scattering detection (SEC-MALS) measurements, and activity in an oxidative burst assay. In present study, we investigate the effects of the degree of branching of β-1,3-D-glucan produced by the Smith degradation on the triple helical conformation and the cooperative order-disorder transition in aqueous solution using SEC-MALS and differential scanning calorimetry (DSC). The degree of branching was determined from high performance liquid chromatography after the enzymatic degradation with *exo*-β-1,3-glucanase.[6,33] The molar mass dependence of the radius gyration obtained from SEC-MALS was analyzed in terms of the wormlike chain model. Binary mixtures of deuterium oxide (D_2O) and hexadeuterodimethylsulfoxide (DMSO-d_6) were used as the solvents to shift CODT to higher T_r. T_r and ΔH_r were analyzed quantitatively with the LCT theory in terms of the dissociation-association equilibrium between the side chain and DMSO-d_6 in the mixed solvent system to clarify the stabilization effects of adding DMSO-d_6 on the ordered structure. β-1,3-glucans have been investigated in detail for the antitumor activity. It has been known that the human immune cell has the membrane protein receptor for β-1,3-

glucan, such as Dectin-1, which stimulates the immunity by the recognition with the triple helix of β-1,3-glucan.[1] However, the molecular mechanism between the triple helix and receptor remains obscure. This work may provide the unique way for the quantitative analysis of the molecular interaction between the triple helix and the solvent molecules, concerned with the molecular recognition of β-1,3-glucans.

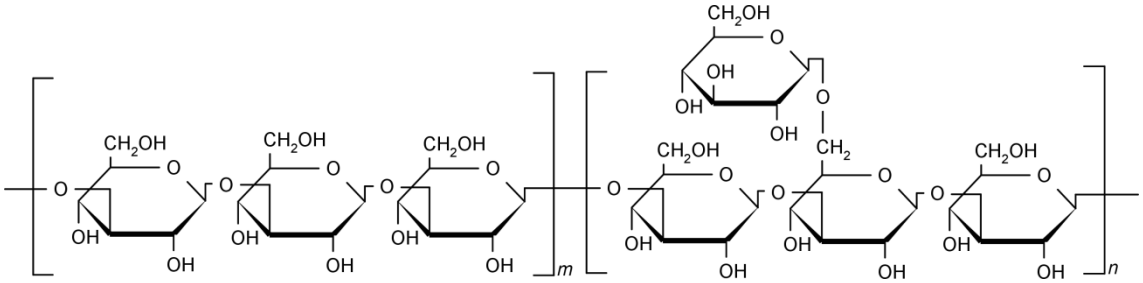

Figure 1 The chemical structure of the repeating unit of Smith degraded β-1,3-D-glucan prepared from schizophyllan. The removal of side chains is assumed to be random, resulting in random distribution of residual side chains.

2. Materials and Methods

2-1. β-1,3-D-Glucan Samples with Different Degree of Branching

A stock sample of schizophyllan (Taito Co., now Mitsui Sugar Co., Tokyo, Japan) was dissolved in deionized water (Organo Co., Tokyo, Japan), and 100 ml of 1.0% (w/v) aqueous solution of schizophyllan was sonicated by Branson Sonifier 250 (Branson Ultrasonic Co., Danbury, USA) for 12 hours in ice bath. The sonicated sample was purified by the fractional precipitation from the aqueous solution with ethanol as the precipitant.[9] The sample was fractionated into three fractions. The fractionation was carried out twice for the middle fraction before lyophilization. Two partially Smith-degraded samples were subsequently prepared. The reaction scheme is shown elsewhere.[31,32] Predetermined amounts of 30 mM $NaIO_4$ (0.2 and 0.5 moles per repeating unit) was dripped into 100ml of 0.1% (w/v) aqueous schizophyllan to obtain the corresponding dialdehydes. The oxidation was performed in dark at 4°C for 48 hours. The reaction mixture was then dialyzed repeatedly against deionized water. 0.1 M NaOH was added into the dialyzed solution to be adjusted to pH 10. $NaBH_4$ (100mg) was added and the mixture was stirred for 24 hours at room temperature to reduce the oxidized samples to the corresponding alcohols. The solution was neutralized with acetic acid, and then the solution was dialyzed against deionized water repeatedly. Oxidized/reduced side chains were selectively removed by mild acid hydrolysis in 0.1 M H_2SO_4 (48 hours, T = 25°C). The reaction solutions were subsequently neutralized with $NaHCO_3$, and dialyzed

repeatedly against deionized water and lyophilized.

All chemicals except for SPG were purchased from Fujifilm Wako Pure Chemical Co.. Since the degree of branching was controlled by the amounts of $NaIO_4$ at the initial oxidation, the Smith degraded sample was designated as SD-*X*, where *X* indicates the molar ratio of $NaIO_4$ in the repeating unit of schizophyllan.

2-2. Determination of the degree of branching with exo-β-1,3-glucanase

Schizophyllan and Smith-degraded schizophyllan were degraded by the enzymatic hydrolysis with *exo*-β-1,3-glucanase from *Trichoderma virens* (Megazyme, Bray, Ireland). 10 units of the enzyme were mixed with 3 ml of 0.2% (w/v) polysaccharide solution in 20 mM sodium acetate (pH 4.5), and the mixture was kept at 37 °C for 24 hours. After the enzymatic hydrolysis, the solution was filtered (Vivaspin 20, Sartorius Co., Goettingen, Germany, MWCO = 10,000 g mol^{-1}) to remove the enzyme. The filtrate was diluted with acetonitrile to adjust the solvent composition for the following HPLC analysis. The degraded products in the filtrate were detected by a high-performance liquid chromatography system LC-2000plus (JASCO Co., Tokyo, Japan) at the flow rate of 0.8 ml min^{-1} at 35°C. Acetonitrile-water mixture (65/35, v/v) was used as the eluent. Polyamine II column (YMC Co. Ltd., Kyoto, Japan) was employed to detect the degraded products by a RI-2031plus differential refractive index (RI) detector (JASCO, Tokyo, Japan). Commercially available saccharides, glucose and gentiobiose (Tokyo Chemical Industry, Co., Ltd, Tokyo, Japan) were used for constructing the calibration curve. The data were analyzed by ChromNAV software (JASCO, Tokyo, Japan).

2-3. Size Exclusion Chromatography with Multi-Angle Light Scattering Detection (SEC-MALS)

The size exclusion chromatography with a multi-angle light scattering detection (SEC-MALS) was performed to determine the molar mass and the radius gyration. SPG and SD-*X* were dissolved in 0.1 M NaCl containing 0.01 M NaOH to prepare the injecting solution. Three SEC columns, Shodex OHPak SB-G, two Shodex OHPak SB806MHQ (Showa Denko K.K., Kanagawa, Japan) were serially connected in a GPC-101 SEC system (Showa Denko K.K., Kanagawa, Japan). A Dawn Heleos II (Wyatt Technology Co., Santa Barbara, USA) was employed for the MALS detector, connected below the SEC system. Aqueous 0.1 M NaCl was used as an eluent. The flow rate was fixed at 1.0 ml min^{-1} and the columns were kept at 40 °C. The solution was injected (injection volume 100 μl) after filtration with DISMIC-25AS (pore size 0.80 μm, Toyo Roshi Co., Ltd., Tokyo, Japan). The measurement was operated by ASTRA® ver. 5.3 software (Wyatt Technology Co., Santa Barbara, USA) to obtain the elution curves from the differential refractive index (dRI) detector and MALS detector. The output data from dRI detector

were converted to the mass concentration (c) with the refractive index increment, $(\partial n/\partial c)_{\mu}$=0.141 ml g^{-1}.

2-4. Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry (DSC) was performed on a Pyris-1 differential scanning calorimeter (Perkin Elmer Co., Massachusetts, USA). The dried sample and the solvent were mixed in a large volume stainless steel capsule (Perkin Elmer Co., Massachusetts, USA), and the DSC pan was sealed. D_2O and DMSO-d_6 (99.9 atom% D, Sigma-Aldrich Co. LLC, St. Louis, USA) were used as the solvents without further purification. The measurements were performed at the scanning rate of 2.0 K min^{-1} at N_2 gas flow of 20 ml min^{-1}. The solvent was used as a reference for the measurement. The temperature scale of the calorimeter was calibrated with the melting temperature of cyclohexane and indium, and the heat flow was corrected with the enthalpy of fusion of indium. A Pyris® software (Perkin Elmer Co., Massachusetts, USA) was used to operate the measurement. The measurements were performed repeatedly to confirm the heat flow curves to reproduce each measurement within the experimental error.

3. Results

In the first step of the Smith degradation (periodate oxidation), the glucose side chains may consume up to two molecules of periodate because of two periodate-sensitive vicinal diols between C2 and C3, and C3 and C4, with the release of C3 as formic acid.[34] For this reason, the molar ratio of added periodate does not agree with the degree of residual branching (DB). The latter should therefore be assessed by an independent method. We therefore applied enzymatic hydrolysis with an *exo*-β-1,3-glucanase, which produces two moles of glucose and one mole of gentiobiose for each repeating unit.[6] Because glucose and gentiobiose represent the corresponding linear and branched units in β-1,3-D-glucan, the molar ratio of these degraded products enable us to determine DB of the Smith degraded schizophyllan. Figure 2 shows the elution curve of SPG (black), SD-0.2 (blue) and SD-0.5 (red) in acetonitrile-water mixture at 35°C after the enzymatic hydrolysis with *exo*-β-1,3-glucanase from *Trichoderma virens*. Two sharp peaks for SPG, SD-0.2 and SD-0.5 were found around 10.1 min and 15.5 min, which correspond to the degraded products. Indeed, these retention times well agreed with those for glucose and gentiobiose. For glucose, the peak height for SD-0.2 and SD-0.5 is larger than that for SPG, while the peaks of gentiobiose have almost the same peak height as SPG, reflecting the difference of DB. The molar ratio of glucose and gentiobiose of the samples was determined with the calibration curves for glucose and gentiobiose from the peak area of the elution curve. DB was calculated according to the chemical structure in Figure 1. The values of DB are

listed in Table 1. The values of DB are consistent with the correlation curve constructed by Koumoto *et al.*[31] with *exo*-β-1,3-glucanase from *Basidiomycete* QM-806.

Table 1. The number average molar mass (M_n), the weight average molar mass (M_w), and the degree of branching (DB) of schizophyllan (SPG) and Smith degraded schizophyllan (SD-*X*) samples.

Sample	M_n / g mol^{-1}	M_w / g mol^{-1}	DB
SPG	228,000	259,000	0.333
SD-0.2	242,000	284,000	0.301
SD-0.5	216,000	244,000	0.257

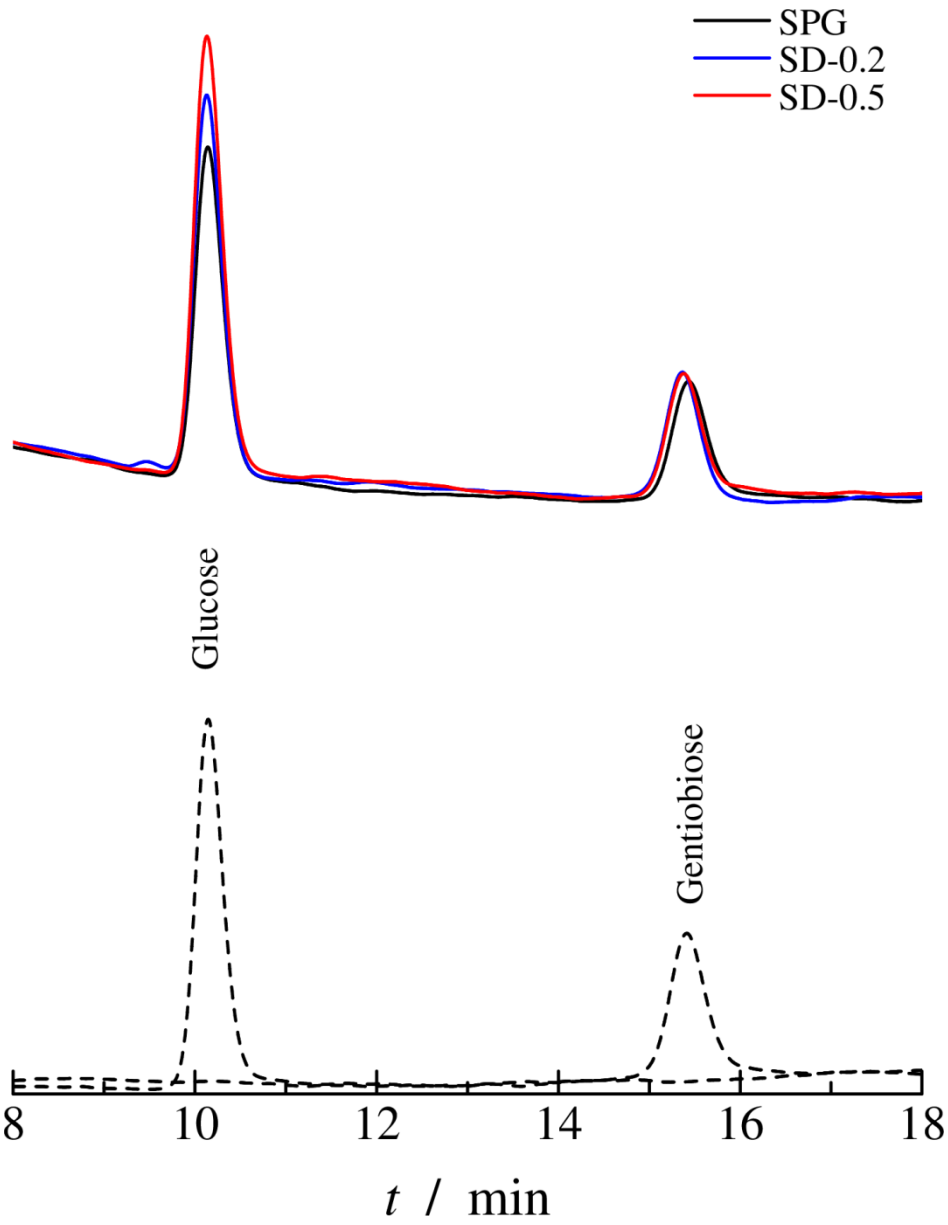

Figure 2 HPLC chromatograms of schizophyllan (SPG) and Smith-degraded samples (SD-0.2 and SD-0.5) in acetonitrile-water (65:35, v/v) after the enzymatic hydrolysis with *exo*-β-1,3-glucanase. The elution curves for glucose and gentiobiose are plotted by dashed curves as references for the retention time.

Figure 3 shows the SEC chromatograms consisting of the mass concentration (c) and the molar mass (M) data plotted against the elution volume (V_e) for schizophyllan and Smith-degraded samples in aqueous 0.1 M NaCl at 40 °C. The scattering intensity at 90° (LS(90°)) from MALS detector and output data from dRI detector were given in Supplementary information (Figure S1). The c-V_e curves (bottom side) and M-V_e curves

(upper side) for SD-0.2 and SD-0.5 are overlapped with those for parent SPG. As seen in Table 1 calculated from these SEC-MALS data, the number average molar mass (M_n) and the weight average molar mass (M_w) for SD-0.2 and SD-0.5 were almost the same as SPG. Magree *et al.*[32] studied the aggregation behavior of scleroglucan with a different degree of branching synthesized by the Smith degradation. Since they used highly modified samples (DB=0.17 and 0.12), the Smith degraded scleroglucan appears to aggregate in the aqueous solution. Indeed, the solubility of β-1,3-D-glucans to water is largely affected to degree of branching. However, our samples maintain good solubility to aqueous solutions because of relatively high degrees of branching (Table 1). Therefore, the SEC-MALS results indicate that these samples maintain the triple helical conformation and the molar mass distribution is unchanged after the Smith degradation.

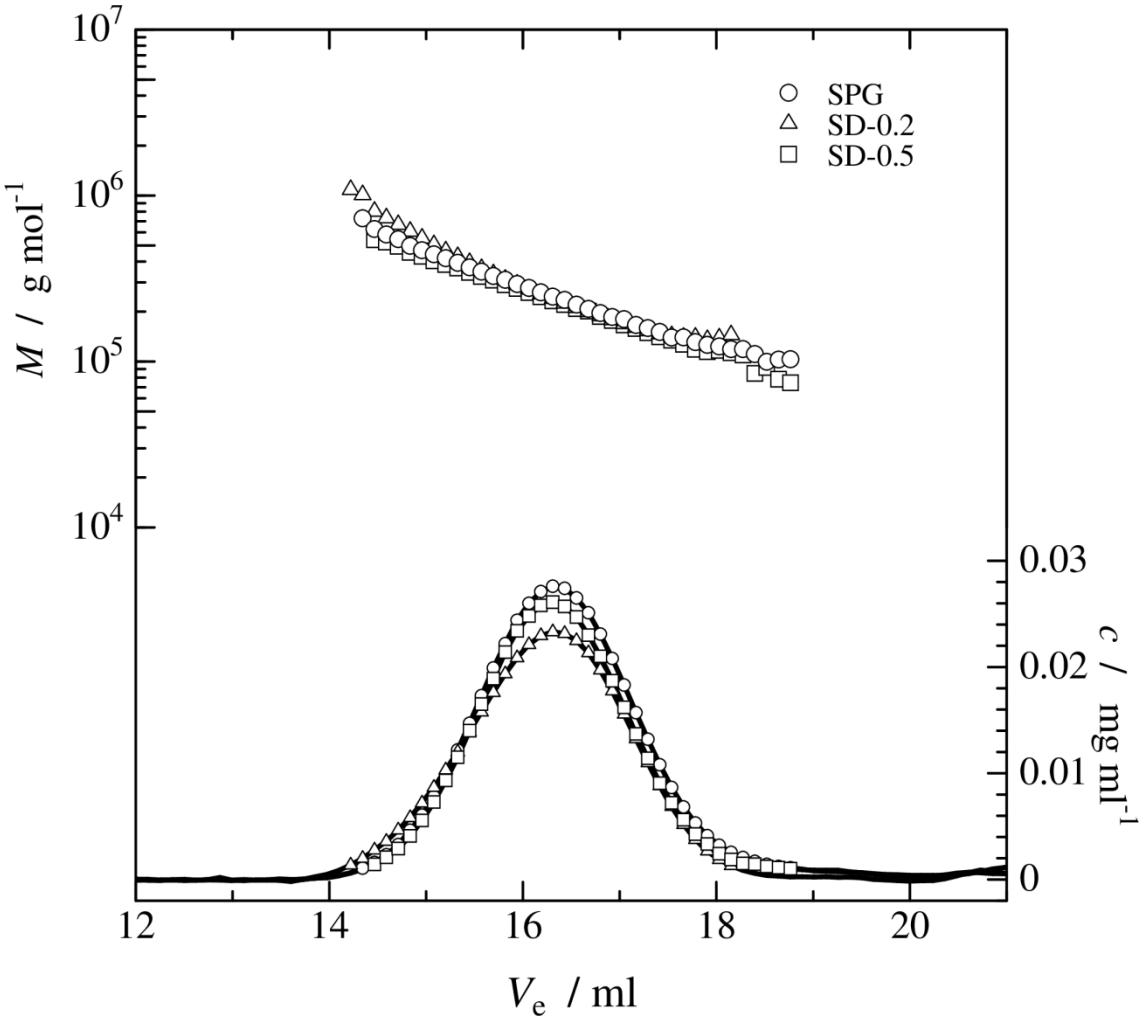

Figure 3 SEC-MALS chromatograms for SPG, SD-0.2, and SD-0.5 in 0.1 M NaCl at 40°C; bottom side, the concentration profile (c-V_e curves); upper side, the molar mass

profile (M-V_e curves),.

DSC curves for SPG, SD-0.2 and SD-0.5 in the CODT region are shown in Figure 4. Here, x_D represents the mole fraction of the mixed solvent. A well-defined single peak was detected in all the DSC curves. The peak temperature was taken as T_r, and ΔH_r was calculated from the peak area of the DSC curve, where the baseline was determined from the flat regions at low and high temperature sides. With increasing x_D, T_r was shifted to higher T, and ΔH_r became larger than those in D_2O, as reported by Hirao *et al.*[26] Table 2 summarizes T_r and ΔH_r for SPG, SD-0.2 and SD-0.5 obtained from DSC measurements. With increasing DB, T_r was shifted to lower T, and ΔH_r was decreased. These changes of the transition behavior well resemble those induced by the effects of carboxylation in our previous study on the carboxylated derivative ('sclerox').[35,36] However, there are several differences in the DSC curves between these derivatives. Firstly, T_r of sclerox in pure D_2O is depend strongly on the degree of carboxylation. Secondly, peak broadening of the DSC curve (or C_p curve) occurs after carboxylation. However, these effects observed for carboxylated samples were too little extent observed for the present samples prepared by the Smith degradation at the corresponding degree of modification.

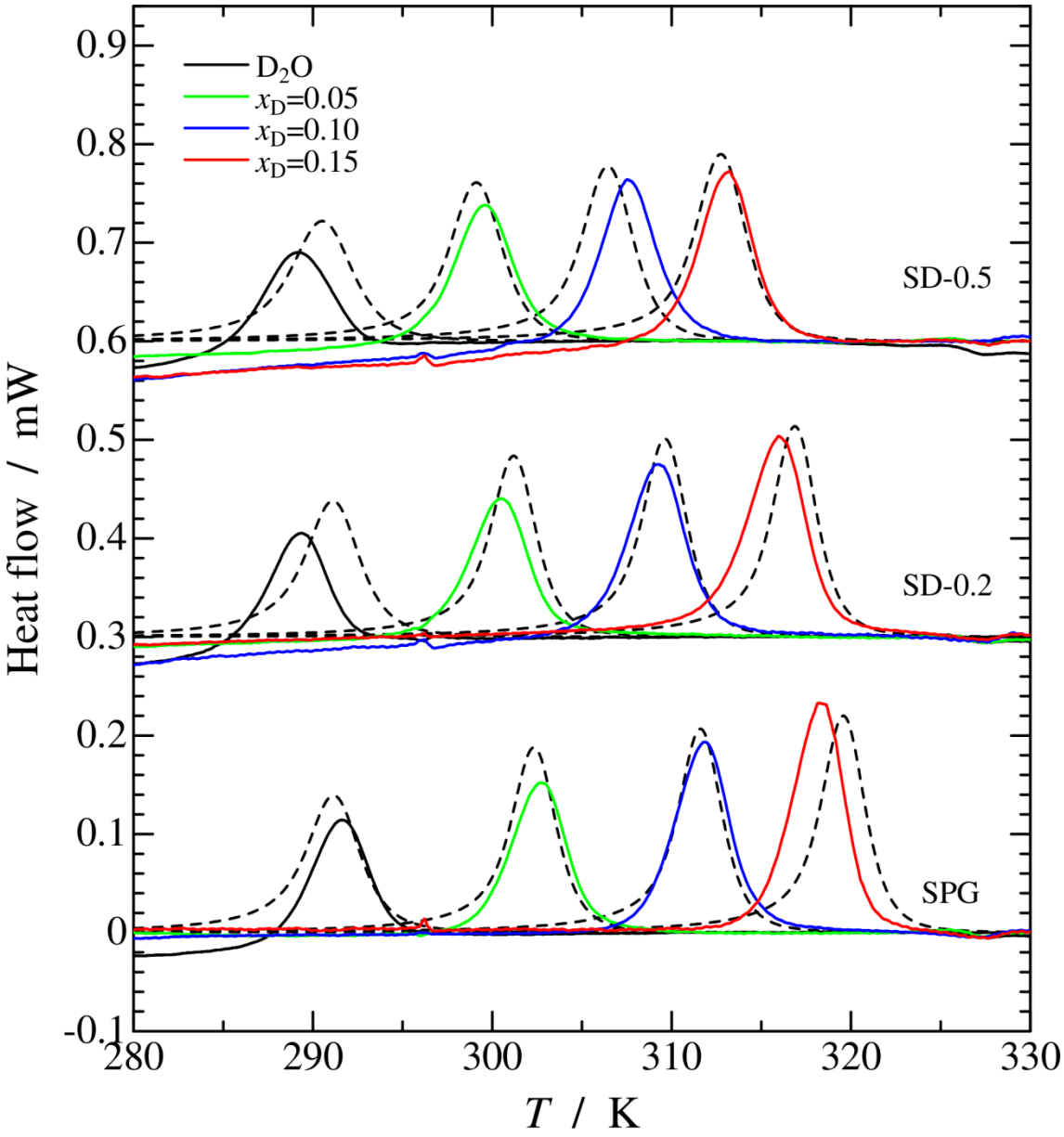

Figure 4 DSC curves for SPG, SD-0.2 and SD-0.5 in the mixture of D_2O-DMSO-d_6. (10 *wt*%) The DSC curves are colored according to the mole fraction of DMSO-d_6 (x_D). Dashed curves are the reduced ΔC_p curves calculated by the LCT theory. DSC curves are shifted by the samples for clarity.

Table 2 Thermodynamic quantities from DSC measurements for the Smith-degraded β-1,3-D-glucans from schizophyllan

	SPG		SD-0.2		SD-0.5	
x_D	T_r / K	ΔH_r / Jg^{-1}	T_r / K	ΔH_r / Jg^{-1}	T_r / K	ΔH_r / Jg^{-1}

0	291.3	5.92	289.0	5.48	288.8	5.33
0.050	302.4	7.02	300.3	6.37	299.3	6.03
0.100	311.5	8.16	309.0	7.67	307.4	6.78
0.150	318.1	9.06	315.9	8.21	312.9	6.99

4. Discussion

4-1. Chain Stiffness of Triple Helix of Smith-Degraded β-1,3-D-Glucans

In Figure 5, the radius gyrations for SPG, SD-0.2 and SD-0.5 were plotted against the degree of polymerization (N_0) from SEC-MALS to compare the chain stiffness in aqueous 0.1 M NaCl. Here, N_0 was calculated from the molar mass M divided by the average molar mass ($M_{0,G}$) per main-chain glucose residue. Such $<S^2>^{1/2}$-N_0 data can be modelled according to the wormlike chain (WC) theory, with the persistence length (q) and the helical pitch (h) as main parameters.[37] For SPG, the molar mass dependency of the radius gyration can be represented with q = 100 nm and h = 0.28 nm, as indicated by the solid curve. Further, data for SPG, SD-0.2 and SD-0.5 overlapped, indicating that SD-0.2 and SD-0.5 have the same rigid conformation. These data mean that SD-0.2 and SD-0.5 maintain the molecular conformation of the triple strand. Kitamura *et al.*[38] showed the rod-like conformation of branched β-1,3-D-Glucans (DB=0.25) extracted from *Cryptoporus volvatus* from TEM results. They estimated the wormlike chain parameters as q = 140 nm and h = 0.30 nm. DB for SD-0.5 in present study is almost the same as that for their β-1,3-D-Glucans. Nearly the same value of q and h confirms the validity of our analysis for Smith-degraded β-1,3-D-glucans. Data points for SD-0.2 in high N_0 region slightly deviate downward from the solid curve. This may be due to a little amount of aggregation at high N_0 in the solution.

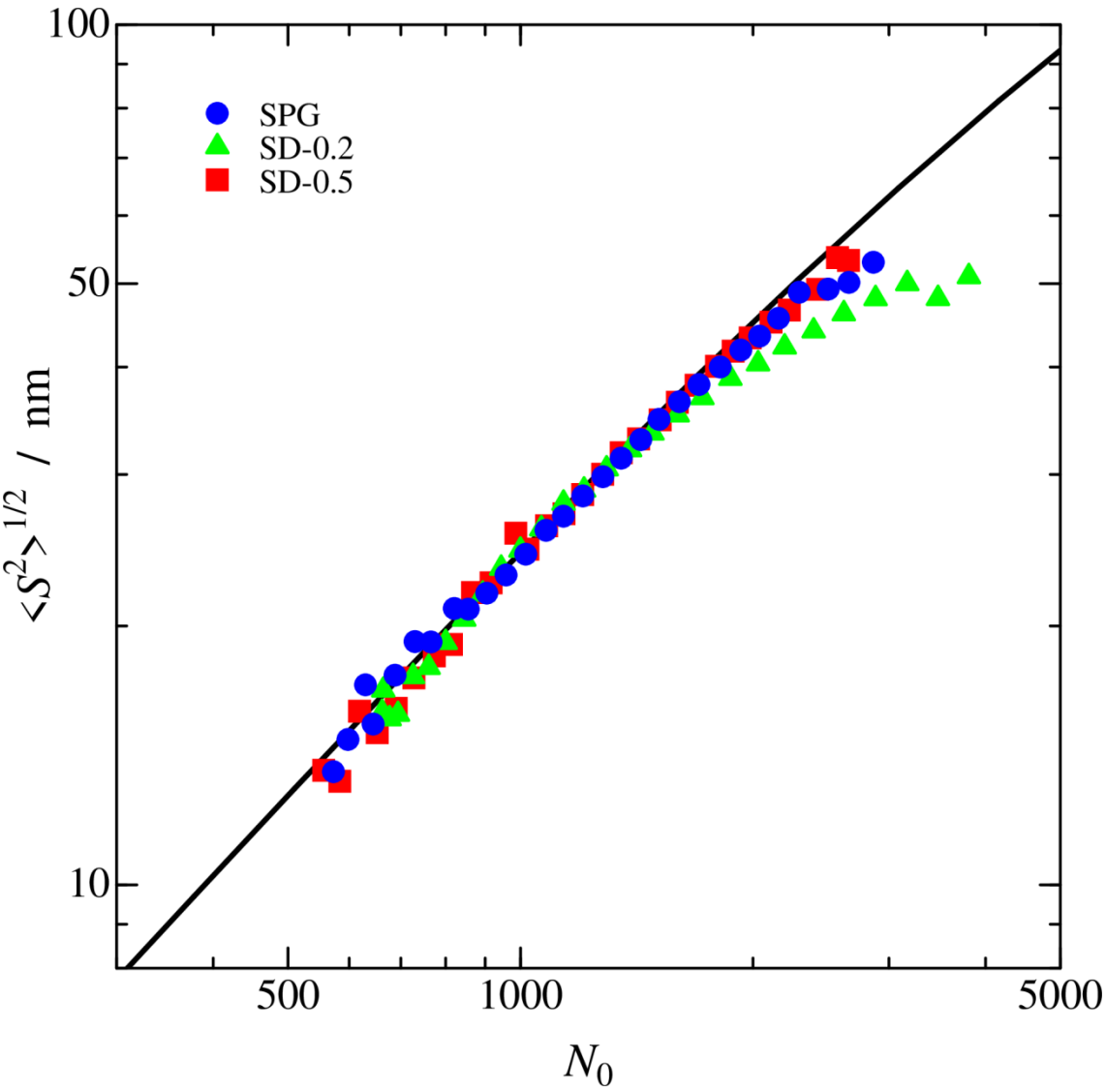

Figure 5 Radius gyration vs. degree of polymerization in 0.1 M NaCl at 40 ºC for SPG, SD-0.2, and SD-0.5. The solid curve represents the theoretical curve by the wormlike chain theory with q = 100nm and h = 0.28 nm. N_0 is the degree of polymerization calculated from M divided by the average molar mass per main chain glucose.

4-2. LCT theory for Smith-Degraded β-1,3-D-Glucans

The cooperative order-disorder transition (CODT) of aqueous β-1,3-D-glucans has been investigated mainly on aqueous schizophyllan in H_2O[17,22], D_2O[18-21,23] and their mixtures with DMSO[25,26]. At low temperatures, the triple helix of β-1,3-D-glucans forms an ordered structure between the side chains and water molecules. With an increase in temperature, the ordered structure (Triple Helix I) is turned to disordered structure (Triple Helix II). CODT has a strong molar mass dependency of the transition

temperature.[17,18,21,26] This is derived from the fact that the ordered and disordered units coexist in a triple helix with some cooperative length at the mid-temperature of the transition. Such a linear cooperative system can be expressed by the LCT theory. Recently, we have extended the LCT theory to apply it to the carboxylated derivative (sclerox) to investigate the effects of the chemical modification on CODT.[35.36] The degree of carboxylation (DS) was introduced into the theory to express the reduction of the number of unmodified side chain. In this case, the carboxylated side chains cannot take the ordered state, so that the transition temperature is lowered, and the transition enthalpy is decreased with increasing DS. However, the molecular conformation of the sclerox trimer is affected by carboxylation.[39] The chain stiffness becomes flexible, and the helical pitch is extended, which may hinder a long sequence of the ordered structure. On the other hand, three main chains of the present Smith degraded β-1,3-D-glucans maintain the original triple helix, and there is no ionic substituent to alter the interaction between the triple helix and the solvent molecule. These are essentially different from sclerox.

The LCT theory is a statistical thermodynamic theory for CODT, which is characterized with three theoretical parameters; the number of the theoretical transition units (N), the transition enthalpy at infinite N (ΔH_r^∞), and the cooperative parameter ($\sigma^{1/2}$). The statistical weight for the ordered state ($s(T,0)$) in a single solvent system is expressed by

$$\ln s(T,0) = \frac{3\Delta H_{r,0}^{\infty}}{R}\left(\frac{1}{T} - \frac{1}{T_{r,0}^{\infty}}\right) \qquad (1)$$

where $\Delta H_{r,0}^\infty$ and $T_{r,0}^\infty$ represent the transition enthalpy and transition temperature at infinite N. R and T are the gas constant, and the absolute temperature, respectively. In mixed solvent systems, aprotic solvents, such as dimethylsulfoxide (DMSO), become associated with the side chains to stabilize the ordered structure. In this case, the dissociation-association equilibrium for the associating molecule at the side chain is introduced into the LCT theory to analyze the stabilizing effects on CODT. [26,36] The equilibrium constant ($K(T)$) for the dissociation-association is defined by

$$K(T) = B\exp\left(-\frac{\Delta H_{a-m}}{RT}\right) \qquad (2)$$

Two association parameters, the enthalpy of association ($\Delta H_{a\text{-}m}$) and the pre-exponential factor (B), are concerned with the affinity of the associating molecules to the triple helix. The degree of association of the triple helix ($\xi_{ass}(T)$) depends on the temperature as follow,

$$\xi_{ass}(T) = \frac{K(T)x_D}{1 + K(T)x_D} \qquad (3)$$

where x_D is the mole fraction of DMSO-d_6 in the mixed solvent. For Smith-degraded

samples, the degree of association becomes lower because of the removal of the side chains. According to our previous work on the sclerox solution,[36] we can formulate the theoretical equations for the Smith-degraded schizophyllan solution. The statistical weight in the mixed solvent system is related with eq.1 by

$$s(T,x_D)=s(T,0)\left[1-\gamma_{SC}\xi_{ass}(T)\right]^{-3} \qquad (4)$$

where γ_{SC} is the relative degree of branching defined as the ratio of DB to the parent schizophyllan. Eq. 4 satisfies the following condition at T_r^{∞},

$$\frac{\Delta H_{r,0}^{\infty}}{R}\left(\frac{1}{T_r^{\infty}}-\frac{1}{T_{r,0}^{\infty}}\right)=\ln\left[1-\gamma_{SC}\xi_{ass}(T_r^{\infty})\right] \qquad (5)$$

The enthalpy of transition at infinite N in the mixed solvent (ΔH_r^{∞}), per mole of the repeating unit, is calculated with T_r^{∞} by

$$\Delta H_r^{\infty}=\Delta H_{r,0}^{\infty}-\gamma_{SC}\xi_{ass}(T_r^{\infty})\Delta H_{a-m} \quad (6)$$

A set of T_r^{∞} and ΔH_r^{∞} is given by eq. 5 and 6 to calculate the theoretical curve for the solvent effects of CODT at finite N. Let us consider that the triple helix is divided into N transition units. According to the LCT theory,[27] the partition function of the triple helix (Z_N) is represented by

$$Z_N=\mathbf{A}\mathbf{M}^{N-2}\mathbf{B} \qquad (7)$$

Here, **M** is the statistical weight matrix defined by

$$\mathbf{M}=\begin{pmatrix} u_{\mathrm{M}} & vu_{\mathrm{P}} \\ vu_{\mathrm{M}} & u_{\mathrm{P}} \end{pmatrix} \qquad (8)$$

where u_M and u_P are the statistical weight for M state and P state, respectively, and v represents the transition probability from M state to P state. In the case of CODT, u_M, u_P and v correspond to s, 1, and $\sigma^{1/2}$, respectively. **A** and **B** in eq. 7 are the vectors to specify the state of the terminal units, defined formally by

$$\mathbf{A}=\begin{pmatrix} au_{\mathrm{M}} & u_{\mathrm{P}} \end{pmatrix},\quad \mathbf{B}=\begin{pmatrix} b \\ 1 \end{pmatrix} \qquad (9)$$

In CODT, these may be simplified for the present system by the following molecular consideration. Although the ordered structure is formed between the nearest neighbor units, there is no neighboring unit outside the terminals. In other words, the terminal units can be considered as the disordered state throughout the transition. In such case, a and b

are taken as 0 and 1. The fraction of the ordered unit (f_N) can be calculated by the numerical formulas for the LCT theory summarized by Teramoto,[27] so that T_r at finite N can be determined from the peak temperature of the theoretical heat capacity (ΔC_p) curve.[36]

4-3. Analysis of DSC data for β-1,3-D-glucans in D_2O-DMSO-d_6 mixtures

Hirao *et al.*[26] discussed the theoretical transition unit of CODT in detail. The transition unit for the triple helix of schizophyllan has been defined as three times of the repeating unit (M_0),[18,21] so that the number of the transition unit is calculated as $N = M/(3M_0)$. In this model, the three side chains in the transition unit are considered as a unity, because the three saccharide chains constituting one unit are not independent because of the triple helical conformation. Above definition for the theoretical unit can be applicable to the present system. Because the removal of side chains is assumed to occur randomly, the number of side chains in a triplex is reduced from $3N$ to $3\gamma_{SC}N$ following the Smith degradation. The average number of side chains per one transition unit is decreased after the Smith degradation, whereas both the modified and unmodified units can participate in the transition because of the other unmodified side chains in the unit. On this assumption, we can calculate N for Smith-degraded samples; $N = (\gamma_{SC}M/3)[\gamma_{SC}M_0+(1-\gamma_{SC})M_1]^{-1}$. M_0 and M_1 express the molar mass of the repeating unit of unmodified and modified unit of Smith-degraded schizophyllan, respectively; $M_0 = 648.6$ g mol^{-1} and $M_1 = 486.5$ gmol^{-1}. The number of the transition unit is calculated as $N = 117$ for SPG, $N = 115$ for SD-0.2 and $N = 91$ for SD-0.5 from the number average molar mass and DB obtained. The other theoretical parameters for the LCT theory have been analyzed in previous study on schizophyllan solutions,[26] so that the theoretical curve for T_r and ΔH_r at finite N can be calculated without any fitting parameters.

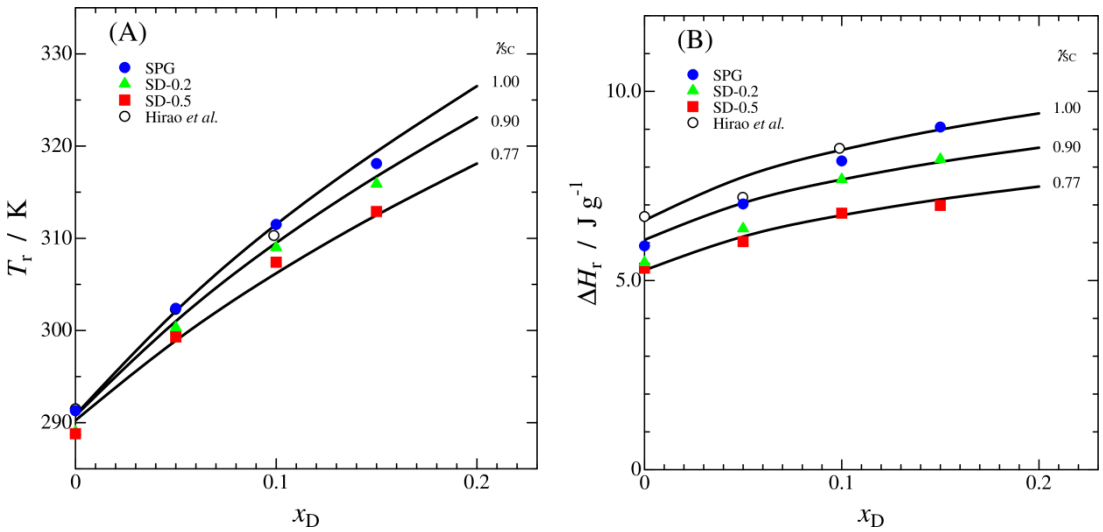

Figure 6 Transition temperatures (A) and transition enthalpies (B) of the cooperative order-disorder transition of Smith-degraded β-1,3-D-glucan in D_2O-DMSO-d_6 mixtures. The data by Hirao *et al.*[26] were added. Solid curves represent the theoretical curves with the relative degree of branching (γ_{SC}) calculated by LCT theory with $\Delta H_{a\text{-}m}$ = -8.60 kJ mol^{-1} and B = 0.0488 for SPG (N=117), SD-0.2 (N=115) and SD-0.5 (N=91). Theoretical ΔH_r was calculated from ΔH_r^{∞} multiplied by f_N (280 K) and γ_{SC}.

Dashed curves in Figure 4 represent the theoretical ΔC_p curves calculated by the LCT theory for SPG (N=117), SD-0.2 (N=115) and SD-0.5 (N=91), adapting $\Delta H_{r,0}^{\infty}$ = 4.60 kJ mol^{-1}, $T_{r,0}^{\infty}$ = 292.3 K and $\sigma^{1/2}$ = 0.01 except for those in pure D_2O ($\sigma^{1/2}$ = 0.0135).[18,26] The ΔC_p curves in Figure 4 were reduced to compare the experimental DSC data. To calculate the ΔC_p curves in D_2O-DMSO-d_6 mixtures, T_r^{∞} and ΔH_r^{∞} was determined by eq. 5 with γ_{SC} and the known parameters; $\Delta H_{a\text{-}m}$ = -8.60 kJmol^{-1} and B = 0.0488.[26] Theoretical T_r is determined from the peak temperatures of the ΔC_p curves. Figures 6A and 6B show the plots of T_r and ΔH_r against the mole fraction of DMSO-d_6 (x_D) in the solvent for CODT of the Smith degraded β-1,3-D-glucan in D_2O-DMSO-d_6 mixtures. The data for R-212 and R-4 by Hirao *et al.*[26] were added in Figures 6A and 6B to confirm the analysis. The solid curves represent the theoretical x_D-T_r curves determined from Figure 4. Figure 6B shows the increment of ΔH_r of CODT in D_2O-DMSO-d_6 mixtures. ΔH_r increases at low x_D but levels off at high x_D. As mentioned in our previous study,[34] the values of ΔH_r at low x_D are affected by the tailing of DSC curve. Since the number of side chains per triplex is decreased by γ_{SC}, the theoretical ΔH_r^{∞} curve may be corrected by multiplying γ_{SC} and f_N(280 K) at 280 K. The solid curves are, therefore, the theoretical curves with eq. 6 and the above corrections. All the theoretical parameters for the LCT theory are the same as those in the calculation of T_r above. The theoretical curves of both

T_r and ΔH_r well reproduce the DSC results within the experimental error. These agreements with the LCT theory indicate that the primary structure of β-1,3-glucan, such as repeating branched structure, is not so sensitive to T_r and ΔH_r, but the 'average' degree of branching is effective to T_r and ΔH_r. Because the degree of branching is relatively high in our samples, the triplex unit of the lateral structure is preserved by the unmodified side chains and nearby solvent molecules to form the ordered structure. Consequently, the longitudinal order along the triplex, in other words, the cooperativity of the transition, is maintained after the Smith degradation.

Conclusion

The triple helical conformation of β-1,3-D-glucan with different degrees of branching varied by the Smith degradation of schizophylan was investigated by SEC-MALS and DSC measurements. The degree of branching is reduced by the Smith degradation. The molecular conformation of the Smith-degraded schizophyllan with low degree of modification in water is unchanged, which has a comparable stiffness to the original triple helix analyzed by the wormlike chain theory. The stabilizing effects of the cooperative order-disorder transition in D_2O-DMSO-d_6 mixtures were analyzed by the linear cooperative transition theory. The triplex unit of the Smith-degraded schizophyllan is constructed by both the solvent molecule and the residual side chains in the unit, so that the cooperativity of the transition is independent of the degree of branching.

Supplementary information

The LS90° and dRI data obtained from SEC-MALS measurements were given.

Acknowlegements

K. Y. thanks Professor Takahiro Sato, Graduate School of Science, Osaka university, for his valuable comments on this work. The author thanks Dr. Yuya Tachibana, Graduate School of Science and Technology, Gunma University, for his permission to perfom HPLC measurements.

References

1. G. D. Brown, D. L. Williams, In *Chemistry, Biochemistry and Biology of (1→3)-β-Glucans and Related Polysaccharides* (Eds. A. Basic, G. B. Fincher, B. A. Stone), Academic Press, Amsterdam, 2009; Chapter 4.5.2, p 579.
2. M. Kanagawa, T. Satoh, A. Ikeda, Y. Adachi, N. Ohno, Y. Yamaguchi, *J. Biol. Chem.*

2011, *286*, 29158–29165.
3. K. Sakurai, S. Shinkai, *J. Am. Chem. Soc.* **2000**, *122*, 4520–4521.
4. K. Kobiyama, T. Aoshi, H. Narita, E. Kuroda, M. Hayashi, K. Tetsutani, S. Koyama, S. Mochizuki, K. Sakurai, Y. Katakai, Y. Yasutomi, S. Saijo, Y. Iwakura, S. Akira, C. Coban, K. J. Ishii, *Proc. Natl. Acad. Sci. U.S.A.* **2014**, *111*, 3086-3091.
5. S. Kikumoto, T. Miyajima, S. Yoshizumi, S. Fujimoto, K. Kimura, *J. Agricul. Chem. Soc. Jpn.* **1970**, *44*, 337-342.
6. S. Kikumoto, T. Miyajima, K. Kimura, S. Okubo, N. Komatsu, *J. Agricul. Chem. Soc. Jpn.* **1971**, *45*, 162-168.
7. Y. Takahashi, T. Kobatake, H. Suzuki, *Rep. Prog. Polym. Phys. Jpn.***1984**, *27*, 767-768.
8. T. Okobira, K. Miyoshi, K. Uezu, K. Sakurai, S. Shinkai, *Biomacromolecules* **2008**, *9*, 783-788.
9. T. Norisuye, T. Yanaki, H. Fujita, *J. Polym. Sci. Polym. Phy. Ed.* **1980**, *18*, 547-558.
10. Y. Kashiwagi, T. Norisuye, H. Fujita, *Macromolecules* **1981**, *14*, 1220-1225.
11. T. Itou, A. Teramoto, *Macromolecules* **1984**, 17, 1419-1420.
12. T. Itou, K. Van, A. Teramoto, *J. Appl. Polym. Sci. Appl. Polym. Symp.* **1985**, *41*, 35-48.
13. K. Yoshiba, A. Teramoto, N. Nakamura, T. Sato, *Macromolecules* **2003**, 36, 2108-2113.
14. T. Norisuye, *Makromol. Chem. Suppl.* **1985**, 14, 105-118.
15. K. Okamura, M. Suzuki, T. Chihara, A. Fujiwara, T. Fukuda, S. Goto, K. Ichinohe, S. Jimi, T. Kasamatsu, N. Kawai, K. Mizuguchi, S. Mori, H. Nakano, K. Noda, K. Sekiba, K. Suzuki, T. Suzuki, K. Takahashi, K. Takeuchi, S. Takeuchi, A. Yajima, N. Ogawa, *Cancer* **1986**, *58*, 865-872.
16. T. Asakawa, K. Van, A. Teramoto, *Mol. Cryst. Liq. Cryst.* **1984**, *116*, 129-139.
17. T. Itou, A. Teramoto, T. Matsuo, H. Suga, *Macromolecules* **1986**, *19*, 1234-1240.
18. T. Itou, A. Teramoto, T. Matsuo, H. Suga, *Carbohydr. Res.* **1987**, *160*, 243-257.
19. A. Teramoto, H. Gu, Y. Miyazaki, M. Sorai, S. Mashimo, *Biopolymers* **1995**, *36*, 803-810
20. Y. Hayashi, N. Shinyashiki, S. Yagihara, K. Yoshiba, A. Teramoto, N. Nakamura, Y. Miyazaki, M. Sorai, Q. Wang, *Biopolymers* **2002**, *63*, 21-31.
21. K. Yoshiba, T. Ishino, A. Teramoto, N. Nakamura, Y. Miyazaki, M. Sorai, Q. Wang, Y. Hayashi, N. Shinyashiki, S. Yagihara, *Biopolymers* **2002**, *63*, 370-381.
22. K. Yoshiba, A. Teramoto, N. Nakamura, K. Kikuchi, Y. Miyazaki, M. Sorai, *Biomacromolecules* **2003**, *4*, 1348-1356.

23. K. Yoshiba, A. Teramoto, N. Nakamura, Y. Miyazaki, M. Sorai, T. Shikata, Y. Hayashi, N. Miura, *Biomacromolecules* **2004**, *5*, 2137-2146.
24. G. Bocchinfuso, A. Palleschi, C. Mazzuca, T. Coviello, F. Alhaique, G. Marletta, *J. Phys. Chem. B* **2008**, *112*, 6473-6483.
25. S. Kitamura, T. Kuge, *Biopolymers* **1989**, *28*, 639-654.
26. T. Hirao, T. Sato, A. Teramoto, T. Matsuo, H. Suga, *Biopolymers* **1990**, *29*, 1867-1876.
27. A. Teramoto, *Prog. Polym. Sci.* **2001**, *26*, 667-720.
28. S. Kitamura, M. Ozasa, H. Tokioka, C. Hara, S. Ukai, T. Kuge, *Thermochim. Acta* **1990**, *163*, 89-96.
29. X. Wang, Y. Zhang, L. Zhang, Y. Ding, *J. Phys. Chem. B* **2009**, *113*, 9915-9923.
30. A. S. Perlin, In *The Carbohydrates. Chemistry and Biochemistry Second Edition*; (Eds. Pigman, W., Horton, D. Wander, J. D.), Academic Press: New York, 1980; Vol. IB, Chapter 25, p 1167.
31. K. Koumoto, R. Karinaga, M. Mizu, T. Anada, K. Sakurai, T. Kunitake, S. Shinkai, *Biopolymers* **2004**, *75*, 403-411.
32. A. S. Magee, R. R. Langeslay P. L. Will, M. E. Danielson, L. R. Wurst, V. A. Iiams *Biopolymers* **2015**, *103*, 665-674.
33. K. Martin, B. M. McDougall, S. Mcllroy, Jayus, J. Chen, R. J. Seviour, *FEMS Microbiol. Rev.* **2007**, *31*, 168-192.
34. B. E. Christensen, E. Aasprong, B. T. Stokke, *Carbohydr. Polym.* **2001**, *46*, 241-248.
35. K. Yoshiba, S. Okamoto, T. Dobashi, H. Oku, B. E. Christensen, T. Sato, *Carbohydr. Polym.* **2017**, *168*, 79-85.
36. K. Yoshiba, T. Dobashi, A.-S. T. Ulset, B. E. Christensen, *J. Phys. Chem. B* **2018**, *122*, 6551-6558.
37. H. Yamakawa, Y. Yoshizaki, *Helical Wormlike Chains in Polymer Solutions. Second Edition,* Springer, Berlin, 2016.
38. S. Kitamura, T. Hori, K. Kurita, K. Takeo, C. Hara, W. Itoh, K. Tabata, A. Elgsaeter, B. T. Stokke, *Carbohydr. Res.* **1994**, *263*, 111-121.
39. K. Yoshiba, T. Sato, T. Osumi, A.-S. T. Ulset, B. E. Christensen, *Carbohydr. Polym.* **2015**, *134*, 1-5.