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Abstract

Among various kinds of polysaccharides known to form hydrogels, locust bean gum (LBG) consisting of a mannose backbone and galactose side chains has unique characteristics, since LBG forms hydrogels by freezing and thawing. In this study, effect of thermal history on gelation was investigated by differential scanning calorimetry (DSC) and thermomechanical analysis (TMA). Gel/sol ratio calculated by weighing method was found to be affected by sol concentration, freezing rate and the number of freezing and thawing cycle (n). Once LBG hydrogels are formed, they are thermally stable, although syneresis was observed when n increased. Dynamic Young's modulus (E') of hydrogels measured by TMA in water increased with increasing n and decreasing freezing rate. Non freezing water calculated from DSC melting peak of ice in the gel decreased with increasing n and decreasing freezing rate. Morphological observation of freeze-dried gels was carried out by scanning electron microscopy (SEM). The above results indicate that weak hydrogel having large molecular network structure transformed into strong gel with densely packed network structure by increasing n and decreasing freezing rate.

Key words: galactomannan polysaccharide; locust bean gum; hydrogel; DSC; TMA

1. Introduction

Various kinds of polysaccharide are known to form physical gels in aqueous media, for example, agalose [1], κ -carrageenann [2,3], and curdlan [4] form transparent hydrogel by cooling, in contrast, methylcellulose forms hydrogel by heating [5]. Molecular mechanism of crosslinking of the above polysaccharides has been investigated by many researchers relating with the role of water molecules which concern molecular assembly of polysaccharide molecules. Locust bean gum (LBG) is a galactomannan polysaccharide which consists of a 1,4- β -D-mannose backbone and 1,6- α -D-galactose side chains. LBG has been known as non-gelling polysaccharide as it is. In order to obtain LBG hydrogel, multi-valent ions, such as Boron has been added to solutions in order to establish inter-molecular linking [6]. In our previous studies, we suggested that LBG forms hydrogels by freezing and thawing [7-9] similar to poly(vinyl alcohol) (PVA) which is well known to form hydrogel by freezing and thawing [10,11]. The difference of above two gels is that once LBG

hydrogel is thermally stable and no gel-sol transition is observed, while PVA gels formed by freezing and thawing transform into sol state by heating [12]. On this account, the thermal irreversibility is a characteristic feature of LBG hydrogel.

Guar gum (GG) and tara gum (Tara-G) are also categorized as galactomannan polysaccharides having similar structure of LBG, consisting of a 1,4- β -D-mannose backbone and 1,6- α -D-galactose side chains [13-15]. A difference between the three gums is the galactose/mannose ratio, which is 1:2, 1:3 and 1:4 for GG, Tara-G and LBG, respectively. Among above three gums, only LBG forms hydrogels by freezing and thawing [7-9], in contrast no gelation is found for Tara-G and GG. It is thought that an appropriate chemical structure for the formation of hydrogen bonding between the hydroxyl groups is crucial in order to form a network structure.

In our previous studies, we introduced thermomechanical analysis (TMA) in order to measure viscoelastic properties of polysaccharide hydrogels in water [16-18]. Using the above technique, both static and dynamic mechanical properties of hydrogels at various temperatures can be measured by controlling water temperature [19]. In this study, LBG hydrogels were prepared by freezing and thawing by varying the cooling rate and the effect of cooling rate on gelation is investigated by TMA in water. By differential scanning calorimetry (DSC), melting of ice in the gels and melting enthalpy are also related with cooling rate. Based on thermal data, gelation mechanism of LBG by freezing and thawing is discussed.

2. Experimental

2.1. Sample preparation

LBG in powder form was purchased from Sigma Chemical Co., USA. LBG extracted from Carob seeds (*Caratonia siliqua*). The molecular weight was ca. 3.1×10^5 according to the manufacturer. LBG was solved in deionised water at 25 °C to obtain 0.5 ~ 10 % solutions. The solutions were annealed at 105 °C for 2 hours. After annealing at 105 °C, 5, 10, 20, 30 or 50 ml of LBG solutions were put in a 100 ml polyethylene container (49 ϕ x 72 mm). The LBG solution in PE container were directly transferred to a freezer whose temperature was -20 °C and maintained for approximately 18 hours. Cooling rate was measured by thermometer. Obtained cooling rate was 3.0, 2.5, 1.5, 1.0 and 0.7 °C min⁻¹, respectively. Frozen samples were thawed slowly at 25 °C. This process took more than 6 hours. The above freezing and thawing cycles is stated as “*n*”. After freezing and thawing, the samples were stored at 25 °C.

The gel and sol were centrifuged at 1,000 rpm for 5 min. The gels were removed from the centrifugal vessel and weighed quickly. The gel ratio was calculated according to following equation.

$$\text{Gel ratio (g g}^{-1}\text{)} = (\text{mass of gel}) / (\text{mass of gel} + \text{mass of sol}) \quad (1)$$

The gel or sol was taken out from the container and dried at 120 °C for 10 hours in an oven and dry mass of the gel was recorded. Polymer concentration in the gels was calculated according to following equation [8].

$$\text{Polymer concentration (\%)} = (\text{mass of dry gel}) / (\text{mass of gel}) \quad (2)$$

The mass of samples was measured using a Sartorius micro-balance (MC210S). Precision was $\pm 1.0 \times 10^{-5}$ g.

2.2 TMA measurements in water

A SII Nano Technology Inc. thermomechanical analyser (TMA, SII Nano Technology Inc. TMA/SS 150) equipped with a newly designed sample holder was used. Quartz rod with uniform cross-sectional area ($5.09 \times 10^{-5} \text{ m}^2$) was used as a probe. The sample holder was immersed in water whose temperature was controlled 25 °C. The gel sample was placed in a quartz sample pan with diameter 9 mm (inner diameter) and height 5mm and immersed in 70 ml water. And the sample was compressed by quartz probe.

Dynamic measurements were carried in water at 25 °C by using a quartz rod probe. Operating frequency was 0.05 Hz. Measurements were carried out for 5 min. The conditions were employed for all measurements to facilitate comparison of the variation in dynamic modulus (E') and $\tan \delta$. From Lissajous diagram, E' and $\tan \delta$ were obtained [16]. 1 % LBG hydrogel was used as sample for TMA measurements.

2.3. DSC measurements

1 % LBG aqueous solution was used as sample for DSC. A SII Nano Technology Inc. differential scanning calorimeter (DSC) EXSTAR 6000 equipped with a cooling apparatus was used. Temperature and enthalpy calibrations were carried out using water. Dry nitrogen was used as a

purge gas and the flow rate was 30 ml min⁻¹. The sample mass was ca. 3 mg and an aluminium sealed-type sample-pan was used. The sample-pan was hermetically sealed and the total mass of the LBG sol recorded. A Sartorius ultramicro-balance ($\pm 0.1 \times 10^{-6}$ g) was used for sample mass measurements. The cooling rate was from 2 °C min⁻¹ to 50 °C min⁻¹, and heating rate was 10 °C min⁻¹. The sample was cooled from 25 °C to -80 °C and heated from -80 °C to 60 °C (1st-run measurement). Then from 2nd-run to 5th-run was cooled from 60 °C to -80 °C and heated from -80 °C to 60 °C. The first to 5th repeated runs were designated as $n = 1, 2, 3, 4$ or 5 , respectively. Liquid nitrogen was used a coolant. The starting temperature of melting endothermic was used as melting temperature (T_m). The melting endothermic peak (T_{pm}) was also used as criteria for comparison and the melting enthalpy (ΔH_m) was calculated area of melting endothermic peak.

2.4 Morphological observation

A JEOL JSM 35CF scanning electron microscope (SEM) was used. Samples for SEM were prepared as follows; samples in glassware were put in liquid nitrogen and freeze dried. Fracture surface was coated by Au-palladium in vacuum using a sputtering apparatus.

3. Results

The rate of crystallization of water depends on volume [20]. Freezing rate was changed according to the procedure described in the experimental section. Figure 1-(a) shows relationships between gel ratio of LBG hydrogels prepared at $n=1$, LBG concentration and cooling rate. Figure 1-(b) shows relationships between polymer concentration in gel of LBG hydrogels ($n=1$), LBG concentration and cooling rate. At other cycles (n), similar results were also obtained. A turbid gel was formed by the freezing and thawing process, and transparent liquid was separated from the gel at the time of thawing, i.e. syneresis occurred. In Figure 1-(a), gel ratio increased with increasing concentration and increased with decreasing cooling rate. When concentration is in a range from 0.5 to 2 %, LBG concentration in gel decreased with increasing cooling rate. When concentration is larger than 3 %, turbid gel without sol was formed which indicates that gel ratio is 1.0 g g⁻¹ and LBG concentration in gel is same value. As shown in Figure 1-(b), values of polymer concentration in gel are equal to sol concentration when concentration is greater than 3 %. This indicates that all LBG molecules are involved in network structure regardless of cooling rate. The dotted lines shown in Figure 1-(b) indicate that the polymer concentration in the gel when the whole solution transforms to gel and no

syneresis is observed. Values of polymer concentration in gel are larger than value of chain line in a concentration range from 0.5 to 2 %. When concentration was in a range of 0.5 ~ 2 %, polymer concentration in gel decreased with increasing cooling rate. Polymer concentration in sol was measured after gel portion was separated, however no LBG was found in the sol.

Figure 1 (a) Relationships between gel ratio, LBG concentration and cooling rate of LBG hydrogels with $n=1$.

Figure 1 (b) Relationships between LBG concentration in gel, LBG concentration and cooling rate of LBG hydrogels with $n=1$.

Figure 2-(a) shows relationships between gel ratio of LBG hydrogels prepared from 1 % aqueous solution, n and cooling rate. Gel ratio decreased with increasing n and decreasing cooling rate. Figure 2-(b) shows relationships between polymer concentration in gel of LBG hydrogels prepared from 1 % aqueous solution, n and cooling rate. Polymer concentration in gel increased with increasing n and decreasing cooling rate. Similar results were also obtained if concentration was varied.

Figure 2 (a) Relationships between gel ratio, number of freezing and thawing cycle (n) and cooling rate of LBG hydrogels with 1 %.

Figure 2 (b) Relationships between LBG concentration in gel, number of freezing and thawing cycle (n) and cooling rate of LBG hydrogels with 1 %.

Figure 3-(a) shows relationships between E' or $\tan \delta$ calculated from dynamic TMA, and n . E' increased and $\tan \delta$ decreased with increasing n . Figure 3-(b) shows relationships between E' or $\tan \delta$ obtained by TMA, and cooling rate. E' increased and $\tan \delta$ decreased with increasing cooling rate.

Figure 3 (a) Relationships between E' or $\tan \delta$ by TMA, and number of freezing and thawing cycle (n) of LBG hydrogels with 1 %.

Solid circle ; E' , open circle ; $\tan \delta$

Figure 3 (b) Relationships between E' or $\tan \delta$ by TMA, and cooling rate of LBG hydrogels with 1 %.

Solid circle ; E' , open circle ; $\tan \delta$

Phase transition of water in hydrogels by DSC. Figure 4 shows stacked DSC heating curves at various repeated runs (n). Figure 4-(a) shows DSC heating curves of sample cooled at $2^{\circ}\text{C min}^{-1}$ and (b) is those of sample cooled at $50^{\circ}\text{C min}^{-1}$. Heating rate was maintained at constant $10^{\circ}\text{C min}^{-1}$. Melting peak temperature of water in gels decreased with increasing n . The endothermic melting peak of ice in the gels prepared by cooling rate at $50^{\circ}\text{C min}^{-1}$ was broader than that of gels prepared by at $2^{\circ}\text{C min}^{-1}$.

Figure 4 Stacked DSC heating curves of LBG sol with 1 % at various scanning times. Heating rate was constant at $10\text{ }^{\circ}\text{C min}^{-1}$. (a) cooling rate = $2\text{ }^{\circ}\text{C min}^{-1}$ (b) cooling rate = $50\text{ }^{\circ}\text{C min}^{-1}$

Figure 5 shows relationships between T_m and ΔH_m and n at various cooling rates. T_m and ΔH_m decreased with increasing n . Furthermore, T_m increased with increasing cooling rates and ΔH_m decreased with increasing cooling rates.

Figure 5 Relationships between T_{im} and ΔH_m of LBG sol with 1 %, and scanning times. Heating rate was constant at $10\text{ }^{\circ}\text{C min}^{-1}$. Solid circle ; T_m for cooling rate at $2\text{ }^{\circ}\text{C min}^{-1}$, solid diamond ; T_m for cooling rate at $50\text{ }^{\circ}\text{C min}^{-1}$, open circle ; ΔH_m for cooling rate at $2\text{ }^{\circ}\text{C min}^{-1}$, open diamond ; ΔH_m for cooling rate at $50\text{ }^{\circ}\text{C min}^{-1}$

Figure 6 shows SEM photographs of LBG hydrogels at various cooling rates. As shown in the photographs, pores are observed. With increasing cooling rate, pore walls become thinner and the size of the pore decreases. It is clear that morphology of gels varies by cooling rate.

Figure 6 SEM photographs of 1 % LBG gels at $n=1$ of different cooling rates. (a) cooling rate = $0.7\text{ }^{\circ}\text{C min}^{-1}$ (b) $2.5\text{ }^{\circ}\text{C min}^{-1}$ (c) $3.0\text{ }^{\circ}\text{C min}^{-1}$

4. Discussion

Among various factors affecting on freezing and thawing gelation of LBG, in this study, we paid special attention to three factors, such as concentration of sol, cooling rate and repeated cycles of freezing and thawing. As shown in Figures 1 and 2, polymer concentration in each gel linearly increased with increasing sol concentration, i.e. 100 % LBG molecules were participated in network structure when sol concentration was higher than 3 %. Once the sol concentration was diluted more than this characteristic value of 3 %, syneresis occurred, i.e. water was spontaneously separated from network at the time of thawing. An excess amount of water in the gel was excluded and gel portion contains always 3 % of LBG. It was noteworthy that no LBG remains in sol, even sol concentration decreased until 0.5 %. This kind of characteristic point was also observed in PVA hydrogel formed by freezing and thawing. The characteristic value of PVA was reported to be 5 % [11]. The difference was thought to come from that LBG had bulky side chain of galactose and PVA had simple linear structure. Number of the hydroxyl group and complex conformation of LBG were attributable to thermal irreversible property of LBG hydrogel.

It was thought that molecular motion of LBG chains in water was necessarily ceases when water surrounding each LBG molecular chain starts to freeze. With decreasing temperature, ice grew and inter-molecular distance between LBG molecules necessarily decreased. Galactose side chain made a contact with adjacent galactose and the hydroxyl groups participate in hydrogen bonding. Cooling rate played a crucial role in above freezing procedure in a concentration range lower than characteristic point, since an excess amount of water presented in the system. With increasing cooling rate, gel ratio increased (Figure 2-b), Young's modulus increased (Figure 3-a) and ΔH_m decreased (Figure 5). The above results strongly suggested that small and dense network structures were formed by rapid cooling. The fact that ΔH_m of slowly cooled gel was smaller than that of rapid cooled gel, indicated that number of bound water molecules restrained by network structure of LBG molecules was larger than that of rapid cooled gel. As shown in Figure 5, T_m shifted to the low temperature side with decreasing cooling rate. We had classified hydrated water of polymers as follows: freezing (crystalizable) water which could be observed as the first order phase transition was categorized into (1) free water whose melting temperature was observed at 0 °C and (2) freezing bound water which melting temperature observed at a temperature lower than 0 °C and strongly affected by the matrix polymer. In addition to freezing water there existed (3) non-freezing water, i. e. water which was non-crystallizable due to strong molecular interactions with the matrix polymer. Both freezing bound water and non-freezing water were categorized as bound water. The results obtained

in this study indicated the presence of freezing bound water in LBG hydrogels [21,22]. Micrographs (Figure 6) also showed that quenched gel had small pore whose cell wall was thin. It was reported that dynamic rigidity (G') values obtained from corn-plate type rheometer of hydrogels formed by rapid cooling are higher than those formed by slow cooling [8]. The above results indicated that the strength of the LBG hydrogel was strongly related to the cooling rate on freezing, *i. e.* the rate of crystallization of ice in the LBG-water system. It was appropriate to consider that molecular diffusion in a long range was disturbed by the presence of small size of ice formed by rapid crystallization. Accordingly, three dimensional densely packed network structure was established.

Loose crosslinking networks were formed in the first freezing and thawing and the segment exhibited limited thermal fluctuation around fixed average positions [7]. On this account, the number of crosslinking points increased and molecular chains aggregate with increasing freezing and thawing cycles. As shown in Figure 2, the gel ratio slightly decreased and polymer concentration increased with increasing n . Figure 3-(a) showed that E' increases with increasing n . The above results suggested that the cell wall of LBG hydrogels becomes thicker with increasing freezing and thawing cycles. At the same time, the facts that T_m and ΔH_m decreased with increasing n strongly suggested that molecular equilibration occurred by freezing and thawing cycles. By increased of freezing and thawing cycle, residual free molecular chains were successively included in junction zones.

5. Conclusion

LBG hydrogels were prepared by freezing and thawing. The above hydrogels was thermally irreversible and no gel-sol transition was observed. By increasing freezing and thawing cycles, Young's modulus measured in water by TMA increased. At the same time, melting temperature and enthalpy of ice formed in the gels decreased. It is thought that LBG molecular assemblies consisting cell wall becomes thicker during melting and crystallization of ice accompanied with syneresis. It was found that crystal size of ice directly affect on network structure of LBG hydrogels.

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