Colloid Polym Sci 275:67–72 (1997)

ORIGINAL CONTRIBUTION

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Effects of hydroxy compounds on gel formation of gelatin

Received: 18 April 1996
Accepted: 23 July 1996

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Abstract Network formation of gelatin gel is known to consist of three-dimensionally cross-linked triple helices among polypeptide chains. The effects of added low molecular weight mono-ols, diols and polyols on the higher-order structure formation of gelatin chains were investigated using the following measurements: melting temperature, viscoelasticity and spin-lattice relaxation time (T_1) of $H_2^{17}O$ of gels, and circular dichroism spectra of diluted gelatin solutions. Furthermore, hydration behaviors of these hydroxy compounds were evaluated from the dynamic hydration numbers ($\mathbf{n}_{DHN}$) derived from T_1 of $H_2^{17}O$ in the solutions. It was found that network structures of gelatin gels containing hydoxy compounds were influenced by the number and position of hydroxyl groups as well as the number of carbon atoms of these coexisting compounds. The effect of hydroxyl groups of hydroxy compounds was considered to stabilize the helices among gelatin chains. Especially, the addition of polyols with large number of hydroxyl groups increased the number of cross-linking junctions in the gel networks, which consist of the aggregation among the helices. On the contrary, the effect of carbon atoms of hydroxy compounds is to disturb the formation of the helices and the aggregation among the helices.

Key words Gelatin – gel formation – NMR – viscoelasticity – circular dichroism

Introduction

It has been recognized that sugars and polyols stabilize higher-order structures of polypeptide chains. Consequently, they are widely applied to the either biochemical research or food processing as indispensable reagents and preservatives for various proteinaceous materials. Although the preservative functions of sugars and polyols have not been sufficiently clarified, it seems to converge to a common concept that these effects are primarily mediated by the effects of these compounds to the water structure [1–6]. Gekko et al. [5, 6] studied the stabilization of globular proteins by sugars and polyols from a thermodynamic point of view, and concluded that the proteins would be stabilized through a promotion of the hydrophobic interaction originated by the character of these compounds to form water-structure. Some fibrous proteins are also known to be stabilized by the same promotion mechanisms [7–10]. However, some compounds, such as polyethylene glycol and 2-methyl-2,4-pentanediol are known to act as destabilizers for some globular proteins [11, 12]. The partially hydrophobic nature of these compounds is considered to cause the binding to the hydrophobic region of the proteins, while being forbidden to interact with the surface-localized charged residues of the proteins. Therefore, formation of the higher-order structure of polypeptides would be governed partly by the chemical structure of coexisting organic compound.

Thus, an investigation was carried out in order to clarify the effects of hydroxyl groups and carbon atoms in the coexisting low molecular weight hydroxy compounds on the gelation process of gelatin.

Materials and methods

Materials

Gelatin was a commercial product from Fluka Chemie AG. (from porcine skin, high strength, Bloom number 250). Alcohols (mono-ols) and dihydric alcohols (diols) were reagent grade and purchased from Tokyo Kasei Co. (Tokyo, Japan). The names and abbreviations of these compounds are given in Table 1 along with experimentally obtained dynamic hydration numbers ($\mathbf{n}_{\mathrm{DHN}}$). Glycerol was a special reagent grade product and sorbitol was a reagent grade product both from Kanto Chemicals Co. (Tokyo, Japan). Xylitol was a special reagent grade product from Tokyo Kasei Co. (Tokyo, Japan). All reagents were used without further purification. Water was deionized and distilled.

Dynamic hydration number ($\mathbf{n}_{\mathrm{DHN}}$)

The dynamic hydration number ($\mathbf{n}_{\mathrm{DHN}}$) is derived from the following relation (1) [13, 14], which expresses the hydration behavior of a low molecular weight compound in the aqueous solution,

$$\mathbf{n}_{\mathrm{DHN}} = n_{\mathrm{h}} \left(\tau_{\mathrm{c}}^{\mathrm{h}} / \tau_{\mathrm{c}}^{\mathrm{o}} - 1\right) \tag{1}$$

where n_{h} is the number of coordinating water molecules around a solute molecule. $\tau_{\mathrm{c}}^{\mathrm{h}}$ and $\tau_{\mathrm{c}}^{\mathrm{o}}$ are the rotational correlation time of the water molecules in the hydration share and in the bulk phase, respectively. $\mathbf{n}_{\mathrm{DHN}}$ of hydroxy compounds were derived from the spin-lattice relaxation times (T_1) of $H_2^{17}O$. According to the method of Uedaira et al. [13, 14], $\mathbf{n}_{\mathrm{DHN}}$ is obtained from the value of B in the following empirical equation,

$$T_1^{\mathrm{o}} / T_1 = 1 + Bm + Cm^2 \tag{2}$$

where T_1^{o} and T_1 are the spin-lattice relaxation times of $H_2^{17}O$ in the pure water and the aqueous solution of a hydroxy compounds, respectively, and m is the molarity of the solute (0 ~ 2.0 mol/l, pH 3.5). NMR measurements of naturally abundant oxygen-17 were performed using a JEOL GSX-400 spectrometer operating at 52.4 MHz. A sample solution of 5 ml was put in a 10 mm diameter tube. The temperature was maintained at 30.0 ± 0.1 °C.

Preparation of gels

A given amount of gelatin was dissolved into distilled water at 45 °C, thus preparing a stock solution with a definite concentration. The gelatin stock solution was diluted with an aqueous hydroxy compound solution to the desired concentration at 45 °C. Well-mixed sample solutions were poured into the glass tubes and stored at 10 °C for 24 h to complete formation of gel networks.

Table 1 Abbreviations of hydroxy compounds and the $\mathbf{n}_{\mathrm{DHN}}$ values at 30 °C

	Compounds	Abbreviation		$\mathbf{n}_{\mathrm{DHN}}$	
mono-ols	Methanol	C_nOH	$n = 1$	6.0	(6)*
	Ethanol		$n = 2$	10.4	(10.4)*
	n-Propanol		$n = 3$	16.0	(16.3)*
polyols	Ethylene glycol	$C_n(OH)_n$	$n = 2$	7.3	
	Glycerol		$n = 3$	8.7	
	Xylitol		$n = 5$	11.9	
	Sorbitol		$n = 6$	14.0	
α, β-diols	Propylene glycol	$\alpha, \beta\text{-}C_n(OH)_2$	$n = 3$	11.9	
	1,2-Butanediol		$n = 4$	17.8	
	1,2-Pentanediol		$n = 5$	22.8	
α, γ-diols	1,3-Propanediol	$\alpha, \gamma\text{-}C_n(OH)_2$	$n = 3$	10.9	
	1,3-Butanediol		$n = 4$	18.9	
	3-Methyl-1,3-butanediol		MBD	19.2	
α, ω-diols	1,4-Butanediol	$\alpha, \omega\text{-}C_n(OH)_2$	$n = 4$	15.0	
	1,5-Pentanediol		$n = 5$	18.5	
	1,6-Hexanediol		$n = 6$	24.1	

*cited from Hertz HG (26).

Measurement of melting temperature of gels

A gel sample of 4 ml, containing gelatin of 5 wt/vol% and a hydroxy compound of 2 mol/l was prepared in a glass tube (diameter of 10 mm and length of 100 mm). Sample tubes were kept in a water bath controlled at 20 °C for 1 h. Then, small stainless steel balls (2 mm diameter and 60 mg weight) were placed on the top of each gel. The temperature was increased at a rate of 0.1 °C/min. The melting temperature (T_m) of the gel was defined as the temperature at which the ball passed down through the middle point of the gel [15].

Measurement of the degree of triple helix formation

The degree of triple helix formation in a dilute gelatin solution was evaluated by circular dichroism (CD) spectroscopy with a JASCO-J720 w spectro polarimeter. The ellipticity measurement was performed for gelatin solution (0.1 wt/vol%) containing a hydroxy compound of 2 mol/l at 10 °C, after being stored at the same temperature for 72 h. Relative helix content ($[C]_{rel}$) was calculated by Eq. (3) using ellipticity values of the samples at 222 nm according to the method by Nishio et al. [16].

$$[C]_{rel} = (\theta_s - \theta_h)/(\theta_h - \theta_r) \tag{3}$$

in which θ_s is the ellipticity of a gelatin solution containing a hydroxy compound and θ_h is the ellipticity of a gelatin solution without any hydroxy compounds at 10 °C after being stored at the same temperature for 72 h. θ_r is the ellipticity of a gelatin solution without any hydroxy compounds at 45 °C after being stored at the same temperature for 5 h, which is the unfolded state of gelatin chains.

Measurement of viscoelasticity of gels

Viscoelasticity of the gel was evaluated using a HAAKE RS-100 controlled stress rheometer fitted with a profiled plate (diameter of 20 mm) having a plate gap of 1.3 mm Disc-shape molded gels (diameter of 20 mm and thickness of 2 mm) were stored at 7 °C for 72 h. Storage modulus (G'), loss modulus (G'') and mechanical loss tangent (tan δ) of the gels were measured by using a linear temperature increase program (rate: 0.4 °C/min, range: from 7° to 37 °C, frequency: 1.00 Hz, stress: 5.00 Pa). Linearity of the viscoelastic behavior was checked at 10 °C across a stress range of 1–1000 Pa at a frequency of 1.00 Hz.

Measurement of spin-lattice relaxation time (T_1) of $H_2^{17}O$ in gels

Spin-lattice relaxation time (T_1) of $H_2^{17}O$ in the gel was measured using a JEOL GSX-400 spectrometer operating at 52.4 MHz at 20 °C. A sample gel of 5 ml containing gelatin of 2 ~ 6 wt/vol% and a hydroxy compound of 2 mol/l was put in a measuring tube of 10 mm diameter.

Results and discussion

Dynamic hydration number ($\mathbf{n}_{DHN}$)

The $\mathbf{n}_{DHN}$ values of each hydroxy compound are listed in Table 1. Hydroxy compounds were classified into three groups that included mono-ols, diols and polyols. Diols were further divided into α, β-diols, α, γ-diols and α, ω-diols based on the position of hydroxyl groups. $\mathbf{n}_{DHN}$ correlates closely with the restricted mobility of coordinating water molecules around a solute molecule in an aqueous solution [13, 14]. As a general rule, $\mathbf{n}_{DHN}$ increased with the increase in the number of both hydroxyl groups and carbon atoms of hydroxy compounds. However, diols such as $C_3(OH)_2$ or $C_4(OH)_2$, which have the same molecular weight, showed the diversified $\mathbf{n}_{DHN}$ values depending on the position of hydroxyl groups. This indicates that the chemical structures of hydroxy compounds directly influence the mobility of the coordinating water molecules around these hydroxy compounds.

Melting temperature of gel

Figure 1 shows melting temperatures (T_m) of the gels as a function of $\mathbf{n}_{DHN}$ of coexisting hydroxy compounds. Melting temperatures (T_m) of the gels containing polyols increased with the increase in $\mathbf{n}_{DHN}$. In contrast, T_m of the gels containing mono-ols or diols decreased with the increase in $\mathbf{n}_{DHN}$. Effects of mono-ols and diols on T_m of the gels depended on the position of hydroxyl groups. These results indicate that carbon atoms of these hydroxy compounds conversely influence the thermal stability of gelatin gels. In the case of mono-ols and diols, the thermal stability of the gels is considered to be influenced by the compensatory functions between hydroxyl groups and carbon atoms.

Triple helix formation

Figure 2 shows the relative helix content ($[C]_{rel}$) of a gelatin solution (0.1 wt/vol%) containing a hydroxy

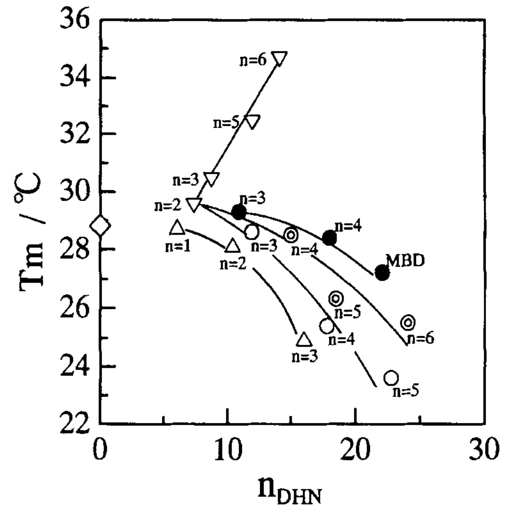

Fig. 1 Effects of the coexisting hydroxy compounds (2 mol/l) on the melting temperature (T_m) of gelatin gels (5 wt/vol%) as a function of the n_{DHN} values of these hydroxy compounds. Symbols refer to the hydroxy compounds, ◇; without additives, △; mono-ols [C_nOH], ▽; polyols [$C_n(OH)_n$], ○; α, β-diols [α, β-$C_n(OH)_2$], ●; α, γ-diols [α, γ-$C_n(OH)_2$], ◎; α, ω-diols [α, ω-$C_n(OH)_2$]

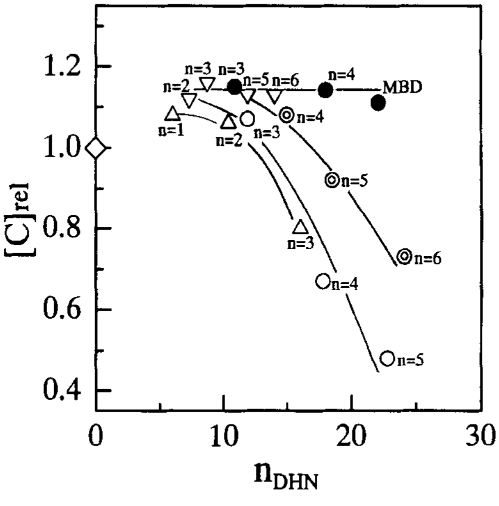

Fig. 2 Effects of the coexisting hydroxy compounds (2 mol/l) on the relative helix content ($[C]_{rel}$) of gelatin solutions (0.1 wt/vol%) as a function of the n_{DHN} values of these hydroxy compounds at 10 °C. Symbols refer to the hydroxy compounds, ◇; without additives, △; mono-ols [C_nOH], ▽; polyols [$C_n(OH)_n$], ○; α, β-diols [α, β-$C_n(OH)_2$], ●; α, γ-diols[α, γ-$C_n(OH)_2$], ◎; α, ω-diols [α, ω-$C_n(OH)_2$]

compound of 2 mol/l as a function of n_{DHN} of coexisting hydroxy compounds. For mono-ols, α, β-diols and α, ω-diols, $[C]_{rel}$ decreased with the increase in n_{DHN}. For α, γ-diols, the dependence of $[C]_{rel}$ on the n_{DHN} is small. From these results, it is considered that one possible factor that disturbs the formation of the helix is the length of the carbon chain free from the hydroxyl groups in added hydroxy compounds. Addition of polyols increased $[C]_{rel}$ of gelatin solutions regardless of their n_{DHN}. Mono-ols and diols having relatively small n_{DHN} also increased $[C]_{rel}$ similar to polyols. Gekko [17, 18] clarified that methyl alcohol and ethyl alcohol also stabilize hydrogen-bonding among polypeptide chains. Therefore, the helices among gelatin chains are considered to be strengthened by the effect of hydroxyl groups of hydroxy compounds, regardless of the number of hydroxyl groups.

It is widely accepted that the cross linking junction of gelatin gel consists of three dimensional aggregation of the helices among gelatin chains [19–23]. From that aspect, thermal stability of gelatin gel seems to be subject to the amount of aggregates among helix structures. For mono-ols, α, β-diols and α, ω-diols, positive correlation was found between $[C]_{rel}$ in the dilute solution and T_m of the gel with increasing n_{DHN}. This result indicates that addition of the hydroxy compound with longer carbon chain disturbs the helix formation and hence decreases the cross-linking junctions of the gel. However, no positive correlation was found between the effect of polyols on $[C]_{rel}$ and that on T_m. Gekko et al. [10] studied the effects of sorbitol and glycerol on the gel formation of gelatin and in the diluted gelatin solution. They reported that the order in viscosity-decreasing effect in the dilute gelatin solution (water < glycerol < sorbitol) is not consistent with the order of helix-forming ability in the solution (water < sorbitol < glycerol), but rather with that of gel-stabilizing ability. Actually, the effects of hydroxy compounds in dilute solutions do not directly manifest themselves in stabilization of the gel. On the other hand, it is known that the helix is stabilized by hydrogen bond, while both electrostatic forces and hydrophobic interactions are important in aggregation of the helices [10, 24]. Gekko et al. [17, 18] have revealed that sorbitol could more effectively enhance the intermolecular hydrophobic interaction of globular proteins compared to glycerol. Thus, sorbitol seems to promote the aggregation of the helices more effectively than glycerol. Such aggregation should be reflected in viscoelasticity of the gel.

Crosslinking junctions of gel networks

Figure 3 shows temperature dependence of the viscoelasticities of gelatin gels with various concentrations. The values of storage modulus (G') of the gelatin gels decreased with the increase in temperature and increased with the increase in gelatin concentration, meanwhile the mechanical loss tangent (tan δ) remained constant within the temperature range from 7° to 20 °C. In the case of gelatin gel, storage modulus (G') and loss modulus (G'') are considered to represent a character of crosslinking junctions

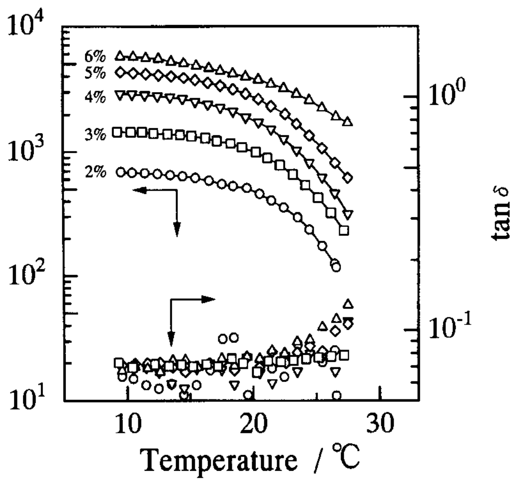

Fig. 3 Temperature dependence of the storage modulus (G') and the mechanical loss tangent (tan δ) of gelatin gel with various concentrations of gelatin (2–6 wt/vol%). Symbols refer to various concentrations of gelatin gels, ○; 2%, □; 3%, ▽; 4%, ◇; 5%, △; 6%

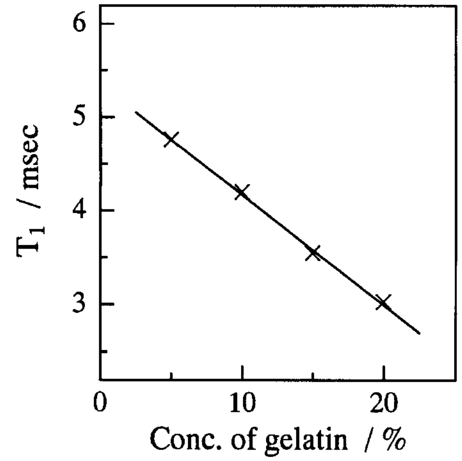

Fig. 4 Spin-lattice relaxation time (T_1) of $H_2^{17}O$ in gelatin gel as a function of gelatin concentration at 20 °C

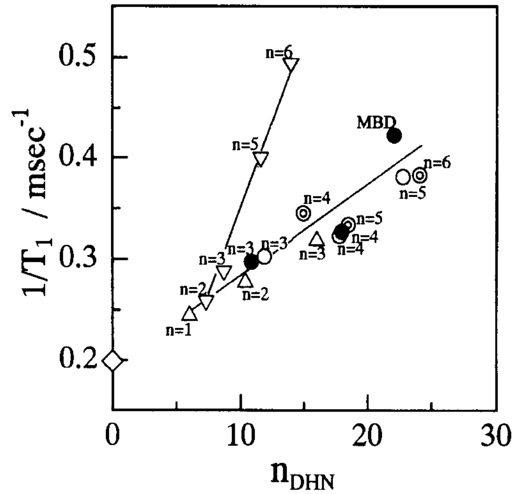

Fig. 5 Effects of the coexisting hydroxy compounds (2 mol/l) on the mobility of water molecules in gelatin gels (5 wt/vol%) as a function of the n_{DHN} values of these hydroxy compounds at 20 °C. Symbols refer to the hydroxy compounds, ◇; without additives, △; mono-ols [C_nOH], ▽; polyols [$C_n(OH)_n$], ○; α, β-diols [α, β-$C_n(OH)_2$], ●; α, γ-diols [α, γ-$C_n(OH)_2$], ◎; α, ω-diols [α, ω-$C_n(OH)_2$]

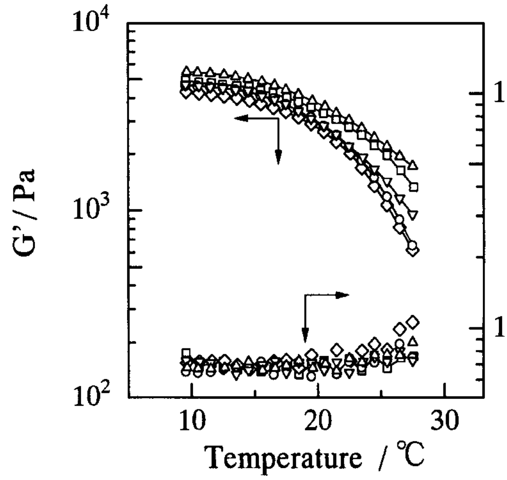

Fig. 6 Temperature dependence of the storage modulus (G') and the mechanical loss tangent (tan δ) of the gels containing gelatin (5 wt/vol%) and polyols (2 mol/l). Symbols refer to the hydroxy compounds, ◇; without additives, ○; Ethylene glycol, ▽; Glycerol, □; Xylitol, △; Sorbitol

in the gel networks and a character of the flexible chains released from the junctions, respectively [19–23]. Figure 4 shows the gelatin concentration dependence of T_1 of $H_2^{17}O$ in the gel. It is possible that an increase in the gelatin concentration leads to increase in the number of cross-linking junctions in the gel networks, and simultaneously restrains the mobility of water molecules in the gel.

Figure 5 shows $1/T_1$ of $H_2^{17}O$ in the gels containing a hydroxy compound as a function of n_{DHN} of the added hydroxy compounds. $1/T_1$ increased proportionally with the increase in n_{DHN} of mono-ols and diols. However, the addition of sorbitol was found to restrain the mobility of water molecules in the gel more strongly than diols. Figure 6 shows G' and tan δ of the gels containing polyols of 2 mol/l as a function of temperature. Polyol with large number of hydroxyl groups brought about larger values of G' and similar values of tan δ compared to the gel without any additives. Temperature dependence of G' of the gels containing sorbitol or xylitol (Fig. 6) resembles that of G' of the gels which contains gelatin more than 5 wt/vol% (Fig. 3). These results indicate that the addition of polyols with large number of hydroxyl groups increases the amount of cross-linking junctions in gelatin gel. Oakenfull et al. [9] and Nishinari et al. [25] have estimated the increase in the number of cross-linking junctions of gelatin gel by the addition of sucrose. Sorbitol seems to show a similar effect to sucrose. Thus, polyols with large number of hydroxyl groups are considered to promote the aggregation among the helices of gelatin chains, and increase the number of cross-linking junctions in the gel.

It can be concluded that network structures of gelatin gels are influenced by the compensatory influence between hydroxyl groups and carbon atoms of the coexisting hydroxy compounds as follows. The effect of carbon atoms of the hydroxy compounds is to disturb the formation of helices among gelatin chains and the aggregation among the helices, and consequently decrease the thermal stability of the gel. On the other hand, the effect of hydroxyl groups of these hydroxy compounds is to increase the helix formation among gelatin chains and the thermal stability of the gels. The addition of polyols with large number of hydroxyl groups is considered to increase the number of cross-linking junctions in the gel, which consist of the aggregation among the helices.

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