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CHEMICAL CROSS-LINKING OF GELATIN GEL

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Gelatin is cross-linked chemically using a newly designed cross-linking agent. The cross-linking reaction is made both under the gel state (at 2°C) and sol state (at 40°C) of gelatin. The swelling behaviors of the gel that prepared in the gel state show that the physical cross-linking domains in the gel are stabilizes by the chemical cross-links.

Key Words: Gelatin, Chemical cross-linking, Helix-coil transition, Sol-gel transition, Gel

INTRODUCTION

Gelatin is a typical biological polymer that prepared from collagen in the skin, the tendon, and the bone. The conformation of gelatin changes from coil to helix when the temperature is lowed from higher temperature. Then the helix regions behave as the physical cross-linking point and hence the solution transforms into the gel state [1]. Although many studies have been made to clarify the sol-gel transition of gelatin solution, the entire aspects of transition have yet to be clarified. Recently, we have designed and synthesized a new cross-linking agent as shown in Fig 1. The cross-linker prepared here is capable of reaching with the amino group on the gelatin chain. Besides, the reaction proceeds even under lower temperature such as 0°C. By using this cross-linking agent gelatin can be cross-linked both in the sol state and in the gel state. The gel, which cross-linked chemically, is insoluble in water. It, however , showd the volume phase transition in response to the changes of external conditions. It has been well established that the swelling behaviors of gels are related to the structure, the electrostatic interaction within the polymer network, and so forth. Here we report swelling behaviors of chemically cross-linked gelatin gels. Then we discuss about the relationship between the swelling behavior and the structure of polymer network.

MATERIALS AND METHODS

The sample of gelatin was obtained from Nitta Gelatin Co, Ltd. Japan. This gelatin contains 9.0 % (mol/mol) of basic amino acid in the polymer chain. The compositions of amino acid in the sample and their dissociation constants are listed in Table 1.

The cross-linkers were synthesized under

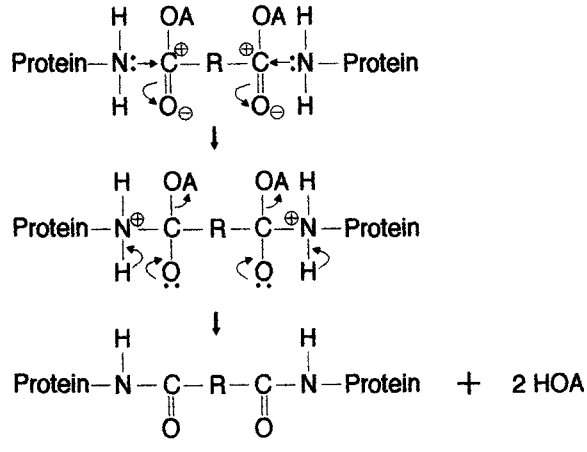

Fig. 1 The chemical structure and reaction mechanism of cross-linking agent. R represents aliphatic hydrocarbon chain. The cross-linker reacts with the amino groups of protein.

TABLE 1 The composition of amino acids in the gelatin and their dissociation constants

	Amino acid	The composition of amino acids (mol percentage)	Dissociation constants k (M) [1]	
Basic amino acid	Arginine	5.0	Basic dissociation constants	12.48
	Lysine	2.7		10.53
	Histidine	0.5		6.0
	Hydroxy-lysine	0.7		
				9.6 (N-terminal)
Acidic amino acid	Glutamic acid	0.8	Acidic dissociation constants	4.25
	Asparanic acid	4.6		3.86
	Tyrocine	0.4		10.07
				2.3 (C-terminal)
Other amino acid	Glycine	33.9		
	Prorine	13.1		
	Alanine	10.7		
	Hydroxy- prorine	0.9		
	Serine	2.9		
	Leucine	2.4		
	Valine	2.3		
	Threonine	1.6		
	Phenylalanine	1.3		
	Isoleucine	1.0		
	Metionine	0.3		

1) The dissociation constants are the values of the side chain in polypeptide, its amino-terminal and corboxyl-terminal.

appropriate conditions and then purified by re-crystallization. The details of synthesis and purification procedure will be reported elsewere.

The chemically cross-linked gelatin gels were prepared from the sol state (2°C) and from the gel state (40°C) under the various concentrations of gelatin and cross-linkers. The gelatin and the cross-linker were dissolved into the distilled and de-ionized water at desired concentrations. The pre-gel solution was kept at 40°C for 1hr to ensure the complete dissolution. Then triethylamine was added to the solution, which initiatethe cross-linking reaction, and kept for one day at 40°C. The chemically cross-linked gelatin gels, which preparated from the solution state, were obtained by this procedure. On the other hand, the pre-gel solution was cooled at a temperature of 2°C. The solution was kept for one day to ensure the complete gelation by the physical cross-linking. Then the gelatin gels, which are in the gel state, were soaked in the triethylamine solution to initiate the chemical cross-linking reaction. The gels were kept at 2°C for two days to ensure the complete reaction. In both cases, the micripipetts of known diameter (do=261 μm) were immersed into the reaction solution to obtain the cylindrical sample gels. The gels thus obtained were taken out of micropipetts, washed the residual chemicals, and then used further experiments.

The equilibrium diameter d of the gel was measured as a function of pH and the concentration of urea. Then the swelling ratio of the gel was calculated as follows.

$$\frac{V}{V_o} = \left(\frac{d}{d_o}\right)^3$$

RESULTS AND DISCUSSION

Figure 2 shows the pH dependence of the swelling ratio of chemically cross-linked gelatin gels. The swelling ratios of both gels, which prepared from solution state and gel state of gelatin, show similar pH dependence. It is clear from this figure that both gels

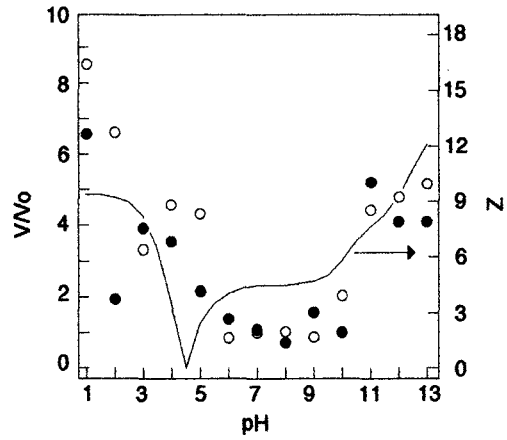

Fig. 2 The pH dependence of swelling ratio of the chemically cross-linked gelatin gel. The symbols indicate ○: gel cross-linked in the solution state and ●: gel cross-linked in the gel state. Solid line indicates the pH dependence of the effective number of charge on the gelatin chain.

swell at higher and at lower pH regions. The gels, in contrast, collapses at intermediate pH region near the neutral pH. This swelling behavior of the gel is the characteristic of the amphoteric polyelectrolyte gels. It is obvious that gelatin contains both the acidic amino acid residues and the basic amino acid residues as shown in Table 1. The basic amino acid residues are charged when the pH of external solution in lowered. On the other hand, the dissociation of acidic amino acids residues is restricted at lower pH regions. The polymer chains are, therefore, positively charged at lower pH regions. In contrast, the polymer chain is negatively charged at higher pH regions because the basic amino acid residues are un changed and the acidic amino acid residues are charged. It may be natural to assume that the swelling behaviors of the gelatin gels are effected by the dissociation of acidic and basic amino acid residues. The mean field theory of the volume phase transition yields that the swelling ratio of the gel can be written as follows [2].

$$\tau \equiv 1 - \frac{\Delta F}{kT} = \frac{2\upsilon\nu_0}{N\varphi_0^{\,2}}\left[\left(\frac{\varphi}{\varphi_0}\right)^{-1/3} - \left[f + \frac{1}{2}\right]\left(\frac{\varphi}{\varphi_0}\right)^{-1}\right] + 1 + \frac{2}{\varphi} + \frac{2\ln(1-\varphi)}{\varphi^2},$$

Here, N is the Avogadro's number, k is the Boltzman constant, T is the temperature, υ is the molar volume of the solvent, φ is the volume fraction of the polymer network, ΔF is the excess free energy for the association between polymer segment and solvent, φ_o is the volume fraction of the polymer network at the reference state, ν_o is the number of the elasticity active chains per unit volume at φ_o, and f is the number of dissoiciated counter ions per effective chain. In the present case, the number of change f on the polymer chain plays essential roles in the swelling behaviors of the gel in the acidic and in the basic solution. Since the dissociation constants of amino acids are known, the number of effective charge on the polymer chain can be calculated by the following equation [3].

$$Z = -\sum_i \frac{a_i k_i}{[H^+] + k_i} + \sum_j \frac{b_i [H^+]}{[H^+] + k_j}$$

Here, a_i, b_j, k_i, k_j, and $[H^+]$ represent the number of acidic amino acid residues of i species, the number of basic amino acid residues of j species, the dissociation constant of i species, the dissociation constant of j species and the concentration of proton. By using the dissociation constants that listed in the Table 1, the number of effective charges on the polymer chain is calculated as a function of pH. The results are also shown in Fig 2. It is clear from these results that the number of positive charge and that of negative charge cancels out at pH 4.5 which is the isoelectric point of the gelatin. The pH dependence of swelling ratio of the gel corresponds to that of the number of effective charge on the polymer chain qualitatively. These results strongly suggest that the swelling behaviors of the chemically cross-linked gelatin gels are mainly determined by the electrostatic interaction of the charges on the polymer chain.

The pH dependence of the swelling ratio of gel, however, shows substantial deviation from the pH dependence of the number of charge on the chain. These results, together with the mean field theory of volume phase transition, suggest that the gel collapses only at the isoelectric point. However, the gel collapses

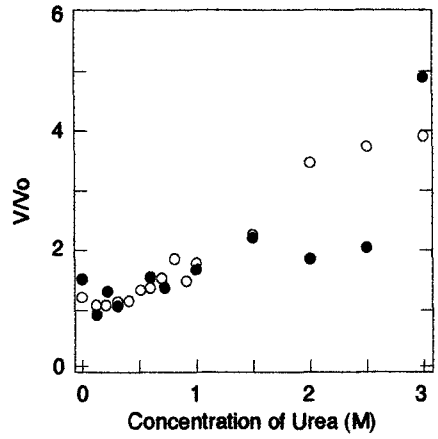

Fig. 3 The urea concentration dependence of the swelling ratio of chemically cross-linked gelatin gel. The symbols indicate ○: gel cross-linked in the sol state and ●: gel cross-linked in the gel state.

in a certain pH region that ranging from pH 6 to 9. Besides, the collapsed region is shifted to higher pH region than the isoelectric point of the gelatin. The difference in the isoelectric point and collapsed region of the gel may be explained as follows. Since the cross-linker reacts with the basic amino acids, it decreases the total number of positive charge in gelatin. Hence, the isoelectric point of the cross-linked gelatin may shift to higher pH region than the intact gelatin. On the other hand, it may be required to assume the presence of attractive interaction between polymer chains to explain the appearance of the collapsed region in the cross-linked gel. The hydrogen bonding between polymer chains may be a candidate for such attractive interaction. The swelling ratio of the gel is, therefore, measured as a function of urea concentration because urea disrupts the hydrogen bond.

In Fig 3, the swelling ratios of the gel are illustrated as a function of the concentration of urea in the external solution. The gel swells upon increasing urea concentration. The swelling of the gel suggests the disappearance of attractive interaction. These results indicate that the hydrogen bonds are formed within the polymer network of the gel at neutral pH. The swelling ratios of these gels are almost the same at lower concentrations of urea. The gel that prepared from the sol state of gelatin swells extensively at the concentration of urea around 2M. On the other hand, the gel, which prepared from the gel state of gelatin, swells at the concentration of urea about 3M. These results indicate that the hydrogen bonds that formed in the cross-linked gelatin gel that prepared from the gel state are much stable than that prepared from the sol state. These results are intuitively explained as follows. The cross-linking reaction occurs between random coil of gelatin chain at sol state. Since the network chains are cross-linked, the helix-coil transition is restricted even if the temperature is lowered below the helix-coil transition temperature. In this case, the hydrogen bonds are randomly formed between neighboring polymer chains. On the other hand, when the cross-linking reaction is made at the gel state of gelatin, the physical cross-linking points are already present in the gel. The physical cross-link of the gelatin is built-up by the bundle of triple helix that stabilized by the hydrogen bonding. In such case, the disruption of the hydrogen bonds occurred cooperatively. Hence, the higher concentration of urea is required to disrupt the hydrogen bonds in the gel that cross-linked in the gel state of gelatin.

CONCLUSION

Gelatin is cross-linked chemically both at the sol state and gel state of gelatin. The pH dependence of swelling ratio is qualitatively explained by the dissociation of the acidic and the basic amino acid residues. Besides, the results suggest the presence of hydrogen bonding within the gel. The urea concentration dependence of the swelling ratio indicates that the hydrogen bonds are much stable in the gel prepared from the gel state of gelatin than that in the gel prepared from the sol state of gelatin. The results indicate that the entire network structure is stabilized by the presence of both chemical and physical cross-links.

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