Characterization of Cross-links Introduced in Gelatin

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ABSTRACT: A method for the determination of the effectiveness of gelatin hardeners was proposed. Formaldehyde (FA) and 1,3-bis(vinylsulfonyl)-2-propanol (BVSP) were used as cross-linking agents for the hardening of gelatin. The cross-linked gelatin showed a typical rubber elasticity in a swollen state with a mixed solution composed of concentrated aqueous LiBr and diethylene glycol monoalkyl ether. The energy components to total retractive forces were below 0.3. The amount of intermolecular cross-links was estimated by applying an usual rubber elasticity theory for the swollen samples. The number of cross-linking sites was also determined by the amino acid analysis of BVSP cross-linked gelatin. The cross-linking efficiency for the reaction of BVSP occurring in a casting film with less swelling conditions was about four times higher than in a swollen gel. Approximately 92% or more of BVSP used was reacted with the free amino groups of lysine and arginine residues. There was a significant difference between the reactivities of these amino groups. The amino groups of lysine residues were more reactive than those of arginine in a gel state; the reactivities, however, reversed in a thin layer of wet film. The effectiveness of FA was extremely low. © 1997 John Wiley & Sons, Inc. J Appl Polym Sci **64:** 1879–1892, 1997

Key words: formaldehyde; 1,3-bis(vinylsulfonyl)-2-propanol; rubber elasticity; cross-linking efficiency; lysine and arginine residues

INTRODUCTION

The fibrous proteins of collagen, keratin, and elastin are complex molecular composites in nature, and their unique properties are closely related to the cross-links occurring in the proteins. A quantitative method for the determination of the cross-link density by measuring the modulus of elasticity of the swollen fibers has been proposed by one of the present authors.[1–5] The number, type, and location of the cross-links in keratin and collagen fibers have been demonstrated widely by combining the results of the force–extension relation of the swollen fibers and the chemical analysis of the groups being close to participate in cross-linkages in the fibers.[6–8]

The cross-linking process of gelatin is important, especially in the medical and photographic industries related to the technology of the hardening of gelatin that has made possible the use of higher solution temperatures. The methods that have been proposed for determining the effectiveness of hardeners are as follows: 1) swelling measurements, e.g., the thickness of a hardened layer versus an unhardened control after immersion in water; 2) melting point, which is determined at a standard rate of heating; and 3) abrasion resistance. The correlation among these different methods is often poor, even in a homologous series of hardeners.[9]

There have been no reliable quantitative methods for determining the degree of cross-linking in gelatin, but there are relative ones, even if they were available to practical use. To determine the cross-linking efficiency going to intermolecular linkage, it is particularly important to analyze the relationship between the structure and properties

of cross-linked gelatin. A mixed solution composed of concentrated aqueous LiBr solution and diethylene glycol monoalkyl ether has a peculiar property for protein fibers, showing a good swellability due to the breaking of hydrogen bonds, the weakening of hydrophobic interaction forces, and the decrease in the reactivity of the functional side chain groups, e.g., the retarding or inhibiting of the thiol/disulfide interchange reactions in keratins,[1] resulting in a stable swollen network of rubbery protein chains.

The aim of this study was to investigate a swelling medium showing an ideal or nearly ideal rubber elasticity for cross-linked gelatin, to determine the cross-link density of gelatin treated with formaldehyde and 1,3-bis(vinylsulfonyl)-2-propanol(BVSP), to evaluate the amounts of inter- and intramolecular cross-linkages, and to clarify the cross-linking site of the residues reacted with BVSP by means of the amino acid analysis of the cross-linked gelatin. The bifunctional BVSP reacts with free amino groups in protein to form the stable C—N bonds to hydrolysis with strong HCl for amino acid analysis.[10] It is of interest to obtain a knowledge concerned with the reaction of BVSP with gelatin since a homologous series of the compounds has been widely used as one of the excellent hardeners in the photographic field.[11]

EXPERIMENTAL

Materials

The gelatin used was a alkali processed bone gelatin with the average molecular weight of 1.1×10^5, which was determined viscometrically by using the equation $[\eta] = 0.64 M_w^{0.38}$, where $[\eta]$ is the viscosity number (mL/g) measured in a molar potassium thiocyanate solution at 40°C.[12] 1,3-Bis-(vinylsulphonyl)-2-propanol (BVSP) used as a cross-link agent was synthesized according to the method of Sera et al.,[13] and the compound purified by recrystallization in ethanol was confirmed by using H-NMR and ^{13}C-NMR, as reported previously.[8] Analytical reagent formaldehyde (FA) solution containing not less than 37% was as received from Wako Chemicals. Lithium bromide used was a reagent grade. Diethylene glycol mono-alkyl ether (AC), methyl (MC), ethyl (EC), and n-butyl (BC) were obtained by distillation of commercial products under reduced pressure, as described in a previous article.[1]

Preparation of Gelatin Film Cross-linked with BVSP

About 8 mL of a mixed solution composed of 25 g of 10% aqueous gelatin solution and 2.5 mL of 0.1M, or 0.05M BVSP acetone solution was cast on a glass plate (11.9 × 16.4 cm) at 45°C, air-dried, and then immersed into 2% sodium carbonate solution for 24 h at 25°C to complete the cross-linking reaction and followed by washing with water. The film was stripped from the glass plate in a wet state and air-dried. The film thus obtained was about 30 μm in thickness. The film was then cut into about 1 mm in width and 40 mm in length and subjected to mechanical tests after swelling. The amount of BVSP used for cross-linking reaction of gelatin was 100 or 50 μmol/g of gelatin.

Preparation of Block Form Gelatin Cross-linked with BVSP

A well-mixed solution composed of 50 g of 10% aqueous warm gelatin solution and 5 mL of 0.1M BVSP acetone solution was poured into a 100 mL Pyrex™ beaker, covered with a glass plate, and followed by standing for 5 h at room temperature. The gelatin gel formed in the beaker was cut by a blade into a block form with about 1 mm in thickness, 2 mm in width, and 40 mm in length. These samples were directly immersed into 2% sodium carbonate solution for 24 h at 25°C, washed with water, and then stored in an aqueous solution containing 50% 2-propanol. The amount of BVSP used for cross-linking reaction of gelatin was 100 μmol/g of gelatin.

Preparation of Gelatin Film Cross-linked with FA

About 8 mL of 10% aqueous gelatin solution was cast on a casein-coated glass plate (11.9 × 16.4 cm), which had been treated previously with a 5% solution of milk casein containing a small amount of aqueous ammonia to prevent adhesion of dry gelatin to glass. The gelatin film obtained was treated in a mixed solution composed of 25% volume of 37% FA solution and 75% methanol for 10 h at 20°C, thoroughly washed with water, and air-dried. The film obtained was subjected to chemical analysis of the amount of FA reacted with gelatin and the mechanical tests for swollen film.

Amino Acid Analysis

The cross-linked samples used for amino acid analysis were both film and block form gelatin

treated with BVSP at the concentration of 100 μmol/g of gelatin. The untreated and BVSP cross-linked gelatin samples were hydrolyzed with HCl for 24 h at 110°C in deaerated condition and dried under reduced pressure. The amino acids in hydrolyzate were converted to their n-butyl esters of N-trifluoroacetyl (BTFA) derivatives according to a method of Gehrke et al.[14] The amino acid compositions were determined by a gas-liquid chromatography (GLC) macro level method using 0.32 w/w% ethylene glycol adipate on chromosorb G (EGA) and 1.5 w/w% phenyl methyl silicone (OV-17) columns for separation of BTFA derivatives.[14,15] A Hitachi Model G-3000 gas chromatograph equipped with hydrogen frame ionization detectors was used.

Combined FA Content of Film

The FA-treated film washed with boiling water for 30 min was hydrolyzed by allowing it to stand overnight in 35% sulfuric acid solution. The released FA was distilled off with water vapor in a solution containing excess sodium bisulfite to form FA-bisulfite adduct, and the residual bisulfite ions in the solution was oxidized with $0.1M$ iodine solution, and the solution was acidified, then back-titrated with the iodine solution to determine the FA content of the sample.[16,17] The FA content of the film used for the present experiment was found to be 1.33% by weight, which corresponds to 440 μmol/g of gelatin.

Preparation of Swollen Sample

The gelatin samples were swollen at room temperature for 24 h in a mixed solution composed of $8M$ LiBr aqueous solution and AC. The swollen samples were then subjected to mechanical measurements. The cross-sectional area of swollen samples S was determined by eq. (1), as follows[8]:

$$S = (W_o/L_s\rho)\,\nu_2^{-1} \qquad (1)$$

where W_o is the weight of dry sample; L_s, the swollen length of the sample; ρ, the density of dry sample; and ν_2, the volume fraction of gelatin in swollen sample. The ν_2 value was determined by assuming the additivity of specific volume of gelatin and diluent in the sample, as in eq. (2):

$$\nu_2 = W_o\rho_d/[W\rho - W_o(\rho - \rho_d)] \qquad (2)$$

where W is the weight of swollen sample equilibrated at 25°C, which was measured after removing an excess liquid on the surface of the sample by a filter paper; and ρ_d, the density of the mixed solution.

Stress–Strain Measurements

The stress–strain relations of the swollen sample of about 20 mm length in the mixed solution were measured at a constant temperature and at a constant extension speed of 2 mm/min using the same apparatus as reported in a previous article.[8] Stresses referred to the swollen cross-sectional area were used.

Stress–Temperature Relationship

Equilibrium forces were measured at constant temperatures and at constant strains. Strain ratios λ were expressed by eq. (3), as follows:

$$\lambda = 1 + L/L_0 \qquad (3)$$

where L is the strained length of the sample, and L_0 is the unstrained zero length of the sample equilibrated at the measurement temperature. The equilibrium forces f and λ relationships were constructed at constant temperatures. The temperature was varied at intervals of about 5 to 10°C in temperature-descending and -ascending processes. The relationships between the temperature and the equilibrium forces at constant strain ratios were thus obtained. The detailed experimental procedure was described in a previous article.[8]

RESULTS

Rheological Properties of Cross-linked Gelatin Film in Swollen State

Figure 1 shows the stress relaxation–time curves at different temperatures for the swollen gelatin films cross-linked with BVSP at 50% extension in a mixed solution composed of 40% $8M$ LiBr aqueous solution and 60% BC by volume. Variation of the forces in the initial relaxation stage is more or less characteristic of viscoelastic bodies. As temperature is raised, the magnitude of the stress in the latter stage is considerably increased at the temperatures below about 50°C. This suggests that the stress produced in the swollen film is a rubbery
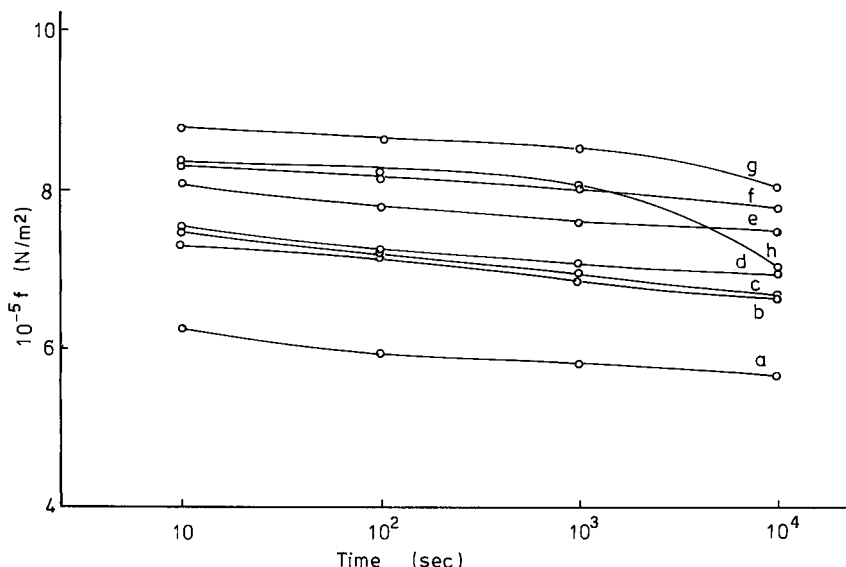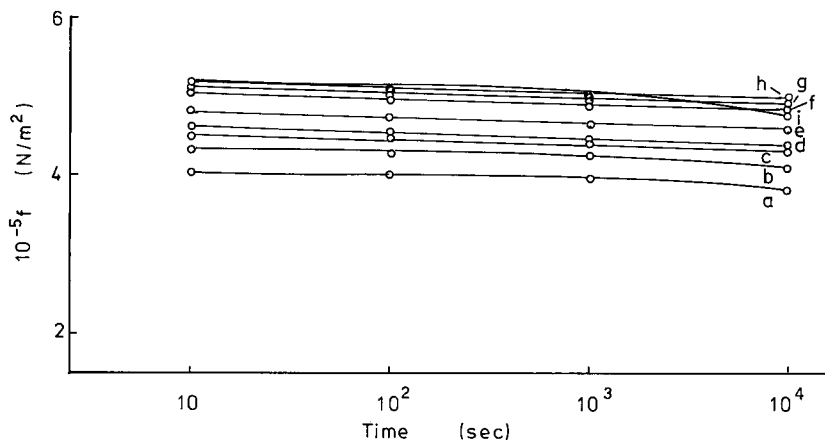
Figure 1 Stress relaxation curves at different temperatures for BVSP cross-linked gelatin film at 50% extension state under swelling in a mixed solution composed of 40% 8M LiBr and 60% BC by volume. Temperature (°C): (a) 1.0, (b) 18.5, (c) 27.3, (d) 36.0, (e) 43.5, (f) 52.4, (g) 61.2, and (h) 71.5.

force that is entropic. At the temperature around 70°C, the rate of relaxation increases at 10^3 s and the force at 10^4 s is greatly decreased more than the forces at lower temperatures. This may be due to a chemical stress–relaxation resulting from the hydrolytic breakage of covalent bonds occurring in the swollen network chains since the cross-link introduced by the reaction of BVSP with a basic

amino group is stable[10] and seems to be unlikely to be decomposed at this temperature.

Figure 2 shows the stress relaxation–time curves at different temperatures for swollen gelatin films cross-linked with BVSP at 50% extension in a mixed solution composed of equal volumes of 8M LiBr aqueous solution and BC. The stress relaxation scarcely occurs over the range of the

Figure 2 Stress relaxation curves at different temperatures for BVSP cross-linked gelatin film at 50% extension state under swelling in a mixed solution composed of equal volumes of 8M LiBr and BC. Temperature (°C): (a) 0.8, (b) 7.5, (c) 15.5, (d) 22.1, (e) 30.2, (f) 37.5, (g) 44.8, (h) 52.0, and (i) 60.0.
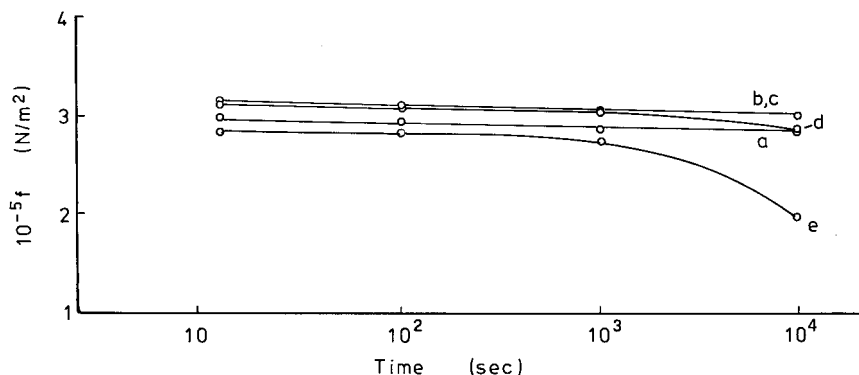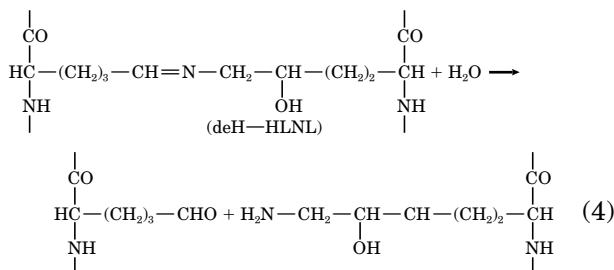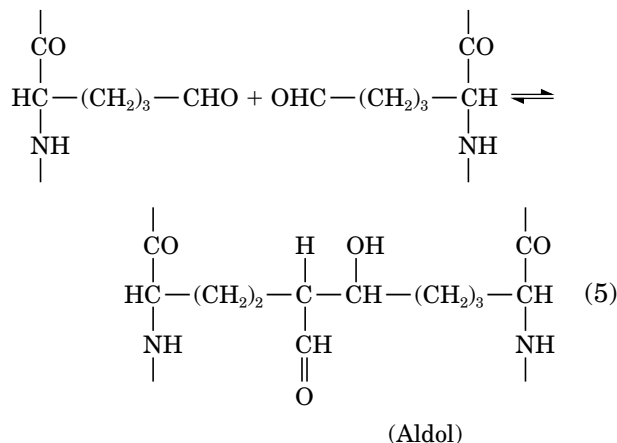
Figure 3 Stress relaxation curves at different temperatures for BVSP cross-linked gelatin film at 50% extension state under swelling in a mixed solution composed of 60% 8M LiBr and 40% BC by volume. Temperature (°C): (a) 21.1, (b) 29.8, (c) 41.7, (d) 51.6, and (e) 62.8.

time measured except in the case of the higher temperature of 60°C, and the magnitude of the stress increases with the increase of the temperature. This behavior is similar to that of typical rubbers and elastomers. However, a great decay of the stress is observed at 60°C and above 10^3 s.

Figure 3 shows the stress relaxation–time curves at 50% extension for the swollen gelatin films cross-linked with BVSP in a mixed solution composed of 60% 8M LiBr aqueous solution and 40% BC by volume. At the temperatures around 50 and 60°C, a considerable stress relaxation is observed. In contrast to the swelling systems as described above with higher content of BC, the entropy component of the elastic stress tends to decrease even at lower temperatures in order to enhance the chemical stress–relaxation, which may be due to the cleavage of the hydrolytically unstable bonds in the branched regions of highly ramified molecules of gelatin, which are derived from the cross-linked tropocollagens.[12] One is a hydrolytic decomposition of dehydro-hydroxylysi-nonorleucine (deH-HLNL) (Schiff base) cross-links present in native collagen.[18,19] The Schiff base cross-links are quite easily hydrolyzed back to amines as shown in eq. (4)[20]:

$$
\begin{array}{c}
\underset{\underset{NH}{|}}{\overset{\overset{CO}{|}}{HC}}-(CH_2)_3-CH=N-CH_2-\underset{\underset{(deH-HLNL)}{OH}}{\overset{}{CH}}-(CH_2)_2-\underset{\underset{NH}{|}}{\overset{\overset{CO}{|}}{CH}} + H_2O \longrightarrow \\[2em]
\underset{\underset{NH}{|}}{\overset{\overset{CO}{|}}{HC}}-(CH_2)_3-CHO + H_2N-CH_2-\underset{\underset{OH}{|}}{\overset{}{CH}}-CH-(CH_2)_2-\underset{\underset{NH}{|}}{\overset{\overset{CO}{|}}{CH}} \quad (4)
\end{array}
$$

Another is an interchange reaction due to an equilibrium process of aldol cross-linkages under the strained network, as shown in eq. (5):

$$
\begin{array}{c}
\underset{\underset{NH}{|}}{\overset{\overset{CO}{|}}{HC}}-(CH_2)_3-CHO + OHC-(CH_2)_3-\underset{\underset{NH}{|}}{\overset{\overset{CO}{|}}{CH}} \rightleftharpoons \\[2em]
\underset{\underset{NH}{|}}{\overset{\overset{CO}{|}}{HC}}-(CH_2)_2-\underset{\underset{\underset{O}{\|}}{CH}}{\overset{H}{C}}-\underset{}{\overset{OH}{CH}}-(CH_2)_3-\underset{\underset{NH}{|}}{\overset{\overset{CO}{|}}{CH}} \quad (5)
\end{array}
$$

(Aldol)

The aldol cross-linkage occurs as an intermediate of the stable cross-links in collagen.[18,19]

As the BC content in the mixed solution increases the value of ν_2, being inversely proportional to the degree of swelling for the cross-linked film, greatly increases, namely, 0.332, 0.380, and 0.625 for 40, 50, and 60% BC content, respectively. The increase of the BC content leads to an increase in the interaction forces arising between the network chains and to a decrease in the rate of reactions associated with the chemical stress relaxation. For the estimation of cross-link density by mechanical means, it is primarily necessary to confirm the stability of network under extension.

Figure 4 shows the stress relaxation–time
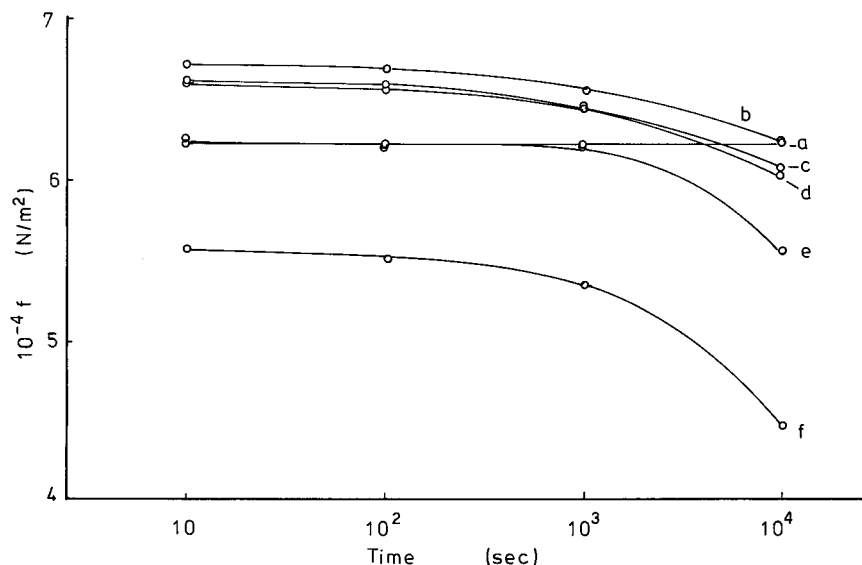
Figure 4 Stress relaxation curves at different temperatures for FA cross-linked gelatin film at 30% extension state under swelling in a mixed solution composed of equal volumes of $8M$ LiBr and BC. Temperature (°C): (a) 6.0, (b) 14.0, (c) 22.2, (d) 30.0, (e) 38.4, and (f) 46.0.

curves at 30% extension for gelatin films cross-linked with FA in a mixed solution composed of equal volumes of $8M$ LiBr and BC. At the temperature of 6°C, no substantial change of the stress is observed over the all range of the time measured. It is noted, however, that stress relaxation occurs and the rate of relaxation increases markedly with an increase of the temperature. This suggests that the network becomes unstable at temperatures over about 10°C. This may be due to the chemical stress relaxation caused by the hydrolytic cleavage of the cross-links formed in gelatin.

The reaction of FA with wool proteins, which probably indicates most clearly the types of compound formed with gelatin, were studied under extremely mild conditions by Caldwell and Milligan[21] using ^{14}C-FA; and the product was enzymatically hydrolyzed. Only two products were separated, and the compounds were identified as δ-N-hydroxymethylglutamine and ε-N,ε-N'-methylenedilysine. The latter represents a type of methylene cross-link. Davis and Tabor[22] showed that the first stage in the formation of a FA cross-link is the reversible reaction of a FA molecule with a side chain amino group to form a methylol compound. This compound then reacts with the methylol compound of another group to give a cross-link such as methylene bridge ($-CH_2-$) or methylene ether cross-link ($-CH_2-O-CH_2-$).

Jopling[23] kinetically studied an FA-treated gelatin film by using a method of intermittent stress relaxation in a solution mixture of 85% methanol and 15% water at 60°C. From the fact that methylene ether cross-links are broken under stress but reformed under the unstressed state, he pointed out that the cross-linking reaction is reversible, as shown in eq. (6):

$$-NH-CH_2-OH + HO-CH_2-NH- \rightleftharpoons$$
$$-NH-CH_2-O-CH_2-NH- + H_2O \quad (6)$$
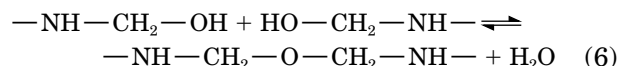
Further suggestion was also made that the methylene cross-link is more stable than the methylene ether cross-link.

A marked stress relaxation observed in our experiments at a relatively lower temperature is considered to be due to the cleavage of hydrolytically unstable methylene ether cross-linkage formed in gelatin. It should be emphasized that the scission of the unstable cross-links could be completely inhibited even at 30% extension state in the solution composed of equal volumes of $8M$ LiBr and BC at 6°C.

Thermal Expansion Coefficient

Figure 5 shows the change of the unstrained zero length (L_0) with temperature. The length of the BVSP cross-linked gelatin film is slightly ex-
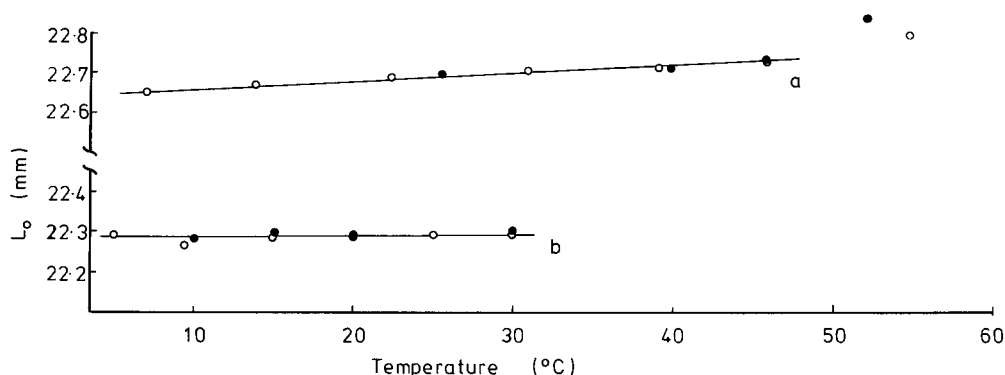
Figure 5 Relationships between unstrained zero length L_0 and temperature for BVSP cross-linked gelatin film and block swollen in a mixed solution composed of $8M$ LiBr and BC. (a) Swollen film in 50% $8M$ LiBr and 50% BC; (b) swollen block in 55% $8M$ LiBr and 45% BC by volume: ($\bigcirc$) temperature descending; ($\bullet$) temperature ascending.

panded in the mixed solution composed of equal volumes of $8M$ LiBr and BC [Fig. 5(a)]. The calculated linear coefficient of thermal expansion was 7.79×10^{-5} deg^{-1}. As suggested from the stress relaxation curve shown in Figure 2, the plots at the temperature above 50°C deviate from the straight line. In the temperature range from 5 to 50°C, straight lines were obtained for the BVSP cross-linked film in both diluent systems of $8M$ LiBr/EC and $8M$ LiBr/MC. The linear thermal expansion coefficient was 2.17×10^{-4} for the former and 1.58×10^{-4} for the latter. A similar order of magnitude in the thermal expansion was obtained for the block form sample in the corresponding diluent system.

It was found that the decrease in the content of BC tends to decrease the thermal expansion coefficient. In a diluent system of $8M$ LiBr/BC being 55/45, the thermal expansion coefficient for block form sample was approximately zero, as shown in Figure 5(b). However, the linear relation only exists up to about 30°C, suggesting that either the change in the compositions of diluent in the sample or the decrease in the stability of swollen network occurs at the temperature above 30°C.

Force–Temperature Relationship

Figure 6 shows the force–temperature curves for BVSP cross-linked film in a mixed solution composed of equal volumes of $8M$ LiBr and BC. Even at the higher strain ratios, linear relationships are obtained. Figure 7 shows the force–temperature curves of the BVSP cross-linked block form sample in a mixed solution composed of $8M$ LiBr/

BC = 55/45 (%v/v). The data taken at decreasing and increasing temperature coincide on the straight lines at constant extensions.

DISCUSSION

Calculation of f_e/f for Swelling System

For simple elongation of the swollen sample, the thermodynamic relationships at a fixed composition is given by eq. (7), as follows:

$$f = (\partial E/\partial L)_{V,T,N} + T(\partial f/\partial T)_{V,L,N} \qquad (7)$$

where f is the total force; L, the length of the specimen; and the subscript N denotes the constancy of composition. The internal energy contribution f_e is therefore shown by eq. (8), as follows:

$$f_e \equiv (\partial E/\partial L)_{V,T,N} = f - T(\partial f/\partial T)_{V,L,N} \quad (8)$$

From the elastic equation of state for the Gaussian network, f is expressed by eq. (9), as follows[24]:

$$f = (\nu kT/L_i)(\langle r^2\rangle_i/\langle r^2\rangle_0)(\alpha - \alpha^{-2}) \qquad (9)$$

where ν is the number of chains in the sample; and k, the Boltzmann constant. Here, the strain ratio $\alpha = L/L_i$, where L_i is the length in the isotropic state at the volume V corresponding to the deformed sample of length L. The quantity $\langle r^2\rangle_i$ is defined as the mean square length of the chains in the undistorted state of the network at volume V, and the indepen-
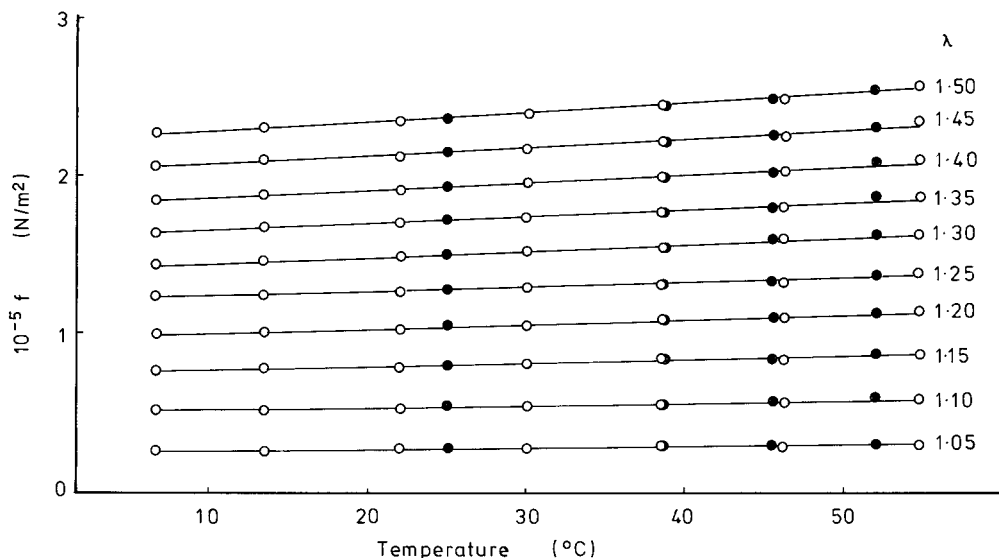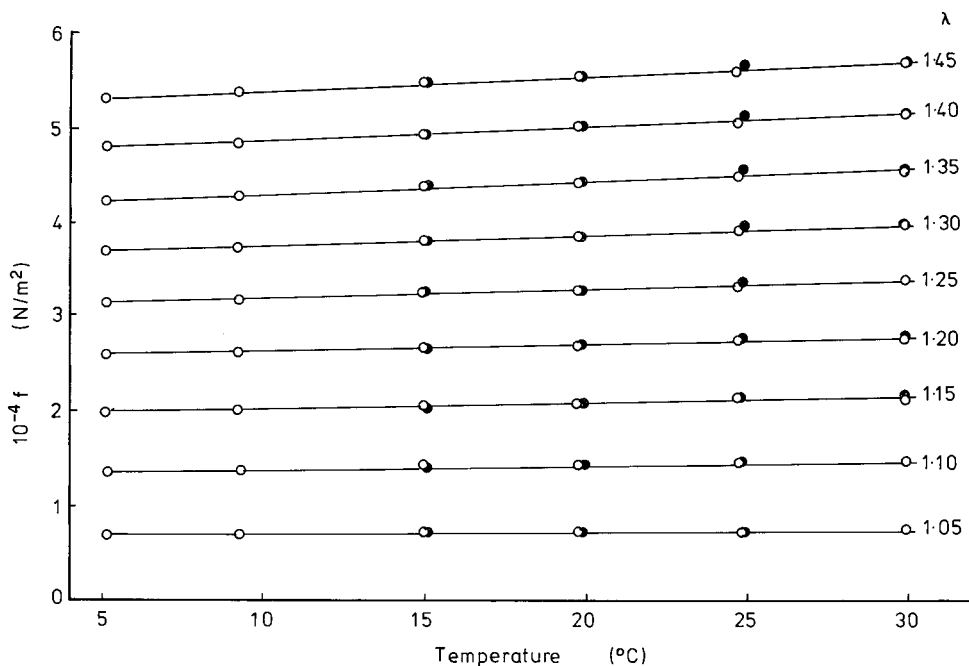
Figure 6 Relationship between equilibrium stress f and temperature at different extension ratio λ for BVSP cross-linked gelatin film swollen in a mixed solution composed of 50% $8M$ LiBr and 50% BC by volume: ($\bigcirc$) temperature descending; ($\bullet$) temperature ascending.

dent quantity $\langle r^2 \rangle_0$, represents the mean square length of the corresponding free chains.

The quantity of force–temperature coefficient at a constant pressure and a constant strain ra-

tio, and at swelling equilibrium $(\partial f/\partial T)_{P,\alpha,\text{eq}}$ must be converted to $(\partial f/\partial T)_{V,L,N}$. According to Hoeve and Flory,[25] the relationship between the two quantities may be obtained from the rela-

Figure 7 Relationship between equilibrium stress f and temperature at different extension ratio λ for BVSP cross-linked gelatin block in a mixed solution composed of 55% $8M$ LiBr and 45% BC by volume: ($\bigcirc$) temperature descending; ($\bullet$) temperature ascending.

tionship $f = g(T, L, V, N)$ as in eq. (10), as follows:

$$(\partial f/\partial T)_{P,\alpha,\text{eq}} = (\partial f/\partial T)_{V,L,N}$$

$$+ (\partial f/\partial L)_{T,V,N}(\partial L/\partial T)_{P,\alpha,\text{eq}}$$

$$+ (\partial f/\partial N)_{T,L,\text{eq}}(\partial N/\partial T)_{P,\alpha,\text{eq}} \quad (10)$$

Hoeve and Flory[25] argue that the last term of the right-hand side of eq. (10) is negligible. The quantity $(\partial L/\partial T)_{P,\alpha,\text{eq}}$ $[=\alpha(\partial L_i/\partial T)_{P,\alpha,\text{eq}}]$ is given by eq. (11):

$$(\partial L/\partial T)_{P,\text{eq}} = \alpha V^{1/3}\beta_{\text{eq}}/3 \quad (11)$$

where β_{eq} is the bulk coefficient of thermal expansion. The quantity $(\partial f/\partial L)_{T,V,N}$ is obtained by differentiating eq. (9) as eq. (12), as follows:

$$(\partial f/\partial L)_{T,V,N} = f/\alpha V^{1/3} \quad (12)$$

If this is the case, we obtain eq. (13):

$$(\partial f/\partial T)_{V,L,N} = (\partial f/\partial T)_{P,\alpha,\text{eq}} - f\beta_{\text{eq}}/3 \quad (13)$$

The ratio of the energy component to the total equilibrium force can be therefore expressed by eq. (14):

$$f_e/f = 1 - (T/f)(\partial f/\partial T)_{P,\alpha,\text{eq}} + T\beta_{\text{eq}}/3 \quad (14)$$

In our present experiment, the force-temperature coefficient actually measured $(\partial f/\partial T)_{P,\lambda,\text{eq}}$, which will differ from $(\partial f/\partial T)_{P,\alpha,\text{eq}}$, but the difference is only to a negligible extent.[26] When this assumption is made, eq. (14) is rewritten by eq. (15), as follows:

$$f_e/f \cong 1 - (T/f)(\partial f/\partial T)_{P,\lambda,\text{eq}} + T\beta_{\text{eq}}/3 \quad (15)$$

At $\beta_{\text{eq}} = 0$, f_e/f is expressed simply by eq. (16):

$$f_e/f \cong 1 - (T/f)(\partial f/\partial T)_{P,\lambda,\text{eq}} \quad (16)$$

Table I shows the result obtained for gelatin film cross-linked with BVSP in a mixed solution composed of equal volumes of $8M$ LiBr and BC. The quantity f_e/f calculated by eq. (15) is almost independent of extension ratios, and the average value is 0.29, which is similar to the values reported for cross-linked natural rubbers,[26,27] and swollen collagens[8] and keratins[2,3] in the concentrated LiBr and BC systems.

Table II shows the result obtained for the block form gelatin cross-linked with BVSP when the mixed solution of $8M$ LiBr/BC = 55/45 (%v/v) was used as a swelling solution. The average value for f_e/f estimated by applying eq. (16) is 0.20. These results show that an usual rubber elasticity theory for swollen network polymer can be applicable to the cross-linked gelatin in the diluent system of $8M$ LiBr-BC.

Degree of Cross-links in Gelatin

The degree of cross-links of an elastic network in a swollen system can be deduced from the relationship between the stress referred to the swollen cross-sectional area in equilibrium f and the strain λ. The formula may be written by eq. (17), as follows[26,28]:

$$f = G(\lambda - \lambda^{-2}) \quad (17)$$

The shear modulus G is expressed by eq. (18):

$$G = (\rho RT/M_c)\nu_2^{1/3}[1 - (2M_c/M)] \quad (18)$$

where ρ is the density of the polymer; R, the gas constant; T, the absolute temperature; ν_2, the volume fraction of polymer in swollen sample; M_c, the average molecular weight between cross-links; and M, the molecular weight of primary molecule of polymer.

With respect to the molecular weight of alkali-processed gelatins, the constants in Staudinger–Mark–Houwink–Sakurada equation have been determined by Janus et al.[12] and expressed by eq. (19):

$$[\eta] = 0.64M_w^{0.38} \quad (19)$$

where $[\eta]$ is the viscosity number (mL/g) measured at 40°C in a molar potassium thiocyanate. The low value of exponent in eq. (19) has been explained by the presence of highly ramified molecules of gelatin, which are derived from the parts

Table I   Thermoelastic Data for BVSP Cross-linked Gelatin Film in Equilibrium Swelling with a Solution Mixture of Equal Volumes of 8M LiBr and BC in the Temperature Range 5–55°C[a]

$\lambda_{40°C}$	$10^{-4} f$ (N/m^2)	10^{-2} $(\partial f/\partial T)_{P,\,\lambda,\mathrm{eq}}$	$(T/f)(\partial f/\partial T)_{P,\lambda,\mathrm{eq}}$	f_e/f
1.05	2.82	1.09	1.21	(−0.18)
1.10	5.57	1.41	0.792	0.23
1.15	8.24	1.98	0.748	0.27
1.20	10.8	2.40	0.696	0.27
1.25	13.2	3.29	0.780	0.24
1.30	15.6	3.72	0.746	0.27
1.35	17.8	4.10	0.721	0.30
1.40	19.9	4.61	0.725	0.30
1.45	22.2	4.82	0.680	0.34
1.50	24.5	5.77	0.737	0.29
			ave.	0.29

[a] The concentration of BVSP used for cross-linking reaction to prepare gelatin film was 100 μmol/g of gelatin.

of more than one tropocollagen molecule. For the sake of such a complexity, dangling end correction term $(1 - 2M_c/M)$ may be omitted from eq. (18), and the calculation for the degree of cross-linking is therefore performed according to eq. (20), as follows:

$$f = (\rho RT/M_c)\nu_2^{1/3}(\lambda - \lambda^{-2}) \qquad (20)$$

The stress–strain curves of gelatin samples conditioned by three cycles of loading and unloading at about 30% extension in a variety of the mixed solution showed excellent elastic recoveries and no substantial energy losses. The results plot-

ted in Figure 8 show linear relationships between the stress and the quantity $(\lambda - \lambda^{-2})$ as required from eq. (20).

The values of M_c calculated from the slope of these curves are shown in Table III. The measurement temperatures for mechanical test are different among the diluent systems. As indicated from the stress-relaxation properties, stabilities on the hydrolytic cleavage of gelatin molecule and for the cross-link itself depend largely on the composition of the swelling liquid and the temperature. The test conditions were adopted within a temperature range with no chemical stress-relaxation even at 10^4 s in

Table II   Thermoelastic Data for BVSP Cross-linked Gelatin Block in Equilibrium Swelling with a Solution Mixture of 55% 8M LiBr and 45% BC by Volume in the Temperature Range 5–30°C[a]

$\lambda_{20°C}$	$10^{-3} f$ (N/m^2)	10^{-1} $(\partial f/\partial T)_{P,\,\lambda,\mathrm{eq}}$	$(T/f)(\partial f/\partial T)_{P,\lambda,\mathrm{eq}}$	f_e/f
1.05	7.2	1.64	0.666	0.33
1.10	14.4	3.31	0.674	0.33
1.15	20.6	6.51	0.926	0.07
1.20	26.6	7.29	0.710	0.29
1.25	32.4	8.87	0.803	0.20
1.30	38.4	11.1	0.846	0.15
1.35	43.6	13.3	0.893	0.11
1.40	50.0	14.5	0.853	0.15
1.45	55.3	15.2	0.807	0.19
			ave.	0.20

[a] The concentration of BVSP used for cross-linking reaction to prepare gelatin film was 100 μmol/g of gelatin.
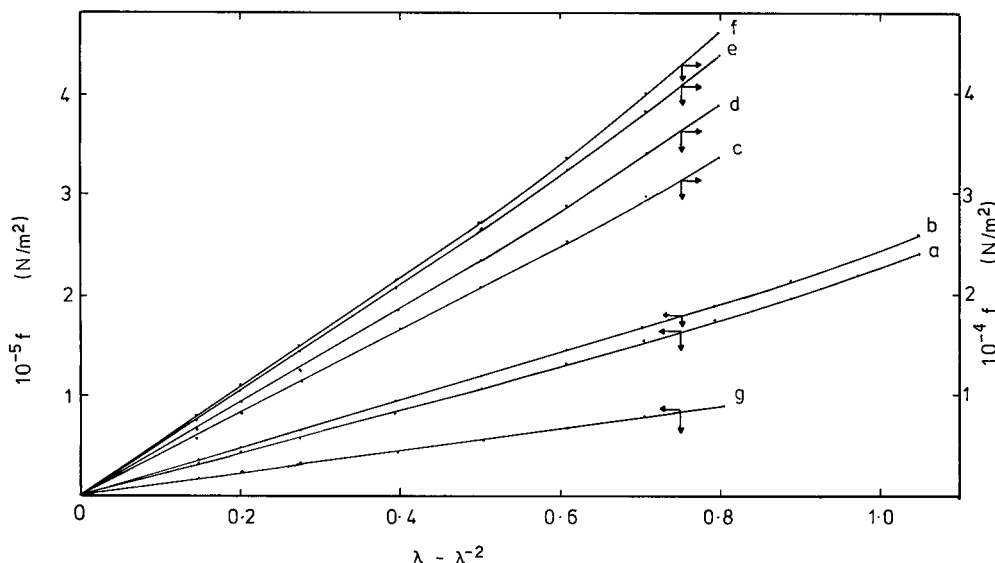
Figure 8 Relationships between equilibrium stress f and the quantity of $\lambda - \lambda^{-2}$ for cross-linked gelatin film and block in a variety of swelling systems. The measurement temperature is shown in parenthesis, and the percentage value for the composition of AC in a swelling solution containing $8M$ LiBr is shown by volume: (a) BVSP film, 100 μmol/g [BVSP], 50% BC(40); (b) 50 μmol/g [BVSP], 50% BC(25); (c) BVSP block, 50% EC(40); (d) 50% MC(40); (e) 45% BC(20); (f) 50% BC(40); (g) FA film, 50% BC(6).

the respective diluent. With respect to the cross-linking reaction of bifunctional BVSP, the amounts of intermolecular cross-links cal-culated as $10^6/2M_c$ are 45.5 and 40 μmol/g for the film samples prepared at the concentra-tions of 50 and 100 μmol/g of BVSP per gram of

Table III   Number of Intermolecular Cross-links Estimated by Mechanical Measurement in Various Diluents

Samples			Diluents						
Cross-linking Agents	Sample Form	ρ (g/cm³)	Percent Composition of AC[a]	ρ_d (g/cm³)	ν_2	$10^{-4}G$[b] (N/m²)	$10^{-4}Mc$ (g/mol)	$10^6/2Mc$[c] (μmol/g)	Cross-linking Efficiency[d] (%)
BVSP 50[e]	film	1.342	50BC	1.243	0.313	20.5 (25)	1.10	45.5	90.9[f]
BVSP100[g]	film	1.342	50BC	1.243	0.380	20.2 (40)	1.25	40.0	43.7
FA[h]	film	1.325	50BC	1.243	0.331	10.4 (6)	2.05	24.4	5.5
BVSP100[g]	block	1.345	50BC	1.243	0.316	5.38 (40)	4.42	11.3	11.9
BVSP100[g]	block	1.345	45BC	1.261	0.249	5.12 (20)	4.02	12.4	13.1
BVSP100[g]	block	1.345	50EC	1.272	0.203	4.13 (40)	4.99	10.0	10.5
BVSP100[g]	block	1.345	50MC	1.286	0.175	5.12 (40)	3.81	13.1	13.8

[a] Percentage value shown by volume for the composition of diethylene glycol monoalkyl ether (AC) in a swelling solution containing $8M$ aqueous LiBr.

[b] Measurement temperature (°C) was shown in parenthesis.

[c] The number of intermolecular cross-links per gram of gelatin.

[d] The percentage ratio of the intermolecular cross-links to the total amount of cross-links determined by chemical analysis (see Table V).

[e] The amount of BVSP used for cross-linking reaction was 50 μmol/g of gelatin.

[f] The percentage ratio of intermolecular cross-links to the amount of BVSP used for cross-linking reaction.

[g] The amount of BVSP used for cross-linking reaction was 100 μmol/g of gelatin.

[h] The amount of FA reacted with gelatin was 440 μmol/g of gelatin.

Table IV   Amino Acid Compositions of Untreated and BVSP Treated Gelatin[a]

| | Untreated Gelatin | | BVSP Cross-linked Gelatin | | | |
| | | | Film | | Block | |
Amino Acids	A[b]	B[c]	A[b]	B[c]	A[b]	B[c]
Lys	221	26	164	18	80	10
Arg	197	23	71	9	148	18
Asp	331	39	371	41	364	44
Thr	141	16	159	18	145	17
Ser	300	35	291	32	327	39
Glu	492	58	576	64	522	63
Pro	1045	22	1067	118	1034	125
Hpr	1006	118	1039	115	949	114
Gly	3160	371	3532	389	3091	372
Ala	995	116	1016	112	1003	121
Val	312	37	339	37	308	37
Ileu	109	13	122	13	123	15
Leu	128	15	197	22	100	12
Phe	98	11	110	12	110	13
Lys + Arg	418	49	235	27	228	28

[a] The concentration of BVSP used for cross-linking reaction to prepare both gelatin film and block was 100 μmol/g of gelatin.
[b] In μmol/g.
[c] In residues/1000 residues.

gelatin, respectively. The corresponding values obtained for the block form sample measured in various diluents are approximately similar. The mean value is 11.7 μmol/g, which corresponds to only about 30% to the amount of the cross-links formed in the film. Although all of the diluent systems of 8*M* LiBr/AC are applicable to evaluate the degree of cross-linking in gelatin, a mixed solution composed of equal volumes of 8*M* LiBr aqueous solution and BC is the most preferable system from the point of view of the stability of the swollen network.

Number, Type, and Site of Cross-links in Gelatin Treated with BVSP

The vinyl double bond is activated by an adjacent strong electron-withdrawing group of sulfone and reacts easily with basic amino, imidazol, and guanidino groups of lysine (Lys), histidine (His), and arginine (Arg) residues in protein, respectively.[10,17] The carbon–nitrogen bonds formed in reactions with Lys, His, or Arg are stable to hydrolysis with strong HCl.[10] Consequently, reaction of these amino acids can be determined by amino acid analysis.

Table IV shows the amino acid compositions of BVSP-treated and untreated gelatin. The contents of Lys and Arg in the treated gelatin decrease; however, the other amino acid content seems to be unchanged. This is evidenced more clearly from the compositions represented by residues per 1000 residues. The total amount of bifunctional BVSP used for cross-linking reaction was 100 μmol/g of gelatin. This corresponds to the amount of 200 μmol/g as the total number

Table V   Quantities of Lysine and Arginine Residues Reacted with BVSP in Film and Block Form Gelatin in Micromole per Gram of Gelatin

| | Amount of the Amino Acid Residues Reacted with BVSP | | | |
| | Film | | Block | |
Amino Acids	(mol/g)	(%)[a]	(mol/g)	(%)[a]
Lys	57	25.8	141	63.8
Arg	126	64.0	49	24.9
Lys + Arg	183	43.8	190	45.5

[a] Based on the amount of corresponding amino acid residues in the untreated gelatin: Lys = 221 and Arg = 197 in μmol/g, as shown in Table IV.

of reaction sites, provided that all of the BVSP molecules were cross-linked with gelatin. The total quantity of Lys and Arg residues lost by the reaction with BVSP is 183 μmol/g for the film and 190 μmol/g for the block form gelatin, as shown in Table V. The values correspond to 91.5 and 95.0% of the total number of vinyl groups in the BVSP used, respectively. It is considered that the reacted BVSP molecule was converted to either the type of cross-linkage or the pendant group with an unreacted vinyl group. The cross-linking reaction of amino groups of Lys and Arg residues with BVSP is shown by eq. (21), as follows:
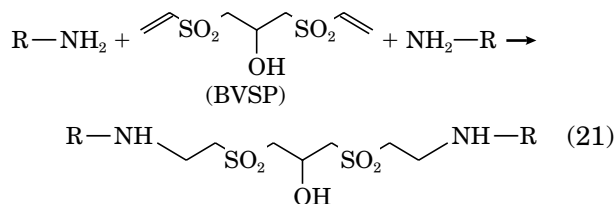
As shown in eq. (22), the pendant groups may be formed from Lys and Arg residues located on a peptide chain with a lack of the juxtaposed Lys or Arg residue existing in a sterically favored conformation for the reaction with another vinyl group of the BVSP molecule.
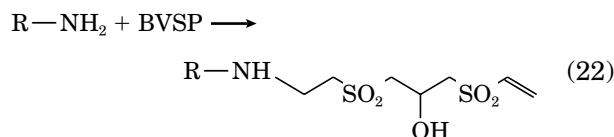
It can be presumed, however, that the reacted BVSP molecules form the type of the cross-links rather than the pendant groups since there were excess amounts of reactive groups in gelatin (414 μmol/g) to those of BVSP used (200 μmol/g).

When it is assumed that all of the BVSP molecules form cross-links, the efficiency of the cross-links going to intermolecular linkages can be obtained from the total number of cross-links determined by chemical analysis. The result is shown in the last column of Table III. The efficiencies of the intermolecular cross-linking are 90.9 and 43.7% for the films cross-linked with 50 and 100 μmol of BVSP, respectively. The higher the concentration of BVSP results, the lower the efficiency. This is considered to be due to the formation of a nonuniform structure of network including densely cross-linked domains. The network chain between the cross-links introduced into the highly cross-linked domains does not act as a usual rubbery chain. The amount of intermolecu-

lar cross-links calculated from the result of mechanical measurements for the cross-linked sample with a higher concentration of BVSP, such as the 100 μmol/g, would be underestimated, resulting in a lower efficiency even if the intermolecular cross-links were actually formed within the domain regions.[29]

An extremely lower efficiency is found for FA film. This clearly suggests that the major part of the cross-links introduced by FA are intramolecular linkages. The average efficiency of the block form gelatin cross-linked with BVSP is 12.3%, which is much lower than that for the case of the film. This suggests that the formation of intermolecular linkages is greatly concerned with the orientation of gelatin molecules during cross-linking reaction. It is of interest that Arg residues are more reactive than Lys residues under the oriented state of gelatin molecules in a parallel array to the film surface, while the reactivity reverses under the unoriented state of the molecules.

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