Crosslinking Structure of Keratin. VI. Number, Type, and Location of Disulfide Crosslinkages in Low-Sulfur Protein of Wool Fiber and Their Relation to Permanent Set

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SYNOPSIS

Crosslinked structures of the permanent set wool fiber treated with boiling water at a 40% extension state and the control fiber were studied by analyzing the rubberlike force-extension curve of the swollen fiber in a mixed solution composed of equal volumes of 8*M* LiBr and butyl carbitol. The thiol and disulfide contents of set fibers were also determined. It was found that (1) the disulfide (SS) bonds in low-sulfur (LS) microfibril protein transform into new crosslinkages in boiling water, but the SS bonds in high-sulfur matrix protein remain intact, (2) the SS bonds in α-helical segments becomes reactive only at the extension state of fiber and produces a free thiol group, and (3) intramolecular SS bonds may exist in the α-helical segments. Discussion was also made about the closeness of the number of crosslinkage sites of SS bonds obtained from the present rubber elasticity theory and from the theoretical analysis of the amino acid sequence of the intermediate filament. The crosslinking structure model in LS protein was proposed. It was suggested further that the setting mechanism for new crosslinkage theory seems to be unsatisfactory, since the new crosslinkages do not contribute to stabilize the extended conformation of the wool chain.

INTRODUCTION

The setting process is important in the wool industry for the finishing of wool fibers and fabrics. The setting mechanism was studied for many years by many workers. However, it remains an unresolved problem. Various mechanisms have been proposed to explain the permanent set obtained by steaming extended wool fibers with or without setting agents. The various mechanisms claimed can be divided into three principal theories as thiol (SH)/disulfide (SS) interchange theory, hydrogen-bond theory, and new crosslinkage theory.

Zahn et al.[1] stated that SH/SS interchange occurs during setting, but that the stabilization of set is due to the formation of lanthionine which occurs concomitantly. On this basis, they supported the crosslinkage stabilization theory of set postulated by Speakman et al.[2-5] Speakman et al. proposed that permanent set is due to SS breakdown brought about either by boiling water or by reducing agents, followed by linkage rebuilding to stabilize the extended conformation. This theory was also extended and supported by Robson et al.[6] who reported that the content of lysinoalanine and lanthionine increases in proportion to the degree of set retained.

In previous articles,[7-10] characterization of crosslinkages in a variety of keratins was performed by analyzing the force-extension relation of the swollen and decrystallized fibers and the distribution of crosslinks in keratin fibers has been widely demonstrated. This study is focused on the number and

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the type of crosslinkages formed by the setting treatment in boiling water and on the location of crosslinks in microstructures of wool fibers.

EXPERIMENTAL

Materials

Lincoln wool fibers were degreased by a Soxhlet extraction with acetone for 24 h, washing with ethanol at room temperature for 24 h, washing with cold distilled water, and then air-drying. Tri-*n*-butylphosphine (TBP) used as reducing agent of SS groups and *N*-ethylmaleimide (NEMI) used as a blocking agent of free SH groups were special reagent grade. Diethylene glycol mono-*n*-butyl ether (BC) was obtained by distillation as reported previously.[11]

Setting Treatment

Single fibers of the Lincoln wool, about 4 cm long, were mounted in a setting frame and stretched in water at 40°C by exactly 40% of the initial length. They were immersed in boiling water for a required time of set, $\frac{1}{4}$, $\frac{1}{2}$, 1, 2, 3, 4, and 5 h, and allowed to relax in the boiling water for 1 h. This condition was defined by Astbury and Woods[12] as "permanent set." After these treatments, their lengths were measured after air-drying. The degree of set (*DS*) was calculated as the percentage of the length change ($L_1 - L_0$) to the initial length (L_0) as eq. (1):

$$DS(\%) = 100(L_1 - L_0)/L_0 \quad (1)$$

About 10 specimens were tested for each sample and the average value was recorded.

The same procedure as described above was applied for the bundles of approximately 200 Lincoln wool fibers. Control fibers were also obtained by the treatment in boiling water at no extension for a corresponding time of set plus 1 h. These samples obtained were subjected to analyses of SH and SS contents, reduction treatments, and mechanical measurements.

Determination of Disulfide (SS) and Thiol (SH) Contents

SS and SH group contents in set and control samples were analyzed by a polarographic method using methylmercury iodide.[13] The analyses were carried out for each three samples and the average value was taken.

Preparation of Reduced Fibers

The set and control fibers (20 single fibers each) were reduced with a 2% TBP solution containing 1-propanol (5 mL) and borate–phosphate buffer adjusted at pH 9.6 (5 mL) for 24 h at 25°C, washed three times with the same buffer containing 1-propanol (10 mL), and then treated with a 10^{-2} *M* NEMI solution composed of 1-propanol (5 mL) and the same buffer (5 mL) for 24 h at 25°C to block free SH groups. These reduction and blocking cycles were carried out three times for the preparation of a completely reduced and NEMI-blocked fiber. The treated fibers thus obtained were thoroughly washed with water and air-dried.

Preparation of Swollen Fibers and Mechanical Tests

Swollen fibers were prepared by the treatment with an 11*M* LiBr aqueous solution containing 10^{-2} *M* NEMI at 90°C for 1 h and then immersed in a mixed solution composed of equal volumes of 8*M* LiBr and BC. The force-extension property of the swollen fiber was measured in the mixed solution at 50°C as reported previously.[7,8] Four or five specimens were tested for each sample to construct stress–strain curves.

Analysis of the Force-Extension Curve

The force-extension curve of swollen fiber was analyzed by a two-phase model in which the swollen fiber is assumed to be composed of a continuous lightly crosslinked rubbery phase and density crosslinked microdomains which act as reinforcing filler particles in rubber.[8,10] The assumption corresponds to the structure of swollen fiber including the globular high-sulfur (HS) matrix proteins dispersed in the swollen network of low-sulfur (LS) proteins. A theoretical relationship between the elastic forces originating from the network with such a nonuniform structure, *F*, and the extension ratios, α, is shown by eq. (2)[14]:

$$F = G(\sqrt{n}/3)\{L^{-1}(\alpha/\sqrt{n}) - \alpha^{-3/2}L^{-1}(1/\sqrt{\alpha n})\} \quad (2)$$

where α is the extension ratio of the rubbery chain, $L^{-1}(X)$, the inverse Langevin function; *n*, the number of segments in network chain; and *G*, the shear modulus which is represented by eq. (3)[8,15]:

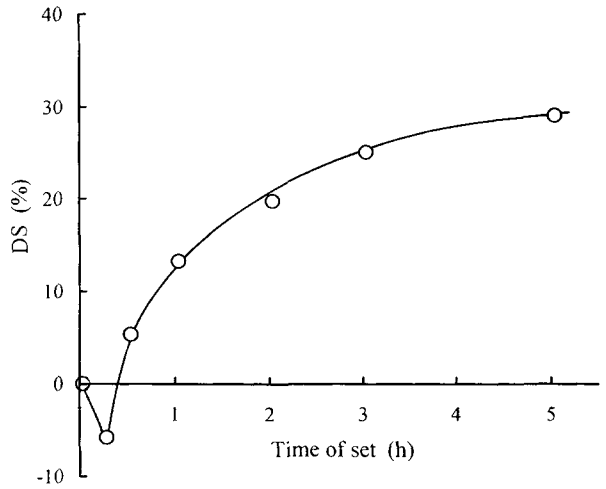


Figure 1 Relationship of degree of set (DS) vs. time of set.

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

where ρ/M_c is the crosslink density in the rubber region; ρ, the density of the polymer; M_c, the molecular weight between crosslinks in LS protein; R, the gas constant; T, the absolute temperature; ν_2, the volume fraction of polymer in the swollen polymer; M, the molecular weight of the primary molecule; ϕ_d, the volume fraction of domains in the swollen sample; and γ, the filler effect of domains in the swollen network, which is represented by eq. (4)[16]:

$$\gamma = 1 + a\kappa\phi_d + b\kappa^2\phi_d^2 \quad (4)$$

where κ is the shape factor defined as the length: breadth ratio for rodlike filler. The extension ratio of the swollen sample, λ, can be related by the extension ratios of the rubber region, α, as eq. (5)[8]:

$$\alpha = (\lambda - \phi_d)/(1 - \phi_d) \quad (5)$$

In the swollen keratin system, the following assumptions were made[8]: (1) the domains are rigid and stable to the swelling and the deformation of fiber, (2) the molecular weight of segment for the keratin chain comprising LS protein, n_r ($=M_c/n$), is a constant in the value of 1250, where n is the number of segments between crosslinks, (3) the number-average molecular weight of the primary molecule of the keratin chain in the rubbery network consisting of LS protein (M) is 50,000, (4) the filler particles are near spherical as $\kappa \geq 1$, and (5) the values of two constants a and b in eq. (3) are equal to the values given by Guth[16] for the spherical shape of $\kappa = 1$, namely, $a = 2.5$ and $b = 14.1$. The values of ρ/M_c, ϕ_d, and κ were obtained as parameters by fitting the experimental force-extension data to eq. (2). The densities for both set and reduced fibers, ρ, were assumed to be 1.30 g/cm^3. The fitting method applied was described in a previous article.[10]

According to the above assumption (1), the volume fraction of domains in unswollen fiber, ϕ'_d, can be calculated by eq. (6):

$$\phi'_d = \phi_d/\nu_2 \quad (6)$$

The value of ϕ'_d represents the volume fraction of the matrix of HS proteins in the fiber.

RESULTS

The relationship between DS and time of set is shown in Figure 1. Supercontraction can be observed near $\frac{1}{4}$ h, and DS reaches about 30% after 5 h set. The result obtained was approximately the same as reported by Robson et al.[6]

Figure 2 shows the relationships between equilibrium stress, F, and strain, λ, for the untreated Lincoln wool and the set fiber at 40% extension for 1 h. These are shown as typical examples of many samples tested. The solid lines fitted to the experimental data are also shown.

Structural Parameters for Set Fibers

Table I shows the results of structural parameters obtained for no-extension set (control) and 40% extension set fibers. The observed values for G are

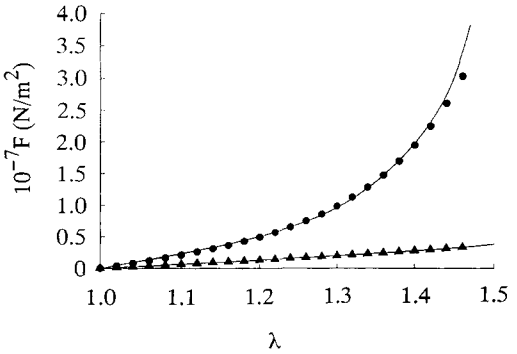


Figure 2 Relationship of equilibrium stress, F vs. strain, λ: (●) untreated Lincoln wool fiber; (▲) set fiber at 40% extension for 1 h. The solid lines fitted to the experimental data by eq. (2).

greatly different between the fibers set at no-extension and extension states. It is also shown that the difference is due mainly to the differences between the amounts of crosslinkages in LS protein (ρ/M_c) and the values of the shape factor (κ).

Figure 3 shows the relationship between ϕ'_d and the time of set for no-extension and extension set fibers. The values of ϕ'_d are approximately constant and independent of the setting conditions. This implies that the matrix structure was not changed by the setting treatment with boiling water even at a 40% extension state for a prolonged period of time. As compared with the untreated wool, the magnitude of the ϕ'_d obtained for set fibers is somewhat lower. This may be caused by deaggregation of globular matrix proteins due to some breakdown of the SS bonds between the globular proteins and also between the globular and microfibrillar proteins.

The κ-factor is concerned with the shape of globular matrix proteins. The κ values are approximately constant and independent of the time of set for each control and extension set fibers. From the magnitude of κ, it is indicated that the form of the globular matrix proteins are nearly spherical in the control fibers, while rather elliptical in the extension set fibers. It is also suggested that from the comparison

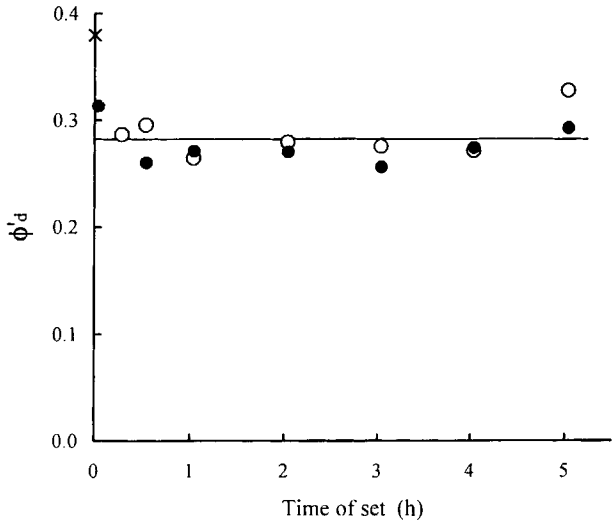


Figure 3 Relationships of ϕ'_d vs. time of set: (●) no-extension set (control) fiber; (○) 40% extension set fiber; (×), untreated Lincoln wool fiber.

with the shape of the matrix in the untreated fiber a considerable deaggregation of the matrix occurs during setting treatment, especially at the condition of no-extension set. Although the difference between the values of ϕ'_d is a relative measure of the deag-

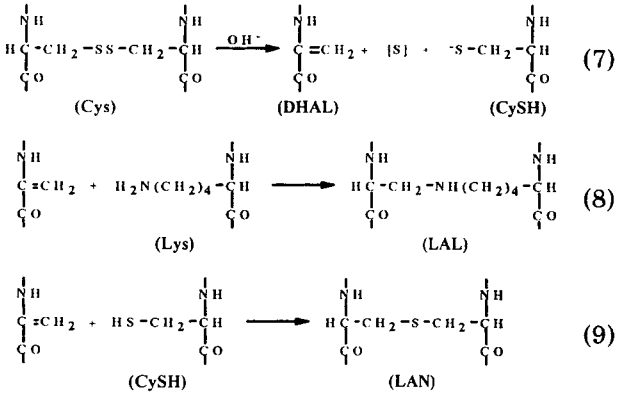
Table I Structural Parameters for No-extension and 40% Extension Set Fibers

Setting Conditions	Time of Set (h)	$10^{-6}\,G$ (N/m^2)	ν_2	$10^4\,\rho/M_c$ [a] (mol/cm^3)	M_c [b] (g/mol)	κ	ϕ_d	ϕ'_d	$10^6/2M_{c,LS}$ [c] (μmol/g)
Untreated	—	4.91	0.580	3.51	3700	1.70	0.220	0.379 ($=\phi'_{d0}$)	135
No extension (control)	0	2.59	0.648	2.98	4360	1.20	0.203	0.313	115
	$\frac{1}{2}$	2.90	0.632	2.99	4350	1.30	0.164	0.260	115
	1	2.40	0.630	2.84	4570	1.20	0.171	0.271	109
	2	2.07	0.560	3.00	4330	1.10	0.156	0.270	116
	3	2.21	0.671	2.91	4470	1.10	0.172	0.256	112
	4	1.80	0.650	2.72	4780	1.10	0.178	0.274	105
	5	2.06	0.658	2.80	4640	1.10	0.192	0.292	109
40% extension	$\frac{1}{4}$	1.82	0.646	2.16	6010	1.50	0.185	0.286	83
	$\frac{1}{2}$	1.77	0.651	2.13	6090	1.50	0.192	0.295	82
	1	2.02	0.701	2.30	5640	1.50	0.185	0.264	89
	2	1.81	0.671	2.12	6140	1.60	0.187	0.279	82
	3	1.78	0.630	2.10	6190	1.50	0.173	0.275	82
	4	1.43	0.595	1.95	6660	1.50	0.161	0.271	75
	5	1.69	0.640	2.01	6470	1.50	0.209	0.327	78
									82

[a] The crosslink density of SS and X crosslinkages in LS proteins.
[b] The number-average molecular weight between crosslinks in LS proteins.
[c] The no. SS + X crosslinkages per gram of LS protein.

gregation of the matrix proteins, there exists little difference between the levels of the values obtained for the control and the extension set fibers as shown in Figure 3. This seems to be inconsistent with the interpretation for the structural change of the matrix proteins based on κ. The difference in the shape of the matrix may result from the difference in the location of the SS bonds suffered under no extension and extension of the fiber. However, at present, there is no clear explanation about this.

It has been pointed out by Robson et al.[6] that during the setting treatment lanthionine (LAN) and lysinoalanine (LAL) residues were formed as the transformation products from cystine (Cys) residues via dehydroalanine (DHAL) residues as shown in eqs. (7)-(9). They also showed that the extent of SS-bond scission balanced the new crosslinkages formed by the setting treatment. These new crosslinkages due to LAN and LAL residues were designated as X crosslinkages in the text:



The number of intermolecular crosslinkages in the LS protein can be calculated by $10^6/2M_c$ in μmol/g. The values are shown as $10^6/2M_{c,LS}$ in the last column of Table I. The number of intermolecular crosslinkages in LS protein for the untreated wool is 135 μmol/g. A similar value has been obtained for human hair as 132 μmol/g,[10] although ϕ'_d and SS contents are very different between the two keratins. These results accord with the fact that the SS content and as well the structure of LS proteins among keratins are approximately the same. Figure 4 shows the relationship between the number of the crosslinkages in LS protein ($10^6/2M_{c,LS}$) and the time of set. The number of these crosslinkages is the sum of SS and X linkages. It is of interest to note that the number of intermolecular crosslinkages, $[SS + X]_{inter}$ is approximately constant and independent of the time of set. However, the values obtained for extension set fibers (82 μmol/g) are considerably less than for the control fibers (112 μmol/g). This means that the amount of the intermolecular crosslinkages was decreased by the extension of the fiber. The amount of the intermolecular X crosslinkages formed during the setting treatment can be evaluated by applying the same fitting method to the stress–strain curve measured in the swollen state of the set fibers in which the residual SS bonds have been completely reduced with TBP and blocked with NEMI.

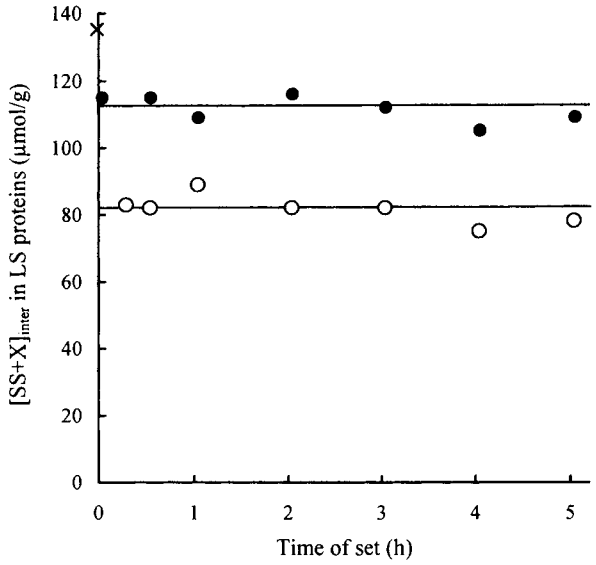


Figure 4 Relationships of the number of intermolecular crosslinkages, $[SS + X]_{inter}$ in LS protein vs. time of set: (●) no-extension set (control) fiber; (○) 40% extension set fiber; (×) untreated Lincoln wool fiber.

Structural Parameters for Reduced Set Fibers

Table II shows the results of the characterization of the swollen network for the reduced fibers. The values of ϕ'_d are substantially zero for all of the reduced fibers. It is suggested, therefore, that there exist no rigid domains within the network of the LS proteins. The G and ν_2 of the reduced fibers for both control and set wools increase with the time of set. The increasing tendencies for G is clearly due to the increase of the amount of X crosslinkage as indicated by the increase of the values of ρ/M_c (fifth column in Table II). It should be noted that the reduced fiber contains both species of the crosslinked LS proteins and the noncrosslinked materials originated from the globular HS proteins as a result of reduction treatments. Therefore, we must evaluate true crosslink density of X linkage in LS proteins. Assuming the similarity of the densities (ρ) for the two-component proteins, the number-average mo-

lecular weight between X crosslinkages in LS proteins, $M_{c,LS}$, can be calculated by eq. (10)[10]:

$$M_{c,LS} = (1 - \phi'_{d0})M_c \qquad (10)$$

where ϕ'_{d0} is the volume fraction of domains in the untreated wool fiber and M_c is the number-average molecular weight of the chain obtained for the two-component system (sixth column in Table II). Here, the value of $\phi'_{d0} = 0.379$ (Table I). The number of intermolecular X crosslinkages in LS protein is shown in the last column of Table II.

Number of Intermolecular SS and X Crosslinkages in LS Proteins

Figure 5 shows the relationship between the number of intermolecular X crosslinkages, $[X]_{inter}$ and the time of set together with the curvers plotted for the number of $[SS + X]_{inter}$ in Figure 4. The rate of formation of X crosslinkages and their amounts are not so different between the set and control fibers. It is noted that at 5 h set the amounts of X crosslinkages in both fibers are almost the same (77–78 μmol/g). This fact suggests that X crosslinkages is located in the region of LS proteins where no conformational change occurs during the fiber extension, since a considerable difference will be expected if the formation of X linkages is concerned with the region of the α-helix being unfolded by the extension of the fiber. The amount of intermolecular SS linkages, $[SS]_{inter}$, in each set fiber is equal to the difference between the values of $[SS + X]_{inter}$ and

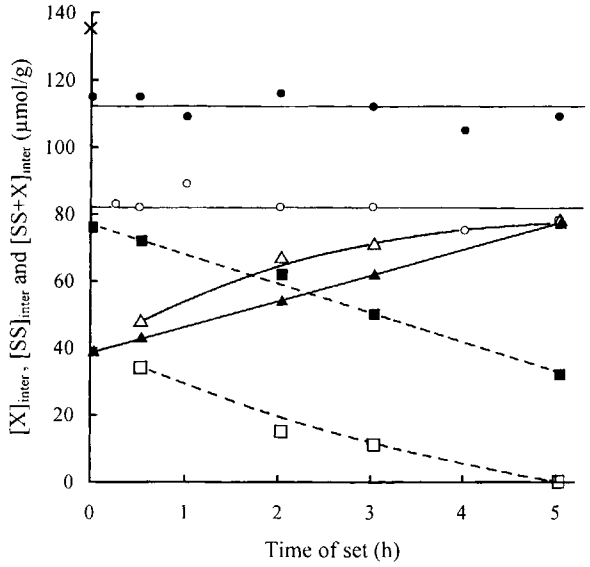


Figure 5 Relationships of the number of intermolecular crosslinkages, $[X]_{inter}$ and $[SS]_{inter}$, in LS protein vs. time of set. $[X]_{inter}$: (▲) no-extension set (control) fiber; (△) 40% extension set fiber. $[SS]_{inter}$: (■) no-extension set (control) fiber; (□) 40% extension set fiber. The curves plotted for the number of $[SS + X]_{inter}$ in Figure 4 are also shown: (●) no-extension set (control) fiber; (○) 40% extension set fiber; (×) untreated Lincoln wool fiber.

$[X]_{inter}$. These plots are shown by dashed lines. It should be noted that all the SS groups in the LS proteins disappear at 5 h extension set. The amounts of SS linkages in the control and set fibers are decreased approximately parallelly with increasing the time of set. The difference between the amounts of

Table II Structural Parameters for Set Fibers Reduced Completely with TBP

Setting Conditions	Time of Set (h)	$10^{-4}\,G$ (N/m^2)	ν_2	$10^5\,\rho/M_c$[a] (mol/cm^3)	M_c (g/mol)	κ	ϕ_d	$M_{c,LS}$[b] (g/mol)	$10^6/2M_{c,LS}$[c] (μmol/g)
No extension (control)	0	1.26	0.097	6.25	20,800	1.0	0.00	12,900	39
	½	2.28	0.128	6.92	18,800	1.0	0.00	11,700	43
	2	4.02	0.167	8.67	15,000	1.0	0.00	9300	54
	3	7.31	0.176	10.0	13,000	1.0	0.00	8100	62
	5	13.7	0.282	12.5	10,400	1.0	0.00	6500	77
40% extension	½	2.97	0.05	7.65	27,000	1.0	0.00	10,500	48
	2	9.02	0.208	10.7	12,100	1.0	0.00	7500	67
	3	11.4	0.265	11.5	11,300	1.0	0.00	7000	71
	5	15.1	0.392	12.6	10,300	1.0	0.00	6400	78

[a] The crosslink density of X crosslinkages based on whole wool protein.
[b] The number-average molecular weight between X crosslinkages in LS proteins calculated by $M_{c,LS} = (1 - \phi'_{d0})\,M_c$ (= 0.621 M_c), where ϕ'_{d0} is the volume fraction of domains in unswollen wool fiber.
[c] The no. X crosslinkages per gram of LS protein.

$[SS]_{inter}$ in both set fibers clearly corresponds to the amount of the SS bonds lost by the extension of the fiber. The extent of the intermolecular SS bonds lost by extension can be estimated to be 39 μmol/g as the average value for the differences at a definite time of set. In other words, the SS bonds amounting to 39 μmol/g may be located in the region accompanying the conformational changes in the fiber extension, i.e., from X-ray evidence,[17,18] the ordered α-helical section in the LS proteins, since these SS bonds will be reactive only in the extended state of the fiber. It is considered that the SS bonds lost by the extension might be converted to either the intramolecular *X* bonds or free SH groups. This will be discussed in the next section. As shown in Table I, the 23 μmol/g of intermolecular SS bonds in the untreated wool is lost by the treatment with boiling water within 1 h at the no-extension state. The transformation products may probably be intramolecular *X* linkages.

SH and SS Contents of Set Fibers

Table III shows the SH content for set fibers. An approximately constant amount of SH is produced by the extension of the fiber at various times of set. The production of SH is 20 μmol/g as the average value, which corresponds to 33 μmol/g (= [SH]/$(1 - \phi'_{d0})$) on the basis of the LS protein. This figure is very similar to the value of 39 μmol/g for the intermolecular SS linkages lost by extension. It is considered, therefore, that 1 mol of each free SH group and DHAL residue is originated from an intermolecular SS bond under the deformation of LS proteins, and the formation of *X* crosslinkages may be inhibited sterically within and between the extended α-chains. Again, in Table I or in Figure 4, the net decreased amount of intermolecular crosslinkage on fiber extension processes is about 30 μmol/g (= 112 − 82). This quantity seems to be consistent with the amount of the SS linkages converted to SH groups. The stable and unreactive intermolecular SS bond located in the region inaccessible to water is thought to become reactive with boiling water as a result of reduced order in chain conformation brought about by the extension of the fiber.

Table IV shows the SS content for control and set fibers. The SS content is decreased from 420 to 289 μmol/g by the setting treatment for 5 h at a 40% extension state. The net decreased amount is 131 μmol/g. Robson et al. reported that under the same setting conditions the sum of the amounts of LAN and LAL formed is about 140 μmol/g of Lincoln wool and balances the amount of the SS bond scission. It seems to be appropriate to assume, therefore, that in the authors' experiment the LAN and LAL linkages were formed quantitatively from the stem of Cys. The SS contents of the extension set fibers are decreased as compared with the no-extension set fibers. The decreased amount of SS groups are approximately constant independent of the time of set, namely, 44 μmol/g as the average value. This means that these SS bonds are located within a region of LS proteins accompanying the conformational changes with fiber extension since the amount of the lost SS bonds is probably concerned with only the extension processes. The extent of the SS bonds lost by the extension reaches to 72 μmol/g of LS protein as the average value (designated as *A* in Table IV). As shown in the preceding discussion, the number of SS bonds lost by the extension includes the SS bonds converted to free SH groups amounting to 33 μmol/g (B). Accordingly, the remaining SS

Table III SH Contents of Wool for Set Fibers and the Quantities of SH Groups Produced by 40% Extension in Micromole Per Gram

Samples		Time of Set (h) 0	½	2	3	5	Ave
Untreated wool	19						
No-extension set fibers		10	9	7	8	8	8
40% extension set fibers		—	27	28	29	28	28
SH produced by 40% extension							
Based on whole protein			18	21	21	21	20
Based on LS protein[a]			29	34	34	34	33

[a] The calculated values as $[SH]/(1 - \phi'_{d0})$, where ϕ'_{d0} is the volume fraction of LS protein in unswollen Lincoln wool used in this experiment, which equals 0.379.

Table IV SS Contents of Wool for Set Fibers and the Quantities of SS Groups Lost by 40% Extension in Micromole Per Gram

Samples		Time of Set (h) 0	$\frac{1}{2}$	2	3	5	Ave
Untreated wool	420						
No-extension set fibers (control)		343	330	337	329	332	
40% extension set fibers		—	290	288	284	289	
SS lost by 40% extension							
Based on whole protein			40	49	45	43	44
Based on LS protein (A)[a]			65	79	73	69	72
SS converted to SH groups (B)[b]			29	34	34	34	33
SS converted to X groups (C)[c]			36	45	39	35	39

[a] The calculated values from $[SS]/(1 - \phi'_{d0})$, where ϕ'_{d0} is 0.379.
[b] The values listed in Table III.
[c] (C) = (A) − (B), which equals the amount of intramolecular X crosslinkages formed in LS protein.

bonds (C) calculated as 39 μmol/g ($A - B$) might be converted to X linkages. Considering the fact that the rate of formation of the intermolecular X crosslinkages are almost independent of the setting conditions, the 39 μmol/g (C) might be presumed to be present in the native fiber as mechanically inactive intramolecular linkages, and they are transformed to intramolecular X linkages and, as a result, the SS content decreases.

DISCUSSION

It is of interest to compare the SS contents of the untreated and the 5 h set fibers which contain no intermolecular SS groups in LS proteins (Fig. 5). The difference in the SS content between the untreated and the set wool is 131 μmol/g (= 420 − 289). This value corresponds to 213 μmol/g ($= [SS]/(1 - \phi'_{d0})$) on the basis of LS protein. This figure is very similar to the value obtained from the analysis of the sulfur content in the LS proteins fractionated from soluble S-carboxymethylated (SCM) keratins, namely, 400 μmol/g as the content of SCM cysteine,[19-21] which corresponds to 200 μmol/g as Cys.[7] This also strongly supports the result presumed from the constancy of ϕ'_d that during the setting treatments in boiling water no or little change of the matrix structure occurs (Fig. 4). It is, thus, emphasized that such crosslinking reactions in boiling water are specific for microfibril proteins.

The diffusion and adsorption of water to the reaction sites are essential for the formation of X linkages. Therefore, as a relative meaning, the microfibril proteins are considered to be more hydrophilic than are the matrix proteins, while the α-helical region of the microfibril becomes accessible to water only when the α-helix was unfolded by the fiber extension. It is further concluded, therefore, that the most hydrophilic region in microfibril proteins and in the wool cortex as well is the non-helical regions of the microfibril, namely, N- and C-terminals of the LS proteins.

With respect to the adsorption sites of water in keratin fibers, Spei and Zahn[22] reported that on the basis of the low-angle X-ray diffraction data the ratio of the intermicrofibrillar distance in the wet-to-dry state increases with increase in the ratio of the microfibril to the matrix components of keratin. This suggests that the microfibrillar component is more hydrophilic than is the matrix. This evidence seems to support the present result. However, the nature of the surface of the matrix has been unknown since the concentration of SS bonds in the surface is too low to differentiate from the reactivities among the SS bonds in the other parts of the microstructures.[7,10]

Distribution of SS and X Crosslinkages in LS Protein of Wool and Set Fibers

Figure 6(a) shows the distribution of the different types of SS linkage in LS proteins of wool. They contain 135 μmol/g of intermolecular (Table I) and 39 μmol/g of intramolecular linkages (Table IV), which correspond to 63.4 and 18.3% of the total content of SS linkages (213 μmol/g), respectively. The bonding pattern of the residual linkage desig-

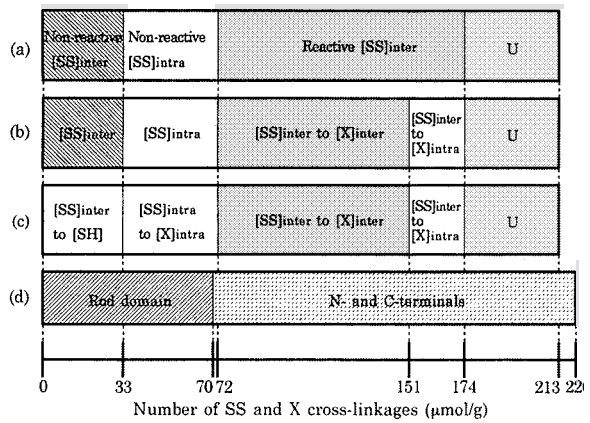


Figure 6 Distribution of SS and *X* linkages in LS proteins of Lincoln wool: [SS] and [*X*] designate the number of SS and new crosslinkages formed by the setting treatment, respectively; [SH], the thiol content; *U*, the crosslinkage type being unknown; and the subscripts, inter and intra denote inter- and intramolecular linkage, respectively. (a) Distribution of different type of SS linkage: nonreactive and reactive mean the reactivities of SS linkage with boiling water. (b) Distribution of SS and *X* linkage in fiber set at no-extension state for 5 h: inter- and intra-molecular *X* linkages are produced from the reactive intermolecular SS linkages. (c) Distribution of SH, SS, and *X* groups in fiber set at 40% extension state for 5 h: free SH groups and intramolecular *X* linkages are produced from the nonreactive inter- and intramolecular SS linkages, respectively. (d) Distribution of SS linkage in the α-helical rod domain and N- and C-terminal domains in the IF molecule.

nated as *U*, being 39 μmol/g (= 213 − 135 − 39), is uncertain. This reason is due to the fact that the type of crosslinkage could not be determined by mechanical means if the crosslinkages occurred in the environment of a higher crosslink density. The intermolecular SS crosslinkage is divided into two groups: one is nonreactive linkages being located in a region inaccessible to water, and the other is reactive in a hydrophilic environment. The number of crosslinkages in the former was 33 μmol/g (Table III) and 102 μmol/g (= 135 − 33) in the latter.

Figures 6(b) and (c) show the distribution of SS and *X* crosslinkages in the fibers set at no extension and extension states for 5 h, respectively. Total amount of reactive intermolecular SS bonds reaches to 102 μmol/g. Either at no extension or extension set, about 78% of the SS bonds go to intermolecular *X* linkages and the residual 23 μmol/g transform to intramolecular *X* linkages (Table I). Only at the extension conditions are the 39 μmol/g of intramolecular SS bonds which are nonreactive in boiling water converted to intramolecular *X* linkages, and the 33 μmol/g of intermolecular SS bonds which are nonreactive in boiling water change into free SH groups, but they do not further transform to *X* crosslinkage.

Number, Type, and Location of SS Crosslinkages in LS Protein of Wool Keratin

Keratin microfibrils constitute a form of intermediate filaments (IF) and the matrix proteins, the so-

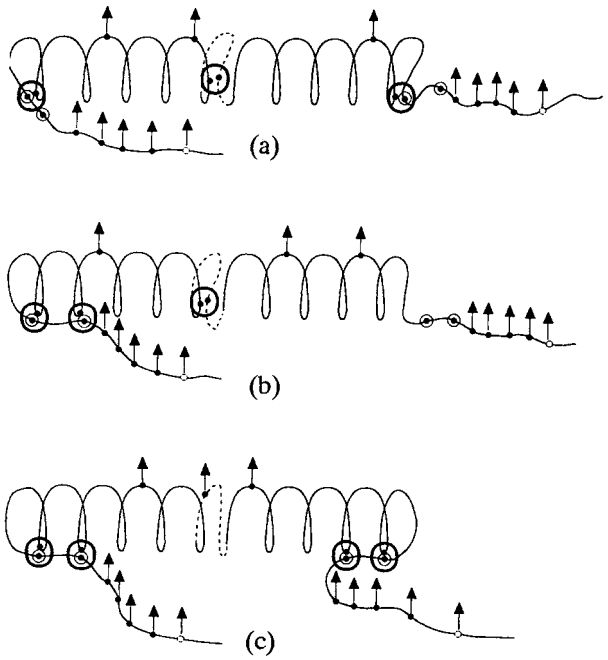


Figure 7 Schematic representation of possible crosslinked structures of the disulfide bonds in wool IF chain. The structures (a), (b), and (c) were constructed by combining the different three types of intrachain linkage sites occurring in the α-helical rod domain. The location of intramolecular crosslinkage sites in IF chain is indicated by a large circle. (●→) Crosslinkage site going to adjacent IF chain: Three sites are included in the rod domain and eight sites in the terminal domains. (○→) Crosslinkage site going to adjacent IF chain or IFAP: Two sites are involved in the terminal domains and these change into the sites of intrachain X linkage by setting treatment at the no-extension state. (◉) Crosslinkage type being unknown: These two crosslinkage sites may be located within the region of a higher crosslink density in the terminal domains, and as shown in (a), (b), and (c), these were presumed to be the sites of intrachain linkage to form a closed loop which makes no contribution to the network elasticity. The crosslinkage sites in the nonhelical section of the two terminal domains were assumed to be equally distributed in each domain.

called intermediate filament-associated proteins (IFAP), aggregate with IF to form macrofibril.[23-26] The wool IF consist of heteropolymers formed by interaction of type I and type II proteins.[27] There are four protein species in each of these two classes.[28] The amounts of each four species are approximately equimolar.[29] The IF chains aggregate into two-stranded, coiled-coil rope molecules.[28] These proteins have been considered to be bonded with IF–IF and IF–IFAP types of SS linkage.[30] It has been pointed out that intrachain and intrarope SS bonds are not possible in a regular α-helical coiled-coil structure, but interrope SS bonds are possible, especially between 2B segments of the IF rod domain consisting of helical segments 1A, 1B, 2A, and 2B and nonhelical linking segments L1, L2, and L12.[30]

The numbers and locations of cysteine residues in wool IF chains for sequenced Type I species (8c-1, 8a) and Type II species (7c, 5) are known,[30,31] i.e., the rod domains of the IF chain contain 8, 5, 9, and 7 cysteine residues in the components 8c-1, 8a, 7c, and 5, respectively, and the corresponding contents for N- and C-terminal nonhelical domains are 17, 10, 21, and 11 residues. The numbers of cysteine residues in each domain are considerably different among them. When the equality in the amount of each species in microfibrils is assumed, the average numbers of cysteine residues in the rod domain and the terminal regions are calculated to be ~ 7 and ~ 15 residues, respectively. The molecular weight range of Type I proteins is $4.2–4.6 \times 10^4$ and that of Type II proteins is $5.6–6.0 \times 10^4$ (Ref. 27). The average value can be assumed to be 5.0×10^4. Almost all the cysteine residues may exist as Cys residues in the native filament.[32] Therefore, the total Cys content in IF protein is calculated to be 220 μmol/g and as 70 μmol/g for the rod domain and 150 μmol/g for terminal domains. The suggestions from these calculations based on only four IF proteins sequenced at the present time are, of course, an indication of the possibilities on the distribution of Cys residues in the IF proteins. The calculated result is shown in Figure 7(d). It is worth noting that (1) the value obtained as 213 μmol/g for a total number of SS bonds in LS protein is very similar to the Cys content (220 μmol/g) in IF protein and (2) the quantity of the nonreactive Cys residues estimated as 72 μmol/g of LS protein in the set fibers at the no-extension state is approximately equal to the Cys content (70 μmol/g) in the rod domain. Although the closeness of the number of crosslinkage sites obtained from the present rubber elasticity theory and from the sequence analysis must be regarded as fortuitous, it implies that, when wool fiber is boiled in water, the crosslinking reaction is confined substantially in the microfibrillar proteins.

When it is assumed that both of the inter- and intrachain SS linkages occur in the rod domain of the IF chain, the number of crosslinkage sites in the rod-domain segments is calculated to be about 3 mol for interchain and 4 mol for intrachain linkages. Intrachain SS linkages will occur within a single IF chain to give rise to a closed loop linked between the two sites: One is located on the helical segments and the other on the nonhelical segments, since it is most improbable that intrachain SS linkages occur within the regular α-helical structure.[30]

Three types of intrachain linkages can be presumed as follows: (1) the linkage between the two sites located on the helical and the nonhelical rod-domain segments in wool IF chains that contain one or two cysteine residues in the L1 segment,[30,31] (2) the linkage between the two sites on the helical section of the rod domain and the nonhelical section of terminal domain existing near the linking region of the rod and terminal segments, and (3) the crosslinkages among the two sites on the helical section and their paired sites closely positioned on the nonhelical section of the terminal domain. These two paired intrachain linkages will act like a loop and they are mechanically inactive, but chemically determinable as two intrachain bonds. The distance between two such proximate sites on the nonhelical section of terminal domain may be less than the order of magnitude of n_r, representing a segment length of network molecules in the swollen keratin. The n_r has been estimated to be 1250 as the molecular weight per segment, which corresponds to about 11 amino acid residues.[8]

Figure 7 shows possible crosslinked structures of the wool IF chain consisting of the rod-domain segments with three interchain and four intrachain linkage sites and the nonhelical terminal segments with 10 interchain and four unknown type linkage sites. As based on the three types of intrachain linkage, the crosslinked structures can be constructed from the combinations of types (1) and (2), types (1) and (3), and types (2) and (3). The proposed structures are shown schematically as (a), (b), and (c), respectively. However, at present, it is uncertain whether the formation of these intrachain linkages is sterically acceptable.

CONCLUSION

From this study, it was concluded that the intermolecular SS crosslinkages in the α-helical rod do-

main are changed into free thiol groups by the extension of the fiber. The new crosslinkage theory seems to be unsatisfactory to explain the permanent set obtained by boiling extended wool fibers.

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