Cross-linking Structure of Keratin. IV. The Number of Cross-linkages in Low-Sulfur Components and the Volume Fraction of High-Sulfur Domains in Various α-Keratin Fibers

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SYNOPSIS

Various α-keratin fibers that had been treated with an 11*M* LiBr solution containing *N*-ethylmaleimide showed typical rubberlike elasticity in a solution composed of equal volumes of 8*M* LiBr and diethylene glycol mono-*n*-butyl ether. Stress–strain relations of the swollen fibers were treated with a two-phase model: a mechanically stable phase of higher cross-linked domains and a rubber phase with lower cross-link density. Stress–strain curves for a variety of keratins (three different human hairs, six different wools, mohair, cashmere, llama, alpaca, angora, and opossum) were analyzed by applying non-Gaussian chain statistics to the swollen keratin network, including microdomains, which act as reinforcing filler particles in rubber. The phase structures of unswollen domains and swollen rubber were considered to originate from different structural components characteristic of α-keratin, namely, the high-sulfur matrix and the low-sulfur microfibrils being randomized by swelling. It has been suggested that (1) the modulus of swollen fibers increases with increase of the content of disulfide (SS) in keratins, (2) the volume fraction of high-sulfur domains increases with increase of SS content, and (3) the number of intermolecular cross-links in the rubber region of low-sulfur proteins is virtually the same among keratins and reaches about 65–75% of the SS linkages in the corresponding proteins. Some discussion has been made on the SS bonding *in situ,* namely, SS linkages between the low-sulfur proteins, between the low-sulfur and the high-sulfur proteins, and between the high-sulfur proteins in keratins. © 1993 John Wiley & Sons, Inc.

INTRODUCTION

Keratin fiber, which consists of α-helical microfibrils and globular matrix, has been considered as a heavily cross-linked and nonisotropic molecular system containing disulfide cross-linkages. The filamentous microfibrils of low-sulfur proteins are arranged in the fiber direction and embedded in the matrix of high-sulfur proteins. The content of sulfur in keratins and the proportion of microfibril and matrix components differ considerably. Gillespie and Inglis[1-3] showed that from the results of the amino acid analysis of the extracted protein fractions from a variety of keratins each keratin contains low-sulfur proteins that have a similarity to each other, whereas the keratins differ greatly in the type and amount of high-sulfur proteins that they contain.

In the field of basic research on keratin structure, there has been a great advance during the last years since the finding that wool and hair microfibrils constitute a form of intermediate filaments (IF) in epithelial cells and that the matrix proteins, the so-called intermediate filament-associated proteins (IFAP), aggregate with keratin IF to form macrofibril.[4-7]

Fraser et al.[8] reported the disulfide bonding within and between the subunits constituting IF and discussed it on the basis of the sequential and conformational structure of the proteins. Zahn[9] pointed

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out that for future problems in keratin structural research the cross-linking structure of the disulfide linkages in the intact proteins in macrofibril has remained an interesting uncharted problem.

A number of reports to assess cross-linking in keratins have been presented.[10-14] However, there has been no reliable quantitative method for determining the number of cross-linkages in keratin. One of the methods for the determination is the application of rubber elasticity theory for swollen keratins.[10,15-18] The swollen keratin fibers prepared by treatment with a concentrated LiBr aqueous solution show a rubberlike elasticity during extensions. However, the nonisotropic molecular structure of keratin made up of α-helical and globular regions still remains, even when the fiber attained a highly swollen state. Therefore, application of conventional rubber elasticity theory to such a heterogeneous and densely cross-linked system becomes clearly inaccurate.

It has been evidenced that the swollen hair and wool keratins prepared by the treatment with an $11M$ LiBr solution containing N-ethylmaleimide show typical rubberlike elasticity in a mixed solution composed of equal volumes of $8M$ LiBr aqueous solution and diethylene glycol mono-n-butyl ether, namely, entropy-dependent retractive forces at extensions up to ca. 30%[19,20] and no crystallization during higher extensions.[19] A suggestion has been made that the swollen fiber thus obtained consists of a two-phase structure similar to A–B–A block copolymer[20]: One is a mechanically stable phase of a densely cross-linked domain and the other is a continuous rubbery phase with a lower cross-link density. According to this structural model, the equation of state for the network containing microdomains has been derived and applied to swollen hair and wool keratins to analyze the stress–strain relationships.[21] The results obtained showed that (1) the difference in the modulus of the swollen fibers is due mainly to the volume fraction of domains in keratin that act as reinforcing filler particles in a usual rubber network and (2) the cross-link density of the rubber region in both swollen keratins is approximately similar.

The aim of this study was to estimate the volume fraction of the domains and the number of disulfide cross-linkages of the swollen network in various keratins, to demonstrate the relations between each structural parameter and the disulfide content, and to discuss the intermolecular disulfide bonding between low-sulfur proteins, between low-sulfur and high-sulfur proteins, and between high-sulfur proteins.

EXPERIMENTAL

Materials

Keratin fibers used were purified by a Soxhlet extraction with acetone for 24 h and followed by extraction with ethanol for 24 h at room temperature, washing with distilled water, and then air-drying. Keratin fibers tested were human hairs, wools (Suffolk, Merino Medium Sociality, Border Leicester, Dorset, Leicester, Lincoln), mohair, cashmere, llama, alpaca, angora, and opossum.

N-Ethylmaleimide (NEMI) used as an inhibiting agent of thiol/disulfide interchange reactions was special reagent grade. Diethylene glycol mono-n-butyl ether (BC) was obtained by distillation as described in a previous paper.[21]

Determination of Disulfide (SS) Content

The SS contents of keratins were determined by a Leach's polarographic method using methyl mercury iodide.[22] The polarographic analyzer used was a Yanako Polarograph Model P-900. All analyses were performed three times, and the average value was taken.

Preparation of Swollen Fibers

The fibers (50 mg) were treated with an $11M$ LiBr aqueous solution containing 10^{-2} M NEMI (2 mL) at 90°C for 1 h and, subsequently, immersed in a mixed solution composed of equal volumes of $8M$ LiBr and BC at room temperature. The swollen fibers thus obtained were subjected to mechanical tests.

Mechanical Tests for Swollen Fibers

Stress–strain relations for the swollen fibers were obtained in the mixed solution at a constant temperature of 50 or 70°C. Five samples were elongated at a rate of ca. 10%/min to break for each keratin, and the average value of the breaking elongation was determined. The sample was elongated up to an extension corresponding to about 70% of the average value of breaking elongation and conditioned by repeated loading and unloading. Equilibrium forces were measured at constant elongations at intervals of ca. 2–4% extension. The sample was elongated at a constant extension ratio, λ, and allowed to relax for a time until an equilibrium force, F, was obtained. The relaxation times required for measurement of equilibrium forces were different among the

fibers under extension at different strain ratios. The equilibrium forces measured at 70°C were obtained after much shorter relaxation times as compared to the measurements at 50°C.[20] The equilibrium stresses referred to the average cross-sectional area of the swollen and unstrained fiber were used for construction of the stress–strain curve. The mechanical tests were performed for five to eight specimens of each keratin. Uniform and no medullary fiber specimens were selected before testing under a microscope by measuring the fiber diameter in a longitudinal direction. The measurements of the cross-sectional area were carried out by an optical microscope method.[20] The volume fraction of keratin materials in swollen samples, v_2, was determined as described in a previous paper.[20]

Determination of Structural Parameters of Swollen Keratins

On the basis of a two-phase system that the swollen keratin is composed of densely cross-linked domains dispersed in a continuous lightly cross-linked rubbery phase, the relationship between the equilibrium forces, F, and the extension ratios of the rubbery chain, α, has been derived using eq. (1)[21]:

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

where the shear modulus of the swollen keratin sample, G, represents $(\rho RT/Mc)\{(v_2 - \phi_d)/(1 - \phi_d)\}^{1/3}(1 - 2Mc/M)\,\gamma$; n is the number of segments in network chain; $L^{-1}(x)$, the inverse Langevin function; ρ, the density of unswollen sample; Mc, the number-average molecular weight between cross-links in rubbery region; M, the number-average molecular weight of the primary molecule; R, the gas constant; T, the absolute temperature; v_2, the volume fraction of polymer in a swollen sample; ϕ_d, the volume fraction of high-sulfur domains in swollen keratin; and γ, the filler effect of domains existing in the rubbery region. Here, the filler effect, γ, is a function of k and ϕ_d, where k is the shape factor as the length : breadth ratio for rodlike filler. In the case of swollen hair and wool, a near-spherical particle in shape has been assumed. As given by Guth[23] and recently by Leonard,[24] the filler effect can be expressed by eq. (2):

$$\gamma = 1 + 2.5k\phi_d + 14.1k^2\phi_d^2 \quad (2)$$

According to our simplified model, the relationship between α and the extension ratios of the swollen keratin sample, λ, can be described as eq. (3):

$$\alpha = (\lambda - \phi_d)/(1 - \phi_d) \quad (3)$$

Fitting the experimental data, F, G, λ, and v_2 for eq. (1), with a suitable choice of parameters, ϕ_d, ρ/Mc or Mc, and k, we can evaluate the values of these parameters. With the aid of computer, it was attempted to fit the equation to the experimental data with these adjustable parameters using a Damping–Gauss method of nonlinear least squares. These parameters can be obtained by using the data over the range of extensions to the inflexion point observed at a higher extension of the stress–strain curve. The least-squares refinement was executed by repeating the cycles on the three parameters under the condition that one was fixed by the computer program. Here, two assumptions were made: (1) that the segment length of keratin chains, n_r ($= Mc/n$) is a constant, and (2) that the number-average molecular weight of the primary molecule of the keratin chain (M) is 5.0×10^4, being the same for low-sulfur proteins in wool keratin. The n_r value can be obtained from the $F - \lambda$ relationship for reduced keratin, which contains a small number of SS bonds. Reduced and S-β-cyanoethylated human hairs that contain only 91.6 μmol/g of SS groups were prepared and it was assumed that they contain no domains. By fitting eq. (1) for the experimental data of the reduced keratin at the condition of $\phi_d = 0$ and, therefore, $\alpha = \lambda$ and $\gamma = 1$, the n_r value was thus obtained as 1250. It was assumed that the same value is applicable for the other keratins as the length equivalent to the random chain segment.[21]

RESULTS

Figure 1 shows the results obtained by fitting eq. (1) for the stress–strain data from different keratins. The k values determined by a good fitting were approximately similar to the value of 1.7 ± 0.1 for all keratins studied. The SS content, v_2, and G values obtained experimentally and the ρ/Mc and ϕ_d values evaluated as parameters in eq. (1) are shown in Table I. It is noted that the shear modulus, G, increases with increase of the SS content in keratins. As shown in Figure 2, the cross-link density in the rubbery phase, ρ/Mc, is almost the same for various keratins, although two of three human hairs, Leicester and Suffolk, have relatively lower values than do the others. This similarity implies that the

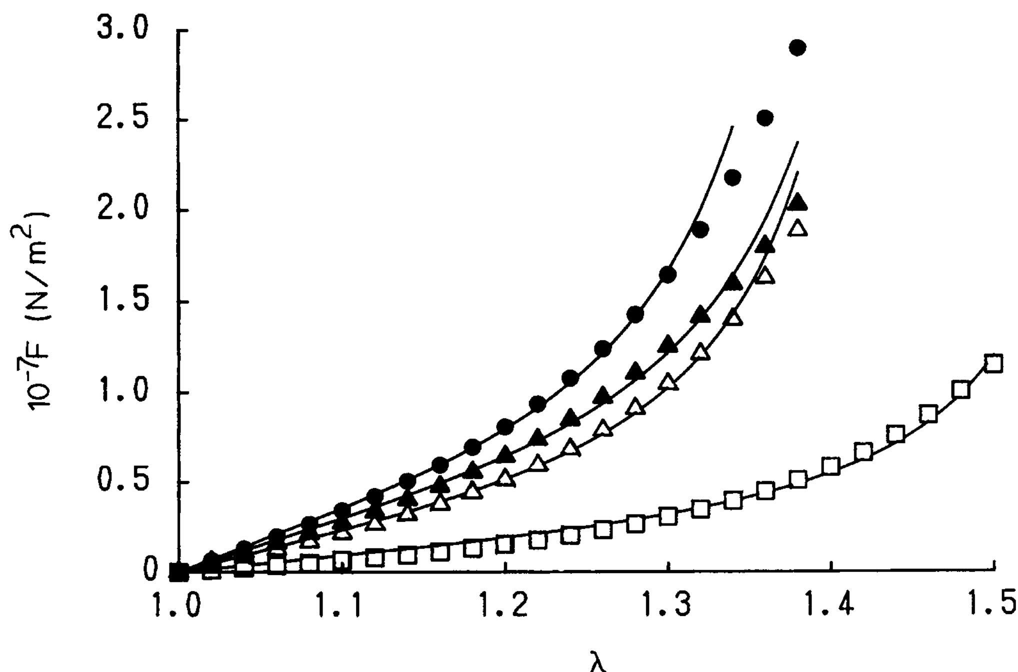

Figure 1 Relationships between equilibrium stress, F, and strain, λ, for swollen keratin fibers; (●) human hair; (▲) alpaca; (△) llama; (□) opossum; (——) lines fitted to experimental data by eq. (1).

number of intermolecular SS cross-links in the network composed of low-sulfur protein chains is substantially the same among keratins, whereas the ϕ_d values increase linearly with increase of the SS content, as shown in Figure 3. These results show that the difference in the shear modulus of swollen ker-

Table I Results Obtained from Eq. (1)

Sample No.	Samples	[SS] (μmol/g)	10^{-6} G (N/m^2)	v_2	10^4 ρ/M_c (mol/cm^3)	10^{-3} Mc^a (g/mol)	ϕ_d	ϕ'_d
	Human hairs							
1	Japanese female	598	12.0	0.71	3.0	4.3	0.41	0.58
2	Japanese female	588	12.0	0.76	3.0	4.3	0.39	0.51
3	Japanese female	572	13.1	0.80	3.5	3.7	0.35	0.43
	Wools							
4	Suffolk	494	6.49	0.68	3.1	4.2	0.25	0.37
5	Merino Medium Sociality	481	7.04	0.64	3.7	3.5	0.23	0.36
6	Border Leicester	438	6.22	0.58	3.5	3.7	0.24	0.42
7	Dorset	438	6.90	0.65	3.6	3.6	0.24	0.37
8	Leicester	421	3.84	0.78	2.9	4.5	0.26	0.40
9	Lincoln	420	4.91	0.60	3.5	3.7	0.22	0.36
10	Mohair	404	4.63	0.74	3.6	3.6	0.18	0.24
11	Cashmere	313	3.98	0.74	3.6	3.6	0.16	0.22
12	Angora	496	9.12	0.57	3.4	3.8	0.34	0.60
13	Llama	468	8.25	0.52	3.7	3.5	0.29	0.56
14	Alpaca	418	8.96	0.65	3.7	3.5	0.28	0.43
15	Opossum	379	5.43	0.50	3.9	3.3	0.19	0.38

[a] The Mc values were calculated from the ρ/Mc values by assuming that the fiber densities, ρ, are 1.30 g/cm^3 for all keratins studied.[21]

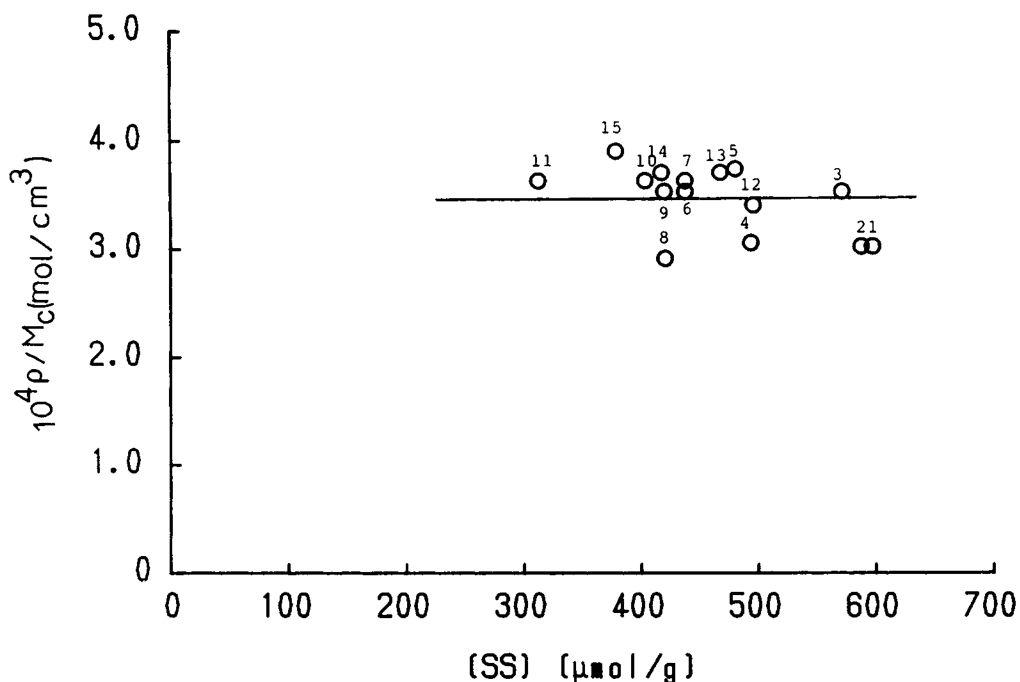

Figure 2 Relationship between cross-link density of the rubbery region in swollen keratin, ρ/Mc, and content of disulfides, [SS]; the number designated for each plot corresponds to the sample number in Table I.

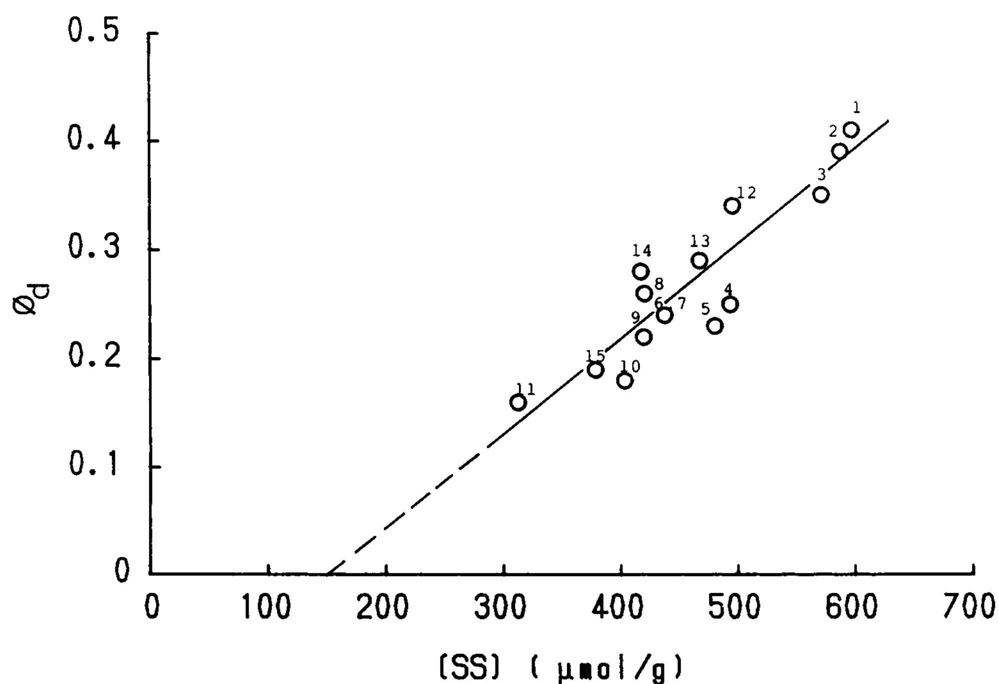

Figure 3 Relationship between volume fraction of domains in swollen keratin, ϕ_d, and content of disulfides [SS]; the number designated for each plot corresponds to the sample number in Table I.

atin fiber is due to the difference in the volume fraction of domains and, therefore, to the difference in the amount of the high-sulfur proteins acting as filler particles in the swollen network.

DISCUSSION

Number of Cross-links in Low-Sulfur Proteins

The amounts of low-sulfur and high-sulfur proteins in keratins and the sulfur content in each protein have been extensively studied by applying a fractional precipitation method for soluble *S*-carboxymethylated (SCM) keratins.[1-3,25-27] It has been shown that the sulfur content in low-sulfur proteins is approximately constant and the average value is 1.3% for SCM keratins with different sulfur content ranging from 1.9% (rhinoceros horn) to 5.5% (raccoon hair).[2,3] The sulfur content analyzed as SCM cysteine includes the sulfur from free thiol (SH) groups. However, the SH groups present in keratin fibers are relatively small and the amount is only about 5% or less of the total SS content in keratins. We can assume, therefore, that the sulfur content analyzed as 1.3% in low-sulfur proteins is mainly from the SS groups in keratins, and, therefore, ca. 200 μmol/g ($= 1.3 \times 10^4/64$) of SS linkages are included in low-sulfur proteins from a variety of keratins.

As the cross-linkages other than SS cross-linkage, lanthionine,[28] lysinoalanine,[28,29] and isopeptide linkages[30-33] have been found and their content is different among keratins, but they are minor constituents relative to SS cross-linkages. Acid labile bonds have also been reported and considered to be probably ester cross-linkages.[34,35] From the stress–strain data of the swollen Lincoln wool fiber reduced thoroughly with thioglycolic acid and then blocked with iodoacetate, but not with acrylonitrile as reported previously,[20] no perceptible cross-links in keratin have been suggested.[36] The cross-linking structure of keratin proposed from the present study is, therefore, concentrated on SS cross-linkages.

From the results in Figure 2, it is quite reasonable to assume that the number of intermolecular cross-links in the network composed of low-sulfur protein segments is approximately the same among keratins, namely, the average Mc value is 3800 (Table I). On the basis of this assumption, the content of the SS bonds participating to the formation of intermolecular cross-links can be calculated to be about 130 μmol/g ($= 10^6/2Mc$). This value corresponds to about 65% of the total SS content of the low-sulfur proteins.[2,3] The calculated value seems to be reasonable for the following reasons: (1) the actual number of cross-links is less than the analytical values for SCM cysteine, which include the entities from free cysteine residues existing in the intact proteins; (2) mechanically ineffective SS linkages such as intrachain linkages may be involved in the keratin system; and (3) the distribution of SS cross-links is not random[8,37] and they occur in nearby sites to play a role as different interchain cross-links.

Relationship between Volume Fraction of High-sulfur Domains and SS Content

A linear relationship between the volume fraction of domains and SS content in keratin is notable (Fig. 3). From the intercept of the extrapolation of the linear line to the axis of SS content, we can evaluate the SS content of a keratin presumed as it contains no high-sulfur domains, but consists of only low-sulfur protein components. The value obtained is about 148 μmol/g. It should be noted that this is approximately similar to the value calculated from the average Mc value of low-sulfur proteins as described in the preceding discussion. It is inferred, therefore, that the SS bonds over this value may occur in the domains of high-sulfur matrix proteins.

It should be emphasized that the number of intermolecular cross-links in low-sulfur proteins is approximately constant and independent of the amounts of low-sulfur and high-sulfur proteins that are greatly different among keratins. This implies that from the point of view of cross-linking these two different proteins occur in a separate fashion as cross-linked systems independent of each other. It is likely to be considered, therefore, that there must be no or only a few cross-links between the two different proteins. In general, extraction of these component proteins from keratin fibers is unsuccessful without scission of SS bonds.[9] From the insolubility of α-keratin, it has been considered that cross-links must be present between these different chemical constituents in keratin.[8] However, even for cross-link-free molecules, the extraction might be extremely difficult from a highly cross-linked gel system such as swollen keratin, as pointed out for the case of the extractions of homopolymers occluded within graft copolymers.[38,39] At present, exact treatment for the SS cross-linkages between the two different proteins could be impossible. It can be concluded, however, that the proportion of the SS bonding between the two different components is low even if the cross-links occur.

The volume fraction of domains in the unswollen sample, ϕ'_d, can be calculated by eq. (4):

$$\phi'_d = \phi_d / v_2 \qquad (4)$$

A linear relationship between the ϕ'_d values and SS content is shown in Figure 4. Although the plots tend to scatter as compared to the plots for the ϕ_d values in Figure 3, an approximately linear curve can be constructed to give a value of ca. 148 μmol/g in SS content at the intercept of the extrapolation of the curve.

According to the data tabulated in Ref. 3, the weight fractions of high-sulfur proteins to high-sulfur plus low-sulfur proteins, ϕ are recalculated and plotted against the total sulfur content of keratins, as shown in Figure 4. A good linear relationship can be observed. The linear curve intercepts on the axis of SS content at about 200 μmol/g, which equals the SS content in the low-sulfur proteins from various types of keratins. It is of interest that the SS content in the low-sulfur proteins evaluated physically from the relationship of ϕ'_d vs. SS content is about 75% to that value obtained by the chemical method based on amino acid analysis of the fractionated low-sulfur proteins. This also suggests that, as in the conclusion derived from the preceding discussion on the cross-link density, a large amount of the SS bonds occurring within the low-sulfur components consist of intermolecular or interchain cross-linkages. Further, this suggestion accords well with the consideration for the SS bond formation in the higher-order structure of IF proteins.[8]

Assuming the similarity of the density for microfibrils and matrix proteins in keratin,[40] the weight fraction ϕ values may probably equal the values corresponding to the volume fractions, ϕ'_d. A considerable difference, however, can be observed between the slopes in Figure 4. In other words, with the increase of the SS content, the actual domain volume increases more rapidly than does the volume of high-sulfur protein components.

Recent advances in the knowledge of the sequence and structure of IF proteins shows that IF subunits

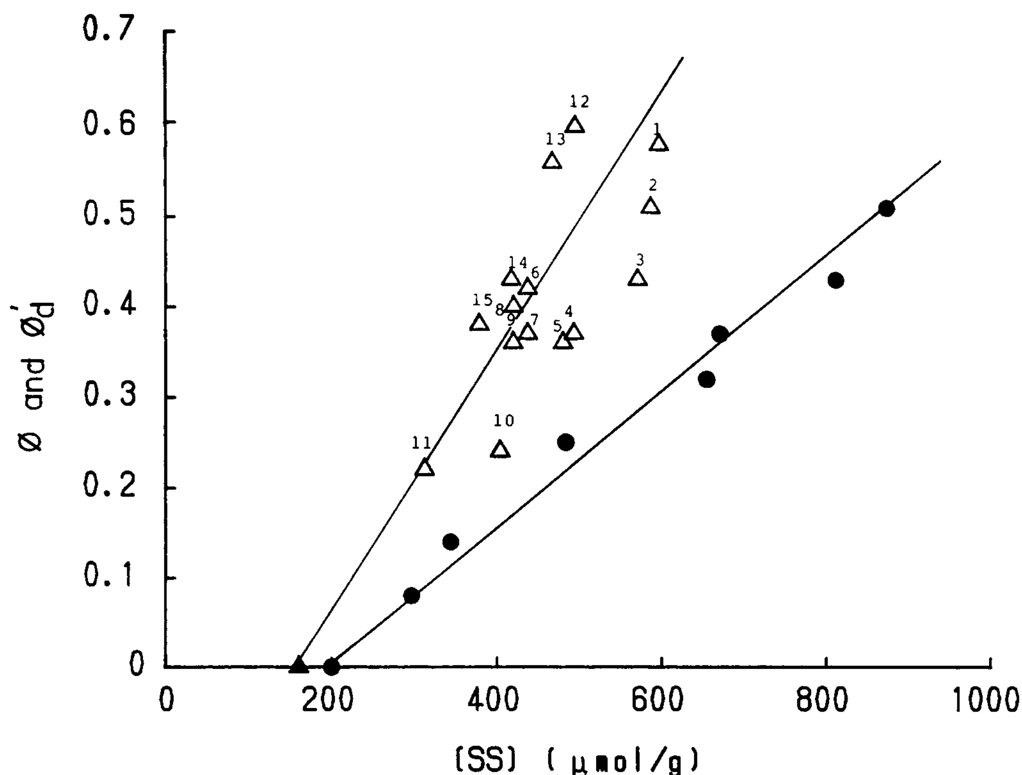

Figure 4 Relationships between volume fraction of domains in dry fiber, ϕ'_d, and content of disulfides, [SS], and between weight fraction of high-sulfur proteins to high-sulfur plus low-sulfur proteins in keratins, ϕ, and content of disulfides, [SS]; here, ϕ values were obtained from the data in Ref. 3; (△) ϕ'_d; (▲) the value obtained by the extrapolation of ϕ_d values to SS content in Figure 3; (●) ϕ; the number designated for each plot corresponds to the sample number in Table I.

are composed of a central α-helical domain and two non-α-helical domains at N and C terminals.[7,41] It has been considered that the nonhelical parts may occur in part within the matrix and interact with the surface of the globular matrix.[42–44] These situations may lead to a net increase in the domain volume, provided that the interactions occur through the SS cross-linkages between the nonhelical parts of IF and the high-sulfur matrix molecules. These effects, however, must be decreased with increase of the SS content, because there is a relative decrease in the amount of low-sulfur proteins or IF components when the SS content is increased. Contrary to this, the domain volume is markedly increased with increase of the SS content (Fig. 4).

It has been suggested that from the swellability and mechanical properties of keratins, some interchain SS cross-linkages must be present in the matrix proteins.[45,46] The aggregation of the proteins constituting the matrix through the interchain SS bonding may result in a volume increment of the matrix components, since the volume of aggregates will be larger than the total volume of the constituent matrix proteins with no interchain cross-linkages. The volume increment of the aggregates must be increased with increase of the SS content because the increase of the SS content results from the increase in the amount of high-sulfur matrix proteins (Fig. 3). Although it is too complex to give a reasonable explanation of the difference between the slopes observed on the relationships of ϕ'_d and ϕ vs. SS content, it is likely to be explained in terms of the formation of aggregates of the matrix protein, namely, the volume fraction of domains corresponding to the aggregates (ϕ'_d) must be larger than that of the high-sulfur matrix proteins (ϕ).

The heterogeneous nature of high-sulfur proteins exists in the number, type, and distribution of the components that are different for each keratin.[3] Although we might expect differences in the actual domain volume and the nature of domains among keratins under the variability in content of high-sulfur proteins, the result in Figure 4 seems to indicate the possibility that some proportion of the interchain SS cross-linkages is present in the high-sulfur matrix proteins. With respect to this, Crewther[47] suggested in his keratin fiber-structure model that SS bonding exists between the globular matrix proteins. However, further investigation is needed for this problem.

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