Doctoral Dissertation (Shinshu University)

Studies on the disulfide cross-linked structure and mechanical properties of keratin fibers

September 2015

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CONTENTS

Abstract

Influence of the disulfide (SS) cross-linked structure of keratin fiber on its mechanical property was investigated in this dissertation. Relevance of microstructure of keratin fibers to its mechanical behavior has been unclear. Since mechanical degradation of the keratin fiber is the problem related to hair damage by hair-coloring, permanent waving and hair straightening and so on, this study is also very interesting in the view of cosmetic technology. We attempted to clarify the number, type, and location of SS cross-links and identify the cross-links related to its mechanical properties. The contents in this dissertation are summarized below:

In chapter 1, the background and the aim of this study are introduced. In keratin, there are a lot of cystine residues that exist as inter- or intramolecular SS cross-links, that is, keratin has huge polymeric network structure composed of a large number of SS cross-links. Keratin fibers from mammal such as wool and human hair constitute a complicated hierarchical structure composed of many cells, and shows characteristic mechanical properties. Since SS cross-linked structure of the keratin fiber seems to be related to its various mechanical behavior, understanding the SS cross-link structure in more detail and clarifying the relationship to its mechanical properties are very important in the view of not only protein chemistry but cosmetic industry.

In chapter 2, the SS cross-linked structure of hair and wool keratin fibers is discussed. Rubber elasticity theory was applied to the force–extension curves of swollen fibers in a diluent mixture of concentrated aqueous lithium bromide and diethylene glycol mono-*n*-butyl ether. On the basis of a two-phase structural model for the globular matrix keratin-associated protein (KAP) dispersed in the swollen network of intermediate filament (IF) proteins, structural parameters were obtained by fitting the force–extension curve to theoretically derived relations between elastic forces originating from nonuniform network and the extension ratios. The parameters extracted are the number of intermolecular SS bonds in the IF, the volume fraction of matrix proteins in the fiber, and the shape of the matrix domain. The number, type, and location of SS cross-linkages in the IF proteins were estimated using the difference in the

conversion rate from disulfide to lanthionine and lysinoalanine induced by the treatment with boiling water, because it depends on the location of the SS cross-links in IF and KAP structure. The total number of SS cross-linking sites in the IF chain of an average molecular mass of 50000 is determined to be twenty-one moles. The twenty-one moles are divided into thirteen moles comprising intermolecular cross-linkages, which are subdivided to three moles between rod domains, eight moles between terminal domains and two moles between terminal and KAP domain, and eight moles comprising intramolecular cross-linkages, which are subdivided into four moles in the terminals and four moles at the interface region between rod and terminal domains. It was also found that a KAP molecule with an assumed molecular mass of 20000 involves seventeen moles of intramolecular SS bonds and 4–5 sites on the surface of the KAP molecule of hair keratin. These sites are linked to adjacent KAP molecules and form an aggregate of about six KAP molecules against the IF molecule. Finally, we proposed a network model for an IF–KAP structural unit in the hair and wool fiber cortex.

In chapter 3, the SS cross-linked structural change by permanent wave treatment for hair and the structure and mechanical property relationships are investigated using the network structural model proposed in chapter 2. Scission and reformation mechanisms of SS cross-links by the treatments with a reducing agent of thioglycolic acid (TGA) and an oxidizing agent of sodium bromate were demonstrated. It was found that cleavage of the SS cross-links between KAP molecules by preferential attack with TGA leads to the shape change from ellipsoidal form of globular aggregates to near spherical one, and these cross-links and shape are not recovered perfectly by subsequent oxidation. On the other hand, it was also found that nearly complete reformation of SS cross-links occurs between IF proteins through oxidation even when a large number of SS bonds break under strong reducing conditions. An important suggestion was obtained that the extension modulus of the hair fiber in water is highly dependent on the number of intermolecular SS cross-links between KAP molecules around the IF.

In chapter 4, recovery behavior of mechanical property during washing after reduction with TGA is studied. It is well-known that the reduction of SS bonds with thiol is equilibrium reaction. In a permanent waving system consisting of three step processes of reduction with TGA, washing with water and oxidation by sodium bromate,

it was found that the extension modulus and breaking stress of the treated hair fibers in water were increased with increase of washing time. Analysis of the SS cross-linked structure by applying a rubber elasticity theory showed that the integrity of SS cross-linked structure of IF is retained, while the interrmolecular SS cross-links between KAP molecules cleaved by reduction are regenerated by reverse reaction of the equilibrium reactions occurring during washing. Hence, it is concluded that the mechanical properties for hair fibers are controlled by the intermolecular SS cross-links between the KAP molecules.

In chapter 5, the conclusions of this dissertation are summarized. The mechanical importance of the intermolecular cross-links between the KAP molecules is clarified, but theoretical interpretation for mechanical behavior of keratin fibers seems not to be quite simple. It is important that the contribution and mechanism of the SH/SS interchange reaction will be understood in the future.

Chapter 1

General introduction

Chapter 1: General introduction

1.1. Introduction

Keratin is the generic term applied to the resilient structures such as hair, horn, nail, feather, and skin which comprise the integument and appendages of the higher vertebrates and whose prime function is to protect the animals from their environment. They are composed mainly of protein and are characterized by their high cystine content [1] and birefringence [2]. There are a lot of cystine residues that exist as inter- or intramolecular disulfide (SS) cross-links in keratin, that is, keratin has huge polymeric network structure composed of a large number of SS cross-links. Keratin can be categorized into α- and β-type on the basis of the X-ray diffraction pattern [3]. Mammal keratin such as wool and human hair is characterized by the fact that they yield an α-type X-ray diffraction pattern which reverts to a β-type on stretching [4]. In addition, keratin fiber from mammal constitutes a complicated hierarchical structure composed of many cells, and shows various properties owing to the interaction between the conformations.

In textile and apparel industry, these characteristic chemical and mechanical properties arising from the complicated hierarchical structure of keratin lead to distinct functions and feels on fabrics of keratin fibers unattainable on that of synthetic fibers, and therefore, wool fibers have been widely used for various interior goods such as clothes, blankets and carpets [5,6]. Moreover, many studies related to wool keratin structure and properties accumulated in development of wool industry have been now extended to the studies of human hair keratin, in which microstructure is very similar to wool keratin. Therefore, active hair research on the basis of wool science has been carried out for the scientific understanding and the development of some functional products in hair-coloring, permanent waving, and hair-straightening, by which various attractive hairstyles are created [7].

The causal relationships between keratin structure and expressed functions have been the target of interest for many fiber and cosmetic researchers, and investigated by them in detail. In cosmetic science, degradation of hair fiber by hair-coloring and permanent waving is detected as decrease of fiber strength. Therefore, for the

satisfaction of the demand of inhibiting this degradation from the cosmetic market, it is very important to understand the relationship between hair structure and mechanical properties. To explain the relationship between hierarchical structure of keratin fiber, also called supermolecular structure, and its mechanical properties, a lot of mechanical models to simplify the structure have been proposed for the past 80 years [8-21], and the validity has been discussed for each model. However, no model is able to explain mechanical properties of keratin fiber successfully.

It is well-known that SS bond is sensitive for hair-coloring and permanent waving [7]. SS cross-linked structure of keratin fiber seems to be related to its mechanical properties. There are some reports about the distribution of SS cross-links in hierarchical keratin structure [22-25]. The novel method to distinguish and evaluate inter- and intramolecular SS cross-links has steadily advanced the research related to the type, number and location of SS cross-links [26-29]. It is expected that understanding the SS cross-link structure of keratin fiber in more detail and clarifying the relationship to its mechanical properties will not only help the development of technology to inhibit hair degradation by cosmetic treatment, but become a clue for solving the problem of the mechanical models to be the target of interest for many keratin fiber researchers.

The principal purpose of this study is to clarify the influence of the disulfide cross-linked structure of keratin fibers on its mechanical properties

1.2. Hair and wool structure

The hair and the wool are comprised of fibrous protein called α-keratin including much sulfur atom. The sulfur atoms mainly exist as cystine (Cys) residues and form disulfide (SS) cross-links between protein molecules or within molecules. Total amount of SS bonds forming these cross-links is 13-16 mol%. In this way, the keratin fiber is a complicated and huge network polymer with a large amount of SS bonds, and its tissue is high hierarchical structure consisting of a cuticle, cortex and cell membrane complex (CMC).

Hierarchical structure of hair is shown in Figure 1.1 [30]. The most outer epidermis layer is called cuticle and comprised of flat overlapping cells. Each cell is 0.4-0.5 μm

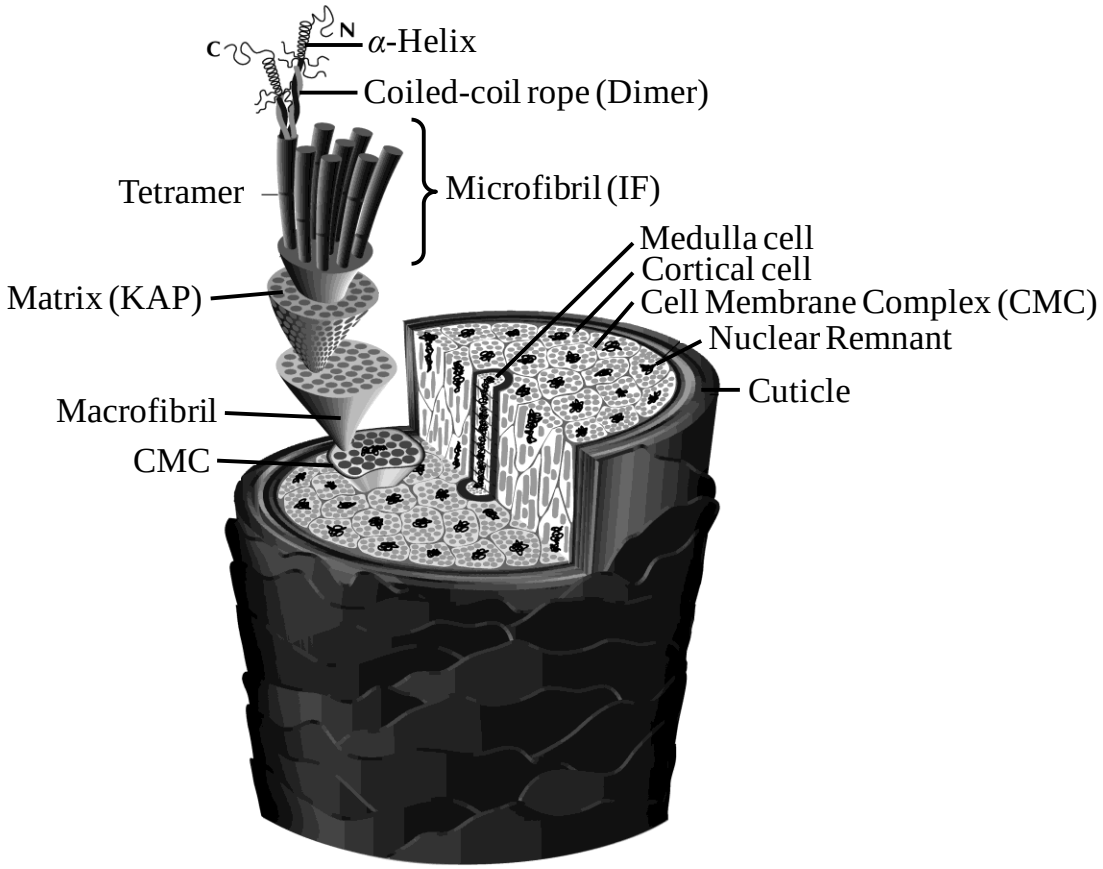

Figure 1.1. Hierarchical structure of human hair [30].

thickness, and they are piled up in 1-2 layers on the surface of the wool fiber and 6-10 layers on that of the hair fiber [31]. One cuticle cell consists of three layers such as A-layer, exocuticle and endcuticle and the SS content of them are reported to be approximately 17.5, 7.5 and 1.5 mol% respectively [32,33]. In addition, the hardness of each layer is reported to depend on the SS content, and A-layer is the most hard and the endcuticle is soft.

The cortical cell is composed of aggregation of the macrofibril, and they have spindle-shaped form of 3 μm in diameter and 100 μm in length, and are arranged in the same direction. On the border between adjacent cells, the CMC which consists of 3 layers is located and exists to circumscribe a cortical cell. The CMC runs between cells in succession from root to tip of hair fiber like blood vessels, and serves as the way of the materials and as the adhesive to fix cortical cells each orther [32]. The cortex of the hair and the wool is broadly separated into two kinds of protein such as the low sulfur (LS) protein with the low cystine content and the high sulfur (HS) protein with the high cystine content [34]. The former is called the microfibrillar protein because it constitutes the microfibril, and the latter is called the matrix protein because it is a component of globular matrices in which a microfibril is embedded. Furthermore, the former has the structure same as the intermediate filament protein included in the cell of the mammal so that is called as IF (Intermediate filament protein), and the latter is called as KAP (Keratin associated protein).

The IF protein is molecules of which an average molecular weight is approximately 50000 and its considerable part has spiral form (α-helix). These two molecules wind around each other to form a rope (coiled-coil rope, IF molecules), and then one pair of rope gathers to form 4 molecules aggregation (tetramer). This aggregation becomes a unit, and 8 units gather cylindrically to constitute a microfibril [35-39]. The end of the rope (N- and C-terminal) does not have α-helix structure, and proline and cystine residue are included abundantly there.

The KAP protein is molecules with a molecular weight of 10000-22000, and is said to be a spherical protein molecule [40]. They are aggregated to form a matrix. IF filaments of microfibrils implanted in the matrix gather regularly with pseudohexagonal array to form a macrofibril. The IF and KAP gather regularly to become the macrofibril

of 0.3 μm (= 300 nm) in diameter, and macrofibrils gather to be a cortical cell of 3 μm in diameter and 100 μm in length.

The CMC consists of two β-layers and δ-layer between both β-layers. CMC plays a role to join two kinds of cells, cuticle cells and cortical cells, and is classified in three kinds of coupling types [41]. That is, they are between cuticle cells, cortical cells and cuticle and cortical cells. The ratio of the CMC within the wool is estimated at 2.6-3.7 % [42,43], and the CMC is very low as the ratio but is very important as a transfer line of water.

In this way, the wool and the hair fiber have complicated microstructure, and the SS content in each component varies respectively, but it may be said that keratin proteins have huge network structure composed of protein molecules connected continuously by the SS cross-links.

1.3. Mechanical property of keratin fiber

As mentioned above, the wool and the hair are the multicell fiber which has complicated hierarchical structure, but it is very interesting that the stress-strain curve obtained by extension of the fiber has distinctive three liner areas. The mechanical characteristic expressed by the extension is a property of the whole fiber and a characteristic of the cortex that is unrelated to the cuticle. Typical stress-strain curves of the keratin fiber in the air and water are shown in Figure 1.2 [7]. Hydrogen and ionic bonds come loose in water, and the stress is generated by remaining intermolecular forces. Therefore, the qualitative and structural knowledge about the cross-linked density by the SS bond, the quantity and the integrity of α-crystal can obtained from the characteristic of the stress-strain curve measured in water. The three liner regions are a Hookean region where the strain ratio is less than approximately 2 % and the stress increases linearly, a yield resion where the strain ratio continues within 25-30 % and a post-yield region where the stress increases sharply. It is almost restored in an original structure even from a high extension state when the stress is removed after the extension. It is known that the microstructure of the fiber disrupted by extension is completely restored by the relaxation in water at 52 °C for one hour [44,45]. The ratios of the slope

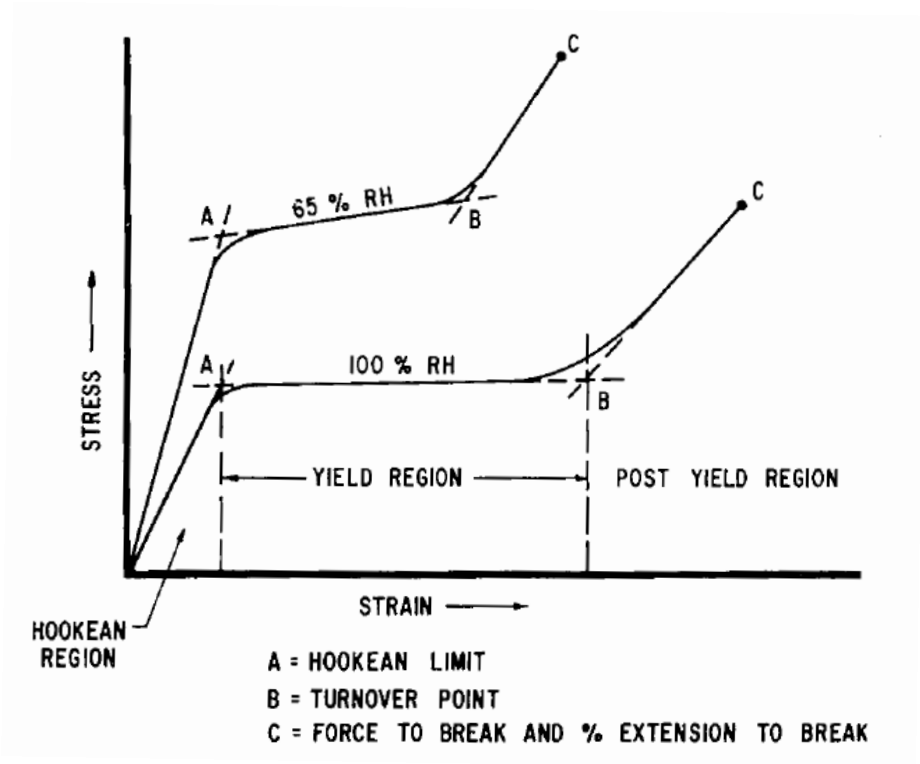

Figure 1.2. Typical stress-strain curves for keratin fibers [7].

in these three regions are 100: 1: 10.

In the Hookean region, it is shown by Astbury et al. that the most transformation of the fiber are based on crystalline elasticity [46]. On the other hand, it is also thought that the stability of α-crystal depends on not only the quantity of the helical material and the integrity of its structure, but also the cross-linked density of the SS network in KAP protein in which IF proteins are embedded [47]. The α-helix structure unholds and β-pleated sheet structure is generated simultaneously in the yield region, and equalization of the stress among the molecular chains in the primary structure is induced by SH/SS interchange reaction between a SS bond and a thiol (SH) which exists slightly in the fiber [48]. However, there are now some interpretations about the mechanism of $\alpha \rightleftarrows \beta$ transformation in the view of molecular theory [49,50]. Speakman showed that the mechanical behavior in the post-yield region is caused by the deformation of the covalent cross-linked network structure including the SS cross-links [44]. In addition, Feughelman confirmed that the mechanical recovery characteristics of the fiber to return to original length decrease rapidly when the fiber is extended to the post-yield region [51].

Even if the stress-strain curve is simple and characteristic, the structural factors related to such mechanical behavior are very complicated. Hierarchical structure of keratin was modelled with simple structure to give the mechanical behavior the interpretation in the view of molecular theory, and it has been argued mainly on the two structured model. The first model is the model that the microfibril and uniformly cross-linked matrix arranged mutually in parallel resist extension [8,9]. In this model, the stress to the yield region depends on structural deformation of the IF which is mainly $\alpha \rightleftarrows \beta$ transformation, and the stress increases continually because the matrix network is restricted in the post-yield region. The second model is the series zone model proposed by Feughelman [10-14]. This is the model that two types of zones (X-zone and Y-zone) showing different mechanical stability are repeated along a fibril direction alternately. The α-helix in the X-zone and the Y-zone is unheld in the yield region and the post-yield region, respectively. It is said that the stress by extension occurs from not only SS cross-links in matrices but also β-sheet structure formed by deformation of the microfibril [15]. In addition, various models were proposed for the successful

interpretation of the stress-strain curve such as the extended two phase model shown by Feughelman et al. [16], the IF extension model by Wortmann et al. [17], the stress transition model by Hearle et al. [18-21], but there are limits in each model and no model is able to explain mechanical properties of the keratin fiber successfully. The main reason is that how the SS cross-links, which form the network structure of the keratin protein and play the important role in the mechanical response of the fiber, exist and function in hierarchical structure of the fiber is unknown sufficiently.

1.4. SS cross-linked structure in keratin fiber

The distribution of SS cross-links in the microstructure of the cortex for keratin fibers has been reported by Fraser et al. [22,37]. The number and location of cystine residues in wool IF chains sequenced for four IF protein species have been summarized in Table 1.1. The IF molecule consists of an α-helical rod domain and nonhelical N- and C-terminals as shown in Figure 1.3 [30,52]. There are two types of protein species in the wool and hair keratin IF, the so-called Type I and Type II proteins. Type I is acidic proteins with a molecular weight range of 4.2-4.6 $\times 10^4$ and Type II is neutral/basic with that of 5.6-6.0 $\times 10^4$. There are 7 cross-linking sites in the rod domain and 15 sites in the N- and C-terminals, and these corresponds to the SS content of 70 and 150 μmol/g, respectively. The total cystine content in IF proteins is calculated to be 220 μmol/g, while it is also reported by Gillespie et al. as 200 μmol/g, regardless of the kind of animal, from the chemical analysis of low-sulfur (LS) proteins fractionated from the keratins of various animals [23-25]. Fraser et al. have proposed the location and type (inter- or intramolecular) of SS cross-links in the rod domain based on the X-ray diffraction data and the knowledge of the SS bond in the globular proteins in which three-dimensional structure have been revealed [22]. It is important to support this proposal experimentally, however, it is unable to differentiate inter- and intramolecular SS cross-links by chemical methods such as amino acid analysis.

Concerning the nature of cross-links in hair and wool, Arai et al. [26] found that fibers treated with a concentrated aqueous lithium bromide (LiBr) solution containing *N*-ethylmaleimide, which serves as a blocking agent for free thiol (SH) groups, show

Table 1.1. Molecular weights of the IF and the number in mole of cysteine residues per IF chain [22,37].

Type of Low-Sulfur Protein Fractions		Domains			Molecular Weight (MW) ($\times 10^{-4}$)
		Rod	N- and C-Terminals	Total	
Type I	8c-1	8	17	25	4.2-4.6
	8a	5	10	15	
Type II	7c	9	21	30	5.6-6.0
	5	7	11	18	
Average Number of Cysteine Residues per IF Chain		7	15	22	Average MW of IF Protein, 5.0×10^4
Average Number of SS Cross-Linkages (μmol/g of IF Protein)		70	150	220	

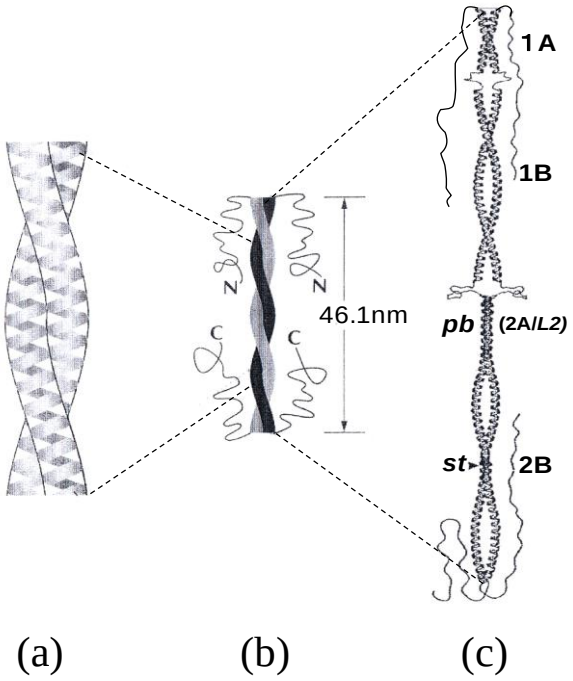

Figure 1.3. Schematic representation of IF chain structure and IF molecules: (a) α-helical region in IF dimer molecules, (b) a Type I + Type II dimer model forming N- and C- terminal structure, (c) a dimer model composed of two arranged IF chains in parallel [52].

typical rubber elasticity in a mixed solution of aqueous 8 M LiBr and diethylene glycol mono-*n*-butyl ether. Therefore, they proposed a semi-quantitative method for determining the SS cross-link density of various keratin fibers. They attempted to evaluate the number of SS cross-links by using a method of Gaussian chain statistics for untreated and thioglycolic acid (TGA)-reduced hair samples in various degrees of reduction and concluded that SS linkages are divided into two groups: the intermolecular linkages group (SS_1 and SS_2) and the intramolecular linkages group (SS_3). SS_1 linkages may be located in water-accessible terminal regions of IF proteins and SS_2 linkages are located on the surface region of the KAP domain, while SS_3 linkages exist in the hydrophobic inner region of the KAP domain.

A quantitative method for analyzing the number, type, and location of SS cross-links in the keratin cortex has been developed by applying a non-Gaussian elastic equation of state to the stress–strain curve for swollen fibers [27-29]. The difference in the reactivity of SS bonds in keratin structures was determined by comparing the analytical results obtained from both chemical and mechanical tests. The difference in the reactivity of SS bonds under fiber extension and non-extension states was utilized for the assignment of bonds existing in water accessible or inaccessible regions, allowing detailed cross-linked structures in microdomains to be determined.

In this way, SS cross-linked structure in keratin fibers has come to be known little by little. However, the relationship to its mechanical properties is not enough clarified. Investigation on the mechanical responses of keratin fibers in which the SS cross-linked structure is systematically modified is expected to elucidate mechanical roles played by each type of SS cross-links and give some findings on the problem of the mechanical model.

1.5. Studies and current state in the cosmetic market for permanent wave

It has been admitted that giving a hair waves makes women so attractive in every time and place. Nessler is considered to be a key person in the invention of permanent wave in the early 1900s [53]. First permanent process was to react alkali or alkaline sulfite solution with a hair at a high temperature [53,54]. The current permanent wave is

very superior to the early hot wave and does not need high temperature, so is called a cold wave. The cold wave develops during World War II and does not change substantially for the next approximately 70 years.

In Japan, the fashionableness including a change of hairstyle and clothes increased from the 1960s and demand for permanent waving products increased more together. With it, development for permanent waving products was more active by each manufacturer. The amount of shipment of the permanent waving product in Japan is shown in Figure 1.4. The amount of shipment increased rapidly from about 1970 and peaked in 1992, but has decreased from then to the present, and the recent amount of shipment falls into about 1/4 of the peak. One reason of this big change on the amount of shipment is that the hair coloring, which began with the boom of brown hair from about 1992, took the place of the permanent wave. However, the important problem was that the unpredicted serious hair damage was caused by applying permanent waving treatment to a colored hair. In this way, beauticians and customers came to keep a distance from the permanent waves. However, Japanese women have a longing strongly for the wave hairstyle like the Westerner, and it may be said that the role of permanent wave prroducts is important. Therefore studies on controlling the damage of hair caused by permanent wave are conducted actively [55-58].

Permanent wave products are prepared based on mercaptan or sulfite solution, and thioglycolic acid is used most commonly. The method of permanent waving consists of a chemical process to reduce SS bonds by applying the first agent (reducing agent) to the hair wound around the rod and convert it into cysteine (SH) residues, and then oxidize using the second agent (oxidation agent) and return it to SS bonds. The wave form of hair fibers is fixed by reforming cross-links at a new position.

The damage of hair caused by the permanent wave is physically detected as a decrease of the stress in the extended hair. Ogawa et al. have studied the mechanical behavior of the hair permed repeatedly [56]. The stress-strain curve for 1-5 times of permanent waved hair is shown in Figure 1.5. The decreases of the initial modulus, the yield stress and the breaking stress in comparison with untreated hair with repetition of treatment are recognized. Hair is always exposed to the irritant factors such as friction of combing, heat from the dryer, or the ultraviolet rays in daily life. Therefore, a

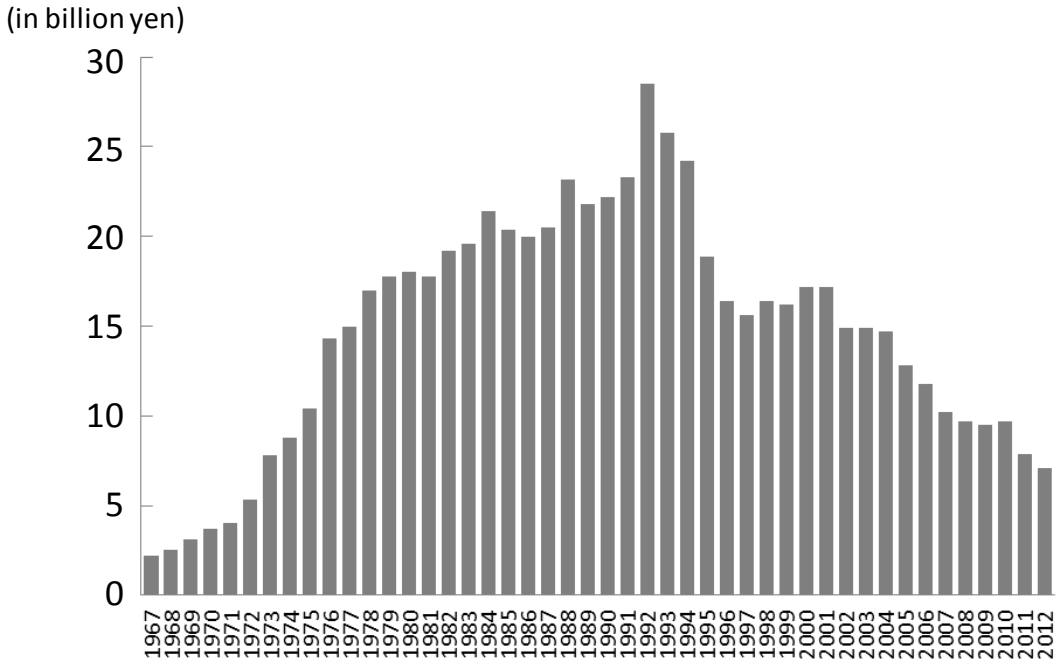

Figure 1.4. The amount of shipments of permanent waving products in Japan (Quoted from Statistics of Production by Pharmaceutical Industry in Ministry of Health, Labour and Welfare).

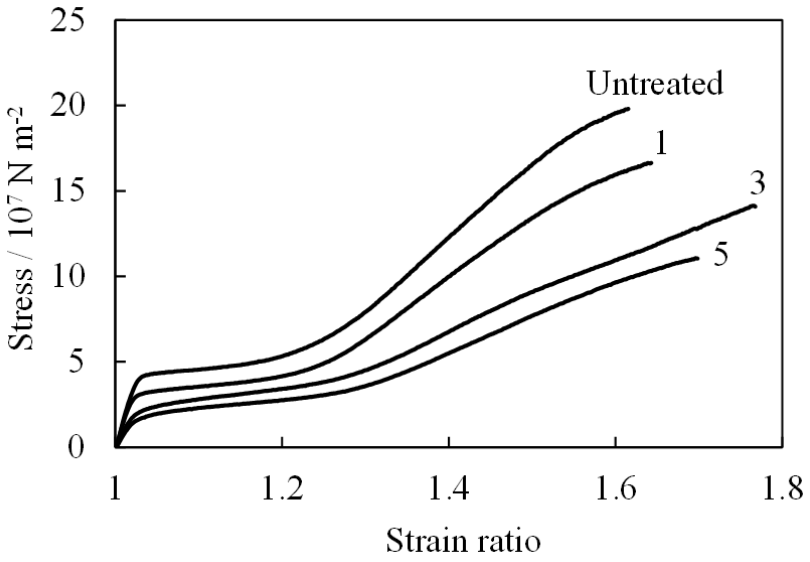

Figure 1.5. Stress-strain curves in water for permed hair samples. Samples designated as 1, 3 and 5 correspond to the times of treatment [56].

decrease of stress means weakening in resistance properties against such irritant, and it is desirable to inhibit the decrease of fiber stress caused by the permanent waving treatment. Incidentally, the change of a curve shown in Figure 1.5 can be seen as the change of SS cross-linked structure in hair cortex induced by permanent waving treatment. Since permanent wave is chemistry for scission and reformation of SS bond, it is expected that clarifying the relationship between SS cross-linked structure in the cortex and the mechanical behavior is related directly to elucidation of hair damage caused by permanent waving treatment, and is helpful in development of the products with superior functions and feels.

1.6. Purpose of this study

The aim of this study is to clarify the influence of SS cross-linked structure in the cortex on mechanical properties of keratin fiber using analysis of the cross-links by application of rubber elasticity theory to the swollen hair. At first, number, type and location of SS cross-links in the cortex of wool and hair have been analyzed, which is known to have different ratio of microfibril / matrix, and a SS cross-linked structural model of keratin fiber have been proposed. Then, the mechanical behavior for hair fibers modified SS cross-linked structure systematically by permanent wave treatment has been measured and influence of the SS cross-linked structure on its mechanical properties has been investigated. In addition, as for water washing process after reduction which has been found to revive its mechanical properties decreased by permanent waving treatment, we have studied the relationship between the recovery behavior of its properties and the changes of the SS cross-linked structure by washing, and clarified the mechanism of its mechanical recovery. These studies are also conducted toward a solution for the problem of hair damage in permanent waving treatment.

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Chapter 2

Disulfide cross-linked network structure of intermediate filament and matrix in hair and wool cortices

Chapter 2: Disulfide cross-linked network structure of intermediate filament and matrix in hair and wool cortices

2.1. Introduction

Cuticle and cortex are the two major morphological components in keratin fibers such as hair and wool. The cortex comprises intermediate filament (IF) proteins embedded in globular matrix proteins (KAP). The architecture of the IF–KAP assembly has been fully characterized using electron microscopy [1,2] and small-angle X-ray diffraction [3-5] on wool and hair keratin fibers. The disulfide (SS) cross-linked structure of in vitro assembled keratin IFs has been studied by using a protein chemical method, and the role of the disulfide bonds in strengthening and stabilizing IFs on the molecular shifting during oxidation has been discussed in detail [6,7]. Further developments of the method on the disulfide cross-linked structure of native keratin fibers are expected because the disulfide cross-linked structure of these network-forming proteins remains uncertain. The primary structural location of the lone cysteine residue has been clearly determined by genetic engineering methods. However, such methods cannot answer where in the IF chain the other lone cysteine residue—a paired thiol (SH) group forming SS bonds—is located and whether or not it is on the chain of the KAP globular protein besides the IF chain.

As mentioned in Chapter 1, a mechanical method for analyzing the number, type, and location of SS cross-links in the keratin cortex has been developed by Arai et al. [8-10]. The objectives of this chapter are to assess the number, type, and location of the network structures of wool and hair, which have a considerable difference in SS content, and to construct a network structural model for the cortex consisting of IF–KAP structural units.

2.2. Materials and methods

2.2.1. Purification of hair and wool fibers

All chemicals used were of reagent grade. Chemically unmodified, 25 cm long, human hair fibers collected from a Japanese female were purified after trimming about 1 cm of the root and about 2 cm from the tips. Hair tress consisting of 50 hair fibers with a

length of 20 cm was purified by immersing in 5 % (w/v) solution (50 mL) of Laureth-9 containing 20 mM EDTA for 1 h at 33 °C, then washing with distilled water, and finally air-drying. A lock of Lincoln wool fibers was purified by Soxhlet extraction with acetone for 24 h and petroleum ether for 24 h after washing with ethanol for 24 h at room temperature, mild washing with cold water after several washings with ethanol, and finally air-drying. The SS and SH contents in the hair and wool fibers were determined by Leach's polarographic method with methyl mercury iodide [11]. All tests were repeated three times and the average value was considered.

2.2.2. Preparation of swollen hair and wool

The hair was immersed in 11 M LiBr solution containing 10^{-2} M *N*-ethylmaleimide (NEM) at 90 °C for 1 h and then immersed and equilibrated in a 55:45 (v/v) solution of 8 M LiBr aqueous solution and diethylene glycol mono-*n*-butyl ether (BC) at room temperature for 1 h. The swollen fiber samples thus obtained were selected for their uniformity and medulla freedom before measuring their cross-sectional area and mechanical properties. A circular cross-section was assumed even for fiber samples with elliptical cross-section. The diameter of the swollen fibers and any significant deviation in ellipticity was measured under a microscope at approximately 20 places randomly selected over the length of the fiber (20 mm). This procedure was performed at room temperature because the diameter of the swollen fibers in the mixed solution was approximately constant irrespective of temperature [12]. The average cross-sectional area of the swollen fibers was calculated from the average value of the swollen fiber diameter D_s. In addition, swollen wool samples for untreated wool fibers were also prepared by the same procedure as described for the hair sample.

2.2.3. Force–extension curve for swollen keratin fibers

Mechanical tests for swollen fibers were performed according to previously described methods [8,9]. A swollen fiber sample of about 20 mm in length was set between the sample holders in a liquid cell. The force–extension properties were measured in a 55:45 (v/v) solution of 8 M LiBr and pure BC at 50 °C after measuring the unstrained zero length at equilibrium L_0. Several loading and unloading cycles were performed to remove

contributions from flow segments and then force–extension curves were measured up to about 40 % extension at an extension speed of 10 %/min. Finally, the extended fiber was retracted to zero force. Next, the mixed solution in the cell was slowly replaced with water and the fiber was held for about 5 min. Then, the length in water at room temperature L_w was also measured in the unstrained state, and the fiber was released from the sample holders and air-dried in a desiccator with P_2O_5 desiccant. The fiber diameter D_d was then measured using laser technology (KL 151A, Anritsu Co.). The volume fraction of keratin materials in the swollen fiber sample v_2 was calculated using the relation $v_2 = (D_d/D_s)^2(L_w/L_0)$, where L_w was assumed to equal the dry length. Note that the stress–strain curves for swollen fibers were constructed using the force values for the average cross-sectional area of the swollen and unextended fibers.

2.3. A structural model for the cross-links

2.3.1. Reversibility in the stress–strain curves for swollen and deswollen fibers

It has been demonstrated that α-helical chains can be randomized by immersing a NEM-treated keratin fiber in a mixed solution of concentrated aqueous LiBr and BC [12]. Figure 2.1 shows the stress versus strain relation of the keratin fibers. Figure 2.1(a) shows the extension and subsequent retraction curves of the swollen hair fibers measured in aqueous 8 M LiBr and BC solution at 50 ºC (solid line 1) and 30 ºC (broken line 2). The shape of the curves is similar to that of rubber and elastomers with a lower modulus elasticity—of about two orders of magnitude less than that of hair in water (see Figure 2.1(b))—excellent elastic recovery, lower energy losses, and significant decrease in energy loss with increasing temperature. These observations clearly indicate that the α-crystallites in IF were destroyed and they transformed to amorphous network material cross-linked with SS bonds. In fact, from the measurements of equilibrium force and temperature relationships in human hair at a higher extension range, the energy component in the retractive forces was analyzed, and it was concluded that there was essentially no energy component at all for such a swollen keratin system [12].

It has also been shown that the randomized IF chains can be subsequently recrystallized in water, and that these conformational changes are substantially reversible [13].

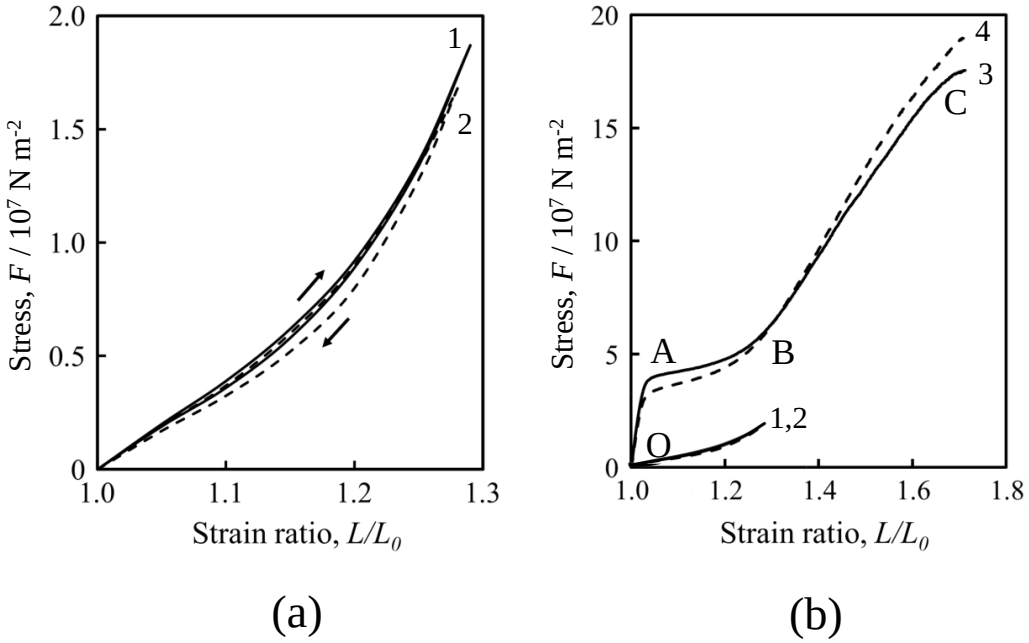

Figure 2.1. Stress versus strain for hair in water and swollen fibers. Figure (a) shows the stress–strain curves at extension and retraction for swollen hair samples in a 55: 45 (v/v) mixed solution of 8 M LiBr and BC at 50 °C (solid line 1) and 30 °C (broken line 2) with characteristic rubbery behavior. The solid line 3 in Figure (b) shows the stress–strain curve of hair in water at 20 °C and the broken line 4 in Figure (b) shows for the deswollen hair fiber, which has been relaxed by rinsing in water at 30 °C for 24 h after swelling in the mixed solution of 8 M LiBr and BC. Lines 1 and 2 in Figure (a) are also shown on the same scale.

The line 3 in Figure 2.1(b) shows the stress–strain curve of the hair in water at 20 °C. The curve has three distinctively characteristic regions: OA, AB, and BC; i.e., Hookean (strain ratio of 1.0–1.02), yield (1.02–1.25), and postyield (≥1.30), respectively [14,15]. The line 4 shows the stress–strain curve in water for the deswollen hair fiber, which had been relaxed by rinsing in water at 30 °C for 24 h after swelling in 8 M LiBr and BC. The shape of the curve is very similar to that of the untreated fibers. Note that under such conditions, almost perfect reformation of hair structure occurs in water from swollen aggregates composed of randomly deformed α-helical chains and cystine-rich globular matrix proteins. This provides evidence that blocking the free SH groups inhibits SH/SS interchange reactions to stabilize the network structure without any change in the relative positions of SS cross-links during the swelling process. It is suggested that (i) the SS bonds between IF proteins affect the reformation of α-crystallites and (ii) the SS bonds between the globular matrix proteins (KAP) control the shape change by retaining their relative positions on the surface and within the KAP molecules.

A two-phase model for the assembly of IF and KAP components was first presented by Feughelman [16], who developed a zone model comprising of an uncross-linked X-zone and a covalent cross-linked Y-zone forming SS cross-links between IF and KAP molecules. A similar model was presented by Crewther [17], who proposed a hypothesis with no covalent cross-links between IF and KAP forming a network cross-linked with SS bonds between globular matrix proteins. However, at present, the detailed cross-linked structure of keratin fibers remains uncertain.

Figure 2.2 shows a two-phase model for (a) unswollen and (b) swollen keratin fibers consisting of IFs and KAP molecules. In the swollen fibers, it was assumed that one phase is stable domain for swelling and extensions, which corresponds to a densely cross-linked matrix of KAP microdomains and the other is a continuous thinly cross-linked rubbery phase corresponding to randomized IF molecules. The rubbery network originates from the IF proteins by hydrogen bond scission. When such a non-uniform structure is stretched, microdomains of KAP serve as reinforcing filler particles in rubber [8].

2.3.2. A mechanical model for the extension of swollen fiber

The rubbery and globular matrix phases were assumed to be arranged in series

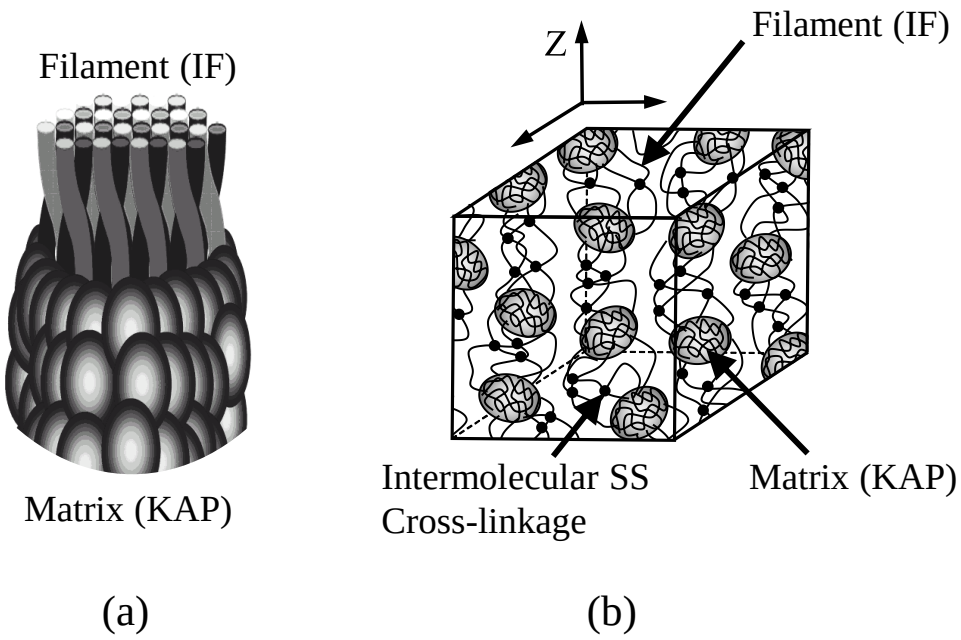

Figure 2.2. Schematic of a two-phase model of the IF–KAP structural unit for unswollen and swollen keratin fibers. (a) Assembly of intermediate IF proteins with eight tetramers embedded in cystine-rich globular matrix proteins—the so-called KAP [14]. IF proteins have been considered to be bonded with IF-IF and IF-KAP types of SS cross-links [18]. (b) The cross-linked structure model in the swollen state comprising the densely SS cross-linked domain phase of KAP and thinly cross-linked rubbery phase of IF array in series [8]. In our model, globular matrix phase of KAP component proteins is an aggregate of KAP molecules cross-linked with SS bonds through four sites on the surface of KAP [9] and was assumed to be attributable to stable during swelling and fiber extensions, namely, swelling and deformation of keratin fiber are only related to the randomly cross-linked rubbery phase with lower cross-link density. A reversible conformational change occurs alternately in the 55:45 (v/v) solution of 8 M LiBr and BC and in water. Reversibility is strongly suggested from the results of the stress–strain properties of the swollen and deswollen samples (see Figure 2.1).

because no energy contribution to the total force was observed up to 20 % or higher extension of the swollen hair in 8 M LiBr and BC [12]. This suggests no change in the bond angles within the globular matrix components arranged in series with the rubbery phase of the IF assembly. In contrast, under such large deformation, large energy contribution can be expected for the parallel array of the two phases owing to the simultaneous deformation of the rubbery IF chains and rigid globular matrix proteins.

For extension of IF-KAP structure shown in Figure 2.2(b), a simple cube model is assumed as shown in Figure 2.3. When the cube of swollen fibers is stretched by force F along the z-axis (fiber axis direction) without any deformation of the KAP domains and when the volume change in the rubbery phase occurs, the volume fraction of the domains in the swollen sample ϕ_d, which characterizes the swollen network is given by equation (2-1) as follows:

$$\phi_d = V_d / (V_d + V_{rs}), \qquad (2\text{-}1)$$

where V_d and V_{rs} are the effective volumes of the domain and the rubbery phase, respectively. The extension ratio α of the rubber network chain is a function of the extension ratio λ of the swollen sample according to equation (2-2):

$$\alpha = (\lambda - \phi_d)/(1 - \phi_d). \qquad (2\text{-}2)$$

NonGaussian chain statistics were applied to the stretched swollen sample, and a theoretical relation between the equilibrium force F and the extension ratio α of the rubber phase was derived as follows: [8,19]

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

where F is the equilibrium stress for the swollen and unstrained fiber cross-sectional area, n is the number of segments in the network chain, $L^{-1}(x)$ is the inverse Langevin function, and the shear modulus G of the swollen sample is $(\rho RT/M_c)\{(v_2 - \phi_d)/(1 - \phi_d)\}^{1/3}(1 - 2M_c/M)\gamma$; where ρ is the density of unswollen sample; M_c is the number average molecular

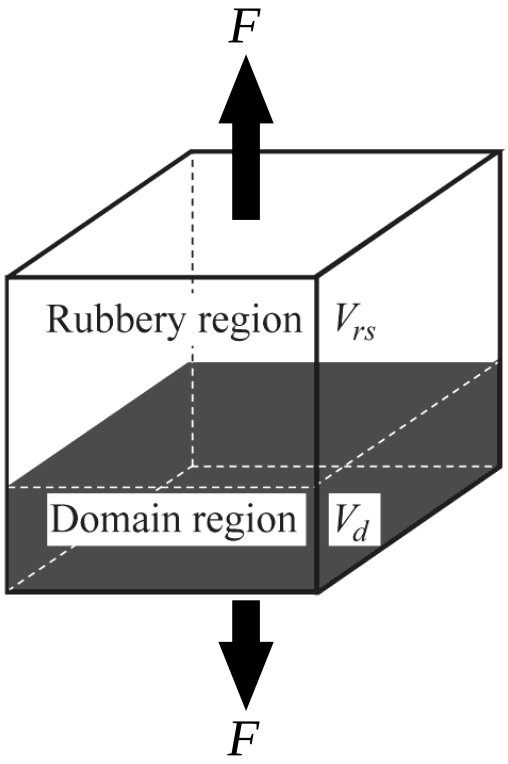

Figure 2.3. Simple extension model in series for a swollen cube containing microdomains and elastic network chains with effective volume V_d and effective volume of rubbery chains V_{rs}.

weight between cross-links in the rubbery region; M is the number average molecular mass of the primary molecule; R is the gas constant; T is the absolute temperature; v_2 is the volume fraction of the globular matrix domains in swollen keratin; and γ is the filler effect of the domains in the rubbery region. The filler effect γ is a function of κ and ϕ_d, where κ is the shape factor (length: width) for the rod-like filler. In the case of hair and wool keratin, a near spherical particle is assumed; i.e., $1 \leq \kappa \leq 2$. According to Guth [20] and Leonard [21], the filler effect can be expressed by equation (2-4) [8] as follows:

$$\gamma = 1 + 2.5\,\kappa\cdot\phi_d + 14.1\,\kappa^2\cdot\phi_d^2. \qquad (2\text{-}4)$$

2.3.3. Structural parameters of the swollen keratin fiber

Based on the series zone model, we attempted to evaluate structural parameters. The characteristic segment length M_c/n in hair proteins was experimentally established as 1250 [8]. This is the equivalent free rotational chain length of keratin protein, which corresponds to ca. 11.9 amino acid residues, because the average molecular mass of amino acid residues of the IF protein is 105 [22].

By fitting equation (2-3) to the stress–strain data for the hair sample, the values of parameters M_c, ϕ_d, and κ can be obtained by using the data of the entropy-dependent extensions up to the inflection point at the high extension range of the stress–strain curve. First, the fitting was performed by repeating the cycles on the two adjustable parameters M_c and ϕ_d under the condition that κ varied between 1 and 2 at 0.1 interval. The square of the deviation Δx between the fitted and experimental force data at constant extension was summed in the whole extension range. The $S = \Sigma(\Delta x)^2$ versus κ plots show that S sharply decreases initially with increasing κ and then levels off. Minimum S was not observed in the above range, which represents the case of near-spherical filler particles. Therefore, we assume that the most probable value of κ at the leveling-off region is the nearest value to unity and is empirically defined by equation (2-5) [9] as follows:

$$(S_{\kappa i+0.1}/S_{\kappa i}) - (S_{\kappa i}/S_{\kappa i-0.1}) = 0.05, \qquad (2\text{-}5)$$

where $S_{\kappa i}$, $S_{\kappa i+0.1}$, and $S_{\kappa i-0.1}$ are the values of S at κ_i, $\kappa_{i+0.1}$, and $\kappa_{i-0.1}$, respectively. κ_i was

computed as the most probable value of the shape factor of the domains in the swollen network. The values of the parameters M_c and ϕ_d can therefore be obtained by the fitting at κ_i.

2.4. Results and discussion

2.4.1. Some reports on the cysteine contents of IF determined by chemical and biotechnological methods

The IF molecule is a heterodimer associated with Type I and Type II proteins, which consist of α-helical rod domains and nonhelical N and C terminals [23-26]. Each of these two types in wool keratin includes four protein species with approximately equimolar concentrations [27], and the two types in hair contain eleven protein species. The SS contents in the IF of wool and hair are approximately the same [23,27]. The number and location of cystine residues in wool IF chains sequenced for each of the four species was originally reported by Fraser et al. [18]. These authors calculated the average number of cysteine (1/2 cystine) per IF chain to seven residues in the rod domain and fifteen residues in the terminal domains as the average of the four protein species in Type I and Type II proteins (see Table 1.1). When the average molecular mass of the IF proteins is assumed 5.0×10^4, the total cystine content $[SS]_{tot}$ in the IF proteins is calculated to be 220 μmol/g ($= 22 \times 10^6/2 \times 5.0 \times 10^4$), which partitions to 70 μmol/g in the rod domain and 150 μmol/g in the terminal domains [18].

From the amino acid analysis of low-sulfur protein fractions, Gillespie [28, 29] showed that the cysteine content analyzed as *S*-carboxymethyl-cysteine (SCMC) groups in low-sulfur (IF) proteins in wool is 400 μmol/g, which corresponds to 200 μmol/g for numerous SS cross-links; namely, 20 residues per IF chain, and the number of SCMC groups in IF (low sulfur) proteins is approximately the same as that in keratin fibers of different SS contents.

2.4.2. Interpretation of structural parameters

Figure 2.4 shows the stress–strain curves of F versus λ for hair and wool samples. We fitted the experimental data with equation (2-3) and suitable choices of parameters M_c, ϕ_d,

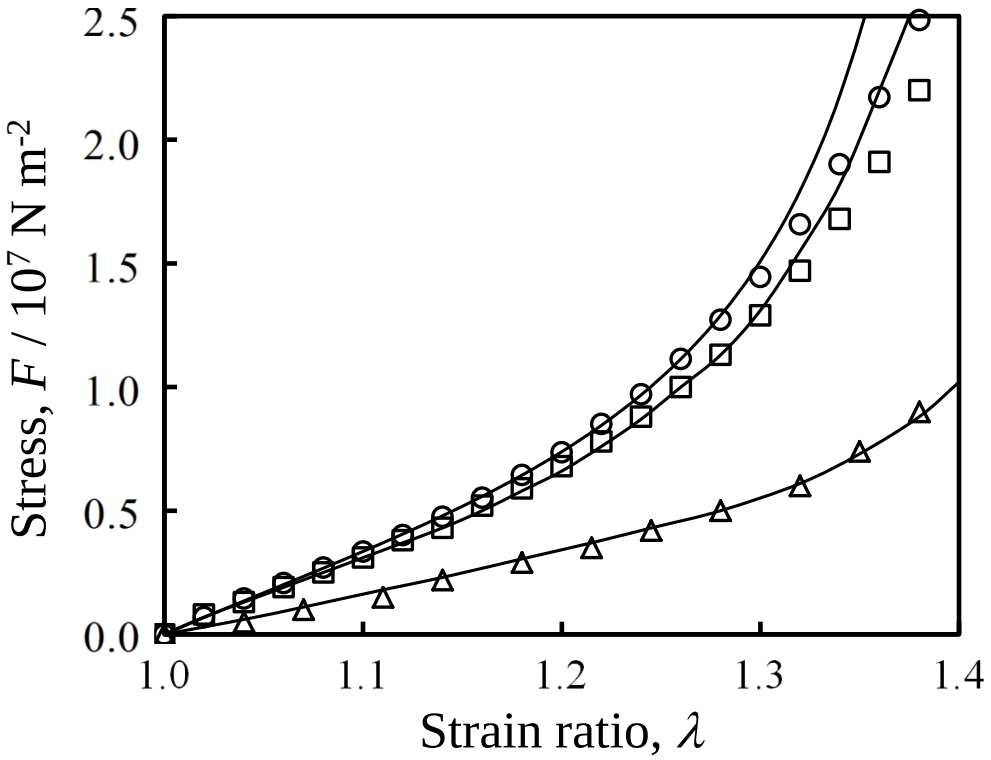

Figure 2.4. Typical stress–strain curves for the swollen untreated keratin fiber samples listed in Table 2.1. Linear fits to experimental data by using equation (2-3): (△) wool sample A, (○) hair sample A, and (□) hair sample B.

and κ. The values of these parameters are listed in Table 2.1.

The amount of disulfide cross-links in the IF proteins is $10^6/2M_c$ μmol/g. The number of intermolecular cross-linking sites on an IF chain, $[SH]_{inter}$, is estimated by using the equation $[SH]_{inter} = 2M_{ave}$ $(10^6/2M_c)$, where M_{ave} is the number average molecular mass of IF proteins, i.e., 5.0×10^4. With respect to the values of the parameters for keratin fibers with different disulfide content, the shear modulus G and the fraction of the domain volume in the cortex at the swollen state ϕ_{d0} and dry state $\phi'_{d0} = \phi_{d0}/v_2$ are considerably different. On the other hand, the values of κ—the shape factor for cylindrical filler particle with average length: width of 1.65: 1 for both hair and wool—and the molecular mass of the network chain of IF (M_c) or the number of SS cross-links in an IF chain ($[SH]_{inter}$) are approximately the same. Compared with hair and wool, the M_c values are the same irrespective of the differences in the disulfide content and G. A similar result has also been obtained for a variety of keratin fibers (three different human hairs, 12 different wools, 2 different ewes, mohair, cashmere, llama, alpaca, angora, opossum, and Shetland sheep dog) with SS contents ranging from 313 to 625 μmol/g [30,31]. Therefore, it is suggested that the value of G for swollen fiber does not depend on the network structure of IF, but depends mainly on the volume occupied by the KAP proteins. However, the exact location of intermolecular cross-links on the α-helical sections and the relative position of the inter- and intramolecular cross-links on the terminal chains remain uncertain.

2.4.3. Cross-linked structure of IF for keratin fibers

The location, type, and number of SS cross-links on an IF chain were determined by Arai et al., who investigated the permanent setting of wool by the formation of new cross-links from disulfide to lanthionine and lysinoalanine during treatment with boiling water [10]. The new cross-links are denoted as X cross-linkage hereafter. Throughout the time of treatments until the setting reaction substantially stopped for 5 h, the SH and SS contents of the set fibers prepared at 40 % extension and unextension (control) states of the fibers were carefully determined by a polarographic method [11], and the distribution of SS and X cross-linkages in IF proteins of wool and set fibers were analyzed by application of our swollen fiber model. The formation rate and location of inter- and intra-

Table 2.1. Structural parameters for the SS cross-links in hair and wool fibers

Keratin samples	SS content (μmol/g)	$10^{-6}G$ (N/m^2)	ν_2	M_c (g/mol)	κ	ϕ_{d0}	$\phi_{d0}/\nu_2 = \phi'_{d0}$	$10^6/2M_c$ (μmol/g)	$[SH]_{inter}$ in an IF chain (residues)
Hair A	581	5.66	0.703	3800	1.70	0.397	0.565	132	13.2
B	627	4.14	0.599	3600	1.65	0.339	0.566	138	13.8
C	663	4.74	0.703	4100	1.60	0.395	0.562	123	12.3
D	625	3.72	0.589	3400	1.55	0.323	0.550	146	14.6
E	648	4.19	0.584	3500	1.66	0.334	0.576	144	14.4
Wool A	420	2.43	0.580	3700	1.70	0.220	0.379	135	13.5
B	407	2.57	0.603	4000	1.60	0.264	0.438	123	12.3
Ave.								**134**	**13.4**

molecular X cross-links stable to reducing agents were estimated by performing the reduction of SS bonds remained in the set fibers [10].

It was found that there are four types of SS cross-links different in reactivity in boiling water, that is, 1) intermolecular SS cross-links which are rapidly transformed to intramolecular X cross-links under unextension condition, 2) intermolecular SS cross-links which are gradually transformed to intermolecular X cross-links under unextension condition, 3) intramolecular SS cross-links which are rapidly transformed to intramolecular X cross-links under 40 % extension condition, and 4) intermolecular SS cross-links which are gradually transformed to SH groups under 40 % extension condition. These types of cross-links were assigned to cross-links between 1) terminal and KAP domains ($SS_{E–KAP}$), 2) terminal domains ($SS_{E–E}$), 3) rod and terminal domains within IF chain ($SS_{R/E}$), and 4) rod domains of adjacent IF molecules ($SS_{R–R}$). The reactivity of SS bonds depends on the location of the cross-links and decreases in the order $SS_{E–KAP}$ > $SS_{E–E}$ > $SS_{R/E}$ >> $SS_{R–R}$. Note that the SS bonds between the rod domains are only reactive when the wool fibers are in the extension state. Experimental results were analyzed by using nonGaussian chain statistics, and the results are summarized in Table 2.2 [9,10].

In Table 2.2, eleven residues per IF chain in the total IF–IF-type intermolecular cross-links (SS_{inter}) to adjacent IF molecules (coiled-coil rope) include three sites in the rod domain that link to an adjacent rod ($SS_{R–R}$) and eight sites in the terminals that link to adjacent terminal domains ($SS_{E–E}$).

Two sites (SS_{inter} in residues per IF chain) in the terminal domains link to an adjacent KAP molecule. The two intermolecular SS cross-links were the most reactive in the setting reaction and changed to new intramolecular cross-links, which have unique behavior than the typical $SS_{E–E}$-type cross-links. Therefore, we assume that $SS_{E–KAP}$-type cross-links are more probable than $SS_{E–E}$-type cross-links.

The type of cross-links in the terminal domains of IF for four residues per IF chain (39 μmol/g of IF protein) is not well known. These types of SS cross-links were previously described as SS_U [10]. The percentage ratio of intermolecular and intramolecular cross-links in IF for hair is calculated as 67: 33 from 134 μmol/g of intermolecular type and 66 μmol/g (= 200 – 134) of intramolecular type in Table 2.1. SS_U was assigned to the intramolecular cross-links in the terminals; i.e., $SS_{E/E}$, because the

Table 2.2. Type, location, and the number of respective SS cross-links in the rod (R) and terminal (E) domains of the IF chain in wool keratin

Type of cross-links	Location	Number of cross-links	
		μmol/g of IF proteins	Cystine residues in an IF molecule
SS_{inter}	$SS_{R–R}$	33	3
	$SS_{E–E}$	79	8
	$SS_{E–KAP}$	23	2
SS_{intra}	$SS_{R/E}$	39	4
	$SS_{E/E}$	39	4
Total content of SS cross-links, $[SS]_{tot} = [SS]_{inter} + [SS]_{intra}$		213	21
$[SS]_{tot}$ as the average value of four protein species in Type I and Type II proteins [a)]		220 [c)]	22
$[SS]_{tot}$ as SS content in low-sulfur proteins [b)]		200	20

a) Published by Fraser et al. [18].

b) Published by Gillespie [28].

c) Total cystine content of 220 μmol per gram was calculated from the average value of total cysteine content determined by Fraser et al. [18] as 22 residues in an IF chain, which were located 7 and 15 residues in rod and terminal domains, respectively. Assuming the average molecular mass of IF chain is 50000, the calculation of $(7 + 15) \times 10^6 / \{2 \times (5 \times 10^4)\}$ shows the value of cystine content in μmol/g.

percentage ratio of $[SS]_{inter}$ = 135 and $[SS]_{intra}$ = 78, i.e., 63: 37, was considerably similar to the value obtained for hair.

Finally, we consider the intramolecular cross-links classified as $SS_{R/E}$. There are two types of SS cross-links in IF rod domains: one is the cross-links between rods which are lost by 40 % extension of fiber to form free thiol groups that correspond to SS_{R-R} and the other is the intramolecular cross-links between rod and terminal domains, which are converted to intramolecular X cross-links under the extension state of the fibers. The contents of SS linkages were estimated to be three moles in IF chain with molecular mass of 50000 in the former and two moles in the latter, which is denoted as $SS_{R/E}$ [10]. It is probable to consider that the four intramolecular cross-link sites are located on the interface regions between the rod and terminal domains, which may exist in the high cross-link density regions of the rod and terminal domains. These cross-links form closed loops with a short network chain length and do not contribute to the network elasticity. This means that the distance between the sites may be limited below the segment length of the keratin proteins that equals the number average molecular mass of the network chain [8,10]. Therefore, the four cross-link sites on the interface regions may be arranged near the terminal chain on either the α-helical 1A or 2B segment of IF. Note that there is none or a small number of cysteine sites on the 1A segment in both Type I and II proteins [27]. As for the sites of the four intra-molecular cross-links $SS_{R/E}$, we chose the α-helical rod domain in the end region of the 2B segment close to the C-terminal chain. Hence, we constructed a model for the number, type, and location of the SS cross-links in the IF chain.

Figure 2.5 shows the schematic of the possible cross-linked structures of the SS bonds in the IF protein chain of keratin fibers.

2.4.4. Cross-linked structures of KAP of wool and hair

Table 2.3 lists the number and type of cross-links in the KAP component of hair and wool. The matrix domain volume corresponding to high-sulfur components in fiber ϕ'_{d0} is considerably different between hair and wool. The SS content of the IF protein in the fiber is calculated with the equation $[SS]_{IF} = 200\ (1 - \phi'_{d0})$ and listed in Table 2.3. Compared with the values for wool and hair, the former is larger than the latter. This

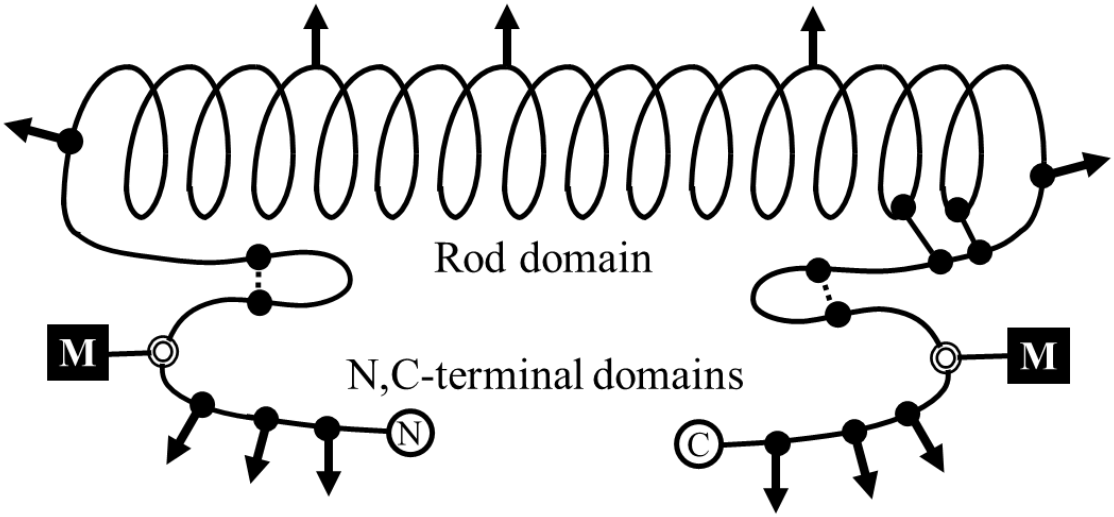

Figure 2.5. Types of intermolecular cross-links on the IF chain. Three $SS_{R–R}$ (➞) cross-link sites on the α-helical rod domain providing links to adjacent rods. Eight $SS_{E–E}$ (●➞) cross-link sites on the N- and C-terminal domains provide links to adjacent terminals; moreover, the cross-link sites in the nonhelical section of the two terminal domains are equally distributed in each domain. Two $SS_{E–KAP}$ (◎—) cross-link sites on the terminal domains linking to matrix (M) aggregates of KAP molecules. Two types of intramolecular cross-links on the IF chain: two $SS_{R/E}$ (●—●) intramolecular links in the interface region between the rod domain of the 2B segment and the C-terminal chain and two $SS_{E/E}$ (●···●) intramolecular links between the terminal domains that assumed to be equally distributed in each domain.

Table 2.3. Results of the number and type of SS cross-links in KAP for hair and wool fibers

Keratin samples	ϕ'_{d0}	SS content, $[SS]_{tot}$ [a] (μmol/g of fiber)	$[SS]_{IF}$ [b] (μmol/g of fiber)	$[SS]_{KAP}$ [c] (μmol/g of fiber)	$[SS]_{KAP}$ [d] (μmol /g of KAP protein)	$[SS]_{KAP.intra}$ [e] (μmol/g of KAP protein)
Hair A	0.565	581	87	494	874	757
B	0.566	627	87	540	966	851
C	0.562	663	88	575	1023	908
D	0.550	625	90	535	952	837
E	0.576	648	85	563	977	862
Ave.	**0.564**	**629**	**87**	**542**	**958**	**843**
Wool A	0.379	420	124	296	781	666
B	0.438	407	112	295	674	559
Ave.	**0.409**	**414**	**118**	**296**	**728**	**613**

a) SS content of fiber.

b) SS content of IF protein in fiber: $200\ (1 - \phi'_{d0})$, where the SS content of the IF protein in the fiber is 200 μmol per gram of IF protein [28].

c) SS content of KAP in fiber: $[SS]_{tot} - [SS]_{IF.}$

d) SS content per gram of KAP protein: calculated by dividing SS content of KAP in fiber by $\phi'_{d0.}$

e) Intramolecular cross-links of KAP molecule: calculated by subtracting 115 from SS content per gram of KAP protein, where the number of intermolecular cross-links between the KAP molecules is 115 μmol per gram of KAP [9].

means that the amount of the IF component is larger in the wool than in the hair because the IF protein is the same for both keratins, as suggested from the values of the cross-link density in Table 2.1. The SS content of KAP in the fiber and the SS content of the KAP molecules are also given in Table 2.3. The SS content of the matrix is significantly different in hair and wool; however, there are no substantial differences in the SS content of both IF proteins. Finally, we estimate the number of intramolecular cross-links in the KAP molecule (Table 2.3). From the reaction of hair with an aqueous solution of potassium cyanide, as a strong nucleophilic reagent, the number of intermolecular cross-links between the KAP molecules, $[SS]_{KAP,inter}$, is 115 μmol per gram of KAP protein [9]. Subsequently, the number of intramolecular cross-links in the KAP molecule, $[SS]_{KAP,intra}$, was calculated by subtracting 115 from the total number of SS links in the KAP molecule (Table 2.3). Note that the SS content in KAP reaches 86.2 % (= 542 / 629) of the total SS content in hair, and a large portion of cross-links, 75.8 % (= 86.2 × 843 / 958), are intramolecular links existing within the globules, while only 10.4 % (= 86.2 – 75.8) links occur as intermolecular links between the surfaces of the globules.

The high- and low-sulfur volume fractions of ϕ'_{d0} and $(1 - \phi'_{d0})$ largely depend on the disulfide content of keratin. Gillespie estimated the weight fractions of both IF and KAP by using the fractional precipitation method for the water-soluble *S*-carboxymethylated keratin proteins derived from keratin fibers with widely different contents of disulfide [28,29]. The obtained mass fractions of IF in IF–KAP in wool and hair were about 75 % and 57 %, respectively [28,30]. Assuming that the density of both IF and KAP materials is the same, these values are somewhat larger than the average data in Table 2.3. The lower percentage of KAP in our data is presumably attributed to the existence of other proteins, such as high-glycine–tyrosine proteins.

In the IF–KAP structural unit, the molecular mass of one IF molecule and five chains of KAP molecules were assumed to be equal by Fraser et al. [18]. We assumed an average molecular mass of the IF molecule $M_{IF,ave} = 10^5$ and $M_{KAP,ave} = 2 \times 10^4$ for the KAP molecule. Here, from the study on the permanent waving hair, it has been suggested that the KAP molecules behave as an ellipsoidal aggregate consisting of some near-spherical KAP molecules associated with a small number of SS cross-links (see Chapter 3). Thus, we can calculate the number of aggregated KAP molecules *P* for hair with $\phi'_{d0} = 0.564$

(Table 2.3) by using equation (2-6):

$$P = M_{\mathrm{IF,ave}}\,\phi'_{\mathrm{d0}}/\{M_{\mathrm{KAP,ave}}\,(1-\phi'_{\mathrm{d0}})\}. \qquad (2\text{-}6)$$

We therefore obtain $P = 6.4$ and about six moles of the KAP protein corresponding to one IF molecule consisting of Type I and Type II proteins. Similarly, for $\phi'_{\mathrm{d0}} = 0.409$, P equals three dot five moles for wool.

Next, we estimate the number of intermolecular cross-links N_{inter} between the KAP molecules and the number of intramolecular cross-links N_{intra} within the KAP molecule in moles per gram of protein by using equations (2-7) and (2-8):

$$N_{\mathrm{inter}} = 10^{-6} M_{\mathrm{KAP,ave}}[\mathrm{SS}]_{\mathrm{KAP,inter}} \qquad (2\text{-}7)$$

$$N_{\mathrm{intra}} = 10^{-6} M_{\mathrm{KAP,ave}}[\mathrm{SS}]_{\mathrm{KAP,intra.}} \qquad (2\text{-}8)$$

In the case of hair, $[\mathrm{SS}]_{\mathrm{KAP,inter}} = 115$ and $[\mathrm{SS}]_{\mathrm{KAP,intra}} = 843$ in μmol/g of KAP, respectively (see Table 2.3). We obtain $N_{\mathrm{inter}} = 2.3$, and this value suggests that about 4–5 cross-link sites (2.3×2) exist on the surface of a globular matrix protein. The number of intramolecular cross-links within the globular matrix protein N_{intra} is 16.9. This means that a cystine residue of about 5.3 ($2 \times 10^4/110 \times 34$) exists as 1/2 cystine in the amino acid residues, assuming that the average molecular mass of the amino acid residues in the KAP molecule is 110. On the other hand, the calculated N_{intra} for wool is 12.3, which corresponds to about 7.6 amino acid residues per cystine (as 1/2 cystine) residue. This value is considerably smaller than that of hair. Despite the small or little difference between the number of cross-links in the IF for wool and hair, the values of N_{intra} and P characteristic of the KAP component proteins in the IF–KAP structural unit are significantly different.

2.4.5. Network structure of the IF–KAP unit in the hair cortex

We have now the number, type, and location of the SS cross-links in the IF chain and KAP molecule. Figure 2.6 shows a predicted cross-linked structure of the IF–KAP

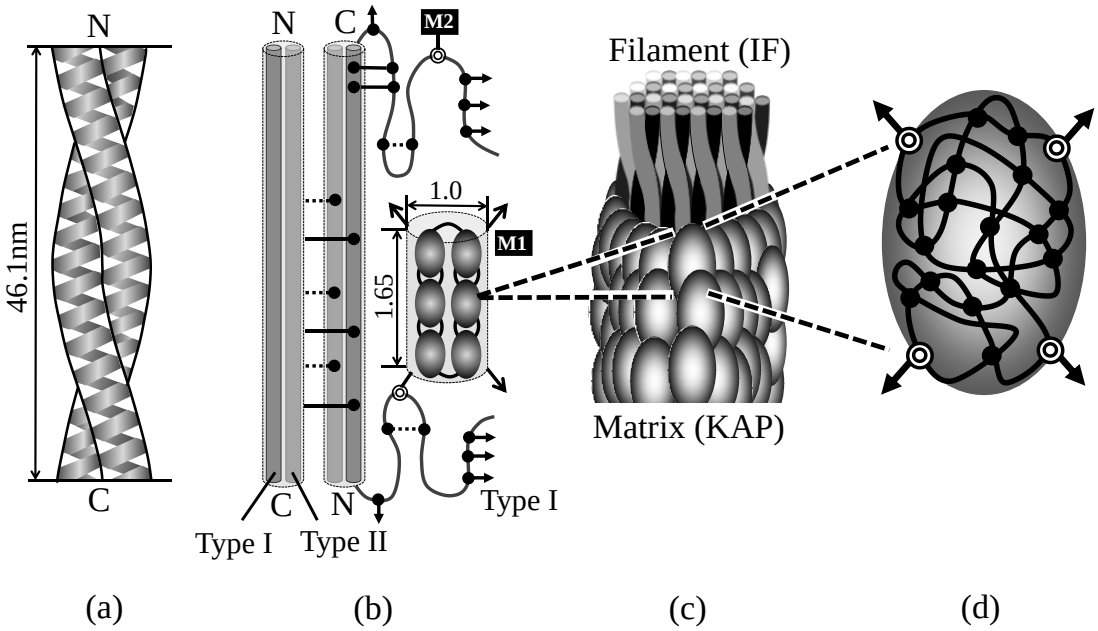

Figure 2.6. Schematic of the SS cross-linked network structure of the IF–KAP structural unit of hair [32,33]. (a) Simple model of an IF rod molecule with a coiled-coil, rope-like structure in which two right-handed α-helices wind around a common axis in a left-handed manner to form a rope-like structure with a repeating unit length of 46.1 nm [6]. (b) Pair of rod domains in the IF molecule composed of Type I and Type II protein chains, which contain three sites linking to adjacent rods and inter- and intramolecular cross-links between and within the N- and C-terminal chains. One site on each terminal chain links to the modeled cylindrical aggregate ellipsoidal particle of six subcomponents of the matrix molecules with shape factor $\kappa = 1.65$. (c) IF–KAP structural unit consisting of sixteen IF molecules embedded in an assembly of globular matrix proteins cross-linked with SS bonds. (d) Four cross-link sites (◎→) on the surface of KAP molecule capable of cross-linking with adjacent KAP molecules and globular KAP molecule with a maximum diameter of ~4 nm [34], which contains about seventeen moles of intramolecular SS cross-links.

structural unit in hair [32,33]. According to the schematic, wool network structure can easily be predicted from the results of the above calculation.

2.5. Conclusions

To understand the cross-linked structure of hair and wool keratin fibers, rubber elasticity theory was applied to the force–extension curve of swollen fiber in a diluent mixture of concentrated aqueous lithium bromide and diethylene glycol mono-*n*-butyl ether. The number, type, and location of SS cross-linkages in IF protein were estimated by using the results obtained from native wool and hair, and the set fibers in boiling water. The total number of SS cross-linking sites is twenty-one moles in the IF chain of an average molecular mass of 50000. The twenty-one moles are divided into thirteen moles comprising intermolecular cross-linkages, which are subdivided to three moles between IF rods, eight moles between terminal domains, and two moles between terminal and KAP, and eight moles comprising intramolecular cross-linkages, which are subdivided into four moles within terminals and four moles within rod and terminal at the interface region between the rod and terminal domains. A KAP molecule with an assumed molecular mass of 20000 involves seventeen moles of intramolecular SS bonds and 4–5 sites on the surface of the KAP molecule of hair keratin. These sites are linked to adjacent KAP molecules and form an aggregate of about six KAP molecules against the IF molecule in the hair, while in the wool, about three KAP molecules with thirteen moles of intramolecular bonds aggregate against the IF molecule. It was found that there is a considerable difference in the KAP structure between hair and wool. In addition, we also proposed a network model for the IF–KAP structural unit in the hair and wool fiber cortex.

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Chapter 3

Effect of permanent wave treatment of human hair with thioglycolic acid on disulfide cross-linked structure and mechanical property

Chapter 3: Effect of permanent wave treatment of human hair with thioglycolic acid on disulfide cross-linked structure and mechanical property

3.1. Introduction

The setting process for permanent waving and straightening of hair is important in the cosmetic industry. The stabilization of curled conformation in permanent waving is accomplished through the cleavage of the cystine SS bonds by reduction and their subsequent recombination by oxidation. Although setting reactions including reduction and oxidation have been studied for many years by various researchers, certain aspects of the setting process are still not well understood. Various mechanisms have been proposed to explain permanent waving achieved by chemical treatment [1–17] and a combination of chemical and heat treatments [18].

The effectiveness of waving on reducing agents was investigated systematically by Okano et al. [1] and a better correlation has been found between the hydrophobic character of thiols and waving efficiency. The diffusion rate of thiols is pH-dependent and is related to the electrostatic interactions between the ionizable functional groups in keratin and thiol [2-7]. Diffusion of thiols into human hair has been investigated extensively by Kuzuhara and Hori [8]. A combined method of microspectrophotometry and FT-Raman spectroscopy was used, and the electrostatic interactions between reducing reagents and hair surfaces or hair cortex have been discussed. The relaxation behavior in bending of hair in a reducing solution was investigated by Wortmann et al. [9,10], and a model for bending set was presented that the degree of reduction in the outer part of the fiber is vital to the degree of set, which in turn the rate of diffusion of the reducing agent is essential. However, views on this model are different among researchers [11-13].

The mechanical degradation of hair fiber by permanent wave treatment is recognized as hair damage. The mechanical behavior of repeatedly permed hair was investigated by Ogawa et. al. [14], and they indicated that the initial modulus and yield stress were decreased with increase of the treatment times. Relationship between types of reductants and mechanical properties of reduced hair fibers were reported by Wortmann et al. [10],

and it was indicated that the reduced fiber with thioglycolic acid expressed the lowest tensile strength. Cannell et al. [15] studied the effects of the concentration of reductant and pH on the mechanical properties related to the slope of the post-yield region in the stress-strain curve of hair fibers. However, the mechanism of the mechanical change induced by permanent wave treatment is still fairly unknown. In order to understand the change in the hair structure induced by permanent wave treatment, it is important to assess the number, type, and location of SS cross-links in the hair cortex.

The objectives of this chapter are as follows: (i) to elucidate the network structure change on IF and KAP induced by reduction and oxidation treatments based on the disulfide cross-link structure in IF and KAP proposed in Chapter 2, (ii) to clarify the number and type of reactive SS cross-links existing in the region accessible to the reducing agent, and (iii) to better understand the mechanical properties relating to intermolecular SS cross-links in the microstructure.

3.2. Materials and methods

3.2.1. Purification of hair fibers

An aqueous ammonium solution of 50 % (w/v) TGA was used as supplied commercially. Other chemicals used were of reagent grade. Chemically unmodified human hair samples (approximately 25 cm in length) collected from a Japanese female were subjected to purification after removing lengths of approximately 1 cm at the root and approximately 2 cm at the tip. A hair tress consisting of 20 hair fibers (approximately 20 cm in length) was purified according to the method described in section 2.2.1. Purified hair fibers were used for reduction and oxidation treatments. The SS and SH contents in hair fibers were 636 and 34 μmol/g, respectively.

3.2.2. Reduced hair fibers

Reduction was performed by winding the hair fibers around a 12-mm-diameter rod and dipping in an aqueous solution (400 mL) containing 0.3, 0.5, 0.75, 1.0, and 1.5 M TGA for 20 min at room temperature at pH 8.7 and 9.3. This was followed by immersing it four times in deionized water (400 mL) for 5 min each at room temperature. Reduced

hair fibers thus obtained were subjected to either oxidation or *N*-ethylmaleimide (NEM) treatment by immersing the rod in the NEM solution.

3.2.3. Reduced and oxidized (reoxidized) hair fibers

Reduced hair fibers prepared under the aforementioned conditions were subsequently immersed in a 0.5 M sodium bromate solution (400 mL) at room temperature for 20 min at pH 6.35 followed by immersing them four times in water (400 mL) repeatedly for 5 min each. Finally, reoxidized hair fibers were blotted and air dried. The reoxidized hair fibers thus obtained represent the so-called permanent waved hair that would be obtained cosmetically.

3.2.4. Reduced and NEM-treated hair fibers

The reduced fibers were treated with a 0.05 M NEM solution for 24 h at pH 8.0 and room temperature to block the free SH groups. The reduced and NEM-treated fibers thus obtained were washed thoroughly with water and air dried. Blocking the free SH groups is necessary at this stage to stabilize the network structure through inhibiting SH/SS interchange reactions during swelling and extension [19,20].

3.2.5. Preparation of swollen hair fibers and determination of structural parameters

Preparation of the swollen hair fibers for the untreated and permanent waved hair fibers was the same as described in section 2.2.2. The force-extension measurement of swollen fiber was presented in section 2.2.3 and the procedure for determining several structural parameters was also described in section 2.3, respectively.

3.2.6. Force–extension curve in water

The untreated and permanent waved hair fibers were immersed in water at 20 °C overnight and then extended in water at 10 %/min. The stress–strain curves were constructed on the basis of the stresses as the forces per cross-sectional area measured using a laser method (KL151A, ANRITSU Co.) in the unstrained state and under room conditions. The initial modulus in water, E_w, was defined as the slope of the initial straight region of the stress–strain curve.

3.2.7. Relative amount of α-crystallites

X-ray diffraction measurements were performed using a RINT X-ray diffractometer (RIGAKU Co.) equipped with a texture goniometer assembly. Exactly 40 straightened hair were fixed on the sample holder. Nickel-filtered CuKα radiation (λ = 0.1541 nm) was used at 40 kV and 100 mA. Diffraction data were collected at 1°/min. The effect of air scattering on the diffraction intensity was performed by subtracting the diffraction intensity in the absence of sample [21]. The peak resolution of the equatorial diffraction profile was computed. The relative amount of α-crystallites in the fiber was defined as the intensity ratio of α-crystallites (2θ = 9º) to that of the total diffraction.

3.3. Results

3.3.1. Stress–strain curve for swollen hair fiber

Figure 3.1 show typical stress–strain curves for the reduction and reoxidation samples. Fitting the experimental data for equation (2-3) with suitable choices of parameters M_c, ϕ_d, and κ, we can evaluate the values of these parameters. The results obtained for untreated and treated hair fibers are shown in Table 3.1.

3.3.2. Swellability of treated hair fibers

As shown in Table 3.1, the values of v_2 (an index inversely related to swellability) decrease with increasing TGA concentration and pH for the reduced hair fibers, and the values were influenced more by the latter as compared to the former. The swellability for reoxidized hair fibers was lower than that for the corresponding reduced fibers. This suggests that the regeneration of intermolecular SS cross-links occurs more or less in the hair fiber during the oxidation step.

3.3.3. Shear modulus of swollen hair fibers

The relationship between shear modulus, G, and TGA concentration at different pH values are shown in Figure 3.2. G decreases steeply in the initial stage of the reduction treatment and then levels off; and tends to recover during the oxidation treatment. The value of G at the highest TGA concentration at pH 9.3 is reduced to only approximately

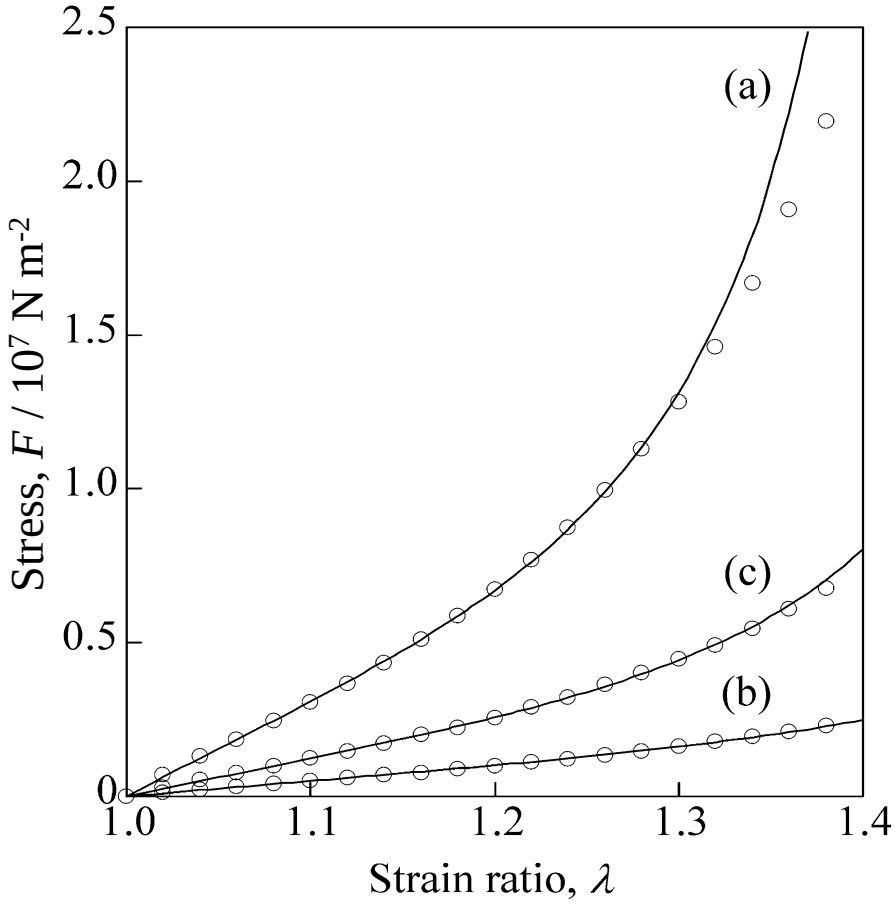

Figure 3.1. Typical stress-strain curves of swollen hair fibers: (a) untreated, (b) reduced with 0.75 M TGA at pH 9.3 and (c) reoxidized samples; lines fitted to the experimental data by equation (2-3).

Table 3.1. Structural parameters for SS cross-links in untreated and permanent treated hair fibers

Treatment	Reduction Conditions		$10^{-6}G$	ν_2	κ	ϕ_d	ϕ'_d	M_c	$10^6/2M_c$
	[TGA] (M)	pH	(N/m^2)						(μmol/g)
Untreated	–	–	4.14	0.599	1.65	0.339	0.566	3600	139
Reduced	0.3	8.7	3.01	0.558	1.47	0.294	0.527	3400	147
	0.5	8.7	2.52	0.559	1.37	0.280	0.501	3500	143
	0.75	8.7	1.84	0.433	1.40	0.219	0.506	3300	152
	1.0	8.7	1.32	0.458	1.15	0.209	0.456	3700	135
	1.5	8.7	1.07	0.435	1.10	0.167	0.384	3700	135
	0.3	9.3	2.61	0.542	1.37	0.286	0.528	3400	147
	0.5	9.3	1.61	0.487	1.23	0.218	0.448	3500	143
	0.75	9.3	0.93	0.387	1.20	0.178	0.460	4300	116
	1.0	9.3	0.61	0.370	1.15	0.151	0.408	5300	94
	1.5	9.3	0.49	0.333	1.20	0.132	0.396	5900	85
Reoxidized	0.3	8.7	3.64	0.615	1.40	0.356	0.579	3500	143
	0.5	8.7	2.80	0.512	1.50	0.284	0.555	3400	147
	0.75	8.7	2.64	0.551	1.40	0.305	0.554	3700	135
	1.0	8.7	2.39	0.521	1.40	0.271	0.520	3500	143
	1.5	8.7	2.15	0.502	1.40	0.259	0.516	3600	139
	0.3	9.3	2.55	0.565	1.30	0.318	0.563	3700	135
	0.5	9.3	2.05	0.434	1.50	0.261	0.601	3700	135
	0.75	9.3	1.85	0.530	1.20	0.285	0.538	3900	128
	1.0	9.3	1.66	0.541	1.10	0.258	0.477	3700	135
	1.5	9.3	1.48	0.509	1.20	0.229	0.450	3900	128

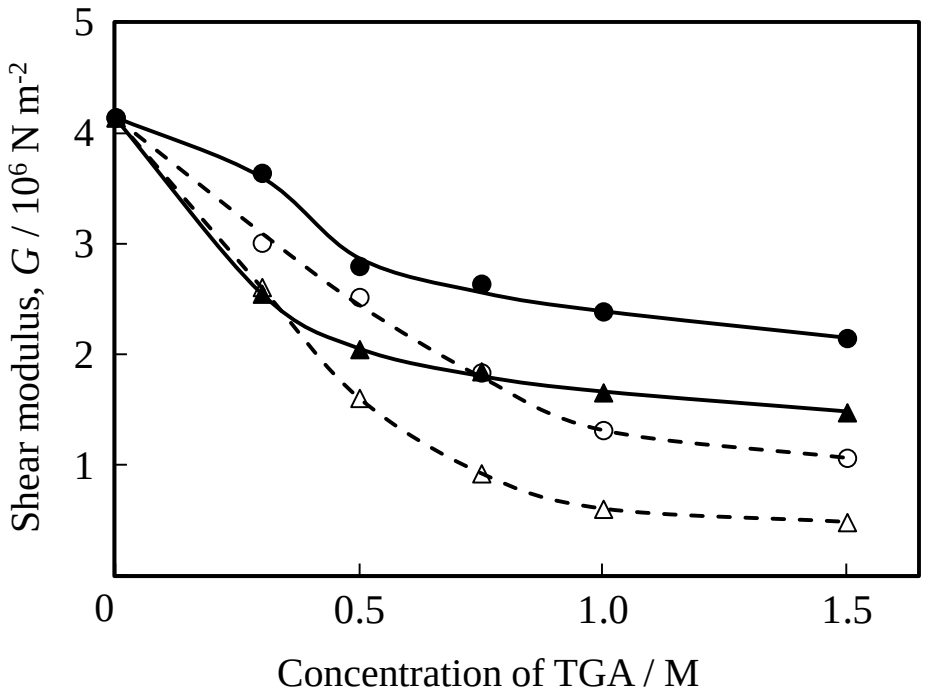

Figure 3.2. The relationships between shear modulus, *G* and TGA concentration at different pH: (○) reduced, 8.7; (△) reduced, 9.3; (●) reoxidized, 8.7; (▲) reoxidized, 9.3.

7 % of the value for the untreated hair fiber, while for the corresponding reoxidized hair fiber, G is restored by only about one-third to the original. This suggests that a large number of SS cross-links disconnected by reduction have remained unrecovered to form intermolecular cross-links. The shear modulus, G, is a function of M_c, ϕ_d, and κ. The number of intermolecular SS cross-links, $[SS]_{inter}$, in IF proteins is represented as $10^6/2M_c$ in μmol/g on the basis of the functionality of the SS bridge.

3.3.4. Molecular weight between SS cross-links in IF

The value of M_c evaluated for the untreated hair fiber is 3600 (Table 3.1). This suggests that the number of intermolecular cross-links in the network of IF proteins is 139 μmol/g (= $10^6/2M_c$) on the basis of IF proteins. The value found from the structural parameter appears to be reasonable because mechanically ineffective intramolecular SS links are involved in hair keratin [21,22], and the distribution of SS cross-links is not random [23] and occur in sites too close to play a role as different intermolecular cross-links.

Figure 3.3 shows the relationship between the number of intermolecular SS cross-links in IF chains, $[SS]_{inter}$ in μmol/g, and TGA concentration. Here usual permanent wave treatment conditions are used with the TGA concentration in the range 0.2–0.7 M, pH in the range 8.5–9.5, and temperatures between 20 and 30 °C. The values of $[SS]_{inter}$ are essentially the same as those for the unreduced condition (untreated hair) except higher reduction above 0.75 M TGA at pH 9.3. This suggests that almost no SS bond scission occurs on the IF chains forming α-crystals, and that the SS bond in the SS-rich segments of the terminal region is highly inaccessible to the reducing agent. It is further noted that a complete reformation of SS bonds occurs without any change in the type of cross-links from the bonds broken in highly reduced conditions. This phenomenon may be related to the ease of the reformation of the SS cross-link on the segment directly linked with a stable rod-like α-helical chain at a sterically favored position of SH groups produced by reduction.

3.3.5. Volume fraction of matrix domains in hair.

Figure 3.4 shows the relationship between the values of ϕ'_d and TGA concentration.

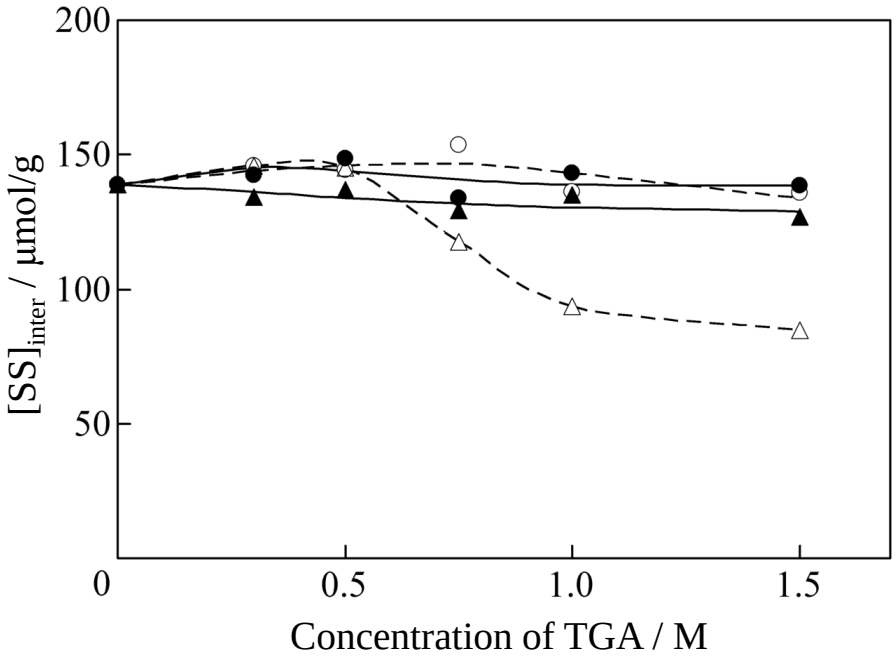

Figure 3.3. The relationships between number of intermolecular SS cross-links in IF chains, $[SS]_{inter}$ and TGA concentration at different pH: (○) reduced, 8.7; (△) reduced, 9.3; (●) reoxidized, 8.7; (▲) reoxidized, 9.3.

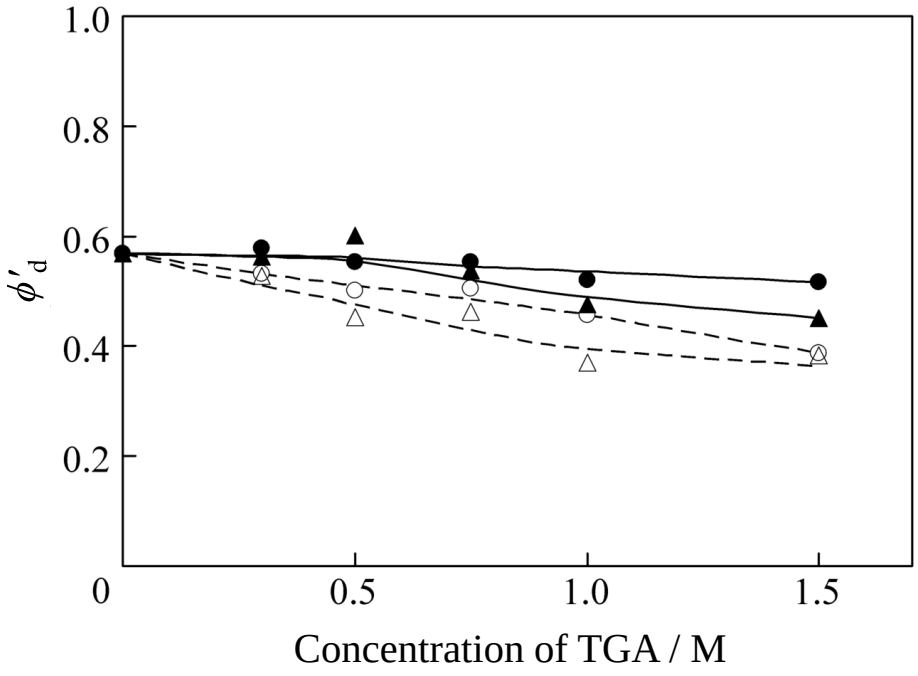

Figure 3.4. The relationships between volume fraction of globular matrix component (KAP), ϕ'_d and TGA concentration at different pH: (○) reduced, 8.7; (△) reduced, 9.3; (●) reoxidized, 8.7; (▲) reoxidized, 9.3.

The domain volume in reduced hair fibers tends to gradually decrease with increasing TGA concentration. At the highest TGA concentration at both pH 8.7 and 9.3, the values of ϕ'_d decrease to approximately 70 % of the value obtained for untreated hair fibers, while for the corresponding reoxidized hair fibers, ϕ'_d recovers to approximately 91.2 % and 79.5 %, respectively. Considerable recovery can thus be observed by oxidation even for highly reduced hair fibers. However, note that there appears to be a limit to the extent to which the globular matrix structure can recover. The decrease in the volume of the matrix may be due to the disconnection of the SS cross-links located at the hydrophilic surface of the globular matrix proteins, because minimal cleavage of SS bonds is anticipated in the hydrophobic internal regions of globular proteins, which are inaccessible to hydrophilic reducing agents such as TGA [24].

3.3.6. Shape change of matrix domain

Figure 3.5 shows the relationship between the shape factor, κ, and TGA concentration at different pH values. The values of κ for reduced hair fibers decrease steeply at the lower TGA concentration range and then reach a plateau. This suggests that TGA attacks the SS bonds between globular matrices and induces the change from an ellipsoidal to a near-spherical form that is associated with some simultaneous decrease of matrix volume. Significant recovery in κ can be observed through oxidation of reduced hair fibers at lower pH, whereas low recovery is observed for the strongly reduced hair fibers at higher TGA concentrations and pH. This indicates a difficulty in the reformation of the deformed matrix shape, and differs from the recovery behavior observed for both matrix volume, ϕ'_d, and number of intermolecular cross-links on IF chains, $[SS]_{inter}$. As a result, the modulus of the swollen fiber, G, is considered to be mainly dependent on the magnitude of κ. In other words, the change in the shape of KAP molecules results from the scission of a small number of intermolecular SS cross-links between globular proteins.

3.4. Discussion

3.4.1. Cross-linked structural change induced by permanent wave treatment

An IF molecule consists of a central α-helical coiled-coil rod domain at the core and

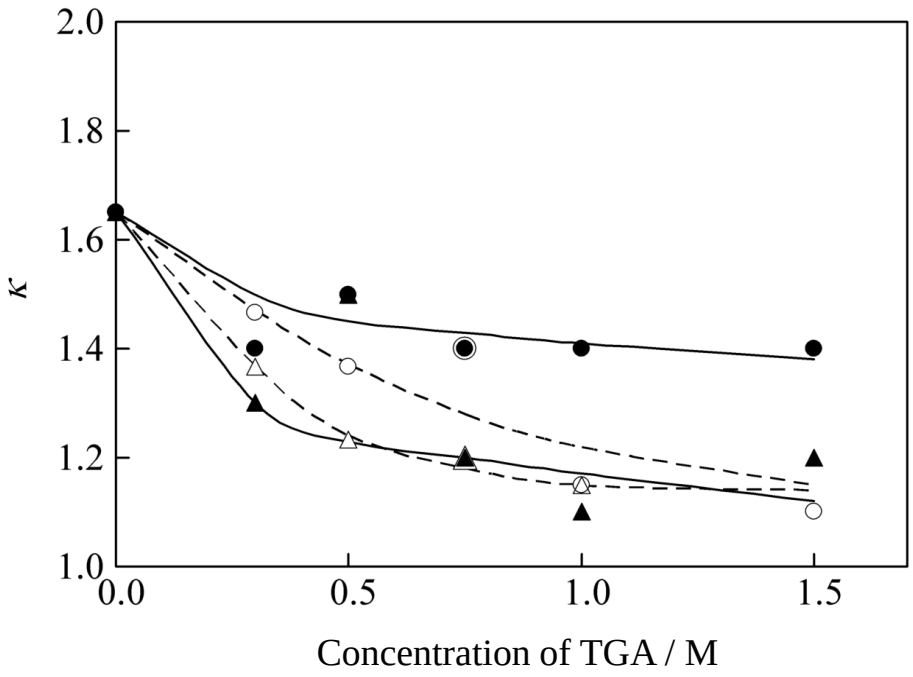

Figure 3.5. The relationships between shape factor, κ and TGA concentration at different pH: (○) reduced, 8.7; (△) reduced, 9.3; (●) reoxidized, 8.7; (▲) reoxidized, 9.3.

relatively cystine-rich N and C terminal domains forming a water accessible region between the core and the globular matrix proteins [22,24,25]. As shown in Figure 3.2, *G* decreases dramatically with increasing TGA concentration, similar to the tendency of κ (Figure 3.5). This suggests that the scission of a small number of intermolecular SS cross-links between the globules has a critical influence on the magnitude of shear modulus. It is reasonable to think that the ellipsoidal KAP molecule is a unit of the matrix component, which is an aggregate consisting of six near-spherical molecules associated through a small number of SS cross-links. With respect to this, it has been found that the number of intermolecular cross-linking sites between adjacent KAP molecules is 4.6 mol, and calculations for both of the number of aggregated KAP molecules, *P* is 6.4 mol for hair (see section 2.4.4) and intramolecular SS cross-links within a KAP molecule is 16.9 mol. In anyway, the shape of aggregated matrix changes from ellipsoidal to spherical. However, reformation to the original form is not attained during oxidation. This means that no perfect reformation of the intermolecular cross-links occurs between the KAP molecules. As interference factors for the recovery to the original structure, some of the intermolecular cross-links between the subcomponent molecules had been changed during the treatment into asymmetrical disulfide, namely mix disulfide (K–SS–CH_2COOH) [26] and cysteic acid (KSO_3H) [18] groups. Consequently, this may lead to the disordering of the KAP molecules around the IF assembly. In other words, a severe problem for the damage of the hair cortex arises mainly from the mixed disulfide groups between globular matrix proteins [14].

Another important finding is that the total number of intermolecular cross-links on the IF chain (139 μmol/g) decreases to 85 μmol/g under the strongest reducing conditions examined (1.5 M TGA concentration) and then recovers up to the level before the treatment after oxidation. Therefore during the oxidation, nearly complete reformation of the SS cross-links in the IF molecules occurs, but no perfect reformation of the regularly arranged structure of KAP molecules takes place as mentioned before. Under strong reducing conditions eight moles of IF–IF-type intermolecular SS cross-links are reactive with TGA and two moles of IF–KAP-type intermolecular SS cross-links in the terminals may be reactive with TGA, whereas three moles of IF–IF-type cross-links in the rod domain are non-reactive with TGA under usual treatment conditions.

Figure 3.6 shows a schematic representation of the possible SS cross-linked structure of IF and KAP proteins in (a) untreated, (b) TGA-reduced, and (c) reoxidized hair fibers. The cleavage and reformation of SS bonds occurring in IF and KAP structures are shown under the usual cosmetic treatment conditions of reduction and oxidation. Targets of TGA attack are the SS bonds associated with globular matrix. Some of the cross-links between the KAP molecules are broken to form free SH groups and mixed disulfide (SSR:–$SSCH_2COOH$) groups, and as a result, ellipsoidal shape of the cross-linked KAP molecule changes into a near spherical KAP molecules. The SH groups reform intermolecular SS bonds and mechanically ineffective intramolecular bonds by subsequent oxidation. However, oxidation of SS bonds may form cysteic acid ($–SO_3H$) groups and the mixed disulfide groups remain in the permanent wave hair fibers. Therefore, no perfect reformation of these cross-links occurs on the surface of the globules.

3.4.2. Mechanical properties of keratin fiber in water and cross-links between KAP

Figure 3.7 shows the stress–strain curves for untreated and reoxidized hair fibers in water at 20 °C. The curve for the untreated hair fiber consists of the three characteristic regions: Hookean, yield, and post-yield ranging from the strains of 0 %–2 %, 2 %–25 %, and beyond 30 %, respectively [27]. Each region can be explained by the underlying molecular event, i.e., the deformation of α-crystallites in IF, the unfolding of the α-helix followed by the formation of the β-pleated sheet structure, and the extension of β-sheets with a complicated contribution of globular KAP components, respectively [28]. The post-yield slope is related to the ease of the deformation of matrix components [28,29].

It can be observed that the post-yield slopes decrease markedly with increased reduction at both pH 8.7 and 9.3. This phenomenon appears to be due to the decrease of κ values following reduction, which is responsible for the deaggregation of the ellipsoidal globular matrix component to near-spherical subcomponents arising through preferential TGA attack on the cross-linking site between the KAP molecules. The initial modulus, yield stress, and breaking stress decrease with increasing TGA concentration and pH. In contrast, break extension increases with the degree of reduction; however, fiber extensibility tends to cease under stronger reducing conditions.

Figure 3.8 shows the relationship between the wet initial modulus, E_w, observed fo

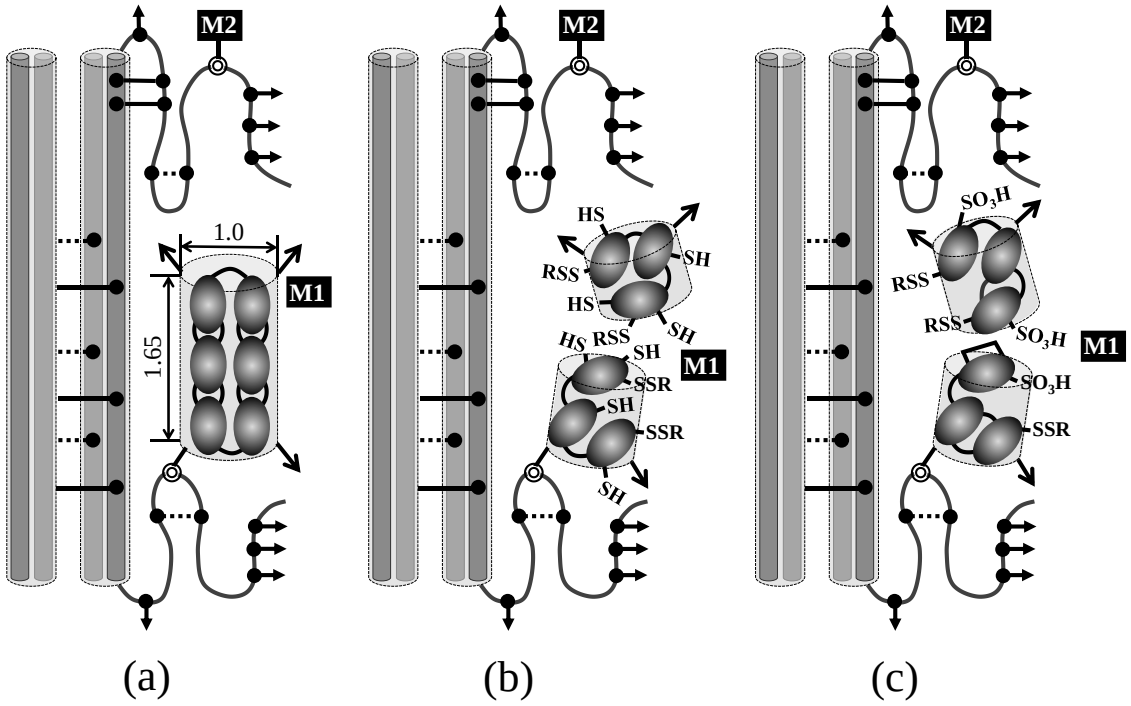

Figure 3.6. Schematic representation of possible SS cross-linked structure of IF and KAP component proteins in TGA-reduced and reoxidized hair fibers. The structures (a) unreduced (untreated), (b) reduced with 0.2 to 0.7 M TGA in a usual cosmetic treatment condition, and (c) reoxidized with BrO_3^- ions, respectively.

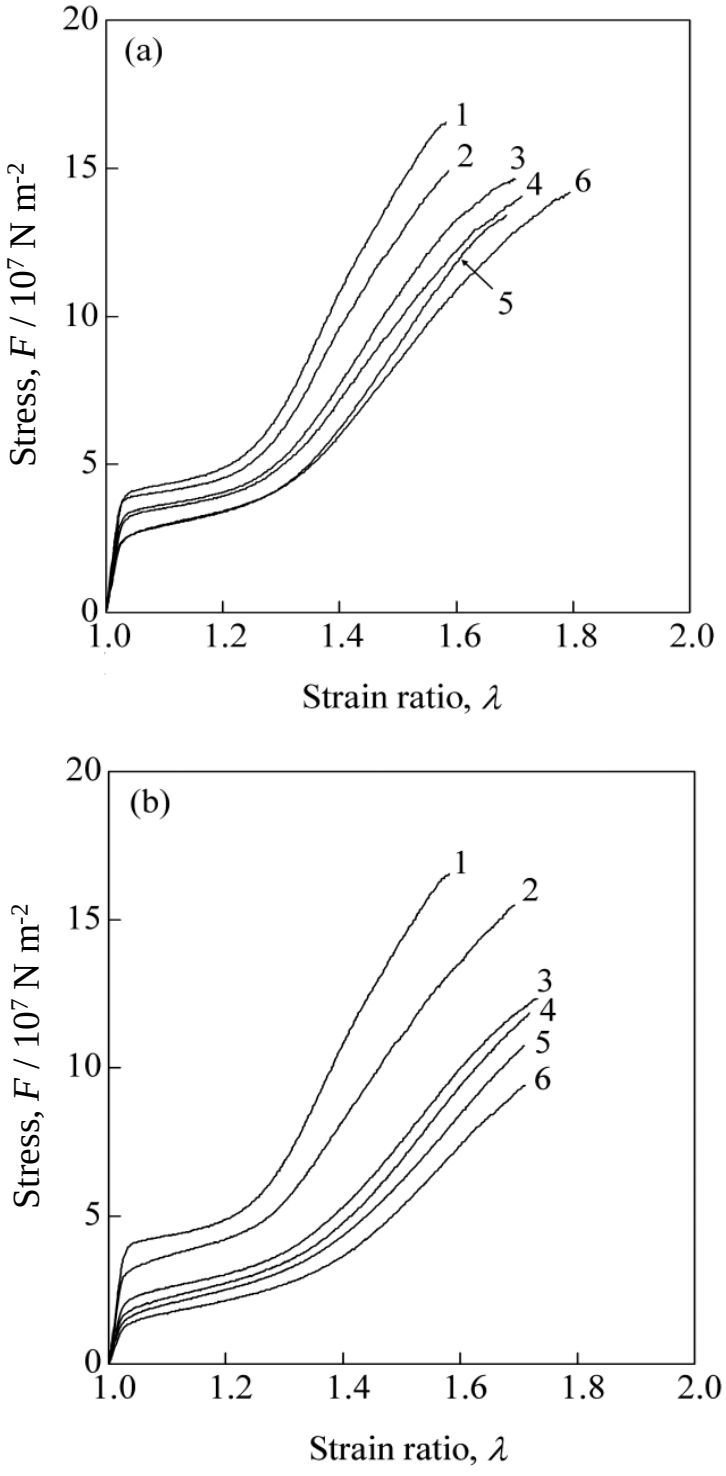

Figure 3.7. Stress-strain curves in water at 20 °C for reoxidized hair fibers after reduction treatments under different molar concentrations of TGA (a) at pH 8.7 and (b) pH 9.3: (1) untreated, (2) 0.3, (3) 0.5, (4) 0.75, (5) 1.0 and (6) 1.5.

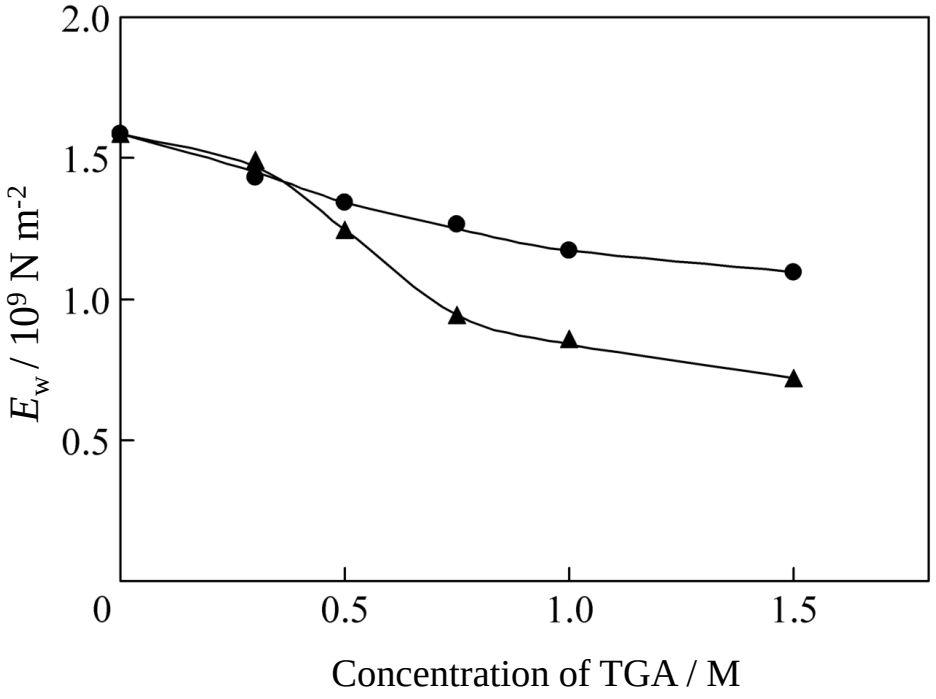

Figure 3.8. Relationships between initial modulus for hair fibers in water, E_w and TGA concentration at different pH: (●) 8.7 and (▲) 9.3.

the reoxidized hair fibers and TGA concentration at different pH values. The curves are very similar to the patterns obtained for the relationships of the shear modulus, G, and the shape factor, κ with TGA concentration (see Figures 3.2 and 3.5). It is suggested from these facts that the extension modulus of the hair fiber in water is highly dependent on the disruption of the cross-links between the KAP molecules, i.e., the arrangement of KAP molecules around the assembly of IF. In contrast, the inter- and intramolecular cross-links between the IF rods or between and within the N and C terminals are unlikely to be affected by the extension modulus, because as described above, almost all the cross-links in the IF rods are unchanged through reduction and subsequent oxidation, and nearly complete cross-linked structure of IF is rebuilt by oxidation after reduction with TGA (Figure 3.3). It has been reported that the extension modulus in water reflects the mechanical stability against the deformation of the α-crystal and that stability depends on not only the integrity of α-crystallites but also the cross-link density around IF molecules [30].

3.4.3. Relative amount of α-crystallites in reoxidized hair

As a measure of the mechanical stability of fibers, a relative amount of α-crystallites was estimated for reoxidized (permanent wave) hair by using the WAXS method. Figure 3.9 shows the equatorial X-ray diffraction intensity versus 2θ for (a) untreated and (b) reoxidized hair obtained by the reduction with 0.75 M TGA at pH 9.3. The simulation of the equatorial intensity was performed assuming Lorentzian line shape. Four lines were obtained for both the untreated and treated hair. The two lines with relatively sharp peaks at 2θ = 9° (line 1) and 19.3° (line 2) correspond to α- and β-pleated sheet crystals, respectively. The third line with a broad peak at nearly 2θ = 24° (line 3) corresponds to amorphous materials. The fourth curve has no peak in the range 5° to 35° (line 4). The relative amount of α-crystallites (R.A.C.) was calculated as the diffraction intensity ratio of α-crystallites (2θ = 9º) to that of the total intensity, which corresponds to the total area for the curves assigned as (1), (2), (3), and (4) with 2θ ranging from 5 to 35°. The results are shown in Figure 3.10.

Relative amounts of α-crystallites in the untreated and permanent wave hair are approximately the same. Therefore, it is suggested that the conformational change in

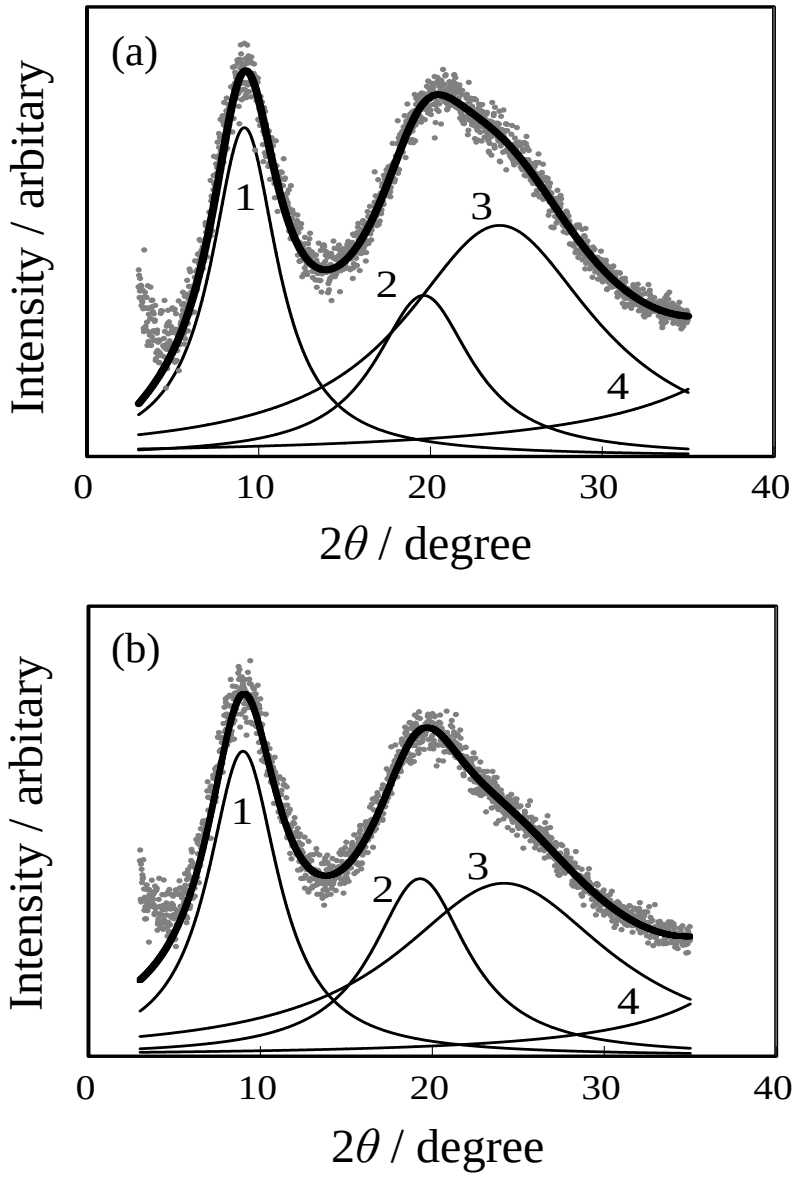

Figure 3.9. Equatorial X-ray diffraction intensity versus 2θ for (a) untreated and (b) treated hair with 0.75 M TGA at pH 9.3. The linear fits have four contributions from (1) a sharp α-peak at $2\theta = 9°$, (2) a relatively sharp β-peak at $2\theta = 19.3°$, (3) a broad amorphous peak at about $2\theta = 24°$, and (4) the no-peak intensity curve, which slight increases with increasing 2θ.

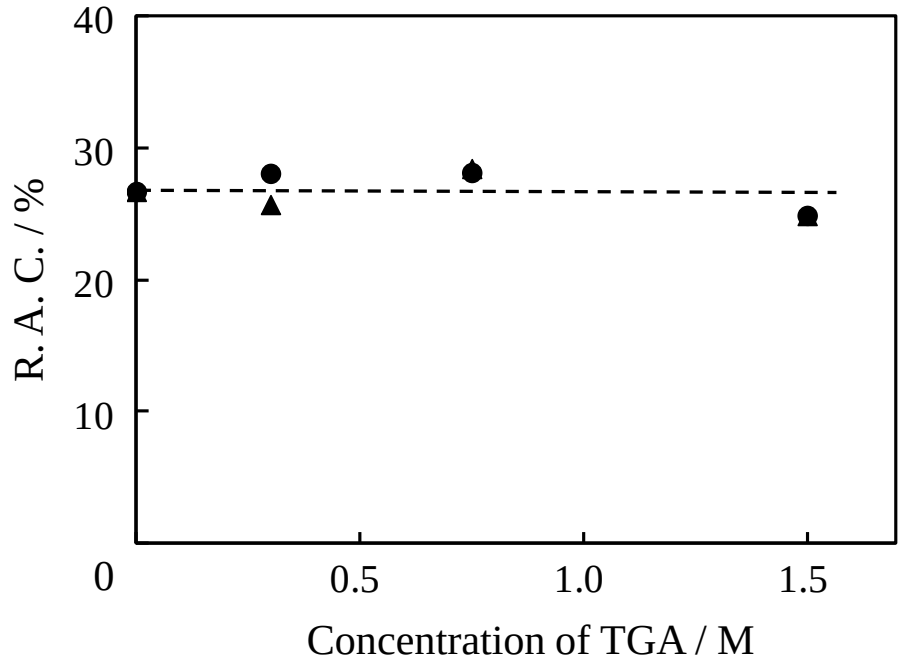

Figure 3.10. Relationships between relative amount of α-crystallites (R.A.C.) and TGA concentration at different pH: (●) 8.7 and (▲) 9.3.

α-helix does not occur in spite of the fact that the wet initial modulus E_w of the permanent wave hair is greatly decreased with increasing reduction.

Fudge and Gosline [31,32] proposed a keratin structural model in which an elastomeric keratin matrix resists IF swelling and maintains the IF stiffness. According to their model, the elastomeric keratin matrix corresponds to our structural model for the matrix, which consists of an assembly of cross-linked globular KAP molecules, as shown in Figure 2.6. The disulfide cross-linked structure and the mechanical properties of the keratin fiber will be addressed in the future.

3.5. Conclusions

Under the conditions for the permanent wave treatment of hair, the reactivities of SS bonds located on IF and KAP molecules with TGA were determined by analyzing the structural parameters calculated by fitting the theoretically derived equation of state to the stress–strain curves measured for reduced and reoxidized hair fibers in a concentrated mixed solution of aqueous LiBr and BC. Under strong reducing conditions eight moles of IF–IF-type intermolecular SS cross-links are reactive with TGA and two moles of IF–KAP-type intermolecular SS cross-links in the terminals may be reactive with TGA, whereas three moles of IF–IF-type cross-links in the rod domain are non-reactive with TGA under usual treatment conditions. Moreover, 2.3 mol of KAP–KAP-type intermolecular links are the most reactive with TGA, whereas 16.9 mol of intramolecular SS cross-links within a KAP molecule are non-reactive.

Following important conclusions were obtained from the correlation of the parameters for reduced and reoxidized hair fibers: (i) TGA attacks preferentially the SS bonds between the KAP molecules, and as a result, the ellipsoidal shape of the globule changes to a near-spherical form, (ii) nearly complete reformation of SS cross-links occurs between IF proteins through oxidation even when a large number of SS bonds break under strong reducing conditions, but no perfect reformation of these cross-links occurs on the surface of the globules, (iii) the extension modulus of the hair fiber in water is highly dependent on the number of intermolecular SS cross-links between KAP molecules around IF, and (iv) the amount of α-crystallites in the reoxidized hair is

approximately constant despite the fact that the extension modulus in water decreases with increasing reduction.

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Chapter 4

Reproduction mechanism of SS cross-links in permed hair by the washing after reduction with thioglycolic acid

Chapter 4: Reproduction mechanism of SS cross-links in permed hair by the washing after reduction with thioglycolic acid

4.1. Introduction

It was found that fibers pretreated with a concentrated aqueous LiBr solution containing *N*-ethylmaleimide, which serves as a blocking agent for free thiol (SH) groups, show typical rubber elasticity in a mixed solution of aqueous 8 M LiBr and diethylene glycol mono-*n*-butyl ether [1], and a semi-quantitative method for determining the SS cross-link density of various keratin fibers was proposed. As mentioned in chapter 3, this method was applied to permanent waved hair fibers with thioglycolic acis (TGA) and cleavage and reformation behavior of the SS cross-links in reduction and subsequent oxidation treatment were considered, that is, it was shown that TGA attacks preferentially the SS bonds between the KAP molecules, and as a result, the ellipsoidal shape of the globule changes to a near-spherical form, and reformation to the original form is not attained during oxidation.

Incidentally, it is well-known that cleavage reaction of the SS bonds by reductants (thiol compounds) is equilibrium reaction which consists of two reaction steps. The balance of this reaction is determined by concentration of the reductant, which is raised in hair fiber during reduction treatment and decreased during subsequent washing with water. Ogawa et al. have reported the influence of washing after reduction on cleavage reaction of SS bonds in hair straightening, and they have showed that a mixed disulfide by-produced by reduction in a bicomponent system of 9 % TGA and 2 % dithiodiglycolic acid is changed into a cystine residue by movement of chemical equilibrium during washing for 1 min [2]. It is reported by Joko et al. that the permanent waving process in which an immersion step in water for a predetermined time is incorporated between reduction and oxidation steps can produce the waving appearance characterized by shorter linear length from root to tip on the hair tress than that of the normal permanent process and shown as if the wavy hair fibers are lifted against gravity [3-5]. They explained this phenomenon as rearrangement of the high-order structure components in

the unreduced area of hair fiber accompanied by the stress relaxation resulting from SH/SS interchange reaction. From these studies it is suggested that washing after reduction may affect the SS cross-links structure. However, the influence of washing after reduction on the microstructure of the hair fiber is little-known because there are few research focusing on washing step. Immediate washing after reduction is called "intermediate washing" in cosmetic industry.

In this chapter, the mechanical properties of hair fibers permed under respective conditions which differ systematically in the washing time are investigated. As discussed in chapter 3, the SS cross-linked structure in IF and KAP is one of the important factors determining the mechanical behavior of the hair fiber. Therefore, we have attempted to clarify the influence of intermediate washing on the SS cross-linked structure by applying a rubber elasticity theory to the corresponding swollen hair fibers. The changes of the SS cross-linked structure induced by intermediate washing are interpreted from the viewpoint of equilibrium theory of reduction, and the mechanism for chemical reaction occurring during intermediate washing is proposed.

4.2. Materials and methods

4.2.1. Purification of hair fibers

An aqueous ammonium solution of 50 % (w/v) TGA was used as supplied commercially. Other chemicals used were of reagent grade. Nitrogen substitution water was prepared by removing dissolved oxygen by bubbling with a dry nitrogen gas from the distilled water which was boiled for an hour and subsequently cooled to room temperature.

Chemically unmodified human hair samples (approximately 25 cm in length) collected from a Japanese female were subjected to purification after removing lengths of approximately 1 cm at the root and approximately 2 cm at the tip. A hair tress consisting of 20 hair fibers (approximately 20 cm in length) was purified according to the method described in section 2.2.1. Purified hair fibers were used for reduction and oxidation treatments.

4.2.2. Permanent waved hair fibers

4.2.2.1. Reduction step

Reduction was performed by winding the hair fibers around a 12-mm-diameter rod and dipping in an aqueous solution (400 mL) containing 0.75 M TGA at pH 8.7 or 1.5 M TGA at pH 9.3 for 20 min at room temperature.

4.2.2.2. Intermediate washing step

Intermediate washing was performed by immersing the reduced hair fibers in the nitrogen substitution water for 1/60 h at 25 °C or for 1/3 to 72 h at 40 °C. Immediately after that, these fibers were subjected to either oxidation or swelling treatment.

4.2.2.3. Oxidation (reoxidation) step

Intermediate washed hair fibers were subsequently immersed in a 0.5 M sodium bromate solution (400 mL) at room temperature for 20 min at pH 6.35 followed by immersing them four times in water (400 mL) repeatedly for 5 min each. Finally, reoxidized hair fibers were blotted and air dried. In addition, the hair fibers oxidized for one to four hours were also prepared.

4.2.3. Reduced and NEM-treated hair fibers

Reduction was performed by winding the hair fibers around a 12-mm-diameter rod and dipping in an aqueous solution (400 mL) containing 0.75 M TGA at pH 8.7 for 20 min at room temperature. The reduced fibers were treated with a 0.05 M NEM solution for 24 h at pH 8.0 and room temperature to block the free SH groups. The reduced and NEM-treated fibers thus obtained were washed thoroughly by immersing in the nitrogen substitution water for a predetermined time at 40 or 60 °C.

4.2.4. Force–extension curve in water

Force–extension measurement in water for the untreated and permanent waved hair fibers was the same as described in section 3.2.6.

4.2.5. Preparation of swollen hair fibers and determination of structural parameters

Preparation of the swollen hair fibers for the untreated and permanent waved hair fibers was the same as described in section 2.2.2. The force-extension measurement of

swollen fiber was presented in section 2.2.3 and the procedure for determining several structural parameters was also described in section 2.3, respectively.

4.3. Results and discussions

4.3.1. Influence of intermediate washing on the force-extension curve in water

Figure 4.1 shows the stress–strain curves for untreated and reoxidized hair fibers in water at 25 °C. It can be observed that the initial modulus, the yield stress and the breaking stress in all reoxidized samples are lowered compared to the untreated fiber. It seems that the post-yield slope is also lowered. These tendencies are owing to the ease of the deformation of matrix components by scission of the SS cross-links on the surface of globular matrix proteins [6,7], and a decline of stability to the extensional deformation of α-crystalline structure. [8]

Furthermore, it is shown clearly by force-extension curves in Figure 4.1 that the initial modulus, the yield stress, the breaking stress and the post-yield slope are increased as the intermediate washing time becomes long. At the condition of 1.5 M TGA (pH 9.3), the stress of the reoxidized fiber is decreased greater than that at 0.75 M TGA (pH 8.7) in comparison of the same strain ratio, while it is recovered more remarkable by intermediate washing for a long time.

Figure 4.2 shows the relationship between the wet initial modulus, E_w, observed for the reoxidized hair fibers and washing time. The values of E_w are increased with increasing time of washing. The values of E_w for the washing time of 1/60 h, as corresponding to lightly washing on the surface of the hair fiber, decrease to approximately 73 % (0.75 M, pH 8.7) and 41 % (1.5 M, pH 9.3) of the value obtained for untreated hair fibers, while E_w of the washing samples for 72 h at 40°C recovers to approximately 93 % (0.75 M, pH 8.7) and 80 % (1.5 M, pH 9.3), respectively. The larger recovery rate for the value of E_w by intermediate washing is shown for the result of more severe reduction condition. It is suggested from these facts that reformation of the SS cross-links in hair cortex during intermediate washing causes the recovery from the mechanical degradation of reduced fiber.

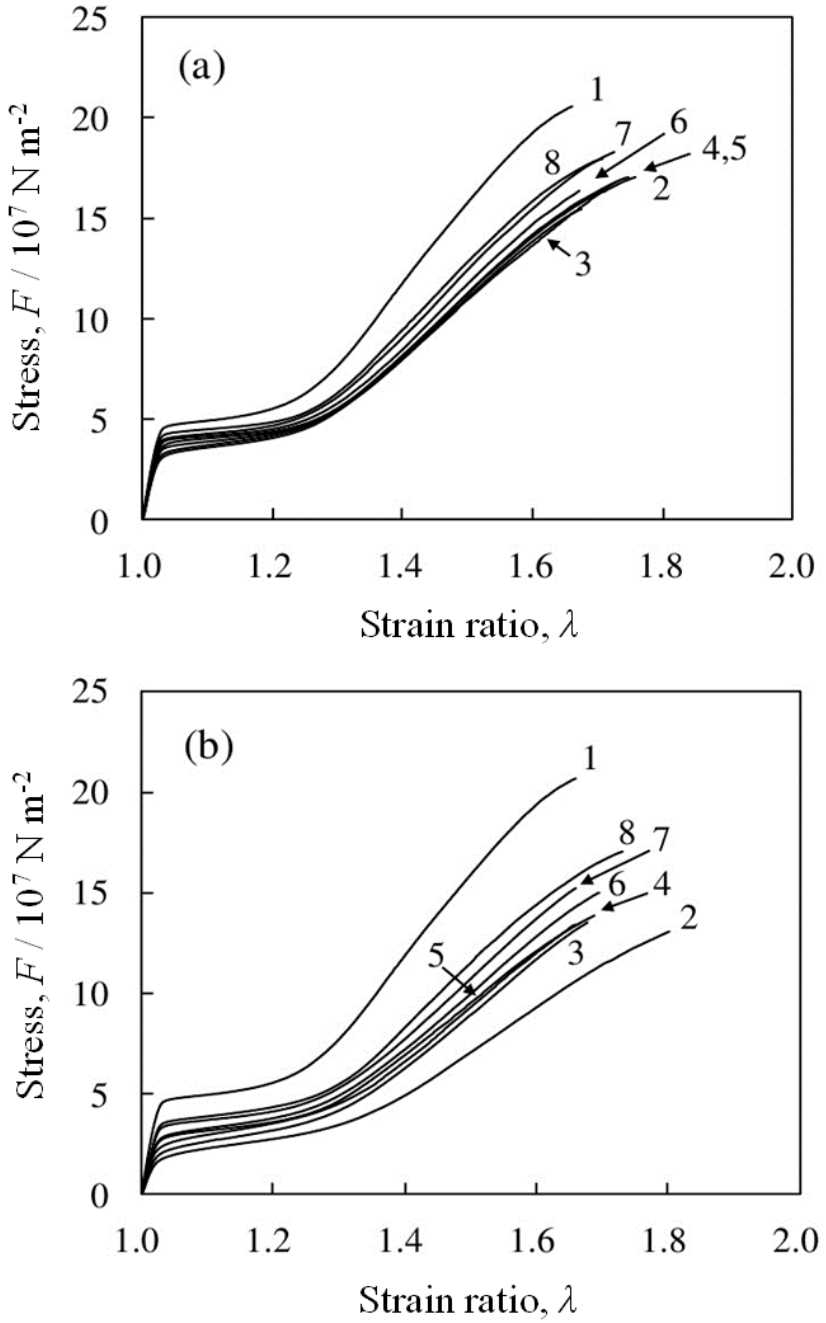

Figure 4.1. Stress-strain curves in water at 20 ˚C for reoxidized hair fibers after reduction followed by different time of washing treatment: (a) 0.75 M TGA (pH 8.7) and (b) 1.5 M TGA (pH 9.3): (1) untreated, (2) 1/60 h, (3) 1/3 h, (4) 1 h, (5) 4 h, (6) 12 h, (7) 24 h, (8) 72 h.

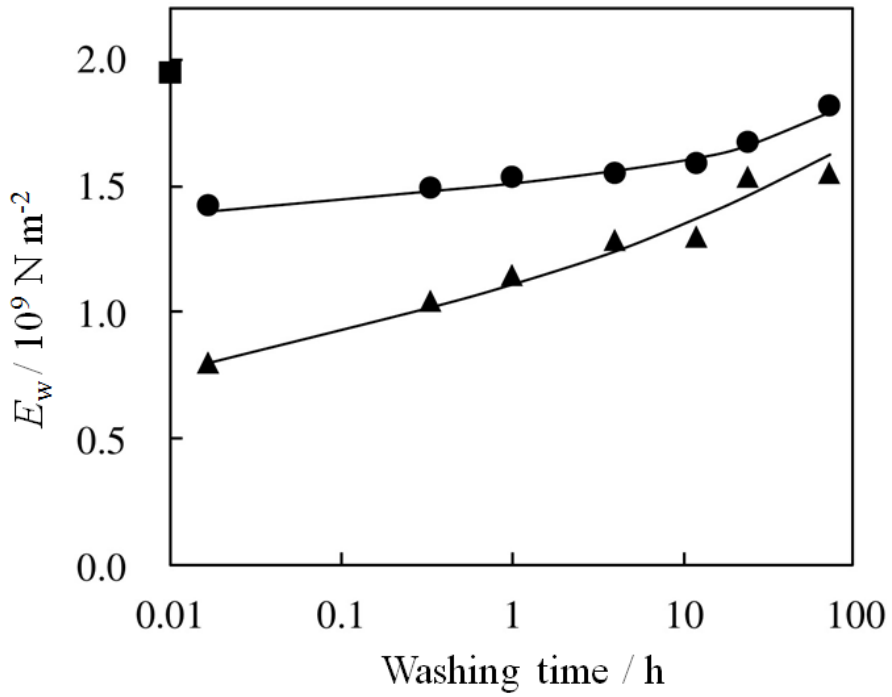

Figure 4.2. Relationships between initial modulus for hair fibers in water, E_w and washing time after reduction: (■) untreated, (●) 0.75 M TGA (pH 8.7) and (▲) 1.5 M TGA (pH 9.3). Washing time is shown by a logarithmic axis in this figure.

4.3.2. Structural parameters of swollen hair fibers

Figure 4.3 shows typical stress–strain curves for the swollen samples from untreated and reoxidized hairs. Fitting the experimental data for equation (2-3) with suitable choices of parameters M_c, ϕ_d, and κ, we can evaluate the values of these parameters. The results obtained for untreated and reoxidized hair fibers are shown in Table 4.1 and 4.2. The values of v_2 (an index inversely related to swellability) for reoxidized hairs are lower than that for untreated hair. As a whole, the values of v_2 for reoxidized hairs corresponding to the reduction with 1.5 M TGA (pH 9.3) are lower than those with 0.75 M TGA (pH 8.7). They mean that no perfect reformation of the SS cross-links cleaved by reduction is attained by oxidation, and the degree of those reformation decreases with increasing of the degree of reduction. A more interesting result is that the values of v_2 at the reduction with 1.5 M TGA (pH 9.3) are increased with increasing washing time, and this indicates that degree of the reformation of the SS cross-links is raised by intermediate washing.

In the shape factor for reoxidized hair fibers, the values of κ being 1.66 for untreated hair decrease through reduction and subsequent oxidation process. This means the deaggregation of the ellipsoidal globular matrix component. However, the values of κ are increased gradually with increasing washing time. This indicates that the decrease in the number of the SS cross-links between the KAP molecules caused by reduction and subsequent oxidation induces the shape change of aggregated matrix from ellipsoidal to near-spherical (see section 3.3.6), while the process including intermediate washing for a long time brings about the recovery into ellipsoidal.

In the volume fraction of matrix domain for reoxidized hair fibers, the values of ϕ'_d corresponding to the reduction with 1.5 M TGA (pH 9.3) are increased with increasing time of washing. This increase of ϕ'_d values seems to be associated with the increase of κ values. This implies that the shape and volume changes of aggregated matrix occur simultaneously. But, the relationship between the values of ϕ'_d corresponding to 0.75 M TGA (pH 8.7) and washing time is unclear, because degree of reduction is lower and the disordering of the SS cross-linked structure in the matrix component is relatively little.

In the number of intermolecular cross-links in the network of IF proteins, the values of $[SS]_{inter}$ for almost all of the samples are essentially the same as those for the untreated hair, 144 μmol/g. Nearly complete reformation of SS cross-links occurs between IF

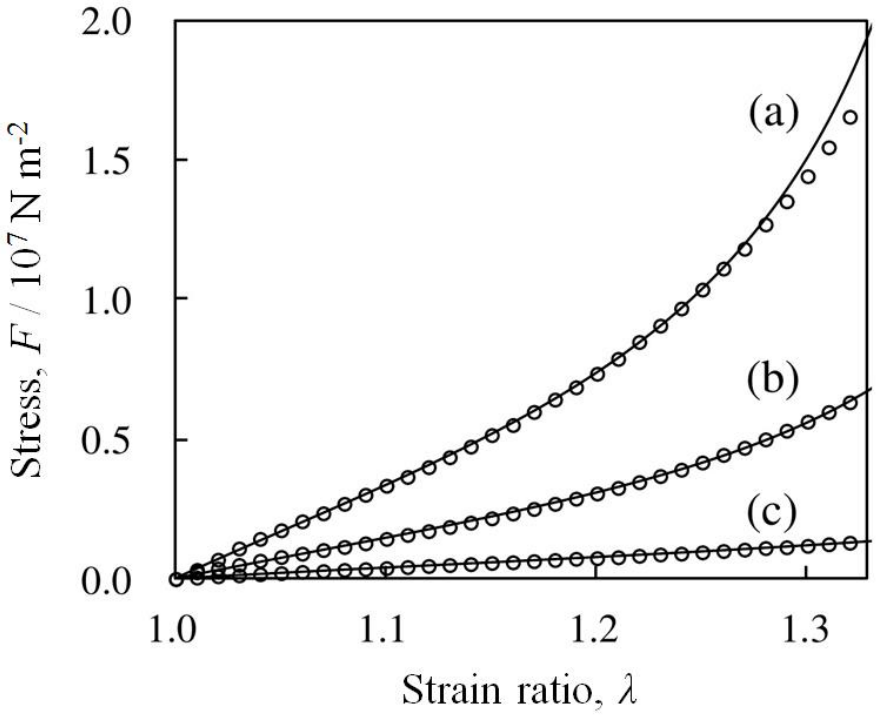

Figure 4.3. Typical stress-strain curves of swollen hair fibers from reoxidized hairs after reduction with different conditions and washing for 1/60 h. (a) untreated, (b) 0.75 M TGA (pH 8.7), (c) 1.5 M TGA (pH 9.3); lines fitted to the experimental data by equation (2-3).

Table 4.1. Structural parameters for SS cross-links in untreated and reoxidized hair fibers after reduction with 0.75 M TGA at pH 8.7 and washing treatments.

Washing Conditions		$10^{-6}G$	ν_2	κ	ϕ_d	ϕ'_d	M_c	$10^6/2M_c$
Temp.	Time (h)	(N/m^2)					(g/mol)	(μmol/g)
Untreated		4.19	0.584	1.66	0.334	0.572	3500	144
25 ˚C	1/60	1.88	0.503	1.33	0.254	0.504	3800	133
40 ˚C	1/3	2.17	0.532	1.38	0.277	0.521	3900	130
	1	2.24	0.459	1.46	0.266	0.579	3500	142
	4	2.26	0.533	1.41	0.278	0.522	3800	130
	12	2.38	0.533	1.40	0.270	0.506	3500	141
	24	2.63	0.563	1.49	0.283	0.502	3800	133
	72	2.86	0.518	1.53	0.284	0.549	3400	145

Table 4.2. Structural parameters for SS cross-links in untreated and reoxidized hair fibers after reduction with 1.5 M TGA at pH 9.3 and washing treatments.

Washing Conditions		$10^{-6}G$	ν_2	κ	ϕ_d	ϕ'_d	M_c	$10^6/2M_c$
Temp.	Time (h)	(N/m^2)					(g/mol)	(μmol/g)
Untreated		4.19	0.584	1.66	0.334	0.572	3500	144
25 ˚C	1/60	0.73	0.370	1.12	0.135	0.365	4300	116
40 ˚C	1/3	1.08	0.400	1.23	0.153	0.383	3600	138
	1	1.17	0.427	1.24	0.177	0.415	3800	132
	4	1.38	0.482	1.26	0.206	0.427	3900	128
	12	1.66	0.510	1.35	0.224	0.439	3900	129
	24	2.07	0.524	1.43	0.249	0.475	3800	133
	72	2.41	0.518	1.52	0.260	0.502	3600	137

proteins through oxidation even when the SS bonds in IF protein break under strong reducing conditions (see section 3.3.4.). Therefore, it is suggested that the decrease in the initial modulus and extension stress through reduction and subsequent oxidation process as shown in Figure 4.1 and 4.2 is caused by cleaving the SS cross-links between the KAP molecules, by which the aggregated structure of globule matrix is constituted, and the recovery from the mechanical degradation by intermediate washing is induced by the reformation of these cross-links.

The shear modulus, G, can be considered as a parameter reflecting the density of the intermolecular SS cross-links in IF and KAP structure since it is a function of M_c, ϕ'_d, and κ. The values of $[SS]_{inter}$ are hardly changed by intermediate washing as mentioned above, and the values of G are changed with the values of ϕ'_d and κ. Thus, the reformation of the SS cross-links in the aggregated matrix by washing is considered as the increase of the values of G.

4.3.3. SS reformation reaction by intermediate washing

Figure 4.4 shows the relationship between the share modulus, G, and time of oxidation for the treated hair with reduction and subsequent washing for 1/3 h at 25°C. The values of G for the oxidation time of 1/3 h are 2.02×10^6 N/m^2、at 0.75 M TGA (pH 8.7), and 0.85×10^6 N/m^2 at 1.5 M TGA (pH 9.3), respectively. It is found that these values in oxidation for up to 4 h are essentially the same as those for 1/3 h. This means that the reformation reaction of the SS bonds by oxidation is ended within 1/3 h. In comparison with the values of G for the reoxidized hair fibers in Table 4.1 and 4.2, the values of G for the hair fibers prepared by reduction and intermediate washing for a sufficiently long time are much higher than those corresponding to reduction and oxidation for 4 h. In general, it is well-known that the SS bond is disrupted by reduction and reformed by subsequent oxidation. It is suggested from these results that the reformation of the intermolecular SS cross-links by intermediate washing is attained by a different mechanism from oxidation.

Accordingly, to reveal this mechanism the shear modulus, G, was measured for the swollen samples prepared from reduced and subsequently washed hair fiber. Figure 4.5(a) shows the value of G for the hair fiber obtained by reduction and the subsequent washing

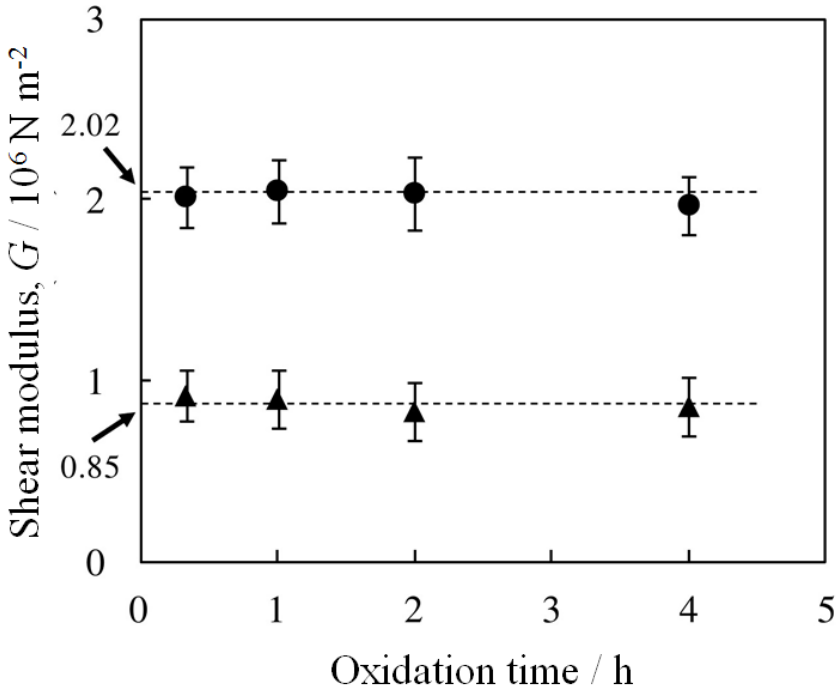

Figure 4.4. The relationships between shear modulus, *G* and oxidation time after TGA-reduction followed by washing for 1/3 h: (●) 0.75 M TGA (pH 8.7), (▲) 1.5 M TGA (pH 9.3). (Mean ± SE).

for 1/60 h at 25 °C, 0.33×10^6 N/m^2, which corresponds to the washing on the surface of the fiber. Figure 4.5(b) also shows the value of *G* by the reduction and the subsequent washing for 24 h at 40 °C, 1.5×10^6 N/m^2. The latter value attains about 5 times of former. Therefore, this increase of *G* means that the number of the intermolecular SS cross-links in the matrix is increased by washing, that is, the SS cross-links between the KAP molecules are cleaved by reduction and reformed by subsequent washing. An interesting result is that the reformation of the SS cross-links is not by oxidation but by intermediate washing.

It is well-known that cleavage reaction of SS bonds by reductants (thiol compounds) is equilibrium reaction which consists of two reaction steps according to equations (4-1) and (4-2) [9]:

$$\mathrm{KSSK} + \mathrm{RSH} \rightleftarrows \mathrm{KSH} + \mathrm{KSSR} \qquad (4\text{-}1)$$

$$\mathrm{KSSR} + \mathrm{RSH} \rightleftarrows \mathrm{KSH} + \mathrm{RSSR} \qquad (4\text{-}2)$$

Here, K represents the keratin chain, and RSH is the reducing agent (TGA). The SS bond between keratins is cleaved and cysteine residue (KSH) and mixed disulfide (KSSR) are generated in the first step reaction. Under the excess amount of RSH, KSSR is more reduced by RSH to be converted into a cysteine residue, and dithiodiglycolic acid corresponding to RSSR is generated simultaneously as shown in equation (4-2). The reformation of the interrupted SS cross-links may be caused not only by reoxidation but by reverse reaction of equation (4-1). In order to estimate the contribution of the reverse reaction to the reformation of the SS cross-links, the shear modulus, *G*, for reduced and NEM-treated hair fibers, namely the hair fibers which are washed under various temperature and time after the treatment with NEM to block the free SH groups produced by reduction, was measured. The results are shown in Figure 4.5 (c), (d), (e) and (f). The values of *G* for the samples treated with NEM after reduction and subsequent washing are almost the same as that for the sample (a) regardless of the washing temperature and time, and different tendency from the increase of the value of *G* by washing such as the sample (b). This means that the treatment with NEM after reduction inhibits the reformation of the SS cross-links due to subsequent intermediate washing, and this is

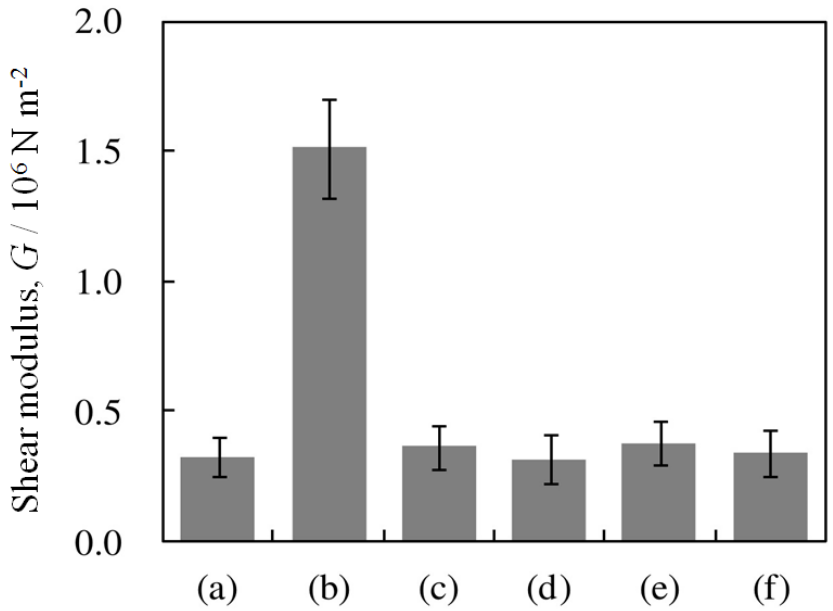

Figure 4.5. Shear modulus, *G* of swollen hair fibers from reduced and washed hairs at different washing conditions: (a) 25 ˚C, 1/60 h, (b) 40 ˚C, 24 h. Swollen hair fibers from reduced, NEM-treated and washed hairs at different washing conditions: (c) 40 ˚C, 1 h, (d) 40 ˚C, 24 h, (e) 60 ˚C 24 h, (f) 60 ˚C, 48 h. (Mean ± SE).

caused by preventing the reverse reaction of equation (4-1) due to blocking the free SH groups with NEM. In other words, the reformation of the SS cross-links by intermediate washing is the reverse reaction of equation (4-1) triggered by removal of RSH molecules by washing which penetrating during reduction step in the hair cortex.

4.3.4. The removal mechanism of mixed disulfide by intermediate washing

It is considered that one of the effect by intermediate washing is decrease of RSH concentration in the hair cortex. The equilibrium reactions of equations (4-1) and (4-2) may come back to the left hand side due to the decrease of RSH concentration in the reaction field by its desorption and removal. Note that the contribution of reverse reaction of equation (4-1) to the reformation rate of the SS cross-links is larger than that of equation (4-2), because the latter is reaction between small molecules and macromolecules while the former is between macromolecules and remarkably lower than the latter in the rate of reverse reaction. Therefore, when RSH molecules leave the reaction field by those diffusion, RSH concentration is lower there and the KSSK cross-links are reformed.

With respect to desorption mechanisms of dye for wool and various fibers, the desorption rate analyzed from the relationship between dye concentration and the square root of desorption time, $t^{1/2}$, has been reported by many researchers [10-13]. The shear modulus、*G*, reflects the amount of the intermolecular SS cross-links in the matrix as mentioned above. The value of *G* when restoring the SS cross-linked structure completely is presumed to the same as that for untreated hair, G_0, and the relationship between the ratio of the intermolecular SS cross-links, G/G_0, and the square root of intermediate washing time, $t^{1/2}$, is shown in Figure 4.6. Excelent linearities are obtained in both reduction case of 0.75 M and 1.5 M TGA. Since the slope of the plots corresponds approximately to the diffusion coefficient for removing RSH molecules in the hair cortex, and it is found that the coefficient in the case of 1.5 M TGA is larger than that in 0.75 M TGA. These linearities mean that the reformation of the SS cross-links by intermediate washing depends on the decrease of RSH concentration in the reaction field by its diffusion. In addition, it can be said from the slope of plots that an enormous amount of time is necessary for removing TGA completely by the washing which penetrating in hair

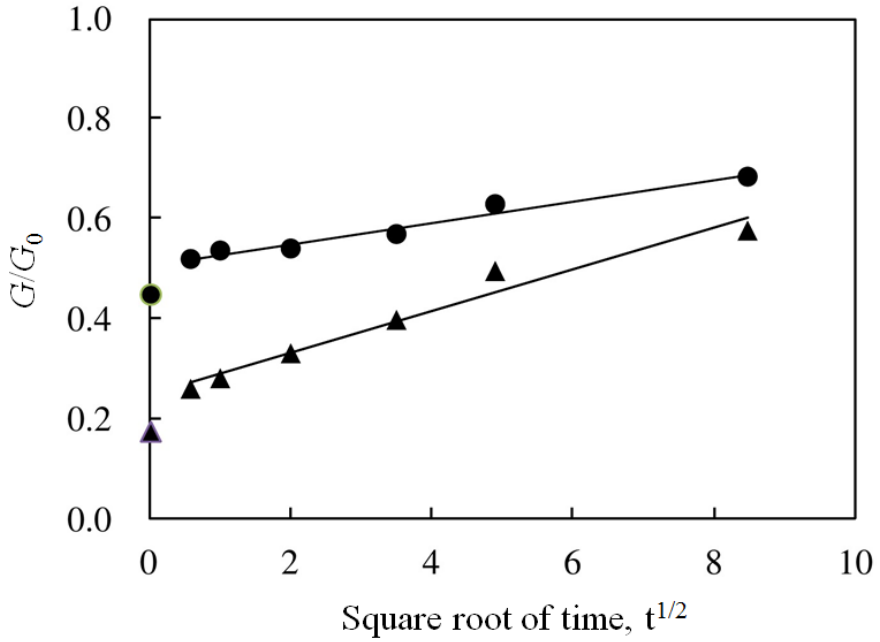

Figure 4.6. Relationships between G/G_0 and the square root of washing time, $t^{1/2}$ after reduction at different conditions: (●) 0.75 M TGA (pH 8.7) and (▲) 1.5 M TGA (pH 9.3).

cortex during reduction. For example, it may be estimated that the degree of recovery due to washing for a week is merely 80 % ($G/G_0 \approx 0.8$) in both reduction conditions. It suggested that the amount of RSH molecules in which the complete reformation of the SS cross-links is not attained remains after washing for a week. The further investigation relating to the remaining state of reductant molecules in hair cortex and the recover property of the cross-linked structure is expected in the future.

4.4. Conclusions

In permanent waving process, some changes of mechanical properties induced by intermediate washing have been clarified in this chapter. In addition, in order to estimate the change of the SS cross-linked structure by the washing, the swollen hair fiber prepared by the treatment with concentrated LiBr aqueous solution was extended in a mixed solution of aqueous 8 M LiBr and diethylene glycol mono-*n*-butyl ether, and the four structural parameters related to the network structure of IF and KAP were decided by fitting the theoretical relation between equilibrium force and extension ratio to the experimentally obtained stress-strain curve. These parameters have been compared with one another in relation to the washing time to find the changes of the cross-linked structure by the washing. Furthermore, the chemical reaction caused by the washing has been discussed in the view of equilibrium reaction of reduction. The conclusions obtained are summarized below:

i) Initial modulus and breaking stress decreased by reduction are restored so as to approach to the properties for untreated hair by intermediate washing.
ii) The SS cross-links between the KAP molecules cleaved by reduction are reformed by the washing.
iii) This reformation is due to the reverse reaction of reduction induced by removing reductant molecules from equilibrium system by those diffusion.

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Chapter 5

General conclusions

Chapter 5: General conclusions

Influence of the disulfide cross-linked structure of keratin fibers on the mechanical properties was investigated in this dissertation. Relevance of microstructure of keratin fibers to its mechanical behavior has been unclear. Many types of mechanical models to explain this have been presented by many workers, but none of them has been successfully accepted. We attempted to clarify the number, type, and location of SS cross-links and identify the cross-links related to its mechanical properties. The results obtained in this study are summarized below:

In chapter 2, the disulfide (SS) cross-linked structure of hair and wool keratin fibers is discussed. Rubber elasticity theory was applied to the force–extension curves of swollen fibers in a diluent mixture of concentrated aqueous lithium bromide and diethylene glycol mono-*n*-butyl ether. On the basis of a two-phase structural model for the globular matrix keratin-associated protein (KAP) dispersed in the swollen network of intermediate filament (IF) proteins, structural parameters were obtained by fitting force–extension curves to theoretically derived relations between elastic forces originating from nonuniform network and extension ratios. The parameters extracted are the number of intermolecular SS bonds in the IF, the volume fraction of matrix proteins in the fiber, and the shape of the matrix domain. The number, type, and location of SS cross-linkages in the IF proteins were estimated using the difference in the conversion rate from disulfide to lanthionine and lysinoalanine induced by the treatment with boiling water, because it depends on the location of the SS cross-links in IF and KAP structure,. The total number of SS cross-linking sites in the IF chain of an average molecular mass of 50000 is determined to be twenty-one moles. The twenty-one moles are divided into thirteen moles comprising intermolecular cross-linkages, which are subdivided to three moles between rod domains, eight moles between terminal domains and two moles between terminal and KAP domain, and eight moles comprising intramolecular cross-linkages, which are subdivided into four moles in the terminals and four moles at the interface region between the rod and terminal domains. It was also found that a KAP molecule with an assumed

molecular mass of 20000 involves seventeen moles of intramolecular SS bonds and 4–5 sites on the surface of the KAP molecule of hair keratin. These sites are linked to adjacent KAP molecules and form an aggregate of about six KAP molecules against the IF molecule. Finally, we proposed a network model for the IF–KAP structural unit in the hair and wool fiber cortex.

In chapter 3, the SS cross-linked structural change by permanent wave treatment for hair and the structure and mechanical property relationships are investigated using the network structural model proposed in chapter 2. Scission and reformation mechanisms of SS cross-links by the treatments with a reducing agent of thioglycolic acid (TGA) and an oxidizing agent of sodium bromate were demonstrated. It was found that cleavage of the SS cross-links between KAP molecules by preferential attack with TGA leads to the shape change from ellipsoidal form of globular aggregates to near spherical one, and these cross-links and shape are not recovered perfectly by subsequent oxidation. On the other hand, it was also found that nearly complete reformation of SS cross-links occurs between IF proteins through oxidation even when a large number of SS bonds break under strong reducing conditions. An important suggestion was obtained that the extension modulus of the hair fiber in water is highly dependent on the number of intermolecular SS cross-links between KAP molecules around the IF.

In chapter 4, recovery behavior of mechanical property during washing after reduction with TGA is studied. It is well-known that the reduction of SS bonds with thiol is equilibrium reaction. In a permanent waving system consisting of three step processes of reduction with TGA, washing with water and oxidation by sodium bromate, it was found that the extension modulus and breaking stress of the treated hair fibers in water were increased with increase of washing time. Analysis of SS cross-linked structure by applying a rubber elasticity theory showed that the integrity of the SS cross-linked structure of the IF is retained, while the intermolecular SS cross-links between KAP molecules cleaved by reduction are regenerated by reverse reaction of the equilibrium reactions occurring during washing. Hence, it is concluded that the mechanical properties for hair fibers were controlled by the intermolecular SS cross-links between the KAP molecules.

Contriburion of microstructure in keratin fibers to their extension behavior is quite complicated. In this study, the mechanical importance of the intermolecular cross-links between the KAP molecules has been clarified. In the end of chapter 3, we point out that as one of the functions of the keratin matrix these cross-links may be essential to resist IF swelling and maintain the IF stiffness. However, it seems not to be quite simple to model the mechanical behavior on the basis of the SS cross-link network structure, because SH/SS interchange reactions should be considered in extending the keratin fiber. It is important that the primary structural location of the lone cysteine residue will be clearly determined by genetic engineering method, and the development of these biological technique may play an important role for solving the contribution and mechanism of the SH/SS interchange reaction to extension, compression and bending of keratin fibers.

List of paper

Papers

1. K. Suzuta, S. Ogawa, Y. Takeda, K. Kaneyama and K. Arai, Intermolecular disulfide cross-linked structural change induced by permanent wave treatment of human hair with thioglycolic acid, *J. Cosmet. Sci.*, **63**, 177-196 (2012).
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Proceedings of International Conference

1. K. Suzuta, S. Ogawa, K. Fujii, K. Kaneyama, and K. Arai, Application of rubber elasticity theory to swollen hair for elucidating the disulfide cross-linked structure of cosmetically treated hair, *Proc. 24th Congress of International Federation of Societies of Cosmetic Chemists (IFSCC)*, Osaka, Japan, pp.352-353 (2006).
2. K. Suzuta, M. Yoshida, S. Ogawa, Y. Takeda, K. Kaneyama, and K. Arai, Effect of crystallinity on the modulus of permanent waved hair, *12th International Wool Research Conference (IWRC)*, Shanghai, China, vol.2, pp.649-652 (2010).

Acknowledgement

It is my pleasure to express my gratitude to all those who have directly or indirectly contributed to the creation of this thesis. First of all, I would like to thank my superviser Professor Kunihiro Hamada in Shinshu University, for giving me the opportunity to study and for supporting me with a lot of useful advice.

I would like to express my heartful respect to Dr. Kozo Arai, Director of KRA Wool Research Laboratory, for his rigorous instruction with professional knowledge to help me throughout the whole process of accomplishing this thesis.

I am particularly grateful to Professor Kyohei Joko in Sugiyama Jogakuen University, and Associate Professor Yuichi Hirata in Shinshu University for the discussion and helpful suggestions and comments. My deepest appreciation goes to Professor Toshihiro Fujii and Professor Limin Bao in Shinshu University for the insightful comments as referee members. I also would like to thank Mr. Hidekazu Komatsu in Gunma Industrial Technology Center for his successful contribution on the development of fitting method.

Helpful support by Milbon Co. Ltd. was essential to accomplish this study. I am deeply grateful to Mr. Katsumi Kaneyama, Senior Managing Director of Milbon Co. Ltd., and Mr. Yasufumi Takeda, Director of Milbon Co. Ltd., for giving me the opportunity to study and supporting me. I also sincerely thank Dr. Masato Yoshida, Chief Researcher of Milbon Co. Ltd., for technical collaboration on WAXS investigation and useful discussion.

Thanks go to all members in Central Research Institute of Milbon Co. Ltd. for their general advice and daily support for me. I also wish to thank all members of Hamada Laboratory in Shinshu University for their kind helpful experimental support.

Finally, I am grateful to my family for their understanding, encouragement and support.

September 2015

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