Journal of Colloid and Interface Science **240,** 30–39 (2001)
doi:10.1006/jcis.2001.7673, available online at http://www.idealibrary.com on IDEAL®

Stabilization of Solutions of Feather Keratins by Sodium Dodecyl Sulfate

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Received November 6, 2000; accepted April 30, 2001; published online June 21, 2001

Feather keratins were extracted from chicken feathers with aqueous solutions of urea and 2-mercaptoethanol. After filtration of the insoluble residue, a feather keratin solution was obtained. Removal of 2-mercaptoethanol and urea by dialysis resulted in aggregation of the keratin polypeptide chains and oxidation of the cysteine residues to afford a gel. The addition of sodium dodecyl sulfate (SDS) to the keratin solution prior to dialysis prevented extensive aggregation of the keratin chains. The effect of the addition of various quantities of SDS on the rate of aggregation of the polypeptide chains and the rate of oxidation of cysteine residues during dialysis was studied. With size exclusion chromatography, it was found that lower initial SDS/keratin ratios (0.125–0.5 g SDS/g keratin) resulted in larger SDS–keratin complexes. This indicates that more intermolecular cross-links had formed. Higher SDS/keratin ratios (1–2 g SDS/g keratin) resulted in small SDS–keratin complexes, comparable in size to the keratin monomer. High amounts of SDS seemed to prevent the oxidation reaction between different keratin chains, resulting in more intramolecular disulfide bond formation.

Key Words: **feather keratins; SDS–protein complexes; micelles; binding isotherm.**

INTRODUCTION

Currently, the interest for the development of products for environmentally sustainable applications from protein waste streams is growing (1). Research has focused to a large extent on the excellent film-forming capacity of proteins and the good gas barrier properties of these films. Possible applications are compostable packaging, agricultural films, or edible films and coatings (2–7). Feather keratins have received little attention in this field.

For the application of feather keratins in films and coatings a stable keratin solution is required. An important feature of keratins is the occurrence of a large amount of disulfide bonds when compared to other major structural proteins in vertebrates, such as collagen, elastin, and myofibrillar proteins. Because of this extensive disulfide cross-linking and a high amount of hydrophobic amino acids, keratins are insoluble in polar solvents like water, as well as in apolar solvents. Keratins can only be extracted from feathers if the disulfide and hydrogen bonds are broken. A mild extraction procedure, without significant peptide bond hydrolysis, involves the use of thiols, like 2-mercaptoethanol, in concentrated urea solutions at a moderately alkaline pH (8). When feathers were solubilized following this procedure a stable feather keratin solution was obtained (9). Removal of 2-mercaptoethanol and urea from this solution by dialysis against distilled water resulted in aggregation of the keratin polypeptide chains and reoxidation of the cysteine residues, yielding a white, opaque gel.

To increase the solubility of the extracted keratins in the absence of reducing and disruptive agents, many different approaches have been followed, most of which involve chemical modification of the cysteine residues of the keratin (9–14). A method that does not involve chemical modification is the addition of an ionic surfactant to the keratin solution. Yamauchi *et al.* investigated the extraction of wool keratins with an aqueous solution of urea, 2-mercaptoethanol, and sodium dodecyl sulfate (SDS) (15). They found that the surfactant, SDS, accelerated the extraction and increased the extraction yield. It also stabilized the aqueous protein solution after removal of urea by dialysis against water containing 2-mercaptoethanol (0.08 wt%). The surfactant forms a complex with the keratin and is removed by dialysis much slower than other low molecular mass compounds.

Knowledge of the binding behavior of surfactants to keratinous substrates, such as *stratum corneum*, wool, and hair, is of special interest in cosmetic and textile applications. It is difficult, however, to compare the interactions of surfactants with different keratin types, because of large differences in structural arrangement, molecular mass, tertiary and secondary structure, and the amount of cross-linking through disulfide bonds (16–18).

As far as the interactions of surfactants with feather keratins are concerned, only a few reports were found. Feather keratins could be extracted with SDS solutions, in the presence of a reducing agent (19). The binding of a mixture of anionic surfactants, sodium alkylbenzenesulphonates, to reduced feather

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0021-9797/01 $35.00

keratins was studied by Ward *et al.* (20). The resulting keratin solution contained an unknown amount of reoxidized cysteine (12) and was "polymerized" to some extent, as they estimated the molecular mass to be 34 to 40 kDa instead of 10 kDa for the keratin elementary chain. After treatment of the keratin solution with salts, such as sodium sulfate, a mixture useful for fiber spinning was obtained.

From these studies it is clear that keratin solutions can be stabilized by forming a complex with anionic surfactants. Solution properties differ however, depending on the type of keratin and anionic surfactant used. Here, we report on the chemical and physical characterization of feather keratin solutions stabilized with SDS. The effect of adding varying quantities of SDS to the extraction medium on the extraction yield was studied. In other experiments, SDS was added to the feather keratin solutions prior to dialysis after which protein aggregation as well as the degree of cysteine reoxidation during dialysis were investigated. The size of the SDS–keratin complexes was measured with size exclusion chromatography (SEC).

MATERIALS

White body feathers from broilers, 70 days old, were supplied by Hago Rijssen (the Netherlands). All chemicals were of analytical grade and were purchased from Merck (Darmstadt, Germany). Whatman 54 hardened cellulose filters were supplied by Fisher Scientific (Zoetermeer, The Netherlands). PD-10 desalting columns and Sepharose CL-6B were supplied by Pharmacia Biotech (Uppsala, Sweden). The molecular mass markers for size exclusion chromatography, thyroglobulin (bovine thyroid) 669 kDa, ferritin (horse spleen) 440 kDa, catalase (bovine liver) 232 kDa, and aldolase (rabbit muscle) 158 kDa, were purchased from Pharmacia Biotech. Carbonic anhydrase (bovine erythrocytes) 29 kDa was purchased from Sigma (St. Louis, MO, USA). Spectra/Por dialysis membranes (MWCO 6-8,000) were purchased from Spectrum Medical Industries (Laguna Hills, CA, USA). All spectrophotometric measurements were performed with a Uvikon 930 (Kontron Instruments, Milan, Italy).

METHODS

Pretreatment of the feathers. Freshly plucked wet chicken feathers were cleaned, cut, and degreased following the procedures described previously (9).

Solubilization of feather keratin. Per gram of pretreated feathers, 25 ml of a buffered solution containing 6 M urea, 3 mM EDTA, and 1.4 M of 2-mercaptoethanol (2-ME) were used. The buffers used (0.2 M) were KCl–NaOH (pH 10.0), $NaHCO_3$ (pH 9.0), and tris(hydroxymethyl)-aminomethane (Tris buffer) (pH 7.0). All buffers were adjusted to the appropriate pH with either 6 N HCl or 6 N NaOH. To one of these solutions, 1.4 g SDS was added per gram of feathers. To another, no SDS was added. The mixture was agitated at 40°C under a nitrogen atmosphere for at least 1 h. In each experiment, the insoluble material was removed by filtration using Whatman 54 hardened cellulose filters with a pore size of 20–25 μm. Filters were washed three times with water (250 ml), air dried, and weighed. The amount of keratin in the filtrate, the extraction yield, was expressed as a percentage of the total weight of feathers used. Based on the keratin content of feathers, the maximal extraction yield is approximately 90%. The amount of keratin in the filtrate was also determined using a modified Lowry procedure and bovine serum albumin as a standard (21, 22).

Preparation of a 3% (w/v) solution of feather keratins. Feathers (30 g) were solubilized under optimized conditions (750 ml aqueous solution, 8 M urea, 3 mM EDTA, 125 mM 2-mercaptoethanol, 200 mM Tris, pH 9.0) (9). After filtration a 3% (w/v) keratin solution was obtained.

Turbidity and cysteine oxidation as a function of dialysis time. The 3% (w/v) keratin solution was split into different parts of 100 ml and varying quantities of sodium dodecyl sulfate were added (0.125, 0.25, 0.5, 1, and 2 g SDS/g keratin). These parts were dialysed against 5 liters of distilled water. After 17, 23, 42, and 65 h, and 14 days samples were taken from the keratin solutions (10 ml), after which the dialysate was replaced with distilled water (5 liters). The turbidity of the keratin solutions at 540 nm was measured. Free thiol groups from unoxidized cysteine in the samples were carboxymethylated with an excess of iodoacetic acid (30 mM) in the presence of 8 M urea. The samples were dialyzed extensively and lyophilized. The amount of disulfide bonds plus any remaining free thiol groups in the lyophilized samples was measured by using a NTSB-assay (disodium 2-nitro-5-thiosulfobenzoate) (23, 24). Lyophilized, unmodified feather keratin was used as a blank. With a DTNB-assay (Ellman's reagent, 5,5'-dithiobis(2-nitrobenzoic acid)) it was found that less than 5% of the cysteine residues of lyophilized feather keratin samples were present as free thiol groups (9). Therefore, the amount of free thiol groups measured by the NTSB-assay was neglected in the calculation of the degree of cysteine modification, which could hence be calculated as

$$100 * \frac{(SS_{unmod} - SS_{mod})}{SS_{unmod}}, \qquad [1]$$

where SS_{unmod} and SS_{mod} are the amounts of disulfide bonds in lyophilized unmodified and modified feather keratin, respectively.

Estimation of the rate of SDS removal by dialysis and of the amount of bound SDS. In a separate experiment, the 3% (w/v) keratin solution, obtained by solubilizing feathers under optimized conditions, was split into different parts of 10 ml, and varying quantities of sodium dodecyl sulfate were added (0, 0.063, 0.125, 0.25, 0.5, 1, and 2 g SDS/g keratin). The same quantities of SDS were also added to 10-ml aliquots of the extraction buffer, containing no keratins (8 M urea, 3 mM EDTA, 125 mM 2-mercaptoethanol, 200 mM Tris, pH 9.0). Each part was dialyzed against 500 ml of distilled water. After 17, 23, 42,

and 65 h the conductivity of the dialysates was measured, after which the dialysates were replaced with distilled water (500 ml). After 65 h, the dialyzed keratin solutions were lyophilized.

To estimate the concentration of SDS in the dialysates using conductivity measurements, calibration curves were used, obtained by adding small aliquots (0.25 ml) of a concentrated aqueous SDS solution (60 mg/ml) to 100 ml of the dialysates from the solutions containing no SDS and measuring the increase in conductivity. The amount of SDS removed from the SDS solutions after a certain dialysis time could hence be calculated.

To estimate the amount of SDS still bound to the feather keratins after 65 h of dialysis, the nitrogen content of the lyophilized samples was measured by elemental analysis using an EA 1108 Elemental Analyser (Fisons Instruments, Rodano, Italy). The N content of a lyophilized keratin sample without SDS was used as a blank. The amount of protein was then calculated by dividing the N content of the sample by the N content of the blank. The difference was ascribed to the presence of SDS.

Size exclusion chromatography. To compare the hydrodynamic radius of SDS–feather keratin complexes, size exclusion chromatography (SEC) was performed with a column of Sepharose CL-6B (2.6×28.5 cm^2). The eluent contained 50 mM Tris and 0.02% sodium azide. The pH of the eluent was set at 8.5 with 6 N HCl. The flow rate of the eluent in the column was maintained at 20–25 ml/h with a P-1 peristaltic pump (Pharmacia, Biotech, Uppsala, Sweden). The column was kept at 25°C. The absorbance of the eluent was continuously measured at 280 nm and the void volume V_o of the column was determined by applying Blue Dextran 2,000. The total liquid volume of the column V_t was measured by applying a sample of oxidized dithiothreitol (10 mM). The column was calibrated with thyroglobulin 669 kDa, ferritin 440 kDa, catalase 232 kDa, aldolase 158 kDa, and carbonic anhydrase 29 kDa. Samples were injected using a Bio-Rad MV-6 sample loop injector valve that allows a constant sample volume (about 0.5 ml) to be applied. The distribution coefficient K_d of the solutes was calculated according to

$$K_d = \frac{V_e - V_o}{V_t - V_o}, \qquad [2]$$

where V_e is the elution volume of the solute, V_o the void volume, and V_t the total liquid volume of the column.

Feather keratin solutions (3% (w/v), 100 ml), which contained different amounts of SDS prior to dialysis (0.125, 0.25, 0.5, 1, and 2 g SDS/g keratin) were dialyzed against distilled water (5 liters). The dialysate (5 liters) was replaced with distilled water after 17 and 23 h. After 42 h of dialysis, samples were collected and applied on this column. Chromatography of the SDS–keratin complexes was performed in the presence of 3.4 mM SDS in a high ionic strength buffer (50 mM Tris, 0.02% NaN_3, pH 8.5). In this medium the SDS concentration is above the critical micelle concentration. The elution volume V_e was measured and the distribution coefficient K_d was calculated using Eq. [2].

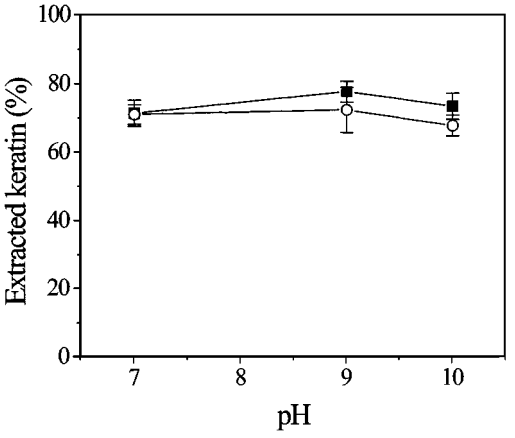

FIG. 1. Extraction yield of feather keratins, solubilized with an aqueous solution of 2-mercaptoethanol and urea under nitrogen at 40°C, as a function of pH, without (■) or with (□) the addition of 1.4 g SDS/g keratin.

RESULTS

Extraction yield with and without the addition of SDS. In Fig. 1 the extraction yield of feather keratins is shown as a function of pH, with or without the addition of 1.4 g SDS per gram of feathers. Reaction times longer than 1 h did not result in higher yields. At pH 9.0, about 77 ± 3% (w/w) and 72 ± 6% (w/w) of the feather keratins were extracted without or with the addition of SDS, respectively. Under the conditions used, SDS and pH appeared to have no significant influence on the extraction yield.

Turbidity and free thiol groups during dialysis. The effect of the addition of SDS to the keratin solution on the rate of aggregation of the polypeptide chains during dialysis was studied. Protein aggregation was measured qualitatively by following the turbidity change in time. In Fig. 2 it is shown that there was a

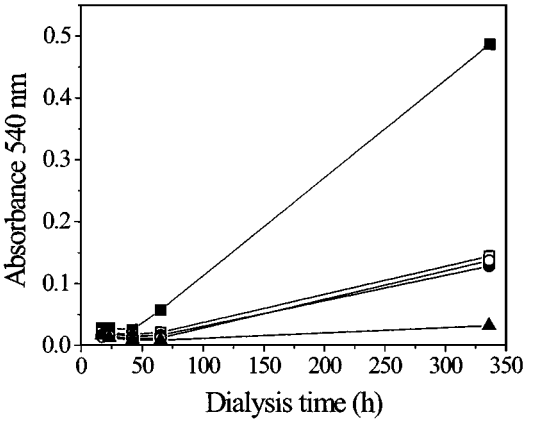

FIG. 2. Turbidity at 540 nm measured as a function of dialysis time for feather keratin solutions (100 ml, initially 3% w/v, 8 M urea, 3 mM EDTA, 125 mM 2-mercaptoethanol, 200 mM Tris, pH 9.0) with different amounts of SDS added prior to dialysis: ■, 0.125; □, 0.25; ●, 0.5; ○, 1; and ▲, 2 g SDS/g keratin. Dialysis was done against distilled water (5 liters) and the dialysate was replaced by distilled water after every measurement.

small decrease in turbidity during the first 42 h of dialysis. Prolonged dialysis resulted in a significant increase in turbidity. The aggregation occurred faster for the lower SDS/keratin ratios. The solution with 2 g SDS/g keratin added prior to dialysis showed no increase in turbidity, even after 2 weeks of dialysis.

The effect of the addition of SDS to the keratin solution on the rate of cysteine oxidation during dialysis was studied. The total amount of unoxidized cysteine residues per gram of feather keratins before dialysis in the presence of 2-mercaptoethanol is approximately 700 μmole (9). After addition of SDS, only 70–80% of the cysteine residues could be carboxymethylated prior to dialysis (Fig. 3a). During the first 42 h there was a large decrease in the amount of free thiol groups. The oxidation appeared to be faster when lower amounts of SDS were present. Figure 3b shows the percentage of unoxidized cysteine as a function of the amount of SDS prior to dialysis, for several dialysis times. It can be seen that after 23 and 42 h of dialysis

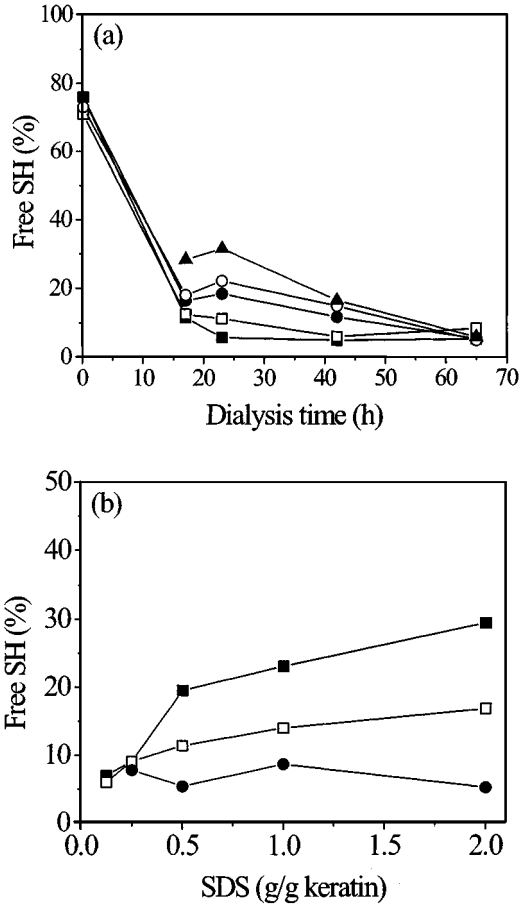

FIG. 3. Free SH groups of SDS–keratin complexes (a) as a function of dialysis time for different amounts of SDS added prior to dialysis and (b) as a function of the amount of SDS prior to dialysis for different dialysis times. Complexes were formed by adding different amounts of SDS to a 3% (w/v) feather keratin solution (8 M urea, 3 mM EDTA, 125 mM 2-mercaptoethanol, 200 mM Tris, pH 9.0). Dialysis was done against distilled water (5 liters) and the dialysate was (a) ■, 0.125; □, 0.25; ●, 0.5; ○, 1; and ▲, 2 g SDS/g keratin. (b) ■, 23; □, 42; and ●, 65 h. replaced by distilled water after every measurement.

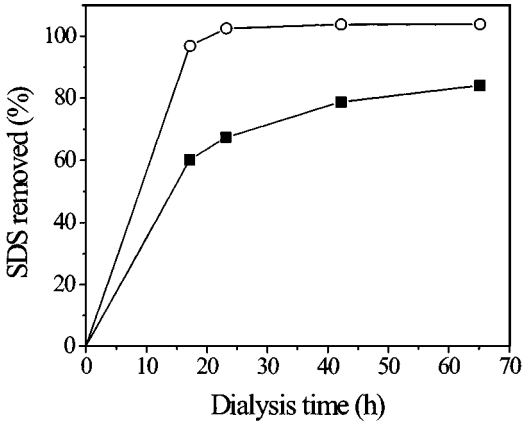

FIG. 4. Percentage of sodium dodecyl sulfate (SDS) removed by dialysis against 500 ml of distilled water from an aqueous SDS solution (10 ml, 15 mg/ml SDS, 8 M urea, 3 mM EDTA, 125 mM 2-mercaptoethanol, 200 mM Tris, pH 9.0) in the presence (■) or absence (□) of feather keratins (30 mg/ml). The dialysate was replaced with distilled water after each measuring point. The concentration of SDS in the dialysate was calculated using calibration curves.

the percentage of free thiol is higher for increasing amounts of SDS. The percentage of free, unoxidized cysteine after 42 h of dialysis time varied from 6% for a solution containing 0.125 g SDS/g keratin to 17% for a solution containing 2 g SDS/g keratin. After 65 h of dialysis, however, this effect becomes less pronounced: all molecules possess approximately the same percentage of free cysteine (5–9%).

Estimation of the amount of SDS bound to keratin. In a separate experiment, the amount of surfactant removed by dialysis against distilled water from aqueous SDS solutions, containing urea, Tris-buffer, and 2-mercaptoethanol, in the presence or absence of feather keratins, was measured by using conductivity. From Fig. 4 it can be seen that the presence of feather keratins has a considerable effect on the rate at which SDS is removed from the solutions. After 23 h of dialysis, all SDS was removed from the solution containing no keratins, while for the same amount of SDS in the presence of keratin, only 67 wt% of the amount of SDS added prior to dialysis was removed.

After 65 h the dialyzed keratin solutions were lyophilized. The amount of SDS that remained per gram of keratin after dialysis was determined by elemental analysis of lyophilized samples (Fig. 5a). For mass ratios of SDS to protein from 0.25 to 2 there is a linear relationship between the amount of SDS in the keratin solution prior to dialysis and after 65 h of dialysis. The percentage of SDS removed by dialysis is shown in Fig. 5b. About 80 wt% of the SDS is lost upon dialysis, using the conditions applied. For an SDS/keratin mass ratio of 0.063 and 0.125 a lower amount (60 wt%) is lost by dialysis. For the solutions, the number of SDS moles per mole of feather keratin, ν, was estimated from the molar mass of SDS and keratin and the total mass of the compound present. Using a molecular mass of 10,000 for the keratin, the number of moles of SDS per mole of keratin ranged from 1.6 for a mass ratio of 0.04 g SDS/g keratin to 10.8 for a mass ratio of 0.31 g SDS/g keratin.

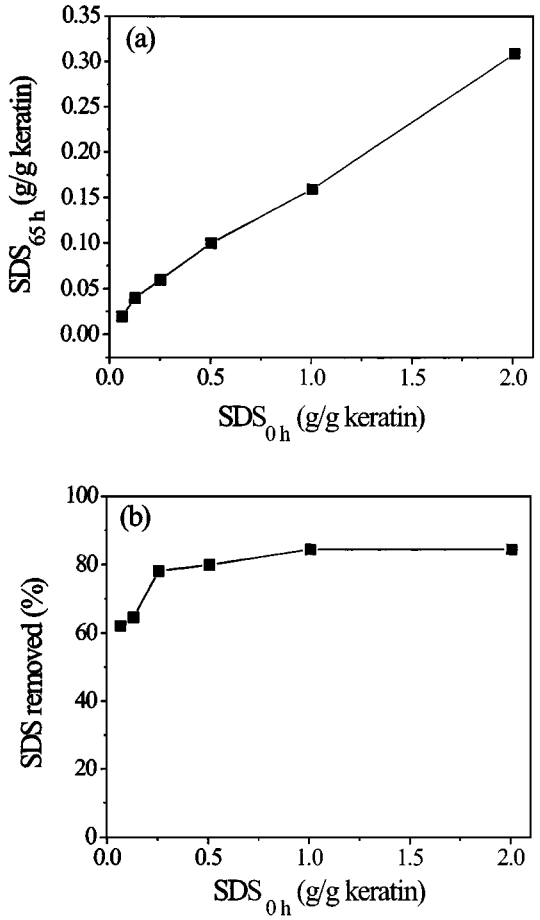

FIG. 5. (a) Amount of sodium dodecyl sulfate (SDS) remaining in feather keratin solutions after 65 h of dialysis and (b) the percentage of the total amount of SDS lost upon dialysis as a function of the amount of SDS prior to dialysis (0 h). Dialysis was done against distilled water (500 ml) and the dialysate was replaced by distilled water after 17, 23 and 42 h.

Size exclusion chromatography. With SEC the hydrodynamic radius of SDS–keratin complexes in samples collected during dialysis was determined. The column was calibrated with proteins of known molecular mass and Stokes radius, using Tris-buffer as eluent (50 mM Tris, 0.02% NaN_3, pH 8.5). Table 1 shows the molecular mass M_w, the intrinsic viscosity-based Stokes radius R_{vis}, the elution volume V_e, and the distribution coefficient K_d of the calibration proteins. In Fig. 6 the calibration curve, obtained by plotting $\log M_w$ against the distribution coefficient K_d, is shown, which yields a sigmoid curve that may be approximated in the middle range by $K_d = a - b \log M_w$ (25). A universal calibration curve was constructed by plotting the calibration proteins' intrinsic viscosity-based Stokes radius as a function of K_d, which gives an approximately linear relationship (26).

After calibration, a carboxymethylated sample of feather keratin ($M_w = 10.4$ kDa) was applied on the column under the same conditions as the molecular mass markers, but with 1 mM dithiothreitol added to the eluent to prevent any oxidation that might still occur in the column (50 mM Tris, 1 mM DTT, 0.02% NaN_3, pH 8.5) (9). The elution volume was found to be 130.1 ml, which corresponds to a calculated R_{vis} of 5.8 Å.

TABLE 1

Molecular Mass M_w, Intrinsic Viscosity-Based Stokes Radius R_{vis} (40), Elution Volume V_e and Distribution Coefficient K_d of the Calibration Proteins[a]

	M_w (kDa)	R_{vis} (Å)	V_e (ml)	K_d
Carbonic anhydrase	29	22	115.9	0.64
Aldolase	158	46	108.6	0.56
Catalase	232	52	96.5	0.44
Ferritin	440	64.2	88.8	0.36
Thyroglobulin	669	79	81.9	0.29

[a] As measured on a Sepharose CL-6B column (2.6 × 28.5 cm^2) using Tris buffer as eluent (50 mM Tris, 0.02% NaN_3, pH 8.5, flow rate 25 ml/h). The void volume of the column was 54 ml, the total liquid bed volume 151.3 ml.

When the SDS–keratin complexes were subjected to SEC with the eluent used for the column calibration, the keratin precipitated in the column. To prevent the SDS–keratin complexes from precipitating, 3.4 mM of SDS was added to the elution buffer after calibration (50 mM Tris, 3.4 mM SDS, 0.02% NaN_3, pH 8.5). This increased the stability of the feather keratin solutions, and no precipitates were formed.

The elution profile of a selection of samples collected after 42 h of dialysis against distilled water (5 liters) is presented in Fig. 7 (the dialysate was replaced with distilled water after 17 and 23 h). Most SDS–keratin complexes showed sharp peaks. A shoulder was observed in the elution profile of the sample that contained 0.125 g SDS per gram of keratin prior to dialysis. Table 2 shows the elution volume V_e, the distribution coefficient K_d, and the calculated Stokes radius R_{vis} of these samples. The Stokes radius was calculated, using the universal calibration

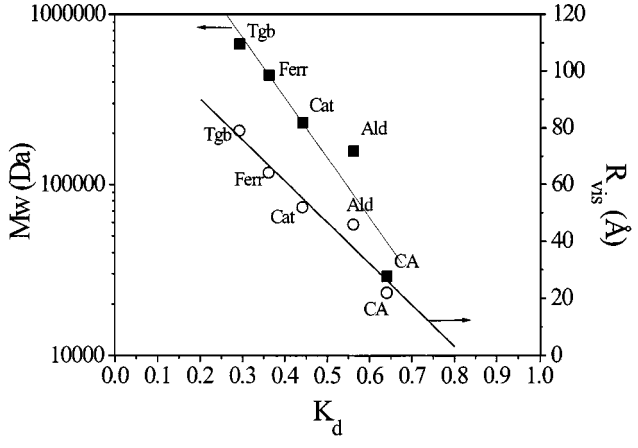

FIG. 6. Molecular mass M_w (■) and viscosity-based Stokes radius R_{vis} (○) versus partition coefficient K_d on a Sephadex CL-6B column (2.6 × 28.5 cm^2) for water-soluble globular proteins. Chromatography was performed in Tris buffer (50 mM, 0.02% NaN_3, pH 8.5). CA, carbonic anhydrase; Ald, aldolase; Cat, catalase; Ferr, ferritin; Tgb, thyroglobulin.

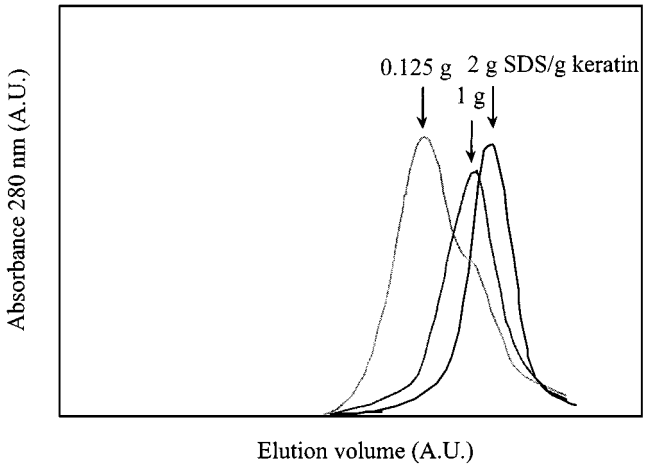

FIG. 7. Elution profiles of different SDS–keratin complexes in 3.4 mM SDS and 50 mM Tris buffer (0.02% NaN_3, pH 8.5) on a Sepharose CL-6B column (2.6×28.5 cm^2). Complexes were formed by adding different amounts of SDS (0.125, 1, and 2 g SDS/g keratin) to a 3% (w/v) feather keratin solution (8 M urea, 3 mM EDTA, 125 mM 2-mercaptoethanol, 200 mM Tris, pH 9.0) and dialyzing this solution for 42 h against 3 volumes of distilled water (5 liters).

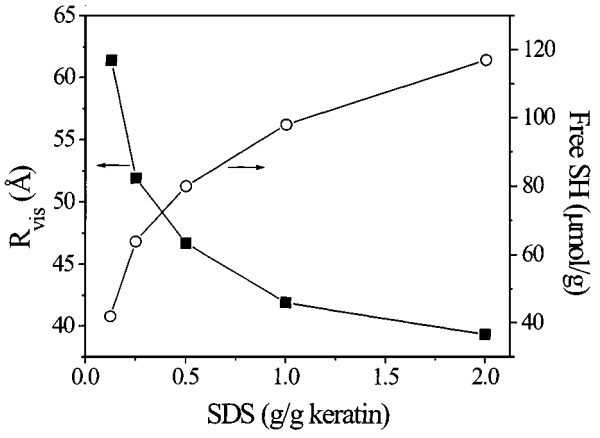

FIG. 8. Viscosity-based Stokes radius (R_{vis}, ■) and amount of free SH groups (○) of SDS–keratin complexes as a function of the amount of SDS per gram of protein added prior to dialysis, after dialyzing for 42 h against 3 volumes of distilled water (5 liters). Complexes were formed by adding different amounts of SDS to a 3% (w/v) feather keratin solution (8 M urea, 3 mM EDTA, 125 mM 2-mercaptoethanol, 200 mM Tris, pH 9.0). Dialysis was done against distilled water and the dialysate was replaced after 17 and 23 h.

curve of Fig. 6. A fully reduced sample was obtained by adding 10 mM of dithiothreitol (DTT) to the SDS–keratin complex containing 0.25 g SDS/g keratin before dialysis and applying the sample on the column equilibrated with a buffer containing 1 mM DTT to prevent oxidation in the column (50 mM Tris, 1 mM DTT, 0.1% SDS, 0.02% NaN_3, pH 8.5). The Stokes radius of this fully reduced SDS–keratin complex is about 40 Å. It is also apparent that the Stokes radii of the samples that contained 1 and 2 g of SDS per gram of keratin before dialysis are about the same as that for the reduced sample. These proteins all elute at approximately the same volume and are considered to be feather keratin monomers. In Fig. 8 the Stokes radius of the different SDS–keratin complexes and the amount of free SH groups after 42 h of dialysis is plotted against the amount of SDS added prior to dialysis. For increasing amounts of SDS, a decrease in the complex's Stokes radius and an increase in the amount of free SH can be seen.

TABLE 2

Elution Volume V_e, Distribution Coefficient K_d, and Calculated Stokes Radius R_{vis} of SDS–Keratin Complexes, with Different Amounts of SDS Added before Dialysis[a]

SDS (g/g keratin)		V_e (ml)	K_d	R_{vis} (Å)
0.125	peak	92.7	0.40	61.4
	shoulder	103	0.50	46.1
0.25	peak	99	0.46	51.9
0.5	peak	102.6	0.50	46.7
1	peak	105.8	0.53	41.9
2	peak	107.6	0.55	39.3
0.25 DTT[b]	peak	107.2	0.55	39.8

[a] As measured on a Sepharose CL-6B column (2.6×28.5 cm^2) using Tris buffer as eluent (50 mM Tris, 0.1% SDS, 0.02% NaN_3, pH 8.5, flow rate 25 ml/hr).

[b] Measured by adding 10 mM of dithiothreitol (DTT) to the SDS–keratin complex with 0.25 g SDS/g keratin added before dialysis and applying the sample on the column, equilibrated with the same buffer as used for the other SDS–keratin complexes, except for the additional presence of 1 mM DTT, to prevent oxidation in the column.

DISCUSSION

It is well known that proteins in aqueous solution bind ionic surfactants (27–29). Most knowledge of the binding behavior of surfactants to proteins comes from studies on water-soluble proteins. The binding of a charged surfactant, like SDS, to such a protein is believed to take place in two distinct stages. Initially, the surfactant ligands bind to specific sites on the protein surface. For SDS an electrostatic interaction of the sulfate anion with the positively charged side chains is expected, as well as a hydrophobic interaction between the dodecyl chain of the surfactant and nonpolar regions of the protein surface adjacent to cationic sites. For proteins in which all disulfide bonds are reduced by 2-mercaptoethanol, Reynolds and Tanford report that in this binding stage, independently of the type of protein, 0.4 g of SDS per gram of protein is bound or one surfactant molecule per seven amino acid residues (30).

In the second stage of binding by ionic surfactants, the cooperative binding, the protein unfolds to expose its hydrophobic interior and hence further potential binding sites. As the free surfactant concentration approaches the critical micelle concentration (CMC), a dramatic increase in the average number of surfactant ions bound per molecule of protein is usually seen over a relatively small range of free surfactant concentrations (31). Cooperative binding is weaker than specific binding. In this nonspecific cooperative binding region, the main driving force is the hydrophobic interaction between the surfactant tails

and the nonpolar residues of the unfolded protein. When the surfactant approaches the CMC, the structure of the protein–surfactant complex will closely resemble that of a surfactant micelle containing solubilized protein. Reynolds and Tanford report that after this stage, for proteins in which all disulfide bonds are reduced by 2-mercaptoethanol, 1.4 g of SDS is bound per gram of protein or one surfactant molecule per two amino acid residues (30).

Here, we present a scheme for the interactions that are involved during dialysis of SDS–keratin complexes. SDS was added to the feather keratin solution prior to dialysis, in the presence of 2-mercaptoethanol and 8 M urea. During dialysis, all low molecular mass compounds, like urea and 2-mercaptoethanol, are removed fast by diffusion through the dialysis membrane. The SDS micelles and the SDS associated with the protein are in equilibrium with free surfactant molecules inside the dialysis membrane. These free surfactant molecules will also be removed by diffusion. Evidence for the formation of SDS–keratin complexes is presented in Fig. 4, where the SDS is removed more slowly in the presence of keratin.

In the presence of 2-mercaptoethanol and 8 M urea, all potential binding sites are exposed to the surfactant. This implies that, most probably, no cooperative binding of the surfactant to the protein takes place due to denaturation. From amino acid analyses it was found that there are approximately 5 to 6 positively charged amino acid residues in feather keratin, all arginine (32). These form high-affinity binding sites for SDS ligands in the specific binding regime (33). According to the empirical relationship found by Reynolds and Tanford (30), a completely reduced feather keratin elementary chain consisting of 96 amino acids has approximately 48 potential binding sites for SDS. Approximately 5 of these sites form high-affinity binding sites for specific binding and 43 form low-affinity binding sites for nonspecific binding. A hypothetical binding isotherm for SDS and denatured, reduced feather keratin, in the absence of cooperative binding, is described by the equation (34)

$$\nu = \frac{n_1 K_1 [\mathrm{SDS}]_{\mathrm{free}}}{1 + K_1 [\mathrm{SDS}]_{\mathrm{free}}} + \frac{n_2 K_2 [\mathrm{SDS}]_{\mathrm{free}}}{1 + K_2 [\mathrm{SDS}]_{\mathrm{free}}}, \qquad [3]$$

where ν is the number of moles of SDS bound per mole of keratin, $[\mathrm{SDS}]_{\mathrm{free}}$ is the molar concentration of unbound SDS, and K_1 and K_2 are equilibrium binding constants for n_1 identical high-affinity binding sites and n_2 identical low-affinity binding sites, respectively. Such a binding isotherm, with the number of binding sites taken as above and hypothetical equilibrium binding constants, $K_1 = 10^5$ and $K_2 = 2 \times 10^3$, is presented in Fig. 9. Clearly, the high-affinity binding sites dominate the behavior at low SDS concentrations and the low-affinity sites are important at higher concentrations.

In SDS–keratin complex formation, three different cases can be discerned. The first case corresponds to region A in Fig. 9 and to Fig. 10a. All binding sites on the protein are occupied by an SDS ligand. Some SDS molecules interact through specific

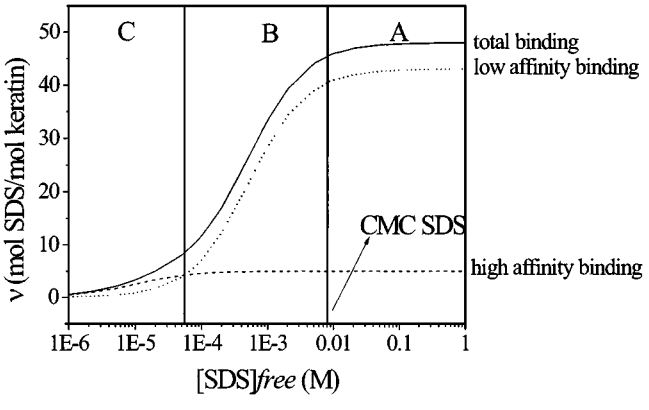

FIG. 9. Hypothetical binding isotherm for SDS to feather keratin for a molecule with high- and low-affinity binding sites, in the absence of cooperativeness, using Eq. [3], with $n_1 = 5$, $n_2 = 43$, $K_1 = 10^5$ and $K_2 = 2 \times 10^3$. The arrow indicates the critical micelle concentration (CMC) of SDS in distilled water. See text for the explanation of the three regions.

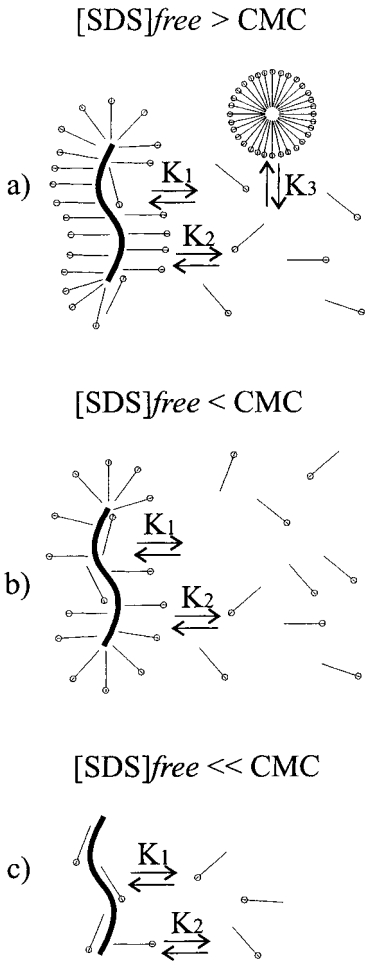

FIG. 10. Schematic representation for the interactions that are involved during dialysis of SDS–keratin complexes, for varying SDS/keratin molar ratios ν. In case (a) $\nu > 48$, in (b) $9 \leq \nu \leq 35$, and in (c) $\nu = 4$.

(ionic) interactions, but most of the SDS interacts nonspecifically (hydrophobically). SDS molecules bound to the protein are in equilibrium with free SDS. The equilibrium binding constants for specific and nonspecific binding are represented by K_1 and K_2 respectively in Fig. 10a. The concentration of free SDS, $[SDS]_{free}$, is above the CMC and micelles of SDS with a size of approximately 2 nm are formed. Micelles are in equilibrium with free SDS monomers. The equilibrium constant for micelle–monomer exchange is represented by K_3 in Fig. 10a. The equilibrium between micelles and monomers in surfactant solutions is established very rapidly. Relaxation times for micelle–monomer exchange of 10^2 to 10^6 s^{-1} were reported for SDS (35). Free SDS monomer is removed by dialysis. It can be seen in Fig. 9 that, as long as the SDS concentration is higher than the CMC, the binding ratio of SDS to keratin changes only slightly. This case applies to the solution where 2 g of SDS is added per gram of feather keratin prior to dialysis. Theoretically, not all of the SDS can be bound because there are only 48 binding sites and 69 moles of SDS were added per mole of keratin prior to dialysis (Table 3). This leaves about 63 mM SDS free in solution. The CMC of SDS was about 8.5 mM in distilled water (measured conductometrically) and decreases with increasing salt concentrations, so micelles will form in this solution.

The second case corresponds to region B in Fig. 9 and to Fig. 10b. The amount of bound SDS molecules is still high, but not all binding sites are occupied. The free SDS concentration is below the CMC. It can be seen from Fig. 9 that in region B the binding ratio of SDS to keratin changes drastically.

The third case corresponds to region C in Fig. 9 and to Fig. 10c. In this case, specific (ionic) interactions between SDS and the protein dominate. The concentration of free SDS is very low. It was observed that for 0.25 to 2 g SDS/g keratin about 80% (w/w) of the added SDS could be removed in equal dialysis times (Fig. 5). For the lowest amount of SDS (0.125 g/g keratin) only 60% (w/w) could be removed by dialysis in the same time interval. In this case almost all SDS is probably specifically bound to the keratin ($\nu = 4$), as in the situation described in Fig. 10c and the concentration of free SDS is very low compared to the other situations (region C in Fig. 9). This means that there is only a very small driving force for diffusion through the dialysis membrane, which leads to a low flux of SDS.

TABLE 3

Amount of Sodium Dodecyl Sulfate (SDS) Added Prior to Dialysis to a 3% (w/v) Keratin Solution[a] and Measured Amount of SDS Bound to Keratin after Dialysis[b]

$SDS_{added,\ t=0}$ (g/g keratin)	$SDS_{added,\ t=0}$ (mM)[c]	$SDS_{added,\ t=0}$ (mol/mol)	$SDS_{bound,\ t=end}$ (mol/mol)[d]
0.125	13.0	4	1.6
0.25	26.0	9	1.9
0.5	51.9	17	3.5
1	103.8	35	5.4
2	207.6	69	10.8

[a] 10 ml, containing 8 M urea, 3 mM EDTA, 125 mM 2-mercaptoethanol, 200 mM Tris, pH 9.0.

[b] Dialysis was done for 65 h against 500 ml of distilled water and the dialysate was replaced with distilled water after 17, 23, and 42 h of dialysis.

[c] Molecular mass of SDS and feather keratin were taken as 289 and 10,000 g/mol, respectively.

[d] As calculated from N analysis of the lyophilized samples.

After 65 h of dialysis against distilled water and at least three changes of dialysis water, most of the low molecular mass compounds will have disappeared, while some of the SDS molecules remain associated with the protein (Table 3, ν = 1.6 to 10.8, depending on the amount of SDS added). In this way the SDS molecules stabilize the feather keratin in solution and hinder aggregation of the protein. When more SDS is added prior to dialysis, the solution is stabilized for longer dialysis times, as after a certain dialysis time more SDS molecules remain associated with the protein (Fig. 2).

In the presence of 2-mercaptoethanol, all keratin cysteines are in the reduced state, as the thiol reduces every disulfide bond present. This means that the total amount of free SH per gram of keratin before dialysis is approximately 700 μmole (9). Only about 75% of this amount could be quantified by carboxymethylating the free cysteines and measuring the degree of modification after extensive dialysis and lyophilization by an NTSB-assay (Fig. 3a). This is possibly due to steric inhibition of the modification reaction by the bound SDS molecules. In the absence of SDS, up to 95% of the cysteines could be modified (9). When 2-mercaptoethanol is removed by dialysis, many keratin cysteines reoxidize rapidly with the formation of disulfide bonds. Again, this appears to occur earlier when smaller amounts of SDS are added prior to dialysis. SDS is thus not only able to hinder aggregation, but also oxidation. The oxidation is kinetically controlled and after 65 h of dialysis in three volumes of distilled water (5 liters), all SDS–keratin complexes are reoxidized to approximately the same degree (Fig. 3b).

With SEC, the size of the SDS–keratin complexes was compared after universal calibration of the column with proteins with a known viscosity-based Stokes radius (36). Le Maire *et al.* reported that for native water-soluble globular proteins and guanidinium hydrochloride- (GuHCl) and SDS-denatured peptides there is hardly any difference between the elution positions and consequently the K_d values of the different types of protein conformation below a molecular radius of 50 Å (37). It is only for larger SDS–protein complexes that the elution deviates markedly from those of the native and GuHCl-denatured proteins, being characterized by a relatively large R_{vis} for the same elution volume. Hence for most of the SDS–keratin complexes, i.e., those with a radius below 50 Å, a reliable estimate of the viscosity-based Stokes radius can be made by comparison to a universal calibration with proteins not complexed with SDS. For the larger SDS–keratin complexes, the calculated value will probably be an underestimate of the true value.

By performing chromatography of the SDS–keratin complexes in the presence of 3.4 mM SDS, some changes will occur

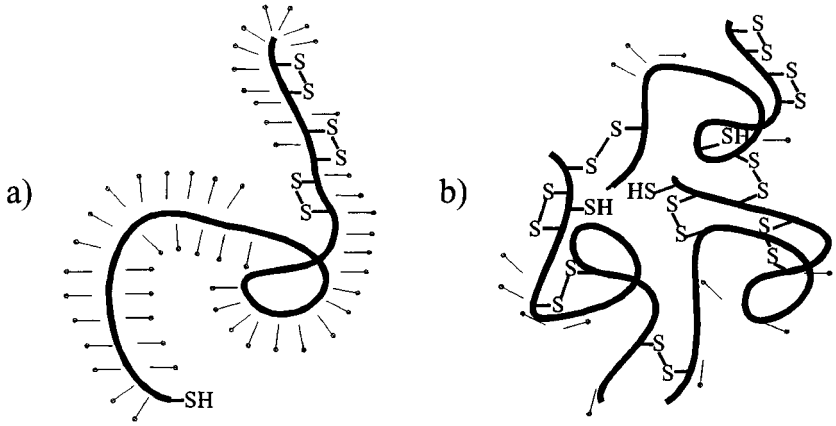

FIG. 11. Schematic representation for (a) SDS–keratin complexes with a high amount of SDS added prior to dialysis, which results in more intramolecular disulfide bond formation, and (b) complexes with a low amount of SDS added prior to dialysis, which results in more intermolecular cross-linking, here represented as three keratin chains.

in the amount of surfactant bound by the feather keratin. In the presence of 50 mM Tris buffer, the surfactant concentration in the eluent is higher than its CMC. It is expected that the SDS–keratin complexes will all bind approximately the same, maximal amount of SDS, independently of the amount of SDS added prior to dialysis in the experiment. Moreover, the SDS in the eluent prevents aggregation processes from taking place in the column. Consequently, differences in elution position are only reflecting differences in molecular size due to covalent bonding of molecules, not due to different amounts of bound SDS or aggregates formed during dialysis and/or chromatography.

The feather keratin monomer is expanded to a large extent by the addition of SDS. The Stokes radius of the monomer has increased from about 6 Å for the carboxymethylated species, under reducing conditions, to 40 Å for the SDS–keratin complex, under the same reducing conditions (0.25 DTT, Table 2). This effect of SDS association on complex size was also reported for SDS–bovine serum albumin complexes, where the hydrodynamic radius increased from 31 to 60 Å, while the SDS concentration increased (38).

The keratin cysteine groups were found to be highly reoxidized (Fig. 3a) after 42 h of dialysis. The difference in size between a complex of SDS with a totally reduced sample (0.25 DTT, Table 2) and a complex with a highly reoxidized sample, containing a high initial amount of SDS (1 or 2, Table 2) is very small. In the latter case the disulfide bonds probably do not compact the molecule to a large extent. Cysteines are located at the chain ends of the feather keratin. The B-4 chain of chicken feather barbs has 6 cysteines located at the N terminus, while there is only 1 cysteine residue at the C terminus (32). Other feather keratins have a similar primary sequence (39). For high initial amounts of SDS, intramolecular cross-linking of the cysteines located at the N terminus is expected as in Fig. 11a. In this way cross-linking only marginally affects the overall conformation of the chain in the presence of SDS and therefore the size measured chromatographically. Very small (Stokes radius < 45 Å) cross-linked keratin particles are formed, surrounded by a layer of SDS molecules. It is only after extensive dialysis that a sufficient amount of this SDS is removed to allow the complexes to aggregate further (Fig. 2). This is probably not accompanied by a further decrease in the amount of free SH.

For lower amounts of SDS added prior to dialysis, more intermolecular disulfide bond formation is allowed and the complexes become increasingly larger (Table 2, Fig. 11b). This can be explained by a decrease in sterical hindrance of the oxidation reaction by the associated SDS molecules. A compact, disulfide bonded network is formed, with a radius of 45 to 60 Å. This difference in inter- and intramolecular cross-linking is also apparent when the Stokes radius and the amount of free SH of the different SDS–keratin complexes after 42 h of dialysis are plotted against the amount of SDS added prior to dialysis (Fig. 8). This graph indicates that the larger complexes (Stokes radius 45–60 Å) are oxidation products of the feather keratin monomer. For increasing initial amounts of SDS, the degree of reoxidation is lower after a certain dialysis time. It was indicated before, however, that this effect is probably only kinetically controlled as longer dialysis times result in about the same degree of reoxidation for all the complexes, although no further increase in complex size was observed (Fig. 3).

CONCLUSION

When feathers are extracted in aqueous solutions of urea and 2-mercaptoethanol, the addition of SDS to the extraction medium has no significant influence on the extraction yield. Adding SDS prior to dialysis aids in the stabilization of feather keratin solutions during dialysis by complexation to the denatured feather keratin chain. Aggregation is prevented to a large extent. Aggregation of feather keratins and reoxidation of cysteine to disulfide bonds during dialysis appear to occur faster when lower amounts of SDS are added prior to dialysis. Higher

amounts of SDS result in smaller SDS–keratin complexes with more intramolecular disulfide bond formation.

ACKNOWLEDGMENT

This work was supported by Fleischmehlfabrik Brögbern (Brögbern, Germany).

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