Partially Carboxymethylated Feather Keratins. 1. Properties in Aqueous Systems

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Feather keratins were extracted from chicken feathers with an aqueous solution of urea and 2-mercaptoethanol. The keratin solution obtained was dialyzed to remove the reagents. Upon dialysis, extensive protein aggregation occurred. To obtain stable solutions or dispersions in water, cysteine residues were modified prior to dialysis with iodoacetamide, iodoacetic acid, or bromosuccinic acid, thereby blocking free thiol groups and introducing hydrophilic groups. For the development of biodegradable materials with good mechanical properties from these biopolymers, disulfide bonds between the keratin molecules are needed. Therefore, cysteine residues were only partially modified by using different reagent/cysteine molar ratios. The reaction rate constants of iodoacetate with glutathione and 2-mercaptoethanol were successfully used to predict the degree of modification of keratin cysteine. It was shown that, for carboxymethylated keratin, fewer aggregates were formed for higher degrees of cysteine modification, while more protein was present as oligomers. Aggregates and oligomers were stabilized through intermolecular disulfide bonds.

Keywords: *Feather; keratins; chemical modification; protein aggregation*

INTRODUCTION

Feathers are an important waste product of the poultry industry, causing an environmentally difficult disposal problem. It is estimated that in 1998 more than four million tons of feathers were available worldwide. They consist of 90% of the structural proteins, the keratins (Fraser et al., 1972).

Currently, the interest for the development of products for environmentally sustainable applications from protein waste streams is growing (Fritz et al., 1994). 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 (Brandenburg et al., 1993; Bietz and Lookhart, 1996; Gennadios and Weller, 1990; Gennadios and Weller, 1991; Gennadios et al., 1994; Kester and Fennema, 1986, Zhang et al., 1998). Feather keratins have received little attention in this field. An increased understanding of the relationships between the molecular structure of feather keratins and their functional properties is regarded as important for tuning the properties of materials containing these proteins. A distinctive feature of keratins, when compared to other major fibrous proteins, such as collagen, elastin, and myofibrillar proteins, is the occurrence of a large amount of cysteine residues, mainly present as the disulfide bonded dimeric amino acid cystine. Because of this extensive cross-linking and a high amount of hydrophobic residues, keratins are insoluble in polar solvents such as water, as well as in apolar solvents.

The avian β-keratins form a multigene family of about 20 proteins, which are coordinately synthesized during growth and differentiation in the embryonic feather (Presland et al., 1989a; 1989b). Only minor differences in amino acid composition of different feather parts have been reported (O'Donnell, 1973). Feather keratins have a molecular mass of approximately 10 kDa (Woodin, 1954). A high degree of homology exists between the amino acid sequences of feather proteins that have been determined so far. The distribution of residues is highly nonuniform, with the basic and acidic residues and the cysteine residues concentrated in the N- and C-terminal regions. The central portion is rich in hydrophobic residues and has a β-sheet conformation (Arai et al., 1983).

Several procedures are described in the literature to dissolve feather keratins. Solubilization methods with concomitant peptide bond scission include acid and alkali hydrolysis (Nagai and Nishikawa, 1970a), reduction of disulfide bonds with alkaline sodium sulfide solutions (Van Ingen et al., 1998), a combination of enzymatic and chemical treatment (Dalev, 1994; Virkki et al., 1995), and the use of ammonium copper hydroxide (Nagai and Nishikawa, 1970c; 1970b). Procedures without significant peptide bond scission in which only disulfide bonds are split include sulfitolysis or oxidation of disulfide bonds with performic acid (Woodin, 1954; 1956; Harrap and Woods, 1964a; 1964b).

Another mild procedure involves the use of thiols, like 2-mercaptoethanol, to reduce the disulfide bonds in concentrated urea solutions at a moderately alkaline pH (Jones and Mecham, 1943). When feathers are solubilized, following this procedure, a stable feather keratin solution is obtained. Removal of 2-mercaptoethanol and urea from this solution by dialysis results in aggregation of the keratin polypeptide chains and reoxidation of the cysteine residues to yield a white, opaque gel. For the

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development of biodegradable materials from feather keratins, like films for compostable packaging or paper coatings, water-soluble derivatives are needed (Anker et al., 1999; Brandenburg et al., 1993; Fairley et al., 1996; Gennadios et al., 1994; Kester and Fennema, 1986). Chemical modification of proteins has been used frequently to improve their functional properties (Feeney et al., 1982; Hwang and Damodaran, 1996; Dua et al., 1996; Means and Feeney, 1971; Ryan, 1977). To increase the solubility of extracted keratins in aqueous solvents many different approaches have been followed, most of which involved complete chemical modification of the cysteine residues of the keratin. Oxidation of cysteine into cysteic acid residues with performic acid or conversion of cysteine into *S*-sulfocysteine by sulfitolysis resulted into a water-soluble keratin derivative (Woodin, 1954; 1956; Harrap and Woods, 1964a; 1964b). Modification of cysteine with iodoacetic acid after cystine reduction has been generally used to block all the cysteine thiol groups present (Harrap and Woods, 1964a; 1964b).

Films prepared from solutions of completely modified feather keratins have poor mechanical properties because of the relatively low molecular mass of the keratin elementary chain and the absence of cross-links between the polypeptide chains. Partial alkylation of the cysteine residues, leaving the remaining thiol groups free to reoxidize to disulfide bonds, may well be used in the preparation of stable keratin dispersions. Films prepared from these dispersions will probably have improved mechanical properties.

The objective of the present study was to obtain stable aqueous dispersions of feather keratins which can be used for film casting. The influence of pH, time, and concentration of urea and 2-mercaptoethanol on the extraction yield of keratins from whole feathers was investigated. Partial modification of cysteine in feather keratins was performed with iodoacetamide, iodoacetic acid, or bromosuccinic acid, in concentrated aqueous urea solutions, in the presence of 2-mercaptoethanol. The influence of the reagent/cysteine ratio on the degree of cysteine modification, the solubility, and the particle size was investigated.

MATERIALS AND METHODS

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), except for bromosuccinic acid, which was purchased from Fluka (Buchs, Switzerland), and 5,5′-dithiobis(2-nitrobenzoic acid), which was obtained from Sigma (St. Louis, MO). Sepharose CL-6B was 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. Spectra/Por dialysis membranes (MWCO 6−8,000) were purchased from Spectrum Medical Industries (Laguna Hills, USA). All spectrophotometric measurements were performed on a Uvikon 930 (Kontron Instruments, Milan, Italy). The centrifuge used was a Biofuge13 (radius 55 mm) from Heraeus instruments (Hanau, Germany).

Pretreatment of the Feathers. Freshly plucked wet chicken feathers were cleaned following a procedure based on a standard test method for wool content of raw wool (ASTM D 584). Wet feathers were dried in a tumble drier and cut in a cutting mill (Fritsch Pulverizette 15) to small filaments with a length of 10 μm to 1 mm. Of this material, 30 g were Soxhlet extracted for approximately 12 h with petroleum ether (boiling range 40−60 °C) to remove grease. The petroleum ether was evaporated and the dry feathers were stored at room temperature in open containers.

Solubilization of Feather Keratins with 2-Mercaptoethanol. As a Function of Time and pH. To evaluate the solubilization as a function of time and pH, buffered solutions (25 mL) containing 6 M urea, 3 mM EDTA and 1.4 M of 2-mercaptoethanol were used per gram of pretreated feathers. The (0.2 M) buffers used were KCl−NaOH (pH 10.0), $NaHCO_3$ (pH 9.0), tris(hydroxymethyl)-aminomethane (Tris-buffer) (pH 7.0) and citric acid (pH 5.0 and pH 3.0). All buffers were adjusted to the appropriate pH with either 6N HCl or 6N NaOH. The mixture was agitated at 40 °C under a nitrogen atmosphere for 5, 10, 30, 60, and 120 min.

As a Function of pH and Urea Concentration. As a furher evaluation of keratin solubilization, buffered solutions (25 mL) containing different concentrations of urea (8 M, 6 M, 5 M, 3 M, 2 M and no urea), 3 mM EDTA, and 1.4 M 2-mercaptoethanol were used per gram of pretreated feathers. The buffers used were 0.2 M KCl−NaOH (pH 11.0), $NaHCO_3$ (pH 9.0), Tris (pH 7.0), and citric acid (pH 5.0 and 3.0). All buffers were adjusted to the appropriate pH with either 6 N HCl or 6 N NaOH. The mixture was agitated at 40 °C under a nitrogen atmosphere for 2 h.

As a Function of Time and 2-Mercaptoethanol Concentration. A final evaluation of keratin solubilization concentrated on solutions of Tris-buffer (25 mL, 0.2 M, pH 8.5) containing 6 M urea, 3 mM EDTA, and different concentrations of 2-mercaptoethanol (1.5 M, 750 mM, 500 mM, 250 mM, 125 mM, and 50 mM), were used per gram of pretreated feathers. The mixture was agitated at 40 °C under a nitrogen atmosphere for 5, 15, 30, and 60 min.

Following these reactions, 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 3 times with water, air-dried, and weighed. The amount of protein in the filtrate was expressed as a percentage of the total weight of feathers used. The amount of protein in the filtrate was also measured by using a modified Lowry procedure (Lowry et al., 1951; Peterson, 1977).

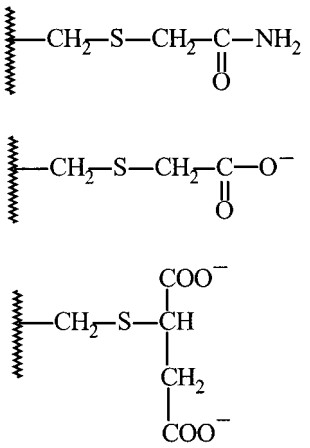
The filtrate was transferred to dialysis tubing and extensively dialyzed against distilled water. The white product obtained after lyophilization, referred to as unmodified feather keratins, was stored at 4 °C in a desiccator over P_2O_5.

Preparation of a Solution of Feather Keratins. Feathers (20 g) were solubilized under optimized conditions (500 mL aqueous solution, 8 M urea, 3 mM EDTA, 125 mM 2-mercaptoethanol, 200 mM Tris, pH 9.0). After filtration a 3% (w/v) keratin solution was obtained. This solution was divided in parts of 25 mL, containing 750 mg of keratins (525 μmol of cysteine residues, based on 7 mol of cysteine per mole of feather keratin, with a molecular mass of 10 kDa).

Chemical Modification of Cysteine Residues. This method was based on a procedure described by Crestfield et al. (Crestfield et al., 1963). Different amounts of monoiodoacetamide (I-AAm), monoiodoacetic acid (I-AA), or monobromosuccinic acid (Br-SA) were added to 25 mL of a 3% (w/v) keratin solution and the resulting mixture was stirred for 30 min at room temperature. Different reagent/cysteine molar ratios were used (0.25/1, 0.5/1, 0.75/1, 1/1, 1.25/1, 1.5/1, 1.75/1, 2/1, 5/1, 10/1) to give *S*-(carboxamidomethyl)keratin, *S*-(carboxymethyl)keratin, or *S*-(succinyl)keratin, respectively (Scheme 1).

The reaction was performed in the presence of a 4-fold excess of 2-mercaptoethanol with respect to the amount of cysteine, which also reacts with the modifying reagents. Iodine or bromine anions were released in solution during reaction and the reaction had to be performed in the dark, to prevent the formation of iodine or bromine. At high ratios of I-AA or Br-SA to cysteine, the pH dropped significantly and was adjusted again to 9.0 with 6 N NaOH after 15 min of reaction time. After reaction, the mixture was dialyzed extensively against distilled water. The dialysate (30 L) was replaced after 16 and

Scheme 1. *S*-(Carboxamidomethyl)keratin (1), *S*-(Carboxymethyl)keratinb (2) and *S*-(Succinyl)keratin (3)



24 h, and dialysis was stopped after 40 h. The turbidity of the resulting dispersions was measured at 540 nm. Subsequently, the keratin derivatives were lyophilized.

Free −SH after Dialysis. The amount of free thiol groups of cysteine residues from (partially) carboxymethylated feather keratin after dialysis was measured by using a DTNB-assay (Ellman's reagent or 5,5′-dithiobis(2-nitrobenzoic acid)). To 100 μL of an aqueous feather keratin dispersion (~2% w/v), 2.4 mL of a solution containing 8 M urea, 50 mM Tris, 3 mM EDTA, and 10 mM DTNB was added. The absorbance was continuously measured at 412 nm. A plateau value was reached after approximately 10 min. A molar extinction coefficient of 13 600 M^{-1} cm^{-1} was used to calculate the amount of free thiol groups.

Degree of Modification. Free thiol groups from cysteine residues in partially modified and unmodified feather keratins were fully reoxidized to disulfide bonds by lyophilization, as measured with a DTNB-assay (data not shown). The degree of modification of the partially modified keratins could hence be calculated by determining the amount of disulfide bonds formed after lyophilization using an NTSB-assay (disodium 2-nitro-5-thiosulfobenzoate) (Thannhauser et al., 1984; 1987):

$$\text{degree of modification} = 100^* \frac{(SS_{\text{un mod}} - SS_{\text{mod}})}{SS_{\text{un mod}}} \quad (1)$$

where $SS_{\text{un mod}}$ and SS_{mod} are the amounts of disulfide bonds in lyophilized unmodified and modified feather keratin, respectively. For unmodified keratin, $SS_{\text{un mod}}$ is approximately 350 μmol per gram of lyophilized material.

Aggregated Protein. After dialysis of partially modified *S*-(carboxymethyl)- and *S*-(carboxamidomethyl)keratin, samples were taken from the dialyzed solution. Aggregated feather keratin was pelleted in a centrifuge (40 min at 13 000 rpm). The amount of soluble keratin derivative was determined by measuring the protein concentration in the supernatant, using a Biuret-assay (Itzhaki and Gill, 1964). The percentage of aggregated protein was calculated from the total amount of protein and the amount of soluble protein.

Size Exclusion Chromatography (SEC). Size exclusion chromatography (SEC) was performed with a column of Sepharose CL-6B (2.6 × 28.5 cm). 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 by 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

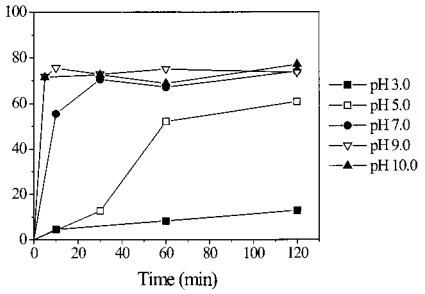


Figure 1. Extraction of keratins from feathers as a function of time and pH in aqueous solution containing 6 M urea and 1.4 M 2-mercaptoethanol at 40 °C. Feathers (4g) are contacted with 100 mL of solution.

$$K_d = \frac{V_e - V_o}{V_t - V_o} \quad (2)$$

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.

Samples from dispersions of partially modified *S*-(carboxymethyl)keratin in water were filtered through a 0.45 μm filter and applied on the column immediately after dialysis. The elution volume V_e was measured and the distribution coefficient K_d was calculated by using eq 2.

RESULTS AND DISCUSSION

Keratins, as present in feathers, are water-insoluble proteins because of a high disulfide bond content and a high amount of hydrophobic amino acids. Extraction of keratins from feathers without peptide bond scission is only possible by breaking the disulfide bonds. A mild procedure involves the use of thiols to reduce the disulfide bonds under moderately alkaline conditions in the presence of urea to break the hydrogen bonds (Jones and Mecham, 1943). To determine the keratin extraction yield, the protein concentration in the filtrate was measured by using a modified Lowry assay. Weight analysis of the insoluble residue gave similar results, indicating that only keratins are extracted.

The average sulfur content of chicken feathers that have been used as a starting material was 2.3% (w/w), as determined with elemental analysis. This value comprises the sulfur of cystine disulfide bonds (−SS−), cysteine free thiol groups (−SH), and methionine. The amount of free thiol groups present in feathers is reported to be less than 5% (w/w) of the total cysteine (Arai et al., 1993). The low content of methionine in feathers has also been reported (Akahane et al., 1977). It is assumed that the 2.3% (w/w) sulfur measured is mainly from disulfide linkages and therefore 1 g of feathers contains approximately 360 μmol of disulfide groups. When all the disulfide bonds have been broken 720 μmol of cysteine residues are present per gram of feathers.

To reduce the disulfide bonds, a 100-fold molar excess of 2-mercaptoethanol with respect to the disulfide bonds was used. The rate of dissolution of keratins from feathers in solutions with different pH is presented in Figure 1. In neutral or alkaline solutions the feathers were solubilized to a constant, high level (about 75%, based on the starting weight of the feathers), within 5−10 min. The remaining material probably consisted

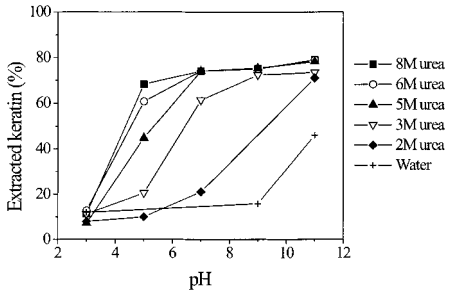


Figure 2. Extraction of keratins from feathers as a function of pH and urea concentration in aqueous solutions containing 1.4 M 2-mercaptoethanol at 40 °C. Feathers (4 g) are contacted with 100 mL of solution for 2 h.

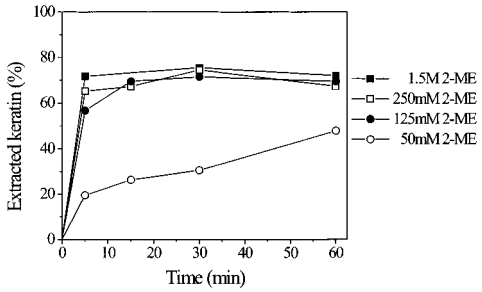


Figure 3. Extraction of keratins from feathers as a function of time and 2-mercaptoethanol (2-ME) concentration in aqueous solutions (pH 8.5) containing 6 M urea at 40 °C. Feathers (4 g) are contacted with 100 mL of solution.

of cellular envelopes and ϵ-*N*-(γ-glutamyl)lysyl cross-linked material (Rice et al., 1994). Under acidic conditions the rate of dissolution was much lower showing that the attack of the 2-mercaptoethanol thiolate anion, present at high pH values, initiates the reduction of disulfide bonds (Torchinsky, 1981). The pK_a of 2-mercaptoethanol is about 9.5 and therefore at low pH values virtually no 2-mercaptoethanol is in the ionized form, while at pH 9.5, 50% of the thiol groups is in the ionized, reactive form. In Figure 2, the amount of extracted material is presented as a function of pH for different urea concentrations. Minimal urea concentrations of 5 M and pH values higher than 7.0 appeared to be necessary to solubilize the feather keratins to a maximum value of 75% within 2 h reaction time. When less than 5 M urea was used, the extraction yield could only be increased by applying a higher solution pH. For buffered solutions containing no urea, there was an increase in solubility of the feathers above pH 9.0. The diffusion rate of 2-mercaptoethanol into the feather filaments depends on the urea concentration and the pH (Leon, 1975). Urea increases the accessibility of the disulfide bonds by breaking up the hydrogen-bonded structures and thus swelling the feather filaments. When less urea is present, a higher solution pH is necessary to establish equal extraction yields. At high pH, the reduction rate of disulfide bonds by 2-mercaptoethanol is increased, due to the higher concentration of the reactive thiolate anion. In addition, at high pH, disulfide bonds are hydrolyzed by hydroxide ions (Torchinsky, 1981). This results in the formation of cysteine thiolate anions, which increase the swelling of the feather filaments and thus the diffusion rate of 2-mercaptoethanol into the filaments. However, degradation of the protein through peptide bond scission and deamidation of asparagine and glutamine residues cannot be excluded at pH 10.0 or higher.

The rate of dissolution of feather keratins at various 2-mercaptoethanol concentrations was determined (Figure 3). At a pH of 8.5 and a urea concentration of 6 M, maximum extractability was reached after 15−30 min at concentrations of 2-mercaptoethanol of 125 mM or higher, corresponding to a 10-fold excess with respect to the number of disulfide bonds. Lower 2-mercaptoethanol concentrations required reaction times over 1 h to reach the same solubilization yield.

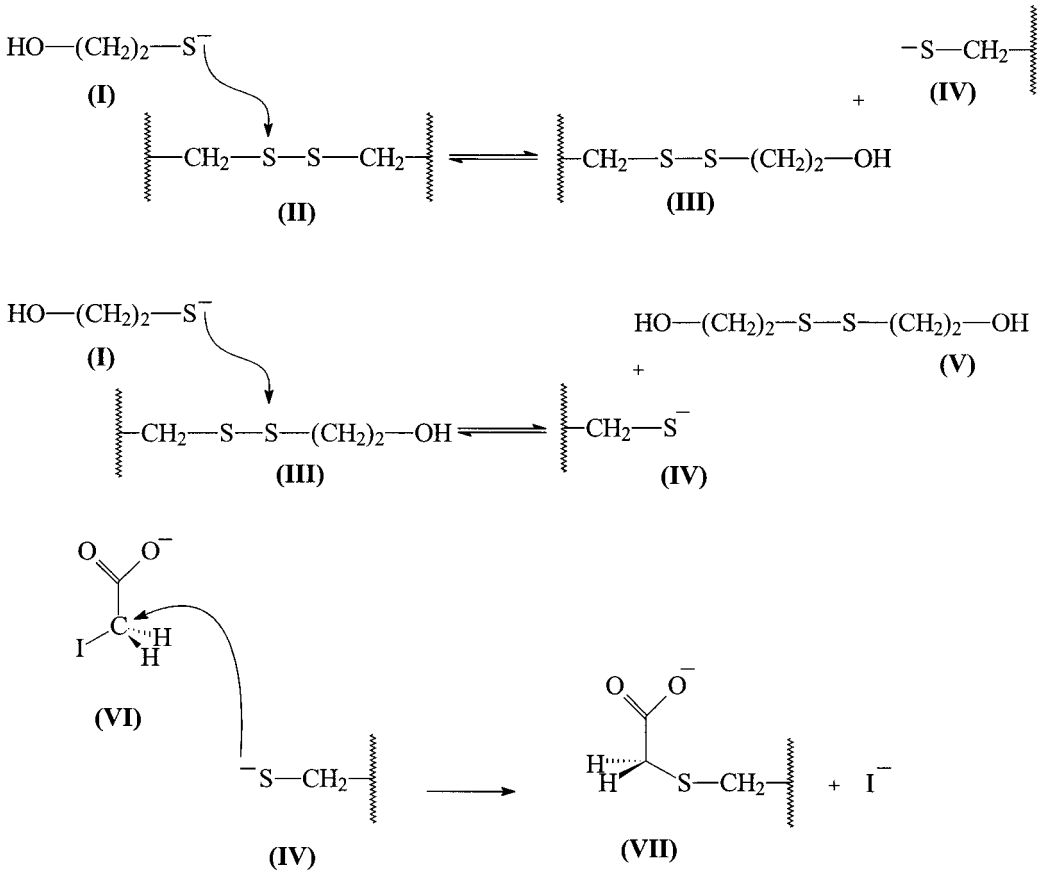
Optimal conditions for extracting up to 75% of the keratins from feathers thus appeared to be a reaction time of 30 min at 40 °C, a pH of 7.0−9.0, a urea concentration of 5 M or higher, and a 10-fold molar excess of 2-mercaptoethanol with respect to the number of disulfide bonds present.

Scheme 2 represents the reactions that occur during the keratin extraction. Cystine **(II)** reacts with the 2-mercaptoethanol thiolate anion **(I)** to yield a cysteinyl anion **(IV)** and a mixed disulfide intermediate **(III)** by nucleophilic substitution. The mixed disulfide intermediate subsequently reacts with another 2-mercaptoethanol thiolate anion, with the formation of 2,2′-dithiobis(ethanol) **(V)**. Reaction rates are generally low and therefore a 10-fold excess of 2-mercaptoethanol with respect to the amount of cysteine was used.

Cysteine residues in feather keratin readily oxidize to disulfides in the absence of reducing agents. A gel is rapidly formed as a result of cross-linking of the polyfunctional keratin chains. To avoid the oxidation of the cysteine thiol groups, these may be blocked by alkylation. When the alkylation of feather keratin is performed with compounds containing a hydrophilic group, water-soluble derivatives can be obtained. Moreover, chemical modification of cysteine thiol groups may be a well controlled methodology because no other nucleophilic groups such as amines (lysine, histidine) are present in the feather keratin polypeptide chain. Carboxymethylation has been applied to prepare keratin solutions from feathers and wool (Harrap and Woods, 1964a; 1964b; MacLaren and Sweetman, 1966; MacLaren et al., 1968). Iodoacetic acid **(VI)** or chloroacetic acid have been used for this modification to give *S*-(carboxymethyl)keratin **(VII)**. An excess of these reagents is generally applied to modify all cysteine thiol groups. In a similar way, other reagents containing hydrophilic groups can be used for the modification of cysteine thiol groups. The reagents iodoacetamide (I-AAm), iodoacetic acid (I-AA), and bromosuccinic acid (Br-SA) were selected for studying the effects of nonionizable groups and various numbers of ionizable groups on the properties of keratin. To the feather keratin solutions, containing urea and 2-mercaptoethanol as well as 2,2′-dithiobis(ethanol), I-AAm, I-AA, or Br-SA was added to give *S*-(carboxamidomethyl)keratin, *S*-(carboxymethyl)keratin or *S*-(succinyl)keratin, respectively.

First, solutions of feather keratin were treated with a 10-fold excess of I-AAm, I-AA, or Br-SA to establish full conversion of the cysteine thiol groups. After dialysis and lyophilization, all keratin derivatives were suspended in water at neutral pH. As a result of the introduction of amide and carboxylic acid groups, a different isoelectric point (pI) was expected for different keratin derivatives. Isoelectric focusing was used to determine the pI of these derivatives (data not shown). It was shown earlier that there are more than 20 different feather keratins, which have approximately the same molecular mass, that can be fractionated electrophoretically and chromatographically (Presland

Scheme 2. Reduction of Cystine (II) with 2-mercaptoethanol (I) and Alkylation of Cysteine with Iodoacetate (VI)



et al., 1989a; 1989b; Akahane et al., 1977; Murayama et al., 1977; Murozono et al., 1977). This heterogeneity of the protein mixture was also apparent in this experiment, where the modified proteins displayed different pI values, within a relatively broad pH range. However, the pI of the protein shifted to more acidic values when acetamide, acetate, or succinyl groups were introduced, respectively. The pI was in the range of 3.5−4.70, 3.2−4.75, and 2.9−4.8 for keratin modified with I-AAm, I-AA, and Br-SA, respectively. This was also the pH range in which these proteins precipitated in water.

Films formed by casting solutions of fully carboxymethylated feather keratin derivatives were water-soluble and had little mechanical strength. This is due to the relatively low molecular mass of the feather keratin monomer (10 kDa) and the absence of disulfide bonds in the films. Partial modification of the cysteine residues will leave the remaining thiol groups free to reoxidize and to form intermolecular disulfide cross-links during film formation. These films are expected to have better mechanical properties and become unsoluble in water.

In Figure 4 the degree of cysteine modification of feather keratin is shown as a function of different reagent/cysteine ratios. The degree of cysteine modification could be measured accurately by using an NTSB-assay. Up to reagent/cysteine ratios of about 2, there was a sharp increase in the degree of cysteine modification to 80% for I-AA and I-AAm and a more gradual increase to 40% cysteine modification for Br-SA. The maximum degree of cysteine modification was 95% for I-AA and I-AAm, using a reagent/cysteine ratio of 5 and 80% for Br-SA, using a reagent/cysteine ratio of 10. It appeared that the relative reactivity for feather keratin cysteine residues toward modification with I-AA, I-AAm, and Br-SA varied approximately as 4:2:1 for reagent/cysteine ratios up to 1. Most importantly, the method shows that it was in all cases possible to control the degree of cysteine modification.

In the reaction mixture an excess of 2-mercaptoethanol is present, which also reacts with the alkylating reagents under the reaction conditions employed. The reaction rate of cysteine and 2-mercaptoethanol is highly dependent on the pH. The pK_a of the 2-mercaptoethanol thiol group is 9.5, while the pK_a of thiol from protein cysteine is generally around 8.5 (Lundblad, 1991). Thus, at pH 9.0 and for equal concentrations, the concentration of cysteine thiolate anions will be an order of magnitude higher than that of 2-mercaptoethanol thiolate anions. The apparent rate constants, k_{app}, at

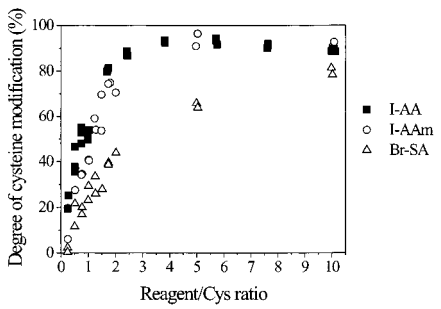


Figure 4. Degree of feather keratin cysteine modification as a function of reagent/cysteine molar ratios for iodoacetic acid (I-AA), iodoacetamide (I-AAm), and bromosuccinic acid (Br-SA). Reagents were added to 25 mL of a feather keratin solution (3% (w/v), 8 M urea, 3 mM EDTA, 125 mM 2-mercaptoethanol, 200 mM Tris, pH 9.0) and stirred for 30 min at room temperature.

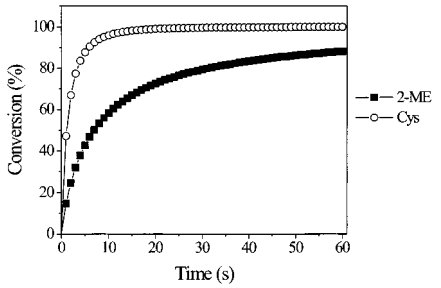


Figure 5. Conversion of 2-mercaptoethanol (2-ME) and peptide cysteine (Cys) as a function of time during modification with iodoacetate (reagent/Cys ratio = 5) at pH 9.0, by numerically solving equation (**5**), using k_{app} values of 1.4 and 4.5 M^{-1} s^{-1}, for 2-ME and Cys, respectively.

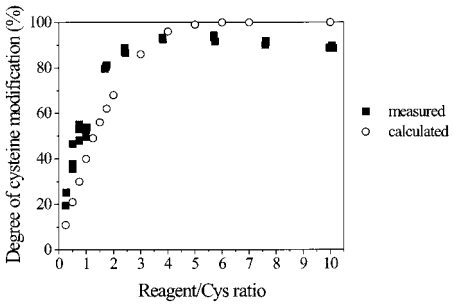


Figure 6. Calculated and measured degree of cysteine modification for the reaction of iodoacetate with keratin cysteine in the presence of an excess of 2-mercaptoethanol. For the measurements, iodoacetate was added to 25 mL of a feather keratin solution (3% (w/v), 8M urea, 3 mM EDTA, 125 mM 2-mercaptoethanol, 200 mM Tris, pH 9.0) and stirred for 30 min at room temperature.

this pH, for the alkylation of 2-mercaptoethanol and the tripeptide glutathione, γ-Glu-Cys-Gly, with iodoacetate, were reported to be 1.4 and 4.5 M^{-1} s^{-1}, respectively (37 °C) (Dahl and McKinley-McKee, 1981). As the reaction of thiols with alkyl halides is an S_N2 reaction, the alkylation rate of free SH with I-AA can be written as

$$-\frac{d[SH]}{dt} = k_{app}[IAA][SH] \qquad (3)$$

where [IAA] and [SH] are the concentrations of iodoacetate and free SH, respectively, t is the reaction time, and k_{app} the apparent rate constant at a specific pH. In Figure 5 the conversion of feather keratin cysteine to *S*-(carboxymethyl)cysteine with iodoacetate in the presence of 2-mercaptoethanol is presented, by numerically solving eq 3 and using the k_{app} values for glutathione and 2-mercaptoethanol as given above. The same initial concentrations of 2-mercaptoethanol and cysteine were used as under the experimental conditions. The concentration of 2-mercaptoethanol was corrected for the formation of 2,2′-dithiobis(ethanol), a reaction product formed during reduction of cystine to cysteine (Scheme 2). For a 5-fold excess of reagent, the theoretical degree of cysteine modification after 20 s of reaction time was 99%. Consequently, the experimentally applied reaction time of 30 min appeared to be sufficient to establish full conversion of cysteine.

Figure 6 shows the calculated and measured degree of cysteine modification at different reagent/cysteine ratios. The overall reaction rate of 2-mercaptoethanol with iodoacetate is approximately equal to that of cysteine because of its higher initial concentration. Therefore, when less than a 5-fold excess of iodoacetate with respect to cysteine was used, no full conversion of cysteine could be established. The calculated values compared well with the experimentally obtained results. Up to a reagent/cysteine ratio of about 4, the calculated values were somewhat lower than the experimental values. This could be due to a higher reactivity of cysteine residues in feather keratin compared to that of cysteine in glutathione, which was used as a model peptide. At high ratios, complete alkylation of the cysteine was predicted, which is close to the experimentally observed degree of modification. It is possible that steric inhibition strongly decreased the reactivity of some individual cysteine residues, which are very close to each other (Dahl and McKinley-McKee, 1981). This could also be the reason it became increasingly difficult to alkylate the feather keratin cysteines with Br-SA, by which a succinate side chain is introduced containing two negative charges at neutral pH.

Feather keratin was extracted from feathers using 2-mercaptoethanol (125 mM) as a disulfide bond reducing agent in concentrated aqueous urea solutions (8 M), which disrupt the protein's secondary structure. Dialysis against distilled water of unmodified feather keratin solutions afforded a white gel when the protein concentration was higher than ∼3% (w/v) or a white dispersion below this protein concentration. This is due to extensive aggregation of the denatured keratins and reoxidation of cysteine residues into cystine. Hydrophobic residues, exposed during denaturation, are believed to be the major cause of aggregation (Cleland et al., 1993). After dialysis of solutions of feather keratin derivatives (3% w/v) modified with different alkylating reagents and different reagent/cysteine ratios, large differences in the stability of the resulting dispersions were observed. The effect of the introduction of different amounts of carboxylic acid groups on the aggregation and solubility of the keratin derivatives was compared with the effect of the introduction of uncharged amide groups. For keratin modified with I-AAm and I-AA, aggregation was measured qualitatively as the absorbance at 540 nm, immediately after dialysis (Figure 7a). For *S*-(carboxamidomethyl)keratin, the aggregates were finely dispersed at low degrees of cysteine modification (20−30%), resulting in a milky, white dispersion with a high turbidity. Higher degrees of modification resulted in large aggregates that precipitated. Therefore, these dispersions displayed a relatively low turbidity. When more than 80% of the cysteine was modified, an increase in turbidity was observed. For *S*-(carboxymethyl)keratin, low degrees of cysteine modification (<30%) resulted in a white dispersion with a high turbidity. Feather keratin with more than 30% cysteine modification had a low turbidity.

These observations were also reflected in the amount of aggregated (Figure 7b) keratin derivatives. In all cases, the amount of aggregated protein was higher for keratin modified with I-AAm than for keratin modified with I-AA. For *S*-(carboxymethyl) keratin, with more than 25% of the cysteine residues modified, the amount of aggregated protein was less than 20%. However, more than 40% of *S*-(carboxamidomethyl)keratin was aggregated for all degrees of cysteine modification. Obviously, the increase in net charge at neutral pH on the keratin through alkylation of cysteine with iodoacetic acid was responsible for the formation of fewer and

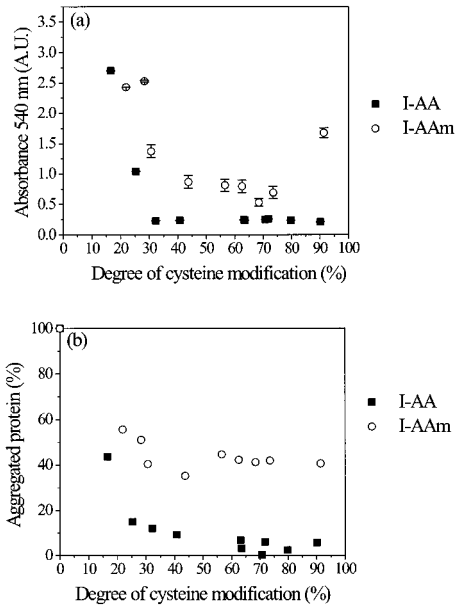


Figure 7. Absorbance at 540 nm (a) and percentage of aggregated protein (b) of feather keratin, modified with iodoacetic acid (I-AA) and iodoacetamide (I-AAm) as a function of the degree of cysteine modification.

smaller aggregates when compared to cysteine alkylation with iodoacetamide.

For the preparation of films by solution casting, a stable feather keratin dispersion is needed in which no extensive aggregation occurs. Dispersions from feather keratin derivatives in which more than 25% of the cysteine residues was carboxymethylated appeared to be suitable for solution casting. The proteins displayed little aggregation (Figure 7b). For this reason, these derivatives were chosen for further experiments. In these dispersions, the formation of aggregates is probably accompanied by oxidation of unmodified cysteine residues to disulfide bonds. Therefore, the amount of free, unoxidized cysteine after dialysis was measured with a DTNB-assay (Ellman, 1958; 1959). For feather keratin derivatives with a degree of cysteine modification of 32 and 90%, the amount of unmodified cysteine was 480 and 70 μmol/gram of keratin, respectively. For these derivatives, the amount of free SH groups after dialysis was 16 and 2 μmol/g of keratin, respectively. This means that as soon as 2-mercaptoethanol was removed by dialysis, over 95% of the unmodified cysteine reoxidized to form disulfide bonds.

The size distribution of *S*-(carboxymethyl)keratin in dispersions obtained after dialysis was determined by size exclusion chromatography (SEC). The column was calibrated with proteins of known molecular mass and Stokes radius. 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. The calibration curve, obtained by plotting log M_w against the distribution coefficient K_d, yields a sigmoid curve that may be approximated in the middle range by $K_d = a - b \log M_w$ (Hagel, 1989). 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 (Laurent and Killander, 1964).

After calibration, dispersions of *S*-(carboxymethyl)-keratin with different degrees of cysteine modification

Table 1. Molecular Mass M_w and Intrinsic Viscosity-based Stokes Radius R_{vis} (Horiike et al., 1983); 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) 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.

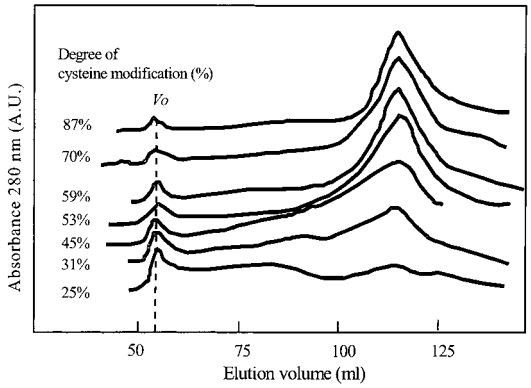


Figure 8. Elution profiles of *S*-(carboxymethyl)keratin with different degrees of cysteine modification, in Tris-buffer (50 mM, 0.02% NaN_3) on a Sepharose CL-6B column (2.8 × 28.5 cm).

Table 2. Elution Volume V_e, Distribution Coefficient K_d, and Calculated Stokes Radius R_{vis}, of *S*-(Carboxymethyl)keratin, with Different Degrees of Cysteine Modification[a]

deg of cysteine modification (%)	V_e, (mL)	K_d	R_{vis} (Å)
25	78−85	0.26−0.33	71−81
	117	0.66	25
31	67−91	0.16−0.40	62−96
	117	0.65	26
45	116	0.65	27
53	115	0.64	27
59	115	0.63	27
70	115	0.63	28
87	115	0.63	28
25 DTT[b]	128	0.77	9
70 DTT[b]	132	0.81	3

[a] As measured on a Sepharose CL-6B column (2.6 × 28.5 cm) using Tris-buffer as eluent (50 mM Tris, 0.02% NaN_3, pH 8.5, flow rate 25 mL/h). [b] Measured by adding 10 mM of dithiothreitol (DTT) to the 70% *S*-carboxymethylated keratin dispersion and applying the sample on the column equilibrated with Tris-buffer and DTT (50 mM Tris, 1 mM DTT, 0.02% NaN_3, pH 8.5).

were filtered through a 0.45-μm filter and applied on the column, using Tris-buffer as eluent (50 mM Tris, 0.02% NaN_3, pH 8.5). No guanidinium chloride or SDS was added to the eluent to prevent the separation of aggregates present in the dialyzed solutions. The elution profiles are presented in Figure 8. The elution volume V_e of ranges or peaks in the SEC-profiles, the calculated distribution coefficient K_d, and Stokes radius R_{vis} are shown in Table 2. All samples showed a peak at the exclusion volume, which was ascribed to a fraction of relatively large aggregates. This fraction became smaller at higher degrees of cysteine modification. At higher elution volumes, a peak became more apparent as the

degree of cysteine modification increased. The Stokes radius of the proteins in this peak was estimated to be 26−28 Å. Apparently, oligomers of this size were present for all degrees of modification. Possibly, the charge density at the surface of these oligomers prevented further aggregation (Blank, 1994).

For 25% cysteine modification, feather keratin molecular species with a broad size distribution were present (Stokes radius 70−80 Å). For 87% cysteine modification, only a small fraction of aggregates was formed and the major fraction was present as oligomers. For degrees of modification between 25 and 87%, a gradual decrease in the amount of larger aggregates and an increase in the amount of oligomers was seen.

When dithiothreitol (DTT) was added to the eluent (50 mM Tris, 1 mM DTT, 0.02% NaN_3, pH 8.5) and to a dispersion of *S*-(carboxymethyl)keratin with 70% cysteine modification (10 mM DTT) most of the aggregates present disappeared. For 25% modification, a peak still appeared at the exclusion volume, but many of the intermediate protein oligomers disappeared. For both modified keratins (25% and 70%), most of the protein appeared as a peak close to the elution volume of the feather keratin monomer (Table 1). This, together with the information on the high amount of reoxidation of unmodified cysteine, shows that the aggregates and oligomers formed were stabilized by disulfide bonds. For *S*-(carboxymethyl)keratin with low degrees of cysteine modification it was clear that the oligomers contained a large amount of disulfide bonds, considering the low amount of free SH. These disulfide bonds were intermolecular as, by addition of DTT, many aggregates disappeared. By reducing the disulfide bonds with DTT, free thiolate anions were formed, which aided in separating the protein aggregates. It appeared that, for *S*-(carboxymethyl)keratin with high degrees of cysteine modification, the few remaining unmodified cysteine residues still played an important role in stabilizing the keratin oligomers, as addition of DTT led to their disruption and the proteins eluted at higher volumes, close to the elution volume expected for a feather keratin elementary chain.

Through partial carboxymethylation of cysteine in feather keratin, it has become clear that during dialysis complex aggregation and oxidation processes were involved which seemed to be highly specific for each degree of modification. At low degrees of cysteine modification a polydisperse system of aggregates and oligomers stabilized by intermolecular disulfide bonds was formed. At higher degrees of cysteine modification the system gradually became less polydisperse and oligomers are predominantly formed.

CONCLUSIONS

Optimal conditions, without amide bond scission, for extracting up to 75% of the keratins from feathers appeared to be a reaction time of 30 min at 40 °C, a pH of 7.0−9.0, a urea concentration of 5 M or higher, and a 10-fold molar excess of 2-mercaptoethanol with respect to the number of disulfide bonds present. Feather keratin cysteine could be partially modified, in aqueous solutions of urea and 2-mercaptoethanol, with iodoacetamide, iodoacetic acid, or bromosuccinic acid, through nucleophilic substitution. A high control in degree of cysteine modification was achieved by carefully choosing the reaction pH and the concentration of 2-mercaptoethanol and by changing the reagent/cysteine ratios. Iodoacetic acid and iodoacetamide were more effective in alkylating cysteine than bromosuccinic acid. The increased net charge at neutral pH on the keratin through alkylation with iodoacetic acid was responsible for higher protein solubility and the formation of fewer and smaller aggregates when compared to alkylation with iodoacetamide. For *S*-(carboxymethyl)keratin, fewer aggregates were formed for higher degrees of cysteine modification, while more protein was present in an oligomeric form. Aggregates and oligomers were stabilized through intermolecular disulfide bonds. Stable dispersions were obtained from partially carboxymethylated feather keratins, which could be used for producing biodegradable films and coatings.

ABBREVIATIONS

DTT, dithiothreitol; I-AAm, iodoacetamide; I-AA, iodoacetic acid; Br-SA, bromosuccinic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NTSB, disodium 2-nitro-5-thiosulfobenzoate.

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