Partially Carboxymethylated Feather Keratins. 2. Thermal and Mechanical Properties of Films

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Free cysteine thiol groups of keratin extracted from chicken feathers were partially carboxymethylated with iodoacetic acid (25−76% cysteine modification). Stable dispersions were used for the preparation of films by solution casting. Glycerol was used as a plasticizer (0.05−0.47 g/g of keratin), and films were stored at a constant relative humidity (20, 30, 50, 70, or 90%). The degree of crystallinity in the films was higher when more cysteine residues were carboxymethylated. The films displayed an optimum in mechanical properties at ∼50% cysteine carboxymethylation. The tensile strength at this optimum was 25 MPa, the *E* modulus, 350 MPa, and the elongation at break, 50%. Probably, this optimum was the result of both a decreasing amount of disulfide bonds and an increasing degree of crystallinity for higher degrees of cysteine modification. The influences of a higher amount of glycerol and of different storage conditions on the mechanical properties of films from keratin with a defined degree of cysteine modification were also investigated.

Keywords: *Feather; keratins; films; chemical modification; thermal properties; mechanical properties*

INTRODUCTION

Over the past 15 years, poultry production in the European Union increased by 5% annually, which led to a growing waste stream of feathers. In 1996, >770000 tons of chicken feathers was available as a byproduct of the poultry industry. Feathers are mainly composed of the structural proteins keratins and are generally transformed into hydrolyzed feather meal. Feather meal has little added value and is used as an organic fertilizer or as an additive to animal feed. The function of feathers as a tough, insoluble, fibrous material that provides a protective outer covering indicates the potential of the feather keratin molecule for applications in which these properties are desirable. Water insolubility and mechanical strength are mainly due to the occurrence of a large amount of hydrophobic amino acids and cysteine residues, which are mainly present as the disulfide bonded, dimeric amino acid cystine, and to the structural organization of the keratin molecules in the feather. Recently, there has been an increased interest in the use of proteins as a renewable resource for the development of biodegradable films, for example, for compostable packaging, agricultural film, or edible film applications (*1*−*6*). Only limited attention has been given to keratin in this field (*7*−*11*).

Feather keratins are composed of ∼20 proteins, which differ by only a few amino acids. These proteins have approximately the same molecular weight of 10.4 kDa (*12*). The distribution of amino acids 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 crystalline β-sheet conformation (*13*).

There are essentially two types of keratin, traditionally classified as either “soft” or “hard” (*14*). The soft keratins, with a low content of disulfide bonds, are found in the *stratum corneum* and callus, whereas the hard keratins are found in epidermal appendages such as feathers, hair, nails, and hoofs and have a high disulfide content. Apparently, the amount of disulfide bonds determines largely whether a keratinous material is soft, flexible, and extensible, like the epidermis, or hard, tough, and inextensible, like hair or feathers (*15*).

For film preparation by solution casting, stable solutions or dispersions are needed. For feather keratins it has been demonstrated previously that such solutions or dispersions can be prepared by partially carboxymethylating the cysteine residues (*16*). To obtain films with a high *E* modulus and tensile strength, it appears to be appropriate to decrease the amount of intermolecular cross-links in keratin by partially modifying the cysteine residues, leaving the remaining cysteine free to oxidize during the film-forming process. In this study we report on the preparation of solution cast films from partially carboxymethylated feather keratin dispersions. The effect of the degree of cysteine modification, added glycerol as a plasticizer, and water on thermal and mechanical properties of these films 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 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), which was obtained from Sigma (St. Louis, MO).

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Spectra/Por dialysis membranes (MWCO 6000−8000) were purchased from Spectrum Medical Industries (Laguna Hills, CA). For all spectrophotometric measurements, a Uvikon 930 (Kontron Instruments, Milan, Italy) was used.

Pretreatment of the Feathers. Freshly plucked, white chicken feathers were cleaned, cut, and degreased following the procedures described previously (*16*).

Preparation of a Feather Keratin Solution. Feathers (30 g) were solubilized under optimized conditions (750 mL, 8 M urea, 3 mM EDTA, 125 mM 2-mercaptoethanol, 200 mM Tris, pH 9.0, room temperature). After separation from the insoluble residue, a 3% (w/v) keratin solution was obtained. The keratins were pure samples, as measured using size exclusion chromatography in an eluent containing guanidine hydrochloride (6 M) and dithiothreitol. Keratins isolated from the feathers contained 7 mol of cysteine/mol of feather keratin, with a molecular weight of 10 kDa (*13*).

Partial Carboxymethylation of Feather Keratins. This method was described previously (*16*) and was based on a procedure presented by Crestfield et al. (*17*). To 3% (w/v) keratin solutions were added different molar ratios of monoiodoacetic acid (I-AA) with respect to the cysteine residues to obtain different degrees of modification. Molar ratios of I-AA/cysteine of 0.5:1, 0.75:1, 1:1, 1.25:1, 1.71:1, and 2:1 were used. 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 I-AA. After 30 min of reaction, the mixture was dialyzed extensively against distilled water. The dialysate (30 L) was replaced after 16 and 24 h, and dialysis was stopped after 40 h.

Degree of Modification. Free thiol groups from cysteine residues in partially and unmodified feather keratins were fully reoxidized to disulfide bonds upon lyophilization, as measured with a DTNB assay (Ellman's reagent or DTNB) (*16*). 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) (*18, 19*):

$$\text{degree of modification} = 100 \times \frac{SS_{unmod} - SS_{mod}}{SS_{unmod}} \quad (1)$$

where SS_{unmod} and SS_{mod} are the amounts of disulfide bonds in lyophilized unmodified and modified feather keratin, respectively. For unmodified keratin, SS_{unmod} is ∼350 μmol/g of lyophilized material.

Preparation of Keratin Films. After dialysis, the protein concentration of the dialyzed feather keratin dispersions was measured by using a biuret assay (*20*). Glycerol was subsequently added to the dialyzed keratin dispersions (0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.33, and 0.47 g/g of keratin). The dispersions were poured into Petri dishes, and these were placed in a ventilated oven at 40 °C. The resulting films consisted of ∼20 mg/cm^2 of feather keratin. After 4 days, no further weight loss was observed. The Petri dishes were stored in a desiccator at room temperature at a constant relative humidity (20, 30, 50, 70, or 90% RH).

Water Absorption. Water absorption of the films was measured by weighing the Petri dishes after drying for 4 days in a ventilated oven at 40 °C and following the water uptake as a function of time by measuring the change in weight, when placed in a desiccator at room temperature over $Mg(NO_3)_2 \cdot 6H_2O$ (RH = 45−50%). At room temperature, constant relative humidity conditions were created by using saturated solutions of different salts in a desiccator: $CaCl_2 \cdot 2H_2O$ (RH = 20%), $CaCl_2 \cdot 6H_2O$ (RH = 30%), $Mg(NO_3)_2 \cdot 6H_2O$ (RH = 50%), $Na_2S_2O_3 \cdot 5H_2O$ (RH = 70%), and $Na_2CO_3 \cdot 10H_2O$ (RH = 90%).

Mechanical Properties. Films conditioned for at least 7 days at various relative humidities were mechanically tested using a Zwick tensile tester. All films had reached a plateau value of absorbed water, except for a film of keratin with a degree of cysteine modification of 52%, containing 0.30 g of glycerol/g of keratin and conditioned at 90% RH. *E* modulus, tensile strength, and elongation at break were measured. Film specimens 4 mm wide and 80 mm long were cut with a metal model cutter. The thickness was measured with a Mitutoyo micrometer and was between 180 and 200 μm. The *E* modulus was calculated from the linear part of the curve, between 0.5 and 2.0% elongation. Tensile strength was calculated automatically by dividing the peak load by initial specimen cross-sectional area. Elongation at break is expressed as the percentage of change of the original length of the specimen between the grips. The initial grip separation was 40 mm. The cross-head speed was 7.5 mm/min. Each tensile strength, elongation at break, and *E* modulus value was measured six times on specimens cut from the same film. One film was used for each treatment. Film specimens were tested within 10 min after they had been removed from the desiccator to minimize effects due to absorption or loss of water.

Table 1. Degree of Cysteine Modification As Measured with an NTSB Assay for Different I-AA/Cysteine Molar Ratios

I-AA/Cys ratio:	0.5:1	0.75:1	1:1	1.25:1	1.71:1	2:1
degree of modification (%):	25	39	50	57	70	76

Extractable Protein in Water. The percentage of extractable keratin from the films in water was measured. From films with various degrees of cysteine modification, containing glycerol (0.30 g/g of keratin), samples of 70 ± 10 mg were weighed before immersion in 5 mL of demineralized water at 20 °C. After 1, 3, 6, and 24 h, the extracted keratin was quantified by measuring the protein concentration in the extract, using a modified Lowry procedure (*21, 22*).

Differential Scanning Calorimetry (DSC). The thermal properties of the proteins were determined using a Perkin-Elmer DSC-7 differential scanning calorimeter, calibrated with indium and gallium. Round specimens were cut from the conditioned keratin film, and 10−15 mg of this material was put in high-pressure DSC pans. Generally, the samples were heated from −50 to 200 °C at a heating rate of 10 °C/min and next quenched to −50 °C at a rate of 300 °C/min. A second heating curve was then recorded (10 °C/min). Glass transition temperatures and first-order denaturation endotherms were determined from the first heating curve. The integration of the peak area of the endotherm of the feather calamus was taken as 100% native material. The degree of secondary structure recovery of the modified keratins was calculated by integrating the peak area of the first-order denaturation endotherms of the keratins and dividing this area by the area obtained for the calamus.

RESULTS

In a first series of experiments, the degree of modification of cysteine in feather keratin was varied by adding different amounts of I-AA to the keratin solution (3% w/v) containing urea (8 M) and 2-mercaptoethanol (125 mM). The solutions obtained did not gel when urea and 2-mercaptoethanol were removed by dialysis, but became slightly turbid. The degree of modification of the feather keratin was measured with an NTSB assay. Depending on the I-AA/cysteine ratio, 25−76% of the cysteine was carboxymethylated (Table 1). From the dialyzed solutions, films were prepared by the addition of a fixed amount of glycerol (0.30 g/g of keratin) and drying for 4 days at 40 °C. These films were subsequently conditioned for 3 weeks at a constant RH of 50%. The water content did not vary significantly with the degree of modification and was 9.5 ± 0.3 wt %. All films formed were transparent.

The amount of protein that can be extracted from partially carboxymethylated feather keratin films gives an indication of the extent of disulfide bond formation in the film. Keratin extractability for different films was tested as a function of time by measuring the protein concentration after immersion in water for 1−24 h. In

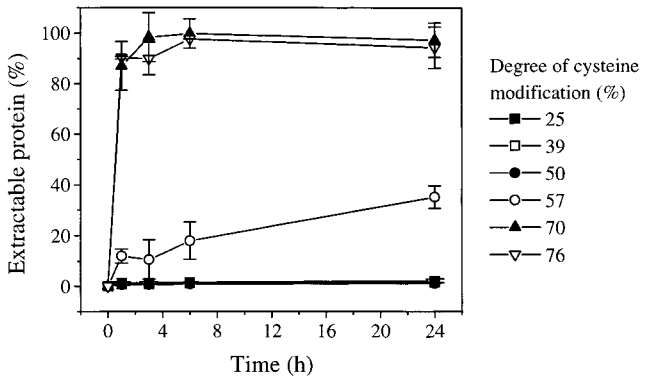

Figure 1. Extractable protein expressed as percentage of total protein present for feather keratin films with different degrees of cysteine modification as a function time, in films containing 0.30 g of glycerol/g of keratin.

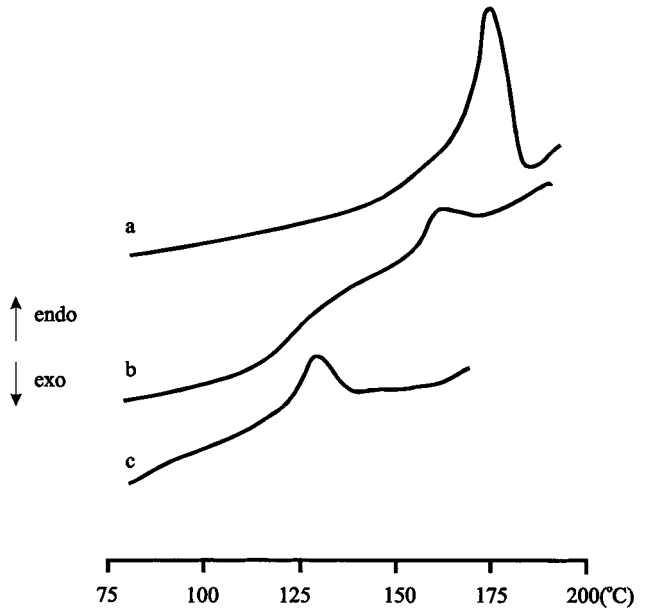

Figure 2. DSC diagram of (a) feather calamus and (b) a film of feather keratin with a degree of cysteine modification of 50% without glycerol, measured immediately after drying, and (c) a film of feather keratin with a degree of cysteine modification of 50% with glycerol (0.30 g/g of keratin), conditioned at 50% RH. The heating rate was 10 °C/min.

Figure 1 the percentage of extractable keratin from these films is shown. After 24 h, <2% could be extracted from films with 25−50% carboxymethylated cysteine thiol groups. Equally, keratin could not be extracted from these films with 8 M aqueous urea solutions (data not shown). About 35% keratin from films with 57% cysteine modification was extracted in water in 24 h. Films with a higher degree of cysteine modification dissolved completely in water.

Thermal properties of feathers and feather keratin films were investigated using DSC. Typical thermograms of whole feather and feather keratin films with or without glycerol are presented in Figure 2. Samples were heated at a rate of 10 °C/min. For feather calamus, the portion of the feather shaft located under the skin, no second-order transition was observed between −50 and 260 °C. However, a broad endothermal first-order transition due to denaturation was revealed between 148 and 183 °C, with a peak at 175 °C. The transition enthalpy was 15.3 J/g. Thermal degradation started at ∼230 °C.

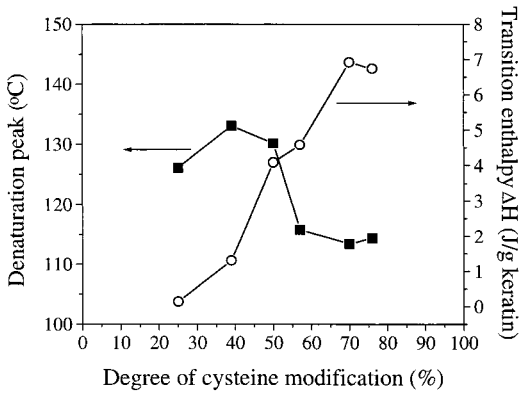

Figure 3. Denaturation temperature and corresponding transition enthalpy of feather keratin with different degrees of cysteine carboxymethylation in films containing glycerol (0.30 g/g of keratin) and water (9.5 wt %). Enthalpies were corrected for the presence of glycerol and water.

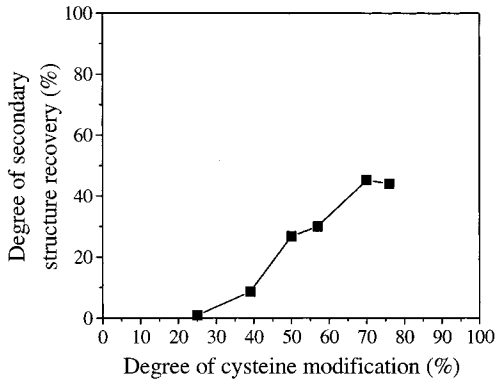

Figure 4. Degree of secondary structure recovery as a function of the degree of cysteine modification in films from partially carboxymethylated feather keratin dispersions, containing glycerol (0.30 g/g of keratin) and water (9.5 wt %).

Feather keratin films comprising different degrees of cysteine modification (39, 50, and 57%) were very brittle. First-heating curves of dried films showed a second-order transition at ∼121 ± 3 °C with a heat capacity jump, ΔC_p, of 0.3 ± 0.1 J/g·°C (Figure 2b). The denaturation temperature was ∼170 °C and decreased slightly with increased cysteine modification.

In a similar way, feather keratin films with different degrees of cysteine modification, containing glycerol (0.30 g/g of keratin), were investigated. The water content was 9.5 wt %. In the thermograms no clear second-order transition was observed between −50 and 170 °C. However, an endothermal first-order transition due to protein denaturation appeared between 110 and 130 °C. This transition became more prominent as the degree of modification increased. This peak was not observed in second-heating runs. In Figure 3 the denaturation temperature and the corresponding transition enthalpy are presented as a function of the degree of modification. Between degrees of cysteine carboxymethylation of 25 and 76%, the denaturation temperature decreased from 126 to 114 °C and the transition enthalpy increased from 0.1 to 6.7 J/g of keratin. From the transition enthalpy of feather keratin in films and in the calamus, the degree of secondary structure recovery compared to the native keratin could be calculated by assuming that the secondary structure formed in the films is the same as in the feather (Figure 4) (*23*). The degree of secondary structure recovery varied between 1 and 44% for feather keratin with

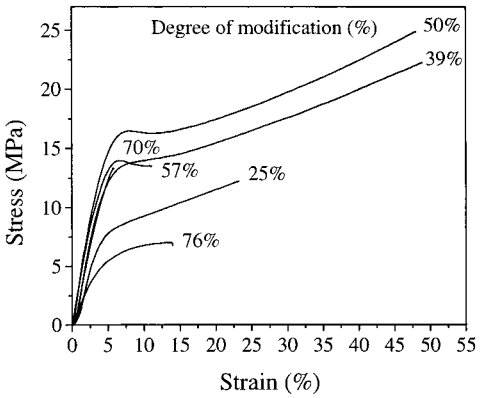

Figure 5. Typical stress−strain curves of films from partially carboxymethylated feather keratin dispersions with different degrees of cysteine modification. Films contained glycerol (0.30 g/g of keratin) and water (9.5 wt %).

degrees of cysteine modification of 25 and 76%, respectively. Thus, the degree of crystallinity varied between 0.3 and 13%, on the basis of a degree of crystallinity of the native feather keratin of ∼30% (*24*).

The mechanical properties of films containing glycerol (0.30 g/g of keratin) and water (9.5 wt %) were determined from stress−strain curves (Figure 5). As the degree of modification increased, the elongation at break and the tensile strength increased to an optimum for 50% cysteine modification. The keratin films with 25, 39, and 50% cysteine modification showed strain hardening behavior above 10% elongation. In panels a and b of Figure 6 the *E* modulus and tensile strength are shown as a function of the degree of modification. The film with 25% cysteine modification had an *E* modulus of ∼280 MPa. For higher degrees of modification, the *E* modulus increased and remained about 350 MPa for a degree of modification of 39−70%. For 79% cysteine modification, it decreased sharply to 170 MPa. The tensile strength clearly showed an optimum of ∼25 MPa at 50% modification and decreased sharply to 7 MPa at higher degrees of modification. The elongation at break (Figure 6c) also increased to an optimum of ∼50% elongation for 50% modification and decreased again to 5−10% for increasing degrees of cysteine modification.

In a second series of experiments, the amount of glycerol was varied in feather keratin films with a degree of cysteine modification of 52%. All films were transparent and were more brittle as the amount of glycerol decreased. Figure 7a shows the weight increase of these films as a function of time, at a constant RH of 50%. Films containing a higher amount of glycerol also absorbed a higher amount of water. This is illustrated in Figure 7b, where the absorbed water after 332 h of conditioning time at 50% RH is shown as a function of the amount of added glycerol. The amount of absorbed water varied from 3 wt % for films containing 0.05 g of glycerol/g of keratin to 9 wt % for films containing 0.30 g of glycerol/g of keratin.

The thermal properties of these films were studied, using DSC. Film samples were heated at a rate of 10 °C/min. For a brittle film containing 0.05 g of glycerol/g of keratin and water (3 wt %), in the first-heating curve a second-order transition was present at ∼57 °C as was an endothermal first-order transition due to denaturation at ∼130 °C (Figure 8). The sample was quenched and reheated immediately after the first scan, after annealing for 24 h at 40 °C and for 5 days at 40 °C. The

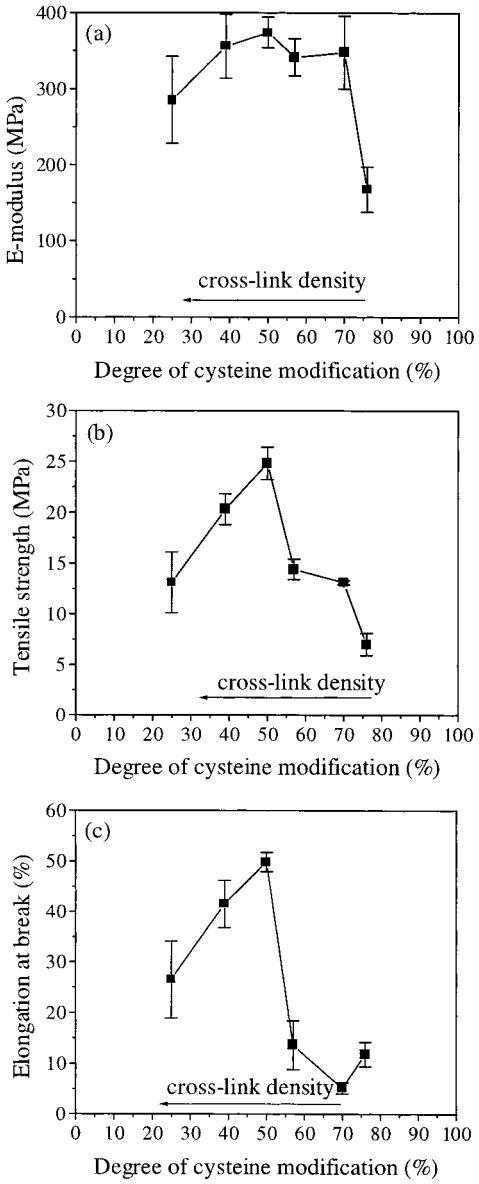

Figure 6. *E* modulus (a), tensile strength (b), and elongation at break (c) as a function of the degree of modification of feather keratin films modified with monoiodoacetic acid, containing glycerol (0.30 g/g of keratin) and conditioned for 1 week at 50% RH. The cross-link density indicated refers to the reconstitution of disulfide bonds in the film.

first-order transition at ∼130 °C had disappeared in the subsequent heating curves.

For the second-order transition, the heat capacity jump, ΔC_p, in these heating curves was lower than in the first-heating curve. In the second-heating curve, the endothermal overshoot in the second-order transition region had disappeared. For annealing times of 24 h and 5 days, the endothermal peak in the second-order transition region gradually reappeared. The film was also heated at different rates, and the second-order transition could be extrapolated to zero heating rate to determine the actual second-order transition temperature, which was 30 °C.

First-heating curves of feather keratin films containing different amounts of glycerol and conditioned at 50% RH were determined. The apparent second-order transition temperature varied between 54 and 44 °C for a film containing 0.05 and 0.30 g of glycerol/g of keratin, respectively. Actual second-order transition tempera-

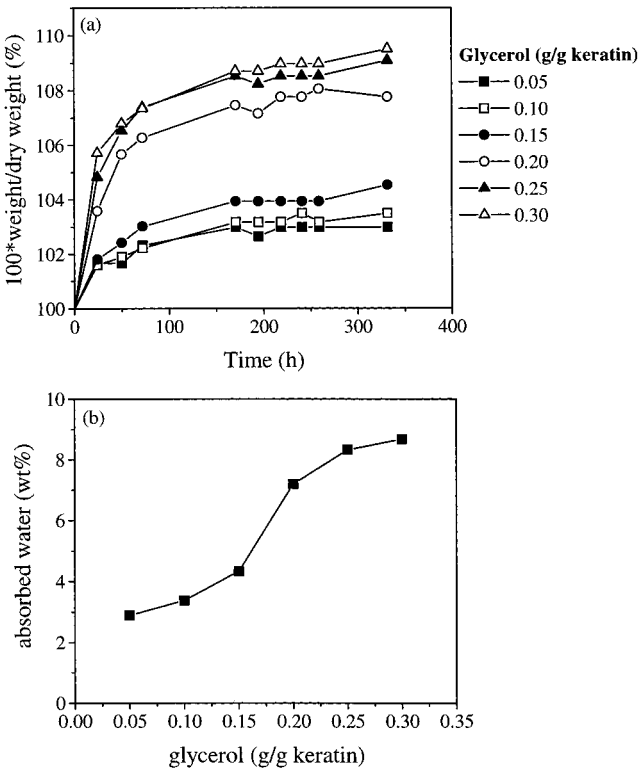

Figure 7. Weight increase as a function of time (a) and water content as a function of the amount of added glycerol after 332 h of conditioning time at an RH of 50% (b) for feather keratin films with a degree of cysteine modification of 52%.

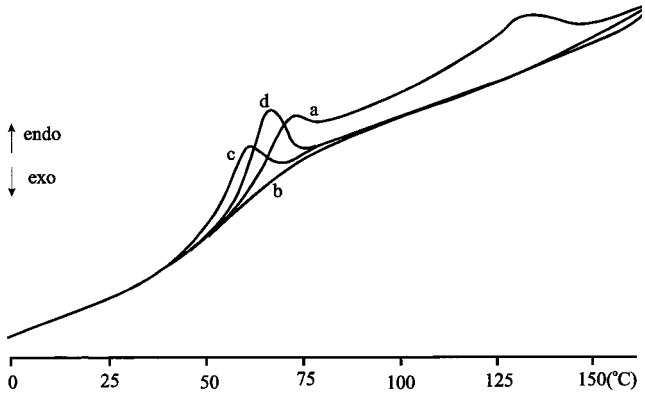

Figure 8. DSC of a feather keratin film with a degree of cysteine modification of 52%, containing glycerol as a plasticizer (0.05 g/g of keratin) and conditioned at 50% RH: (a) first-heating run; (b) second-heating run immediately after cooling at a rate of 300 °C/min; (c) third-heating run after annealing for 24 h at 40 °C; (d) fourth-heating run after annealing for 5 days at 40 °C. The heating rate was 10 °C/min.

tures were determined by extrapolating to zero heating rate, and the results are presented in Figure 9. For these films the denaturation endotherm decreased from 130 to 111 °C.

The mechanical properties of the films were tested after conditioning at 50% RH for 14 days. Films containing 0.05 and 0.10 g of glycerol/g of keratin were too brittle to measure the mechanical properties. The *E* modulus of films containing 0.15−0.47 g of glycerol/g of keratin decreased from 1344 to 145 MPa (Figure 10a). Tensile strengths remained almost constant (30 MPa) for films containing 0.15−0.23 g of glycerol/g of keratin and decreased to 6.6 MPa for films containing higher

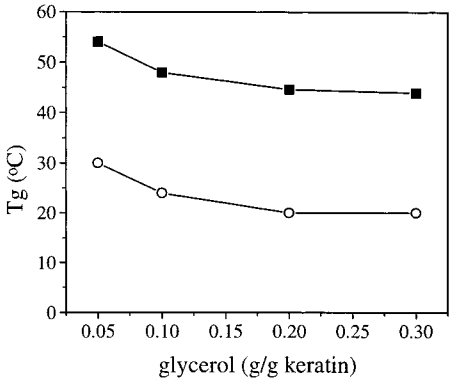

Figure 9. Apparent (■) and actual (○) glass transition temperature of feather keratin films with a degree of cysteine modification of 52%, containing different amounts of glycerol as a plasticizer and conditioned at 50% RH. Apparent glass transition temperatures were measured using DSC at a heating rate of 10 °C/min. Actual glass transition temperatures were determined by extrapolation to zero heating rate.

amounts of glycerol (Figure 10b). The elongation at break of these films (Figure 10c) increased from 3 to 13%.

In a third series of experiments the amount of water in feather keratin films with a degree of cysteine modification of 52%, containing a fixed amount of glycerol (0.30 g/g of keratin), was varied. Films stored for 7 days at an RH of 50% were transferred to desiccators at various RH values from 20 to 90%. The weight increase of these films was observed in time (Figure 11a). In Figure 11b, the amount of absorbed water after 14 days of conditioning at various RH values is shown. The amount of absorbed water increased at a high RH of 90%, and a weight increase of 26% was obtained after 2 weeks. At lower RH, plateau values were found after ∼5 days.

The thermal properties of these films revealed a decrease in the apparent second-order transition temperature from ∼45 to −20 °C for films containing 2 and 26 wt % of water, respectively, and glycerol as a plasticizer (0.30 g/g of keratin).

The mechanical properties of the films were tested after conditioning at various RH values for 14 days. A strong decrease in both the E modulus (Figure 12a), from 863 to 250 MPa, and in the tensile strength (Figure 12b), from 40 to 24 MPa, was observed for films conditioned at an RH up to 50% (8 wt % water). Storing films at higher RH resulted in a slow decrease in *E* modulus and tensile strength. The elongation at break increased from 25 to 57% as the RH varied from 20 to 90% and thus for increasing amounts of water present (Figure 12c).

DISCUSSION

Currently, interest in the development of products for environmentally acceptable applications from protein waste streams is growing. Keratin has received limited attention in this field. To investigate the possibilities of using keratin in such applications, a mild extraction procedure for keratin from whole chicken feathers, using 2-mercaptoethanol (125 mM) as a disulfide bond reducing agent and urea (8 M) as a disruptive agent at a moderately alkaline pH (9.0), has recently been studied and optimized (*16*). Under these conditions, ∼75 wt % of the feather keratin was extracted and no protein degradation occurred. An insoluble residue remained,

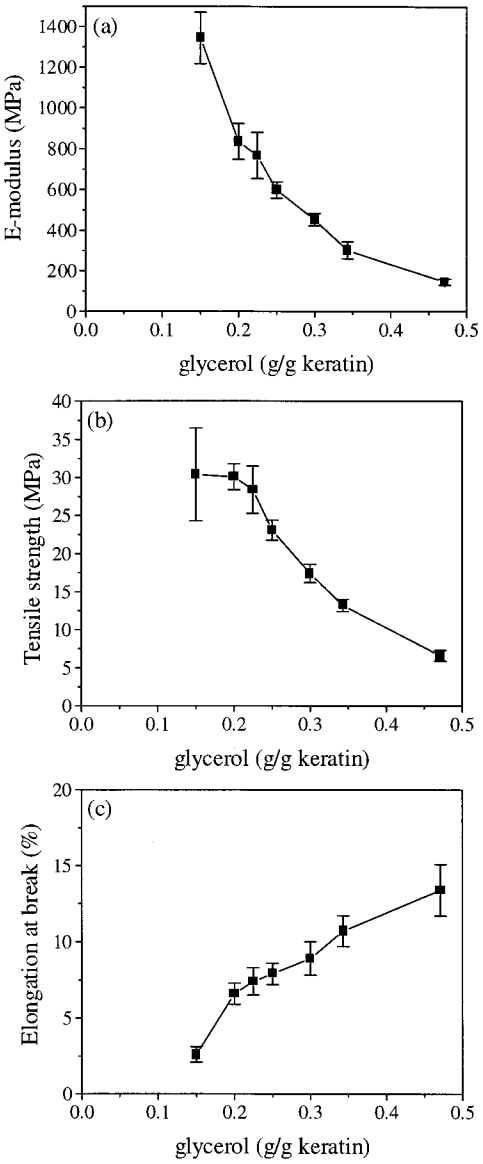

Figure 10. *E* modulus (a), tensile strength (b), and elongation at break (c) as a function of the amount of added glycerol for feather keratin films with a degree of cysteine modification of 52%, conditioned at 50% RH.

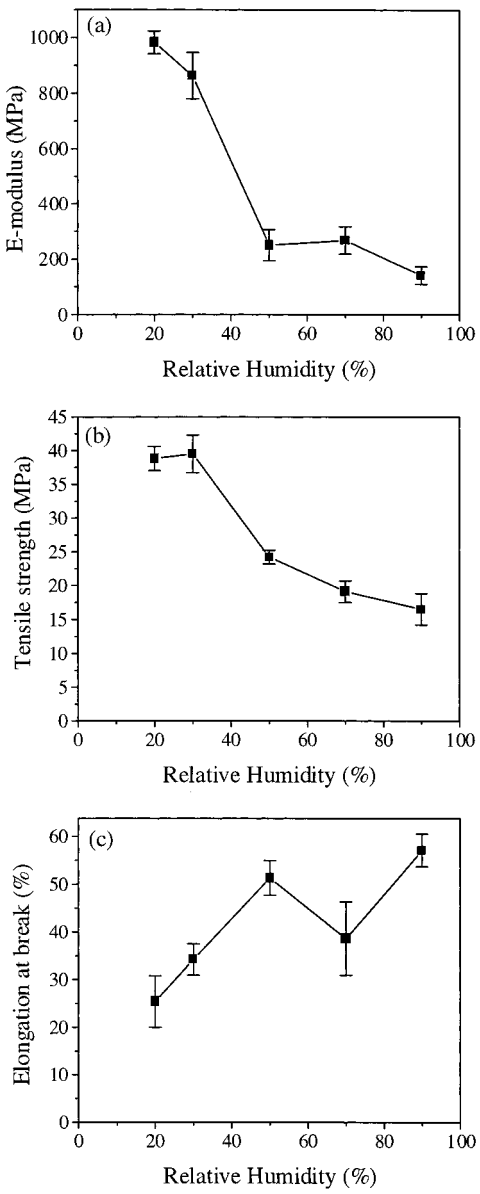

Figure 11. Weight increase as a function of time (a) and water content as a function of RH after 500 h of conditioning time (b) of feather keratin films with a degree of cysteine modification of 52%, conditioned at varying RH, containing a fixed amount of glycerol (0.30 g/g of keratin).

which probably consisted of cellular envelopes and ϵ-*N*-(γ-glutamyl)-lysyl cross-linked material (*25*). Removal of 2-mercaptoethanol and urea from the feather keratin solution by dialysis resulted in aggregation of the keratin polypeptide chains and oxidation of the cysteine residues. For the development of biodegradable materials, such as films for compostable packaging or paper coatings, stable keratin solutions or dispersions in water can be used. Ideally, regeneration of disulfide bonds in these materials may be used as a method to impart water insolubility and good mechanical properties.

To obtain a stable solution or dispersion of the extracted keratins in the absence of a reducing and disruptive agent, many different approaches have been followed. One method involves the addition of sodium dodecyl sulfate (SDS), an anionic surfactant, to the keratin solution prior to dialysis (*10*). The surfactant forms a complex with the keratin and prevents extensive protein aggregation when reducing and disruptive agents are removed by dialysis. Other methods to stabilize feather keratin solutions involve complete chemical modification of the cysteine residues. Oxidation of cysteine residues into cysteic acid with performic acid or conversion of cysteine into *S*-sulfocysteine by sulfitolysis results in a water-soluble keratin derivative (*12*, *26*−*28*). Complete modification of cysteine with I-AA after cystine reduction has also been used frequently to obtain water-soluble keratin (*27*, *28*). Feather keratin films prepared by solution casting of these water-soluble keratin derivatives cannot be stabilized by disulfide bonds. Because of the relatively low molecular weight of the feather keratin monomer (10.4 kDa) and the absence of cross-links, these films are water soluble and have little mechanical strength.

In previous work it has been found that partial carboxymethylation of cysteine residues prior to dialysis resulted in stable aqueous dispersions after dialysis against distilled water (*16*). These dispersions can be used for producing solution cast films. In these films, unmodified cysteine residues can oxidize readily to form

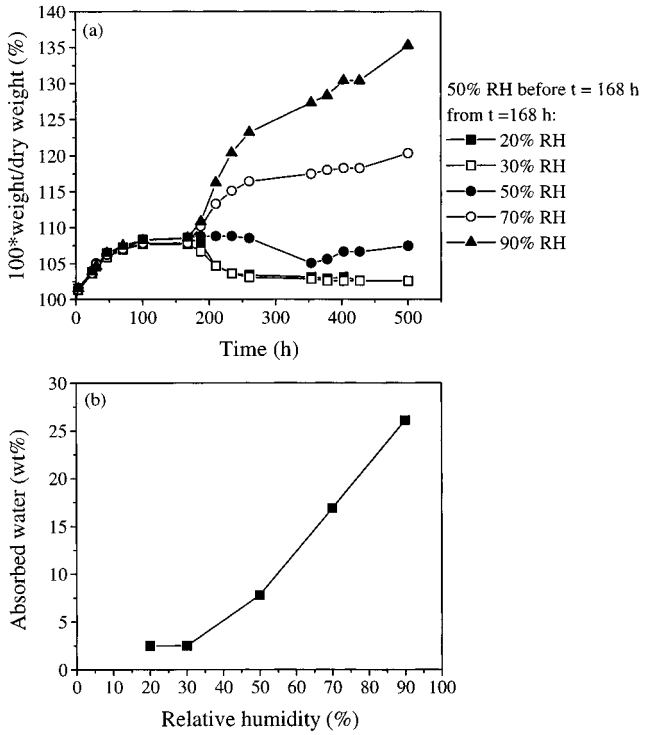

Figure 12. *E* modulus (a), tensile strength (b), and elongation at break (c) of feather keratin films with a degree of cysteine modification of 52%, conditioned at varying RH, containing a fixed amount of glycerol (0.30 g/g of keratin).

disulfide bonds that impart water insolubility and mechanical strength.

In the feather keratin dispersions obtained after dialysis, >95% of the unmodified cysteine residues were already reoxidized to disulfide bonds and only a very few cysteine thiol groups remained (*16*). Upon drying of the dispersions, further oxidation of these thiol groups led to the formation of cross-links between aggregates and oligomers. The amount of water-extractable keratin from the resulting films gives an indication of the amount of disulfide bond formation between the aggregates and oligomers in the film (Figure 1). Despite a small difference in free thiol groups that can still form disulfide bonds upon drying, a large difference in protein extractability from the films was observed. No keratin could be extracted in water or concentrated aqueous urea solutions from films with a degree of cysteine modification up to 50%. All keratin was extracted from films with 70% or more of carboxymethylated cysteine residues.

It is well established that at neutral or alkaline pH and in the presence of a catalytic amount of free thiol groups, thiol/disulfide interchange can occur. In this process, free thiol groups interchange with existing disulfide bonds to generate new, energetically more favorable, intra- and intermolecular disulfide bonds (*29*):

$$R_1S{-}SR_2 + R_3SH \rightleftharpoons R_1SH + R_2S{-}SR_3$$

Recently, thiol/disulfide interchange during heat-induced aggregation of whey proteins has been investigated (*30*−*32*). For partially carboxymethylated feather keratins, the number of possible sites where this reaction can occur, and thus the possibility of forming a network, is greatly reduced at high degrees of cysteine modification. This could explain the high extractability of keratin from these films.

It is commonly known that protein refolding occurs spontaneously under appropriate conditions after denaturation (*33*, *34*). After dialysis of completely carboxymethylated feather keratin, it has been observed frequently that spontaneous assembly of the keratin into filaments occurred, which had an X-ray diffraction spectrum comparable to that of the keratin in the feather (*35*−*38*). DSC of feather calamus revealed an endotherm at ∼175 °C due to denaturation of the β-sheet portions of the feather keratin. Films from partially carboxymethylated feather keratin dispersions without glycerol displayed a denaturation endotherm between 157 and 169 °C when measured immediately after drying. The presence of glycerol and water resulted in a shift of the denaturation temperature to lower temperatures, between 110 and 130 °C (Figure 2). Increasing degrees of modification resulted in a decreased denaturation temperature as well as an increased transition enthalpy (Figure 3). Apparently, denatured and partially carboxymethylated feather keratin was able to refold into a regular conformation during dialysis or during the film-forming process. It cannot be excluded that a secondary structure other than the native β-sheet is formed, although the tendency of known feather keratin sequences to form α-helices is low (*39*, *40*). More secondary structures were formed or more proteins were completely refolded when more cysteine residues were modified, indicated by a higher transition enthalpy. This implies that the feather keratin could refold to a greater extent when the formation of disulfide bonds is hindered or inhibited as often observed for other proteins (*41*). It is not known whether these secondary structures are formed during dialysis or during film formation. A hypothetical model is proposed to account for both effects, assuming that the same structure is formed as in the feather (Figure 13). If the keratins remain random coils during dialysis, a dispersion of protein aggregates stabilized by disulfide bonds is obtained. When β-sheets are subsequently formed during film formation, these will have only limited possibilities to interact with each other to form physical cross-links (Figure 13a). On the other hand, when the secondary structures are formed during dialysis, this could result in the aggregation of β-sheets as well as the formation of physical cross-links and concomitant reconstitution of disulfide bonds (Figure 13b). This effect has been observed in the feather and in films from completely carboxymethylated feather keratins (*24*). The amount of chemical and physical cross-links and the extent of refolding of partially carboxymethylated feather keratin influence the mechanical properties of keratin films. At higher degrees of cysteine modification the amount of chemical cross-links decreases. Generally, *E* modulus and tensile strength decrease and the elongation at break increases when the cross-link density decreases. Higher degrees of cysteine modification result in a higher extent of refolding and thus a higher crystallinity (Figure 4). The *E* modulus and tensile strength generally increase and the elongation at break decreases when a material becomes more crystalline. It is also possible that the number of physical cross-links increases for higher degrees of modification, as indicated in the hypothetical model (Figure 13). The decrease in the amount of chemical cross-links for higher degrees of cysteine modification, together with an increase in crystallinity, possibly accompanied by more physical cross-links, could

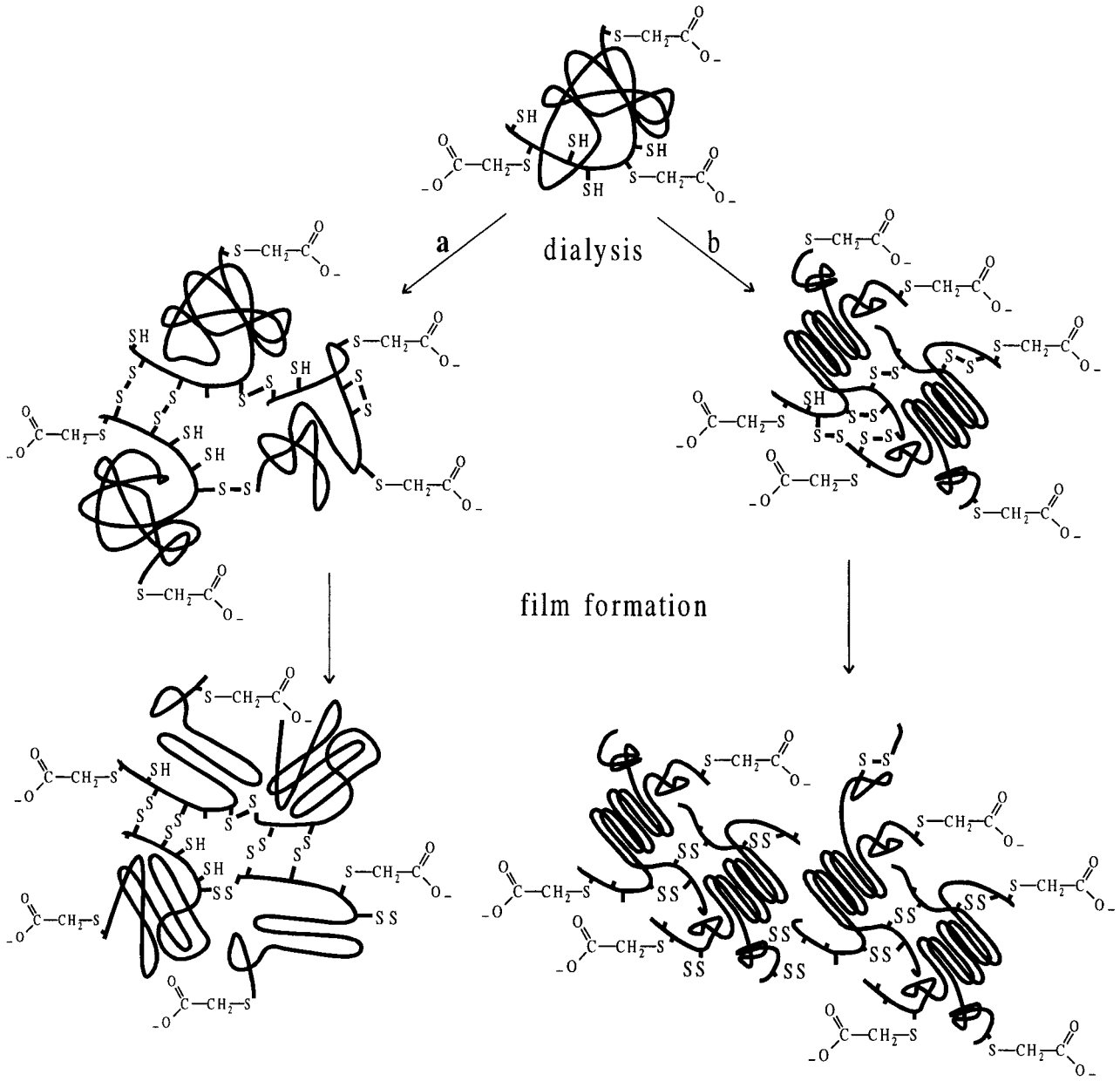

Figure 13. Hypothetical model for the formation of physical cross-links in feather keratin films. In (a) β-sheets are formed during film formation, and disulfide bond formation prevents the refolded structures from interacting with each other as in (b), where the secondary structures are formed upon dialysis and both physical and chemical cross-links are formed.

explain the observed optimum in the mechanical properties of these materials (Figure 6).

Films prepared from proteins such as gluten, casein, and soy as well as keratin are very brittle and need plasticization because of the presence of extensive hydrogen bonding with ionic and hydrophobic interactions. Addition of a plasticizer to a polymer generally results in a decreased elastic modulus and tensile strength, a lower glass transition temperature, and an increased elongation and impact strength. In protein films the most commonly used plasticizers are polyols, mono-, di-, or oligosaccharides, lipids, and derivatives (*3*). Feather keratin with a degree of cysteine carboxymethylation of ~50% was used in further experiments, as films from this material had optimal mechanical properties, under the conditions used (0.30 g of glycerol/g of keratin, 50% RH). The effect of varying the amount of glycerol on the thermal and mechanical properties of the films was investigated. Figure 7 illustrates that the concentration of glycerol in the film strongly influences the amount of absorbed water. A sigmoidal relationship was observed between the quantity of absorbed water and the concentration of glycerol. The solubility of water in nonporous polymeric films depends strongly on the state of the polymer and is generally much lower in the glassy state than in the rubbery state. Probably, at the inflection point of the sigmoidal curve, the total amount of glycerol and water had shifted the glass transition temperature to below room temperature. This is accompanied by an increase in free volume and accounts for an increased solubility of water in the film. Investigation of the thermal properties of feather keratin films with DSC revealed second-order transitions. The effect of annealing on the intensity of the endothermal peak accompanying the second-order transition clearly indicated that the observed transition had all of the characteristic features of the glass transition in amorphous polymers (Figure

Table 2. Comparison of Mechanical Properties of Different Polymer Films

material	*E* modulus (MPa)	tensile strength (MPa)	elongation at break (%)
LDPE[a]	100−250	7−16	100−800
MDPE[a]	200−350	9−21	50−600
HDPE[a]	900−1200	25−45	50−900
PP[b]	1100−1500	31−41	100−600
cellulose acetate[a]	1200	45	8
CM-keratin[c]	300−400	12−25	25−50

[a] Fritz et al. (*43*). [b] 0.38 g of dimethyl phthalate/g of polymer (*43*). PP is polypropylene. [c] Films from partially carboxymethylated feather keratin dispersions with a degree of cysteine modification of 25−50%, 0.30 g of glycerol/g of keratin, and 9.5 wt % water LDPE, MDPE, and HDPE are low-density, medium-density, and high-density polyethylene, respectively.

8). This has also been observed for wheat gluten (*42*). The actual glass transition temperature for the feather keratin films shifted to values close to room temperature for increasing concentrations of glycerol (Figure 9). The mechanical properties also showed that the material gradually became less brittle when more glycerol was added (Figure 10). Conditioned films containing ≤0.10 g of glycerol/g of keratin could not be tested because they were too brittle.

In biopolymer-based plastics, the effect of water absorption on the thermal and mechanical properties is of great importance to the ultimate usability of the material (*43*). The effect of water on the glass transition behavior of several proteins has been investigated previously. The apparent glass transition temperature of silk fibroin films decreased from 179 to 39 °C as the water content increased from ∼0 to 20−23 wt % (*44*). For wheat gluten proteins the glass transition temperature decreased from 120 to 10 °C for water contents of 1 and 15 wt % (*42*). For dry films from partially carboxymethylated feather keratin a glass transition temperature of 121 °C was found when measured immediately after drying. For films containing 0.05 g of glycerol/g of keratin and 3 wt % of water the glass transition temperature was 30 °C. These values indicate the large differences that exist between proteins and the strong influence of water on the thermal properties.

Here, the influence of RH on water absorption and thermal and mechanical properties of feather keratin films was investigated for films with a degree of cysteine modification of ∼50%, containing glycerol as a plasticizer (0.30 g/g of keratin). Specific interactions of water with the matrix and glycerol are responsible for the substantial moisture uptake observed (Figure 11b). Because of the presence of an important amount of glycerol, the actual glass transition temperature for films conditioned at 20 or 30% RH was ∼20 °C and for films conditioned at an RH of 50% or higher, <20 °C. This is also reflected in the large difference in mechanical properties between these films, measured at room temperature (Figure 12). A high *E* modulus and tensile strength were observed for films conditioned at 20 and 30% RH. For films conditioned at an RH of ⩾50%, the *E* modulus and tensile strength had decreased considerably. Nevertheless, properties at high RH are still comparable to those of LDPE, except for the elongation at break (Figure 12; Table 2). Probably, it is the optimum in the number of cross-links and in the degree of crystallinity that accounts for these much improved properties.

ABBREVIATIONS USED

I-AA, iodoacetic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); MWCO, molecular weight cutoff; NTSB, disodium 2-nitro-5-thiosulfobenzoate; RH, relative humidity; DSC, differential scanning calorimetry.

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