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Determination of the renaturation level in gelatin films

D. Achet* and X. W. He

Capsule Research and Development, Lilly France SA, 67640 Fegersheim, France
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The renaturation level of gelatin films was determined by calorimetry and polarimetry. Good agreement was observed for films made with conventional gelatins. A significant discrepancy was found on desiccated films (4% water), on films where KSCN had been added, and on films made with hydrolysed, chemically modified, or crosslinked gelatins. These results are discussed in relationship to the two techniques used and to the renaturation mechanism in steps. The discrepancy was attributed to the fact that some gelatin strands were frozen in intermediate states.

(Keywords: gelatin films; renaturation; conformation)

INTRODUCTION

Gelatin is perhaps best known and most widely used for its gelling ability. It is unique among hydrocolloids in forming thermo-reversible gels with a melting point close to body temperature. This property is particularly significant in edible and pharmaceutical applications.

At the molecular level, the gelation of a gelatin solution results from the renaturation of random gelatin strands to the triple-helix structure that exists in native collagen. The renatured triple helices act as junctions from which a three-dimensional network is formed. The network structure and the physical properties of gelatin gels are mainly conditioned by their renaturation level. It has been convincingly demonstrated that the mechanical properties of gelatin films are closely related to this parameter[1]. The phenomenon of gelatin renaturation is still being actively studied and the determination of the renaturation level is of great interest for both fundamental understanding of the process and for application purposes[2–11].

Many techniques have been used to monitor gelatin renaturation: polarimetry, differential scanning calorimetry (d.s.c.), ultra-violet (u.v.) spectrometry and X-ray diffraction. Polarimetry and d.s.c. appear to be the most convenient and widely used methods. However, the principles on which these two techniques work are different: polarimetry is conformational and d.s.c. is energetic. In some cases, renaturation levels determined by these two methods show significant discrepancies. This paper reports and discusses the results of the measurement of the renaturation level on gelatin films. The discussion focuses on the principle of the determination of the renatured gelatin fraction by the two methods and on the renaturation mechanism.

* To whom correspondence should be addressed

EXPERIMENTAL

The gelatins used to prepare films are listed in *Table 1*. Gelatin samples 1 to 4 are typical pharmaceutical grades. Chemically modified gelatin samples were provided by gelatin suppliers. A gelatin solution of 26.5% was matured at 55 C for 15–18 h and cast into a film. The wet film

Table 1 Renaturation level measurement results of gelatin films

Gelatins	h (%)	ΔH (cal g^{-1})	$-[\alpha]$ (deg dm^{-1})	$\chi^{[\Delta H]}$ (%)	$\chi^{[\alpha]}$ (%)
Limed bone gelatin 1	16.3	6.5	685	50	52
Limed bone gelatin 2	16.2	6.4	669	50	51
Acid pig-skin gelatin 3	15.8	7.0	720	55	55
Acid pig-skin gelatin 4	16.2	6.8	722	52	56
70/30 blend of gelatins 1 and 3	16.3	6.5	677	50	51
50/50 blend of gelatins 1 and 3	16.2	6.6	712	51	55
30/70 blend of gelatins 1 and 3	16.2	6.6	724	51	56
70/30 blend, 2% of KSCN[a]	14.8	5.6	654	44	49
Succinylated limed bone gelatin I	15.3	5.0	642	39	48
Succinylated limed bone gelatin II	16.9	4.6	572	36	42
Phthalated limed bone gelatin	16.8	3.3	523	25	37
70/30 blend, 90 C/4 h[b]	16.4	5.2	665	41	50
Limed bone H gelatin[c]	16.7	5.0	664	39	50
Gelatin 2[d]	3.9	2.5	608	19	45
Limed bone H gelatin[d]	4.0	1.8	588	14	43

[a] 2% of KSCN was added to the gelatin solution
[b] Gelatin solution has been maturated at high temperature (90 C) for 4 h instead of 55 C
[c] Limed bone H gelatin is characterized by a high content of microgel and a high viscosity: η(6.66%, 60 C) = 10.4 mPa s
[d] Gelatin films were additionally dehydrated in desiccators containing silica gel for two days

was dried in a climatic chamber set at 20°C 40% r.h. for 1 h and then equilibrated at 20°C 50% r.h. for 24 h prior to tests. The films were controlled to a thickness of $100 \pm 5\ \mu m$ and the moisture content (h) to approximately 16%, as determined by the Karl Fischer method. This technique is based on an electrometric system where the water reacts chemically with the Karl Fischer reagent and allows the measurement of the water content of gelatin films with an accuracy of $\pm 0.2\%$. A film of native type I collagen of bovine origin (supplied by Coletica) was used as a reference in the calculation of the renaturation level obtained by each technique.

Calorimetric measurements were performed using a Perkin Elmer DSC-4. Thermograms were obtained by using sealed stainless-steel d.s.c. pans to prevent any loss of water during heating at a rate of $10°C\ min^{-1}$.

Optical rotation measurements were carried out on an automatic Autopol III Rudolph Research polarimeter at a wavelength of 589 nm. A goniometer was mounted near the detector to perform optical rotation measurements on dried films. This configuration allowed the film to be maintained flat. The film sample was placed perpendicularly to the polarized beam during the measurement.

RESULTS AND DISCUSSION

Experimental results are reported in *Table 1*.

The renaturation level monitored by polarimetry was given by the following standard equation:

$$\chi^{[\alpha]} = \frac{[\alpha] - [\alpha]_a}{[\alpha]_{col} - [\alpha]_a} \quad (1)$$

where $[\alpha]_{col}$ and $[\alpha]_a$ represent respectively the specific rotation of the native collagen film ($1210\ deg\ dm^{-1}$) and that of a fully amorphous gelatin film prepared at 60°C for which $\Delta H = 0$ ($113\ deg\ dm^{-1}$).

The renaturation level determined by d.s.c. was calculated from the equation:

$$\chi^{[\Delta H]} = \frac{\Delta H}{\Delta H_{col}} \quad (2)$$

where ΔH_{col} is the melting enthalpy of the native collagen film, which was given as $12.9\ cal\ g^{-1}$.

The gelatins listed in *Table 1* can be divided into two groups. The first group represents conventional gelatins and their blends, for which $\chi^{[\Delta H]}$ and $\chi^{[\alpha]}$ are in good agreement. The second group includes the modified gelatins and those subjected to a special treatment, for which a significant discrepancy was observed. Measurement by d.s.c. gives much lower values of χ than those obtained by polarimetry. This observation, which will be discussed further, can be attributed to intermediate states in the renaturation mechanism.

The three-step renaturation mechanism, which had been the most commonly accepted in the literature, was proposed almost simultaneously by von Hippel and Harrington (1959)[12] and Flory and Weaver (1960)[13]. This three-step scheme has been written as:

$$C \xrightarrow{\text{initiation}} NC \xrightarrow{\text{growth}} I \xrightarrow{\text{stabilization}} \tfrac{1}{3}(H)$$

Here C is the largely disordered random-coil state. NC is the conformation of the chain after initiation of the poly(L-proline) II type of helix in sections of the chain that are particularly rich in imino acids, and is the centre or nucleus from which the helix grows. I is an ordered chain intermediate having the form of a poly(L-proline) II type helix along the gelatin chain, through growth from the nucleated regions. Finally $\frac{1}{3}(H)$ is the chain in the collagen-like structure after interchain stabilization by hydrogen bonds. According to this mechanism there are four possible states in gelatin films, and only the strand fraction in the state $\frac{1}{3}(H)$ has a real collagen-like structure. Evidence has accumulated to favour the Flory–Weaver view that state I is a transient, unstable intermediate that is transformed into a collagen structure much more rapidly than it is formed. Thus the number of gelatin strands in state I could be ignored and the growth phase is the rate-determining step. The rate is a function of the composition of the chains, their cross-linking degree, the solvent environment and the temperature[14]. In fact, the absence of state I reduces this mechanism to that proposed by Harrington and Rao[15] and is the currently accepted mechanism[16].

The fact that gelatin is an active optical laevo-rotatory substance makes optical rotation sensitive and non-destructive for the measurement of the concentration of gelatin solutions. Because the rotatory dispersion curves of native and denatured protein solutions are typically quite different, the measurement of the optical rotation of a given sample has frequently been used to determine the renaturation level. The specific rotation $[\alpha]$ can be expressed by the sum of two terms[17]:

$$[\alpha] = (100/nM)\sum[R_i] + \chi_{conf}[\alpha]_{conf}$$

where $[R_i]$ is the intrinsic residue rotation of the ith amino acid residue, n is the number of residues in the chain, M is the mean residue weight, and χ_{conf} and $[\alpha]_{conf}$ are the fraction and specific rotation of gelatin chains having helical conformation. The first term depends only on the gelatin composition and corresponds to the value determined on the completely amorphous or denatured gelatin sample $[\alpha]_a$. The second term is the contribution of any conformational order, i.e. collagen-fold helix. According to the above three-step renaturation mechanism, we have:

$$\begin{aligned}[\alpha] &= [\alpha]_a + \chi_{conf}[\alpha]_{conf} \\ &= [\alpha]_a + \chi_{NC}[\alpha]_{NC} + \chi_I[\alpha]_I + \chi_{\frac{1}{3}(H)}[\alpha]_{\frac{1}{3}(H)}\end{aligned}$$

Consequently the renaturation level determined by this method is given by the sum of the gelatin strand fractions in these states NC, I and $\frac{1}{3}(H)$. Thus equation (1) becomes:

$$\chi^{[\alpha]} - \chi_{conf} = \chi_{NC} + \chi_I + \chi_{\frac{1}{3}(H)} \quad (3)$$

Equation (3) is obtained from the hypothesis that $[\alpha]_{conf} = [\alpha]_{NC} = [\alpha]_I = [\alpha]_{\frac{1}{3}(H)}$. This will be discussed further.

In contrast to the optical rotation measurement, the determination of the melting enthalpy ΔH by d.s.c. is destructive; all the gelatin chains are transformed into the random-coil state by increasing the temperature:

$$\tfrac{1}{3}(H) \xrightarrow{\Delta H_{\frac{1}{3}(H)}} C$$

$$I \xrightarrow{\Delta H_I} C$$

$$NC \xrightarrow{\Delta H_{NC}} C$$

and we have

$$\Delta H = \chi_{NC}\Delta H_{NC} + \chi_{I}\Delta H_{I} + \chi_{\frac{1}{3}(H)}\Delta H_{\frac{1}{3}(H)}$$

However, owing to the absence of interchain stabilization in the states NC and I, their conformation could be considered as one of the possible conformations of a random coil, and the energy level difference between states NC, I and C could be neglected: $\Delta H_{NC} \simeq 0$, $\Delta H_{I} \simeq 0$.

It has been reported that in systems where denaturation does not involve any significant change in solvation of buried hydrophobic groups (e.g. the collagen–gelatin system[18,19]), the enthalpy of the helix → coil transition may be considered to arise solely from hydrogen-bond interactions[20]. Hence, the renaturation level determined by d.s.c. reflects the gelatin strand fraction in a real collagen-like structure $\frac{1}{3}$(H):

$$\Delta H = \chi_{\frac{1}{3}(H)}\Delta H_{\frac{1}{3}(H)}$$

and equation (2) becomes:

$$\chi^{[\Delta H]} = \chi_{\frac{1}{3}(H)}$$

Equations (3) and (4) show clearly that $\chi^{[\Delta H]}$ will be in agreement with $\chi^{[\alpha]}$ only when $\chi_{NC} \simeq 0$ and $\chi_{I} \simeq 0$. These conditions are generally true, because of the absence of strands in state I, as mentioned above, and because of the small size of the nuclear centres NC, and are confirmed by the values of $\chi^{[\Delta H]}$ and $\chi^{[\alpha]}$, which were found to be in agreement in the case of the first group of conventional gelatins (see *Table 1*).

Significantly lower renaturation levels obtained by d.s.c. for the gelatin films included in the second group in *Table 1* were found to comply with their more marked brittle behaviour[1]. However, this result was not confirmed by optical rotation measurements: $\chi^{[\alpha]}$ is significantly higher than $\chi^{[\Delta H]}$. This discrepancy can be explained by the fact that some strands were blocked in an intermediate state during the renaturation process and were still accounted for in the renatured fraction by optical rotation measurement. Beier and Engel have demonstrated that optical rotation alone can be a misleading indication of the collagen renaturation[21].

The presence of KSCN, which is a hydrogen-bond-disrupting agent, should interfere with the formation of hydrogen bonds so that a significant number of strands are blocked in state I: a decrease in renaturation level measured by d.s.c. from 50% to 44% was observed when 2% of KSCN was added to the gelatin solution. However, because the strands in state I are accounted for by the renatured fraction of the optical rotation measurement (equation (3)), $\chi^{[\alpha]}$ gives an overestimate of the renaturation level: $\chi^{[\alpha]}$ decreases only from 51% to 49%.

In the three-step mechanism, the growth step NC → I implies that three segments of gelatin strands simultaneously take a helical conformation and come together in register to form a triple helix. We suppose that this process might be disrupted in particularly unfavourable conditions, and an additional intermediate state NCI between NC and I could be involved:

$$C \rightarrow NC \rightarrow NCI \rightarrow I \rightarrow \tfrac{1}{3}(H)$$

In such a state, the gelatin segments would individually take the helical conformation but would not yet have come together in register, and the kinetics of registration that transforms state NCI to state I would depend upon the primary structure of the protein and the mobility of the gelatin chain, which is a function of the thermodynamic conditions such as temperature, concentration, etc. In the case of unfavourable conditions for the registration, some chains could be blocked in state NCI, and the chain fraction in NCI could become significant ($\chi_{NCI} \neq 0$). This would consequently explain why the measurement of optical rotation provides an overestimate of the renaturation level in such cases.

The following three cases support the explanations based upon the disruption of the growth step and the presence of an intermediate state NCI leading to an overestimate of the renaturation level by polarimetry, $\chi^{[\alpha]} > \chi^{[\Delta H]}$:

(1) The significant change in the chemical structure of gelatin strands by succinylation and phthalation alters the physico-chemical properties of the modified gelatin, especially the renaturation ability as shown by the $\chi^{[\Delta H]}$ values. Results of $\chi^{[\Delta H]} < \chi^{[\alpha]}$ observed for all three modified gelatins suggest that the loss of native chemical structure of the gelatin strands or the substitution of $-NH_2$ functions by more voluminous functions of different nature has disrupted probably both the registration and stabilization steps, causing some gelatin strands to be blocked in intermediate states NCI and I. In addition $[\alpha]_a$ was experimentally found to be 101° for the succinylated gelatin instead of 113°. This change is not significant enough to impact $\chi^{[\alpha]}$.

(2) Ripening of the gelatin solution at 90°C for 4 h led to a significant decrease in intrinsic viscosity, from 47.4 to 37.3 ml g^{-1}. Viscosity is related to the molecular weight, and this observed drop in intrinsic viscosity is mainly due to the hydrolysis of the gelatin strands causing cleavage on specific sites. It is well known that highly hydrolysed gelatin loses its gelation ability. In the present case of partial hydrolysis, if the cleavage occurs in the helical section of protein strands, the helical growth could be disrupted and some gelatin strands would be blocked in the intermediate state NCI as supported by the results obtained: $\chi^{[\Delta H]} = 41\%$ *versus* $\chi^{[\alpha]} = 50\%$.

(3) The result obtained in the case of the high-viscosity gelatin, containing a significant content of microgel components, can be explained by a disturbed registration due to the reduction of chain mobility and the steric hindrance that results from the high level of crosslinking or branching of the protein strands.

The existence of the intermediate state I is strongly supported by measurements on desiccated films. The collagen-like structure of gelatin can only exist if water molecules are included in the crystals formed[22,23]. A definite amount of water is believed to be necessary to maintain the native conformation of collagen molecules[24–26]. The structural water, directly bound to the gelatin strands by hydrogen bonds, contributes to the stabilization of collagen-like structure. In the case of dry gelatin films, desiccated by silica gel at 20°C to 4% of residual moisture, most of the collagen-like helices will have lost their crystal structure because of the removal of structural water. However, under these conditions the gelatin strands do not have enough mobility to recover the random-coil state, so the helices collapse on the spot with the memory of their triple-helical conformation retained:

$$\tfrac{1}{3}(H) \rightarrow I$$

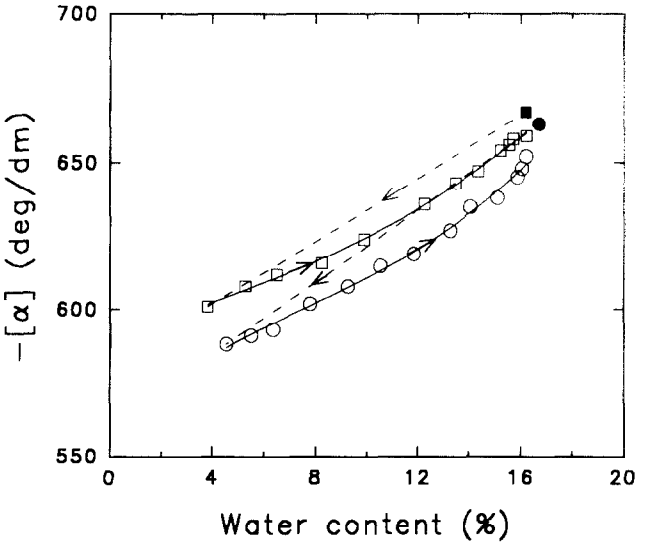

Figure 1 Evolution of specific rotation *versus* gelatin film moisture during its take-up after desiccation: (□, ■) gelatin 2; (○, ●) lime bone H gelatin

The d.s.c. measurement shows a significant decrease in renaturation level from 50% (LB gelatin 2) and 39% (LB-H gelatin) to 19% and 14%, respectively, reflecting the real fraction of gelatin strands remaining in a collagen-like structure after the desiccation. Polarimetry shows a much more moderate decrease, from 51% and 50% to only 45% and 43%. The most significant difference ($\chi^{[\alpha]}-\chi^{[\Delta H]}$) observed on the desiccated films confirms the abundance of gelatin strands in state I formed after the removal of structural water.

On the other hand, although the optical rotation $-[\alpha]$ is somewhat reduced by film desiccation, the retention of the memory of the triple-helical conformation in the latter case is convincingly demonstrated by the recovery of the optical rotation after the films have been remoisturized.

Figure 1 shows the change in the specific rotation as a function of the film moisture, caused by putting the dehydrated films in an environment at 20°C/50% r.h. Under such conditions, any helical growth cannot occur quickly enough, because the glass transition temperature of the system (about 45°C for a moisture content of 16%) is much higher than the storage temperature and thus the segmental mobility is significantly reduced[27]. The observed increase in $-[\alpha]$ results from the stabilization of the gelatin strands by the water molecules captured from the environment, in state I as previously existed and caused by the removal of structural water during the desiccation. Based on the almost total recovery of the original optical rotation after remoisturization, the observed difference in $-[\alpha]$ between the films before and after desiccation could be mainly attributed to the difference in specific rotation between states I and $\frac{1}{3}$(H), i.e. $-[\alpha]_I$ is somewhat smaller than $-[\alpha]_{\frac{1}{3}(H)}$. In the light of this observation, the preceding hypothesis that the states NC, I and $\frac{1}{3}$(H) have identical specific rotation should be modified to $[\alpha]_{NC}=[\alpha]_I=[\alpha]_{\frac{1}{3}(H)}(1-\xi)$, and equation (3) becomes:

$$\chi^{[\alpha]}=(\chi_{NC}+\chi_I)(1-\xi)+\chi_{\frac{1}{3}(H)}$$

As $\xi \simeq 15\%$ according to *Figure 1*, it would imply that $\chi^{[\alpha]}$ is an approximate value of χ_{conf} when protein strands are partly blocked in the intermediate states

$$\chi^{[\alpha]} \simeq 0.85(\chi_{NC}+\chi_I)+\chi_{\frac{1}{3}(H)}$$

or

$$\chi_{conf} \geq \chi^{[\alpha]} \geq 0.85\chi_{conf}$$

and

$$\chi^{[\alpha]}-\chi^{[\Delta H]} \simeq 0.85(\chi_{NC}+\chi_I)$$

CONCLUSION

The renaturation level of gelatin films can be conveniently determined by both d.s.c. and polarimetry. D.s.c. (based on the energy state) reveals the fraction of gelatin strands in a collagen-like stabilized structure, whereas polarimetry (based on the conformational state) gives the total fraction of gelatin strands having a helical conformation. The agreement in results obtained by these two methods reflects the absence of gelatin strands in intermediate states of the renaturation process, which occurs in steps. In the case of unfavourable renaturation conditions, such as the introduction of KSCN, the chemical modification of gelatin strands, the steric hindrance due to crosslinking or the removing of structural water by desiccation, the presence of gelatin strands in the intermediate states became significant and leads to an overestimate of the renaturation level as measured by polarimetry. Thus calorimetry is more suitable to monitor the real fraction of gelatin chains in a collagen-like structure, and it is mainly upon the value of this fraction that the mechanical properties of gelatin films depend.

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