Quantitative structural analysis and physical properties of silk fibroin hydrogels

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The effect of dehydration on the molecular structures and physical properties of fibroin gels was investigated. The physical properties that are discussed include strength, water content and thermal stability. It was found that the molecular structure of fibroin was not changed by the change in water content of the gel, while the physical properties of the gel, however, were changed significantly. A quantitative analysis of the molecular structures was carried out by separating the components of the i.r. spectra. The spectral parameters of the amide I, II, and III absorption bands, corresponding to the silk I, β, and random coil conformations, respectively, have been derived. In addition, the fibroin gels were characterized by their water contents, strength and thermal stability.

(Keywords: fibroin gel; water content; molecular structure)

INTRODUCTION

Recent studies[1-3] have shown that silk fibroin has good biological compatibility. These results indicated that it could be used both as a food component and also as a biomaterial in the medical field. We have produced fibroin gel and studied its various properties, and, in particular, the structure of the gel has been studied by using infra-red (i.r.) spectroscopy.

I.r. spectroscopy was one of the first experimental methods to be recognized as being potentially useful for estimating the secondary structures of polypeptides and proteins. The application of i.r. spectroscopy to the analysis of the secondary structure of proteins is based on the sensitivity of the peptide group absorption bands to the polypeptide chain conformation. I.r. spectroscopy covering the range of amide vibrations has been widely used for qualitative evaluation of the character of the chain secondary structures in polypeptides and proteins. A theoretical analysis of the nature of the frequency splitting of the amide bands, and experimental determination of their intensities, have made it possible to use i.r. spectroscopy for quantitative analysis[4]. A similar method for the estimation of protein and polypeptide secondary structures, on the basis of contour-shape analysis of the amide bands, was also suggested by other workers[5-7]. The method included a quantitative measurement of the intensities and other spectral parameters of the absorption bands of the polypeptides in their ordered and random conformations, and also the amino acid side chains. A similar method can also be used for silk fibroin gel.

The i.r. spectrum of silk has been of interest to many research workers over the past few years. The greatest efforts have been made in attempts to explain the observed vibrational frequencies and intensities of silk in its silk I and silk II states. Many studies concerning the structure of fibroin have indicated that the physical properties of these silk materials are influenced by their structural features. For example, Pauling[8] suggested that the softness and water holding capacity of a fibroin membrane might be controlled by its geometrical structure. The vibrational frequencies and their corresponding intensities have received considerable attention in the fibre, film and membrane states, but very little in the gel state, as only a very few studies concerning the structure of fibroin gel have been reported. A thorough understanding of the gel structure in these systems is, therefore, an essential requirement.

It was found that the β-structure is responsible for the gelation of fibroin solutions[9]. It was also qualitatively assumed that the β-structure is dependent on the pH of the fibroin gel solution[10]. Therefore, a quantitative understanding of the β-form, fibroin gel structure under different conditions appeared to be an interesting study. In this paper, based on the above considerations, we have quantitatively analysed the characteristic molecular vibrations of fibroin gel. The purpose of this present study is to investigate, in a quantitative manner, the secondary structure of dehydrating fibroin gel by analysing the amide I, II, and III regions of the i.r. spectra. The conformational characteristics, with relationship to the physical properties of the gel, were also studied.

EXPERIMENTAL

Samples

Cocoon shells of the silkworm, *Bombyx mori*, were refined by first treating with 50 times their amount of water (vol/wt) for a period of 20 min. Degumming was then carried out using sodium carbonate solution (0.5 wt%), and the refining was continued for another

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20 min at 90–100°C, after which the sample was washed with warm water to obtain the refined fibroin. This was dissolved in a mixture of ethanol and calcium chloride solution[11], and the solution was separated from any foreign particles by centrifugation at 1000 *g* for 10 min. A regenerated fibroin solution was obtained by filtering the centrifuged solution and by dialysing the filtrate for 3 days using cellulose tubes. After determining the concentration of the protein, the solution was kept in an air-conditioned room at 20°C, at a relative humidity of 56–64% for gel formation to occur. Details of the various samples used are summarized in *Table 1*.

Measurements

A Tensilon UMT-II universal tensile testing instrument (Toyo Measuring Instruments Co. Ltd, Japan), was used for determining the strength of the gel (which was prepared in a commercially available ice-cream cup). A gel obtained from 25 ml of fibroin solution was taken and its strength measured by inserting a modified probe (with a diameter of 1 mm), down to a depth of 5.0 mm. The insertion was carried out by elevating the cup on the cross-head of the machine, which was moving at a speed of 10 mm min^{-1} (chart speed = 200 mm min^{-1}). The strength of the gel was measured at different water contents. The water contents of the various gels were calculated by using the following equation:

$$w = 100 - [(W_0 - W_1)/W_0](\times 100\%) \qquad (1)$$

where w is the water content of the gel at a certain dried state, W_0 is the initial weight of the gel and W_1 is the weight of the gel at this particular dried state.

Thermogravimetric analysis (t.g.a.) was carried out in air using a Shimadzu DT-30 thermal analyser, with a heating rate of 10°C min^{-1}. The decomposition temperatures and weight losses of the gels were evaluated by measuring the intersection points of the tangents for the various t.g.a. curves.

I.r. spectra were recorded at room temperature on a Shimadzu IR-435 spectrophotometer. The proportions of the different molecular conformations were calculated by measuring the areas under their characteristic peaks, as reported previously[12].

RESULTS AND DISCUSSION

It was found that the fibroin gel shrank on dehydration, with a corresponding change in colour. In addition, the strength of the gel was also changed significantly. In order to obtain an insight into this phenomenon, and also to compare this gel with the most widely used gels that show swelling properties, we first attempted to study the swelling properties of dehydrated fibroin gels with different initial water contents. It was generally observed that the initial yellow colour of the dehydrated gel became a fainter colour, and showed only a little swelling. The gel at the final (equilibrium) water content level (at ambient pressure), absorbed ~20 wt% water, with a little swelling, after the gel had been kept in the water for 48 h, and the colour had become a faint yellow. Therefore, it can be said that water molecules had penetrated into the gel without any significant effects on the swelling properties, with the water molecules probably filling the pores that remained after dehydration.

Table 1 Weights of samples of fibroin gel at different time intervals[a]

	Sample weight (g)					
Time (h)	1	2	3	4	5	6
0	26.00	25.50	25.80	25.00	25.60	25.60
21	19.50	19.13	19.35	18.76	19.21	19.20
40	15.60	15.30	15.48	15.01	15.35	15.36
65	10.40	10.21	10.33	10.00	10.24	10.25
95	6.11	6.00	6.06	5.88	6.02	6.01
125	5.61	5.48	5.55	5.35	5.51	5.50

[a] Underlined samples were used for i.r. spectroscopy and thermal analysis

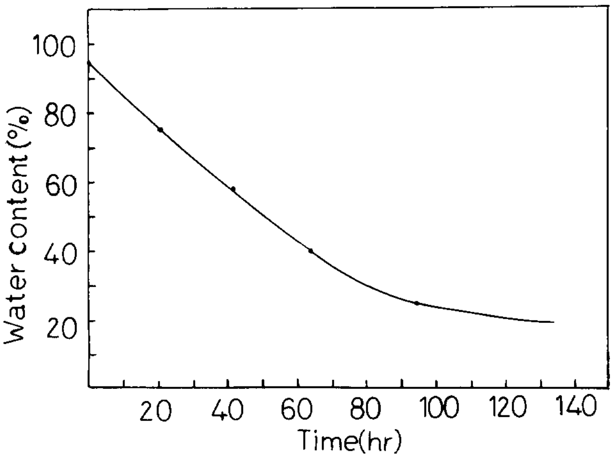

Figure 1 Loss in water content of the fibroin gel as a function of time

Figure 1 shows the change in water content of the gel as a function of time. The dehydration rate was high until ~65 h, but became slower after this time. The higher dehydration rate indicated that the water molecules on the surface of the gel network evaporated during the initial stage, after which period the water molecules inside the collapsed gel network evaporated at a much slower speed. It can be concluded that the evaporated water molecules were being held by the network in the fibroin gel. The remaining water in the gel at the final stage of dehydration, i.e. when the gel reached its equilibrium state with respect to the water content, might have some relationship to the physical properties of the gel, as it was found that the dehydrated gel, after drying in an oven, became brittle in nature.

In the next stage, the following experiments were performed in order to investigate the physical properties and structures of samples of fibroin gel with different initial water contents.

The strength of gels with different water contents was measured by compressing the samples. *Figure 2* shows the relationship between the strength of the fibroin gel and the time. As can be seen from this figure the strength of the gel increased as the time increased. From the dehydration rate, the water content was measured, and the relationship between the water content and the gel strength was studied. *Figure 3* shows the results obtained. The gel strength gradually increased with a decrease in water content, with the strength starting to increase rapidly after the water content had reached 40 wt%, after a period of 65 h. The strength of the gel at the equilibrium state was not within the measurable range of the testing equipment. The highest measured strength was 30.60 kg cm^{-2}, after 95 h, when the water content was 23.50 wt%. When the intermolecular spaces of the fibroin

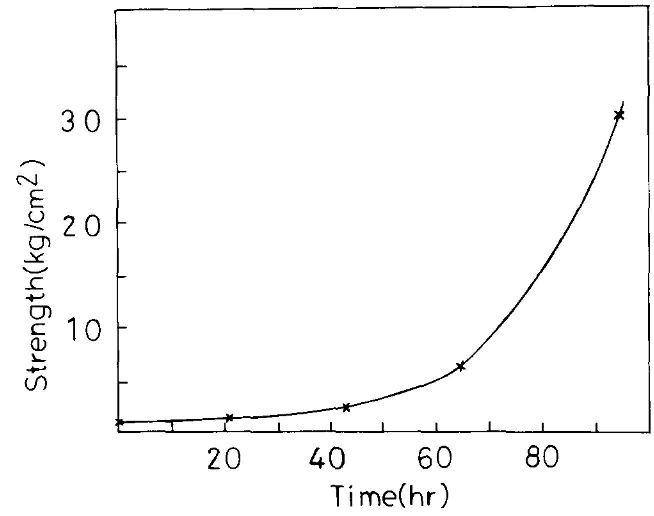

Figure 2 Increase in the fibroin gel strength as a function of time

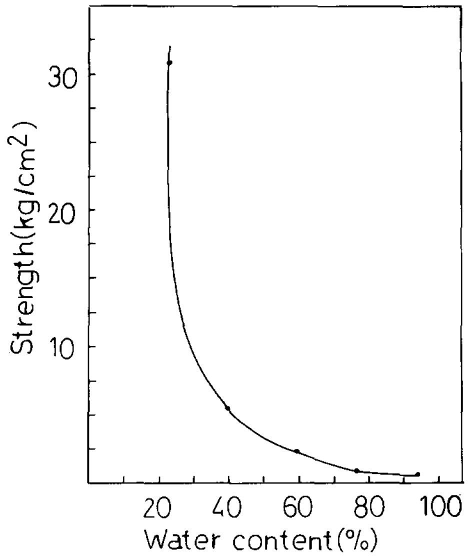

Figure 3 Relationship between the strength and water content of the fibroin gel

molecules are occupied by large numbers of water molecules, the strength is reduced: when the amount of water molecules was reduced on dehydration an increase in strength was observed, as a result of the close contact of the fibroin molecules. Therefore, it is concluded that the increase in strength might be due to the dense aggregation of fibroin molecules on dehydration. The water molecules only prevent the gel from collapsing into a compact mass.

Thermogravimetric measurements were carried out in order to analyse the changes in the thermal properties of the silk fibroin hydrogels during the dehydration process. *Figure 4* shows t.g.a. thermograms of silk fibroin hydrogels with different water contents. Significant variations in decomposition temperatures were not observed for the different gels. All samples showed two decomposition temperatures: the decomposition at ~300°C is attributed to a disintegration of the intermolecular side chains during the crystalline melting process, while that at ~550°C is attributed to a main chain disintegration, coupled with simultaneous carbon atom rearrangements[13]. It was also reported[14] that the decomposition at 300°C indicated the low crystallinity of the unoriented β-type configuration and, therefore, it can be said that there is less possibility of obtaining a crystalline β-structure, which occurs in the temperature range 325–330°C. It was found that the weight losses at 500°C were low for the gels with low water contents. This suggests that the fibroin molecules come into close contact with each other during the dehydration process and form a dense aggregate which could mechanically resist carbon atom rearrangements, thus resulting in low weight losses. This phenomenon of the fibroin molecules could be a possible reason for the thermal stability of the dehydrated fibroin gel.

It was also believed that the structural conformations do not change during the removal of water molecules from the silk fibroin hydrogels. Therefore, a quantitative conformational analysis was carried out in order to obtain an insight into the gel structure. *Figure 5* shows the i.r. spectra of fibroin gel samples with different water contents. In these spectra, peaks at 1620–1626 cm^{-1} (the amide I band), at 1508–1512 cm^{-1} (the amide II band), and at 1228–1230 cm^{-1} (the amide III band) indicate that the conformation is transformed into the β-form in the case of samples 1 and 2. However, it was found that the contour shapes of the other samples varied with their water contents. All of the peaks observed in the three amide band regions varied in their width and intensities.

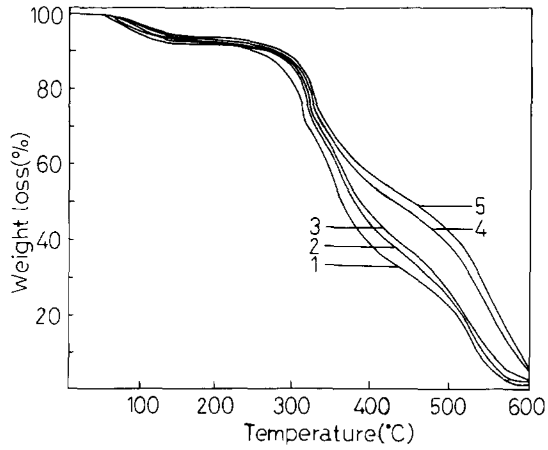

Figure 4 T.g.a. thermograms of fibroin gels with different water contents (details of the samples used are given in *Table 1*)

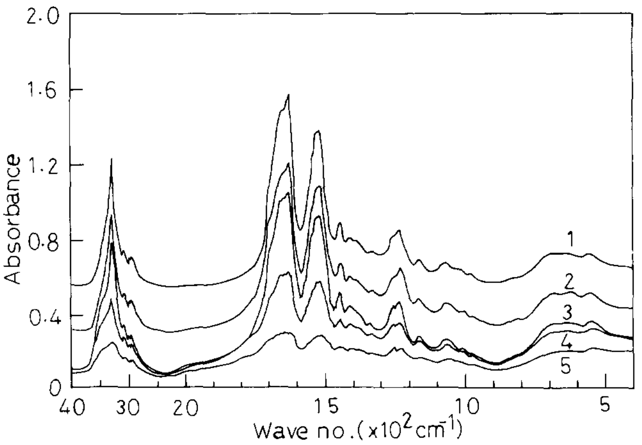

Figure 5 Infra-red spectra of fibroin gels with different water contents (details of the samples used are given in *Table 1*)

Table 2 Amounts of the various molecular conformations in samples of fibroin gels, calculated from their i.r. spectral peak areas

		Amide I			Amide II			Amide III		
	Sample[a]	Random	Silk I	β	Random	Silk I	β	Random	Silk I	β
σ		1666	1652	1695	1528	1548	1512	1257	1280	1230
(cm^{-1})	1			1626						
Content										
(%)		41.3	11.7	47.0	41.4	11.3	47.3	41.2	11.6	47.2
σ		1670	1650	1700	1528	1550	1508	1253	1275	1228
(cm^{-1})	5			1620						
Content										
(%)		45.0	11.6	43.4	45.0	11.6	43.4	44.9	11.7	43.4

[a] Sample details given in *Table 1*

In particular, a significant variation was observed for the mixed vibration, amide III band. A single peak in this region for the gel containing 95 wt% water began to change at a water content of 60 wt% (after 40 h), at which stage the gel strength started to increase rapidly. A separation into two peaks was observed, which became more clearly defined as the dehydration process continued. Two peaks were clearly observed for the gel which contained 23.5 wt% water, after the highest strength of 30.60 kg cm^{-2} was recorded (after 95 h). To summarize, the peak, which appears at 1235 cm^{-1} for the gel with a maximum water content (sample 1), splits into two peaks, at 1230 and 1255 cm^{-1}, when the water content is lowest (samples 3–5).

The spectroscopic results indicate, qualitatively, changes in the structural conformations of the gels with variations in the water contents. Therefore, in order to verify this fact, the three amide bands for gel samples 1 and 5, containing high and low water contents, respectively, were quantitatively measured using the same method as previously reported in the case of fibroin membranes[12]. The proportions of the conformations found in this way are summarized in *Table 2*. It was found that the amount of the random conformation was increased by 4–5%, while the content of the β-conformation was decreased by the same amount in the case of sample 5. The amount of the silk I conformation remained unchanged, with a value of $\sim 11.60\%$ obtained from all of the amide bands. These minor quantitative variations in both conformation and frequency could not be considered as evidence of structural changes, if we take into account the instrumental or operational errors that can occur during these measurements. This indicates that a change in contour shape does not always suggest a quantitative variation in the conformation, as has been shown here in the case of fibroin gels with different water contents. The contour shape was different due to shrinkage of the gel on dehydration, but the proportions of the various conformations remained unchanged. The reason behind this might be due to variation in the vibrational changes or the resisting force generated by the close contact of the fibroin molecules after dehydration. The percentages of the various conformations were almost the same in all cases, and were always independent of water content.

CONCLUSIONS

The results indicated that the conformations of the silk fibroin hydrogels remain (quantitatively) unchanged at different dehydration levels, with the structure independent of the water content. The higher the concentration of the aqueous fibroin, the higher is the gelation rate[10], indicating that close contact of fibroin molecules is of prime importance in the gelation process. Similarly, close contact of fibroin molecules rendered both a high strength and a high thermal stability to the dehydrated fibroin gels. A solvent such as water is required in the gelation of aqueous fibroin, in order to obtain a gel with a required volume and shape. The gel, once formed, has a β-oriented network structure, as a result of intermolecular hydrogen bonding between the molecular components: this stabilizes the conformation with an additional rotational stability, thus holding the water molecules in the said structure.

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