Physical properties and structure of silk: 4. Spherulites grown from aqueous solution of silk fibroin

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Spherulite formation in silk fibroin films cast from aqueous solution has been studied for crystallization conditions such as drying temperature, drying rate and pretreatment (freezing). Negatively birefringent spherulites in the α-form are observed in films cast between 0° and 40°C, and with a high drying rate at 20°C; positive β-form spherulites appear at higher temperatures up to 80°C and with a low drying rate at 20°C. Positive β-form spherulites are also obtained by freezing fibroin solution at −2° to −18°C and then drying at 20°C. It is found that positive β-form spherulites grow at 20°C on the surface of well-oriented β-form silk fibroin filaments (degummed silk) immersed in fibroin solution.

INTRODUCTION

Three conformations have been found for silk fibroin by X-ray diffraction and infra-red spectroscopy[1–4]: random coil[1], α-form[2] (Silk I[3]), and β-form[2] (Silk II[3]). Previously, we have reported that crystallization of α-form silk fibroin takes place when an aqueous solution of silk fibroin is cast at 0° to 40°C: crystallization of the β-form takes place at 60°C and above[5]. We also found[11] that native silk fibroin solution contains a small amount of α-form crystal nuclei at 20°C [11]. Iizuka *et al.*[6] found that the β-form crystals can be obtained by mechanically stirring an aqueous solution of silk fibroin.

Spherulites of silk fibroin were found by Ishikawa[7] and Hirabayashi[12], but they did not establish details of the relations between crystallization conditions and the structure of the spherulites. Therefore in this work we have clarified the effects of crystallization conditions, such as drying temperature, drying rate and pre-treatment (freezing), on the chain conformation and texture of the spherulites in films of silk fibroin cast from aqueous solution.

EXPERIMENTAL

Materials

Native silk fibroin solution was prepared from the posterior part of the middle division of the silk gland in full-grown larvae (one day before spinning and cocooning) of the silkworm, *Bombyx mori.* The gel-like silk material (concentration about 25%) was dispersed and dialysed against deionized water. Crystalline fibroin film was prepared by casting the solution (~4% concentration) on a glass plate at a predetermined temperature. Drying rate was controlled by blowing air over the solution in a thermostat with a fan. Pretreatment of fibroin solution was carried out by quenching the solution (~0.8% concentration) in a cooling bath kept at a predetermined temperature. The frozen solution was allowed to melt and then dried at 20°C over a period of a day.

Measurements

Wide-angle X-ray diffraction patterns were recorded on a Weissenberg camera at 35 kV and 20 mA, using a Rigaku–Denki Type 4042 apparatus with copper target and nickel filter. The texture of the spherulites was observed with a Nikon microscope equipped with polarizer and analyser.

RESULTS AND DISCUSSION

Effect of drying rate

When fibroin films were crystallized by evaporating water from the native fibroin solution at 20°C and 65% r.h. at various drying periods, two kinds of spherulite were observed according to the drying temperature. Negatively birefringent spherulites in the α-form appeared when the water was evaporated over a period of less than 3 days. The other type of spherulites in the β-form with positive birefringence appeared when the evaporation was carried out slowly over a period of more than 4 days.

Figure 1 shows schematically the effect of drying rate on the conformation of silk fibroin and the birefringence of the resulting spherulites. Crystallization to both negative α-form and positive β-form spherulites occurs with intermediate drying rate.

Figure 2a shows a micrograph of a spherulite crystallized at 20°C over a period of a day. A straight extinction cross and negative birefringence are observed under these crystallization conditions. Conformation of fibroin in the spherulite as α-form is observed from the microbeam X-ray diffraction pattern (*Figure 2b*). When the evaporation was carried out more slowly over a period of 2 days, smaller spherulites were

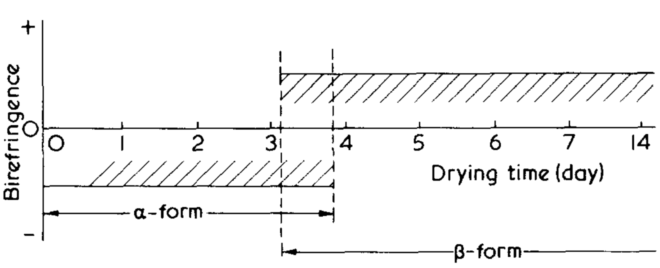

Figure 1 Schematic illustration of the effect of drying time on the texture of spherulites of silk fibroin crystallized by casting from aqueous solution at 20°C

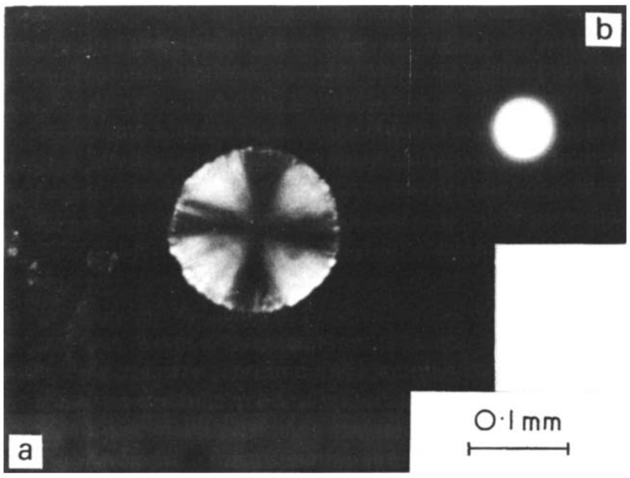

Figure 2 Micrograph as observed between crossed polarizers (a) and microbeam X-ray diffraction pattern (b) of a spherulite crystallized by casting from aqueous solution at 20°C over a period of a day

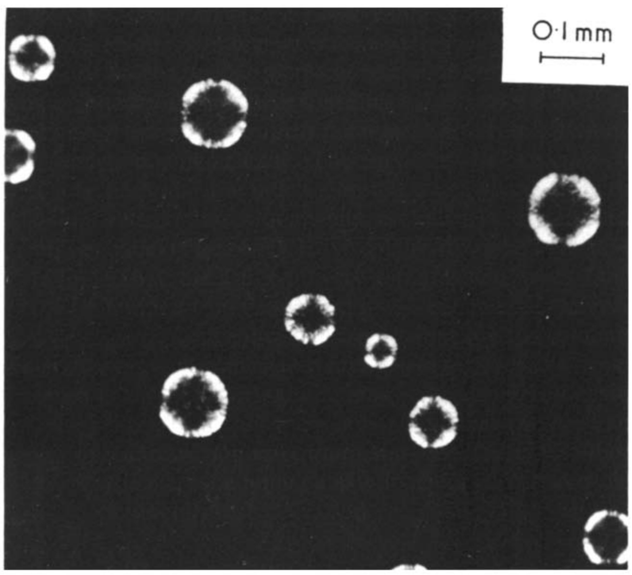

Figure 3 Micrograph as observed between crossed polarizers of spherulites of silk fibroin crystallized by casting from aqueous solution at 20°C over a period of 2 days

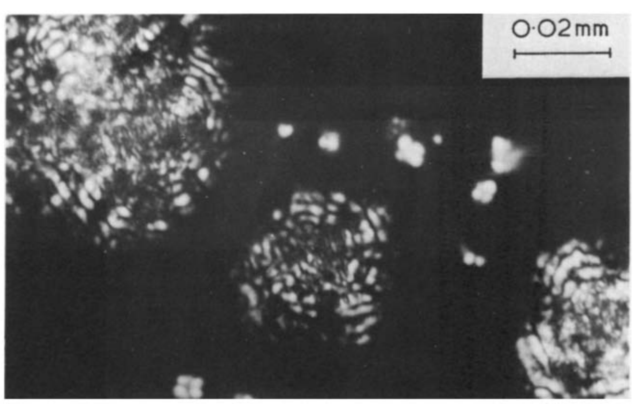

Figure 4 Micrograph as observed between crossed polarizers of spherulites of silk fibroin crystallized by casting from aqueous solution at 20°C over a period of 3 days

obtained (*Figure 3*). Although the central portions of each spherulite are dark, extinction crosses are observed. These spherulites are also negative ones in the α-form. With a 3-day drying period, extinction crosses and zig-zag extinction rings appear in the spherulites with rather irregular shapes (*Figure 4*); the spherulites are in the α-form and negatively birefringent.

When the water in the fibroin solution was evaporated very slowly over a period of 7 days, positive β-form spherulites with needle-like branches were obtained (*Figure 5*). Sometimes dendritic spherulites grow in the radial direction and overlap each other. The texture of the dendritic spherulites of silk fibroin is very similar to those of polypropylene[8] and nylon[9]. With the slowest drying rate (a drying period of 14 days), small β-form spherulites appear in the interstices of needle-like crystals (*Figure 6*). Extinction crosses are observed vaguely in the spherulites, which are positively birefringent.

As described above, under constant temperature and humidity conditions (20°C and 65%), negative spherulites in the α-form appear with a drying period of less than 3 days, whereas positive β-form spherulites grow with a drying period of more than 4 days. The spherulites crystallized over a period of less than 3 days grow from the α-form crystal nuclei originally existing in the solution[11] and have a negative birefringence, i.e. the larger refractive index lies in the direction of the radius. Since the axis of the molecular chain is assumed to have the largest refractive index, the silk fibroin molecules in the α-form spherulites must be perpendicular to the radius of the spherulites. On the contrary,

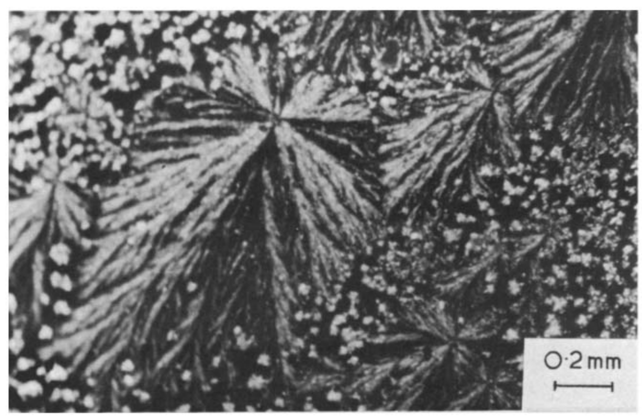

Figure 5 Micrograph as observed between crossed polarizers of spherulites with needle-like branches of silk fibroin crystallized by casting from aqueous solution at 20°C over a period of 7 days

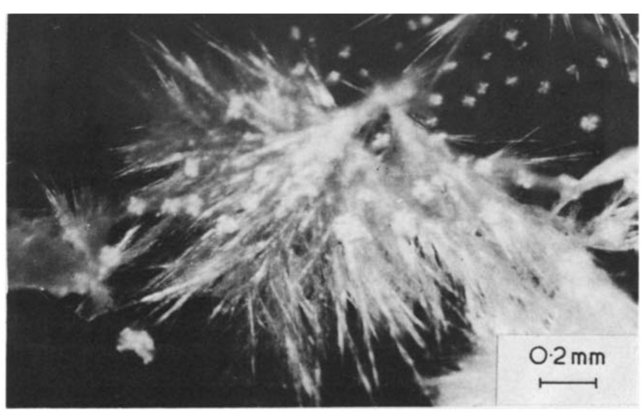

Figure 6 Micrograph as observed between crossed polarizers of a mixture of spherulites and needle-like crystals of silk fibroin crystallized by casting from aqueous solution at 20°C over a period of 14 days

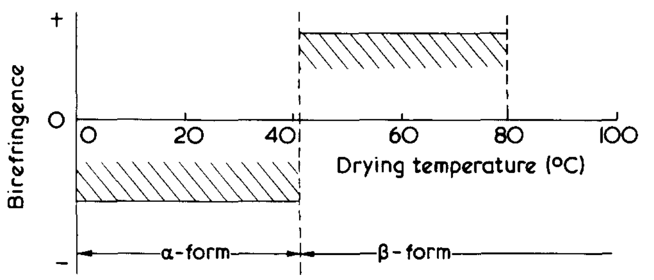

Figure 7 Schematic illustration of the effect of drying temperature on the texture of spherulites of silk fibroin crystallized by casting from aqueous solution

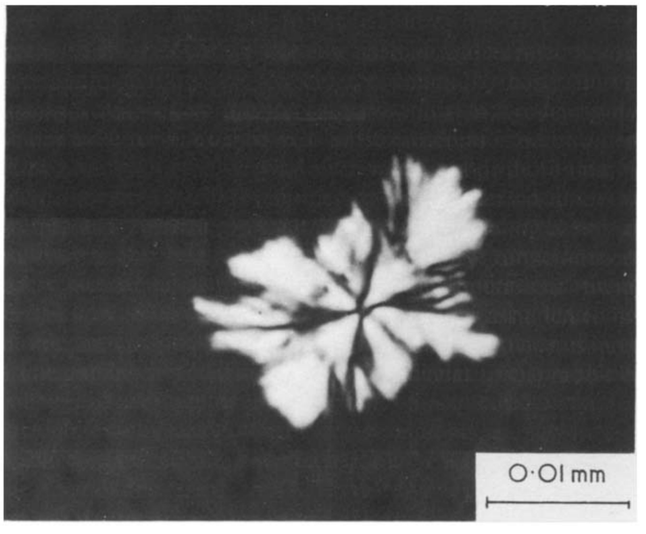

Figure 8 Micrograph as observed between crossed polarizers of a fibrillar spherulite of silk fibroin crystallized by casting from aqueous solution at 2°C over a period of 2 days

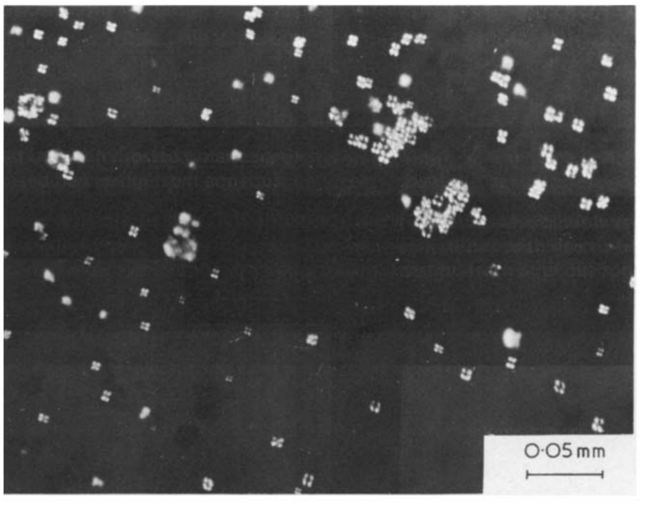

Figure 9 Micrograph as observed between crossed polarizers of spherulites of silk fibroin crystallized by casting from aqueous solution at 40°C for a day

the β-form spherulites have a positive birefringence. Therefore the silk fibroin chains must be aligned radially in the α-form spherulites.

Previously, we detected free amino-acids such as glycine and alanine by paper chromatography in silk fibroin solution left for more than 3 days[11]. This is attributed to the degradation of silk fibroin by the attack of some enzyme in the solution, or of bacteria in air, and the molecular weight of fibroin in the solution is fairly reduced compared to that of native silk fibroin. The α crystal nuclei originally existing in the solution are destroyed coincidentally, β crystal nuclei form, and α crystallization proceeds. The amounts of glycine and alanine in the solution increase with standing time, indicating more severe degradation of the silk fibroin. Therefore, fibroin crystallizes from the solution dried over a period of 14 days as needle-like crystals which consist of degraded fibroin with lower molecular weight.

Effect of drying temperature

When crystallization of fibroin was carried out by evaporating water from a 4% aqueous solution at various temperatures between 0° to 100°C, negative α-form spherulites appeared with drying temperature between 0° to 40°C, and positive β-form spherulites grow in films cast at higher temperatures. With drying temperatures of 80°C and above no spherulites were observed. These results are schematically illustrated in *Figure 7*.

Casting of the fibroin solution at 2°C over a period of 2 days results in fibrillar negative spherulites in the α-form as shown in *Figure 8*. With a casting temperature of 40°C small α-form spherulites with irregular shape grow; they show extinction crosses and are negatively birefringent (*Figure 9*). The spherulites crystallized at 60°C are in the β-form as seen from the microbeam X-ray diffraction pattern (*Figure 10b*). The curving of the spherulite cross implies that the index ellipsoid is changing direction along a given radius, along which growth would continue to be radial (*Figure 10a*). No spherulites are observed in a film cast at 80°C but fine crystals appear (*Figure 11*). At this temperature the drying rate is too high for spherulites to grow. The conformation of fibroin in these microcrystals was determined to be the β-form by X-ray diffraction.

With a casting temperature below 40°C negative spherulites grow from the α crystal nuclei originally existing in the solution. On the other hand, positive spherulites result from the β crystal nuclei newly formed, which are stable at these temperatures[5].

Effect of freezing

When a 0.8% aqueous fibroin solution was frozen at −2° to −18°C before drying at 20°C, the β spherulites with positive birefringence were obtained, whereas no spherulites were observed by freezing the solution below −20°C and

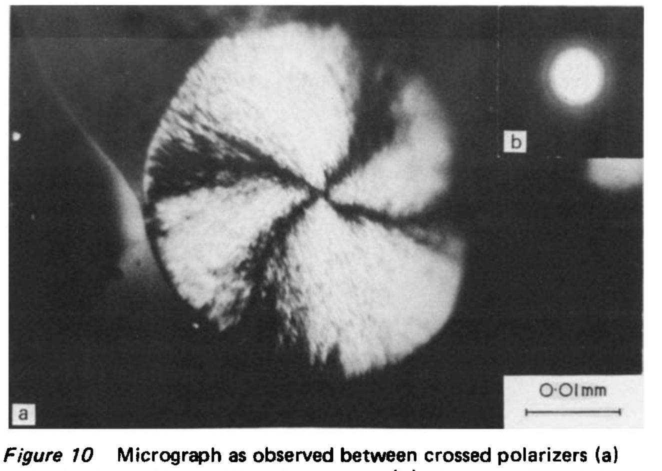

Figure 10 Micrograph as observed between crossed polarizers (a) and microbeam X-ray diffraction pattern (b) of a spherulite of silk fibroin crystallized by casting from aqueous solution at 60°C over a period of a day

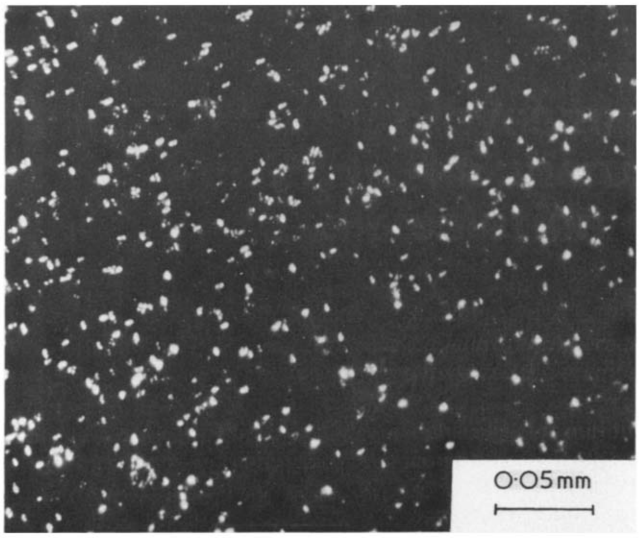

Figure 11 Micrograph as observed between crossed polarizers of spherulites of silk fibroin crystallized by casting from aqueous solution at 80°C over a period of 10 h

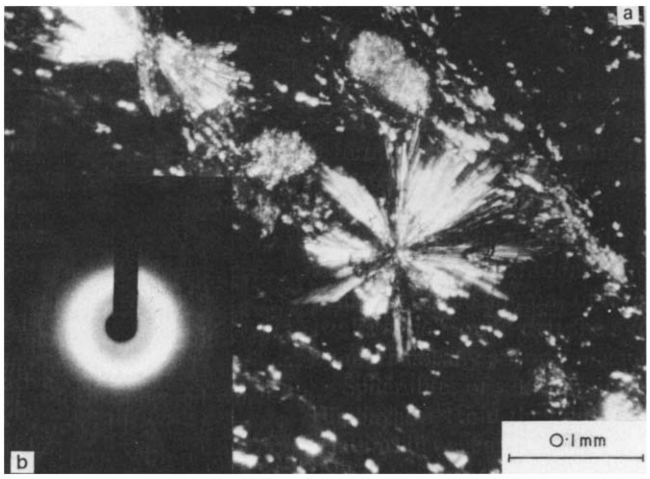

Figure 12 (a) Micrograph as observed between crossed polarizers of spherulites and sheaf-like crystals of silk fibroin crystallized by drying from aqueous solution at 20°C over a period of a day after frozen at −16°C. (b) X-ray diffraction pattern showing β-form conformation

then drying at 20°C. The micrograph of a film frozen at −16°C and then dried at 20°C shows sheaf-like crystals in addition to spherulites (*Figure 12*). We have previously reported that these spherulites have a 'shish-kebab' structure as observed by scanning electron microscopy[10]. The films dried at 20°C after the solution was frozen below −20°C were found to be amorphous and showed random coil conformation by X-ray diffraction and infra-red spectroscopy.

When the concentration of fibroin in solution is very low, the α crystal nuclei cannot exist in the solution. When the water in the solution is frozen, shear stress is applied to fibroin molecules so that the conformation of fibroin locally becomes the β-form[10]. Therefore, in the course of drying, the sequences of β conformation act as the β crystal nuclei and β-form crystallization proceeds. This crystallization mechanism is very similar to the β crystallization of silk fibroin induced by mechanically stirring a dilute aqueous solution of fibroin. On the other hand, when the solution is quenched to a temperature below −20°C, water in the solution is frozen too promptly for the formation of the β crystal nuclei to occur. Therefore, the resulting films are amorphous and in the random coil conformation.

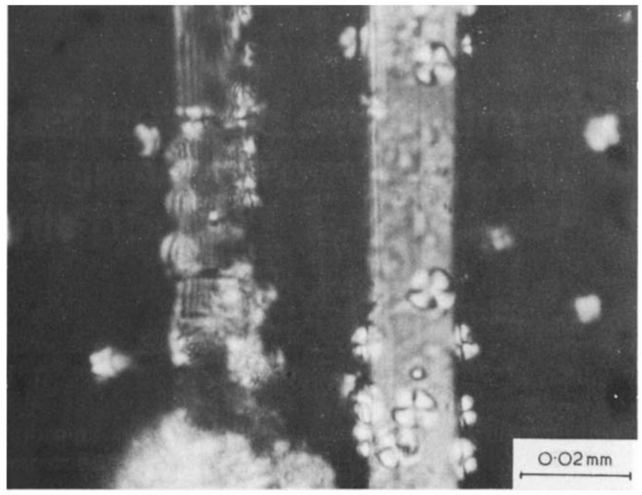

Figure 13 Micrograph as observed between crossed polarizers of spherulites of silk fibroin crystallized by casting from aqueous solution in the presence of well-oriented (β-form) filaments at 20°C over a period of 2 days

Effect of the presence of well-oriented silk fibroin filament

When well-oriented β-form silk fibroin filaments (degummed silk) were immersed in a 0.8% fibroin solution and crystallization was carried out by evaporating water at 20°C over a period of 2 days, positive spherulites showing maltese crosses were observed on the well-oriented fibroin filaments (*Figure 13*). As these spherulites were very thin, the conformation could not be determined by X-ray diffraction. However, the conformation in the spherulites is attributed to the β-form due to their positive birefringence.

As mentioned above, the α-form spherulites with negative birefringence are obtained in the absence of the β-form fibroin filaments under the same casting conditions. Accordingly the crystalline regions of the β-form fibroin filaments act as the β-form crystal nuclei for the crystallization of fibroin dissolved in the solution; or, formation of the β-form crystal nuclei is favoured by the presence of the β-form filaments.

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