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Lattice deformation and thermal stability of crystals in spider silk

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Abstract

The X-ray diffraction of dragline silks, produced by *Nephila* and *Cyrtophora* spiders, were measured by synchrotron radiation in their original states or in situ during stretching and heating. *Nephila pilipes* spiders construct a two-dimensional orb web that must be rebuilt in one or 2 days, but *Cyrtophora* spiders form a three-dimensional tent web that can exist for several weeks in a tropical forest. Diffraction patterns of *N. pilipes* and *Cyrtophora* draglines resemble each other. Crystals of two kinds are identified in these draglines; one is aligned parallel to the silk direction and another is less oriented. The less oriented crystal in *Cyrtophora* dragline is aligned better than that in *N. pilipes* dragline, which generates about three times stronger diffract intensity. Crystals in *N. pilipes* and *C. moluccensis* dragline silks have remarkable thermal stability. Equatorial reflections remain undiminished until 350 and 450 °C for *N. pilipes* and *C. moluccensis*, respectively. In contrast, the meridional reflections *S* and (0 0 2), which are parallel to the silk thread, disappear at a temperature less than 100 °C for *C. moluccensis* but remain for *Nephila* up to 100 °C. Meridional reflections *S* and (0 0 2) shift to a smaller angle during stretching, whereas equatorial reflections remain constant in a range 1.0–1.3 times the original length. The position of the *S* reflection shifts rapidly in the first 10% of elongation from the original length but remains constant during subsequent stretching, whereas the (0 0 2) reflection shifts rapidly during the first 5% elongation from the original length and continues to shift subsequently. In contrast, the features of *N. pilipes* dragline alter insignificantly during stretching. Examination of the composition of amino acids of the draglines of *N. pilipes* and *C. moluccensis* indicates that a dragline of *N. pilipes* contains more glycine, but much less alanine, than that of *C. moluccensis*.

Keywords: X-ray diffraction; Spider silk; Lattice deformation; Thermostability

1. Introduction

As research on spider silk generally focuses on dragline produced by species that build typical orb webs, draglines produced by taxa that exhibit other foraging strategies and web architectures have attracted little attention. Orb-weaving species of genera *Nephila* and *Araneus* have received intensive investigation of aspects ranging from physical properties to genetic engineering [1–6]. Those species build two-dimensional vertical orbs consisting of radial dragline and concentric sticky spirals. Planar orb webs absorb kinetic energy generated on interception of a prey through vibration of the orb panel via elastic silks, and retain the prey in the web by the hydrophilic glue on sticky spirals [2]. Because of the planar architecture of the orb and the nature of the sticky substance, the orb is unable to withstand a strong environmental impact such as heavy rain, and the orb must be taken down and rebuilt each foraging bout to maintain stickiness [7]. Orb weavers such as various species of genus *Cyrtophora* build modified orb webs that operate differently. *Cyrtophora* spiders build a three-dimensional web that

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consists of a horizontal orb and a tangle structure above the orb; such a web intercepts an insect by knocking down its prey through its complicated upper structure. The horizontal orb, not at all sticky, serves to intercept the knocked down prey [8]. Whereas orbs built by *Nephila* or *Araneus* can be completed in less than one hour, completion of a web, composed of much silk, of *Cyrtophora* requires several hours [9]. Silk of this large and complex architecture enables *Cyrtophora* webs to occupy open habitats unsuitable for *Nephila* or *Araneus* and enables them to be ready for foraging almost all the time [8]. *Cyrtophora* webs might endure more than a month in tropical forests [9], whereas in species such as *Nephila pilipes* the orbs are only used for few days [10]. This property indicates that dragline silks produced by *Cyrtophora* can withstand an environmental impact such as rain, wind and temperature fluctuation better than those produced by *Nephila* and *Araneus*. So far nobody has investigated how the great durability of *Cyrtophora* silk is achieved.

Spider dragline silk is a semi-crystalline material that has been examined with SAXS [11], WAXD [12–16], NMR [17–19] and other methods of characterization. In this work, we examined patterns of X-ray diffraction and composition of amino acids in dragline silks produced by *Nephila* and *Cyrtophora*. The spider dragline was measured both in its original state and in situ during stretching and heating. The *Nephila* spider weaves an orb web whereas *Cyrtophora* constructs a space web. The crystallinity, thermal stability and elasticity of webs of spiders of these two species are compared on the basis of their diffraction patterns. We also analyzed the composition of amino acids of these two silks with HPLC to acquire a preliminary understanding of a relationship between their physical properties and their molecular structure.

2. Materials and methods

Dragline silks of spiders of three species were selected for study of their crystalline behaviour under stretching or heating. Spiders were first trapped on a platform with ventral side up; dragline silk was pulled from the spinneret under a dissecting microscope. Dragline silks were force-pulled in air from *N. pilipes*, *Cyrtophora moluccensis* and *Cyrtophora unicolor* with a drawing speed 1–2 m min^{-1} for 30–90 min. The spider silk was swathed on a steel frame ($\sim 1 \times 10\,mm^2$) for several thousand rounds; a sheet of silk subsequently formed was studied with X-ray diffraction when fresh or after storage (23 °C and 50% humidity) for days or months. X-ray diffraction patterns of dragline silks from these three species were measured at the BL17A1 and BL17B2 wiggler beamlines of National Synchrotron Radiation Research Center (NSRRC), Taiwan, with wavelengths 1.3263 and 1.1271 Å, respectively. The beam size was 0.5 mm $V \times 2$ mm H for BL17A1; the diameter was 0.2 mm for BL17B2. A diffraction pattern was recorded with a flat imaging plate and a typical exposure period ∼20 min. Diffraction profiles and intensities were extracted from two-dimensional diffraction images with Xpress software. A custom-designed motorized stretchable holder with a steel frame was used to elongate the spider dragline silk as X-ray diffraction proceeded. To study the thermal stability of crystallinity in dragline silks, the temperature was elevated to 450 °C with a stream of hot air generated with a computer-controlled heater. As the temperature of silk was not measured directly, the actual temperature might be several degrees less than the readings of the heater.

Silks used for analysis of composition of amino acids were obtained also by force-silking. The pulled silk thread was tapped on a rotor turned with a motor and pulled at a speed 1–2 m min^{-1} for 60–90 min. These silks were cut from the rotor and kept first in HFIP then were analyzed with HPLC. The mass of silk in each sample was ∼0.1 mg. These silks were cut from the rotor and dissolved by HFIP (500 μl mg^{-1} silk) and then the silk solution was carefully examined to make sure that no suspended particles existed. Silk solutions were taken to the Instrument Center at the Department of Chemistry, National Tsing Hua University, Taiwan, for HPLC analysis. The silk solution samples were first dried and then hydrolyzed at 115 °C in 6N HCl for 24 h. The resulting product was transferred to a Waters PICO.TAG Amino Acid Analysis System to obtain the amino acid composition.

3. Results and discussion

The composition of amino acids of dragline silks drawn from *N. pilipes* and *C. moluccensis* is presented in Table 1. A dragline of *N. pilipes* contains greater glycine, glutamate and proline than silk from *C. moluccensis*, but a dragline of *C. moluccensis* contains twice as much alanine as that from *N. pilipes*. Patterns of X-ray diffraction of dragline silks of *Nephila* and *Cyrtophora* were measured with synchrotron radiation (Fig. 1); these patterns resemble those previously

Table 1
Composition (%) of amino acids in dragline silks from *Nephila pilipes* and *Cyrtophora moluccensis*

Species	Gly	Ala	Glu	Pro	Ser	Tyr	Leu	Arg
Nephila pilipes	41.61	18.99	12.19	9.67	4.62	3.79	2.73	1.42
Cyrtophora moluccensis	33.36	38.19	5.27	3.88	4.39	4.21	1.05	1.81

Species	Val	Thr	Asp	Ile	Cys	Met	Phe	Lys
Nephila pilipes	1.10	0.96	0.74	0.64	0.51	0.36	0.35	0.30
Cyrtophora moluccensis	0.99	0.85	2.09	0.67	Trace	1.23	0.67	0.78

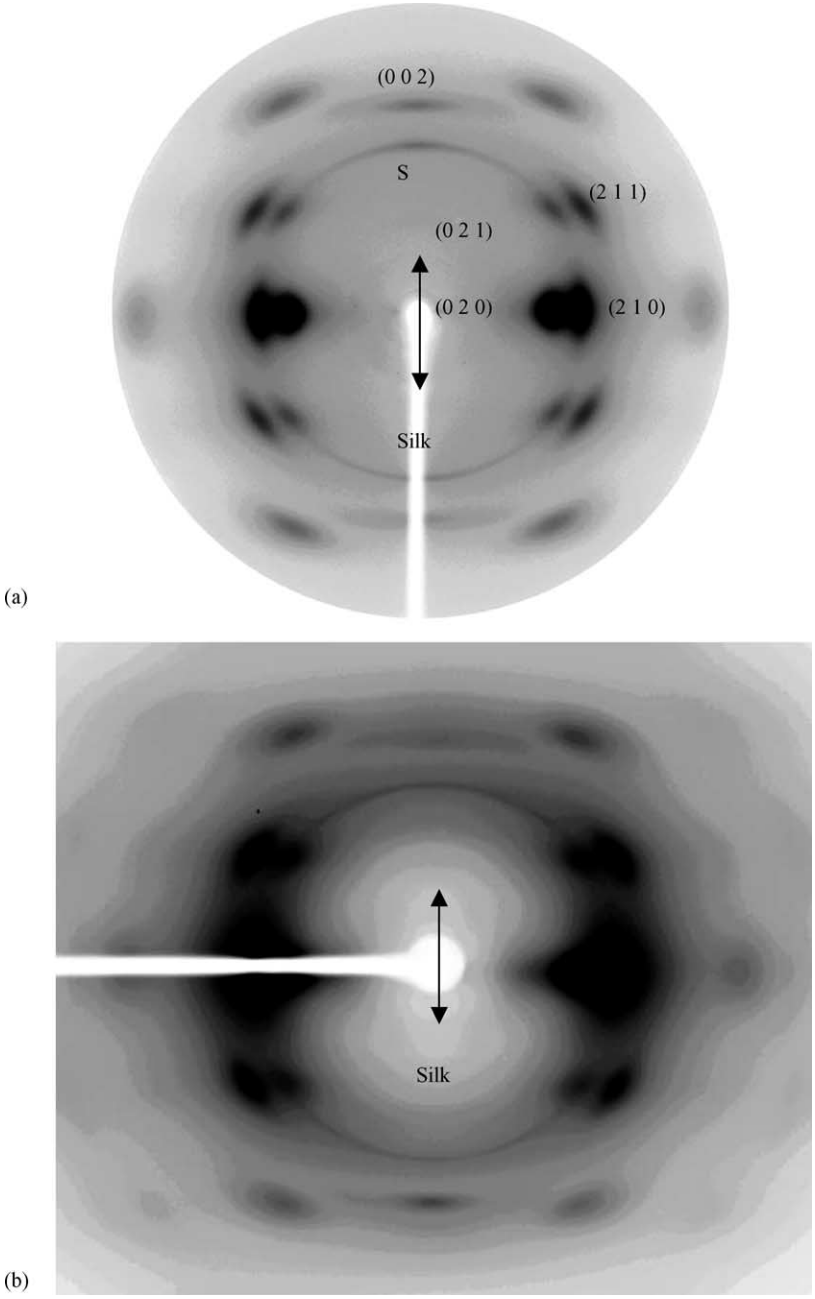

Fig. 1. Two-dimensional X-ray diffraction images of: (a) *Cyrtophora unicolor* and (b) *Nephila pilipes* dragline measured with synchrotron radiation using an imaging plate, at wavelength 1.1271 Å.

reported [12–16]. Intense reflections occur in a direction perpendicular to the orientation of the silk, but weak reflections also occur parallel to the orientation of the silk. These patterns indicate that the crystalline component of spider silk has a well-oriented structure of texture. All reflections are assignable to the same unit cell, known to be formed with the β-sheet aligned parallel to the silk thread, except the *S* reflection. β-sheets pack together to form crystals of nm size dispersed in the dragline silk. *D*-values of principal reflections of three spiders' dragline are listed in Table 2. Diffraction patterns taken from silk freshly drawn or after storage near 23 °C for more than 12 months show no significant differences. This result indicates that the biomaterial from a spider dragline exhibits exceptional stability in retaining its crystallinity for at least a year. Diffraction patterns of *N. pilipes*

Table 2
D-values (Å) of observed diffraction data of spider dragline at original state

Species	(0 2 0)	(2 1 0)	(0 2 1)	(2 1 1)	(0 0 2)	*S*
Nephila pilipes	5.396	4.432	4.264	3.751	3.506	4.236
Cyrtophora moluccensis	5.423	4.481	4.272	3.741	3.489	4.272
Cyrtophora unicolor	5.399	4.489	4.275	3.779	3.500	4.298

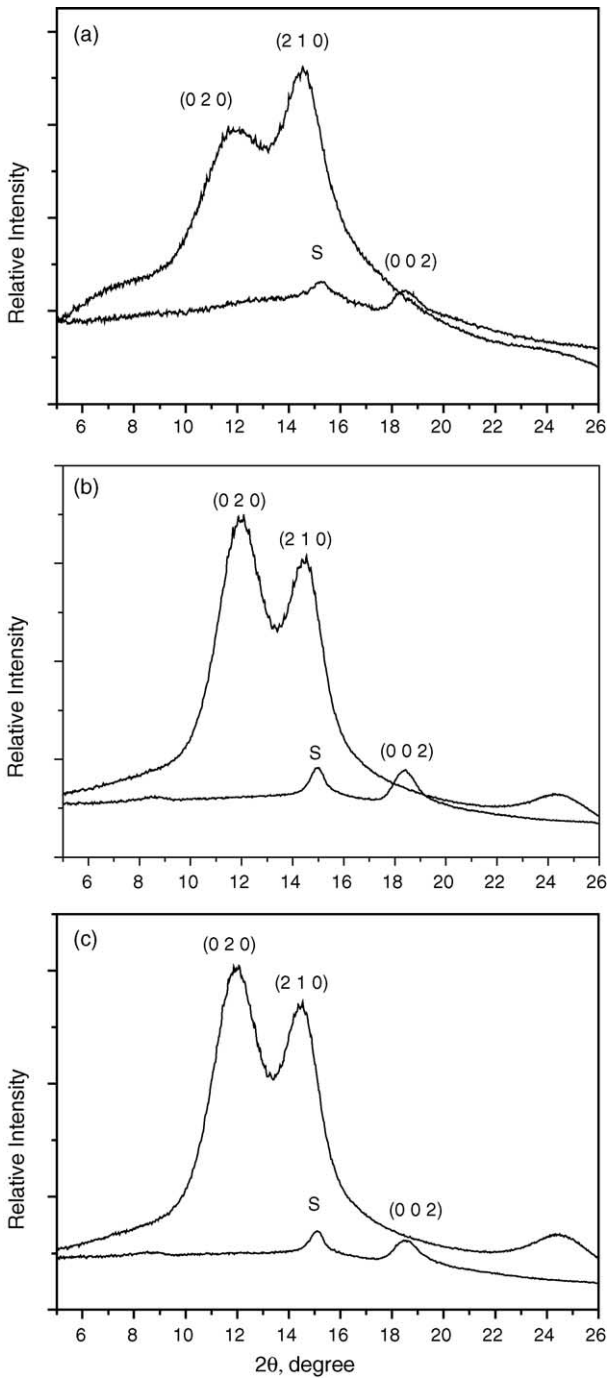

Fig. 2. (0 2 0), (2 1 0), (0 0 2) and *S* reflections of spider draglines. (a) *Nephila pilipes*; (b) *Cyrtophora moluccensis*; and (c) *Cyrtophora unicolor*.

and two species of *Cyrtophora* are similar, but differ in relative intensities (Fig. 1). The profile and relative intensity of reflections differ slightly between *Nephila* and *Cyrtophora* spider silks (Fig. 2). Most diffraction patterns generated with the β-sheet parallel to the silk direction had comparable intensities for the two spider species. The most intense reflections, indexed as (0 2 0) and (2 1 0), respectively, are both perpendicular to the silk. The ratio of (0 2 0) and (2 1 0) reflections differs slightly between *Nephila* and *Cyrtophora* spider silks, perhaps resulting from a slight twist of the β-sheet in the unit cell or from varied composition of amino acids in the β-sheet region. Five structural silk categories were recognized by Warwicker on the basis of X-ray diffraction studies and amino acid analyses. The silk of *N. pilipes* and *Cyrtophora* were classified as group 3 with 10.8 Å inter-sheet packing which is consistent with a high alanine content. Furthermore the full-width at half-maximum (FWHM) ratio of (0 2 0) and (2 1 0) (denoted as E2 and E3 peaks in Warwicker's paper) are 1.48 and 1.27 for *N. pilipes* and *Cyrtophora*, respectively. It indicated that *N. pilipes* belong to class 3b and *Cyrtophora* dragline belong to class 3a. The peak width ratio of E2/E3 for *N. pilipes* is 1.48, which is close to that of *N. clavipes* of 1.50. To reveal the relationship between the physical properties and the molecular structure, determination of the protein sequence in draglines of these species is currently in progress. *S* reflections of dragline silks of two *Cyrtophora* spiders are much more intense in diffraction than for *N. pilipes*, whereas no difference was found between the two *Cyrtophora* species. This result indicates that the same amount of dragline silk exposed to X-rays of the same dosage yields an intensity of *S* diffraction for *Cyrtophora* three times that of *N. pilipes*. The *S* diffracted a more or less circular shape for *Cyrtophora*, but with some intense peak at longitudinal direction, which indicated that the "*S* crystalline" component has some order orientation. As the width of an *S* reflection is only half that of a (0 0 2) reflection in 2θ indicates that the grain size of a crystal is much larger in a *S* crystalline component than in a β-sheet, which is parallel to the silk direction. The *S* reflection might diffract from a crystalline component other than the parallel β-sheet. Simmons et al. [19] reported that of regions rich in alanine of two types, one is highly oriented and the other is poorly oriented and less densely packed. The poorly oriented crystallites might be important in effectively coupling the highly oriented crystalline domains and amorphous regions, thereby producing a biomaterial with exceptional toughness.

Diffractions of X-rays from *C. unicolor* and *N. pilipes* draglines were measured in situ when the silks were elongated up to 1.3 times their original lengths. The two forms of crystallite of *C. unicolor* – β-sheet and *S* crystallite – respond differently to elongation. Reflections (0 0 2) and *S* of *C. unicolor* shift to smaller angles during stretching, indicating that interplane spacing increased (Fig. 3). The d-spacing of reflection *S* increases abruptly on initiation of stretching the silks, and attains a stable state when the silks are elongated to 1.1 times their original length. In contrast, the (0 0 2) reflection shifts rapidly during the first 5% of elongation, and continues subsequently to shift roughly linearly up to 1.3 times elongation. A shift of reflection *S* during the first 10% of elongation might correlate with a change in the elastic region. A rapid shift of the (0 0 2) reflection during the first 5% of elongation might correlate with enlargement of the unit cell in the c direction through a weak interaction; further elongation requires more energy. A tetrahedral carbon with amino and acid functional groups connects the peptide backbone. Twisting of the hydrogen bond in the β-sheet or the tetrahedral bond length

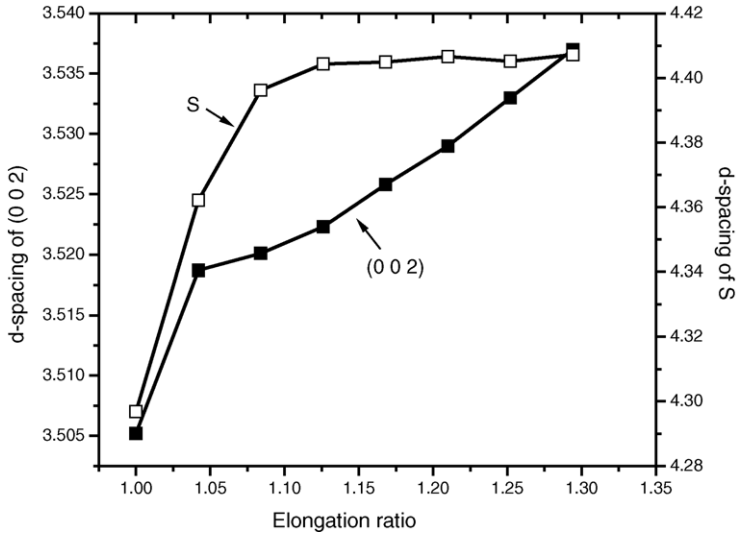

Fig. 3. d-Spacing of *S* and (0 0 2) reflections of *Cyrtophora unicolor* as a function of fraction of elongation.

or angle of the peptide bond might increase the length, but the former process requires less energy than the latter. The composition of amino acids in the β-sheet along the silk direction is known to differ from that of less orientation. (0 2 0), (2 1 0) reflections shifted 0.05° in 2θ during silk elongation (Fig. 4), that is only 0.01 Å changed in term of cell dimensions. In conclusion, the dimensions of *a* and *b* axes exhibit no significant alteration in either *N. pilipes* or *Cyrtophora*, whereas the dimension of the c axis increases in *Cyrtophora* during stretching of the silk thread.

Grubb reported [20] that the X-ray diffraction image of *N. clavipes* dragline, on becoming decreased to 54% of its original length by supercontraction, greatly alters its textural properties from those of the original state. The peak positions

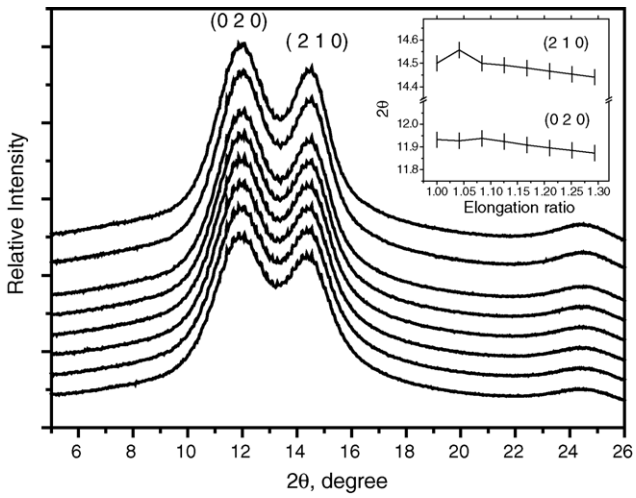

Fig. 4. (0 2 0) and (2 1 0) Reflections of *Cyrtophora unicolor* during stretching. From top down: 1.0, 1.042, 1.084, 1.126, 1.168, 1.210, 1.252, and 1.30 times its original length; points are shifted vertically for clarity. The inset shows the 2θ angle shift during stretching.

(2θ) are unaltered between these two states, indicating that the unit cell does not alter during supercontraction. After a force is applied to stretch the silk to its original length, the X-ray diffraction pattern resumes its original state. Grubb's results demonstrate that the crystalline orientation of spider dragline silk might be reversible during stretching. In our work we found that, on stretching the dragline silk of *Cyrtophora* to 1.3 times its original length, the orientation of the β-sheet in *a* and *b* direction show no difference apart, the change of full-width at half-maximum at azimuthal angle became smaller by only 3°, while the (0 0 2) and *S* reflection became larger by more than 10° during stretching (Fig. 5). This indicates that the crystals align better in the *a* and *b* directions, but worse in the *c* direction after silk elongation. After stretching and then releasing the tension, the silk becomes curled and does not return to its original state. The silks were perhaps stretched beyond their elastic range, so producing an irreversible change in reflections.

X-ray diffraction in situ was applied to assess the thermal stability of crystallinity in dragline silks from *N. pilipes* and *C. moluccensis*. The silk was treated with a stream of dry hot air during diffraction measurements. The intensities of (0 2 0) and (2 1 0) reflections, the two strongest, decrease only after the temperature reaches 300 °C for the *N. pilipes* dragline, whereas after 450 °C for the *C. moluccensis* dragline (Fig. 6). In contrast, reflections parallel to the silk show much less durability to thermal treatment. The (0 0 2) and *S* reflections weaken even at about 30 °C, become still weaker at 50 °C and quickly disappear near 100 °C (Fig. 7). These great disparities in thermal behaviour of reflections parallel and perpendicular to the silk might be related to its packing. Simulations by Fossey and Tripathy [21] base on atomistic modeling demonstrate that the small spider dragline crystallites are connected by interphases, assumed to be thin layers of statistically arranged chains. The molecular packing becomes loose in a direction parallel to the silk above 100 °C;

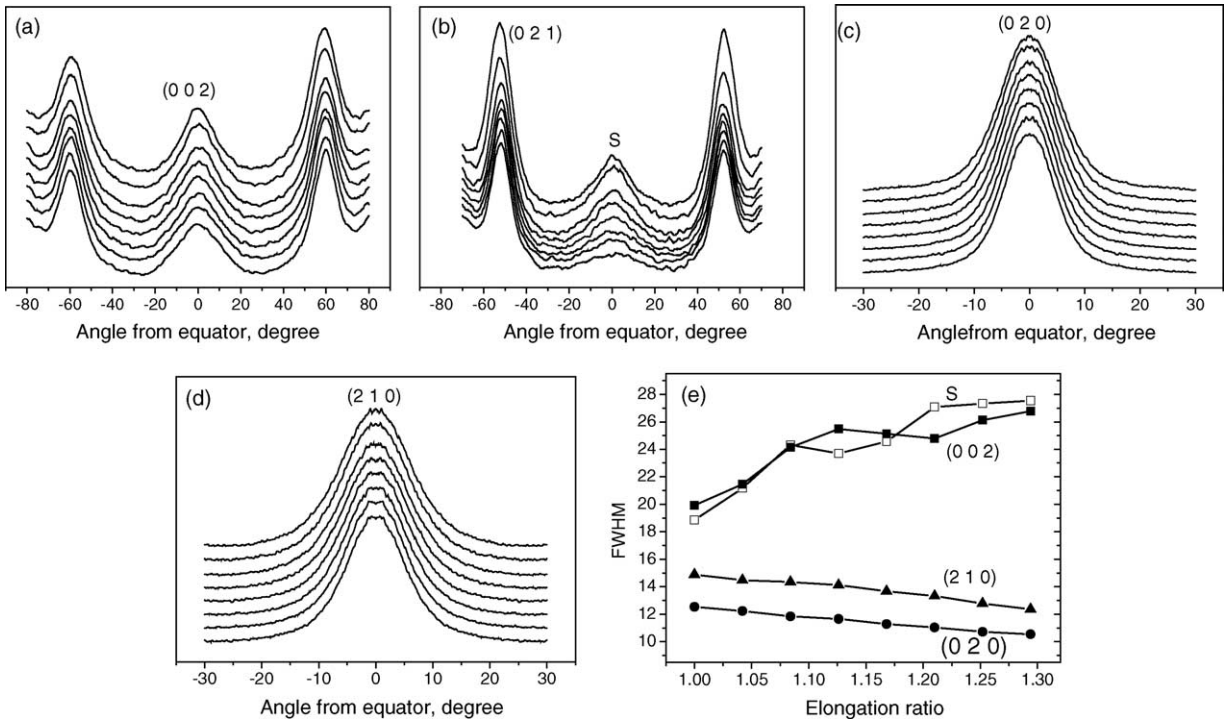

Fig. 5. Intensity of diffraction as a function of azimuthal angle, with zero at the equator. (a) (0 0 2); (b) *S*; (c) (0 2 0); and (d) (2 1 0). From top down: 1.0, 1.042, 1.084, 1.126, 1.168, 1.210, 1.252, and 1.30 times the length of the original fiber. (e) Full-width at half-maximum of four reflections changed during stretching.

the whole molecule was considered to be a rigid body, which is well ordered in a direction perpendicular to the silk up to 400 °C. Hence, the β-sheet is thermally stable because of strong H-bonding, whereas the inter-sheet packing has only weak interactions along the silk thread. The (0 0 2) reflection of *N. pilipes* dragline exhibits no noticeable change upon heating to 100 °C. After thermal treatment both dragline silks become black and loosely packed. Water removed from silk during thermal treatment might diminish its volume, while the decomposition and vaporization of some small organic molecule might play an important role as well.

Although no protein sequence data are available for the dragline of *C. moluccensis* so that the motif cannot be estimated, data on the composition of amino acids correlate well with the observed difference of the diffraction patterns of the draglines of these two genera. Genes encoding major ampullate gland silk of various species of *Nephila* have been extensively studied, and partial sequences are available. At least two genes – MaSp1 and MaSp2 – are involved in the production of dragline in *Nephila* [22]. The composition of amino acids of *N. pilipes* is similar to those reported from *Nephila* species [23]. Existing data shows also that motifs of

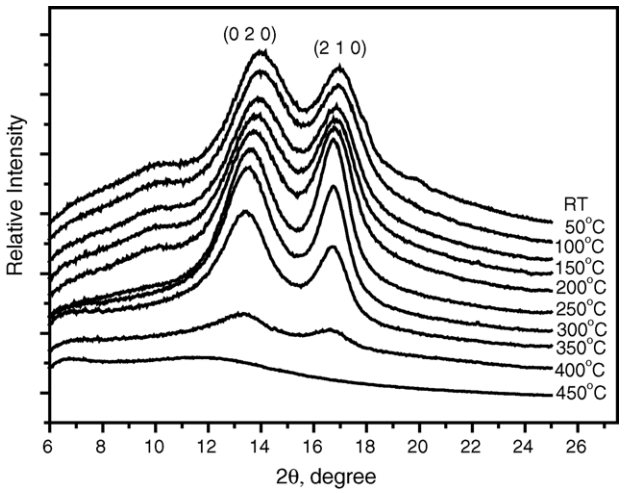

Fig. 6. (0 2 0) and (2 1 0) Reflections of *Cyrtophora moluccensis* dragline at various temperatures. Points are shifted vertically for clarity.

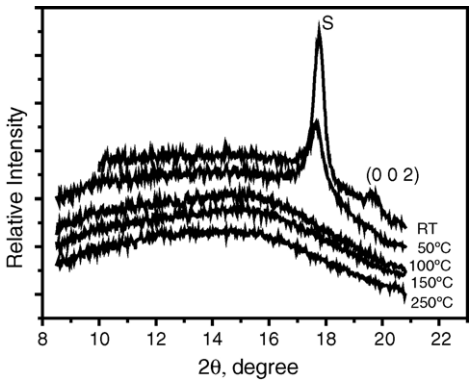

Fig. 7. (0 0 2) and *S* reflections of *Cyrtophora moluccensis* dragline at various temperatures.

draglines genes are similar among the *Nephila* species studied so far [24]. Motifs of partial sequence for major ampullate silk genes of *N. clavipes* show large proportions of poly A, poly GA and GGX in MaSp1 and GPGXX in MaSp2. An amino acid considered responsible for the β-sheet, alanine, was reported to be around 20–25% of the dragline of various *Nephila* species. Results from X-ray diffraction show that the diffraction of the dragline of *C. moluccensis* was significantly greater than that of *N. pilipes* (Fig. 2). Results of amino acid analysis show that the proportion of alanine in the dragline of *C. moluccensis* is twice that of *N. pilipes*, and this might be responsible for the greater diffraction and thermal durability. Molecular studies of *Nephila* dragline genes show that polyalanine appears in the β-sheet regions of major ampullate silks. The crystalline areas of silk were suggested to be formed with polyalanine regions linked with hydrophobic interactions [22,25]. Perhaps the repetitive portion of *C. moluccensis* dragline contains longer and/or more intercalated polyalanine regions thus generates much greater diffraction than that of *Nephila* silk. Future examination of the composition of motifs on repetitive units of dragline genes of *C. moluccensis* will clarify how the dragline of this genera exhibits such greater diffraction and thermal stability than that of *Nephila*.

4. Conclusion

Crystallites of two kinds are identified in the dragline silks of *Cyrtophora* and *N. pilipes* with synchrotron X-ray diffraction. One is the β-sheet that aligns parallel to the silk direction in crystallite of nm size; the other is assigned as *S* crystalline, which in submicrometre size might be also formed with a β-sheet of a different type or a low molecule weight organic/inorganic compounds having less orientation. Those two crystalline responses differ for stretching and heating for *Cyrtophora* and *N. pilipes*. Dimensions of unit cells in *a* and *b* directions remain constant on elongation of the silk, whereas the dimension of the *c* axis increases during stretching for *Cytophora* but not for *N. pilipes*. The thermal stability according to X-ray diffraction also demonstrates that the crystallinity in *Cyrtophora* spider silk can endure a greater temperature than that of *N. pilipes*. The crystallinity in the *c* direction becomes disordered at a low temperature, whereas the packing in *a* and *b* directions is thermally much more stable, possibly correlating with different web structures and foraging strategies of these two genera.

Acknowledgements

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References

[1] Vollrath F. Int J Biol Macromol 1999;24:81.
[2] Vollrath F. Rev Mol Biotechnol 2000;74:67.
[3] Winkler S, Kaplan DL. Rev Mol Biotechnol 2000;74:85.
[4] Hinman MB, Jones JA, Lewis RV. TIBTECH 2000;18:374.
[5] Fahnestock SR, Yao Z, Bedzyk LA. Rev Mol Biotechnol 2000;74:105.
[6] Vollrath F, Knight DP. Nature 2001;410:541.
[7] Foelix RF. Biology of Spiders. 2nd ed. Oxford: Oxford University Press; 1996.
[8] Lubin YD. In: Altevogt R, Hediger H, King JA, Tembrock G, Thomson KS, editors. Forma et Functio, vol. 6. Pergamon Press Inc.; 1973. p. 337.
[9] Tso IM, Severinghaus LL. Zool Stud 2001;39:236.
[10] Tso IM, Severinghaus LL. Anim Behav 1998;56:219.
[11] Yang Z, Grubb DT, Jelinski LW. Macromolecules 1997;30:8254.
[12] Warwicker JO. J Mol Biol 1960;2:350.
[13] Grubb DT, Jelinski LW. Macromolecules 1997;30:2860.
[14] Riekel C, Muller M. Macromolecules 1999;32:4464.
[15] Bram A, Branden CI, Craig CL, Snigireva I, Riekel C. J Appl Cryst 1997;30:390.
[16] Riekel C, Craig CL, Burghammer M, Muller M. Naturwissenschaften 2001;88:67.
[17] Rathore O, Sogah DY. J Am Chem Soc 2001;123:5231.
[18] van Beek JD, Beaulieu L, Schafer H, Demura M, Asakura T, Meier BH. Nature 2000;405:1077.
[19] Simmons AH, Michal CA, Jelinski LW. Science 1996;271:84.
[20] Grubb DT, Ji G. Int J Biol Macromol 1999;24:203.
[21] Fossey SA, Tripathy S. Int J Biol Macromol 1999;24:119.
[22] Hayashi CY. Molecular systematics and evolution, theory and practice. In: Desalle ER, Giribet WW, editors. Switzerland: Birkhauser Verlag; 2002.
[23] Craig CL, Hsu M, Kaplan D, Pierce NE. Int J Biol Macromol 1999;24:109.
[24] Gatesy J, Hayashi C, Motriuk D, Woods J, Lewis R. Science 2001;291:2603.
[25] Hayashi CY, Shipley NH, Lewis RV. Int J Biol Macromol 1999;24:271.