Exploration of the tight structural–mechanical relationship in mulberry and non-mulberry silkworm silks

Guangqiang Fang,\textsuperscript{a} Sunaina Sapru,\textsuperscript{b} Sibaram Behera,\textsuperscript{b} Jinrong Yao,\textsuperscript{a} Zhengzhong Shao,\textsuperscript{a} Subhas C. Kundu*\textsuperscript{b} and Xin Chen*\textsuperscript{a}

The Bombyx mori silkworm is well known as it has been bred by our ancestors with mulberry tree leaves for thousands of years. However, Bombyx mori is not the only silkworm that can produce silk, many other kinds of silkworms can also make silks for commercial use. In this research, we compare the mechanical properties of five different commercial silk fibres including domesticated mulberry Bombyx mori, non-mulberry semi-domesticated eri Samia ricini, and wild tropical tasar Antheraea mylitta and muga Antheraea assamensis. The results demonstrate that the non-mulberry silk fibres have a relatively high extensibility as compared to the mulberry silk fibres. In the meantime, the non-mulberry silk fibres show comparatively unique toughness to the mulberry silk fibres. Synchrotron radiation FTIR microspectroscopy, synchrotron radiation wide angle X-ray diffraction, and Raman dichroism spectroscopy are used to analyze the structural differences among the five species of silk fibres comprehensively. The results clearly show that the mechanical properties of both mulberry and non-mulberry silk fibres are closely related to their structures, such as β-sheet content, crystallinity, and the molecular orientation along the fibre axis. This study aims to understand the differences in the structural and mechanical properties of different mulberry and non-mulberry silk fibres, which are of importance to the related research on understanding and utilizing the non-mulberry silk as a biomaterial. We believe these investigations not only provide insight into the biology of silk fibroins from the non-mulberry silkworms but also offer guidelines for further biomimetic investigations into the design and manufacture of artificial silk protein fibres with novel morphologies and associated material properties for future use in different fields like bioelectronics, biomaterials and biomedical devices.

1. Introduction

Natural silks are regarded as model systems for high-performance polymeric materials that attract considerable interest because of their excellent balance of modulus, strength, and extensibility.\textsuperscript{1,2} Over the last couple of decades, silks have emerged as appropriate and effective biomaterials for use in tissue engineering and regenerative applications.\textsuperscript{3–5} The silks produced by the silkworms are biopolymers, which can be classified mainly into two types, mulberry and non-mulberry. Commercially, the silks are produced from two families of silkworms including Bombycidae (mulberry) and Saturniidae (non-mulberry).\textsuperscript{6} The main commercial silks are from Bombyx mori as mulberry and Antheraea mylitta (tropical tasar), Antheraea assamensis (muga), Samia ricini/Philosamia ricini (eri) and Antheraea pernyi (temperate oak tasar) as non-mulberry.\textsuperscript{7–9} Mulberry silkworms are entirely domesticated, whereas S. ricini is the only semi-domesticated non-mulberry silkworm while others are grown in the wild. A wide range of wild silkworms have evolved independently throughout the world over a long time, and the morphology and properties of each kind of silkworm are slightly different. They adapt to cope with diverse local environments. Although silk fibres show a high degree of similarity in their unique structural features, they have quite different mechanical properties and stretching behaviour. Evidently, this is due to a subtle difference in their hierarchical structures. Many attempts have been made to connect the mechanical properties to the hierarchical structural features of silks in terms of a quantitative structure–property relationship;\textsuperscript{9–15} however, the mystery of the unique animal silk is not yet totally understood.

The unique properties are attributed to the specific secondary structures of repeating units assembled into a hierarchical...
structure depending upon the type and source of the silk. Silk is generally viewed as a semi-crystalline material with the crystallites dispersed in an amorphous matrix, which act as reinforcing junction zones.\textsuperscript{16–18} It is well accepted that the crystallites composed of pleated $\beta$-sheets are preferentially oriented along the fibre axis. The mechanical properties of silk are believed to be closely related to its hierarchical structural assembly at all length scales, including the presence of nanofibrils, the level of crystallinity, the size and distribution of the crystallites, as well as the conformation and orientation of polypeptide chains at the molecular level. Silk can have a large range of mechanical properties with apparently small changes in its chemical structure. What’s more, the nature, season, number of crops in a year, and several other environmental conditions also result in different mechanical properties of different types of silk fibres.\textsuperscript{19} This makes silk an interesting biopolymer for a wide range of important applications such as biomaterials\textsuperscript{20} and biomedical devices,\textsuperscript{9} including medical devices like fracture fixation\textsuperscript{21} and post-surgical infection reduction devices.\textsuperscript{22} Its utility has also diversified into various other fields, for instance, optics\textsuperscript{5,23} and bioelectronics.\textsuperscript{22,24}

In order to biomimic native silks, a profound knowledge of the natural formation process, chemical composition, and relationship between the structure and properties of silk fibres is imperative. The structure–property relationship is one of the most intriguing mysteries of silk fibres. Different investigations suggest that there is a strong connection between the structures of silk fibres and their mechanical properties.\textsuperscript{2,9–11} With the developments in modern analysis techniques, significant progress is made with respect to the structural characterization of silk. However, there is still much more to learn regarding how silks vary within and between individuals. Knowledge of silk structure from different silk cocoons, which have different protein composition, provides an excellent opportunity to study the structure–property correlation. There are many kinds of silk fibres from silkworms with different structure and properties.\textsuperscript{25–27} Thus, silks are suitable to investigate the structure–property relationship for the molecular design of fibres with high strength and elasticity.

To date, many studies focusing on the silk of \textit{Nephila clavipes} and domesticated \textit{B. mori} have established a basic understanding of the relationship between the structure and function in different types of silk fibres.\textsuperscript{10,14,26,28} However, limited studies for such an understanding in wild silk have been carried out. There are reports mostly on \textit{A. pernyi}\textsuperscript{9,11,29} and \textit{S. ricini}.\textsuperscript{30–32} Although the silkworm silks of a given species serve the same general purpose, their mechanical properties are slightly different, allowing them to adapt to their unique ecosystem. Therefore, an extensive study on their variability would be very useful to help the explanation of the excellent mechanical properties of silks and give valuable suggestions when designing advanced artificial and biomimetic silk-like materials. In this study, we aim to compare five different species of silk fibres, including \textit{B. mori} (Bm, domesticated, mulberry, two kinds, white Bm (W) and yellow Bm (Y)), \textit{S. ricini} (Sr, semi-domesticated, non-mulberry), \textit{A. mylitta} (Am, wild, non-mulberry) and \textit{A. assamensis} (Aa, wild, non-mulberry), to understand the differences in structures and mechanical properties between mulberry and non-mulberry, or domesticated and wild silkworms obtained under different conditions. This information should be important to the related studies on understanding and applying non-mulberry silk materials. The research on wild silk will have great implications for bioengineered super-tough silks.

2. Materials and methods

2.1 Materials

The commercial silk threads used as textile materials were chosen as silk samples for the present study. These consisted of two varieties (white and yellow) of \textit{Bombyx mori} (Bm, mulberry) and three non-mulberry including \textit{Samia ricini} (Sr, eri), \textit{Antheraea assamensis} (Aa, muga), and \textit{Antheraea mylitta} (Am, tropical tasar). Depending upon the variety and habitat of \textit{B. mori}, cocoons are white and yellow and they breed twice (bivoltine) and five times (multivoltine) in a year respectively. The silkworm cocoon silk consists of fibroin fibres that are bound together by sercin, a hydrophilic gum-like coating protein. In pre-processing, cocoons were stifled in a hot air oven and sorted. In some cases, cocoons were sun-dried to prevent the emergence of silk moths. The method for different species varies under the conditions used for softening the gum. In general, Bm and Am cocoons were boiled in clean water for 30 min and 40 min, respectively, followed by rinsing in clean water and cooled for 30 minutes. Am cocoons were again immersed in warm water (45–60°C) containing soda (8 g L$^{-1}$), hydrogen peroxide (10 mL L$^{-1}$) and sodium silicate (8 g L$^{-1}$) for 20 minutes. For Aa, cocoons were boiled in clean water containing soda (3–4 g L$^{-1}$) for 10–15 minutes. In the case of Sr, cocoons were tied loosely in a porous cloth to make a bundle. Water (40 L per kg of cocoons) containing soap with a final concentration of 3 g L$^{-1}$ and 0.30 g L$^{-1}$, respectively, was added to the cocoons. The immersed bundle was boiled for 1 h at 95–100°C and then cooled down. The cocoons were boiled again for 30 min (only in water) and washed properly. The processes to obtain the different silk fibres described here are adapted from the protocols developed by the Central Silk Board (India).\textsuperscript{32}

All these conditions varied slightly depending upon the cocoon size, quality and place of production. After softening, semi-dried cocoons were subjected to deflossing (to remove the outermost layer known as silk waste and locate the single free end to obtain the fibre) followed by spinning. \textit{Samia ricini} is not easily reelable and special spinning wheels are used for this purpose.

2.2 Mechanical testing

The mechanical properties of all five silk fibres were tested using an Instron 5565 mechanical testing machine (at 25°C and 45% R.H.; gauge length: 30 mm; cross-head speed: 15 mm min$^{-1}$) with a load cell of 2.5 N. At least 12 replicates from individual fibres were used for mechanical testing. The cross-sectional areas of the silks were measured according to
the method described in detail elsewhere. \textsuperscript{11,33} Briefly, the cross-section of the silks was observed using a Tecnai 5136MM scanning electron microscope (SEM) at 20 kV after sputtering with gold. The cross-section of the silk fibres was obtained by fracturing them perpendicular to the fibre axis under liquid nitrogen. The areas of the silk fibres were calculated using software Atlas 2.9.9.9, provided with the SEM equipment. For each kind of silk fibre, the average area was obtained from more than 12 samples.

2.3 Synchrotron radiation FTIR microspectroscopy (S-FTIR)

The experiment was performed at both Beamline U4 at the National Synchrotron Radiation Laboratory (NSRL) and Beamline BL01B1 at the National Center for Protein Science Shanghai (NCPSS) in Shanghai Synchrotron Radiation Facility (SSRF). The description of Beamline U4 and Beamline BL01B1 can be found in our previous paper.\textsuperscript{34,35} For FTIR measurements, we used a Nicolet 6700 FTIR spectrometer with a KBr beamsplitter and a liquid nitrogen cooled MCT detector coupled with a Nicolet Continuum microscope with a 36× objective. For each measurement, 256 interferograms were coadded and transformed employing a Genzel–Happ apodization function to yield a spectrum with a nominal resolution of 4 cm\textsuperscript{-1}. Deconvolution of amide III bands was carried out by using PeakFit 4.12 according to the method reported in our previous work.\textsuperscript{34,35}

2.4 Raman spectroscopy

The Raman dichroism spectra of five silk fibres were collected using a Renishaw inVia Reflex Raman spectrometer, using the 785 nm wavelength of a He–Ne laser with the energy of 6 mW. Single silk fibres were aligned perpendicular or parallel to the direction of the laser beam. At least 6 individual fibres were used in each Raman measurement.

2.5 Synchrotron radiation wide-angle X-ray diffraction (S-WAXD)

S-WAXD experiments were performed at Beamline BL16B1 in Shanghai Synchrotron Radiation Facility (SSRF). A bundle of fibres were held tautly (but unstretched) and mounted on the sample holder. The detector-to-sample distance was calibrated to 115 mm. The collection time of the data was 100 s for one image. Before the measurement of each sample, a diffraction pattern of the background was collected. FIT2D (v 12.077) software was used to analyse the date. PeakFit 4.12 software was used to deconvolute the 1D WAXD profiles. During the deconvolution process, the numbers and positions of peaks were fixed by using the data reported in the literature.\textsuperscript{16} A Gaussian model was selected for the band shape, and the bandwidth was automatically adjusted by the software.\textsuperscript{37} The Bragg reflections were separated from the broad short-range order background to estimate the crystallinity ($X_c$) of the fibre, which is measured as the ratio of the crystalline area to the total area. Herman’s orientation factor $f$ of five different silks is calculated from the established methods reported in literature studies.\textsuperscript{38,39}

3. Results and discussion

3.1 Mechanical properties of five different silk fibres

The cocoons of silkworm are hierarchical composites exhibiting diversity in size, shape, and colour (Fig. 1). The colours range from creamy white (Bm (W) and Sr) to creamy yellow (Bm (Y)), golden yellow (Aa) and grayish white (Am). The architecture of non-mulberry silkworm cocoons studied in this research differs from the cocoons of both mulberry and other members of the same genus of non-mulberry.\textsuperscript{8,26} To survive natural challenges and environmental hazards, the non-mulberry silk cocoons of genus Antheraea (Aa and Am) possess a ring like structure called pedunche that attaches them to the tree twig/branch and also serves as a sericin reservoir for them. Among them, the Am pedunche is very tough, hard, strong and difficult to remove from the branch or to cut off.\textsuperscript{40} However, domesticated mulberry cocoons of Bm and semi-domesticated nonmulberry cocoons of Sr lack this structure. Both Bm and Sr show similar tiny fibres with smooth surfaces.

Research indicates that silk fibres show a large variability from one sample to another. Thus, the comparison between different types of silks may provide insight into the structure–function relationship. By studying these five different mulberry and non-mulberry silks, we attempt to develop a connection between the secondary structures and mechanical properties of the silk fibres.

Fig. 2 presents the mechanical properties of five different silk fibres. The measurements indicate that different silk fibres exhibit completely different mechanical properties. As shown in Fig. 2, the stress–strain curve shows a monotonic rapid increase for mulberry silk fibres Bm (W) and Bm (Y), but a much milder increase with a flat region like spider dragline silk\textsuperscript{10} for the other three non-mulberry silks. The stress–strain curves of Am, Aa and Sr silk fibres have a distinct yield point followed by obvious strain hardening, which is reminiscent of the oak tasar Antheraea pernyi silk and the spider major ampullate silk.\textsuperscript{9,28} For both Bm (W) and Bm (Y) silk fibres, there do not exist such obvious yield points and they only show mild strain hardening, indicating they are strong but less stretchable, which has already been well-documented in previous research studies.\textsuperscript{41} Table 1 is the summary of the mechanical properties of five different silk fibres. Mulberry silks (Bm (W) and Bm (Y)) stand out as fibres with a high modulus, high strength and low extensibility. However, non-mulberry silks (Sr, Aa and Am)
In order to gain a better insight into the molecular basis of the mechanical properties of the silk fibres, we characterize the structures of mulberry and non-mulberry silk fibres using FTIR, Raman spectroscopy and wide-angle X-ray diffraction to establish the relationship between secondary structures and mechanical properties.

### 3.2 Secondary structures of five different silk fibres analysed by synchrotron radiation FTIR (S-FTIR) microspectroscopy

In order to gain a better insight into the molecular basis of toughness and extensibility, the secondary structure content of these five silk fibres is investigated by S-FTIR microspectroscopy. It is well-known that FTIR is an extremely common technique used to study the protein secondary structures of silk-based biopolymers, including silkworm and spider silks. The amide I, II and III bands are commonly used to infer protein secondary structure. In principle, FTIR spectroscopy is able to provide both qualitative and quantitative information about protein conformations. However, the conventional FTIR technique is hardly used to test single silk fibres because the aperture diameter of the conventional globar light source (usually several millimetres) is too large (three magnitudes large) compared to the diameter of single silk fibres (5–20 μm). Therefore, only a small part of the infrared beam can come through single silk fibres, resulting in a very poor quality and an almost useless spectrum. S-FTIR microspectroscopy, which combines the ultrahigh brightness of a synchrotron infrared source with the powerful magnification of a microscope, is able to provide high quality spectra (a high signal-to-noise ratio) for micrometre-sized samples. In our previous work, we successfully established a practical method to analyse the different conformations and to determine the β-sheet content in a single animal silk fibre using S-FTIR microspectroscopy.34,35

Here we continue to use this method to analyze the secondary structures of five kinds of single silk fibres. Fig. 3A shows a typical S-FTIR spectrum of a single silk fibre in the full middle infrared region, using the Aa silk fibre as an example. Similar to our previous report,34 the S-FTIR spectrum of the single fibre studied in this research has a very good quality, providing a solid basis for qualitative analysis. It is reported that the silk fibroin isolated from a non-mulberry silkworm exhibits significant biochemical differences when compared to that derived from a mulberry silkworm. It is reasonable to believe that different kinds of silk fibres are composed of different amino acid sequences because they belong to different species. The full sequences of Sr and Aa have been recently published.45,46 For the Am fibroin sequence, there is still only a partial sequence.6 We can find that (Ala) motifs exist in all three non-mulberry silk proteins studied here, similar to another well-studied non-mulberry species A. pernyi, but it is different from mulberry B. mori. In addition, the amino acid compositions among Bm, Sr, and Aa are also significant. For instance, the Ala content in Sr (45.4%) and Aa (42.5%) is more than that in Bm (30.2%). The Gly content is just the opposite for Sr, Aa and Bm that is 31.7%, 28.9%, and 45.9%, respectively. For the Ser content, Sr has a very low value (6.7%) compared to Bm.

### Table 1 Mechanical properties of five different silk fibres

<table>
<thead>
<tr>
<th>Sample</th>
<th>Young’s modulus (GPa)</th>
<th>Breaking stress (GPa)</th>
<th>Breaking strain (%)</th>
<th>Breaking energy (MJ m⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bm (W)</td>
<td>9.6 ± 0.6</td>
<td>0.69 ± 0.02</td>
<td>38.5 ± 6.4</td>
<td>187 ± 33</td>
</tr>
<tr>
<td>Bm (Y)</td>
<td>8.5 ± 0.4</td>
<td>0.61 ± 0.03</td>
<td>43.8 ± 3.9</td>
<td>176 ± 28</td>
</tr>
<tr>
<td>Sr</td>
<td>6.3 ± 0.3</td>
<td>0.56 ± 0.02</td>
<td>47.4 ± 6.1</td>
<td>156 ± 24</td>
</tr>
<tr>
<td>Aa</td>
<td>4.5 ± 0.3</td>
<td>0.49 ± 0.02</td>
<td>55.9 ± 5.3</td>
<td>148 ± 16</td>
</tr>
<tr>
<td>Am</td>
<td>3.9 ± 0.2</td>
<td>0.38 ± 0.02</td>
<td>63.5 ± 4.4</td>
<td>139 ± 28</td>
</tr>
</tbody>
</table>

![Fig. 3](A) A representative S-FTIR spectrum of a single silk fibre (sample Aa) and (B) the detailed amide III and fingerprint region of S-FTIR spectra of five silk fibres.
(12.1%) and Aa (10.2%). We know that the primary protein sequence has a major impact on infrared absorption peak positions, especially for those of β-sheet absorption bands. Therefore, these differences may have important consequences on the structural features of these silk proteins and could be responsible for the differences observed in the quality of silk produced by mulberry and non-mulberry silkworms. By comparing the S-FTIR spectra in this work and those we reported in our previous research,

34 we find that the spectra of Bm (W) and Bm (Y) are almost the same as those of the domestic B. mori silk obtained in China. In the meantime, Sr, Aa and Am show similar spectra to the non-mulberry silk found in China.

Fig. 3B shows the amide III band (which is used to calculate the different conformation content

34) and the fingerprint region of five kinds of silk fibres in detail. The spectra of Bm (W) and Bm (Y) are almost identical, which have two characteristic peaks at 1233 cm

1 and 1266 cm

1 (shoulder) in the amide III band. However, for Sr, Aa and Am silk fibres, the spectra are much different. The characteristic peaks in the amide III band are not as clear as those of Bm, but there is an apparent peak at 986 cm

1. The 986 cm

1 peak is assigned to the β-sheet conformation of (Ala)

n that exists in the A. pernyi silk worm and spider silk proteins,

34 so it further implies that Sr, Aa and Am silks are similar to non-mulberry A. pernyi silk, and not to mulberry B. mori. In general, all silkworm silks can be regarded as semi-crystalline biopolymers with highly organized antiparallel β-sheet nanocrystals embedded in an amorphous matrix. The underlying polypeptide sequences of the non-mulberry silk (Ala)

n are more hydrophobic than those in the mulberry silk (Gly-Ala-Gly-Ala-Gly-Ser). As the (Ala)

n β-sheets impart a higher binding energy than (Gly-Ala-Gly-Ala-Gly-Ser) β-sheets, the different sources of β-sheets in different silkworm silks may contribute to the difference in tensile strength between mulberry and non-mulberry silk fibres.

We know that although all the protein conformations in silkworm silks are responsible for the physical properties of silks, the β-sheet is the dominating one.

47 By using the same method we established in our previous work,

34 i.e., to deconvolute the amide III bands in S-FTIR spectra, we are able to calculate the content of different secondary structures in five silk fibres we studied semi-quantitatively. Fig. 4 shows the deconvolution of the amide III band of different silk samples. For Bm (W) and Bm (Y), we deconvolute the amide III band into two components. One is at 1266 cm

1, which is assigned to the β-sheet, another is at 1233 cm

1, which is assigned to the helical and/or random coil conformation (as it is well-accepted that the helical and random coil conformations in the B. mori silk fibroin are hard to distinguish). Thus we are able to calculate the β-sheet content in Bm (W) and Bm (Y) as 28.3% and 26.3%, respectively. The Bm (W) silk is almost the same as the B. mori silk in China tested in our previous work, but the Bm (Y) silk seems to have a low β-sheet content.

The amide III band of Sr, Aa and Am is found to be very similar to that of A. pernyi reported in our previous work,

34 so we deconvolute it into three components, i.e., 1221 cm

1 is assigned to the β-sheet, 1240 cm

1 to the random coil, and 1266 cm

1 to the α-helix. Table 2 shows the percentage of three major conformations in the non-mulberry silk fibroin. The Sr silk fibre has the highest β-sheet content (23.0%), which is almost the same as that of the A. pernyi silk found in China. Other two kinds of non-mulberry silk fibres have low β-sheet content; Aa has a β-sheet content of 19.9% and Am has the lowest β-sheet content of 14.7%. In addition, it is very interesting to find that the α-helix content in Aa and Am is almost the same as the content of the β-sheet. However, the α-helix content in Sr is
much lower than the β-sheet content, and also lower than the α-helix content in both Aa and Am.

The difference in the assignment of the conformations between mulberry and non-mulberry silk fibroins is due to the different motifs that form the secondary structures in these silk proteins. As we mentioned above and in our previous work, the β-sheet structure in mulberry silks (Bm (W) and Bm (Y)) comes from (Gly-Ala-Gly-Ala-Gly-Ser), while for non-mulberry silks (Sr, Aa, and Am), it is from (Ala)n. In addition, also because of the existence of the (Ala)n motif, there is α-helix conformation in non-mulberry silk fibroin, which is not in the case of mulberry silk protein.

From the analysis of S-FTIR spectroscopy, we are able to obtain the order of β-sheet content as Bm (W) > Bm (Y) > Sr > Aa > Am. This order of β-sheet content correlates the mechanical properties of these five different silk fibres (Table 1) very well. With the increase in β-sheet content, the Young’s modulus and the breaking stress increase simultaneously, while the breaking strain decreases. It again proves that the β-sheet plays an important role in determining the properties of silks.2,47,48 The silk fibre is made up of both well-aligned and less-ordered regions in which the interconnected and well-aligned region (β-sheet) may account for the stiffness of the fibre, whereas the less-ordered region (helix and amorphous matrix) accounts for its extensibility.49,50 Therefore, the mulberry B. mori silk fibres (Bm (W) and Bm (Y)) exhibit high modulus and breaking stress because they have a relatively high β-sheet content.51 Conversely, the non-mulberry silk fibres (Sr, Aa, and Am) have a relatively low β-sheet content, thus showing higher extensibility but lower stiffness than the mulberry silk fibres (Bm (W) and Bm (Y)). The lowest β-sheet content silk sample Am (14.7%) has the longest breaking strain (63.5%) but the smallest Young’s modulus (3.6 GPa) and breaking stress (0.38 GPa) values.

The stress–strain curves shown in Fig. 2 also reveal the different extent of strain-hardening behaviour in different mulberry and non-mulberry silk fibres. It is reported that the extent of strain-hardening in silk fibres may be altered by tuning the amount of intramolecular β-sheets.52 Due to the large proportion of β-sheet structure in domesticated mulberry silk fibres (Bm (W) and Bm (Y)), their stress–strain curve is approximately parabolic and slightly convex to load axis up to break. However, a relatively higher proportion of less-ordered structures than β-sheets (helical and amorphous structures) in non-mulberry silk fibres (Sr, Aa, and Am) accounts for their pronounced yield plateau and a distinct concave curve up to break. Since Sr, Aa, and Am have a lower content of β-sheet and more helix structure, they can acquire extra toughness by breaking intramolecular β-sheets based on the ‘β-sheet splitting’ mechanism.52 This coincides with our results as different species of silk fibres have different contents of β-sheet.

### 3.3 Molecular orientation of protein chains in five different silk fibres analysed by Raman dichroism spectroscopy

In conjunction with the protein conformation, the level of molecular orientation is also thought to be very important for the mechanical properties of silk. Raman spectroscopy is already proved to be a powerful tool to investigate the structures of various silk fibres; especially people are able to obtain the information of molecular orientation from the dichroism spectra.33,53–55 That is, the intensity difference in Raman spectra with different beam directions (parallel or vertical) is able to be a label for the estimation of the molecular alignment along the fibre axis. Five Raman dichroism spectra of different silk fibres are shown in Fig. 5. The amide I band (at about 1667 cm⁻¹) of all five silk

![Fig. 5](image_url)
fibres shows distinguishable dichroism. In addition, non-mulberry silk fibres show a peak at 907 cm$^{-1}$, which is also found in the spider silk but absent in the mulberry silk, which is attributed to the ($\text{Ala}_n$) motif. Interestingly but can be imagined, such a band also has some Raman dichroism. The intensity ratio $I(1667)_v/I(1667)_p$ (vertical to parallel) is often used as an order parameter to represent the orientation degree of the molecular chain in silk fibres. Table 3 shows that mulberry Bombyx silks (Bm (W) and Bm (Y)) show the highest orientation, while the non-mulberry Samia silk (Sr) shows the lowest orientation. The non-mulberry Antheraea silks (Aa and Am) are in the middle. Such a result is consistent with the birefringence data, i.e., a more oriented molecular arrangement is found in mulberry silks than in non-mulberry silks.

The lower degree of molecular orientation of the non-mulberry silks may contribute to their higher extensibility than the mulberry silks. The poor orientation of the protein chain in the non-mulberry silks makes it possible to allow the further alignment of the molecular chains without rupture upon drawing the native fibre.

### 3.4 Synchrotron radiation wide-angle X-ray diffraction (S-WAXD) analysis of five different silk fibres

Apart from FTIR and Raman spectroscopy, WAXD is another versatile technique to investigate the structure of the silk fibre. Both crystallinity and molecular orientation along the fibre axis are able to be determined from the WAXD patterns. Fig. 6 shows the high quality two-dimensional (2D) WAXD patterns of five different silks by using a synchrotron radiation X-ray source (the silk fibre is placed along the equatorial direction during the measurement). The diffraction patterns of all silks studied depict a semi-crystalline morphology characterized by Bragg reflections and an amorphous halo, as reported by this and other laboratories previously. That is, the diffraction patterns of Bm (W) and Bm (Y) silks are similar to those reported previously for *B. mori* silks in China, while those of non-mulberry Am and Aa silks are similar to *Nephila* spider silks. The diffraction pattern of Sr is also similar to the reported data, but it is somewhat different from other four kinds of silk fibres. Such a diffraction pattern implies that the molecular orientation along the fibre axis in Sr is worse than in other silks (Bm (W), Bm (Y), Am, and Aa), which is consistent with the Raman dichroism results.

According to the prevalent structural model, silkworm and spider silks have a hierarchical structure wherein each silk fibre is made of many fibrils coaxially parallel to the fibre axis. Each fibril is composed of well-aligned nano-crystalline domains connected by an amorphous matrix and comprised of both oriented and isotropic components. The diffraction patterns...
observed for axially-aligned silk fibres shown in Fig. 6 are similar to those reported in literature studies,36 but have more clear diffraction arcs because we used a brighter synchrotron radiation X-ray source. Intense diffractions are observed along the meridian direction (perpendicular to the fibre axis). For Bm (W) and Bm (Y), only a strong (200) reflection (corresponding to the inter-chain direction and denoted as the \(a\) axis of the crystallite) along the meridian can be seen, which accords with the characteristics of the \(B.\) mori silk.66 While for Sr, Am, and Aa, two strong reflections, i.e., (200) and (120) reflections (corresponding to the inter-sheet distance and denoted as the \(b\) axis of the crystallite) can be found, like in the \(Nephila\) spider silk, which are attributed to the crystalline fraction corresponding to the \(\beta\)-poly(L-alanine) structure and are indexed based on an orthogonal unit cell with the \(c\) axis as the fibre axis.62,67

To analyze the S-WAXD results quantitatively, the crystallinity of different silks is determined from the one-dimensional (1D) profile derived from the 2D WAXD patterns. From Fig. 7, we find that there is an obvious difference in the 1D WAXD profile between mulberry silks and non-mulberry silks. The 1D WAXD profiles of mulberry silks are similar to other \(B.\) mori silks reported in literature studies,33,37 while those of non-mulberry silks are similar to the \(Nephila\) spider silk.38 Interestingly, although the 2D WAXD pattern of Sr is slightly different from those of Am and Aa, their 1D profiles are rather similar. Therefore, we calculated the crystallinity of five different silk fibroins according to the reported method for \(B.\) mori and \(Nephila\) spider silks, and the result is shown in Table 3. The crystallinity of five different silk fibres accords with their \(\beta\)-sheet content very well, which verifies the accuracy of the results from both S-FTIR and S-WAXD techniques. In the meantime, we also calculated Herman’s orientation factor of the crystalline part in five different silk fibres. From Table 3, we can see Bm (W) and Bm (Y) have the largest orientation factor, while Sr has the lowest orientation factor. The values again accord with the Raman dichroism data, indicating that the molecular chains in Bm (W) and Bm (Y) silk fibres align along the fibre axis better than Aa and Am, and the molecular alignment in the Sr silk fibre is the worst among five kinds of silks.

### 3.5 Relationship between the structural and mechanical properties of silk fibres

Generally, all silk fibres we studied in this research have excellent toughness (breaking energy \(> 140 \text{ MJ m}^{-3}\)). Although the mulberry \(B.\) mori silks (Bm (W) and Bm (Y)) exhibit better toughness (\(~ 180 \text{ MJ m}^{-3}\)) than the non-mulberry silks (Sr, Aa, and Am, 140–155 MJ m\(^{-3}\)), the non-mulberry silks are still very competitive in real world application.

The structural characterization of five different silks demonstrates that the mulberry silk has the highest \(\beta\)-sheet content, then the non-mulberry \(Samia\) silk, and the non-mulberry \(Antheraea\) silks show the lowest value. The crystallinity (closely related to \(\beta\)-sheet content in silk fibres) of these silks shows the same tendency. Our data explain the mechanical properties of mulberry and non-mulberry silk fibres well. Although the non-mulberry \(Antheraea\) silk possesses the smallest Young’s modulus and breaking stress value, it shows the highest breaking strain (56–64%), which compensates its relatively low strength, resulting in similar toughness to the mulberry \(Bombyx\) silk.

Among the five kinds of silk fibres studied, the non-mulberry \(Samia\) silk is a little bit special. Actually it has a quite high \(\beta\)-sheet content (\(~ 23\%) or crystallinity (\(~ 53\%)), but it is not nearly as strong as the \(Bombyx\) silk, as its Young’s modulus is only 6.3 GPa. In contrast, it also shows a strain-hardening behaviour like the low \(\beta\)-sheet content (or crystallinity) \(Antheraea\) silk. We find that although its \(\beta\)-sheet content is not low,

---

**Fig. 7** Synchrotron WAXD 1D profiles of five different silk fibres. (A) Bm (W); (B) Bm (Y); (C) Sr; (D) Aa; and (E) Am.
its α-helix (another ordered structure) content is very low (only about 10%), even lower than that in the *Antheraea* silk. One phenomenon that may be more important is the molecular orientation in the *Samia* silk, which is the worst among five kinds of silk fibres studied. Not only the helix/disordered part, but the orientation of β-sheet crystallites is also very poor (Herman’s orientation factor f is only 0.623, and also can be directly seen from the 2D WAXD pattern in Fig. 6). Therefore, during stretching, the molecular chains including those β-sheet crystallites have a great opportunity to rearrange, enabling them to become well-aligned along the fibre axis. The shape of the stress–strain curve of the *Samia* silk is similar to that of the *Antheraea* silk, but its stress in each strain point is significantly larger. As a consequence, the mechanical properties of the *Samia* silk are close to those of the *Bombyx* silk, i.e., with relatively high breaking stress and energy, and moderate breaking strain.

*Mulberry* *B. mori* silkworms have been domesticated for thousands of years. They are now under rearing/cultivation and bred in order to get high quality silks using different modern technologies including selection. Meanwhile, *B. mori* silkworms need not bear the high pressure from nature when they produce cocoons, as they need not think of how to protect their pupae because they always live in a safe place we provide. Therefore, the structure of the *Bombyx* silk evolves to have high β-sheet content (or crystallinity) and high molecular orientation, meeting the requirements of a textile material, which should be strong. At this point, high extensibility is not necessary. In the meantime, we can find that the two kinds of *Bombyx* silk fibres studied here (*Bm* (W) and *Bm* (Y)) and *Bombyx* silk fibres found in China are very similar. The number of crops and environmental conditions do not alter the structural and mechanical properties of the mulberry *Bombyx* silk significantly.

Totally wild non-mulberry *Antheraea* silkworms need to produce silk and make their cocoons strong depending on the harsh natural environment. As the cocoon is just a safe house to protect the pupae, the silks need not necessarily be very strong. However, the cocoons in the wild need to resist the wind blowing them from the tree or prevent animals from biting or tearing, so the *Antheraea* silk is better to be highly stretchable. Therefore, during the evolution, the *Antheraea* silkworms may choose the low β-sheet content (or crystallinity) strategy to achieve this goal. The large portion of stretchable helical and disordered structure provides high extensibility in case of emergency. However, the variety between the different kinds of *Antheraea* silkworms is obvious, which may depend on their living environment. Although two kinds of non-mulberry silks from *A. assamensis* and *A. mylitta* silkworms have similar toughness (breaking energy), the former is stronger (higher Young’s modulus and breaking stress) and the latter has better extensibility (longer breaking strain).

*Samia* is the semi-domesticated non-mulberry silkworm. It is able to be domesticated like *Bombyx*, but it is non-mulberry like *Antheraea*, so it seems like a bridge between the totally domesticated mulberry *Bombyx* silkworm and the totally wild non-mulberry *Antheraea* silkworm. Interestingly, the mechanical properties (Young’s modulus, breaking stress, breaking strain, and breaking energy) of the *Samia* silk are exactly in the middle of *Bombyx* and *Antheraea* silks. Referring to its structure, β-sheet content (or crystallinity) in the *Samia* silk is also in between *Bombyx* and *Antheraea* silks, which could be a good reason to explain the mechanical properties. However, the orientation of molecular chains, especially the β-sheet crystallites, is rather poor in the *Samia* silk. The reason for such a phenomenon during the evolution is not fully understood, may be the *Samia* silkworm uses this method to increase the extensibility of its silk while keeping it strong.

### 4. Conclusions

In this research, we chose five kinds of commercially available silkworm silks (both mulberry and non-mulberry) to compare their mechanical properties. We find that the Young’s modulus and the breaking stress of these five kinds of silk fibres follow the sequence *Bm* (W) > *Bm* (Y) > *Sr* > *Aa* > *Am*, while the breaking strain is in the opposite way, i.e., *Bm* (W) < *Bm* (Y) < *Sr* < *Aa* < *Am* From S-FTIR and S-WAXD analysis, we are also able to obtain the β-sheet content or crystallinity of five kinds of silk fibres as *Bm* (W) > *Bm* (Y) > *Sr* > *Aa* > *Am*, which accord with their mechanical properties well. In the meantime, we found that the molecular orientation in these silk fibres generally follows the sequence of *Bm* (W) > *Bm* (Y) > *Sr* > *Aa* > *Am*, but leaves the *Sr* silk as a distinct one. The molecular orientation, especially the alignment of β-sheet crystallites, in the *Sr* silk is the lowest among the five different kinds of silk fibres studied.

From the results shown above, we may establish the relationship between the mechanical properties and the structures of silk fibres. Irrespective of mulberry or non-mulberry silks, the higher the β-sheet content or crystallinity, the higher the Young’s modulus and the breaking stress, but the lower the breaking strain. In addition, the alignment of molecular chains along the fibre axis may also contribute to the mechanical properties. Good molecular orientation is favourable for strength (Young’s modulus and the breaking stress), but poor molecular orientation may help to improve the extensibility (breaking strain) of the silks.

The different tensile behaviours and properties of the silks indicate that there are mechanical strategies, such as trade-offs between elasticity, yield and hardening regions, for absorbing certain energies. The non-mulberry *Antheraea* silkworms seem to compensate the low strength with high extensibility to make their silk have comparable toughness to the mulberry *B. mori* silk. It is of interest to investigate further the strategies how the silkworms produce silks to balance their mechanical properties to match their multiple applications. The different species of silkworms produce different types of silk fibres, with a completely diverse set of properties, which are able to be applied to various applications. A more thorough understanding of the secondary and hierarchical structure of silkworm silk is required for getting further information on the structure–function relationship. This will guide the development of high
performance bio-synthetic and bio-inspired polymeric materials for different types of applications.

Acknowledgements

X. C. thanks the financial support from the National Natural Science Foundation of China (No. 21274028, 21574023, and 21574024). S. C. K.'s laboratory is financial supported by the Department of Biotechnology, and Indian Council of Medical Research, Government of India. We also sincerely acknowledge the Central Silk Board, Bangalore (India), and their scientists for providing information and assistance in relation to commercial silk threads and their production process. We thank Prof. Zeming Qi at NSRL, Dr Yuzhao Tang and Ms Jiajia Zhong at NCPS-S-SRF, and Prof. Min Chen, Dr Feng Tian and Dr Fenggang Bian at SSRF for their technical support. We also thank Dr Yuhong Yang at Fudan University for her valuable suggestions and discussions.

References