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Differential Scanning Calorimetry of Native Silk Feedstock

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Native silk proteins, extracted directly from the silk gland prior to spinning, offer access to a naturally hydrated protein that has undergone little to no processing. Combined with differential scanning calorimetry (DSC), it is possible to probe the thermal stability and hydration status of silk and thus investigate its denaturation and solidification, echoing that of the natural spinning process. It is found that native silk is stable between −10 °C and 55 °C, and both the high-temperature enthalpy of denaturation (measured via modulated temperature DSC) and a newly reported low-temperature ice-melting transition may serve as useful quality indicators in the future for artificial silks. Finally, compared to albumin, silk's denaturation enthalpy is much lower than expected, which is interpreted within a recently proposed entropic desolvation framework which can serve to unveil the low-energy aquamelt processing pathway.

1. Introduction

Natural silk spinning presents numerous opportunities for technological translation of low-energy aqueous processing routes for polymers.[1] One such avenue for exploration surrounds silk's solidification mechanism which enables a protein feedstock, stored as a liquid gel, to transform into a hierarchically structured fiber.[2] Recently, it has been proposed that this is achieved through the aquamelt pathway—a means by which silk proteins respond to stress by disassociating from their intrinsically bound water and forming more stable intra-/interchain hydrogen bonds and larger scale structures. This has parallels to flow-induced crystallization in polymers, albeit at an inspirational 1000-fold energy saving.[1]

In the animal, the solidification stress occurs as the feedstock is pulled through the silk duct.[3–5] In-depth rheological characterization of this material[1,6–13] allows the calculation of "work to fibrillation"[1] and estimates the activation energies for flow.[9] However, details of the energetics of this process remain elusive. Here we propose to test the hypothesis that one can substitute mechanical stress for thermal stress and thus directly measure the energetics of silk solidification using calorimetry.

Silks have been subjected to calorimetric tests for over 20 years but with the focus either on natural spun fibers[14–21] (which are not directly relevant in the context of this work) or artificial silk feedstocks/films,[22–36] where processing has altered the state of hydration so much that these artificial silks may no longer represent a native unspun silk protein/model aquamelt.[11] However, in the early 2000s, Tanaka et al. pioneered several initial studies where the fundamental thermal properties of the native unspun material have been investigated and thus laid the foundation for our work today.[19,37,38]

Here we present quantitative experimental data from differential scanning calorimetry (DSC) performed on native silk extracted directly from the gland of the silkworm *Bombyx mori*. The data allow us to define the upper and lower temperature limits for the storage and processing of this material, and suggests that the enthalpy of denaturation is a quantitative, useful indicator for the hydration state of a silk feedstock. Furthermore, we compare silk's denaturation enthalpy to that of globular proteins, albumins, and find it to be much lower than one would expect. This surprising result offers two alternative hypotheses for the solidification pathway of silk, both framed within a recently proposed entropic desolvation framework which could serve to unveil the low-energy aquamelt processing pathway.

2. Experimental Section

Native silk feedstock specimens were obtained from 5th instar *B. mori* larvae (commercially bred four-way polyhybrid cross of two Japanese and two Chinese strains) during early stages of cocoon construction and characterized using methods similar to those described previously.[7] Under the Animals (Scientific Procedures) Act 1986 issued by the UK Home Office the invertebrates used in these experiments do not require the authors or institution to hold a relevant licence. However all experiments were carried out under scrutiny from local ethical guidelines. In summary, silk glands were excised and the epithelial membrane was peeled off under cold (≈5 °C) distilled water, using fine tweezers and a dissection microscope. For the initial experiments to determine the optimum portion of the gland to use for measurements,

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the entire gland was stripped of its epithelium and then divided into portions. Fresh hydrated samples ranged from 5 to 10 mg wet weight (using a 5-place Sartorius balance) and were placed in T_{zero} low-mass pans with a hermetically sealed lid.

All DSC tests were performed on a Q2000 DSC instrument (TA Instruments, Delaware), which was fully calibrated with the sample chamber purged with high purity nitrogen (oxygen and moisture free) at a flow rate of 50 cc min^{-1}.

Standard DSC tests were performed from 5 to 95 °C at a heating rate of 5 °C min^{-1}.

Isothermal tests were performed by holding the sample at a set temperature for 2 h and then performing a standard heating ramp of 5 °C min^{-1} from 25 to 90 °C.

Modulated temperature differential scanning calorimetry (MT-DSC) tests were performed between 5 and 100 °C at an underlying heating rate of 3 °C min^{-1} with a modulation amplitude of ±1.5 °C and period of 60 s. These conditions were optimized through an extensive study on *B. mori* silk dope whereby a wide range of MT-DSC conditions were investigated ($n > 40$). The current conditions give good sensitivity to detect heat capacity changes and to quantify the heat of denaturation (please see Section S1, Supporting Information, which details this extensive study).

Freezing studies were performed in a similar manner to the isothermal tests. A set low temperature was determined in which samples were held for 10, 30, or 60 min followed by a standard heating ramp of 3 °C min^{-1} to 95 °C. Note the time held at either 10, 30, or 60 min did not appear to affect the resulting denaturation enthalpy, and so samples were pooled and averages taken.

Dry weight calculations were obtained by making pinholes in the pan lid after being run on the DSC and placed in an oven overnight at a temperature of 60 °C. The weight was then recorded (5-place Sartorius balance) and used to quantify the dry weight and subsequent endothermic denaturation heat flow using the Universal Analysis software.

A complete open access data set for the results presented in this work may be found at https://doi.org/10.15131/shef.data.6397304.

3. Results and Discussion

Tests were first undertaken to determine the optimum sample preparation conditions for thermal analysis. **Figure 1** shows the thermograms from a series of samples prepared from a single *B. mori* silk gland which was sectioned along its length and placed into hermetically sealed pans to ensure any observed effects were not due to water evaporation. This enabled us to determine the heat flow contributions of both the silk fiber–forming proteins, fibroin (our main protein of interest), and sericin, which acts as a lubricant during spinning[39] and as glue to bind fibers together into a nonwoven composite during cocoon construction.[40,41]

Figure 1 shows that the location of sericin in the gland corresponds with both a lower temperature transition between 40 and 45 °C and a shoulder on the main endothermic peak (now referred to as the denaturation endotherm). We define denaturation as a process by which a protein loses its as-synthesized hydration shell, resulting in a structural reconfiguration (which is based on our previously published aquamelt hypothesis and a feature not seen in a standard regenerated/reconstituted silk, Section S2, Supporting Information).[1,42,43] Attributing these lower temperature transitions to sericin is also consistent with visual observations of the presence of sericin during sample preparation, as the introduction of an additional surrounding layer as observed by a change in refractive index of the feedstock along the gland. In samples without the influence of sericin (Figure 1), above ≈62 °C, the following denaturation endotherm is attributed primarily to fibroin. Together, these results agree well with recent studies on the thermal responses of native fibroin and sericin under rheological testing, which show these materials remain stable up to ≈55 °C.[9,39]

The upper temperature limit for native fibroin was confirmed through a subsequent series of isothermal hold tests (**Figure 2**). These demonstrated the presence of the denaturation endotherm at hold temperatures up to 60 °C, suggesting that the proteins present did not undergo any significant changes to their hydration state or structure until this temperature was exceeded. These results extend previous studies where isothermal holds have been performed on native silk by over 1 h, thus increasing the known "safe" time this material can be handled at elevated temperatures.[1,9,44]

Once the effect of gland sampling location and proteins responsible for the observed transitions had been identified, a more detailed characterization of native fibroin was undertaken using MT-DSC (Section S1, Supporting Information).[45] MT-DSC provides more information regarding the status of the material through a thermal transition than standard DSC, as it is able to separate the total heat flow response into its reversing and non-reversing heat flow components.

After determining the optimal methodology to ensure a suitable combination of precision, accuracy, and homogenous heating of the sample, **Figure 3** suggests two potential processes occurring concurrently when native silk fibroin undergoes

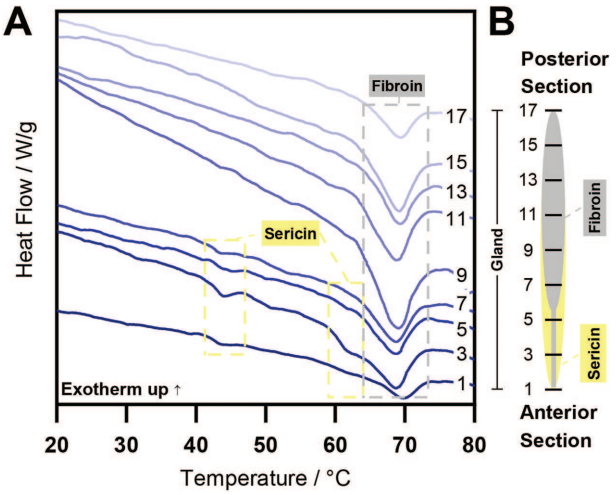

Figure 1. Thermogram of the gland contents of *B. mori* as a function of A) temperature and B) position of sample along the gland. Note that the presence of the "glue" protein sericin correlates with a lower temperature transition and later shoulder (highlighted in yellow) prior to the main endothermic transition observed and attributed primarily to fibroin (highlighted in grey). Small variations in sericin content due to the complexities of sample preparation are most likely to account for the increased endotherms of sample 3. Note exotherm is up.

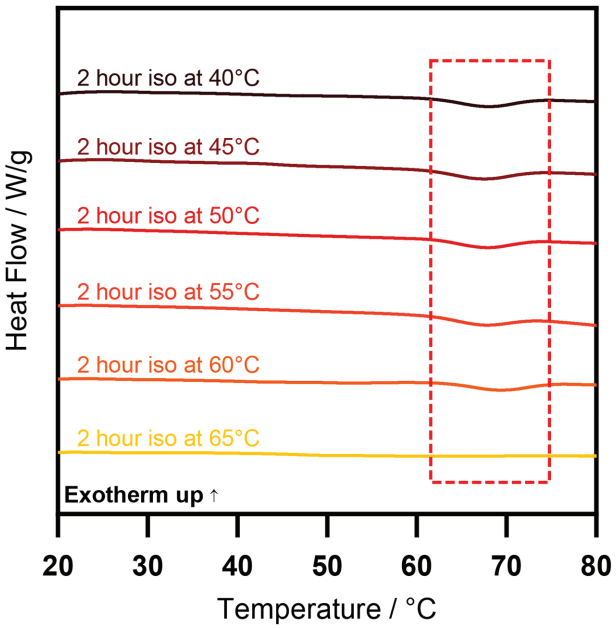

Figure 2. DSC thermograms of native silk samples after preconditioning for 120 min at temperatures ranging from 40 to 65 °C. Note that the denaturation enthalpy decreases and the denaturation temperature range shifts upward slightly as the temperature of the prior isothermal hold increases (dashed red box). Note exotherm is up.

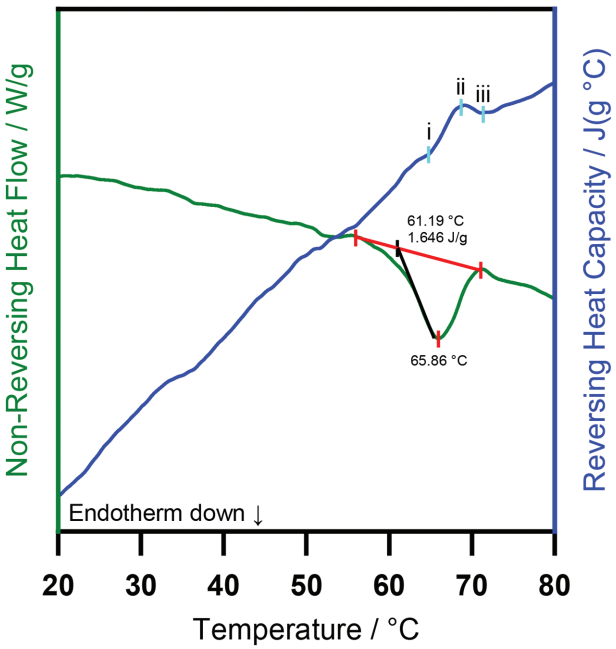

Figure 3. MT-DSC thermogram of a representative native *B. mori* feedstock sample. Non-reversing heat flow is shown in green and the underlying heat capacity is presented in blue. Changes in underlying heat capacity through the denaturation endotherm are labeled as i) onset of dehydration; ii) peak in heat capacity signal due to dehydration and structural reconfiguration; iii) return to heat capacity baseline once denaturation process is complete. Note exotherm is up.

heating—a nonreversible endothermic transition and changes in the underlying heat capacity.

The comparative dominance of the non-reversing signal suggests that this transition is primarily a permanent change in the state of the material. It also suggests that this transition cannot simply be reversed by lowering the temperature, which agrees well with other studies on silk-based proteins[32,46] as well as our own observations of a lack of denaturation endotherm upon reheating once a sample has been heated past this transition (Figure 2 and Section S3, Supporting Information).

Changes in the underlying heat capacity of the material during the denaturation endotherm, as identified by the MT-DSC reversing signal, provide further insight into the molecular processes taking place. Looking at Figure 3, we see a sharp increase in heat capacity from 64 to 68 °C, which then returns to a baseline at 72 °C. This rise is most likely due to the dehydration of the proteins present, and the return to the baseline is due to either the structural reconfiguration of the proteins (e.g., gelation which agrees well with recent rheological studies[9]) or could imply that proteins present are able to become partially rehydrated over the timescale of the experiment (discussed in more detail in Section S3, Supporting Information). This rehydration phenomena may be attributed to sericin itself, which is known to resolubilize more readily than fibroin (perfectly exemplified by degumming)[47–52] or perhaps acting as a renaturing/protective agent for fibroin which is consistent with other recent observations,[53] or finally, that certain portions of the fibroin protein are more hydrophilic than others (Section S3, Supporting Information).

MT-DSC also provides the additional benefit of enabling more precise determination of the energetics of enthalpy of denaturation, which represents a step forward in this area. This is because if one performs peak integration using the total heat flow signal (featured in Figure 1), inaccuracies may arise from an underlying heat capacity change. To guard against this, we opted to use the sensitivity of the MT-DSC reversing heat capacity signal in order to determine the limits of peak integration and the non-reversing heat flow signal for the peak integration (**Table 1**).

This analysis generated a benchmark for the degree of denaturation for these materials that informs not only about the underlying mechanisms, but may also inform about the design of synthetic or biosynthetic polymeric materials aiming an aquamelt process.[1,52]

In order to further probe the hydration status of unspun silk proteins, we investigated another group of fresh, unprocessed proteins, that is, hen egg albumins. The results from the analysis of fresh hen egg white (Section S4, Supporting Information) agreed well with the overall denaturation enthalpy obtained from previous studies (17.0 J g^{-1} for albumin and 4.0 and 13.0 J g^{-1} for conalbumin and ovalbumin proteins, respectively) and lie within the denaturation enthalpy range for most globular proteins,[46,54] thus validating our methodology to obtain quantitative measures of the denaturation enthalpy of a protein solution.[55]

However, when comparing the native silk feedstock to egg white, we note that whilst the maximum peak of the denaturation enthalpy fell within the +60 °C expected range for proteins, silk's denaturation enthalpy was nearly an order of magnitude lower (1.8 J g^{-1}) when compared to albumin (17.0 J g^{-1}) and half that of ovalbumin (4.0 J g^{-1}). Thus, our data present an interesting as well as important observation regarding the metastable state of

Table 1. MT-DSC calculations of different thermal properties of *B. mori* native silk feedstock.

Property measured	Mean value ($n = 27$)	Standard deviation (σ)
Enthalpy of denaturation [J g^{-1}]	1.779	0.245
Onset temperature [°C]	61.21	0.523
Peak maximum [°C]	66.26	0.416

native silk feedstocks that, if considered to be representative of a true aquamelt, may be interpreted one of two ways.

The *first hypothesis* would suggest that silks behave like globular proteins. In this case, the enthalpy of denaturation would represent the full extent of hydration/hydrogen bonding present in the protein. This would imply that, compared to egg white proteins, silk proteins may be only partially hydrated or that only changes in the vicinity of some amino acids contribute to the denaturation endotherm. This interpretation would be indicative of strong selection pressures on nearly all proteins that remain inside the body to maintain their hydration shell. However, we may assume that silk has "evolved to be denatured" and may be considered functionally active in both the hydrated and dehydrated forms.[56]

If we consider that the silk proteins do behave like a typical globular protein, then we should consider the proportion of hydrophilic to hydrophobic amino acids. Kyte and Doolittle developed a means by which the grand average hydropathy index (GRAVY) can be calculated for a protein.[57] With more hydrophobic proteins presenting a more positive score, we find that such calculations for our system give conalbumin a score of –0.3643 (ID: P02789), ovalbumin a score of –0.001 (ID: P01012), and a silk fibroin score of 0.216 (ID: P05790). this relatively positive hydropathy value for native silk fibroin suggests a lower overall affinity to remain in a hydrated state.

Extending this consideration, to interpret our results we can infer that fibroin in the gland is not fully hydrated along its chain length but exists in a partially dehydrated/denatured state. As 94% of the fibroin-heavy chain sequence are Gly-X repeats,[58] these may be entirely denatured, and the observed lower denaturation enthalpy could be the result of only the remaining ≈5% N- and C-termini being hydrated[42] and/or a dissociation of the phenolic –OH group of tyrosine, which constitutes around 5% (molar) of the amino acids in silk fibroin,[58] maintaining hydration.

The *second hypothesis*, perhaps more suitable, would treat silks as generic polymers/fibrillar proteins, and it would interpret the data as a cumulative set of endo- and exotherms from different components which require deconvolution. The two processes proposed are the endotherm as a result of the water being removed and the exotherm of the resulting structural transition and crystallization/aggregation process.[19]

Indeed, consistent with this hypothesis, the MT-DSC results (Figure 3) indicated at least two processes occurring concurrently—one endothermic and the other exothermic. In addition, there is additional supporting evidence for such a two-stage process if it is extended to other types of native silk feedstocks. For example, Magoshi et al.[19,38] observed strong exotherms for "wild" silkworm silks (*Antheraea pernyi*, *Antheraea yamamai*, and *Dugesia japonica*), and our own preliminary studies on other native silk feedstocks have also observed an endotherm, followed by an exotherm (Section S5, Supporting Information).

From a biological perspective, the competing endothermic and exothermic processes may effectively "level the playing field," making it easier for the fibroin to go through the spinning transition/transformation from liquid to fiber as it presents a lower energetic activation threshold for the animal to initiate solidification. This careful balance avoids the need to raise the temperature of the feedstock (which is not feasible given the animals are ectotherms) and enables this transition to be initiated through a rise in free energy of the hydrated protein through a mechanical stress field which is applied by the animal during normal spinning.[6] This is consistent with the view of selection pushing for the animal to minimize the energetic requirements required to navigate through a processing transition where the endothermic penalty for dehydration is offset by the exothermic gain of the resulting crystallization process of the peptides into a more stable lower-energy state.[42]

However, there is evidence to suggest that natural spinning may be more complex than simply two processes. Recently, by manipulating solvent quality in dilute systems, Dicko and collaborators suggest that native silks are able to sample multiple substrates prior to undergoing (or to delay) high-temperature denaturing transition, which may be mediated by a fragile packaging of the silk tertiary structure that is readily lost when the solvent quality changes.[59]

A novel interpretation of the high-temperature denaturing peak was proposed recently by Holland and collaborators, based on the concept of an entropically-driven desolvation mechanism.[1,6] Here, it was suggested that denaturing (observed rheologically as gel formation) above 60 °C represented the hydration shell around the fibroin becoming thermodynamically unstable relative to bulk water. This has also been more recently extended to include the effects of mechanical shear stress and freezing.[6] Hence, heated, frozen, and mechanically stressed systems would be driven by the loss of entropy caused by the restricted movement of the water molecules associated with the gelled (relatively slowly moving) protein.

Moving forward, while thermal gelation of silk feedstock at elevated temperatures is well known and has been investigated in some detail,[6,10,19,20] in contrast, low-temperature gelation (i.e., by freezing) is far less well understood,[6,60] which makes it interesting to examine the effects of freezing on the denaturing endotherm of silk feedstock. This effect would also be important to determine from a processing perspective, as it helps define the range of temperatures in which native silks can be processed without irreversibly affecting their properties.

By conducting a series of downward temperature ramps, we have determined that native silk feedstocks undergo a strong exothermic transition between –12 and 18 °C (data not shown), which we associate with the onset of freezing. Such a transition has been difficult to determine, precisely due to the latent heat of fusion from the water, and the idea will be studied in future work by significantly lowering the cooling rate to <1 °C min^{-1} and potentially accounting for the metal ion content/viscosity of the native silks.[61]

While conducting these freezing experiments, we observed a puzzling phenomenon. If the silk feedstock was taken below its freezing point for any length of time, we observed a reduction in the (high temperature) denaturation enthalpy in

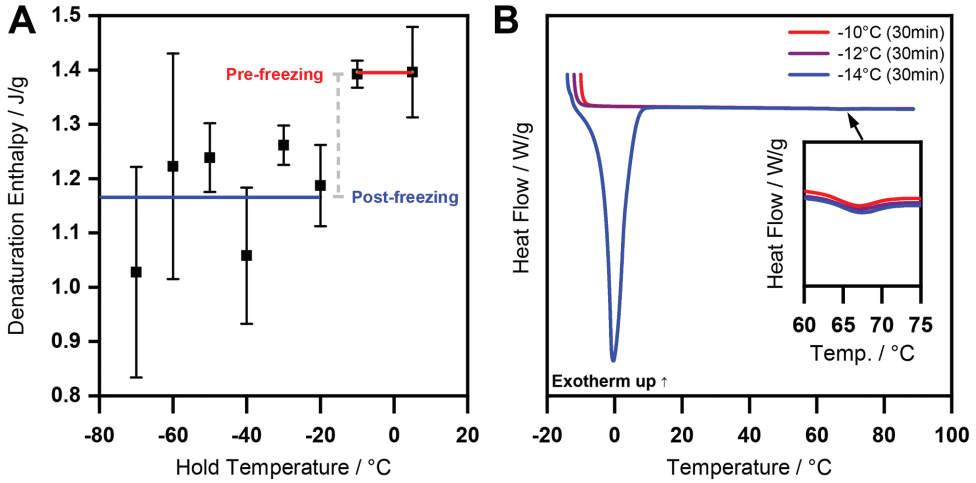

Figure 4. A) Denaturation enthalpy of samples as a result of being held isothermally at different low temperatures. Note the blue line represents the group average for samples held below the freezing point of silk and the red line for those held above. Error bars represent standard deviation ($n = 3$). B) Thermogram of silk samples after being held for 30 min at either –10 °C (red), –12 °C (purple), or –14 °C (blue). Note that for the sample held below the freezing point a new ice-melting endotherm appears, yet denaturation enthalpies are still present (inset). Note exotherm is up.

the subsequent heating ramp (**Figure 4**A). This appears to be a small change, quantitatively, but it accounts for ≈16.4% of the entire peak area, suggesting that on freezing a significant number of water–protein interactions are irreversibly effected. In line with this, previous rheology studies also noted that gelation was initiated by freezing.[6]

Whether or not a sample had frozen at the initial holding temperature was revealed by the appearance of an ice-melting peak upon reheating (Figure 4B). In samples that were stored below their freezing point, a new ice-melting peak appeared in the subsequent temperature ramp. The considerable depression of the freezing point observed may indicate a strong affinity between fibroin and water, while the presence of an ice-melting peak may suggest that some water has adopted a different structure in the sample, presumably by disassociation from the protein to form bulk water. This interpretation is also consistent with the reduction in the enthalpy of the higher-temperature denaturation peak. Such a view would appear to be more diagnostic and may serve as a useful indicator in the future for the status of hydration of aquamelt materials, which avoids being confounded by competing endo- and exothermic processes at higher temperatures. This area of study is of significant interest for the translation of academic insights to commercial practice and will be the subject of continuing work in our collaboration.

4. Conclusions

In this study, we present quantitative data from a combination of differential scanning calorimetry experiments performed on native silk extracted directly from the gland of the silkworm *B. mori*. We found that the temperature range in which this material can be handled without noticeable effects lies between –10 and 55 °C. By measuring the silk's thermal response beyond these temperature limits, we gained insights into the mechanisms by which this material may solidify. We propose that the high temperature enthalpy of denaturation is a useful indicator for the overall hydration state of a silk feedstock and that the presence of a low-temperature ice-melting transition may serve as a useful indicator of protein denaturation in the future. Furthermore, we show that our silk's denaturation enthalpy was much lower than expected than that of globular proteins, such as albumins. This surprising result suggests two alternative hypotheses for the solidification pathway of silk, both framed within a recently proposed entropic desolvation framework which could serve to unveil the low-energy aquamelt processing pathway.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author. A complete open access data set for the results presented in this work may be found at https://doi.org/10.15131/shef.data.6397304.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

Bombyx mori, denaturation, differential scanning calorimetry, protein, silk

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