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Mechanical properties of silkworm silk in liquid media

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Abstract

Tensile tests have been performed on silkworm silk fibres submerged in liquid environments (water, acetone, ethanol and isopropanol). Liquid media were initially chosen in order to weaken non-covalent interactions specifically. However, only immersion in water leads to a decrease in the mechanical properties of silk, indicating the weakening of hydrogen bonds. Immersion in acetone, ethanol and isopropanol leads to an increase in the stiffness of the fibre. In addition, all three organic solvents produce similar force–displacement curves, which can be explained by the desiccating effect that these solvents exert on silk.

These results indicate that water disrupts hydrogen bonds initially present in the amorphous phase, while the other solvents eliminate water and contribute to the formation of new hydrogen bonds in the amorphous phase of silk. This interpretation was developed through the *shear lag* model of the elastic modulus (*E*) of silk, and a good agreement has been found between the model and the experimental values of *E*.

Keywords: Silkworm silk; Elastic modulus; Tensile properties

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1. Introduction

The mechanical properties of many biological materials reflect an abundance of non-covalent (weak) interactions, which play a critical role in the assembly and performance of structures like F-actin in muscles, tubulin in the cytoskeleton, viral capsids, and silk [1]. An improved understanding of how nature uses these weak interactions is needed to clarify the complex relationships between mechanical properties and what is often a hierarchical microstructure in such materials.

The readily available cocoon silk fibre spun by *Bombyx mori* silkworms provides a useful model system. Its microstructure contains only two significant phases: one is highly crystalline, and the other is amorphous. The relative simplicity of the *B. mori* silk microstructure is a direct consequence of the repetitive primary sequence of the constituent protein [2–4]. This polymer (fibroin) features well conserved runs of [-Gly-Ala-Gly-Ala-Gly-Ser-]$_n$. The occurrence of glycine, alanine, and serine in an approximately 3:2:1 ratio allowed Pauling to deduce the first accurate description of β-sheet crystals from X-ray diffraction data [5]. When the chains adopt an extended β-strand conformation, the glycyl and non-glycyl side chains point in opposite directions, nominally perpendicular to the backbone. Folding such a chain into lamellae produces β-sheets with chemically distinct faces: hydrogens on one side, and larger methyl and methanolic groups on the other. Packing of these sheets into three-dimensional structures results in crystals with a bimodal distribution of inter-sheet spacings (3.5 Å for the glycyl–glycyl juxtaposed faces and 5.7 Å for the methyl–methyl juxtaposed faces). Amorphous regions occur where the molecules have an amino acid sequence that does not participate in β-sheet formation. Therefore, the structure and properties of natural silk depend on two types of non-covalent interaction. The β-sheet crystals are stabilised by intra-sheet hydrogen bonds (which occur between the amide hydrogens and carbonyl oxygens on adjacent chains) and inter-sheet van der Waals interactions (which occur between the hydrophobic side chains) [5]. The same two interactions, with the same molecular-level origins as in the crystals, also stabilise the amorphous regions. In order to link the presence or absence of a particular type of bonding to specific features of the stress–strain curve of silkworm silk, it would be particularly helpful if the contributions from hydrogen bonding and van der Waals bonding could be separated.

A possible experimental strategy to achieve this goal

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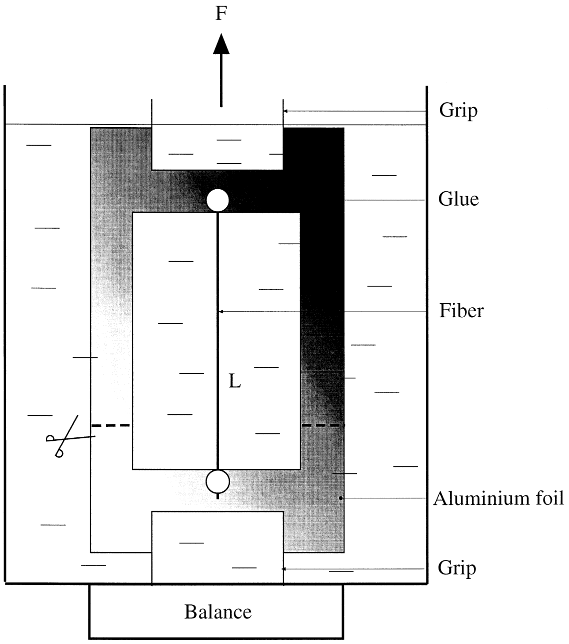

Fig. 1. Experimental setup used for the tensile tests. The fibre is mounted on an aluminium foil frame which is cut prior to immersion.

consists of performing mechanical tests with the fibres submerged in solvents selected to specifically weaken a given bonding type. Four solvents were used in the present study: (1) water, which was expected to disrupt hydrogen bonds initially present between protein molecules in the fibre; (2) acetone (CH_3COCH_3), which may be expected to weaken the van der Waals interactions due to its large dielectric constant ($\epsilon_r = 20.7$); (3) ethanol (CH_3CH_2OH) and (4) isopropanol ($CH_3CHOHCH_3$), which share characteristics of both water (capacity to form hydrogen bonds) and acetone (large dielectric constant, ethanol: $\epsilon_r = 24.3$, isopropanol: $\epsilon_r = 18.3$) [6]. We found, however, that the consequences of immersion in these solvents cannot be described simply in terms of their effect on bonding.

Some noteworthy studies of the influence of water on the major ampullate (dragline) silk of spiders [7–10] and on silkworm silk (*B. mori*) [11] have been undertaken. However, these have focused their attention on the supercontraction effect. Supercontraction of spider dragline occurs when an unconstrained fibre is wetted. The fibre swells, and simultaneously contracts to approximately 50% of its original length; its mechanical properties differ dramatically from those of the dry fibre, yielding a force–displacement curve similar to that exhibited by an elastomer. All these changes are reversible: the fibre recovers its original length and mechanical properties when dried. This behaviour has been explained in terms of the swelling of amorphous regions in the fibre [8]—an explanation that is supported by the stability of the nanocrystals in the supercontracting fibre as observed by X-ray diffraction [12,13]. Also, Raman spectroscopy has been used [14] to identify some qualitative conformational consequences of swelling dragline in water, ethanol, methanol and butanol.

Silkworm silk shows no supercontraction phenomenon. The only reported effect of water is the swelling of the fibre to produce an increase of 18% in the cross-sectional area [15]. The present work is based on a methodology which consists of measuring force–displacement curves of fibres submerged in the different solvents, focusing on the relationship between environment, bonding and tensile mechanical properties.

2. Experimental

Silkworm (*B. mori*) silk fibres (typical length 20 cm) were obtained by boiling (degumming) cocoons in distilled water for 30 min and pulling gently with a pair of tweezers. Care was taken to avoid stretching the fibres plastically during this process. The material recovered in this way is in the form of bave; i.e. the two constituent brins of the cocoon fibre are not separated. Boiling in water does not hydrolyse the fibroin chains, does not lead to coarsening of the silk microstructure, and does not change the volume fraction of crystalline material [16]. Fibres were allowed to dry in air for at least 24 h before performing any mechanical test. The original fibres were cut into smaller (40 mm) pieces. These were mounted across rectangular holes cut in aluminium foil supports to define the gauge length (30 mm) of mechanical test specimens (Fig. 1). Droplets of glue (details will be given below) were used to fix the fibre to the foil at the edges of the holes. The adhesive was allowed to dry overnight before mechanical tests were started.

The foil supports were fixed to the grips of an Instron 4411 mechanical testing machine and then cut, so that the force during testing was exerted directly on the fibre. Loads during testing were measured with an electronic balance (Precisa 6100 C; resolution ±10 mg) under the lower grip. The elongation of the fibre was taken as the displacement of the crosshead (resolution ±10 μm), since we estimate that the compliance of the fibre is at least 1000 times greater than that of the experimental hardware. The crosshead speed was set to give a nominal strain rate of $0.0002\ s^{-1}$. Samples tested in air experienced the following nominal conditions: $T = 20°C$, $RH = 60\%$.

Fibres were submerged in solvent for 2 h before starting the corresponding mechanical test in distilled water, ethanol, isopropanol and acetone. All chemicals were of analytical grade and purchased from Panreac (Barcelona, Spain). No load was exerted on the fibres during the pre-test immersion time, in order to allow for any possible contraction of the fibre. However, no contraction was observed in any of the solvents, consistently with the data found for water [11].

A significant experimental problem related to this methodology is the effect of the solvents on the glue that fixes the fibre to its aluminium support. An adhesive that is resistant to the specific solvent had to be used in each case,

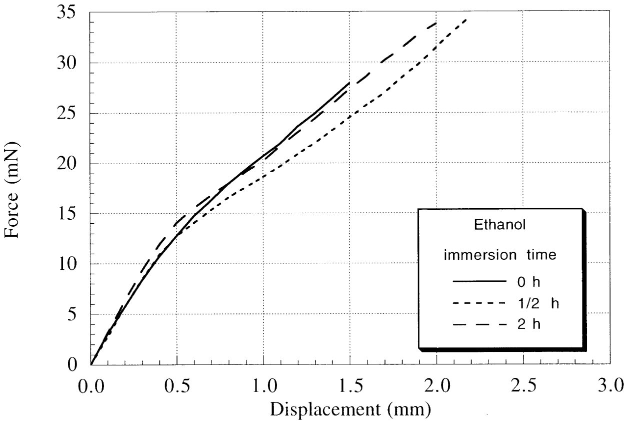

Fig. 2. Comparison of force–displacement curves of fibres immersed in ethanol for different times prior to starting the test. 0 h indicates that the test started immediately after submerging the fibre (gauge length = 30 mm).

since any softening or weakening of the adhesive properties would invalidate the simplification that crosshead displacement is equal to fibre elongation. The first steps to identify suitable adhesives were guided by knowledge of their chemical properties. Cyanoacrylate adhesives have a high shear strength, even in a moderately polar environment [17]. Therefore, Loctite 401 was initially chosen for the tests in ethanol and isopropanol. Acetone, however, quickly degrades the mechanical properties of Loctite 401, so a different cyanoacrylate adhesive (Loctite 496) was proposed for the tests in this solvent. Loctite 496 has a composition similar to Loctite 401, but a lower curing rate; therefore, Loctite 496 has a higher molecular weight and an improved resistance to chemical attack after curing. In highly polar media, the mechanical properties of cyanoacrylates are poor [17], so that adhesives from a different family were proposed for the tests in water. Acrylic adhesives are commonly used in environments with a high moisture content, prompting our choice of Loctite 330 (activated by Loctite 7386) for use under water. The final selection of adhesives was only made after performing controls on Kevlar 49 fibres, since organic solvents have little effect on the mechanical properties of Kevlar 49 [18]. The tensile properties of Kevlar 49 fibres tested in air (control samples) were compared with those obtained from Kevlar 49 fibres immersed in a solvent for 2 h and tested as described above. A particular adhesive was considered to be suitable if the mechanical properties obtained in solvent differed by less than 5% from those measured in air. This methodology confirmed our initial choices, so that Loctite 401, Loctite 496 and Loctite 330 were used for tests in ethanol (isopropanol), acetone and water, respectively.

The intrinsic scatter of tensile test results obtained from silk fibres [19] presents a second significant problem. If the scatter of control samples in air is comparable to or larger than the effect of differences in the immersion medium, no significant conclusion can be drawn from the mechanical tests of immersed samples. Although scatter is reduced if the cross sectional area of each individual test fibre is measured, and stress–strain data rather than load–strain are presented, a variability of 70% in the stress–strain curves is obtained [19], and the observation of trends in the effect of environment would be compromised severely. Fortunately, it has been recognised that *adjacent* fibres (i.e. fibres cut from the same original fibre) show similar force–displacement curves [7]; in other words, the significant variation in tensile properties occurs over fibre lengths that are greater than the gauge length of an individual test sample. We therefore conducted our investigation by comparing results obtained from adjacent samples.

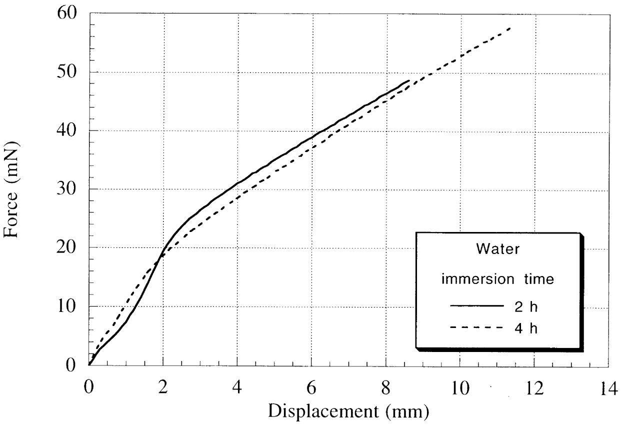

Fig. 3. Comparison of fibres immersed in water for different times prior to starting the test (gauge length = 30 mm).

3. Results

For initial experiments, the fibres were submerged for 2 h in each solvent before starting the test. This duration was chosen so that the fibres could reach an equilibrium state before being tested within the liquid environment. However, additional experiments were performed to investigate the possible effect of the immersion time, since silk fibroin films exhibit behaviour that depends on the duration of prior exposure to solvent [20].

Fig. 2 compares the force–displacement curves of three adjacent fibres tested in ethanol immediately after immersion (0 h), and after immersion for 1/2 and 2 h. No significant differences are apparent, so we can reasonably conclude that equilibrium is reached immediately after immersion.

Fig. 3 compares the force–displacement curves of two fibres submerged in water for 2 and 4 h, respectively before starting the mechanical test. Again, there are no significant differences. These results indicate that 2 h is enough time for the samples to reach an equilibrium state within water before starting the test. Although the immersion time could be considerably shorter, as is the case for ethanol, we preferred to use a consistent time in all solvents; a long

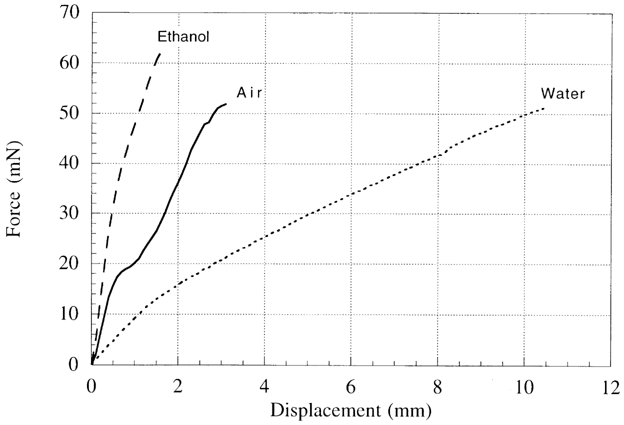

Fig. 4. Force–displacement curves of adjacent fibres submerged in ethanol, water and control sample in air (gauge length = 30 mm).

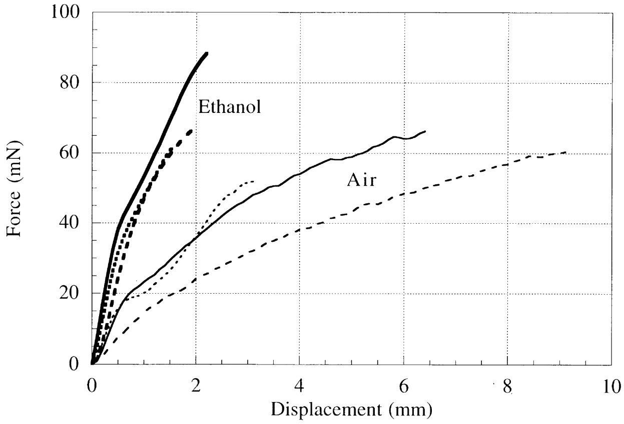

Fig. 6. Force–displacement curves of fibres tested in ethanol compared with control samples tested in air. Curves with the same line symbols correspond to adjacent samples (gauge length = 30 mm).

time can be reproduced with less error, and does not appear to affect the result of the test.

Fig. 4 illustrates the results obtained in ethanol and in water, comparing the force–displacement curve of three adjacent fibres. It is immediately apparent that the sample immersed in water is more compliant than the control sample tested in air, while the sample tested in ethanol is stiffer than the control. Ten tensile tests were performed in each medium (water and ethanol), in every case paired with a control performed in air on an adjacent sample. Some representative curves are shown in Fig. 5 (water) and Fig. 6 (ethanol), where curves with the same line symbols correspond to adjacent samples tested in air and in water (ethanol). These figures further confirm the trends shown in Fig. 4 (we restrict the number of curves shown in these figures so that individual tests can be resolved; note also that the test results obtained in air are different in the two figures due to the pairing of tests obtained from adjacent samples).

Silkworm silk tested in air shows an initial elastic region, followed by a region where permanent deformation is observed if the fibre is unloaded completely. Moreover, the slope of the unloading and reloading steps is similar to the initial slope in the elastic region [19]. Figs. 7 and 8 show unloading–reloading tests on fibres submerged in ethanol and water, respectively. The presence of the initial elastic region is apparent in both the cases, and, in ethanol, the unloading–reloading steps in the non-elastic region proceed with a slope similar to the slope in the elastic region. Unloading–reloading steps in water differ significantly from straight lines, indicating an increased importance of viscoelastic effects under these conditions. This interpretation is consistent with water disrupting the inter- and intramolecular hydrogen bonding in the silk, allowing greater freedom for chains to undergo creep and stress relaxation.

Table 1 presents statistics for: (1) the elastic modulus E; (2) the strain at the proportional limit ϵ_p—a practical definition of yield, at the point where the stress–strain curve intersects a straight line that starts at the origin and has a slope equal to 95% of the initial slope of the curve [21]; (3)

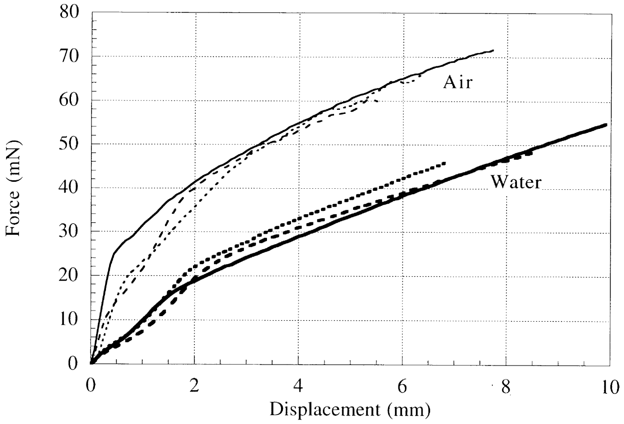

Fig. 5. Force–displacement curves of fibres tested in water compared with control samples tested in air. Curves with the same line symbols correspond to adjacent samples (gauge length = 30 mm).

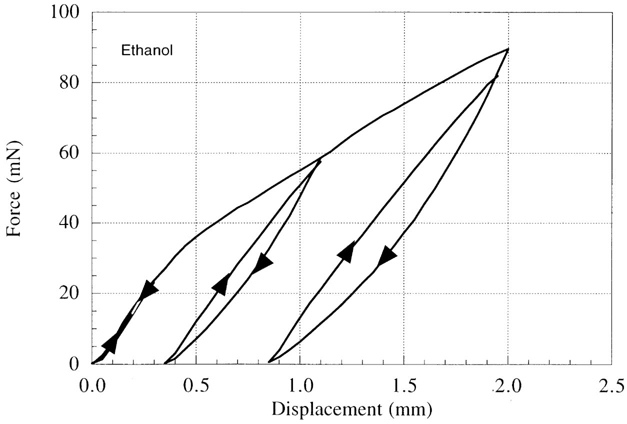

Fig. 7. Tensile test on a fibre submerged in ethanol. Several unloading–reloading steps have been performed during the test (gauge length = 30 mm).

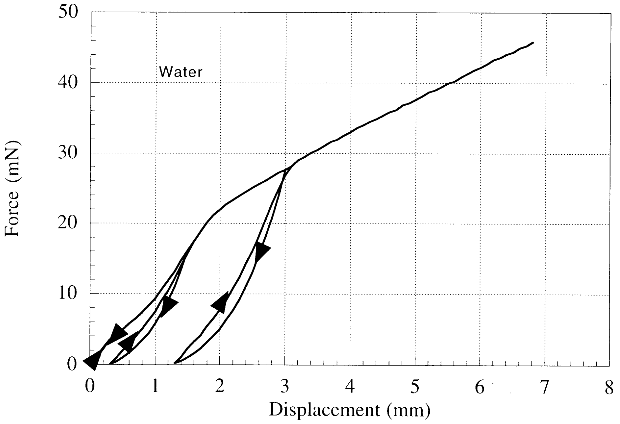

Fig. 8. Tensile test on a fibre submerged in water. Several unloading–reloading steps have been performed during the test (gauge length = 30 mm).

the tensile strength σ_u; and (4) the strain at breaking ϵ_u. Each value in Table 1 is the mean of 10 experimental points. As indicated above, we rely on comparisons between data obtained from fibres tested in different solvents and adjacent fibres tested in air. Therefore, the values of elastic modulus and tensile strength in Table 1 are consistently presented as the relative values (ratios) $E_{\text{solvent}}/E_{\text{air}}$ and $\sigma_u^{\text{solvent}}/\sigma_u^{\text{air}}$, respectively. $E_{\text{solvent}}/E_{\text{air}}$ has been calculated as the ratio between the slopes of the linear region in the corresponding force–displacement curves, a normalisation which implicitly assumes that the cross section of the control sample and the sample tested in solvent are equal. As noted above, this assumption is supported by the similar mechanical properties shown by adjacent fibres [7]. Correspondingly, the ratio $\sigma_u^{\text{solvent}}/\sigma_u^{\text{air}}$ has been determined from the force at the breaking point observed in the force–displacement curves.

Finally, the influence of isopropanol and acetone on the mechanical properties of silkworm silk has been studied. Four tests were performed in each solvent; representative force–displacement curves are shown in Fig. 9 (isopropanol) and Fig. 10 (acetone). In both Figs. 9 and 10, the force–displacement curves are compared with control samples tested in air as well as samples tested in ethanol. It is apparent that isopropanol and acetone have a similar effect to ethanol.

4. Discussion

We consider the three possible ways in which the solvents could affect the tensile properties of silkworm silk: they could simply change the effective concentration of the protein in the fibre; they could allow modifications to the protein conformation (affecting the entropic term of the free energy); and/or they could modify the non-covalent bonds in the fibre (thus affecting the enthalpic term of the free energy).

The first possibility would be an issue if a decreased stiffness were to correlate with swelling by a solvent, while an increased stiffness correlated with a volume shrinkage. If that were the case, one might argue that microstructural rearrangement is facilitated by an increased free volume for chains in the swelled polymer, and retarded by a decreased free volume in the other. However, while water and ethanol have opposite effects on stiffness, they swell the material by comparable amounts: 18% [15] and 20% [22], respectively. The second possibility is thought to dominate in the dragline of spiders [8], and is identified as the underlying cause of supercontraction: swelling by solvent allows the chains to adopt a great variety of conformations, leading to large changes in sample dimensions. As reported in the literature [11], and as further observed in the present work, this is not the case in silkworm cocoon silk, since no change of sample length has been obtained in any of the solvents. Thus, the differences which we observe in the behaviour of silkworm silk tested in air versus silk tested in solvents, can be assigned to changes in the number and/or strength of non-covalent interactions between protein chain segments. In this context, we also justify our avoidance of aggressive hydrogen bond breakers such as urea or guanidinium hydrochloride. Such denaturants would significantly alter the overall conformation of the protein chains, and, therefore, the silk microstructure, so that changes in mechanical properties would not be due to simple changes in bonding.

Our initial expectation was that each of the solvents used would weaken at least one type of non-covalent interaction, i.e. hydrogen bonds or van der Waals bonds. This would lead to a reduction in the initial stiffness of fibres in all cases. However, Figs. 4–6, 9 and 10 clearly show that immersion in the organic solvents produces an increase in the initial modulus. Only water results in a more compliant fibre. The similar results obtained in ethanol and isopropanol demonstrate that the stiffening effect of immersion in the organic solvent is not sensitive to the penetrant size, and the similar results obtained in ethanol and acetone demonstrate that the effect is not a peculiarity of alcohols.

The comparable effects of ethanol, isopropanol and

Table 1
Mechanical properties of silkworm silk tested in different environments

Environment	Property			
	$E_{\text{solvent}}/E_{\text{air}}$	ϵ_p	$\sigma_u^{\text{solvent}}/\sigma_u^{\text{air}}$	ϵ_u
Water	0.27 ± 0.02	0.011 ± 0.001	0.79 ± 0.03	0.27 ± 0.03
Ethanol	1.21 ± 0.04	0.013 ± 0.001	1.20 ± 0.04	0.066 ± 0.004
Air	1.0	0.013 ± 0.001	1.0	0.17 ± 0.02

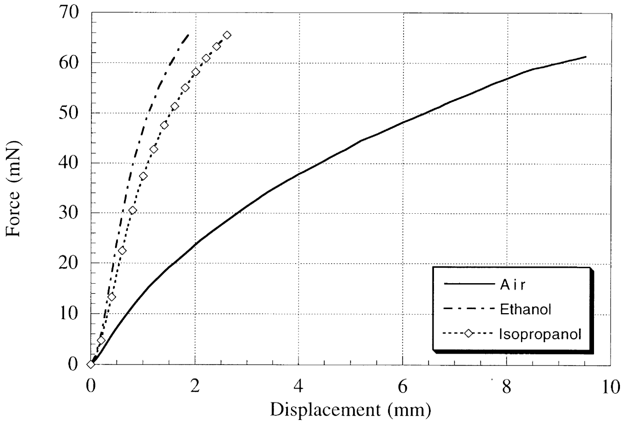

Fig. 9. Comparison of force–displacement curves of adjacent fibres submerged in ethanol, isopropanol and air (gauge length = 30 mm).

acetone indicate that their influence on the silk is exerted through some common mechanism. One feature common to all three solvents is their desiccating ability. Silk that has been dried in air after degumming retains a moisture content of 8% [23]. If this water can be removed by immersion in the organic solvents, this should lead to an increase in the number of hydrogen bonds established between the polymer chains, and thus to the experimentally observed increase in tensile stiffness. In contrast, immersion of the fibres in water would promote the substitution of protein–protein hydrogen bonds by water–protein hydrogen bonds. This substitution should effect a decrease in the tensile modulus of the silk—again consistent with experimental observation.

The information obtained from the tests conducted in ethanol and in water can be used to develop a model for the mechanical behaviour of silkworm silk in its elastic regime. Silkworm silk contains β-sheet microcrystals which represent 60% of the total volume of the fibre [24]. The microcrystals have typical dimensions of 21 nm × 6 nm × 2 nm [25], where the longest axis is aligned with the fibre axis. Moreover, it is accepted that the microcrystals are not affected by immersion in water [15]. Thus, the changes observed in the elastic modulus of samples tested in air and water must be attributed to changes in the mechanical properties of the amorphous regions.

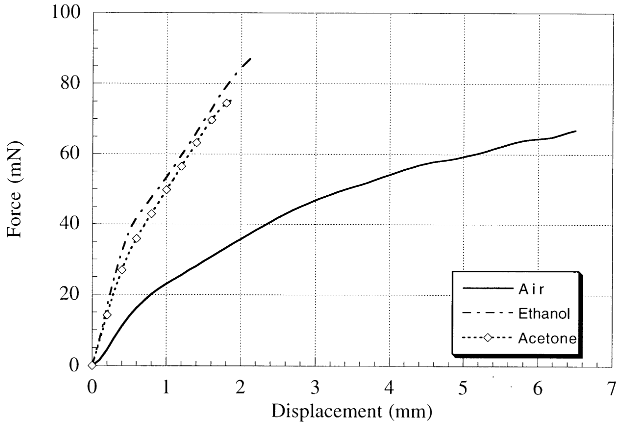

Fig. 10. Comparison of force–displacement curves of adjacent fibres submerged in ethanol, acetone and air (gauge length = 30 mm).

Table 2
Numerical values of the parameters used in the shear lag analysis

Parameter	Value	Reference
f	0.6	[24]
E_{f}	25 GPa	[27,28]
s	3.5	[25]
ν_{m}	0.3	

The dimensions of the β-sheet microcrystals suggest that silkworm silk can be modelled using shear lag analysis [26], since it can be considered as a composite material reinforced with short fibres. This model expresses the elastic modulus of the composite material as:

$$E_{\mathrm{c}} = fE_{\mathrm{f}}\left(1 - \frac{\tanh(ns)}{ns}\right) + (1-f)E_{\mathrm{m}} \tag{1}$$

where E_{f} is the elastic modulus of the reinforcing fibres, E_{m} is the elastic modulus of the matrix, and s is the aspect ratio of the fibres. The coefficient n is defined as:

$$n = \sqrt{\frac{2E_{\mathrm{m}}}{E_{\mathrm{f}}(1+\nu_{\mathrm{m}})\ln(1/f)}} \tag{2}$$

where ν_{m} is the Poisson's ratio of the amorphous matrix. The values substituted into the model are listed in Table 2. Also shown are the corresponding literature references—except in the case of the Poisson's ratio of the amorphous matrix, which has been set to 0.3 on the basis of the following argument. The elastic properties of amorphous polymers do not vary widely [29]. The Poisson's ratio of a fully amorphous polymer such as polystyrene, which equals 0.3 [30], can therefore be used to approximate the Poisson's ratio of the amorphous phase in a composite polymer.

We now seek to demonstrate that, if appropriate assignments of E_{m} are made for silk that has been exposed to different solvents, the model yields values of E_{silk} (E_{c}) which are comparable to experimental values. From the discussion above, the values obtained in ethanol should be representative of the desiccated fibre, with a high density of hydrogen bonds established in the amorphous regions. A typical value of $E_{\mathrm{m}} = 8$ GPa has been proposed for hydrogen bonded solids [31]. The model then predicts a value of $E_{\mathrm{silk}} = 14$ GPa, which is low compared to our experimental value of 19 GPa (a value of $E_{\mathrm{silk}} = 16$ GPa in air has been previously reported [32]). In the case of silk immersed in water, the disruption of inter- and intrachain hydrogen bonds in the amorphous regions should leave van der Waals forces as the dominant interactions. Consequently, E_{m} should be comparable with other solids whose mechanical properties are dictated by van der Waals interactions—such as paraffin [33], with $E_{\mathrm{m}} = 1$ GPa. Using this new value of E_{m}, a value of $E_{\mathrm{silk}} = 4$ GPa is predicted by the model, which is again low relative to our corresponding experimental value (5 GPa).

On the basis of this evidence, agreement between model and experiment appears to be limited. However, we note from Eqs. (1) and (2) that the predictions of the model are sensitive to the value assigned to ν_m. While Poisson's ratio is close to 0.3 for typical amorphous polymers, it can be as low as -0.7 for cellular polymers (foams) [34]. Fine-scale voiding has been observed in silver sulphide "stained" *B. mori* cocoon fibre viewed by transmission electron microscopy [35], suggesting that the matrix in this silk may have a lower Poisson's ratio than is characteristic of fully dense bulk amorphous polymer. If ν_m is set to 0.2 in the model, the predictions for E_{silk} in ethanol and in water are 18 and 5 GPa, respectively, in excellent agreement with the experimental values.

5. Conclusions

The contribution of hydrogen bonds to the mechanical properties of *B. mori* cocoon fibre is highlighted by these studies of the silk immersed in different solvents. Immersion in water disrupts the hydrogen bonds between chain segments in the amorphous phase, leaving van der Waals bonds to dominate, and reducing the initial modulus. Immersion in organic solvents that can desiccate the fibre maximises the number of hydrogen bonds between chain segments, increasing the initial modulus. The latter effect is not sensitive to the exact nature of the desiccating solvent, with similar results being obtained in ethanol, isopropanol or acetone. Values of initial modulus which are comparable to those recorded experimentally are predicted by a model based on a combination of: (a) shear lag analysis; (b) the assumption that the mechanical properties of the amorphous matrix phase are dominated by either hydrogen bonds or van der Waals interactions, depending on whether the medium is hydrating or desiccating; and (c) a choice of matrix Poisson's ratio that recognises the presence of microvoids in the matrix.

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