Laser-Raman spectroscopic studies of the eggshell (chorion) of *Bombyx mori*

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Laser-Raman spectroscopic studies of the eggshell (chorion) of the silkmoth Bombyx mori *reveal that its component proteins consist of 60–70% antiparallel β-pleated sheet and 30–40% of β-turns. The disulphide bonds, which crosslink the (extremely rich in cysteine)-proteins of the outer lamellar eggshell layer, are apparently found in* G–G–G (gauche–gauche–gauche) *and* T–G–T (trans–gauche–trans) *conformation; there is no evidence for the existence of free sulphydryls. The highly localized tyrosine residues appear to form hydrogen bonds, acting as weak proton donors or as acceptors.*

Keywords: Eggshell; chorion; structural protein; laser-Raman spectroscopy; secondary structure determination; disulphide bonds

Introduction

We have chosen the eggshell (chorion) of the silkmoth as a favourable system for studying how proteins self-assemble to form complex, physiologically important structures[1-5].

The architecture of the proteinaceous, lamellar, silkmoth eggshell is helicoidal (like a cholesteric liquid crystal), a structure fairly common in several biological systems[6,7]. The helicoidal structure consists of successive parallel planes, or sheets, of fibrils. Within each plane the fibrils are arranged parallel to each other. From plane to plane the fibril orientation rotates progressively, thus giving rise to a helix with its axis perpendicular to the planes. Recently, we proposed the antiparallel twisted β-pleated sheet as the molecular conformation which dictates the formation of the helicoidal architecture of the silkmoth chorion[5]. Our proposal was based on evidence from X-ray diffraction, laser-Raman and infrared spectroscopy, conventional electron microscopy, freeze fracturing, secondary structure prediction and Fourier analysis of the known amino acid sequences of the A, B and C classes of chorion proteins, which are products of distinct, but related, multigene families[1-5,8,9].

We are currently in the process of building up models of chorion basic structural units from the evolutionarily conserved domains of its constituent proteins[1,10]. We are also trying to find the modes of packing of these units, to simulate the self-assembly of chorion helicoidal architecture[10].

Throughout our studies we were using eggshells from the wild silkmoth *Antheraea polyphemus*. In this work, however, we have chosen to study the eggshell of the domesticated silkmoth *Bombyx mori*, by utilizing laser-Raman scattering. Eggs from the two species have different morphology, differ appreciably in size and, apparently, are deposited by the insects in widely differing environments[11]. Nevertheless, their constituent proteins share extensive sequence homologies[12].

We have undertaken this study seeking: (a) to verify our results in other species of silkmoths, making useful comparisons, (b) to obtain information about important structural features, such as the disulphide bonds, which crosslink chorion proteins and harden the eggshell during the late morphogenetic stages (this information we were unable to obtain in our previous study[2]) and (c) to estimate, quantitatively, the percentage of secondary structures for chorion proteins.

Experimental

Sample preparation

The eggshell (chorion) samples were prepared from follicles dissected from developing female *Bombyx mori* pupae. The follicles were cut in half with fine scissors and washed several times in distilled water to remove the yolky oocyte. Swollen epithelial (follicular) cells were peeled off the surface of the underlying chorion. The insoluble chorions were repetitively washed in 95 and 100% ethanol followed by distilled water to remove the vitelline membrane and were air-dried.

Raman spectroscopy

Raman spectra were measured on a Jobin Yvon Ramanor HG 25 spectrometer. The excitation source was the 514.5 nm line of a Spectra Physics 165 Argon-ion laser operating at 100 mW at the sample. A 90° scattering geometry was employed, with the laser beam hitting tangentially the sample (eggshell) surface. All samples tried showed, initially, a very strong fluorescent background, which was substantially reduced by prolonged laser irradiation of the sample. To reduce the noise level, the spectra were recorded at a scanning speed of 10 cm^{-1}

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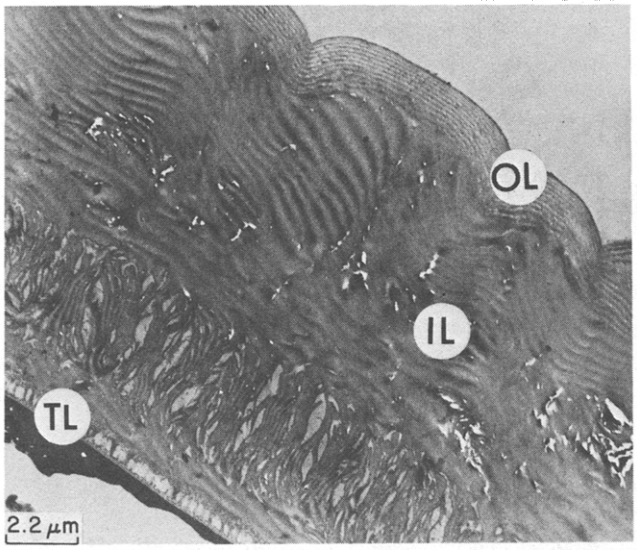

Figure 1 Transmission electron micrograph of a thin section through a mature eggshell (chorion) of *Bombyx mori*. Within the bulk of the chorion three distinct layers can be discerned: nearest to the oocyte the trabecular layer (TL) consisting of pillars surrounding air spaces, the inner layer (IL) and the outer lamellar layer (OL), at the eggshell surface, consisting of thin lamellae which are formed by the unusual (extremely rich in Cys)-proteins (over 30 mol%), which occur uniquely in the silkmoth *Bombyx mori*. This is the eggshell which was used for obtaining the laser-Raman spectrum shown in *Figure 2*

min^{-1} and a time constant of 2 s. The spectral resolution was 5 cm^{-1}

Electron microscopy

The eggshells used for obtaining the laser-Raman spectra were fixed in 2.5% glutaraldehyde, in 0.08 M sodium cacodylate, buffered at pH 7.4, for 90 min at 4°C, postfixed in 1% osmium tetroxide in water for 60 min at 4°C, dehydrated in ethanol and embedded in a modified Mollenhauer's resin (25 g Epon-812; 20 g Araldite-506; 60 g DDSA; and 3 g DMP-30). Thin sections were cut on a MT-1 Porter-Blum ultramicrotome with glass knives and were stained with 7% uranyl acetate for 10 min. Electron microscopy was performed using a Philips EM 200 microscope operating at 60 kV.

Determination of secondary structure of chorion proteins by laser-Raman spectroscopy

The amide I band in the laser-Raman spectrum of the eggshell of *Bombyx mori* was analysed as described by Williams and Dunker[13], to estimate the percentage (%) of secondary structures of chorion proteins. The method is as follows:

At a fixed wavenumber in the amide I band, the observed Raman scattering intensity for a protein may be expressed as $I_e=\sum F_i I_i^{(1)}$, where I_e is the normalized experimental Raman intensity, each F is the fraction of residues in a given type of secondary structure i and each I is the intensity that would be observed for a polypeptide with 100% of the indicated structure type. The normalized intensity at a fixed wavenumber is given by

$$I_e=\frac{I_{\text{observed}}}{\text{Sum over all frequencies of } I_{\text{observed}}}.$$

The normalized intensities were computed at 15, equally spaced, wavenumbers from 1630–1700 cm^{-1} in the amide I band of the Raman spectrum of chorion, and were expressed, using equation (1), as linear combinations of intensity values I taken from the reference spectra given by Williams and Dunker[13] for six types of secondary structure. The six types of secondary structure were monohydrogen bonded α-helix (hm), bihydrogen bonded α-helix (hb), antiparallel β-sheet (ba), parallel β-sheet (bp), β-turns (t) and undefined (u)[13].

To estimate the percentages of each fraction F of the secondary structures, an overdetermined system of 16 linear equations with six unknowns, was solved; (the sixteenth equation was $F(\text{hm})+F(\text{hb})+F(\text{ba})+F(\text{bp})+F(\text{t})+F(\text{u})=1$).

This was done by using the Harwell Library subroutine MA20B which calculates a solution vector $\mathbf{x}=\{x_j\}$ for an overdetermined system of m linear equations with n unknowns, of the form $\sum_{j=1}^{n} a_{ij}x_j=b_i$, $i=1,2,\ldots m$ such that the sum of the absolute values of the residuals

$$G(\mathbf{x})=\sum_{i=1}^{m}\left|b_i-\sum_{j=1}^{n}a_{ij}x_j\right|$$

is minimized, subject to the constraints $x_j\geqslant 0$ (for $j=1,2,\ldots 6$). The routine employs a modification of the standard form of the simplex method to solve the linear programming problem.

Results and discussion

Figure 1 shows the ultrastructure of the eggshell of *Bombyx mori* which was used for obtaining the laser-Raman spectrum presented in *Figure 2*. Obviously, this chorion belongs to an egg at a late developmental stage[11]: several lamellae of the outer lamellar layer, unique in *Bombyx mori*, have already been formed. *Table 1* gives the wavenumbers and our tentative assignments of the bands appearing in the spectrum. Additional peaks are resolved, but not tabulated, because insufficient data are available for unambiguous assignments.

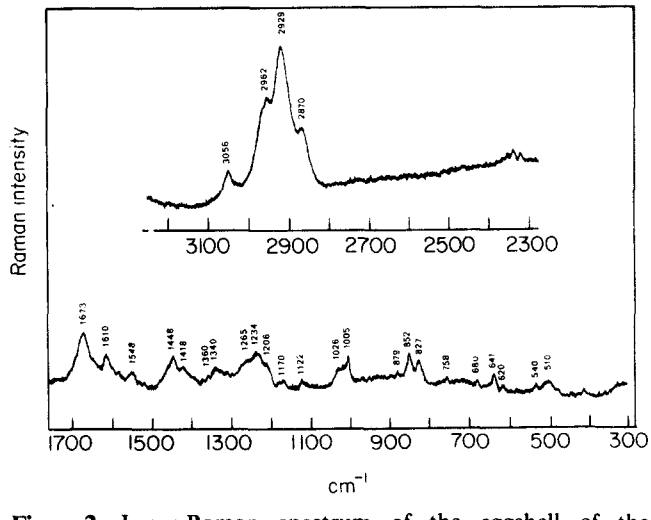

Figure 2 Laser-Raman spectrum of the eggshell of the silkmoth *Bombyx mori*. A 90° scattering geometry was employed, with the laser beam hitting the eggshell surface tangentially (outer lamellar layer). Instrumental conditions: excitation wavelength 514.5 nm; scanning speed = 10 cm^{-1} min^{-1}; time constant = 2 s; spectral resolution 5 cm^{-1}; laser power at the sample = 100 mW

Table 1 Wavenumbers and tentative assignments of bands in the laser-Raman spectrum of the eggshell of *Bombyx mori*

Wavenumber (cm^{-1})	Tentative assignment
510	S–S stretch
540	S–S stretch
620	Phe
641	Tyr
680	C–S stretch? Trp?
758	C–S stretch? Trp?
827 (+)	Tyr
852 (+)	Tyr
879	Trp
1005 (+)	Phe or C–C stretch (β-sheet)
1016	Phe, Trp
1026	Phe
1122	C–N stretch
1170	Tyr
1206	Tyr, Phe
1234 (+)	Amide III (antiparallel β-sheet)
1265 (sh)	Amide III (β-turns? cross-β? α-helix? coil?)
1340	Amide III (β-turns) or Trp
1360	Trp
1418	Trp
1448 (+)	CH_2 deformation
1548	Amide II (β-turns) or Trp
1610	Tyr, Phe, Trp
1673 (+)	Amide I (antiparallel β-sheet)
2800–3100	C–H stretch

(+) indicates a strong peak

Laser-Raman spectroscopy confirms the prevalence of antiparallel β-pleated sheet in the proteins of the eggshell of *Bombyx mori*. The locations of the diagnostic amide I and III bands at 1673 and 1234 cm^{-1} respectively, which are useful indicators of protein or polypeptide structure[14–16], allow us to conclude with certainty that the antiparallel β-pleated sheet conformation is predominant in the *Bombyx mori* chorion. This observation is in agreement with our findings for the eggshell of the wild silkmoth *Antheraea polyphemus*[2,3]. The pronounced similarity of the secondary structure of chorion proteins for the two silkmoth species, *Antheraea polyphemus* and *Bombyx mori* was expected from amino acid sequence homology[12] and secondary structure prediction[1,4,21]. Here, we provide a direct, experimental proof of this fact.

There are several possible explanations for the appearance of a reproducible shoulder at 1265 cm^{-1} in the amide III band. It may be indicative of either cross-β or α-helical conformation or possibly of disordered structure or β-turns[14,15,18,19]. Taking into account the theoretical work of Bandekar and Krimm[20], we previously assigned[2] bands at 1552 cm^{-1} (amide II) and 1342 cm^{-1} (amide III) to β-turns, which are abundant in silkmoth eggshell proteins[1,4]. In the laser-Raman spectrum of *Bombyx mori* chorion (*Figure 2*), these bands appear at 1548 and 1340 cm^{-1} correspondingly. Nevertheless, the origin of these bands is not yet entirely clear: Raman signatures for β-turns are not generally agreed upon; for example Tu and co-workers place the amide III band for β-turns near 1270 cm^{-1} [19]. Recently, Williams and Dunker published an accurate and general method for the determination of the percentage of secondary structure of proteins, by analysing the amide I band of their laser-Raman spectra[13].

Using their data and following their method (*see* 'Methods') we analysed the amide I band of the laser-Raman spectrum of the eggshell of *Bombyx mori*. The analysis suggests that the proteins of chorion consist of 60–70% antiparallel β-pleated sheet and the remainder 30–40% of β-turns.

The chorion proteins, which belong to three different, but related families, A, B, C[4], have a tripartite structure[1,4,5]: a central domain, evolutionarily conserved, highly structured into β-sheet stands – β-turns and two 'arms', more variable, rich in Cys, Tyr and Gly. Presumably, the central conservative regions of the proteins constitute the chorion basic structural units (fibrils), not seen in the low magnification picture of chorion of *Figure 1*, whereas the 'arms' serve in crosslinking[5].

The central conservative domains of chorion proteins consist of tandemly repetitive hexapeptides as has been shown by Fourier analysis of their sequences[5,9,22]. These hexapeptides can be arranged in a characteristic antiparallel β-pleated sheet structure. A schematic example of one such protein is shown in *Figure 3*. In this structure, the ratio of β-sheet strands/β-turns = 2:1, which is in good agreement with the results obtained from the analysis of the amide I band of the laser-Raman spectrum.

In our previous work on the eggshell of *Antheraea polyphemus*[2], it proved extremely difficult to locate bands in the region 500–550 cm^{-1}, typically associated with the S–S stretching mode[14–16] which would indicate the existence of disulphide bonds. On the contrary, there was unambiguous evidence for the existence of free sulphydryls, apparently in diverse environments.

In this work, the evidence for the existence of S–S bonds in the eggshell of *Bombyx mori* is clear, as can be judged from bands appearing at 510 and 540 cm^{-1}.

Lively debate and intense experimentation have attended attempts to correlate S–S stretching frequencies with specific conformations of the C–C–S–S–C–C structural unit of disulphide bonds (for a review *see* ref. 15).

Following Sugeta *et al.*[23] the bands at 510 and 540 cm^{-1} may be assigned to S–S bridges in *G–G–G* (*gauche–gauche–gauche*) and *T–G–T* (*trans–gauche–trans*) conformation respectively. An alternative inter-

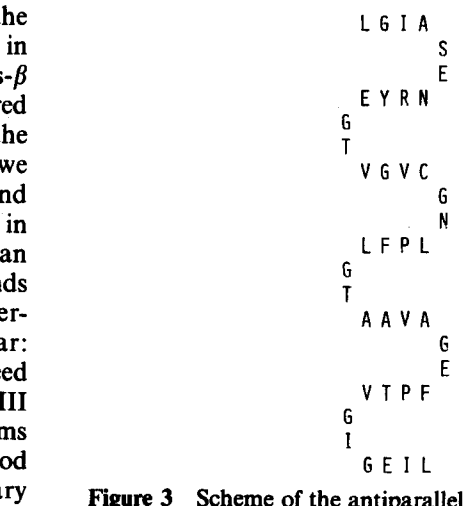

Figure 3 Scheme of the antiparallel β-pleated sheet structure of the central, conservative domain, of the silkmoth eggshell B protein 410, which consists of tandemly repetitive hexapeptides

pretation of these two bands[24] is that the 540 cm^{-1} band arises from the presence of disulphides with conformations with small ($-30°$) S–S–C–C dihedral angles (the so-called A conformations), whereas the disulphides with *trans* and either of two nonequivalent *gauche* conformations about their C–S bonds (S–S–C–C dihedral angles of roughly 180 and $\pm 60°$ respectively), all generate bands due to S–S stretching at about 510 cm^{-1}.

We favour the former alternative since, in proteins, there is a strong preference for the S–S–C–C dihedral angle to adopt values near ± 90 or 180° (*gauche* or *trans*) as has been shown recently[27,28], whereas the C–S–S–C dihedral angle is always $\pm 90°$ (*gauche*) approximately.

The outer lamellar layer, a characteristic feature of *Bombyx mori* chorion, consists of proteins extremely rich in cysteine[11,12]. The great majority of the extra cysteines are located on the protein 'arms'[25,26]. These cysteines are forming disulphide bonds which harden and waterproof chorion, during the unusually long diapause periods, which are essential for the oocyte survival[11].

Whether the bands at 510 and 540 cm^{-1} correspond to intermolecular or intramolecular disulphide bonds (this latter possibility should not be excluded) is not clear. Nevertheless, it is obvious from the geometry of the experiment that they are derived from the proteins of the outer layer of chorion.

No clear evidence was obtained for the existence of free sulphydryls in the 2530–2580 cm^{-1} spectral region, typically associated with the S–H stretching mode[2,17]. This, perhaps, suggests that most cysteines have been oxidized to cystines, which may be an important hint in unravelling the conformation of the protein 'arms' and their packing, considering the large number of cysteines and their exact, periodic, appearance in the amino acid sequences (the 'arms' of the high cysteine proteins consist mainly of tandem repeats of the dipeptide Cys-Gly[25,26]), in conjunction with the conformation of the disulphide bridges.

In our previous work on the moth *Antheraea polyphemus*, the intensity ratio of the Tyr doublet at 850 and 830 cm^{-1}, I_{850}/I_{830}, was found to have low values, approximately 0.3 ± 0.1, suggesting that the tyrosines are buried in a hydrophobic environment, strongly hydrogen bonded[2,29].

In this work, it was consistently found that $I_{850}/I_{827} \approx 1.2$, which probably suggests that most tyrosines act as much weaker hydrogen bond donors or as acceptors to an acidic proton[29]. It should be noted that the great majority of the tyrosines are located in the 'arms' of chorion proteins, in close proximity (at least in the sequence!) to the Cys.

The crosslinking of chorion proteins with disulphide bonds may cause conformational changes in the 'arms', which result in the change of the intensity ratio I_{850}/I_{830} of the Tyr doublet.

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