

Spectroscopic studies of *Manduca sexta* and *Sesamia nonagrioides* chorion protein structure

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The secondary structure of *Manduca sexta* and *Sesamia nonagrioides* chorion proteins has been studied in intact chorions using laser-Raman and Fourier transform infra-red (FTIR) spectroscopy and in a solution containing extracted and reassembled chorion proteins using circular dichroism (CD) spectroscopy. Laser-Raman and IR spectra suggest the predominance of antiparallel β -pleated sheet structure in intact chorion proteins of both Lepidoptera species. The bands at 1673, 1674 cm^{-1} (amide I) and 1234–1238 cm^{-1} (amide III) in the laser-Raman spectra can best be interpreted as resulting from abundant antiparallel β -pleated sheet structure. Analysis of the amide I band suggests that chorion proteins consist of 60–70% antiparallel β -pleated sheet and 30–40% β -turns. Supporting evidence for the prevalence of antiparallel β -pleated sheet in chorion proteins was supplied using FTIR spectroscopy by the observation of a very intense absorption band at 1635 cm^{-1} (amide I) and of a weak band at 1530, 1525 cm^{-1} (amide II) from chorions of both species. Surprisingly, analysis of the CD spectra of extracted and reassembled chorion proteins suggests that, in solution, they retain a regular secondary structure most probably dominated by β -pleated sheet. We therefore suggest that the prominent regular β -sheet structure of chorion proteins may exist in solution and dictate the aggregation and polymerization process *in vivo*.

Keywords: *Manduca sexta*; *Sesamia nonagrioides*; chorion proteins

Lepidopteran chorion, the major component of the eggshell, is a complex extracellular proteinaceous tissue and an excellent model system for studying the self-assembly of structural proteins to form physiologically important structures¹. Silkworm chorion, which has been studied extensively as a model of lamellar helicoidal architecture, consists of proteins predominantly organized as fibres embedded in a matrix^{2,3}, which suggests

analogies with vertebrate keratins and other fibre-matrix systems^{4–9}. The lamellar chorion consists of fibrous layers parallel to its surface. Within individual layers, protein fibrils are oriented more or less parallel to each other. Between successive layers, the fibril direction rotates progressively through a constant angle giving rise to a helix with its axis perpendicular to the layers. This results in a helicoidal structure¹⁰ that is common to most Lepidoptera species studied so far^{11–13}. The helicoidal structure of chorion, a sort of multi-directional 'plywood',

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is a biological analogue of a cholesteric liquid crystal¹⁴. The structure changes during morphogenesis and also varies locally, consistent with the biological complexity and multiple physiological functions of the eggshell³.

Application of the technique of freeze-fracturing, with single-sided and rotary shadowing, has revealed that silkmoth chorion, as well as the chorion of two other *Lepidoptera* species, *Manduca sexta* and *Sesamia nonagrioides*, consists of filaments approximately 3–4 nm in diameter, and has provided direct visualization of the helicoidal arrangement of these basic structural elements in the chorion architecture^{13,15}.

The packing of the filaments as seen by freeze-fracturing is in good agreement with X-ray data obtained from silkmoth chorion^{15,16}. These data also suggest the prevalence of β -sheet structure in silkmoth chorion proteins, in conjunction with evidence from laser-Raman and infra-red spectroscopy^{5,8} and analysis of chorion protein amino acid sequences^{4,9,17}. These data led to the hypothesis that twisted antiparallel β -pleated sheet is the predominant molecular conformation that dictates the formation of a helicoidal architecture in proteinaceous eggshells^{7,17}. The ultrastructural similarities of *M. sexta* and *S. nonagrioides* chorions to silkmoth chorion led us to investigate the chorion protein secondary structure of these two *Lepidoptera* species, utilizing laser-Raman spectroscopy and infra-red spectroscopy. Circular dichroism studies of reassembled chorion proteins in solution were also performed. Apparently, antiparallel β -pleated sheet is the predominant conformation of chorion proteins in both native chorion and reassembled units, which further confirms the suggestion that this structure is the common molecular denominator dictating the formation of proteinaceous chorions with a helicoidal architecture⁷.

Experimental

Preparation of purified chorions

Mature and ovulated follicles were obtained from female *Manduca sexta* and *Sesamia nonagrioides* adult insects. Follicles were cut in half with a razor blade and cleaned ultrasonically in 95% and 100% ethanol to peel the swollen epithelial cells and the vitelline membrane off the underlying and overlying chorion surfaces. Insoluble chorions were selected under a dissecting microscope and air-dried.

Laser-Raman spectroscopy

Raman spectra were measured on a Ramanor HG25 Jobin-Yvon spectrometer. The excitation source was the 488 nm line of a Spectra-Physics 165 argon-ion laser operating at 150 mW at the sample. A 90° scattering geometry was employed, with the laser beam tangential to the chorion surface. All samples investigated initially showed a very strong fluorescent background, which was substantially reduced by prolonged laser irradiation of the sample. The spectra were recorded at a scanning speed of 100 cm⁻¹ min⁻¹ and a time constant of 2 s. The spectral resolution was 5 cm⁻¹. The amide I band in the laser-Raman spectra was analysed as described by Williams and Dunker¹⁸, following the procedure developed by Hamodrakas *et al.*⁸, to estimate the percentage of secondary structure types in the chorion proteins.

Fourier transform infra-red spectroscopy (FTIR)

Samples for IR spectroscopy were in the form of solid KBr pellets. Dried chorions were thoroughly ground in a vibrating mill and mixed with KBr to a final concentration of approximately 2% (w/w). IR spectra were recorded on an FTIR Bruker 113v vacuum spectrometer. Each spectrum is the result of signal-averaging of 100 scans at a resolution of 2 cm⁻¹.

Eggshell solubilization and dialysis

Chorions were solubilized at concentrations ranging from 0.5–5.0 mg ml⁻¹, in 6 M urea, 0.4 M Tris-HCl and 4% 2-mercaptoethanol, pH 8.4. This solvent completely dissolves these chorions. Extracted eggshell proteins were reassembled by 12–18 h dialysis at room temperature against distilled water, pH 6.4. The final concentration was obtained by 30 min dialysis against a 40% sucrose buffer, pH 6.4.

Electron microscopy (EM)

Droplets (3 μ l) of reassembled chorion protein solutions were applied to grids covered with films prepared from 0.5% (w/v) formvar in water-free chloroform. Before drying, they were negatively stained with 1% uranyl acetate for 50 s. EM was performed using a Zeiss EM 9A microscope operating at 60 kV.

Circular dichroism spectroscopy (CD)

Samples of reassembled chorion proteins were diluted to 285 μ g ml⁻¹ for *M. sexta* and *S. nonagrioides* in 10 mM sodium phosphate buffer, pH 6.4, with and without 6 M guanidine hydrochloride. CD spectra were measured at 5°C on a Jasco J-500 spectropolarimeter in a cell with a 0.1 cm pathlength. Fractions of secondary structures were estimated using the secondary structure estimation programs of Jasco Corp.; the spectra of seven standard proteins are provided for calculation of the base spectra. Each observed spectrum was expressed as a linear combination of reference spectra of four types of secondary structure: α -helix (α), β -sheet (β), β -turn (t) and coil (c; random). The linear coefficients representing the secondary structure estimates were calculated by a linear least-squares method. The reference spectra of the four types of secondary structure were calculated from the CD spectra of four proteins with crystallographically known secondary structures: lysozyme (41% α , 16% β , 23% t, 20% c), papain (28% α , 14% β , 17% t, 41% c), ribonuclease A (23% α , 40% β , 13% t, 24% c) and chymotrypsin (9% α , 34% β , 34% t, 23% c). This subset gave the best fit to the data. Secondary structure estimates were also calculated according to the method described by Yang *et al.*¹⁹ and Provencher and Glockner²⁰. The results, in good agreement with those obtained utilizing the secondary structure estimation programs of Jasco Corp., are not presented here.

Results

Secondary structure of chorion proteins in intact chorions

In laser-Raman spectra, the locations of the diagnostic amide I and III bands are useful indicators of protein or polypeptide structure^{21–24}. Table 1 summarizes the diagnostic locations of these bands for α -helical, β -sheet and β -turn structures, and lists the corresponding

Table 1 Summary of diagnostic amide bands in laser-Raman spectra and their observation in chorions of *M. sexta* and *S. nonagrioides* (see Table 2)^{21, 24}

Band nature	Bands characteristic of			Observed in chorion	
	α -helix	β -sheet	β -turn	<i>M. sexta</i>	<i>S. nonagrioides</i>
Amide I	1650–1660	1665–1680	1665, 1690	1673	1674
Amide II	1516, 1545	1535, 1560	(I) 1550–1555 1567 (II) 1545, 1555 1560	1554	1547 1561
Amide III	1260–1290	1230–1240	1290–1330	1234	1238

Table 2 Wavenumbers and tentative assignments of bands in the laser-Raman spectra of chorions of *M. sexta* and *M. nonagrioides*

Wavenumber (cm ⁻¹)		Tentative assignment
<i>M. sexta</i>	<i>S. nonagrioides</i>	
510	515	S-S stretch (<i>g-g-g</i>)
538	–	S-S stretch (<i>t-g-t</i>)
570	578	Trp
615	601	Phe
640	645	Tyr
668	675	C-S stretch? Trp?
752	737, 763	C-S stretch? Trp?
830	830	Tyr
849	849	Tyr
1003	1005	Phe
1025	1032	Phe
1171	1168	Tyr
1206	–	Tyr, Phe
1234	1238	Amide III (antiparallel β -sheet)
1335	1347	Amide III (β -turns) or Trp
1405	1413	Trp
1453	1455	CH ₂ deformation
1554	1547, 1561	Amide II (β -turns) or Trp
1610	1618	Tyr, Phe, Trp
1673	1674	Amide I (antiparallel β -sheet)
2800–3100 (not shown)	2800–3100 (not shown)	C-H stretch

wavenumbers observed in the laser-Raman spectra of the chorion samples.

The laser-Raman spectra of *M. sexta* and *S. nonagrioides* mature chorions are presented in Figures 1a and b, respectively. They are very similar and clearly indicate that antiparallel β -pleated sheet conformation is predominant in chorion proteins. Thus, the most intense peaks at 1673, 1674 cm⁻¹ (amide I region) and 1234, 1238 cm⁻¹ (amide II region) were interpreted as resulting from antiparallel β -sheet structure^{21–23}. In addition, relatively prominent peaks at 1554, 1547 and 1561 cm⁻¹ (amide II) and 1335, 1347 cm⁻¹ (amide III) might suggest the existence of β -turns²⁴.

Table 2 lists the wavenumbers and tentative assignments of bands in the Raman spectra of *M. sexta* and *S. nonagrioides* chorions. Additional minor bands were resolved but are not listed because insufficient data were available for unambiguous assignment.

Secondary structure percentage estimates of chorion proteins were obtained from analysis of the amide I band of the laser-Raman spectra, following the method described by Williams and Dunker¹⁸. According to this analysis, proteins in intact chorions are 60–70% antiparallel β -pleated sheet and 30–40% β -turns (a ratio of 2:1) as shown in Table 3.

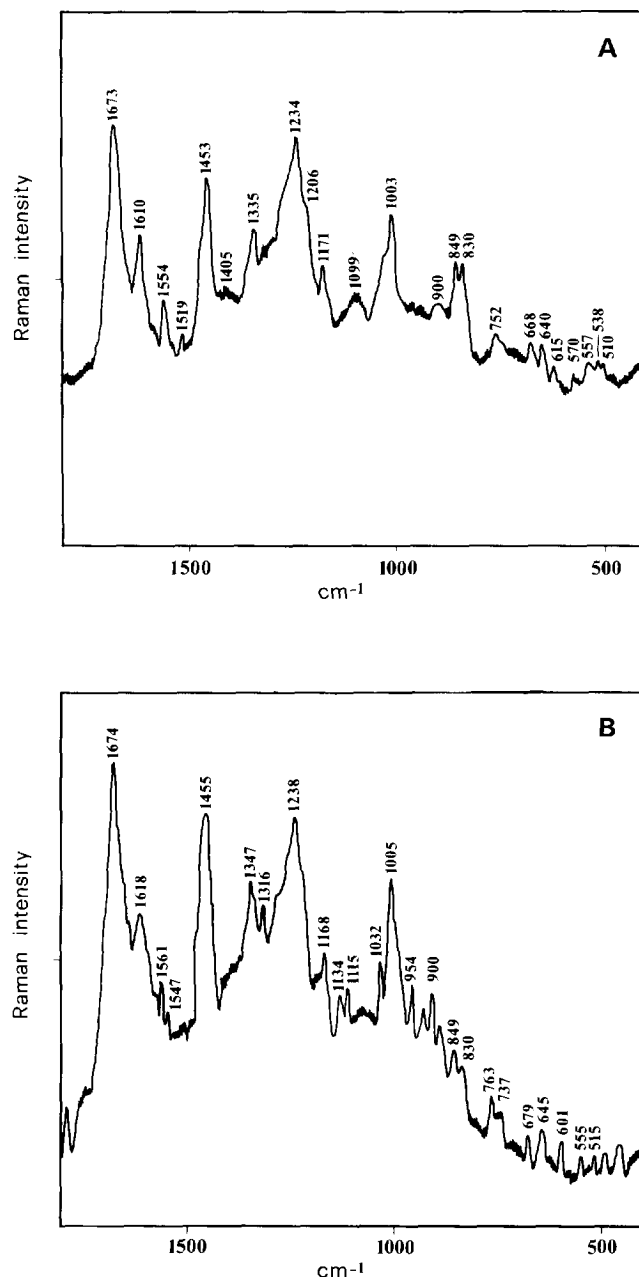


Figure 1 Laser-Raman spectra of (A) *M. sexta* and (B) *S. nonagrioides* mature chorions. A 90° scattering geometry was employed with the laser beam hitting the eggshell surface tangentially. Instrumental conditions: excitation wavelength = 488 nm; scanning speed = 100 cm⁻¹ min⁻¹; time constant = 2 s; spectral resolution = 5 cm⁻¹; laser power at the sample = 150 mW

Table 3 Secondary structure estimates of *M. sexta* and *S. nonagrioides* chorion proteins from analysis of the laser-Raman amide I bands (see Experimental and refs 8 and 18)

Secondary structure	<i>M. sexta</i>	<i>S. nonagrioides</i>
α -helix (mono-hydrogen bonded)	0.000	0.000
α -helix (bi-hydrogen bonded)	0.000	0.000
Antiparallel β -pleated sheet	0.676	0.588
Parallel β -pleated sheet	0.000	0.086
β -turns	0.324	0.326
Coil	0.000	0.000

Tyrosine and cysteine are abundant in lepidopteran chorion³. Peaks at 849, 830 cm^{-1} , in both spectra, are ascribable to tyrosine, and their intensity ratio, approximately 1.0, suggests that most tyrosines act as hydrogen-bond acceptors²⁵. The evidence for the existence of disulfide bonds in chorion is clear, as indicated by bands appearing at 512, 538 and 515 cm^{-1} in the two spectra. These disulfides are apparently found in the conformations *g-g-g* and *t-g-t* (*g* and *t* describe *gauche* and *trans* conformations, respectively), as indicated by the appearance of the two major bands²⁶. In mature chorions, most cysteines are probably cross-linked by disulfide bonds stabilizing chorion, as indicated by the absence of bands in the spectral region of 2530–2580 cm^{-1} (data not shown)²⁷. Bands indicative of the existence of phenylalanine and tryptophan were also seen in the spectra of *M. sexta* and *S. nonagrioides* chorions and are assigned in Table 2^{21–23}.

Supporting evidence for the prevalence of antiparallel β -pleated sheet in the chorion proteins of *M. sexta* and *S. nonagrioides* was obtained by FTIR spectroscopy. The observation of a very intense absorption band at 1635 cm^{-1} (amide I) and of a weak band at 1530, 1525 cm^{-1} (amide II) in the IR spectra of *M. sexta* and *S. nonagrioides* chorions (Figure 2) strongly indicates an antiparallel β -pleated sheet conformation^{28–30}. Table 4 summarizes the diagnostic IR bands of characteristic protein secondary structures and lists the corresponding locations for bands observed in the IR spectra of both *Lepidoptera* chorions^{28–30}.

Secondary structure of reassembled chorion proteins and visualization of reassembled products

Reassembled chorion proteins were studied by negative staining to determine the size and shape of the reconstituted units. Typical electron micrographs of the assembly products are shown in Figure 3. Apparently, solubilized chorion proteins reassemble to form fibrous structures with a beaded appearance. The beads have diameters varying from 5 to 20 nm.

The CD spectra of assembled chorion proteins in solution (Figure 4) from the two *Lepidoptera* species are indicative of β -sheet proteins. They both show a large negative band at approximately 210 nm and a small negative slope between 220 and 240 nm. In both cases, these negative peaks are largely reduced by denaturation with guanidine hydrochloride, with the concomitant appearance of a small positive band at 225 nm. The secondary structure estimates for *M. sexta* and *S. nonagrioides* chorion proteins, calculated as described in Experimental, are 10% α , 48% β , 23% t, 19% c and 14% α , 42% β , 30% t, 14% c, respectively.

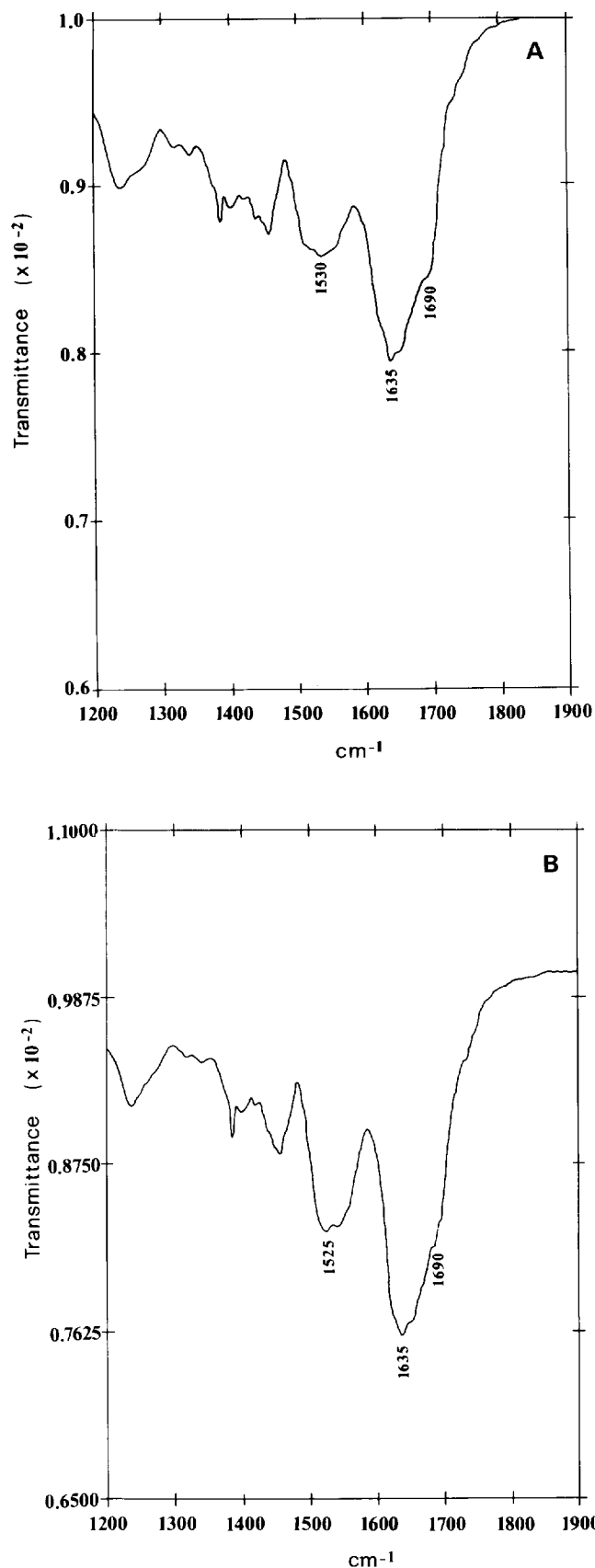


Figure 2 FTIR spectra of the chorions of (A) *M. sexta* and (B) *S. nonagrioides*. The spectra are the result of signal averaging of 100 scans, at 2 cm^{-1} resolution. Samples were in the form of KBr pellets, containing about 2% (w/w) protein, thoroughly ground in a vibrating mill, before mixing with KBr

Table 4 Summary of diagnostic amide bands in infra-red spectra and their observation in FTIR spectra of *M. sexta* and *S. nonagrioides* chorions²⁸⁻³⁰

Band nature	Bands characteristic of			Observed in chorion	
	α -helix	β -sheet	β -turn	<i>M. sexta</i>	<i>S. nonagrioides</i>
Amide I	1645-1662	1615-1638	1662-1695	1635	1635
Amide II	1546	1530		1530	1525

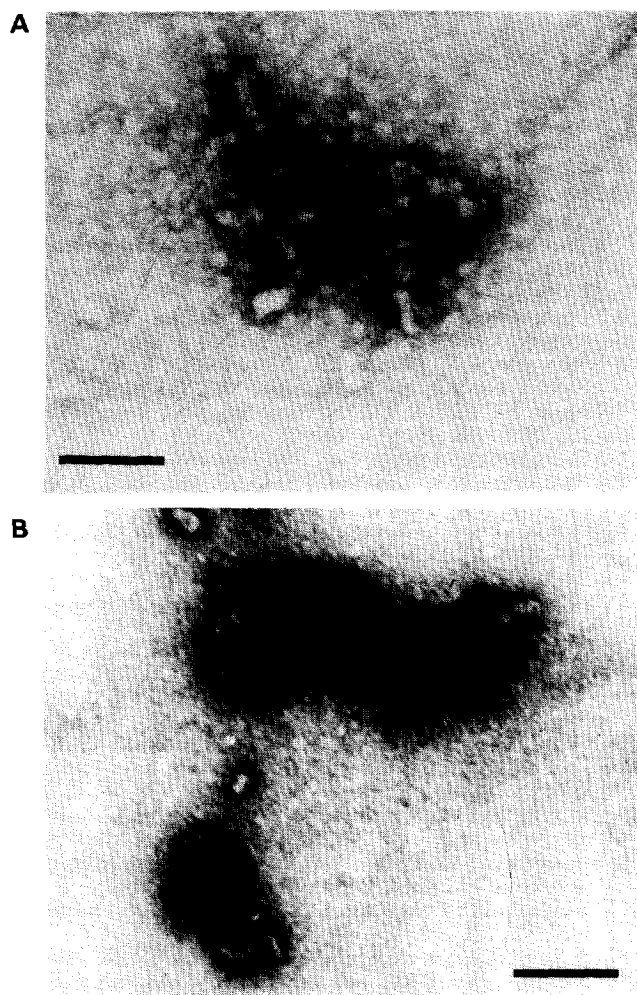


Figure 3 Transmission electron micrographs of chorion proteins extracted in 6 M urea, 0.4 M Tris-HCl, 4% 2-mercaptoethanol, from (A) *M. sexta* and (B) *S. nonagrioides*, reassembled by dialysis at room temperature against water and contrasted by negative staining (1% uranyl acetate). Fibrous structures with a beaded appearance were seen. The beads have 5-20 nm diameters. Bar = 100 nm

The predominance of β -sheet conformation (>40%) for reassembled chorion proteins in solution is clearly indicated.

Since the distinction between random coil and β -sheet structure using far-ultraviolet CD is difficult, the spectra of reassembled chorion proteins were also measured in 6 M guanidine hydrochloride. Denaturation dramatically changed the spectra from that of an ordered to that of a less ordered structure.

Discussion

Simple rules of packing of chorion protein molecules, which are known to depend on the interactions of

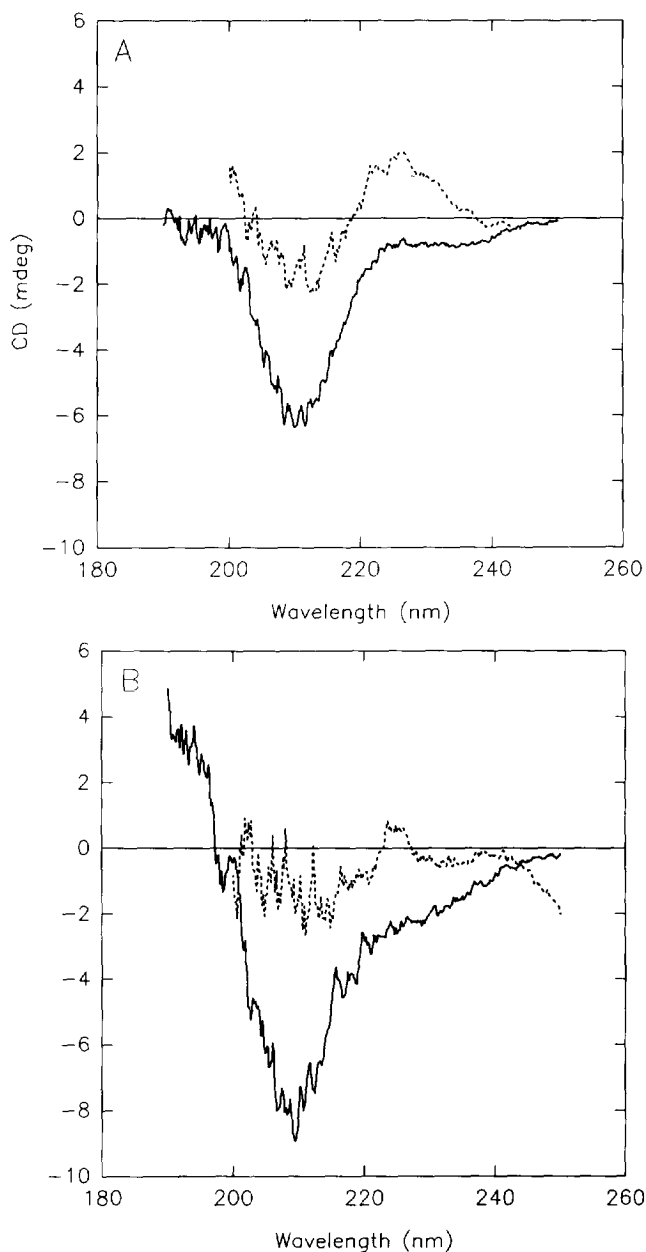


Figure 4 CD spectra of extracted and reassembled chorion proteins (details as in Figure 3) from (A) *M. sexta* and (B) *S. nonagrioides*, in 10 mM sodium phosphate, pH 6.4 (continuous curve) and 10 mM sodium phosphate, pH 6.4, 6 M guanidine hydrochloride (dashed curve). The spectra of denatured proteins are truncated at 200 nm due to guanidine hydrochloride's high absorbance of far-ultraviolet light. Instrumental conditions: scanning speed 10 nm min⁻¹; time constant = 8 s; average of four repeat scans

secondary structure elements, should govern formation of helicoidal chorion architecture through self-assembly mechanisms (ref 31 and refs therein). Since laser-Raman and IR spectroscopy have confirmed the prevalence of antiparallel β -pleated sheet in chorion proteins of the

Lepidoptera species *M. sexta* and *S. nonagrioides*, it appears that this structure dictates the formation of helicoidal architecture in the chorions of both species. The distribution of the phi and psi angles in the antiparallel β -sheets seems to be rather narrow and indicates that they exhibit a uniform structure, since the amide I band at 1673, 1674 cm^{-1} is sharp – its half-width is approximately 30 cm^{-1} in both cases. This can be compared to the values of 27 cm^{-1} observed in very uniform silk fibroin, 76 cm^{-1} observed in less uniform β -keratin (ref 13 and refs therein), 40 cm^{-1} in silkmoth chorion⁵ and 45 cm^{-1} in chorion of the fish *Salmo gairdneri*³¹. The 2:1 ratio of β -sheet/ β -turn secondary structure in chorion proteins as determined from analysis of the amide I band in the Raman spectra suggests similarities of this structure with the model structure proposed for silkmoth chorion proteins¹. However, the amino acid sequences of chorion proteins from the Lepidoptera species studied here have not yet been determined, and a detailed structural model is not possible. Nevertheless, the amino acid composition of *M. sexta* and *S. nonagrioides* chorion proteins shows considerable similarity with that of silkmoth chorion (ref 12 and unpublished data). In the same context, it is interesting to note that a recently determined amino acid sequence from a chorion protein of another Lepidoptera species, the gypsy moth, shares extensive homology with sequences of silkmoth chorion proteins³². Combining this evidence, we predict that the amino acid sequences of chorion proteins from *M. sexta* and *S. nonagrioides* are variants of the same theme elaborated by silkmoth chorion.

The *in vitro* assembly of dissolved chorion proteins was attempted in order to determine structural parameters of the reconstituted units and make useful comparisons with native chorion components. This was done following procedures resembling those for reconstitution of keratin filaments (e.g. ref 33 and refs therein). However, our efforts can only be considered as preliminary. The most surprising finding in these experiments was that, in solution, the proteins which constitute the reassembly products appear to regain regular secondary structure dominated by β -sheet and β -turns. While one must be cautious of quantifying β -sheet structures using CD spectroscopy, the change in spectra obtained by denaturation suggests that it is unlikely that the reassembled chorion proteins are random coils. Moreover, a high percentage of β -sheet structure is consistent with the secondary structure percentages obtained for the proteins in intact chorion. Similar results have been obtained with chorion proteins of the silkmoths (Hamodrakas unpublished results). Based on the results presented here, we find it tempting to suggest that the prominent regular β -sheet structure of chorion proteins may persist in solution, dictating the aggregation and polymerization process. However, comparison of the reconstitution results with chorion fine structure^{13,15} emphasizes that a great deal of caution is required in the interpretation of these data and before drawing any analogies: reconstitution studies are likely to furnish new insights into fibril structure and assembly (in space and time) by a more refined variation of parameters *in vitro*, in order to simulate molecular events occurring in chorion assembly.

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