

Crystal structure of raw pure Mysore silk fibre based on (Ala-Gly)₂-Ser-Gly peptide sequence using Linked-Atom-Least-Squares method

SANGAPPA[†], S S MAHESH and R SOMASHEKAR*

Department of Studies in Physics, University of Mysore, Manasagangotri, Mysore 570 006, India

[†]Department of Physics, Mangalore University, Mangalore 574 199, India

*Corresponding author (Fax, 91-821-2516133; Email, rs@physics.uni-mysore.ac.in)

We have carried out crystal structure analysis of raw pure Mysore silk fibers belonging to *Bombyx mori* on the basis of model parameters of Marsh *et al* using Linked-Atom-Least-Squares technique. The intensity of all the reflections were computed employing CCP13 software. We observe that the molecular modification is essentially same as *b*-pleated structure with antipolar-antiparallel arrangements formed by hydrogen bonds. The essential differences observed in the structure are highlighted and discussed.

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Introduction

Silk fibers are fascinating because of their luster and their extensive use in textile industry. Marsh *et al* (1955), in a classic and elegant paper, reported the crystal structure of silk fibroin, made up of a regular arrangement of antiparallel sheets. In their paper, it is stated that the oriented samples were prepared by maintaining the freshly extracted silk gland with dilute acetic acid and then immediately deforming the contents of the gland by stretching and rolling, which leads to doubly oriented samples for X-ray recordings. Whereas, we have used fibers reeled from cocoons kept in warm water using mono reeling equipment which does not involve any stretching or rolling. The fibers were taken in the form of a bundle of 1 mm thick and then mounted on a rectangular holder in the *just taut* condition without stretching. We would like to emphasize here that we are interested in studying the modifications of crystal and molecular structures of untreated silk fibers. Further a data base of these crystal structure parameters of several varieties of silk fibers developed in Mysore will be of immense help in mapping the structural variations so that one can have a better perspective of structure-property relations in these fibers.

2. Experimental

2.1 Sample preparation

For our study we have used raw pure Mysore silk fibre belonging to *Bombyx mori* family which comes under the classification Multivoltine on the basis of shape, colour, denier and life cycle of the fibers/cocoons. Cocoons were collected from the germ plasma stock of the Department of Sericulture which were then cooked in boiling water (100°C) for 2 min to soften the sericin and transferred to water bath at 65°C for 2 min. Then the cocoons were reeled in warm water with the help of mono cocoon reeling equipment EPPROUVITE. The characteristic features of these fibers are that they are light greenish yellow in colour with an average filament length of 350 meter and denier being in the range 1.8–2.0. These fibers were mounted on rectangular frame in *just taut* condition which does not involve any mechanical stretching of fibers. The whole process, starting from reeling to mounting of fibers, does not involve any type of mechanical deformation.

2.2 X-ray recordings

X-ray diffraction patterns from silk fibers were recorded

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on an imaging plate. In this method, a rotating anode X-ray generator (ULTRA-X, RIGAKU) was operated in a normal focus mode to provide a monochromatic (Mo) beam of wavelength ($\lambda = 0.7107 \text{ \AA}$), at 50 kV and 250 mA. Diffraction data was recorded on a disk shaped imaging plate with the sample to plate distance of 150 mm. The measurement of X-ray diffraction data was implemented by the hard-ware system DIP100S (MACSCIENCE). The intensity values were thereby converted into pixel data in a rectangular coordinate system. A whole area of the imaging plate (diameter $\approx 200 \mu\text{m}$) was divided into 1600×1600 pixels each having a size of 125 nm^2 . For this purpose, the programs available in CCP13 suite were used (CCP13, 2004). For computing, a workstation, OCTANE, version 6.5 with an operating system IRIS 64, was used. We have used Ivanova and Makowshi's (1998) method for background estimation, which is more reliable and consistent. It makes use of the fact that the background of a fiber diffraction pattern is typically composed of lower spatial frequencies than the diffraction maxima. Background subtracted X-ray pattern for pure Mysore silk fiber is given in figure 1.

Each diffraction spot was picked by positioning the mouse on its centre and coordinates were measured using SUN SP/2, a SUN micro system Computer Corporation Business. After determining the centre and inclination angle of the X-ray pattern, the interplanar spacing was obtained by averaging the distances between the centre of the diffraction pattern and the positions of two or four equivalent reflections. The dimensions of unit cells were determined by least squares method with the preliminary cell dimensions being obtained by a trial and error method on the computer display. We found no significant variation in the cell parameters of different silk fibers. The averaged cell parameters are $a = 9.40(2) \text{ \AA}$, $b = 9.20(2) \text{ \AA}$, c (fiber axis) $= 6.97(2) \text{ \AA}$ with $\beta = 90^\circ$. The space group being $P2_1$, which is essentially, the same as that reported by Marsh *et al* (1995) and Takahashi *et al* (1999). The unit cell contains four molecular chains. The whole pattern fitting is carried out in two stages. In the first stage, X-ray diffraction pattern taken on a flat imaging plate system was transformed to reciprocal space using the specimen to film distance, rotation of image, wavelength and tilt of the

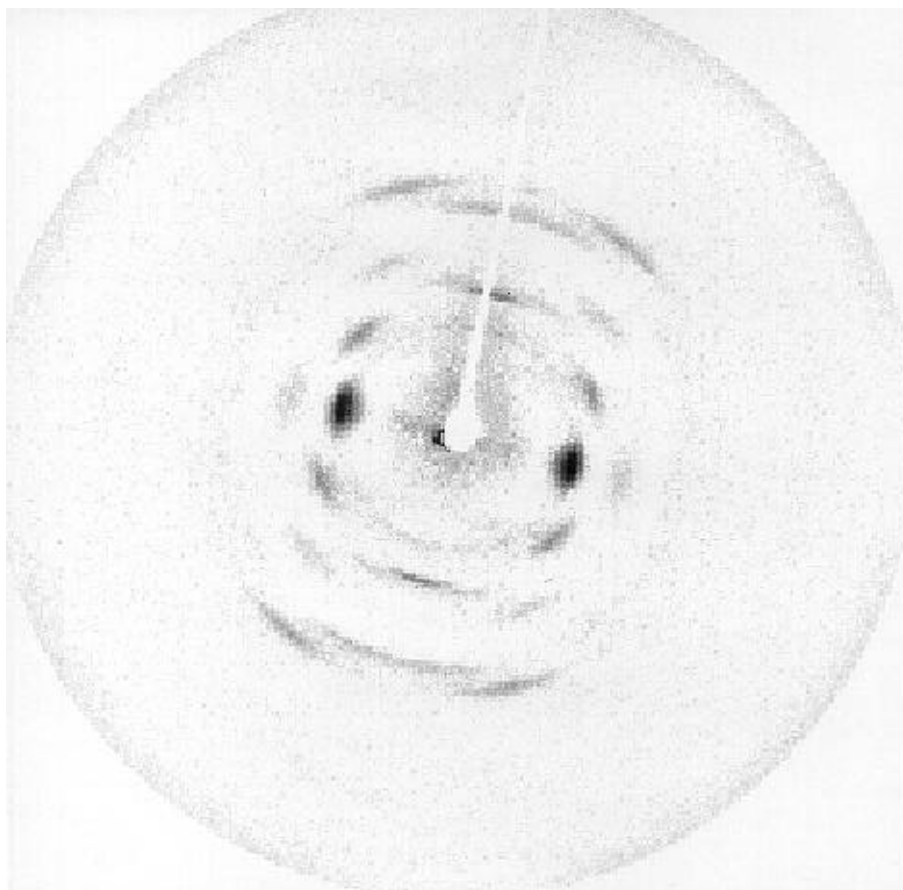


Figure 1. Background-corrected X-ray diffraction pattern of raw pure Mysore silk fiber (multivoltine).

system and employing a program FTOREC, available in the software suite CCP13. In the second step, for the whole pattern fitting, we have used a program LSQINT available in CCP13 suite for which the input file is the output of FTOREC program. By inputting unit cell parameters, the space group symmetry and the profile parameters, further processing of the pattern was carried out. In fact there are six profile parameters. This includes Hosemann's paracrystallinity, which is defined as the extent of disorder of second kind present in the lattice. These six parameters are varied to generate the profiles and then to fit these profiles to the observed pixel intensities by the maximum entropy of Skilling and Bryan (1984) the R-factor is given by

$$R = \sqrt{(\sum(P_o - P_c)^2 / \sum(P_o)^2)}, \quad (1)$$

where P_o and P_c are the observed and calculated pixels respectively. Here, P_o is the background corrected observed pixel values. The R-factor for the pattern fitting in this case was less than 20%. Here it should be noted that the procedure used to simulate equatorial plot do separate the overlapping reflections.

Using CCP13 package 'XCONV' with appropriate file options suitable for DIP image system, we can simulate cylindrical image and also calculate the integrated intensities with standard deviations (Squire *et al* 2003). The output of this routine gives two files: One containing simulated cylindrical pattern and another containing the computed integrated intensities. These intensities were corrected for polarisation and Lorentz factors (Okuyama *et al* 1997). The output file which contains the integrated intensities corresponding to various (hkl) reflections with standard deviations are further used to determine the molecular and crystal structures of Silk fibers in silk-II modification. Figure 2 shows simulated and experimental cylindrical patterns of silk in silk-II modification used to compute integrated intensities of (hkl) reflections. Table 1 shows the experimental integrated intensities of various (hkl) reflections used in this study. However we would like to emphasize that within a layer, in all the cases of overlapping reflections, the percentage of deviation was less than 1% of the mean value of $2 \sin(\theta/\lambda)$.

3. Structure determination

3.1 Molecular model

The amino acid composition of the crystalline fraction of *B. mori* silk fibroin is Gly, 0.48; Als, 0.33; Ser, 0.15; and Tyr, 0.01; by fraction (Lucas *et al* 1957; Strydom *et al* 1997) and they (Ala, Gly, Ser) are in the ratio 3 : 2 : 1. The amino acid sequence in silk-II was Gly-L-Ala-Gly-L-Ala-Gly-[L-Ser-Gly-(L-Ala-Gly) $_n$] $_8$ -L-Ser-Gly-LAla-L-Ala-Gly-L-Tyr (International Tables for Crystallography 1974), where

n is usually 2. Therefore in this study a hexapeptide, L-Ala-Gly-L-Ala-Gly-L-Ser-Gly was used. Molecular models having the appropriate helical symmetry and fiber repeating period were generated using a Linked-atom description with all bond lengths and angles held constant (Momany *et al* 1975; Smith and Arnott 1978). These values are shown in figure 3. The molecular

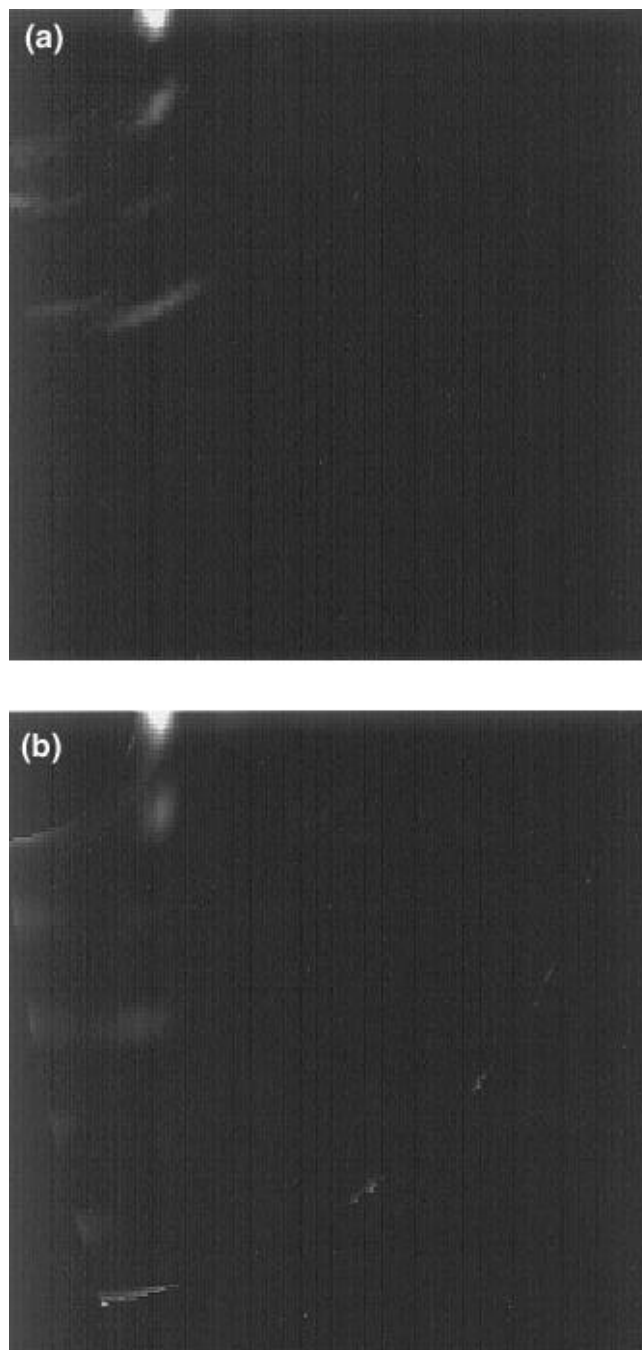
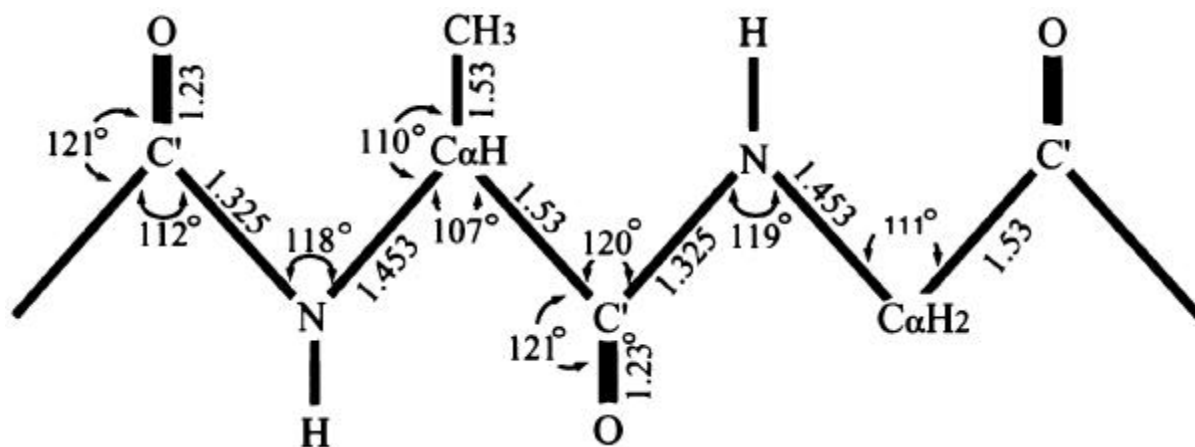


Figure 2. (a) Cylindrical transformed image of experimental X-ray pattern shown in figure 1. (b) Cylindrical transformed image of simulated X-ray pattern of raw pure Mysore silk fiber.

Table 1. Refined details of silk-II with (Ala-Gly)₂-Ser-Gly repeating unit.

Refined parameters	Pure Mysore silk	Marsh <i>et al</i> (1955)
Torsional angle(°)		
f_{Ala}	- 147.99	- 139
γ_{Ala}	143.68	140
w_{Ala}	178.62	176
f_{Gly}	- 144.64	- 139
γ_{Gly}	146.48	140
w_{Gly}	178.19	176
Eulerian angle (°)		
\hat{I}_x	- 88.06	-
\hat{I}_y	128.81	-
\hat{I}_z	- 167.87	-
Other parameters		
S(Å)	42.10	-
m° chain-a	167.31	-
m° chain-b	133.07	-
$u(a); v(a); w(a)$	0.1657; 0.0042; 0.2113	-
$u(b); v(b); w(b)$	0.7018; 0.7712; 0.3018	-
Scale factor	2.783	-
Attenuation factor	- 8.755	-
Hydrogen bonds <i>intra</i> -molecular		
Distance (Å); angle (°)	N(Ala)...O(Ala) (2.74, 165.2)	-
	N(Ala)...O(Ser) (3.1, 136.0)	-
	O(Gly)...N(Gly) (2.7, 161.5)	-
	O(Ser)...O(Ala) (2.8, 89.5)	-
<i>R</i> -factor		
Including unobserved R_c	0.168	-
Reflections R_w	0.172	-

**Figure 3.** Molecular bond angles and bond lengths of silk fiber.

structure of the silk fibroin, with three amino acids Ala, Gly and Ser, has a 1/1-helical symmetry. That is, two chemical repeating units of Ala-Gly are constrained in the fiber repeating period. Actually there is an option in Linked-Arom-Least-Squares (LALS) program to indicate two molecular chains by defining the constrains for only one of them. Here, the geometry for Ala is for the D-Ala and not L-Ala. The molecular conformation must satisfy the both sterical and mathematical requirements. Hence, we have chosen initial \mathbf{f} and \mathbf{y} for Ala and Gly residues to be the same as that of Marsh *et al* (1955) results and the main chain was constructed with an appropriate helical parameters together with bond lengths and angles shown in figure 3.

3.2 Packing models and their refinement

In the following section, the most plausible space group and the number of a chemical repeating unit of (Ala-Gly)₂-Ser-Gly in a unit cell were determined to $P2_1$ and four ($Z = 4$), respectively. The symmetries and four molecular sites in the unit cell with the space group $P2_1$ is shown in figure 4. The molecules at site 1(1') and at site 2(2') are symmetrically independent of each other and form a sheet structure parallel to the *ac*-plane by hydrogen bonds. Two molecules at sites 1 and 2 can be related to the molecules at sites 1' and 2' by the 2-fold screw symmetry, respectively. Takahashi *et al* (1999) have shown that there are four models for the sheet structure formed by hydrogen bonds. They are: (i) polar-antiparallel [PA (1)]; (ii) polar-parallel (PP); (iii) antipolar-antiparallel (AA); and (iv) antipolar-parallel (AP). In the polar model, the methyl groups of alanine residues are on one side of the sheet only. while in the antipolar model, the methyl groups alternately point to both sides of the sheet along the hydrogen bonding direction (Takahashi *et al* 1999). The crystal structure proposed by Marsh *et al* (1955) corresponds to PA sheets (Marsh *et al* 1955). In order to pack four chemical repeating units in a unit cell the helical axis of the molecule must coincide with the *c*-axis which is parallel to 2₁-axis. For the positioning the molecular model in the unit cell, two additional parameters were used to define the relative axial rotation (\mathbf{m}) and translation (w) along the *c*-axis. At each stage in the modelling and refinement of the structure, we minimized the quantity Ω in the following least-squares fashion (Smith and Arnot 1978).

The first summation in Ω ensures the optimum agreement between the observed (F_o) and the calculated (F_c) X-ray structure amplitudes. The w is the weight of each reflection. The second

$$\Omega = \sum w(|F_o| - |F_c|)^2 + S \sum_j \epsilon_j + \sum_h \mathbf{I}_h G_h, \quad (2)$$

ensures the optimization of noncovalent interatomic ($\hat{\mathbf{I}}_j$). S is the scale factor used to adjust the overall weight of the

second term with respect to the first. Third imposes, by the method of Lagrange undetermined multipliers (\mathbf{I}_h), the exact constraints (G_h) we have chosen.

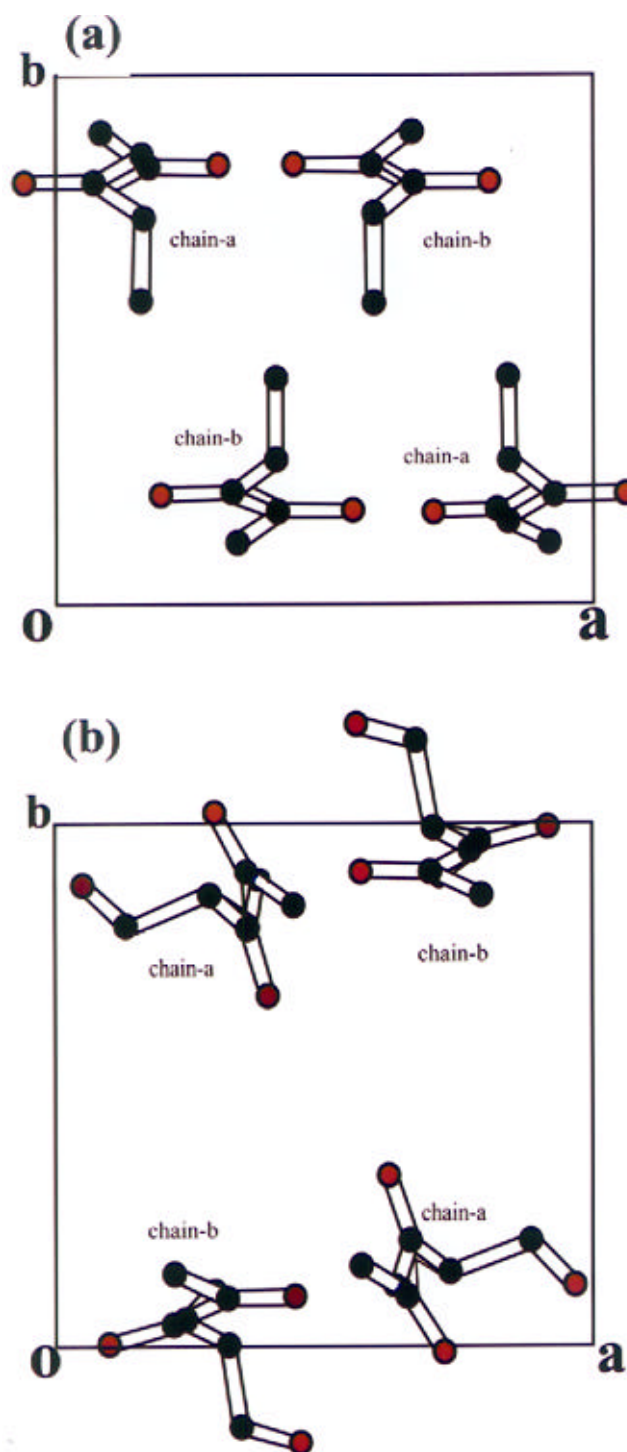


Figure 4. (a) Fiber axis projection of crystal structure of stretched silk fiber obtained by Marsh *et al* (1955). (b) Down the fiber axis projection of untreated and unstretched pure Mysore silk fiber obtained in the present work.

Atomic scattering factors for calculating structure factors were obtained using the method and values given in International Tables for X-ray Crystallography (1974). Computations were carried out using a Linux based PC. We have compiled the software LALS for execution using LINUX operating system.

4. Molecular and crystal structure for the chemical repeating unit of (Ala-Gly)₂-Ser-Gly

The refinement was carried out for the crystal structure in which two antiparallel molecules related by a 2-fold rotation axis parallel to c-axis. In order to get appropriate packing parameters, the discrepancy factors R_c (conventional R factor) and R_w (weighted R_c factor) and shortest contact between non bonded atoms were calculated. Here, R_c and R_w were defined by

$$R_c = \frac{\sum ||F_o| - |F_c||}{\sum |F_o|}$$

$$R_w = \frac{\sum_w (|F_o| - |F_c|)^2}{\sum_w F_o^2} \quad (3)$$

Initially a dipeptide Ala-Gly was used as chemical repeating unit for refinement and later on a hexapeptide (Ala-Gly)₂-Ser-Gly was used at a later stage. In this study, the weight of each reflection, w was fixed to 1.0. The refinement gave far better agreement between the observed and calculated structure factors with the consideration of serine residue by replacing the 1/3 of alanine in the peptide

chain. The R -factor reduced to 16.8%. The refined parameters and the final values are summarized in table 1. Here, the azimuthal angles of the two molecules forming a sheet were also refined independently. The internal rotation angle C(=O)-C(**a**)-C(**b**)-O(H) of serine residue was refined. Table 2 gives the fractional coordinates. Table 3 gives the comparison between the observed and calculated structure factors. The crystal structure of pure Mysore silk in raw form is shown in figure 5.

5. Results and discussion

Here, it is found that, molecular structure is essentially same as what has been proposed by Marsh *et al* (1955), except for the fact that chains 2 and 2' are sheared and twisted along the fiber axis. This essentially implies that the sample used by Marsh *et al* (1955) in their paper is a strained one and the untreated raw fiber used here in this study can be classified as relaxed fiber. A comparison of the structure down the c-axis of the present relaxed raw fiber and the strained fiber used by Marsh *et al* (1955) is given in figure 4. The difference between the relaxed and strained fibers lies in the fact there are conformational changes in the chain either from *gauche* to *trans* in certain portion of the chains 2 and 2'. In fact similar observations were reported in an entirely different material by Hall and Pass (1976). The internal rotation angles w of the glycine and alanine residues about N-C(=O) bonds are 178.7 and

Table 2. Fractional atomic co-ordinates of silk-II for the repeating unit of (Ala-Gly)₂-Ser-Gly.

Atom	x	y	z	x	y	z
	Chain-a			Chain-b		
Ala						
N	0.275	0.032	0.557	0.643	0.198	0.457
H _N	0.381	0.038	0.549	0.594	0.294	0.464
C _a	0.202	0.074	0.381	0.715	0.155	0.632
C _b	0.176	0.238	0.381	0.866	0.216	0.632
H _a	0.110	0.021	0.376	0.716	0.047	0.637
C'	0.296	0.032	0.210	0.632	0.217	0.803
O	0.426	0.043	0.221	0.575	0.337	0.792
Gly						
N	0.224	-0.013	0.059	0.630	0.130	0.954
H _N	0.118	-0.024	0.056	0.675	0.032	0.958
C _a	0.296	-0.053	-0.118	0.560	0.174	1.131
H _{a1}	0.313	-0.161	-0.120	0.461	0.134	1.133
H _{a2}	0.390	-0.002	-0.125	0.556	0.282	1.138
C'	0.204	-0.010	0.711	0.643	0.114	0.302
O(2)	0.073	-0.014	0.723	0.705	-0.003	0.290
Ser*						
O	0.302	0.313	0.324	0.866	0.364	0.689

*Other atomic co-ordinates of Ser residue are same as those of Ala.

Table 3. Observed (F_o) and calculated (F_c) structure amplitudes for (Ala-Gly)₂-Ser-Gly repeating unit.

Data number	<i>hkl</i>	Multiplicity factor	F_c	F_o
1	1 0 0	2		
	0 1 0	2	238.65	240.14
2	1 1 0	4	48.22	36.35
	2 0 0	2		
3	0 2 0	2	119.81	137.74
	2 1 0	4		
4	1 2 0	4	249.78	262.71
	3 0 0	2		
5	0 3 0	2	101.09	67.88
	3 1 0	4		
6	1 3 0	4	170.87	139.36
	3 2 0	4		
7	2 3 0	4	40.13	37.24
	4 0 0	2		
8	0 4 0	2	94.98	111.72
	4 1 0	4		
9	1 4 0	4	46.28	40.13
	1 0 1	2		
10	0 1 1	2	46.94	23.77
	1 1 1	4	48.93	36.38
11	2 0 1	2		
	0 2 1	2	63.11	67.55
12	2 1 1	4		
	1 2 1	4	132.58	141.22
13	2 2 1	4	43.69	56.75
	3 1 1	4		
14	1 3 1	4	53.94	55.16
	3 2 1	4		
15	2 3 1	4	72.45	58.95
	4 0 1	2		
16	0 4 1	2	98.34	118.62
	4 1 1	4		
17	1 4 1	4	53.59	49.21
	4 2 1	4	116.20	128.25
18	1 0 2	2		
	0 1 2	2	47.84	33.15
19	1 1 2	4	26.24	43.56
	2 0 2	2		
20	0 2 2	2		
	2 1 2	4		
21	1 2 2	4	124.44	138.16
	2 2 2	4	60.06	51.88
22	3 1 2	4		
	1 3 2	4	77.48	89.09
23	3 2 2	4		
	2 3 2	4	55.01	102.34
24	1 0 3	2		
	0 1 3	2	39.08	36.85
25	1 1 3	4	27.55	51.60
	2 0 3	2		
26	0 2 3	2	108.74	79.15
	2 1 3	4		
27	1 2 3	4	136.76	122.27

178.2 respectively, which are nearly *trans* conformation. The internal rotation angles f and y for the glycine residue are -144.6° and 146.5° respectively which are between *skew* and *trans* conformations. The internal rotation angles f and y for the alanine residue are -147.9° and 143.7° respectively which are also between *skew* and *trans* conformations. Marsh *et al* (1955) have reported torsion angles for Gly ($-139, 140$) and Ala ($-139, 140$) for the silk-II with mechanically oriented samples. Torsion angles (f, y) of silk-II structure using energy calculations of Fossey's model (Fossey *et al* 1991) has been reported for Ala ($-149, 148$) and for Gly ($-150, 146$) whereas on the basis of solid state NMR for Ala ($-140, 142$) and for Gly ($-139, 135$) (15) (Demura *et al* 1998). In NMR studies, the samples were not subjected to any mechanical treatment. In the final structure, the internal rotation angle $C'-C_a-C_b-O_{ser}$ is 80.9 and the C_b-O_{ser} bond is almost along the chain direction. The stereochemical energy which is repre-

sented by s in silk-II modification turns out to be $2.35E+03$ which is less than the value of $8.25E+03$ for silk-I modification which has a crank-shaft or a S-shaped zigzag arrangement. Here is s given by the sum of the second term of the equation (2) (Okuyama *et al* 2001). The torsional angles and Eulerian angles are the same for chain-a and chain-b as they are symmetric. The intra chain hydrogen bonding network is shown (Spek 2003) in figure 6. In the network, the O and N atoms of glycine are at 2.73 \AA and the bond angles of 161.52° whereas the intra chain hydrogen bondings N-H...O and N-H...O_{ser} in alanine are at bond distances of 2.74 and 3.06 \AA respectively. The corresponding bond angles are 165.21° and 136.02° . Further the intramolecular distances between O atom serine residue and O atom of alanine residue is 2.83 \AA and the bond angle is 89.54° . This may suggest that the OH groups of the serine residues are associated with the hydrogen bonding network forming the sheet

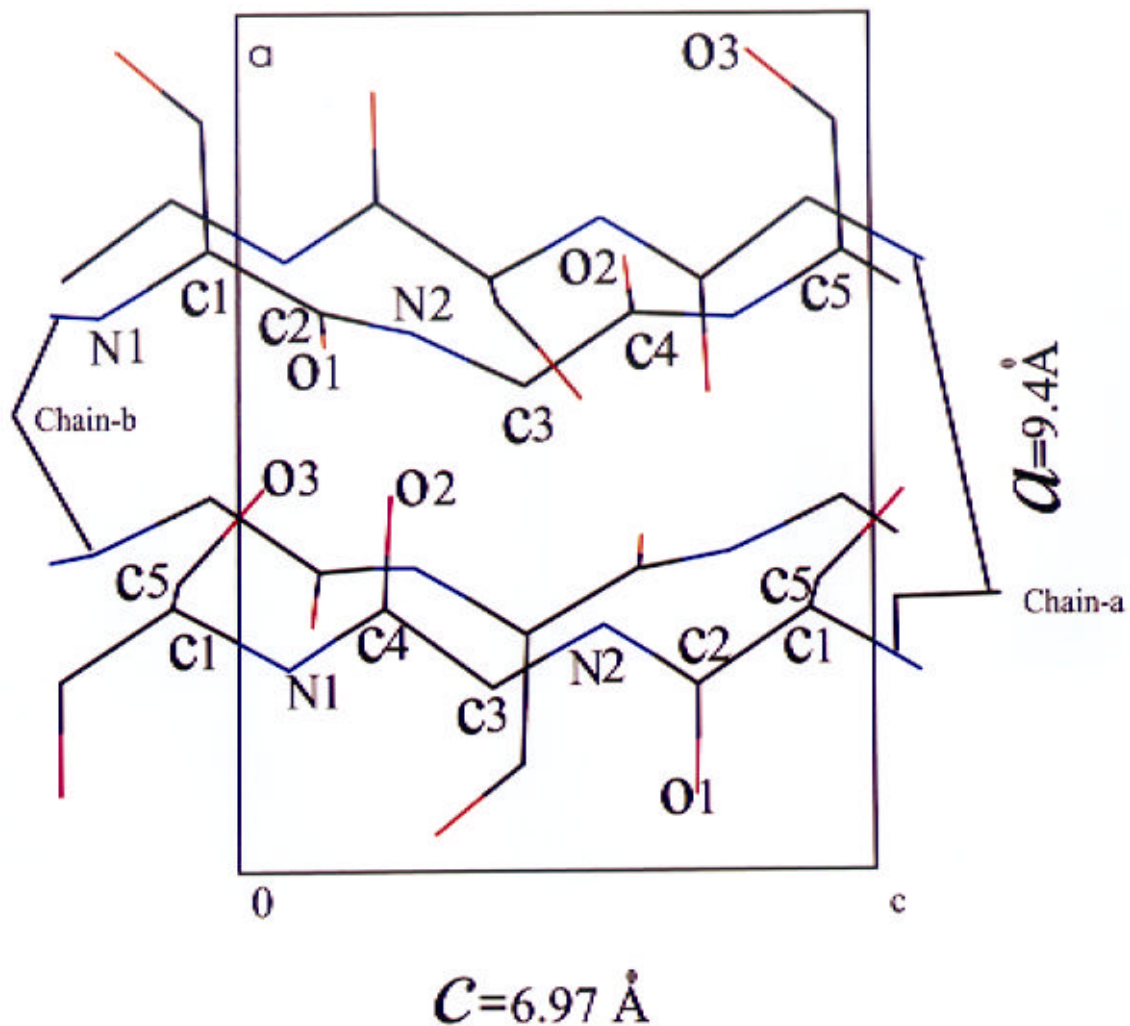


Figure 5. Crystal structure of raw pure Mysore silk down the b-axis.

structure. Jeffrey (1997) has used the concept of strong, moderate and weak hydrogen bonds in order to explain observed hydrogen bonding geometries. The H...A distances are 1.2–1.5 Å, 1.5–2.2 Å and 2.2–3.2 Å for strong, medium and weak bonds and the bond angles are 175–180°, 130–180° and 90–150° respectively. Desiraju and Steiner (1999) have used two categories to describe hydrogen bonds, strong and weak, where H...A is 1.5–2.2 Å and 2.2–3 Å for strong and weak bonds and the bond angles are 130–180° and 90–180° respectively. Most of the hydrogen bonds are medium according to Jeffrey (1997) and weak according to Desiraju and Steiner (1999). Here, we have only highlighted the essential differences and features that arise in structure due to mechanical treatment of silk fibers by studying the untreated silk fibers.

In addition to the NH...O hydrogen bonds found in the poly (1-Ala-Gly), additional hydrogen bond involved with the hydroxyl oxygen in the serine residue is also seen. Infact the main difference between our result when compared with Marsh *et al's* (1955) work is that we have included serine residue in the repeating sequence, which also results in slight improvement in the *R*-factors. Atomic coordinates of Ser residue is given in table 2. The chain conformation is stabilized by the bifurcated hydrogen bond between N of alanine and serine residues. Because of these hydrogen bonds, the molecular conformation is restricted in its degree of freedom by forming the *b*-pleated structure. The hydrogen bonds are the only direct interaction between adjacent layers other than Van der Waals interaction. The results show that the final model

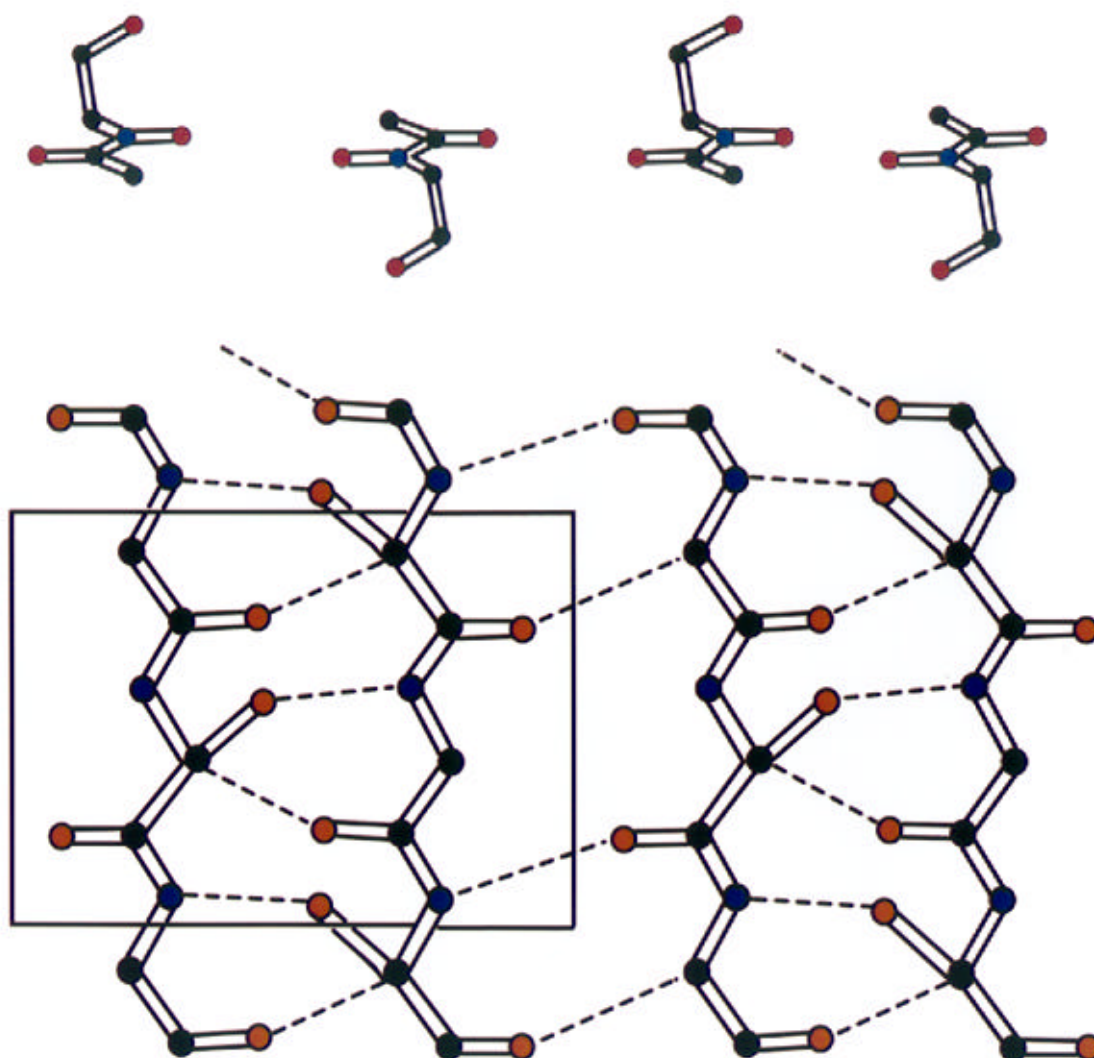


Figure 6. Weak hydrogen bond networks in raw pure Mysore silk fiber.

has a fairly strong intramolecular hydrogen bond among Ala, Gly and Ser residues indicating that the molecular conformation in the silk-II is stabilized mainly by these bonds just like α -helix and tropocollagen.

6. Conclusion

Examination of the crystal structure of untreated raw pure Mysore silk fiber belonging to *B. mori* was carried out by X-ray diffraction method using CCP13 package (to identify and compute integrated reflections) and the Linked-Atom-Least-Squares technique. Four molecular chains are contained in the rectangular unit cell with parameters $a = 9.4 \text{ \AA}$, $b = 9.2 \text{ \AA}$ and c (fiber-axis) $= 6.97 \text{ \AA}$ and the space group being $P2_1$. Comparing our results with Marsh *et al* (1955) wherein they use rolled, well aligned sample, we conclude that in untreated raw silk fiber, the chains 2 and 2' are rotated by an angle 90° with respect to the chains 1 and 1'. The molecular conformation is essentially the same pleated sheet structure as reported by Marsh *et al* (1955). However the sheet structures formed by hydrogen bonds assume the antipolar-antiparallel arrangement, which is in conformity with the results of Takahashi *et al* (1999). Furthermore, quantitatively stereochemical energy of silk-II modification is less than the silk-I modification. This is in conformity with the fact that silk-I is in thermally and physically unstable phase.

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