Crystallinity in Silk Fibers: Partial Acid Hydrolysis and Related Studies

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Synopsis

Four Indian varieties of silk viz., Mulberry, Tasar, Eri, and Muga, have been investigated by x-ray diffraction and infrared spectroscopy. The hydrolysis for Mulberry was carried out using 6*N* HCl at 40°C, whereas the other wild varieties of silk were hydrolyzed partially using 8*N* HCl at 40°C. The x-ray order factor, IR crystallinity index, and crystallite size have been determined for control, 48-hr hydrolysates, and (48 + 48)-hr hydrolysates. The lateral order improvement need not be associated with selective dissolution of amorphous region. This was further verified by short duration hydrolysis. A sort of recrystallization process could be attributed to order improvement. The results are discussed to understand the fine structure of the crystalline region of the fibroin.

INTRODUCTION

The measurement of crystallinity in polymeric materials is an important technological application of different physical methods, such as x-ray diffraction, infrared spectroscopy, density measurements, differential thermal analysis, and isotope exchange. It has become the common experience of polymer scientists that no single method could be used for absolute crystallinity measurements. However, the limitation of individual techniques employed in the estimation of crystallinity need not be considered as a setback for the studies. Collective and comparative studies of the degree of crystallinity by different methods throw light on the structure and hence the end use of individual polymers. Silk fibroins are known for their complexities since they contain different amino acids in their chains such as bulky and polar amino acids. The silk fibroin obtained by cocoons is found to exist mainly in the β-pleated sheet structure form.[1] A survey of methods adopted to measure crystallinity of polymers has been reviewed by Hermans.[2] Magoshi et al.[3,4] have assigned the infrared bands, for the α and β forms. Manjunath et al.[5] have proposed a formula for finding out the lateral order factor. The amino acid composition for different varieties of silk has been studied.[6,7] The chemically resistant fraction of a few varieties of silk after alkali and acid treatment has been investigated by x-ray diffraction[8] and amino acid analysis.[9]

In the present investigation we report data on crystallinity of different Indian varieties of silk as determined by x-ray diffraction and infrared spectroscopy. The residues obtained after partial acid hydrolysis—"hydrofibroins"—have also been studied and compared with the control samples. The changes observed in the x-ray order factor, infrared crystallinity index in the light of mechanism of partial acid hydrolysis, and fine structure are discussed.

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EXPERIMENTAL

Materials

Silk fibroin of different Indian species, viz., Mulberry and Tasar or Tussah, Eri, and Muga, were obtained from different sericultural laboratories in India. The cocoons were degummed by a method described elsewhere.[10] The fibers were removed from the cocoons and were used for the studies.

Hydrolysis

The Mulberry silk was hydrolyzed partially using 6*N* HCl at 40°C. The durations of hydrolysis were 48 hr and (48 + 48) hr. The wild silks, viz., Tasar, Eri, and Muga, were hydrolyzed partially by use of 8*N* HCl at 40°C. The durations of hydrolysis were 48 hr and (48 + 48) hr. The samples were repeatedly washed with distilled water to remove acid. The residues were sieved through sintered-glass filters. The dried samples were in fine powder form and were used for weight loss studies.

Equipment and Procedure

Control samples were cut into fine powder and seived through 300 mesh. A pellet of 1 cm diameter was prepared for the x-ray diffractometric studies. However, hydrofibroins were directly used to prepare the pellet. An x-ray generator model PW 1009 with diffractometric arrangement was used for the studies. A Perkin Elmer model 377 infrared spectrophotometer was used for the investigation. A KBr disk method was adopted for sample preparation for infrared spectral studies.

RESULTS AND DISCUSSION

Weight Loss Studies

The kinetics of hydrolysis with respect to weight loss has been investigated by Shaw and Smith.[9] Acid and alkali hydrolysis were carried out by these authors. Further, it seems from their data that acid hydrolysis using 6–10*N* HCl at 40°C for 48 hr yields fibroin residues which are mostly making up the crystalline part of the control samples. However, optimization of such a hydrolysis process depends on various parameters including the species of silkworm from which the silk has been cultivated. These authors have shown that loss of weight owing to acid treatment is rapid in the beginning, then slows down, and almost saturates at 40 hr. Hence, in the present investigation hydrolysates were studied after 48 hr of acid treatment so that the residues are unaffected as well as representing mostly the crystalline regions of silk fibers. Further treatment for an additional 48 hr on the hydrolysates reveals that a slight weight loss does take place. Table I shows the weight of (hydrofibroins) residues of fibroins owing to partial acid hydrolysis as a function of time (the temperature and concentration of acid being maintained constant). The loss of weight may be attributed to the fact that the fibroins have a less ordered and more accessible amorphous portion as one phase and highly ordered and chemically resistant portion as

TABLE I
Percentage Weight of Residues of Silk after Hydrolysis Using HCl at 40°C

Variety of silk	Percentage of weight of residues or hydrofibroins	
	48 hr, 40°C	48 + 48 hr, 40°C
Mulberry	38	32
Tasar or Tussah	38	33
Eri	39	32
Muga	40	32

another phase. The dissolution may be due to removal of the amorphous portion.

Crystallinity Determination for Control Samples with Various Techniques

Silk fibroin is known to be crystalline and gives rise to x-ray diffraction pattern. However, no attempts have been made to determine its percentage crystallinity. In the present work, an attempt has been made to determine the percentage of crystallinity using three different techniques, viz., x-ray diffraction, electron diffraction, and IR spectroscopy. The determination of crystallinity by x-ray diffraction has been attempted by various workers for polymeric materials such as nylon,[11] PET,[12] and cellulose.[13] Various formulas have been suggested to compute the crystallinity index on the basis of relative intensities of peaks as well as the integrated area under the diffraction profile. However, all the methods are based on the same principle as suggested by Goppel et al.[14] After applying various formulas we found that most of the methods yield essentially the same values. However, the formula proposed by Manjunath et al.[5] was then adopted since it defined the resolution factor and was found better suited. We have used the formula

$$R_f = \frac{m_1 + 2m_2 + m_3 + \ldots + m_{n-1}}{h_1 + h_2 + h_3 + \ldots + h_n} \qquad L_o = 1 - R_f$$

where R_f is the resolution factor; L_o is the lateral order factor; $h_1, h_2, \ldots h_n$ are the respective peak heights and $m_1, m_2, \ldots m_{n-1}$ are the respective heights of minima between the two peaks representing amorphous contribution. The R_f is inversely proportional to the lateral order, i.e., R_f tends to unity when the resolution is completely lost and it tends to zero when the resolution is at maximum. The values obtained as the order factor are tabulated in Table II. It can be noted that the order factor for all the varieties is about 0.42–0.43.

The infrared spectra of silk fibroin has been investigated earlier.[15,16] All the absorption bands have been characterized in terms of amide groups. However, the spectra in the range 1200–800 cm^{-1} is found to be characteristic of individual fibroin.[17] For Mulberry silk[3] the bands assigned to β structure are 1630, 1530, 1265, and 700 cm^{-1} corresponding to amide I, amide II, amide III, and amide V, respectively. Similarly, for the α-random coil conformation the bands assigned are 1660, 1540, 1235, and 650 cm^{-1} corresponding to amide I, amide II, amide III, and amide V, respectively. Recently Magoshi et al.[4] have reported the IR

TABLE II
Crystallinity—Lateral Order Factor Studies for Control Sample

Variety of silk	X-ray order factor	Infrared crystallinity index	Electron diffraction order factor[10]
Mulberry	0.42	0.66	0.62
Tasar or Tussah	0.43	0.50	0.60
Eri	0.44	0.60	0.62
Muga	0.43	0.50	0.63

spectra of Tussah silk obtained from *Antherea perni* silkworm glands. The regenerated film was found to be amorphous with characteristic bands at 1660, 1550, 1310, 1270, 1107, 890, and 650 cm^{-1}. On heating, the film was converted to β form showing characteristic bands at 1630, 1530, 1240, 970, and 700 cm^{-1}. The IR spectra in the case of Eri and Muga was found to be similar to Tasar. The bands at 1235 and 1265 cm^{-1} were used to find the IR crystallinity index. The ratio of the optical density of these bands, viz., D1265/D1235, was used to compute the index. In fact any two bands could have been used; however, the bands at 1660, 1630, 1550, and 1530 cm^{-1} are not well resolved. On the other hand, the 1265 and 1235 bands showed good resolution in the control as well as in hydrolysates. The values obtained are tabulated in Table II. In Mulberry (B. mori) the IR crystallinity index was found to be 0.66, whereas in other wild varieties,the values ranged from 0.5 to 0.62.

It may be noted that Badger et al.[18] determined the β-crystalline component of B. mori by comparing the intensities of a pair of characteristic bands 1636, 1664, and 1528, 1560 cm^{-1}. The values obtained by these workers were 67 and 63%, respectively. Incidentally the value 63% obtained using 1528 and 1560 cm^{-1} agrees well with the value obtained by Drucker et al. by the chemical method.[19] The authors[18] have emphasized that these values are not exact, because the resolution for these bands is not exceptionally good.

Electron diffraction technique was also used to obtain information about the lateral order factor. The technique and the results are reported elsewhere.[10]

Crystallinity Determination for Hydrolysates

It is established that the structure of silk fibroin contains polypeptide chains having antiparallel pleated sheets. The repeat unit along the chain direction is 7 Å (fiber axis), whereas the CO–NH groups are hydrogen bonded between the interchains and from the a axis. The cell parameters, a = 9.44 Å and b = 6.95 Å (fiber axis) being invariant for different species of silk fibroins, c varies from 9.2 and 15.7 Å. Studies on the amino acid composition of fibroin have revealed that most of the fibroins are constituted by simple amino acids such as serine, glycine, and alanine.[6] Further amino acid analysis has revealed that together these three acids form 91% of the composition of Mulberry (control), whereas for the wild varieties, these acids constitute 75–80%. Lucas et al.[18] have shown that the sequence of the amino acids in the crystalline region of the Bombyx mori silk is of the form $[Ser\cdot Gly\cdot Ala\cdot Gly\cdot Ala\cdot Gly]_n$. On the other hand the Tasar (probably Eri and Muga also) is supposed to have mostly an Alanine–Alanine linkage in the crystalline region. On this basis the acid attack was expected to be selective for the amorphous region only.

TABLE III
Values of the X-Ray Order Factor and IR Crystallinity Index Owing to Partial Acid Hydrolysis for Mulberry

Treatment	X-ray order factor	IR crystallinity index
Control	0.42	0.66
Hydrofibroins 6*N* HCl 40°C, 48 hr	0.46	0.67
Hydrofibroins 6*N* HCl, 40°C, (48 + 48) hr	0.48	0.74

TABLE IV
Values of the X-Ray Order Factor and IR Crystallinity Index Due to Partial Acid Hydrolysis for Tasar or Tussah

Treatment	X-ray order factor	IR crystallinity index
Control	0.43	0.50
Hydrofibroins 8*N* HCl, 40°C, 48 hr	0.64	0.66
Hydrofibroins 8*N* HCl, 40°C, (48 + 48) hr	0.77	0.66

Hence partial hydrolysis was carried out. The partial hydrolysis yielded a chemically resistant portion which is supposed to be mostly crystalline. The lateral order factor from the x-ray diffraction and IR crystallinity index for the hydrofibroins have been determined and are tabulated in Tables III–VI. It can be seen that the x-ray order factor has increased for all the varieties after the first hydrolysis (48 hr) and an additional hydrolysis (48 + 48 hr). Similarly, increase in the IR crystallinity index has been observed. The increase in the order factor could be due to the fact that most of amorphous portion has dissolved in the acid treatment. The increase in the order factor was not significant in Mulberry since the Gly–Ala linkage could be easily accessible by acid, thus distorting some of the crystalline portion. Hence the rate of order improvement owing to hydrolysis

TABLE V
Values of the X-Ray Order Factor and IR Crystallinity Index Owing to Partial Acid Hydrolysis for Eri

Treatment	X-ray order factor	IR crystallinity index
Control	0.44	0.60
Hydrofibroins 8*N* HCl, 40°C, 48 hr	0.70	0.62
Hydrofibroins 8*N* HCl, 40°C. (48 + 48) hr	0.72	0.64

TABLE VI
Values of the X-Ray Order Factor and IR Crystallinity Index Owing to Partial Acid Hydrolysis for Muga

Treatment	X-ray order factor	IR crystallinity index
Control	0.43	0.49
Hydrofibroin 8*N* HCl, 40°C, 48 hr	0.63	0.76
Hydrofibroins 8*N* HCl, 40°C, (48 + 48) hr	0.65	0.82

is slower than expected. On the other hand wild varieties have mostly Ala–Ala linkages in the crystalline region which is not labile by acid, and therefore only the amorphous portion is dissolved. A look at the IR crystallinity index for Mulberry shows that it increases similarly as in the case of wild silks.

One may recall that IR spectroscopy[20] is sensitive to short-range order while the x-ray diffraction technique is sensitive to long-range order. The results obtained in Mulberry show that the short-range order has increased but, probably because of the small size of the crystallites, is not reflected in the x-ray values.

The interaction of acid with fibroin may be thought to be either selective dissolution or lateral order improvement or recrystallization. In order to ascertain which of these mechanisms is most probable, a short duration hydrolysis was carried out. Treatment with acid for 3 hr resulted in a weight loss of 2.5% leading to an increased x-ray order factor to 0.57. This shows that for the wild varieties, in addition to chain cleavage, rearrangement of chain is taking place. However, one cannot attribute the hydrolysis mechanism to single process.

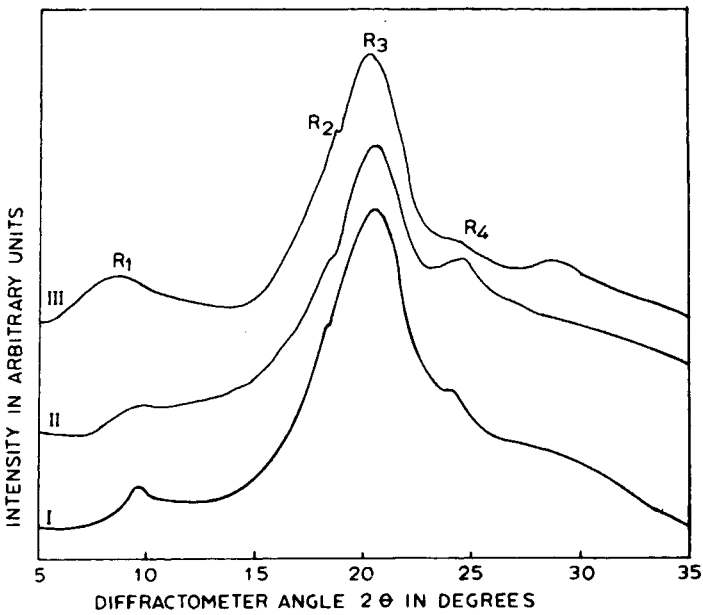

Fig. 1. X-ray diffractograms for (I) Mulberry silk; (II) Mulberry hydrolysate, 48 hr; and (III) Mulberry hydrolysate, (48 + 48) hr.

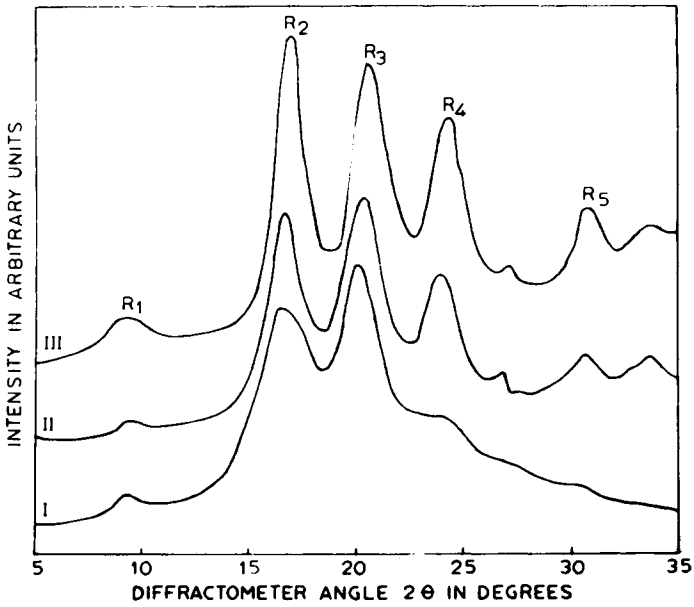

Fig. 2. X-ray diffractograms for (I) Tasar silk; (II) Tasar hydrolysate, 48 hr; and (III) Tasar hydrolysate (48 + 48) hr.

Crystallite Sizes

The crystallite size was determined using the scherrer formula[21]

$$D_{hkl} = \frac{K\lambda}{\beta \cos\theta}$$

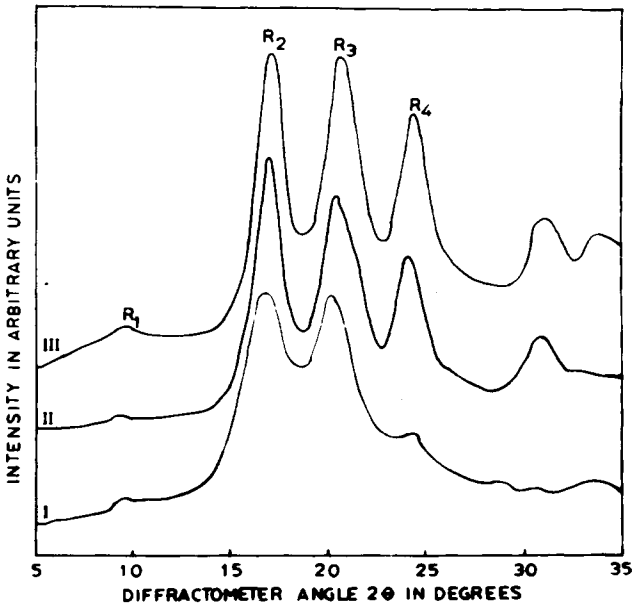

Fig. 3. X-ray diffractograms for (I) Eri silk; (II) Eri hydrolysate, 48 hr; and (III) Eri hydrolysate, (48 + 48) hr.

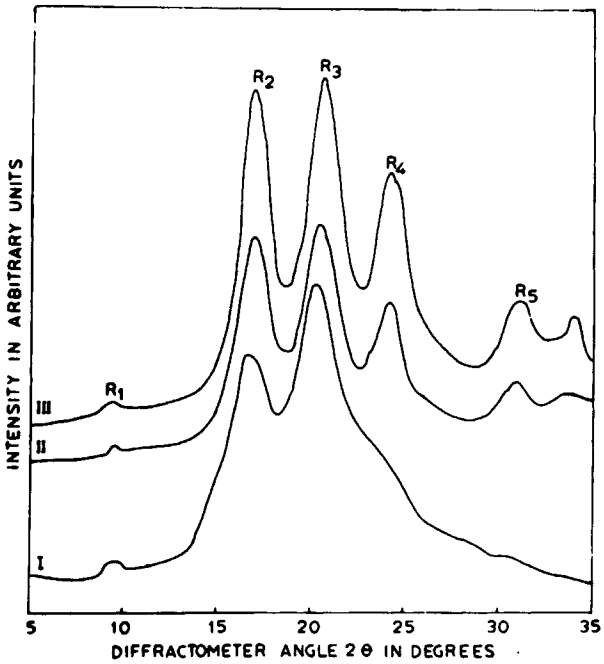

Fig. 4. X-ray diffractograms for (I) Muga silk; (II) Muga hydrolysate, 48 hr; and (III) Muga hydrolysate, (48 + 48) hr.

where K is the shape factor and is generally taken as unity, $\lambda = 1.54$ Å for Cu$K\alpha$ radiation, and β is the half-intensity breadth of reflection profile corresponding to the (hkl) plane. It can be seen from the diffractograms (Figs. 1–4) that the peaks have become quite sharp after hydrolysis in the case of wild silks. However, in Mulberry the peaks are still not resolved and are quite broad.

The crystallite size using the reflections (002) and (201) were calculated and are tabulated in Table VII. It can be seen that in the case of wild silks the increase in crystallite size is quite considerable for (002). However, in Mulberry such a drastic increase was not observed. Though the crystallite size calculated from the (201) reflection did show an increase, it was considerably small. It may be recalled that the (002) reflection arises from the planes whose normals are in the direction of the side chains of the amino acid residues. This indicates that

TABLE VII
Values of the Crystallite Size for Different Varieties of Silk and Their Hydrolysates

Variety of silk	Planes	Control, Å	48-hr hydrofibroins, Å	(48 + 48)-hr hydrofibroins, Å
Mulberry	002	10	15	20
	201	19	20	25
Tasar	002	27	68	60
	201	47	56	52
Eri	002	30	56	56
	201	47	47	60
Muga	002	32	47	59
	201	39	43	47

the recrystallization process must have been occurring by parallel alignment of chains. This process leads further to the Ala–Ala segments aligning parallel to the existing microcrystal and thus causing the crystal to grow. The reflection peak therefore becomes sharper since the other bulky amino acid groups do not interfere (as they have been partially dissolved). According to the results obtained by Warwicker,[8] sharpening of the peaks for the residues (or the increase in the crystallite size) is connected to weight loss owing to hydrolysis. This has been explained on the basis of crystalline and semicrystalline regions lying quite close together. The semicrystalline region is lost during hydrolysis (i.e., bulky and polar acid residues) and closer parallelism is attained for the segments with Ala–Ala linkage in the direction of side chains. Our observation of the increase in crystallite size from (002) reflection and lateral order improvement for short duration hydrolysis give direct evidence for one of the possible explanations given by Warwicker.

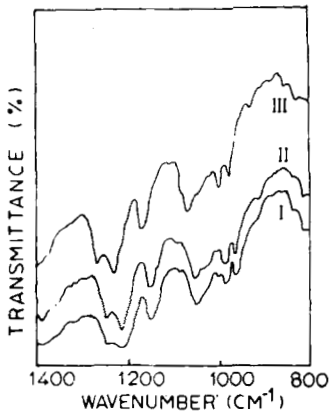

Fig. 5. Infrared spectra for (I) Mulberry silk; (II) Mulberry hydrolysate, 48 hr; and (III) Mulberry hydrolysate, (48 + 48) hr.

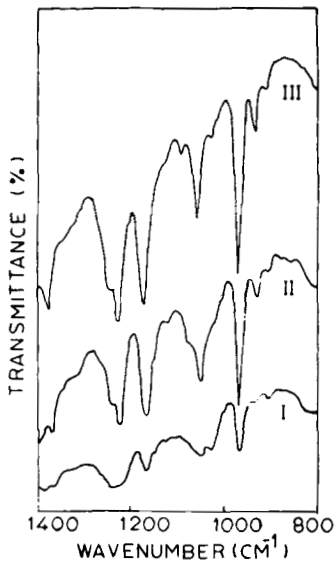

Fig. 6. Infrared spectra for (I) Tasar silk; (II) Tasar hydrolysate, 48 hr; and (III) Tasar hydrolysate, (48 + 48) hr.

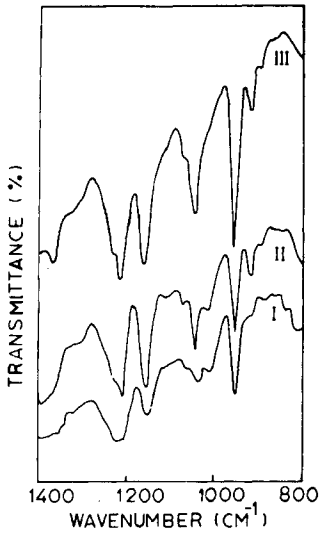

Fig. 7. Infrared spectra for (I) Eri silk; (II) Eri hydrolysate, 48 hr; and (III) Eri hydrolysate, (48 + 48) hr.

The sharpening of the peaks can also be expected because the crystallites are now strain-free and distortions are reduced. However, the large increase in the crystallite size and crystallinity points to the fact that recrystallization is perhaps the main mechanism. However, in Mulberry we do not get sharp peaks even after hydrolysis. This must be due to the fact that the size of the crystallite is very small and also the labile groups such as Ser–Ala and Gly–Ala are within the crystalline region and may get attacked by acid along with the amorphous region. Therefore, though some recrystallization could be possible, the process is competed by dissolution and the crystallite size continues to remain small. Therefore the x-ray crystallinity value does not increase significantly for the hydrolysates.

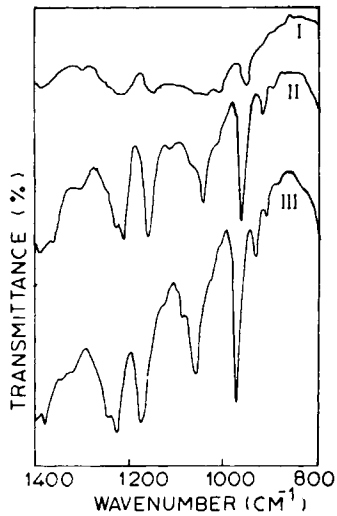

Fig. 8. Infrared spectra for (I) Muga silk; (II) Muga hydrolysate, 48 hr, and (III) Muga hydrolysate, (48 + 48) hr.

However, IR spectroscopy shows improvement in the crystallinity index for Mulberry, the value of which is comparable to the other wild varieties.

Further, the infrared spectra (Figs. 5–8) in the range 1400–800 cm^{-1} shows significant changes owing to the hydrolysis process in relation to amino acid linkage in the crystalline region of the fibroin. The band at 1015 cm^{-1} is characteristic of Gly–Gly linkage and the band at 970 cm^{-1} is characteristic of Ala–Ala linkage. The bands at 998 and 975 cm^{-1} are assigned to Ala–Gly linkages. In Mulberry the bands at 1015 and 970 cm^{-1} are totally absent; also the bands at 975 and 998 cm^{-1} are present and become stronger in the IR spectra of the hydrofibroin of Mulberry. This shows that the crystalline structure of Mulberry must have been formed mostly because of the sequence of glycine and alanine. However, in the case of wild silks, there is a strong band at 970 cm^{-1}, which becomes stronger in their hydrolysates. Further, the band at 1015 cm^{-1} corresponding to Gly–Gly linkage totally disappears in the hydrolysates. These results support our earlier conclusion that nonlabile Ala–Ala linkages mostly form the crystalline portion in the case of Tasar as well as Muga and Eri.

This conclusion is further supported by the estimation of amino acid composition for these hydrolysates.[22] In the case of Mulberry amino acid composition for 48 hr, hydrolysates did not show any significant change. However, the other wild varieties show a high content of alanine (~70%) with traceable glycine and serine. Thus we ascertain that the Mulberry silk is almost a copolymer formed as a result of Gly–Ala, whereas wild silks have Ala–Ala linkage in their crystalline region.

The studies of Zuber[23] and Shaw[24] on various fractions obtained from silk fibroin after chymotryptic and tryptic digestion have revealed that the fibroin from Bombyx mori consists of three phases. The sequence of glycine, alanine, and serine gives rise to phase I which is highly crystalline and gives rise to characteristic x-ray pattern. Phase II is formed mainly as a result of glycine, alanine, valine, and tyrosine which is a mixture of sequence of phase I and other sequences. Phase II, is probably imperfectly ordered and randomly oriented. Phase III consists of the remaining amino acid sequences consisting of mainly polar and high molecular weight residues. Of 100 amino acid residues in fibroin approximately 60 occur in phase I, 30 in phase II, and 9 in phase III. On this basis, we expect the crystallinity to be 60–90%. However, because of environmental constraints of protein material, the ideal situation may not be achieved. The values obtained by infrared spectroscopy and electron diffraction as crystallinity index (0.66) and order factor (0.62), respectively, supplement the idea of phase concept of Zuber[23] and Shaw.[24]

Comparative studies using x-ray diffraction of different varieties of silk made by Warwicker[1] has revealed that those could be classified into five classes. Mulberry has been placed in class 1 and Tasar has been placed in class 3. Further, class 3 has been subdivided into 3a and 3b depending on the intensities of R_2 and R_3 reflections (Figs. 1–4). In this context, Warwicker discussed the disparity of height of peaks R_2 and R_3. Our observations on Tasar, Eri, and Muga showed that the peak height R_2 is less than that of R_3 in control samples and the relative intensities reversed after hydrolysis for Tasar and Eri. However, the intensity reversal was not observed in the case of Muga. Although these three varieties of silk could be placed in Warwicker's group 3a, the different behavior of Muga variety from the other two cannot be properly explained.

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