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COMPOSITION AND MECHANICAL PROPERTIES OF PAKISTANI WHITE AND YELLOW SILK

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Amino-acid composition and mechanical properties of Pakistani white and yellow silk fibroin have been determined, The yellow variety, associated with higher mechanical constants, was rich in alanine, but poor in tyrosine and phenylalanine.

Investigations on composition and mechanical properties of protein fibres, wool and silk, have been of significant assistance in revealing information about their structure. The work on the structure of silk fibroin has been reviewed by Howitt.[1] That the results obtained by the various workers for the amino-acid make up of silk do not totally agree with one another is not surprising in view of both the differences in the techniques of analysis as well as the natural variation associated with most biological products.

Two varieties of silk (Bombyx mori), white and yellow, are grown in West Pakistan. The present study is aimed at a comparison of the mechanical properties of these two varieties vis-a-vis their amino-acid composition.

The desirability of utilizing several solvent systems for paper chromatographic analysis of protein has already been expressed.[2,3,4] An additional aim of the present study is to elucidate a solvent system for such an analysis conducive to obtaining effective separation of the spots and results of acceptable precision.

Materials and Methods

Samples.—Samples of white and yellow mulbery silk cocoons were obtained from the Sericulture Department, Peshawar.

Reeling.—The silk was reeled by steeping the cocoons in a boiling water-bath for 1–2 hr and the free end of the filament was drawn.[5]

Degumming.—The reeled silk was degummed by the soap-alkali process.[6] Each sample was dried at 140°C for one and a half hour before proceeding with subsequent proceedure.

Hydrolysates.—Method adopted was essentially that of Baker and Khan.[7] 250 mg of silk were treated with 5 ml 6N HCl in a pyrex glass test tube for the preparation of the acid-hydrolysate. The tube was sealed and heated at 110°C in an electric oven for 36 hr. The tube was cooled, the seal broken and the hydrolysate evaporated to dryness under vacuum over calcium chloride and sodium hydroxide. The residue was dissolved in distilled water and the solution evaporated to dryness a number of times. This was then dissolved in 2 ml 10% isopropanol and filtered. The residue was thoroughly washed with 10% isopropanol. Finally the pH and the concentration (mg silk/ml) of the hydrolysate were adjusted to the desired value as given in Table 1.

For the preparation of alkali hydrolysate 250 mg of the silk were treated in a glass tube with 1.4g of recrystallised barium hydroxide and 10 ml of distilled water at 120°C for 24 hr. The tube was then cooled and opened and the barium hydroxide neutralized with sulphuric acid. The precipitate of barium sulphate was removed by filtration and was washed four or five times with hot water containing 2 drops of glacial acetic acid per litre. The washings were added to the hydrolysate and the volume reduced to approximately 1 ml by evaporation in a current of hot air. Complete drying was effected in a desiccator over calcium chloride and sodium hydroxide. The dried hydrolysate was dissolved in 5 ml of 10% isopropanol, the pH of the solution was adjusted to 7.0–7.5 and the volume adjusted to 10 ml. Only tryptophane was determined in the alkaline hydrolysate.

Colour Reagents.—Three colour reagents were prepared as described below:

(*i*) *Ninhydrin Reagent No. 1*: five grams of ninhydrin were dissolved in 100 ml n-butanol and 50 ml glacial acetic acid. This solution was added to water-saturated n-butanol. The latter was prepared by mixing 1000 ml of n-butanol with 500 ml of water and shaken in a separatory funnel. The aqueous layer was removed.

(*ii*) *Ninhydrin Reagent No. 2*: For the preparation of this reagent the same method was used as for reagent No. 1, except for the fact that 25 ml of glacial acetic acid were used instead of 50 ml.

TABLE 1.—SOLVENT SYSTEMS AND CONDITIONS EMPLOYED IN THE QUANTITATIVE DETERMINATION OF AMINO-ACIDS.

Solvent system	Composition of solvent	Silk in the hydrolysate solutions mg/ml	pH of hydrolysate	Running time (hr)	Colour developing reagent	Amino-acid determined
A.	96 ml distilled phenol, 4 ml 2-butanol, 100 ml buffer (pH 12)	5,10,25	6.5–7.5	20–24	Ninhydrin reagent No. 1	Aspartic acid, glutamic acid, serine, threonine and alanine
B.	100 ml distilled O-cresol, 100 ml buffer (pH 1.20)	10,25	7.8	24	Ninhydrin reagent No. 1	Histidine
C.	98 ml distilled 2:4 lutidine, 2 ml distilled phenol, 100 ml buffer (pH 6.2)	12.5,25	6.5–7.5	36	Ninhydrin reagent No. 2. Proline developed with reagent No. 3	Lysine, proline, valine and phenylalanine
D.	100 ml distilled 2:4 lutidine, 50 ml water	5,12.5,25	6.5–7.5	24	Ninhydrin reagent No. 2	Arginine, glycine, tyrosine
E.	98 ml distilled 2:4:6 collidine, 2 ml distilled phenol, 100 ml buffer (pH 9.0)	25	7.0–7.5	24	Ninhydrin reagent No. 1	Tryptophane
F.	50 ml n-butanol, 30 ml methyl ethyl ketone, 10 ml water, 10 ml ammonia	10,25	6.5–7.5	2 runs	Ninhydrin reagent No. 2	Leucine, isoleucine

(*iii*) *Isatin Reagent*: 4.0 g of isatin were dissolved in 1000 ml of 95% ethanol containing 40 ml of glacial acetic acid.

Standard Amino Acid Solutions.—Six stock solutions of amino-acids of the following composition were prepared.

No. 1: Aspartic acid, glutamic acid, serine, threoninae and alanine; No. 2: Histidine; No. 3: Lysine, proline, valine and phenylalanine; No. 4: Arginine, glycine, tyrosine; No. 5: Tryptophane; No. 6: Leucine and isoleucine.

All stock amino-acid standard solutions of 14m strength were made, with the exception of that of tyrosine, by dissolving the amino-acids in 10% isopropanol. Tyrosine was prepared by dissolving first in a little quantity of N HCl. Then dilutions of 4mM, 6mM, 8mM, 10mM, 12mM of all the solutions were made with 10% isopropanol to yield the corresponding standard solutions.

Solvent Systems.—The composition of the various solvent systems used in this investigation is given in Table 1. Some of these solvents were used in conjunction with the buffer solutions of pH 6.2, 9 and 12.[2] These solvent systems were prepared by shaking the organic solvent with the appropriate buffer in a separatory funnel.

The mixture was kept overnight and then the aqueous layer which separated from the organic solvent was removed. In the case of solvents D and F, however, a homogenous mixture was obtained.

Analysis.—The chamber manufactured by Shandon Co. London was used for chromatography. The chamber was scrupulously cleaned twice with hot 1% surf-sodium carbonate solution and then repeatedly with warm water in order to remove traces of any of the previous solvents. Whatman No. 1 filter sheets were employed. These were cut into strips (11 × 22 in) and dipped in a buffer solution of the same pH as the solvent in which they were to be used, and finally dried at room temperature. The papers were marked by a line drawn approximately $4\frac{1}{2}$ in from one end of the 11in side. The dots 1 to 8 in number were marked along this line at intervals of 1 in. The hydrolysate solutions of white and yellow silk fibroin were placed on two separate dots and the standard amino-acid solutions of six different concentrations varying from 4mM to 14mM were applied on the remaining dots. The solutions were applied to the paper in 5μl quantities. The spots were allowed to dry in air and the papers were then placed for descending chromartography in the chamber containing the solvent in the trough. The time intervals required for the

development of the chromatograms are given in Table 1.

At the end of the development, the chromatograms were removed and air-dried. These chromatograms were then dipped in the appropriate colour reagent (Table 1) and dried at 60°C for 15 min.

For the estimation of proline, however, the chromatogram after dipping in the isatin reagent was placed in an oven at 100°C for 5 min.

The rows of amino-acid spots were cut out in strips and dipped in 5 ml 75% ethanol in test tubes for half an hour containing 0.2 mg copper sulphate. The colour in the tubes was measured employing the Langels photoelectric cholorimeter. A blank was prepared in the usual manner and the zero reading adjusted.

Standard curves were plotted for the absorbancy against the molarity of the standard solutions of the amino-acids. The percentages of amino-acids were then calculted with reference to these standard curves.

Mechanical Properties.—The tensile characteristics of single silk fibres obtained from different cocoons were determined at 65% r.h. and 21°C, employing Schopper dynamometer. A pretention of 200 mg was used and the experiment was carried out so that the fibre broke within 20 seconds. The diameter of the fibres was evaluated employing a Lanameter at ×500 magnification and recording about 100 readings over a length of about 6 cm of each fibre.

Results and Discussion

The results of the amino-acid analysis are summarised in Table 2. Duplicate hydrolysates of each sample were analysed and in each analysis four determinations of each amino-acid were made. Thus each result in Table 2 represents an average of 8 determinations. It may be pointed out that the duplicate sets of data for the same sample were not found to differ significantly with one another so that it seemed justified to pool all the 8 values in respect of each of the samples.

The significance of the differences in the mean ressults in the case of each amino-acid was tested by the 't' test. The two silk samples were found to differ significantly (Table 2) in the percentages of alanine, phenylalanine and tyrosine at the 1% level, and isoleucine, proline, thereoninae and tryptophane at the 5% level.

The incorporation of 2-butanol to phenol solvents was found to improve the separation of aspartic acid, glutamic acid, serine, threnine and alanine and led to the fromation of distinct and compact round spots. Likewise, the addition of

TABLE 2.—AMINO-ACID COMPOSITION OF SILK (g. OF AMINO-ACID PER 100 g OF SILK)

Sr. No.	Amino acid	White silk	Yellow silk	Values reported in literature	
				Howitt[8]	Howitt
1.	Alanine	25.41	27.31**	26.4	33.00
2.	Arginine	1.11	1.31	1.05	1.00
3.	Aspartic acid	1.38	1.54	2.0	2.75
4.	Glutamic acid	1.13	1.16	2.03	2.15
5.	Glycine	43.45	41.18	43.8	41.2
6.	Histidine	2.75	2.92	0.47	0.37
7.	Isoleucine	1.22	0.94*	—	1.1
8.	Leucine	0.84	0.73	0.8	0.9
9.	Lysine	0.70	0.79	0.88	0.54
10.	Phenyl alanine	5.04	3.37**	1.50	3.36
11.	Proline	0.95	1.18*	1.50	0.7
12.	Serine	12.40	11.65	12.6	16.2
13.	Threonine	1.00	1.20*	1.50	1.57
14.	Tryptophane	0.53	0.81*	—	0.65
15.	Tyrosine	9.24	7.93**	10.6	11.4
16.	Valine	1.23	1.44	3.20	3.6
	Total	108.38	105.46	108.33	120.49

* t value significant at the 5% level; ** t value significant at the 1% level.

phenol to lutidine solvent gave a good separation of lysine, proline, valine and phenylalanine. Tryptophane was more clearly separated when a small amount of phenol was added to the corresponding solvent mixture.

The results of the mechanical tests have been summarised in Table 3. The 2 samples had

TABLE 3.—MEAN VALUES OF MECHANICAL CONSTANTS.

Sample	No. of fibres	Diameter u	Breaking force (g. wt.)	Tensile strength (P.S.I.)
White silk	20	13.0	4.5	48403
Yellow silk	20	12.8	5.1	55395

practically the same diameter but the yellow variety was found to be associated with higher mechanical constants. The tensile strength of this variety was significantly higher than that of the other at the 1% level. Table 2 reveals, on other hand, that the yellow variety is significantly richer (at the 1% level) in the amino-acid alanine, but poorer in tyrosine and phenylalanine. According to the present conception of the structure of silk fibroin,[1] amino-acid residues with bulky side chains cause disalignment of the protein chains and thus give rise to amorphous regions. It follows that the preponderance of amino-acids with bulky side chains would eventually result in lower mechanical constants. The results of the present study are in confirmation of this picture as the white variety richer in tyrosine and phenyalanine exhibited lower mechanical constants.

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