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Isolation of the Smallest Component of Silk Protein

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Silk proteins were solubilized from cocoons with ethylenediamine/cupric hydroxide solution. A series of polymers of the smallest component, detected by polyacrylamide-gel electrophoresis, could be converted into the smallest component by reduction and aminoethylation. Fibroin and sericin fractions were separated by precipitation of sericin at pH 5.5. On gel electrophoresis, sericin showed distinct bands but fibroin did not. The components of fibroin and sericin were fractionated by gel filtration on Sepharose 6B. The smallest component in the sericin fraction was purified by rechromatography and showed a single band on gel electrophoresis. Its mol.wt. was 24000, and its amino acid composition was determined.

In the study of silk proteins the method of separating soluble proteins and insoluble fibres by boiling water or hot 1% Na_2CO_3 solution has usually been used; the soluble protein was defined as 'sericin' and insoluble fibre as 'fibroin' (Dunn *et al.*, 1944; Braunitzer & Wolff, 1955). However, silk proteins seem to be denatured by boiling for a long time, since fibroin and sericin prepared by these methods showed no distinct bands on polyacrylamide-gel electrophoresis (Tokutake & Okuyama, 1972).

In a previous paper it was shown that silk-protein solution that was prepared by solubilization of a whole cocoon with ED/Cu solution showed distinct bands on polyacrylamide-gel electrophoresis in the presence and absence of SDS, and that there exists a series of polymers. These polymers were reduced by 2-mercaptoethanol to the smallest component of silk protein, which had an apparent mol.wt. of 24000 (Tokutake & Okuyama, 1972). This component is noteworthy because it appears to contain cysteine residues.

In the present study, characterization of fibroin and sericin by more up-to-date methods of protein chemistry, and fractionation of silk protein components were attempted, with particular attention being paid to the isolation of the smallest component.

Experimental

Solubilization of cocoons

The cocoons of *Bombyx mori* (silkworm) of mixed strain Shungetsu × Hōshō were used in this experiment. Pupae in cocoons were removed before they became moths, and cocoons were stored in a desiccator over silica gel.

0.13 M-Ethylenediamine/0.06 M-cupric hydroxide (ED/Cu) solution was prepared as described by Coleman & Howitt (1947). The colour of the solution was dark blue, and the pH was 13.6 (equivalent to that of a 1 M-NaOH solution).

Cocoons (10 g) were cut in pieces 5 mm × 5 mm in size and put into a 1-litre beaker. ED/Cu solution (400 ml) was added and stirred with magnetic stirrer for 15–30 min at room temperature. When cocoons were dissolved completely, 50 ml of 50% (v/v) acetic acid was added to neutralize the solution; it was dialysed for 1–2 days in Visking tubing against water to remove ED/Cu solution. The silk-protein solution thus obtained was almost transparent, but was coloured slightly violet with ED/Cu solution, which bound to silk protein.

Reduction and aminoethylation of cysteine residues

Cysteine residues of the components that were produced by polymerization of the smallest component were reduced by 2-mercaptoethanol and aminoethylated by ethyleneimine. Reduction and aminoethylation was carried out with 6 M-urea/0.8 M-Tris/1 mM-EDTA, pH 8.6, in a volume of 1 litre as described by Cole (1967). Reduction was performed in the presence of 0.13 M-2-mercaptoethanol, the mixture being stirred for 3 h under a stream of N_2. Aminoethylation was performed by addition of 0.9 M-ethyleneimine, the mixture again being stirred under N_2 for 1 h.

When aminoethylation was complete, the solution

Abbreviations used: ED/Cu solution, ethylenediamine/cupric hydroxide solution; SDS, sodium dodecyl sulphate.

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was dialysed against water for 3 days to remove urea and reagents used for aminoethylation. With the removal of urea by dialysis, a white precipitate was gradually produced.

Fractionation by precipitation at pH 5.5

When urea and reagents were removed completely, the pH of the dialysis residue was adjusted to 5.5 with 1 M-acetic acid and left for 2–3 h. The precipitate deposited at the bottom of the beaker was collected by centrifugation (3000 *g*, 15 min) after decantation of a large portion of the supernatant. The supernatant was combined with decanted solution, freeze-dried, and is referred to as the 'supernatant fraction'. The precipitate was dissolved in 100 ml of 1% Na_2CO_3/8 M-urea solution with stirring for 30 min. The small amount of undissolved material was removed by centrifugation. The supernatant was neutralized to pH 8 with acetic acid and dialysed against water for 3 days. The dialysis residue was freeze-dried and referred to as the 'precipitate fraction'.

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis was performed as described previously (Tokutake & Okuyama, 1972). In the absence of SDS, the method of Ornstein (1964) and Davis (1964) was modified to contain urea [6 M-urea (pH 9.4)/7.5% polyacrylamide]. In the presence of SDS, the method of Dunker & Ruekert (1969) was modified also to contain urea [0.1% SDS/6 M-urea (pH 7.2)/5% polyacrylamide]. The conditions of electrophoresis, staining and destaining were described previously (Tokutake & Okuyama, 1972).

Amino acid analysis

Samples for amino acid analysis were hydrolysed in redistilled HCl (5.7 M) at 105°C for 24 h. Amino acid analysis was performed on a Hitachi KLA-3B amino acid analyser.

Results

Polyacrylamide-gel electrophoresis of whole silk protein

Fig. 1 shows the stained gel of whole silk protein subjected to electrophoresis on 6 M-urea (pH 9.4)/7.5% polyacrylamide gel. Broad background staining was observed in the low-mobility region. Fig. 2(*a*) shows the gel of whole silk protein on 0.1% SDS/6 M-urea (pH 7.2)/5% polyacrylamide gel. Fig. 2(*b*) is the gel of the sample reduced by 2-mercaptoethanol. Schematic patterns and the molecular weights of the bands were published previously (Tokutake & Okuyama, 1972).

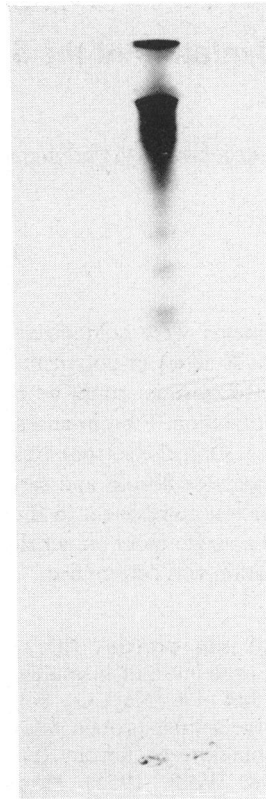

Fig. 1. *Electrophoretic pattern of whole components of silk protein by 6 M-urea (pH 9.4)/7.5% polyacrylamide-gel electrophoresis*

A 50 μl sample of silk-protein solution in 8 M-urea was applied to the gel and electrophoresis was carried out at 3 mA/tube (5 mm × 70 mm) for 3 h. The gel was stained with 1% Amido Black 10B solution in 7% acetic acid, and excess dye was removed by destaining with 7% acetic acid.

Separation of fibroin and sericin by precipitation at pH 5.5

The recoveries of the supernatant and precipitate fractions were calculated from the weight of freeze-dried samples to be 60 and 24% respectively. Both fractions were examined by 0.1% SDS/6 M-urea (pH 7.2)/5% polyacrylamide-gel electrophoresis. Urea (0.5 g) and SDS (10 mg) were dissolved in 0.5 ml portions of both fractions before freeze-drying and subjected to electrophoresis. Distinct bands that were detected in the whole-silk-protein gel were detected in the precipitate-fraction gel. In the supernatant-fraction gel, a broad background staining was observed in the low-mobility region. The smallest component was contained in the precipitate fraction.

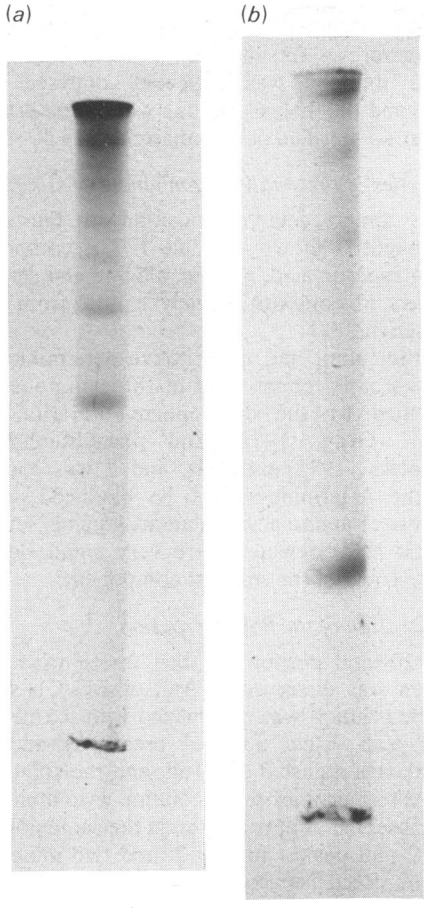

Fig. 2. *Electrophoretic patterns of whole components of silk protein in the absence (a) and presence (b) of 2-mercaptoethanol by 0.1% SDS/6 M-urea (pH 7.2)/5% polyacrylamide-gel electrophoresis*

To 1 ml of silk-protein solution in 8 M-urea, 20 mg of SDS and (for *b*) 80 μl of 2-mercaptoethanol were added and incubated at 36°C for 30 min. A 50 μl portion was applied to a gel. Electrophoresis was carried out at 5 mA/tube for 15 min and subsequently at 8 mA for 75 min. The bands that had fastest mobility in both gels ran to the same position when two gels were subjected to parallel electrophoresis.

Table 1. *Amino acid composition of the supernatant fraction, the precipitate fraction and the smallest component of silk protein*

Contents are expressed as mol% (result for tryptophan not shown). Values for threonine and serine were corrected by assuming that 5% of the threonine and 10% of serine content is destroyed during hydrolysis.

	Supernatant fraction (fibroin)	Precipitate fraction (sericin)	Smallest component
Aspartic acid	1.68	12.9	15.2
Threonine	1.17	5.21	3.66
Serine	9.48	18.9	12.0
Glutamic acid	0.96	4.25	7.50
Proline	0.00	0.69	2.49
Glycine	46.4	24.2	14.7
Alanine	31.6	15.2	13.2
Valine	2.04	3.34	5.54
Methionine	0.00	0.11	0.00
Isoleucine	0.28	1.82	5.50
Leucine	0.22	1.99	5.45
Tyrosine	4.98	4.10	3.70
Phenylalanine	0.00	0.69	2.46
Lysine	0.58	2.07	2.03
Histidine	0.08	0.98	1.45
Arginine	0.29	3.02	3.76
Aminoethylcysteine	0.00	Trace	0.55

The amino acid compositions of two fractions are shown in Table 1. The supernatant fraction was particularly rich in glycine and alanine, which together accounted for 78 mol%. On the other hand, the precipitate fraction was rich in glycine, serine, alanine and aspartic acid, the combined contents of which amounted to 72 mol%. It is thus assumed that fibroin and sericin were separated by precipitation at pH 5.5. The supernatant fraction was therefore later termed the 'fibroin fraction' and the precipitate the 'sericin fraction'.

The results of electrophoresis suggest that components of sericin have definite molecular weights but those of fibroin do not.

Gel filtration of the sericin fraction

A 500 mg sample of freeze-dried sericin fraction was dissolved in 20 ml of 8 M-urea/1% Na_2CO_3 solution. The small amount of undissolved material was removed by centrifugation. The supernatant was neutralized with 50% (v/v) acetic acid to pH 8, and applied to a column (4.5 cm × 75 cm) of Sepharose 6B, which was eluted with 20 mM-Tris/0.1 M-NaCl/6 M-urea, pH 9.0; 10 ml fractions were collected. The elution profile is shown in Fig. 3. Six peaks were observed. The protein components of each peak were examined by 0.1% SDS/6 M-urea (pH 7.2)/5% polyacrylamide-gel electrophoresis. To 1 ml of each peak fraction, 0.1 g of urea and 10 mg of SDS were added, and a 25–50 μl sample was run. The electrophoretic patterns are shown in Fig. 4. In peak 1, only the top of the gel was stained. From peak 6, no bands were detected on the gel. Peak 5 was the smallest component of silk protein.

Peak-5 material was purified by rechromatography. The peak-5 fraction was collected as

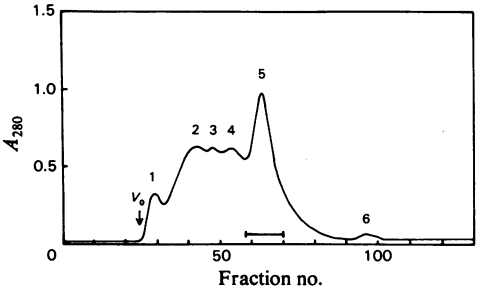

Fig. 3. *Gel filtration of sericin (precipitate fraction) on Sepharose 6B*

A 500mg sample of freeze-dried sericin (precipitate fraction) was dissolved in 20ml of 8M-urea/1% Na_2CO_3 solution. The small amount of undissolved material was removed by centrifugation. The supernatant was neutralized with 50% (v/v) acetic acid to pH8, and applied to a column (4.5cm × 75cm) of Sepharose 6B. The column was eluted with 20mM-Tris/0.1M-NaCl/6M-urea, pH9.0, and 10ml fractions were collected.

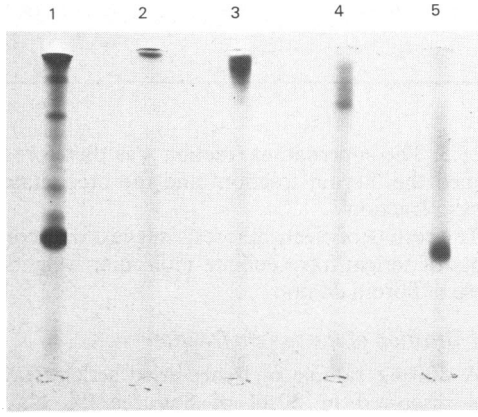

Fig. 4. *Electrophoretic patterns of components of each peak in Fig. 3 by SDS/urea/polyacrylamide-gel electrophoresis*

1, Sericin (precipitate fraction); 2, peak 2; 3, peak 3; 4, peak 4; 5, peak 5. To 1.0ml of each peak fraction, 0.1g of urea and 10mg of SDS were added, and a 25–50μl sample was applied to the gel. The conditions of electrophoresis and staining of the gels were described in Fig. 2.

indicated by the horizontal bar in Fig. 3 and dialysed against water until urea and reagents were removed completely. The dialysis residue was freeze-dried (yield 80mg). It was dissolved in 10ml of 8M-urea solution and applied to the same column. The elution profile was almost symmetrical, and showed a single band on SDS/urea/polyacrylamide-gel electrophoresis (results not shown). The purified peak-5 material was collected, dialysed against water, and the dialysis residue was freeze-dried. The recovery of purified peak-5 material was 40–60mg.

Properties of the smallest component of silk protein

The amino acid composition of the smallest component is shown in Table 1. The component is rich in aspartic acid, glycine, alanine and serine, but it differs in composition only a little from that of total sericin.

Identification and quantitative determinations of the *N*-terminal amino acid of this component were performed by the dinitrophenyl (Narita, 1970), dansyl (Gray, 1972) and phenylthiohydantoin (Schroeder, 1972) methods, and it was considered that the *N*-terminus must be blocked, since the amounts of amino acids (alanine, glycine, serine and aspartic acid) detected were very small (less than 0.09mol/mol of the smallest component).

Gel filtration of the fibroin fraction

A 200mg portion of the freeze-dried fibroin fraction was dissolved in 4ml of ED/Cu solution, and the solution was neutralized with 0.5ml of 50% acetic acid. Then 3.6g of urea was added, the volume was adjusted to 10ml, and the solution was applied to a Sepharose 6B column. Two major peaks were observed at approximately the same position as peak 2 and peak 4 in Fig. 3, and two minor peaks were observed between the major peak. Separation of peaks was poor, and the peaks were broad. Electrophoretic patterns of major-peak components on SDS/urea/polyacrylamide gels showed no distinct band, but the tops of the gels were broadly stained (results not shown).

Discussion

In the present study, fibroin and sericin appeared to be separated by precipitation at pH5.5. The amino acid composition of fibroin fraction (Table 1) was compatible with previously reported results (Schroeder & Kay, 1955; Lucas *et al.*, 1958). However, the content of serine in the sericin fraction and the smallest component was lower than the reported value (Lucas & Rudall, 1968). The difference between results may be due to the difference in preparation methods. It is noteworthy that sericin showed distinct bands on polyacrylamide-gel electrophoresis, whereas fibroin did not. These results suggest that components of sericin, but not fibroin, have definite molecular weights.

Ohara (1933) showed by polarization microscopy that sericin forms three layers surrounding a fibroin fibre, and Shimizu (1941) fractionated sericin into

three components according to their solubility in boiling water and hot 40% urea solution. In the present study, sericin was fractionated into at least four components.

The method of solubilizing sericin from a cocoon at room temperature was investigated. The efficiency of urea, SDS, Na_2CO_3, 2-mercaptoethanol and *p*-hydroxybenzoic acid was examined, and it was observed that an 8M-urea/1% SDS/1% 2-mercaptoethanol solution separated well the soluble protein and insoluble fibre when a cocoon was immersed in this solution overnight. The yields of soluble protein and insoluble fibre was 19 and 81% respectively. The yield of soluble protein was less than that obtained by the boiling-water method (about 30%). On polyacrylamide-gel electrophoresis, distinct bands were detected in the soluble fraction.

ED/Cu solution is strongly alkaline. Nevertheless, it is unlikely that silk protein is denatured by this solution, since liquid silk-protein solution obtained from silkworm-silk gland 1 or 2 days before they make their cocoons showed an electrophoretic pattern similar to that shown in Fig. 1 (result not shown).

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References

Braunitzer, G. & Wolff, D. (1955) *Z. Naturforsch. Teil B* **10**, 404–408
Cole, R. (1967) *Methods Enzymol.* **11**, 315–317
Coleman, D. & Howitt, F. O. (1947) *Proc. R. Soc. London Ser. A* **190**, 145–169
Davis, B. J. (1964) *Ann. N. Y. Acad. Sci.* **121**, 404–427
Dunker, A. K. & Ruekert, R. R. (1969) *J. Biol. Chem.* **244**, 5074–5079
Dunn, M. S., Camien, M. N., Rockland, L. S., Shankman, S. & Goldberg, S. C. (1944) *J. Biol. Chem.* **155**, 591–603
Gray, W. R. (1972) *Methods Enzymol.* **25**, 121–138
Lucas, F. & Rudall, K. M. (1968) *Comp. Biochem.* **26 B**, 475–558
Lucas, F., Shaw, J. T. B. & Smith, S. G. (1958) *Adv. Protein Chem.* **13**, 107–242
Narita, K. (1970) *Mol. Biol. Biochem. Biophys.* **8**, 27–38
Ohara, K. (1933) *Sci. Pap. Inst. Phys. Chem. Res. (Jpn.)* **21**, 104–127
Ornstein, L. (1964) *Ann. N. Y. Acad. Sci.* **121**, 321–349
Schroeder, W. A. (1972) *Methods Enzymol.* **25**, 298–313
Schroeder, W. A. & Kay, L. M. (1955) *J. Am. Chem. Soc.* **77**, 3908–3913
Shimizu, M. (1941) *Sanshi Shikenjo Hōkoku (Bull. Seric. Exp. Stn. Tokyo)* **10**, 441–470
Tokutake, S. & Okuyama, T. (1972) *J. Biochem. (Tokyo)* **71**, 737–741