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Crosslinking of Silk Fibroin by Aqueous Peroxydisulfate

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Synopsis

Silk fibroin is crosslinked by 0.1–1.0*M* ammonium peroxydisulfate at 70°C. or at 40°C. in the presence of silver ion. Induced decomposition of peroxydisulfate in the presence of fibroin is observed. Methylation of tyrosine in fibroin does not slow attack by peroxydisulfate, but crosslinking of the fibroin is prevented. Modification of tryptophan with α-bromo-5-nitro-2-cresol does not prevent crosslinking by peroxydisulfate, however. The nature and sites of crosslinking in fibroin are suggested in light of physical and chemical data, amino acid analyses of crosslinked and control fibroins, and the known mode of decomposition of peroxydisulfate.

INTRODUCTION

Silk fibroin is rendered insoluble in solvents in which it is normally soluble by common crosslinking agents such as formaldehyde,[1] 1,3-difluoro-4,6-dinitrobenzene,[2] and bis(4-fluoro-3-nitrophenyl)sulfone.[3] Recently, Earland and Stell[4] found that fibroin is also crosslinked by reaction with aqueous solutions of simple oxidizing agents such as chlorine, potassium permanganate, chlorine dioxide, sodium hypochlorite, and iodine at room temperature. Earland and Stell[4] presented evidence that oxidation of tyrosine residues in the fibroin result in reactive intermediates that crosslink with other reactive groups in the protein.

In recent studies in this laboratory on the crosslinking of gelatin by aqueous peroxydisulfate,[5] tyrosine appeared to be one of the amino acids forming crosslinks in the gelatin. Since silk fibroin has a high tyrosine content (approximately 10%) and crosslinks in the presence of oxidizing agents,[4] the action of aqueous ammonium peroxydisulfate on fibroin and modified fibroins was studied at 70°C. to determine if crosslinking would take place. The present results show that fibroin is crosslinked by aqueous peroxydisulfate to yield fibers that are partly insoluble in common solvents for fibroin such as 50% aqueous lithium bromide,[7,8] 85% phosphoric acid,[4] and 90% formic acid containing 10% calcium chloride.[4] The observed crosslinking has been interpreted both in light of the known mode of decomposition of peroxydisulfate and of the chemical and physical changes in fibroins and modified fibroins upon treatment with peroxydisulfate.

EXPERIMENTAL

Fibroins and Reagents

Silk fibroin (*Bombyx mori*) was obtained from General Biochemicals, Inc. The fibroin was extracted with ethanol for 8 hr. and then ether for 8 hr. in a Soxhlet apparatus, washed thoroughly with distilled water, dried at 95°C., and finally dried at 1 mm. over sodium hydroxide prior to use.

Methylation of tyrosine in fibroin was by the method of Gordon et al.[9] using dimethylsulfate–aqueous sodium hydroxide and by the method of Rutherford et al.[10] using diazomethane in ether. Tryptophan was modified in fibroin with α-bromo-5-nitro-2-cresol by the procedure of Bewley and Li.[11] All inorganic reagents were Baker analyzed, and organic reagents were Eastman white label grade. Analyses for the above fibroins are found in Table I.

TABLE I
Analyses of Fibroins

Amino acid modification	$(NH_4)_2S_2O_8$ concn., M[a]	Total N, %	Amide N, %	Van Slyke N, %	Total S, %	Methoxyl, %	Tryptophan, %
None	—	18.1	0.26	0.13	0.13	0.0	0.63
None	0.1	18.2	0.33	0.16	0.33	—	0.06
None	0.1[b]	17.7	0.37	0.15	0.37	—	0.17
None	1.0	17.8	0.28	0.27	0.28	—	0.04
Tyrosine $[(CH_3)_2SO_4]$	—	17.7	0.25	0.04	0.06	1.67	0.61
"	0.1	17.4	0.44	0.07	0.21	1.62	0.06
Tyrosine $[CH_2N_2]$	—	17.9	0.49	0.10	0.09	2.10	0.60
"	0.1	17.5	0.38	0.13	0.30	1.90	0.04
Tryptophan	—	17.6	0.21	0.11	0.10	—	0.23
"	0.1	17.8	0.31	0.11	0.22	—	0.04

[a] For 4 hr. at 70.0 ± 0.1°C.
[b] Also 0.006M $AgNO_3$, 2 hr. at 40.0 ± 0.1°C.

Reaction of Ammonium Peroxydisulfate with Fibroins

A 5.0 g. sample of fibroin was immersed in 100 ml. of 0.1 or 1.0M ammonium peroxydisulfate in a 125-ml. Erlenmeyer flask capped with a rubber septum containing a nitrogen inlet and outlet. Nitrogen was bubbled through the solution for 30 min., and nitrogen flow continued throughout the reaction. The reaction flask was placed in a constant temperature bath at 70.0 ± 0.1°C. for 4 hr. The resulting fibroin was washed free of peroxydisulfate with distilled water and dried at 1 mm. over sodium hydroxide. In one experiment, a 0.1M ammonium peroxydisulfate–0.006M silver nitrate solution was reacted with fibroin for 2 hr. at 40.0 ± 0.1°C.

Aliquots were removed from the solutions periodically, and the peroxydisulfate concentration was determined iodometrically by the method of

TABLE II
Reaction of Fibroins with Ammonium Peroxydisulfate[a]

Amino acid modification	$(NH_4)_2S_2O_8$ concn., M	S_2O_8 = reacted, mmole/g. of fibroin	Fibroin insoluble in 50% LiBr, %[b]	Solution	
				η_{inh}, dl./g.[c]	Concn., g./dl.
None	—	—	0.0	0.417	0.500
None	0.1	0.614	54	0.539	0.220
None	0.1[d]	0.314	66	0.730	0.170
None	1.0	4.80[e]	17	0.275	0.415
Tyrosine [$(CH_3)_2SO_4$]	—	—	0.0	0.342	0.500
"	0.1	0.733	26	0.242	0.372
Tyrosine [CH_2N_2]	—	—	Trace	0.228	0.500
"	0.1	0.830	Trace	0.192	0.500
Tryptophan	—	—	6.4	0.192	0.487
"	0.1	0.708	67	0.230	0.164

[a] For 4 hr. at 70.0 ± 0.1°C.
[b] For 20 hr. at 25°C.
[c] At 30.0 ± 0.1°C.
[d] Also 0.006M $AgNO_3$, 2 hr. at 40.0 ± 0.1°C.
[e] Carbon dioxide evolved (0.09 g./g. of fibroin), no CO_2 evolution observed on other reactions.

TABLE III
Amino Acid Composition of Fibroins

Amino acid	Amino acid, mole/10^5 g. protein					
	Control	0.1M $(NH_4)_2S_2O_8$, 4 hr., 70°C.	0.1 M $(NH_4)_2S_2O_8$ + 0.006M $AgNO_3$, 2 hr., 40°C.	1.0M $(NH_4)_2S_2O_8$, 4 hr., 70°C.	Methylated (CH_2N_2)	Methylated (CH_2N_2), 0.1M $(NH_4)_2S_2O_8$, 4 hr., 70°C.
Lysine	4.2	2.7	4.3	2.6	1.5	1.1
Histidine	2.0	1.5	1.0	1.4	0.9	0.6
Arginine	4.9	3.8	4.9	3.4	5.0	2.7
Aspartic acid	20.3	19.2	20.6	13.9	21.2	20.7
Threonine	10.6	10.4	10.2	7.3	12.3	12.5
Serine	123.7	120.3	121.1	92.0	131.3	135.3
Glutamic acid	14.6	14.2	14.4	10.1	15.4	18.0
Proline	6.2	5.0	4.9	2.5	6.4	5.1
Glycine	504.1	480.1	519.9	309.2	470.5	455.8
Alanine	366.7	349.8	363.5	238.1	350.9	355.2
Half cysteine	0.0	0.0	0.0	0.0	0.0	0.0
Valine	27.7	26.2	26.0	19.0	28.7	28.1
Methionine	0.3	0.0	0.0	0.0	0.6	0.0
Isoleucine	7.8	7.1	7.8	5.5	8.4	8.1
Leucine	5.8	5.7	5.6	4.3	6.6	6.6
Tyrosine	56.3	50.9	54.7	29.3	56.0	43.6
Phenylalanine	7.8	8.5	8.4	5.9	9.0	8.9
Tryptophan	3.1	0.3	0.8	0.2	2.9	0.2

Bartlett and Cotman.[12] The difference between the concentrations of peroxydisulfate in the presence and absence of fibroin at completion of reaction was taken as the concentration of peroxydisulfate that reacted with the fibroin. The peroxydisulfate consumed is presented in Table II in millimoles of peroxydisulfate reacted per gram of fibroin. Chemical and amino acid analyses of these treated fibroins are found in Tables I and III.

Solubility of Fibroins in 50% (w/w) Lithium Bromide and Other Solvents

Fibroin samples (0.250 g.) were immersed in 50.0 ml. of 50% (w/w) lithium bromide at room temperatures for 20 hr. Undissolved fibroin was removed by filtration, washed with distilled water, dried, and weighed. Viscosities of the resulting lithium bromide solutions were measured at 30.0 ± 0.1°C. in a Cannon-Fenske viscometer (size 100). Fibroin samples (0.250 g.) also were immersed in 50.0 ml. of 85% phosphoric acid and 50.0 ml. of 90% formic acid in which 5.0 g. of calcium chloride had been dissolved. The solutions were observed periodically to determine when the fibroins had dissolved.

Analytical Methods

All chemical analyses were performed at this laboratory by standard techniques (Table I). Dried fibroin samples weighing 40 mg. were prepared for amino acid analysis by heating for 22 hr. at 120°C. in 10 ml. of 6*N* hydrochloric acid. After removal of hydrochloric acid by flash evaporation, the hydrolyzates were analyzed on a Beckman Spinco amino acid analyzer, Model 120 (Table III). Tryptophan was determined by the method of Graham and Statham.[13] Fibroin samples immersed in 50% lithium bromide were observed at time intervals under a Leitz Dialux-pol polarizing microscope.

RESULTS AND DISCUSSION

Mode of Decomposition of Peroxydisulfate

Peroxydisulfate is known to yield sulfate and hydroxyl radicals on thermal decomposition in aqueous solutions.[14] In the presence of an oxidizable substrate such as protein side chains, induced free-radical decomposition occurs.[14] The reaction of peroxydisulfate with fibroin is thought to occur via such an induced free-radical attack forming reactive groups in the fibroin that are capable of crosslinking.

Chemical Analyses of Fibroin and Modified Fibroins

Chemical analyses for fibroin and modified fibroins are found in Table I. The methoxyl content of fibroin modified by dimethyl sulfate in aqueous sodium hydroxide[9] is nearly equal to the tyrosine residues in the fibroin:

however, treatment of fibroin with diazomethane in ether[10] results in a somewhat higher methoxyl content due to reaction of diazomethane with other reactive groups. Rutherford et al.[10] have shown that reaction of diazomethane with free carboxyl groups in fibroin probably accounts for the higher methoxyl content. Comparison of tryptophan analyses of fibroin treated with α-bromo-5-nitro-2-cresol[11] and control fibroin shows that approximately 65% of the tryptophan has been modified.

Reaction of Fibroin with Ammonium Peroxydisulfate

Induced decomposition of ammonium peroxydisulfate is observed in the presence of fibroin (Table II), and the resulting fibroin is crosslinked. The amount of peroxydisulfate reacting with fibroin is estimated as the difference between the concentrations of peroxydisulfate at reaction completion in the presence and absence of fibroin. This represents the minimum reacted, since noninduced decomposition and subsequent attack of the fibroin by the resultant radicals is also possible. In 0.1*M* ammonium peroxydisulfate, fibroin reacts with 0.614 mmole of peroxydisulfate per gram of fibroin in 4 hr. at 70.0 ± 0.1°C. The resulting fibroin is partially (54%) insoluble in 50% lithium bromide. The soluble portion of the fibroin is crosslinked to some degree, since its inherent viscosity in lithium bromide is greater than that of control.

Fibroin in 1.0*M* peroxydisulfate (4 hr., 70°C.) reacts with 4.80 mmole of peroxydisulfate per gram of fibroin. Degradation of the fibroin, as evidenced by evolution of 0.09 g. of carbon dioxide per gram of fibroin from solution, is noted. Also, only 17% of the resulting fibroin is lithium bromide insoluble, and the viscosity of the resulting fibroin solution is less than that of control solutions. Apparently degradation as well as crosslinking takes place in the fibroin with degradation predominating with high concentrations of peroxydisulfate.

Fibroin reacts with 0.1*M* peroxydisulfate in the presence of 0.006*M* silver nitrate, a known catalyst for peroxydisulfate decomposition,[14] under milder conditions (2 hr., 40°C.) and yields a highly crosslinked fibroin, although only 0.314 mmole of peroxydisulfate per gram of fibroin reacts. The higher degree of crosslinking of this fibroin coupled with its lower consumption of peroxydisulfate compared with fibroin from the uncatalyzed reaction is possibly explained by the milder conditions of silver-catalyzed reaction, which gives less opportunity for degradation.

Fibroins in which tyrosine and tryptophan have been modified react somewhat more rapidly with peroxydisulfate possibly because these modifications open up the fiber structure making the fibroin more accessible to peroxydisulfate attack. Although modification of tryptophan and tyrosine residues in fibroin apparently does not slow the attack of peroxydisulfate on these residues, the capacity for crosslinking of these fibroins is changed.

Methylation of tyrosine greatly reduces the capacity of fibroin to crosslink, methylation by diazomethane being more effective than methylation by dimethyl sulfate. This difference may be due to further reaction of

diazomethane with groups other than tyrosine hydroxyl, thereby protecting such groups as carboxyl from entering into crosslinking. Further, the viscosities of solutions of methylated fibroins which have been reacted with peroxydisulfate are lower than control, indicating that crosslinking is not taking place.

Tryptophan-modified fibroin is still effectively crosslinked by peroxydisulfate, as reflected by the insolubility of the resulting fibroin in aqueous lithium bromide and by the higher viscosity of the soluble portion.

Samples of crosslinked and control fibroins were immersed in 85% phosphoric acid[4] and 90% formic acid containing 10% calcium chloride in order to test their solubilities in other fibroin solvents. Control fibroins dissolved in less than 15 min., while crosslinked fibroins dissolved only after standing 20 hr. or more.

In summary, while protection of tyrosine hydroxyl greatly reduces crosslinking of fibroin by peroxydisulfate, protection of tryptophan nitrogen does not offer such protection.

Chemical Analyses of Crosslinked Fibroins

All fibroins were characterized by chemical analyses and selected fibroins also by amino acid analyses to gain further insight into the nature of the crosslinking reaction. These data (Tables I and III) suggest that crosslinking involves tyrosine and possibly other amino acid sites in the fibroin.

Fibroins reacted with 0.1*M* ammonium peroxydisulfate show little change in nitrogen analyses from control samples; however, a significant increase in the total sulfur is noted, providing further evidence that fibroin-induced decomposition of peroxydisulfate with subsequent grafting of sulfur into the fibroin is occurring. All fibroins reacted with peroxydisulfate show similar increases in total sulfur.

Fibroin reacted with 1.0*M* peroxydisulfate shows evidence of hydrolytic cleavage of the protein main chain, as evidenced by lower total nitrogen and higher Van Slyke nitrogen contents.

Cleavage of methoxyl from methylated fibroins is negligible in peroxydisulfate attack on these fibroins. The methoxyl grouping thereby prevents tyrosine from entering into crosslinking, although attack of tyrosine by sulfate and hydroxyl radicals is apparently still occurring.

Amino Acid Analyses of Fibroins

Fibroin reacted with 0.1*M* ammonium peroxydisulfate (Tables I and III) shows significant loss of tyrosine as well as losses of the lesser amino acids lysine, histidine, arginine, proline, methionine, and nearly total destruction of tryptophan. Attack of these amino acids in other proteins by aqueous peroxydisulfate previously has been observed.[5,13,15] Tyrosine and histidine[16] are known to oxidize to quinone intermediates that can bind to active sites in proteins.[17] In fibroin tyrosine and to a lesser extent histidine are believed to be oxidized to quinone intermediates which condense with reactive sites (amino, hydroxyl, guanidino, histidino, imino, etc.) to yield

crosslinks. Such crosslinking has been suggested previously to account in part for crosslinking of gelatin by peroxydisulfate.[5]

Reaction of peroxydisulfate with fibroin in the presence of silver ion is less discriminant in attack of amino acids. This difference in reactivity in this reaction is probably explained by the competition of silver ion-induced and fibroin-induced decomposition of peroxydisulfate.

Reaction of fibroin with 1.0*M* peroxydisulfate causes a general lowering of the amino acid content with losses being greatest for proline and tyrosine.

Diazomethane-methylated fibroin reacted with 0.1*M* peroxydisulfate shows significant loss of the same amino acids as fibroin reacted with peroxydisulfate. Tyrosine seems to be attacked to even a greater degree in methylated fibroin, although methylated fibroin is not crosslinked by peroxydisulfate. Although peroxydisulfate attack of tyrosine in fibroin is not prevented by methylation, crosslinking may not occur because methylation prevents the formation of quinone intermediates from tyrosine.

In every case, tryptophan in the fibroins is almost completely destroyed by peroxydisulfate (Tables I and III). Further, modification of tryptophan in fibroin does not prevent crosslinking. It therefore appears that tryptophan does not enter into the crosslinking reaction, although it is readily attacked by peroxydisulfate.

Microscopic Investigation of the Fibroins

Periodic examination of samples of crosslinked fibroins in contact with 50% lithium bromide under a polarizing microscope shows that birefringent material in the interior of the fiber dissolves with time, leaving the swollen crosslinked outer portion of the protein. On washing the resulting fiber with water to remove lithium bromide, the swollen fiber returns to near its original size.

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Reference to a company or product name does not imply approval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.

References

1. M. Oku and I. Shimizu, *J. Soc. Textile Cellulose Ind., Japan*, **71**, 537 (1955).
2. H. Zahn, *Kolloid-Z.*, **121**, 39 (1951).
3. H. Zahn and H. Zuber, *Textil-Rundschau*, **9**, 119 (1954).
4. C. Earland and J. G. P. Stell, *Biochim. Biophys. Acta*, **23**, 97 (1957).
5. H. L. Needles, *J. Polymer Sci. A-1*, **5**, 1 (1967).
6. F. Lucas, J. T. B. Shaw, and S. G. Smith, *Advan. Protein Chem.*, **13**, 136 (1958).
7. E. J. Ambrose, C. H. Bamford, Jr., A. Elliot, and W. E. Hanby, *Nature*, **167**, 264 (1961).
8. E. Iizuka and J. T. Yang, *Proc. Natl. Acad. Sci. U.S.*, **55**, 1175 (1966).

9. A. H. Gordon, A. J. P. Martin, and R. L. M. Synge, *Biochem. J.*, **37**, 538 (1943).
10. H. A. Rutherford, W. I. Patterson, and M. Harris, *J. Res. Natl. Bur. Std.*, **25**, 451 (1940).
11. T. A. Bewley, and C. H. Li, *Nature*, **206**, 624 (1965).
12. P. D. Bartlett and J. D. Cotman, Jr., *J. Am. Chem. Soc.*, **71**, 1419 (1949).
13. D. R. Graham and K. W. Statham, *Textile Res. J.*, **30**, 136 (1960); *ibid.*, **32**, 908 (1962).
14. D. A. House, *Chem. Rev.*, **62**, 185 (1962).
15. H. L. Needles, *Textile Res. J.*, **35**, 298, 953 (1965).
16. R. H. Thomson, *Comparative Biochemistry*, Academic Press, New York, Vol. 3, 1962, p. 727.
17. H. S. Mason, *Advan. Enzymol.*, **16**, 105 (1955).

Résumé

La fibroine de soie est pontée par du peroxydisulfate d'ammonium 0.1–1.0*M* à 70°C ou à 40°C en présence d'ions argent. La décomposition induite du peroxydisulfate en présence de fibroine a été observée. La méthylation de la tyrosine dans la fibroine ne diminue pas l'attaque par le peroxydisulfate, mais le pontage de la fibroine est de ce fait empêché. La modification du tryptophane avec l'α-bromo-5-nitro-2-crésol n'empêche pas le pontage par le peroxydisulfate. La nature et les sites de pontage dans la fibroine sont suggérés sur la base d'analyses physiques, chimiques et d'analyses d'acides aminés de fibroines pontées et de fibroines de contrôle, de même que sur la base du mode de décomposition connu du peroxydisulfate.

Zusammenfassung

Seidenfibroin wird durch 0,1 bis 1,0 m Ammoniumperoxydisulfat bei 70°C. oder bei 40°C. in Gegenwart von Silberionen vernetzt. In Anwesenheit von Fibroin wird eine induzierte Zersetzung von Peroxydisulfat beobachtet. Die Methylierung des Tyrosins in Fibroin verlangsamt den Angriff durch Peroxydisulfat nicht, verhindert aber die Vernetzung des Fibroins. Eine Modifizierung des Tryptophans mit α-Brom-5-nitro-2-cresol verhindert dagegen die Vernetzung durch Peroxydisulfat nicht. Vorstellungen über Natur und Ort der Vernetzungsstellen werden im Lichte physikalischer und chemischer Untersuchungen sowie an Hand von Aminosäurenanalysen an vernetzten Fibroinproben und an Vergleichsproben und des bekannten Zerfallsmechanismus von Peroxydisulfat entwickelt.

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