

Presence of Phosphorus in *Nephila clavipes* Dragline Silk

Carl A. Michal,* Alexandra H. Simmons,* Benjamin G. M. Chew,[†] David B. Zax,[‡] and Lynn W. Jelinski*

*Center for Advanced Technology in Biotechnology and [†]Chemistry Department, Baker Laboratory, Cornell University, Ithaca, New York 14853 USA

ABSTRACT Solid-state ³¹P-NMR of *Nephila clavipes* dragline silk indicates the presence of phosphorus in at least two chemically distinct environments. Amino acid analyses of acid-hydrolyzed silk confirm the presence of phosphotyrosine as one of the phosphorus-containing components. The unusual chemical shift (18.9 ppm downfield from 85% H₃PO₄), proton chemical shift, and acid lability of a second component suggest that it is part of a strained five-membered cyclic phosphate that might be found on a β-D-ribose. The five-membered cyclic phosphate is not removed from the silk fibers by exhaustive aqueous extraction. It is absent in nascent silk fibroin from the glands, suggesting that its formation is part of the fiber processing that occurs in the ducts leading to the spinnerets. High-resolution NMR spectra of silk dissolved in propionic acid/12 N HCl (50:50 v/v) show five phosphorus sites assigned to phosphorylated tyrosine residues, phosphorylated serine residues, inorganic phosphate, and two hydrolysis products of the cyclic phosphate compound. The observed posttranslational phosphorylation may be important in the processing and modulation of the physical properties of dragline silk.

INTRODUCTION

Although the amino acid sequence (Lewis, 1992) and structure (Simmons et al., 1994 a,b; Kaplan, 1994) of dragline silk from the spider *Nephila clavipes* are currently under active investigation, the spinning process has not received equal attention (Viney et al., 1993). It is well known that the processing of polymers plays an enormous role in determining the properties of the finished fibers (Zimmerman, 1989). Within the spider, the dragline silk starts as an isotropic liquid in the major ampullate glands, is drawn through long glandular ducts, and emerges from the spinneret as an oriented fiber. It is thought that dehydration is one part of this process (Tillinghast et al., 1984). Physicochemical experiments have suggested that a disorder-to-order transition of *Bombyx mori* (silkworm) silk fibroin takes place under shearing stresses (Iizuka, 1983). More recently, Viney and co-workers (Thiel et al., 1994) have discovered that a large amount of calcium (~0.2 wt %, presumably added during processing) is present in the fibers.

Partial amino acid sequences of dragline silk proteins Spidroin 1 and 2 (Lewis, 1992) indicate a substantial proportion of positively charged amino acid residues, (arginine ≈2%) but only a trace of negatively charged ones (glutamic acid ≈0.3% from sequence). This charge imbalance led us to hypothesize the existence of ionic cross-links, either from exogenous inorganic ions or through posttranslational modifications such as phosphorylation.

Here we describe the application of solid- and solution-state ³¹P-NMR spectroscopy to the detection and identifi-

cation of phosphorus-containing groups in the dragline silk of *N. clavipes*.

³¹P-NMR has been used to study enzyme control and catalysis mechanisms in globular proteins (Brauer and Sykes, 1987). The role of the phosphorus atoms that occur in globular proteins is often dramatic (Lehninger et al., 1993; Creighton, 1993) because the phosphorylation of a single amino acid residue in an enzyme, by altering the three-dimensional structure of the protein, may cause a change in catalytic activity of several orders of magnitude.

The phosphorus nucleus, however, has received little attention in the study of fibrous proteins. Although solid-state NMR has been an important tool in elucidating the structure of several fibrous proteins, for example collagen (Jelinski and Torchia, 1980; Jelinski et al., 1981) and *B. mori* silk (Asakura et al., 1993; Nicholson et al., 1993), ³¹P is a nucleus that has been wholly ignored. Here we present evidence that phosphorus is present in the dragline silk of *N. clavipes* spiders in at least two chemically distinct environments.

MATERIALS AND METHODS

Dragline silk samples were obtained by controlled silking of *N. clavipes* spiders. The silking procedure used is similar to that described elsewhere (Work and Emerson, 1982). Silk was drawn at a rate of ~2 cm/s, comparable to natural draw rates. All samples used in this work were obtained within the first 5 days of captivity of the spiders in order to obtain silk that resembles as closely as possible silk produced in the wild.

Solid-state proton-decoupled ³¹P CP-MAS (cross-polarization magic angle spinning) NMR spectra were acquired on a home-built spectrometer based on a wide-bore 7.05 T magnet (Oxford Instruments, Oxford, UK) operating at 121 MHz. The 90° pulse width was 3.5 μs, and a broad-band triple-resonance probe (Chemagnetics, Otsuka Electronics, Ft. Collins, CO) was used to spin the sample at 5 kHz. Cross-polarization was performed with a 4-ms contact time and a 4-s repetition time between scans. Known amounts of *O*-phosphoserine, sodium phosphate, and *O*-phosphotyrosine were used for spin-counting. Proton-decoupled solution ³¹P spectra were acquired on either a Varian Unity-400 spectrometer at 161 MHz or on a Bruker AM-360 at 145 MHz, both in unlocked mode. The delay

Received for publication 6 March 1995 and in final form 15 September 1995.

Address reprint requests to Dr. Lynn W. Jelinski, Center for Advanced Technology, 130 Biotechnology Building, Cornell University, Ithaca NY 14853. Tel.: 607-255-2300; Fax: 607-255-6249; E-mail: lwj2@cornell.edu.

© 1996 by the Biophysical Society

0006-3495/96/01/489/05 \$2.00

time between acquisitions was 1.8 s. High-resolution proton NMR spectra were acquired on a Varian Unity-400 with a 2.0-s delay between scans. Silk samples were dissolved in propionic acid/12 N HCl (50:50 v/v) (Pierce, Rockford, IL) for solution-state ^{31}P spectra or in formic acid- d_2 (95% in D_2O) (C/D/N Isotopes, Pointe-Claire, Quebec) for proton spectra.

A water extraction of silk was performed with distilled water in a cellulose thimble in a Soxhlet extraction tube for 10 h. The washed silk was then allowed to dry in air for 2 h. The extract was dried in a Rotovapor RE-120 solvent extractor and then redissolved in 0.5 ml D_2O .

Phosphoamino acids used as standards were purchased from Sigma (St. Louis, MO) and used without further purification. Amino acid analyses were carried out on a Waters Pico-Tag amino acid analysis system according to published methods (Heinrikson and Meredith, 1984), which were modified by using propionic acid/12 N HCl (50:50 v/v) for the hydrolysis step.

RESULTS

The solid-state ^{31}P spectrum of unoriented dragline silk fibers is shown in Fig. 1. The spectrum consists of peaks from at least two chemically distinct signals (18.9 and 0.6 ppm) along with spinning sidebands (denoted by *s*). The relative intensity of the downfield to upfield sites is 1:4. By comparing the intensities of this spectrum with ^{31}P standards, we estimate that these sites correspond to 1 ^{31}P nucleus for each 4000 (downfield) and 1000 (upfield) amino acids of silk. Because all the samples were cross-polarized, the spin-counting procedure is not rigorously quantitative. Nevertheless, we obtained excellent agreement between spin counts of sodium phosphate, *O*-phosphotyrosine, and *O*-phosphoserine standards. Additionally, in a simple Bloch decay experiment with a delay time of 60 s between repetitions, the ratio of intensities of the two peaks was the same as in the cross-polarized spectrum.

The sideband intensity pattern of the upfield peak reflects a larger chemical shift anisotropy than that of any of the standards used (*O*-phosphotyrosine, *O*-phosphoserine, *O*-phosphothreonine, and NaH_2PO_4). This may be caused by distortions due to noncovalent interactions within the packed silk fibers. The chemical shift anisotropy of the smaller downfield peak is more difficult to estimate, but it

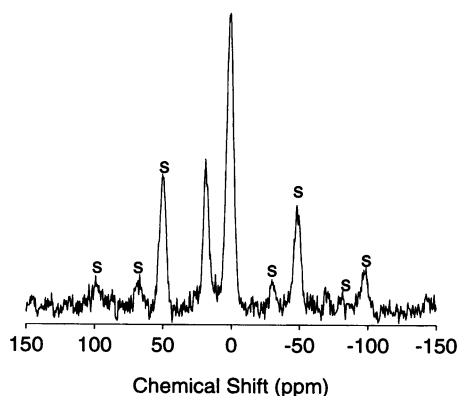


FIGURE 1 Solid-state CP-MAS ^{31}P -NMR spectrum of 123 mg *N. clavipes* dragline silk. Obtained with 18,272 accumulations with a 1-s repetition delay, a contact time of 4 ms, and a 50-kHz spectral width. Spinning sidebands are denoted by *s*.

seems to be of magnitude similar to those of the standards. The components of the chemical shift anisotropy tensors were extracted by a Herzfeld-Berger (Herzfeld and Berger, 1980) analysis and are given in Table 1.

A water extraction was performed to determine whether the observed phosphorus is bound within the silk. Solid-state ^{31}P -NMR spectra of a washed and an unwashed silk sample were obtained. The spectra, collected under identical conditions, show similar phosphorus content for the upfield site, whereas the downfield peak is actually slightly larger in the washed sample. We attribute this difference to the fact that the washed sample was collected from spiders in their first 2 days of captivity, whereas the unwashed sample was collected the following 3 days. We were unable to detect any phosphorus in the extract by solution-state NMR.

Examination by solution-state ^{31}P -NMR of the nascent silk fibroin from dissected major ampullate glands reveals peaks in the ~ 0 ppm region but none in the ~ 19 ppm region.

Amino acid analyses of the silk were performed with various hydrolysis times to identify the phosphorus sites. The chromatograph was calibrated with *O*-phosphoserine, *O*-phosphothreonine, and *O*-phosphotyrosine in addition to the usual standard. Fig. 2 displays the ratio of *O*-phosphotyrosine to total tyrosine detected as a function of hydrolysis time. The data reflect the expected increase in hydrolysis of *O*-phosphotyrosine with increasing reaction time. Because the phosphoamino acids are partially destroyed as the protein is hydrolyzed, it is not possible to quantitate their content accurately from amino acid analysis, and we hesitate to extrapolate the data of Fig. 2 to zero hydrolysis time. Unfortunately, the *O*-phosphoserine peak could not be distinguished from other interfering peaks in the elution. No *O*-phosphothreonine was detected.

A high-resolution solution ^{31}P spectrum of dissolved silk is shown in Fig. 3. Five lines are observed in this spectrum, all with chemical shifts near zero (Table 2). There is no trace of the 18.9-ppm peak observed in the solid-state spectrum. The intensity ratios of the four well resolved lines are $\sim 2:1:1:6$, and the relative intensities do not change with time. The widths of the lines are not all identical (Table 2), indicating a difference in rotational correlation times between the different environments. The line at 0.7 ppm is assigned to inorganic phosphate, inasmuch as a NaH_2PO_4 standard in the same solvent appeared at this position with the same linewidth.

The line at -2.2 ppm is assigned to phosphotyrosine. In our solvent, the free amino acid *O*-phosphotyrosine appeared at -5.4 ppm. A solid-state CP-MAS spectrum of

TABLE 1 Chemical shift anisotropies for *N. clavipes* dragline silk

σ_{iso}^* (ppm)	σ_{11}	σ_{22}	σ_{33}
0.6	-88 ± 5	-5 ± 5	95 ± 10
18.9	-42 ± 15	6 ± 8	93 ± 20

*Downfield from 85% H_3PO_4 .

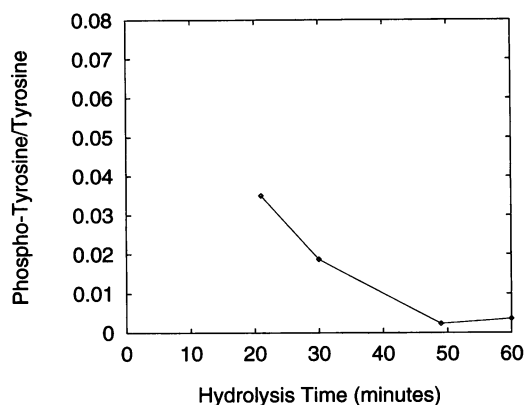


FIGURE 2 Ratio of *O*-phosphotyrosine to tyrosine detected in amino acid analysis of propionic acid/HCl hydrolyzed silk as a function of hydrolysis time.

polycrystalline *O*-phosphotyrosine also revealed an isotropic chemical shift of -5.4 ppm. The chemical shift of this group is known to vary significantly with environment. A titration of free *O*-phosphotyrosine (Moore et al., 1985) showed a variation from -3 to 0.9 ppm between pH 4.5 and 9. The -2.2 -ppm line is also the least motionally narrowed of any in the spectrum, leading us to suggest that it is attached to the high molecular weight silk protein.

The broad, poorly resolved line at -0.7 ppm may be due to phosphoserine. A titration of free *O*-phosphoserine (Vogel, 1989) showed a variation from 0 to 4.5 ppm between pH 8 and 5. In the solid state, we found the *O*-phosphoserine standard at 0.8 ppm.

We propose that the other two sharp peaks in the spectrum correspond to the partial hydrolysis products of the compound responsible for the 18.9 -ppm peak of the solid-state spectrum.

DISCUSSION

There are only two classes of naturally occurring phosphorus compounds with chemical shifts near 19 ppm. The first

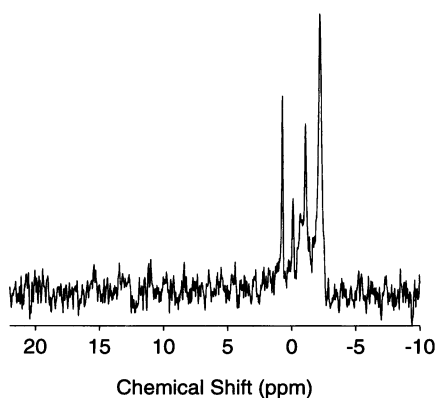


FIGURE 3 Proton-decoupled solution NMR spectrum of 33 mg *N. clavipes* dragline silk dissolved in propionic acid/ 12 N HCl ($50:50$ v/v). Obtained with 8672 accumulations, a 45° tip angle, a 2 -s repetition delay, and a 10 -kHz spectral width.

TABLE 2 Solution chemical shifts* for *N. clavipes* dragline silk

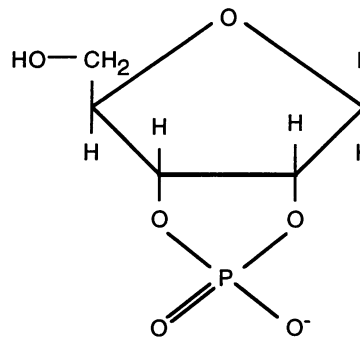
δ^{\ddagger} (ppm)	Intensity	Linewidth § (Hz)	Assignment
0.7	2	11	P_i
-0.1	1	18	Cyclic phosphate hydrolysis product
-0.7	0.3–1.0	≈ 40	Phosphoserine (?)
-1.1	1	18	Cyclic phosphate hydrolysis product
-2.2	6	38	Phosphotyrosine

*Propionic acid/HCl ($50:50$ v/v).

‡ Downfield from 85% H_3PO_4 .

§ Full width at half height.

possibility is a strained five-membered phosphate ring (Gorenstein, 1975). Examples include the $2',3'$ -cyclic nucleoside phosphates (scheme 1) (Fathi and Jordan, 1986; Graham et al., 1987), glycerol $1,2$ -(cyclic) phosphate ($\delta = 19.1$ ppm (Boyd et al., 1987), and other cyclic sugar phosphates (Boyd et al., 1987; Fathi and Jordan, 1986; Gorenstein, 1975; Graham et al., 1987). These compounds undergo hydrolysis under acidic conditions.



Scheme 1

The other class of biologically important phosphorus compounds with chemical shifts in this region is the phosphonates, the most common of which is 2-aminoethylphosphonic acid (Meneses et al., 1987; Hilderbrand et al., 1983). It has been suggested (Hilderbrand et al., 1983) that the role of phosphonates in biological systems is protection from extremes in external chemical environments (for lower marine animals) and from proteolytic enzymes (Zhao et al., 1994). It is known that some peptide phosphonates have antibacterial activity (Allen et al., 1978; Zboinska et al., 1993). The phosphonates are not acid-labile (Hilderbrand et al., 1983).

Two lines of evidence are used to assign the peak at 18.9 ppm in the solid-state spectrum to a five-membered cyclic sugar phosphate. First, on dissolution in propionic acid/HCl, we find that the cyclic phosphate ring is cleaved at either one of its two ester linkages, as predicted by Fathi and Jordan (1986), giving rise to the two slightly broadened resonances observed in the solution spectrum. Second, by high-resolution proton NMR we observe signals corre-

sponding to the ring protons. These signals are of the correct intensity predicted by the ^{31}P data.

The fact that the two ^{31}P resonances from the cleavage products are broadened suggests that the cyclic sugar compound may be associated with protein. The intermediate linewidth suggests that the sugar is either covalently attached to a mobile portion of the protein, perhaps at one end, or that it is more loosely involved. Covalent binding of carbohydrates to globular proteins is common and is predated in fibrous proteins in collagen (Creighton, 1993). The presence of glycoproteins in the droplets of *Araneus diadematus* viscid spiral silk coating has also been reported (Vollrath et al., 1990).

This five-membered cyclic phosphate is not present in the nascent silk fibroin in dissected major ampullate glands, suggesting that this phosphorylation step is part of the processing that occurs as the silk journeys from the glands to the spinnerets.

The remaining phosphorus content of the silk is assigned to phosphorylated tyrosine and serine residues, as suggested by the observation of *O*-phosphotyrosine in amino acid analyses, and to inorganic phosphate. The inorganic phosphate may be present in the silk in inorganic crystallites or may have arisen from one of the other phosphorous environments when the silk was dissolved. Although additional work is necessary to confirm the assignments made here, our assignments are consistent with the positions and relative intensities of all peaks observed in both the solid- and solution-state spectra.

Because we were unable to extract any of the phosphorus with water, we conclude that all of the phosphorus content observed is either covalently bound to the silk protein or is in regions within the silk that are inaccessible to water.

On the basis of a molecular weight of 320,000 (Candelas and Cintron, 1981), we are able to estimate from the spectral intensities that the silk contains five phosphorus nuclei per peptide chain. This corresponds to one inorganic phosphate per chain, three phosphoamino acid residues per chain, and one cyclic sugar phosphate per chain. More recent data (Mello et al., 1994) suggest a molecular weight closer to 200,000, corresponding to somewhat fewer phosphorus nuclei per chain. Because there is some uncertainty about the cross-polarization efficiencies of the silk resonances in the solid-state, these estimates should be regarded as lower bounds.

The origin of the cyclic sugar phosphate is unknown and to our knowledge unprecedented in the study of fibrous proteins. However, carbohydrates have been detected in the silk of *Argiope* spiders (Tillinghast et al., 1981) and more recently in that of *N. clavipes* (Kaplan group, NATICK Army Research Center, personal communication, 1995).

Although the total phosphorus content of the silk is small (<~0.1 wt %), it may be important to the physical properties. Posttranslational phosphorylation of specific amino acid residues in globular proteins is often a crucial step in the development of their activity. This process may also be fundamental to the structural assembly or stability of the

silk. Additional experiments are required to elucidate the role played by this and other trace components in contributing to the physical properties of this remarkable material.

We thank Robert Sherwood and Theodore Thannhauser for performing the amino acid analyses and for useful discussions. We also thank Mary Roberts and Tyler Burt for helpful discussions. Finally, we thank David Hijirida for running some NMR spectra and Steve Lee, Richard Do, Chris Wehman, and Sung Lee for collecting the silk samples. This work was supported by a grant from the National Science Foundation (MCB-9303870). C. A. Michal and A. Simmons acknowledge support from NSERC (Canada). B. G. M. Chew and D. B. Zax acknowledge support from the MRL program of the National Science Foundation under award DMR-9121654.

REFERENCES

- Allen, J. G., F. R. Atherton, M. J. Hall, C. H. Hassall, S. W. Homes, R. W. Lambert, L. J. Nisbet, and P. S. Ringrose. 1978. Phosphonopeptides, a new class of synthetic antibacterial agents. *Nature*. 272:56–58.
- Asakura, T., J. Yeo, M. Demura, T. Itoh, T. Fujito, M. Imanari, L. K. Nicholson, and T. A. Cross. 1993. Structural analysis of uniaxially aligned polymers using solid-state ^{15}N NMR. *Macromolecules*. 26: 6660–6663.
- Boyd, R. K., A. S. W. DeFreitas, J. Hoyle, A. W. McCulloch, A. G. McInnes, A. Rogerson, and J. A. Walter. 1987. Glycerol 1,2-cyclic phosphate in centric diatoms. *J. Biol. Chem.* 262:12406–12408.
- Brauer, M., and B. D. Sykes. 1987. Protein-phosphorus interactions. In *Phosphorus NMR in Biology*. C. T. Burt, editor. CRC Press, Boca Raton, Florida. 153–184.
- Candelas, G. C., and J. Cintron. 1981. A spider fibroin and its synthesis. *J. Exp. Zool.* 216:1–6.
- Creighton, T. E. 1993. *Proteins*. W. H. Freeman and Company, New York.
- Fathi, R., and F. Jordan. 1986. α -D-Ribofuranosyl 1,2-cyclic monophosphate. Isolation, NMR spectroscopic properties, and rates and mechanism of acid and alkaline hydrolysis. *J. Org. Chem.* 51:4143–4146.
- Gorenstein, D. G. 1975. Dependence of ^{31}P chemical shifts on oxygen-phosphorus-oxygen bond angles in phosphate esters. *J. Am. Chem. Soc.* 97:898–900.
- Graham, R. A., R. A. Meyer, B. S. Szwegold, and T. R. Brown. 1987. Observation of myo-inositol 1,2 (cyclic) phosphate in a morris hepatoma by ^{31}P NMR. *J. Biol. Chem.* 262:35–37.
- Heinrikson, R. L., and S. C. Meredith. 1984. Amino acid analysis by reverse-phase high-performance liquid chromatography: precolumn derivatization with phenylisothiocyanate. *Anal. Biochem.* 136:65–74.
- Herzfeld, J., and A. E. Berger. 1980. Sideband intensities in NMR spectra of samples spinning at the magic angle. *J. Chem. Phys.* 73:6021–6030.
- Hilderbrand, R. L., J. Curley-Joseph, H. J. Lubansky, and T. O. Henderson. 1983. Biology of alkylphosphonic acids: a review of the distribution, metabolism, and structure of naturally occurring alkylphosphonic acids. *Top. Phosphorus Chem.* 11:297–338.
- Ilzuka, E. 1983. The physico-chemical properties of silk fibers and the fiber spinning process. *Experientia*. 39:449–454.
- Jelinski, L. W., C. E. Sullivan, L. S. Batchelder, and D. A. Torchia. 1981. Deuterium nuclear magnetic resonance of specifically labeled native collagen. *Biophys. J.* 10:515–529.
- Jelinski, L. W., and D. A. Torchia. 1980. Investigation of labeled amino acid side-chain motion in collagen using ^{13}C nuclear magnetic resonance. *J. Mol. Biol.* 138:255–272.
- Kaplan, D., B. Farmer, and C. Viney. 1994. Silk: biology, structure, properties, genetics. In *Silk Polymers: Materials Science and Biotechnology ACS Symposium Series*, Vol. 544. D. Kaplan, W. W. Adams, B. Farmer, and C. Viney, editors. American Chemical Society, Washington, DC. 176–184.
- Lehninger, A. L., D. L. Nelson, and M. M. Cox. 1993. *Principles of Biochemistry*. Worth Publishers, New York.

- Lewis, R. V. 1992. Spider silk: the unraveling of a mystery. *Acc. Chem. Res.* 25:392–398.
- Mello, C. M., K. Senecal, B. Yeung, P. Vouros, and D. Kaplan. 1994. Initial characterization of *Nephila clavipes* dragline protein. In *Silk Polymers: Materials Science and Biotechnology ACS Symposium Series*, Vol. 544. D. Kaplan, W. W. Adams, B. Farmer, and C. Viney, editors. American Chemical Society, Washington, DC. 67–79.
- Meneses, P., T. Glonek, and T. O. Henderson. 1987. Metabolism of 2-aminoethylphosphonic acid during embryonic development of the schistosomal vector *biomphalaria glabrata*. *Comp. Biochem. Physiol. B Comp. Biochem.* 88:603–612.
- Moore, R. R., C. T. Burt, and M. F. Roberts. 1985. Observation of tyrosine-O-phosphate in *Drosophila melanogaster* larvae by ^{31}P spectroscopy. *Biochim. Biophys. Acta.* 846:394–397.
- Nicholson, L. K., T. Asakura, M. Demura, and T. A. Cross. 1993. A method for studying the structure of uniaxially aligned biopolymers using solid-state ^{15}N NMR: application to *Bombyx mori* silk fibroin fibers. *Biopolymers.* 33:847–861.
- Simmons, A., E. Ray, and L. W. Jelinski. 1994a. Solid-state ^{13}C NMR of *Nephila clavipes* dragline silk establishes structure and identity of crystalline regions. *Macromolecules.* 27:5235–5237.
- Simmons, A., E. Ray, and L. W. Jelinski. 1994b. NMR studies of spider dragline silk. *Polym. Prepr. Am. Chem. Soc., Div. Polym. Chem.* 35 (2):421–422.
- Thiel, B. L., D. D. Kunkel, and C. Viney. 1994. Physical and chemical microstructure of spider dragline: a study by analytical transmission electron microscopy. *Biopolymers.* 35:1089–1097.
- Tillinghast, E. K., F. S. Chase, and M. A. Townley. 1984. Water extraction by the major ampullate duct during silk formation in the spider, *Arigiope aurantia lucas*. *J. Insect Physiol.* 30:591–596.
- Tillinghast, E. K., E. J. Kavanagh, and P. H. Kolbjornsen. 1981. Carbohydrates in the webs of *Argiope* spiders. *J. Morphol.* 169:141–148.
- Viney, C., A. E. Huber, D. L. Dunaway, S. T. Case, and D. L. Kaplan. 1993. Processing natural and reconstituted silk solutions under equilibrium and non-equilibrium conditions. *Mat. Res. Soc. Symp. Proc.* 292: 211–217.
- Vogel, H. J. 1989. Phosphorus-31 nuclear magnetic resonance of phosphoproteins. *Methods Enzymol.* 177:263–283.
- Vollrath, F., W. J. Fairbrother, R. J. P. Williams, E. K. Tillinghast, D. T. Bernstein, K. S. Gallagher, and M. A. Townley. 1990. Compounds in the droplets of the orb spider's viscid spiral. *Nature.* 345:526–527.
- Work, R. W., and D. Emerson. 1982. An apparatus and technique for the forcible silking of spiders. *J. Arachnol.* 10:1–10.
- Zboinska, E., Lejczak, B., and P. Kafarski. 1993. Antibacterial activity of phosphonopeptides based on 4-amino-4-phosphonobutyric acid. *FEMS Microbiol. Lett.* 108:225–230.
- Zimmerman, J. 1989. Polymers for fibers. In *Comprehensive Polymer Science: The Synthesis, Characterization, Reactions, and Applications of Polymers*, Vol. 7. A. Geoffrey, J. C. Bevington, and S. L. Aggarwal editors. Pergamon Press, Oxford. 249–284.
- Zhao, Q., I. M. Kovach, A. Bencsura, and A. Papathanassiou. 1994. Enantioselective and reversible inhibition of trypsin and α -chymotrypsin by phosphonate esters. *Biochemistry.* 33:8128–8138.