

ORIGINAL ARTICLE

Molecular weight of *Nephila clavata* spider silk

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The molecular weight of *Nephila clavata* spider silk was determined to be *ca.* 600 kDa, under unreduced conditions, corresponding to its natural state, using the sodium dodecyl sulfate polyacrylamide gel electrophoresis method and a protein marker with a maximum molecular weight of 500 kDa. The addition of 2-mercaptoethanol into the spider silk solution decreased the molecular weight from *ca.* 600 kDa to *ca.* 270 kDa. Such a dramatic decrease in the molecular weight was ascribed to the presence of 2-mercaptoethanol as a reductant. This finding indicates the possibility that the reduction process cleaved the disulfide bonds formed between the spider silk proteins and then drastically decreased the molecular weight from *ca.* 600 to *ca.* 270 kDa. These results suggest that *N. clavata* spider silk proteins might consist of two proteins with a molecular weight of *ca.* 270 kDa that are crosslinked by disulfide bonds and exist as a dimer of *ca.* 600 kDa.

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INTRODUCTION

Recently, many researchers have become interested in spider silk as a new potential material for textile fibers, sutures¹ and violin strings² due to its excellent physical properties, such as high mechanical strength,^{3–7} heat resistivity⁸ and ultraviolet (UV) resistivity.^{9–12} The physical properties of spider silk, such as mechanical strength, strongly depend on the molecular weight of the polymers.^{13,14} UV irradiation provides the possibility of increasing the molecular weight ascribed to crosslinks,¹⁰ whereas UV rays and thermal treatment decompose spider silk^{9,12,15,16} and generally decrease their molecular weight.^{10,12} Thus the determination of the molecular weight of spider silk proteins is important from the viewpoint of evaluating their physical properties. Moreover, such excellent physical properties have led researchers to focus on the mass production of synthetic silk using genetic engineering, which provides silk-like materials of *ca.* 60 kDa size.¹⁷ Thus an understanding of the molecular weight of natural spider silk is crucial for evaluating silk prepared by genetic engineering.

Previously, the molecular weight of *Nephila clavipes* spider silk was measured by electrophoresis and chromatography methods.^{18–23} The molecular weight was determined to be 195–320 kDa in a reduced state, 275–480 kDa in an unreduced state by electrophoresis^{18,19,23} and 520–720 kDa in an unreduced state by chromatography.^{18,21} Thus the values of the molecular weight ranged widely between 195 and 720 kDa^{18–23} for *N. clavipes* spider silk. The molecular weight values previously reported for *N. clavipes* spider silk^{18,19,21,23} are summarized in Table 1. For comparison, the molecular weight determined for *N. clavata* silk under reduced conditions¹² is also listed in Table 1.

Due to the large diversity in molecular weights, a reliable value for the molecular weight of spider silk must be determined. However, problems stemming from marker proteins and the friction of macromolecules render molecular weight determination by electrophoresis challenging. The first problem is related to the large error in

the estimated molecular weight when determining a high molecular weight from a calibration curve using electrophoresis, because no markers with a molecular weight > 300 kDa are available. The second problem is related to the large error in the position of the bands, because macromolecules with a high molecular weight of > 300 kDa generate strong friction against the polyacrylamide gel mesh used for electrophoresis measurements.

Here we should consider that the data on the molecular weight of spider silk have been restricted to that for *N. clavipes* determined using a marker protein with a molecular weight of 200 kDa²³ or 250 kDa.¹⁸ However, no studies have yet determined the molecular weight of silk from the *Nephila clavata* spider (Japanese golden web spider), which is a different spider species compared with *N. clavipes*, under unreduced conditions (see Table 1).

In our previous paper,¹² we determined the molecular weight of *N. clavata* spider silk to be 272 kDa in a reduced state. In that study, the reducing agent may have induced the chemical cleavage of protein bonds. If a reducing agent is not used, the molecular weight of spider silk in a natural state could potentially be determined.

Here we attempted to resolve the two problems described above by using the following procedure. We used a protein marker with a molecular weight of 500 kDa to reduce the error in molecular weight measurement. Currently, no markers with molecular weights > 500 kDa are commercially available for sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). We also used a 3% polyacrylamide gel with a mesh size larger than that of a 5% polyacrylamide gel¹² to reduce the error arising from the friction of macromolecules. Finally, no reducing agents were used to avoid the reduction of chemical bonds.

This paper describes the measurements of the molecular weight of *N. clavata* spider silk proteins under an unreduced state; this value was

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Table 1 Molecular weights determined for silk proteins of two types of spiders (*N. clavipes* and *N. clavata*) using electrophoresis

Species	Silk protein		Sample	Method	References
	Reduced	Unreduced			
<i>N. clavipes</i>	—	720	Gland	Chromatography	21
	—	520	Thread		
<i>N. clavipes</i>	300–350	550–650	Thread	Chromatography	18
<i>N. clavipes</i>	195, 220	275	Thread, gland	Electrophoresis	19
<i>N. clavipes</i>	220	320	Thread, gland	Electrophoresis	23
<i>N. clavipes</i>	260–320	420–480	Thread	Electrophoresis	18
<i>N. clavata</i>	272	—	Thread	Electrophoresis	12
<i>N. clavata</i>	?	?	Thread	Electrophoresis	Present study Molecular weight/kDa

determined to be *ca.* 600 kDa, which is much larger than that measured under a reduced state.

EXPERIMENTAL PROCEDURE

Sample preparation

Spider silk was prepared by pulling the material out of the abdomens of mature female *N. clavata* spiders. Three milligrams of spider silk was dissolved in 0.3 ml of saturated lithium thiocyanate (Wako Pure Chemical Industries, Ltd., Osaka, Japan) aqueous solution. No insoluble precipitates were detected in the silk solution. Next, the lithium thiocyanate contained in the silk solution was exchanged with urea by dialysis to remove excess lithium thiocyanate electrolytes that could interfere with the electrophoresis. After the dialysis, a 0.188 mol l⁻¹ Tris-HCl (pH 6.8) buffer containing 30% sucrose (Wako Pure Chemical Industries, Ltd.), 6% SDS (Wako Pure Chemical Industries, Ltd.), 16.8% 2-mercaptoethanol (Wako Pure Chemical Industries, Ltd.) and 0.0060% bromophenol blue (Wako Pure Chemical Industries, Ltd.) was added to the urea-exchanged solution. The ratio of buffer to urea-exchanged solution was 1:2 by volume. Next, the silk solution was incubated for 5 min at 373 K to prepare a silk solution sample containing the reductant 2-mercaptoethanol for electrophoresis. SDS is an anionic surfactant that powerfully denatures proteins, and 2-mercaptoethanol cleaves S-S bonds by chemical reduction.²⁴ After dialysis, a 0.221 mol l⁻¹ Tris-HCl (pH 6.8) buffer containing 37.5% sucrose, 7.05% SDS and 0.0071% bromophenol blue was added to the other urea-exchanged solution. The ratio of buffer to urea-exchanged solution was 1:2 by volume. Thus a silk solution containing no 2-mercaptoethanol was prepared as an unreduced sample for electrophoresis by incubation for 5 min at 373 K.

Materials and methods for electrophoresis

Electrophoresis was performed using a 3% polyacrylamide gel with an NA-1010 (Nihon Eido Co., Tokyo, Japan) instrument. Coomassie Brilliant Blue staining (Quick CBB, Wako Pure Chemical Industries, Ltd.) was used to identify band patterns reflecting the existence of proteins in the polyacrylamide gels.²⁵ The intensity of the bands in the polyacrylamide gels was measured using an EPSON GT-F650 scanner (Seiko Epson Co., Nagano, Japan). The retention factor (Rf) was determined from the position of bands relative to the migration distance of the proteins. The molecular weight corresponding to the Rf value was determined from the calibration curve obtained using three different markers with molecular weights of 251, 279 and 500 kDa (HiMark Prestained High Molecular Weight Protein Standard produced by Invitrogen, Carlsbad, CA, USA).

Progress of chemical reduction

The chemical reduction progress was evaluated in terms of the reaction time. Silk solutions in the presence of 0.72 mol l⁻¹ (5.6%) of 2-mercaptoethanol

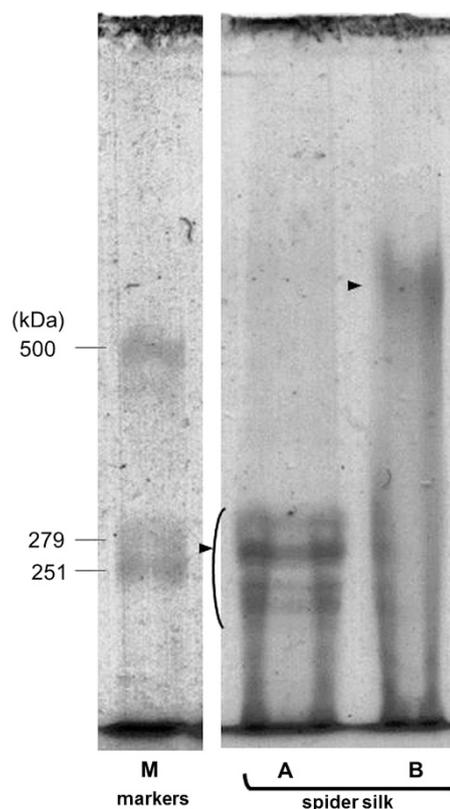


Figure 1 Band patterns for *N. clavata* spider silk proteins and for three different markers in 3% SDS-PAGE. M: marker. (a) Spider silk solution containing 0.72 mol l⁻¹ 2-mercaptoethanol, (b): spider silk solution containing no 2-mercaptoethanol.

were prepared by incubation for 27, 47, 69, 90, 138, 224 or 303 s. The incubation temperature was maintained at 303 K to delay the reaction rate. The chemical reaction was stopped by the addition of iodoacetamide (Wako Pure Chemical Industries, Ltd.). The solution at a reaction time of 0 s corresponded to a sample without 2-mercaptoethanol. Then the electrophoresis measurements were carried out.

The chemical reduction progress was also studied in terms of the amount of reducing agents. Silk solution samples were prepared using the following concentrations of 2-mercaptoethanol: 0, 1.0 × 10⁻⁵, 1.0 × 10⁻⁴, 1.0 × 10⁻³, 1.0 × 10⁻², 1.0 × 10⁻¹, 0.50, and 1.0 mol l⁻¹. The incubation step was performed for 10 min at 373 K to achieve chemical equilibrium. The electrophoresis measurements were also carried out for each silk solution sample.

RESULTS AND DISCUSSION

Figure 1 shows the band patterns of 3% SDS-PAGE for *N. clavata* spider silk and the molecular weight markers. Here we considered two different cases, with and without the reducing agent. Lane A represents a spider silk solution containing 2-mercaptoethanol as the reducing agent. Lane B represents a spider silk solution containing no 2-mercaptoethanol. For a comparison, Lane M is the band pattern of three different marker proteins with molecular weights of 251, 279 and 500 kDa. Multiple broad bands were observed in Lane A and corresponded to spider silk proteins with molecular weights between 220 and 300 kDa. The molecular weight corresponding to the densest band in Lane A was determined to be *ca.* 270 kDa. The molecular weight value determined for Lane A agreed with the value of *ca.* 272 kDa determined for *N. clavata* spider silk reported in our previous paper,¹² which was larger than the 195–220 kDa^{19,23} reported for silk proteins from the *N. clavipes* spider. However, the value of

ca. 270 kDa for *N. clavata* silk was in the range of 260 and 320 kDa, which was observed for *N. clavipes* using a marker protein with a molecular weight of 200 kDa.¹⁸

The molecular weights of the proteins corresponding to the broad, dense band in Lane B were found to be between 560 and 660 kDa. The median molecular weight in Lane B was ca. 600 kDa. The range of 560–660 kDa for *N. clavata* spider silk was much larger than the 420–480 kDa range¹⁸ reported for *N. clavipes* spider silk measured with no reducing agents by electrophoresis, even though the spider species are very different. Here we noted that the value of 420–480 kDa¹⁸ reported for *N. clavipes* was determined using markers with molecular weights <250 kDa. However, markers of 250 kDa are too small in comparison with the 420–480 kDa size determined for *N. clavipes* spider silk. A large difference in molecular weight between the silk proteins and the marker is well known to cause a large error in the measured molecular weight value. Therefore, the reported value of 420–480 kDa¹⁸ for *N. clavipes* likely suffers from large errors. By contrast, the value of 560–660 kDa determined for *N. clavata* in the present study should contain a lower amount of error and serve as a reliable value because the size of the 500 kDa molecular weight marker used here is relatively close to the determined value. The value of 560–660 kDa determined for *N. clavata* spider silk using electrophoresis is comparable with the values of 520 kDa²¹ and 550–650 kDa¹⁸ determined for *N. clavipes* spider silk using chromatography, even though the spider species and the measurement method used differ greatly.

The ratio of the molecular weight of *N. clavata* silk protein obtained in Lane B (ca. 600 kDa) to that in Lane A (ca. 270 kDa) was approximately 2.2. This observation suggests that 2-mercaptoethanol exerted a reducing function by cleaving intramolecular and intermolecular crosslinkages, such as disulfide bonds.²⁴ These results indicate the possibility that *N. clavata* spider silk proteins with a molecular weight of ca. 600 kDa consist of two proteins with molecular weights of ca. 270 kDa that are crosslinked by a disulfide bond. In all of the reported papers,^{23,26–28} the existence of a disulfide bond has not been directly detected but has been estimated from the amino-acid sequence closely related to the DNA gene alignment of spider silk. Similarly, we can assume the existence of the disulfide

bond formed between cysteine residues⁸ contained in spider silk proteins from our study.

Next, the effects of reduction time on the spider silk proteins were studied at 303 K using 2-mercaptoethanol to clarify the reduction process. Figure 2 shows the band patterns for *N. clavata* spider silk in 3% SDS-PAGE after a reduction of 27, 47, 69, 90, 138, 224 or 303 s. A band at ca. 600 kDa was observed at 0 s just before the addition of 2-mercaptoethanol. The intensity of the bands observed at ca. 600 kDa decreased with reduction time. By contrast, a weak band corresponding to ca. 270 kDa appeared after 27 s. Two bands corresponding to 270 and 600 kDa were distinctly observed after a reduction period of 47 s. However, no bands were observed between 270 and 600 kDa (see Figure 2), indicating that no intermediate compounds were generated during the reduction process.

The chemical reduction shown in Figure 2 was completed within 3 min, even at a low temperature of 303 K and a fixed reducing agent (2-mercaptoethanol) concentration of 0.72 mol l^{-1} . Next, spider silk solutions were prepared with different concentrations of 2-mercaptoethanol (between 0 and 1.0 mol l^{-1}) to further evaluate the cleavage process. The chemical reduction procedure was performed by incubation for 10 min at 373 K to achieve chemical equilibrium. Figure 3 shows the band patterns for *N. clavata* spider silk in 3% SDS-PAGE at different concentrations of 2-mercaptoethanol. The intensity of the band corresponding to ca. 600 kDa decreased with increases in the concentration of 2-mercaptoethanol, and the band was not observed at concentrations of $>1.0 \times 10^{-3} \text{ mol l}^{-1}$. By contrast, bands corresponding to ca. 270 kDa appeared at concentrations of $>1.0 \times 10^{-4} \text{ mol l}^{-1}$ of 2-mercaptoethanol. Although the band intensity at ca. 270 kDa increased with an increase in the concentration of 2-mercaptoethanol, no visible bands were observed in the range of ca. 270 to ca. 600 kDa, as shown in Figure 3. The broad bands observed near 270 kDa in Figure 3 agreed with those observed near 270 kDa in Figure 2. The results in Figures 2 and 3 strongly support those of Figure 1. The results suggest that each protein of ca. 270 kDa size is crosslinked by disulfide bonds and constructs a dimer of proteins with a ca. 600 kDa size. The disulfide bond between two

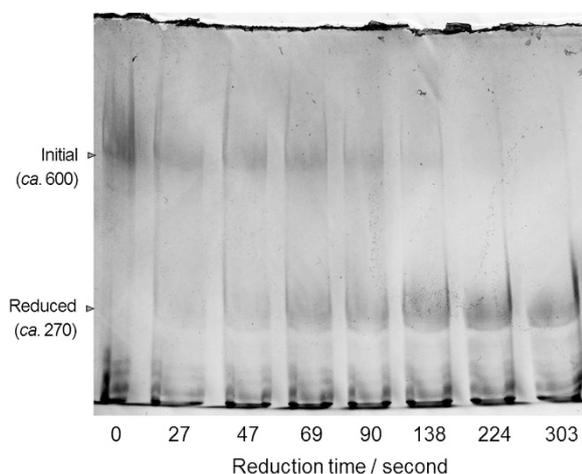


Figure 2 Band patterns for *N. clavata* spider silk protein in 3% SDS-PAGE with different 2-mercaptoethanol reduction times. The reduction times were 0 (without 2-mercaptoethanol), 27, 47, 69, 90, 138, 224 and 303 s at 303 K. The concentration of 2-mercaptoethanol was 0.72 mol l^{-1} just after starting the reduction reaction.

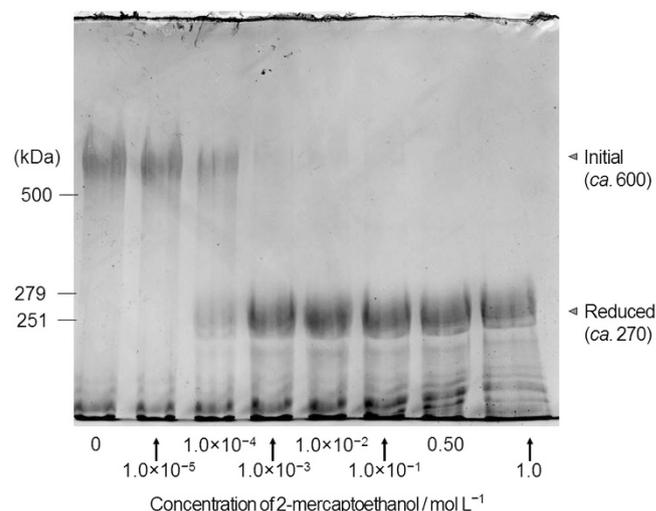


Figure 3 Band patterns for *N. clavata* spider silk protein in 3% SDS-PAGE at different concentrations of 2-mercaptoethanol. The concentrations of 2-mercaptoethanol used were 0, 1.0×10^{-5} , 1.0×10^{-4} , 1.0×10^{-3} , 1.0×10^{-2} , 1.0×10^{-1} , 0.50 and 1.0 mol l^{-1} .

spider silk proteins is generally formed by the oxidation of cysteine residues.⁸

These results indicate that the cleavage of the disulfide bonds strongly depends on the reaction time and the concentration of the reductant. By contrast, the possibility of a chemical association between the two proteins can be ruled out due to the high molecular weight of ca. 600 kDa for *N. clavata* spider silk, as no bands at ca. 270 kDa were observed in SDS-PAGE, even for a diluted solution of proteins without 2-mercaptoethanol.

CONCLUSION

The molecular weight of *N. clavata* spider silk was found to be ca. 600 kDa in the unreduced state by electrophoresis using a marker protein with a molecular weight of 500 kDa, even though the size of reduced *N. clavata* spider silk protein was determined to be 272 kDa in a previous paper.¹² Such a large difference in the molecular weight is ascribed to the use of 2-mercaptoethanol as a reducing agent. This finding suggests that *N. clavata* spider silk protein roughly consists of two proteins with molecular weights of ca. 270 kDa that are cross-linked by disulfide bonds. However, the value of ca. 600 kDa or of 560–660 kDa is not twice the value of ca. 270 kDa. Therefore, the proteins of ca. 600 kDa or 560–660 kDa size may potentially be modified by small amounts of glucides or phosphates. In such a case, the apparent molecular weight may differ slightly from the actual molecular weight of the intrinsic protein. In the near future, these issues will be resolved at the molecular level.

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