Structure and post-translational modifications of the web silk protein spidroin-1 from *Nephila* spiders

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**Highlights**

- We used a gel-based mass spectrometry strategy (ETD and CID fragmentation methods).

- We show for the first time PTMs on the major ampullate silk spidroin-1.

- Phosphorylation was the major PTM observed.

- Dityrosine and 3,4-dihydroxyphenylalanine were also observed.
Our findings may be valuable for understanding the silk physicochemical properties.

Abstract

Spidroin-1 is one of the major ampullate silk proteins produced by spiders for use in the construction of the frame and radii of orb webs, and as a dragline to escape from predators. Only partial sequences of spidroin-1 produced by *Nephila clavipes* have been reported up to now, and there is no information on post-translational modifications (PTMs). A gel-based mass spectrometry strategy with ETD and CID fragmentation methods were used to sequence and determine the presence/location of any PTMs on the spidroin-1. Sequence coverage of 98.06%, 95.05%, and 98.37% were obtained for *N. clavipes*, *Nephila edulis* and for *Nephila madagascariensis*, respectively. Phosphorylation was the major PTM observed with 8 phosphorylation sites considered reliable on spidroin-1 produced by *N. clavipes*, 4 in *N. madagascariensis* and 2 for *N. edulis*. Dityrosine and 3,4-dihydroxyphenylalanine (formed by oxidation of the spidroin-1) were observed, although the mechanism by which they are formed (i.e. exposure to UV radiation or to peroxidases in the major ampullate silk gland) is uncertain. Herein we present structural information on the spidroin-1 produced by three different *Nephila* species; these findings may be valuable for understanding the physicochemical properties of the silk proteins and moreover, future designs of recombinantly produced spider silk proteins.

Biotechnological significance

The present investigation shows for the first time spidroin structure and post-translational modifications observed on the major ampullate silk spidroin-1. The many site specific phosphorylations (localized within the structural motifs) along with the probably photoinduction of hydroxylations may be relevant for scientists in material science, biology, biochemistry and environmental scientists. Up to now all the mechanical properties of the spidroin have been characterized without any consideration about the existence of PTMs in the sequence of spidroins. Thus, these findings for major ampullate silk spidroin-1 from *Nephila* spiders provide the basis for mechanical–elastic property studies of silk for biotechnological and biomedical potential applications. This article is part of a Special Issue entitled: Proteomics of non-model organisms.

Graphical abstract
1. Introduction

Spider silk proteins, known as spidroins, are secreted by specialized abdominal glands connected to spinnerets from which fibers are produced for various task-specific applications, e.g. prey capture, reproduction, and as a lifeline to escape from predators [1] and [2]. The frame and radii of orb webs are made of tough silk fibers that are predominantly composed of the spidroins produced in the major ampullate glands of spiders [3]. Major ampullate silk fibers are one of the most important types of fibers spun by orb-web producing spiders of genus *Nephila*, and are a nanostructured composite material [4] predominantly composed of two structural proteins, designated major ampullate spidroins 1 and 2 [2] and [5], which can be encoded by several gene loci [3] and [6]. Spidroin-1 is present in the fibers in larger quantities than spidroin-2, found relatively uniformly throughout the fiber core, whereas spidroin-2 is inhomogeneously distributed and is clustered in certain core areas, yet missing from the periphery of the fiber core [7] and [8]. Analysis of the amino acid sequence of full-length spidroins showed that these proteins consist of three parts: an N-terminal non-repetitive domain; a highly repetitive central part composed of approximately 100
polyalanine/glycine-rich co-segments; and a C-terminal non-repetitive domain [9] and [10]. The non-repetitive N- and C-termini are known to play important roles in the stability of the proteins in the lumen in which they are stored prior to fiber spinning, and in the hierarchical self-assembly of the proteins during the natural spinning process [11], [12], [13] and [14]. The mechanical properties of major ampullate silk fibers depend on the highly repetitive backbone region, which has a structure that is reminiscent of a block copolymer, composed of alternating blocks of a glycine-rich block followed by an alanine-rich block [3]. Spidroin-1 has GGX, (GA)_n, and (A)_n motifs, while spidroin-2 has (A)_n and GPGXX motifs. The tensile strength is given by the (A)_n and/or (GA)_n repeats that form crystalline intra- and intermolecular β-sheet structures in the fiber, while the elasticity is dependent on the intervening glycine-rich repeats (GGX and GPGXX motifs) [10] and [15]. The glycine-rich segments are postulated to form different structures, such as β-spirals and coil structures [16]. Both spidroins are known to be composed of repetitive short amino acid sequence motifs, predominantly of the amino acids alanine and glycine, however, spidroin-1 contains little proline, whereas spidroin-2 is proline rich [17]. Rauscher et al. [18] identified proline as the primary determinant of elastin-like properties in spidroin-2, while spidroin-1 is proposed to impart tensile strength. The mixture of both proteins in the fiber presents an exceptional combination of tensile strength and extensibility. The appropriate utilization of weak hydrogen bonds by nature, such as those observed in the ‘β-sheet stacks’ commonly reported in spider silk proteins, can be used to produce biological structures which support incredibly high forces, giving new insights for the rational design of novel fiber materials for many different applications [19]. Spider silk proteins have therefore attracted significant interest from academics interested in understanding the properties of these materials on a molecular level, and from scientists interested in their potential for industrial applications (e.g. biomedical/textile industries).

To the best of our knowledge this study represents the first proteomic approach to studying spidroin-1, and information on the major ampullate spidroins currently reported in the literature is based on data obtained from genetic engineering and recombinant DNA technology [3], [10] and [20]. Data on amino acid substitutions and post-translational modifications (PTMs) are limited. Up to now all the mechanical properties of the spidroin have been characterized without any consideration about the existence of PTMs in the sequence of spidroins. Thus, both the natural protein and the recombinant spidroins [21], [22] and [23] have been biochemically and physically characterized as they would have the same (or similar) physico-chemical properties. It was therefore the aim of this study to determine the sequence and presence/location of PTMs and dityrosine for the spidroin-1 produced by three different species of spider, i.e. the orb-web silk producing spiders *N. clavipes*, *N. edulis* and *N. madagascariensis*, and to compare their structures.

**2. Materials and methods**

**2.1. Major ampullate silk samples**

The silk of *N. clavipes* was collected in the campus of the University of São Paulo State at Rio Claro, SP, southeast Brazil. *N. edulis* spiders were purchased from a supplier in Constance, Germany. The spiders were fed a diet of fruit flies in Bayreuth, Germany, and the sample of naturally spun major ampullate silk was obtained by harvesting the silk over a period of several weeks. *N. madagascariensis* silk was donated by Nicholas Godley, New York, U.S.A. The major ampullate silk fibers from the three species of orb-web spiders were
processed as described previously by Chen et al. [24]. About 20 mg of silk fibers were dissolved in 2 mL saturated lithium thiocyanate (38 M LiSCN hydrate, Sigma, Deisenhofen, Germany) at 25 °C for 2 h with shaking. Samples were centrifuged at 14,000 \( \times \)g for 20 min, the supernatants taken and transferred to an Ultrafree-4 centrifugal filter unit (Millipore, Bedford, USA) with the addition of 2 mL urea buffer (20 mM Tris, 7 M urea, 2 M thiourea, 4%w/v CHAPS, 10 mM 1,4-dithioerythritol, 1 mM EDTA, 1 mM PMSF, 0.2% v/v solution containing cocktail of protease inhibitors (CompleteTM from Roche Diagnostics, Mannheim, Germany) and a 0.2% v/v solution containing phosphatase inhibitor cocktail (Calbiochem, San Diego, USA). The sample was centrifuged at 4500 \( \times \)g until the volume was reduced to 0.5 mL. Subsequently LiSCN was removed and exchanged for urea buffer for 2-DE. Protein quantification was determined by the Bradford assay [25].

### 2.2. Two-dimensional gel electrophoresis

Samples of 200 \( \mu \)g spider silk protein were applied by rehydration to 18 cm IPG strips, pH 7–10 nonlinear gradient strips. Isoelectric focusing (IEF) started at 200 V and was gradually increased to 8000 V (approximately 1,500,000 Vh). IPG strips were incubated in equilibration buffer [50 mM Tris–HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS] containing 1% (w/v) DTT for 15 min, followed by equilibration buffer containing 4% (w/v) iodoacetamide for 15 min. The second dimension was run on casted SDS-PAGE gels (7–10% gradient) at a constant potential difference of 50 V overnight, and subsequently at a constant potential difference of 200 V for a further 4 h at 10 °C. Gels were stained with Coomassie Brilliant Blue R-250 (CBB) and were scanned and digitized for documentation.

### 2.3. In-gel digestion

Gel pieces were destained twice for 30 min at 25 °C with 10 mM ammonium bicarbonate/50% (v/v) acetonitrile, dehydrated in acetonitrile, dried, and subsequently treated with six different proteolytic enzymes: 40 ng/mL of trypsin (Promega, Madison, USA) in 5 mM octyl \( \beta \)-d-glucopyranoside (OGP) and 10 mM ammonium bicarbonate pH 7.9 at 37 °C for 18 h; 50 ng/mL of chymotrypsin (Roche Diagnostics) in 5 mM OGP and 25 mM NH\(_4\)CO\(_3\), pH 7.9 at 30 °C, for 2 h; pepsin (Sigma, Deisenhofen, Germany) in 0.1 M HCl, pH 1.0, at 37 °C for 4 h; Subtilisin (Sigma, Deisenhofen, Germany) in 6 M urea, 1 M Tris–HCl, pH 8.8, at 37 °C for 1 h; and Proteinase 10 (syn.: Thermolysin) in 25 mM Tris–HCl, pH 7.5, at 50 °C for 2 h. Peptide extraction was performed with 0.5% formic acid (FA) and 0.5% FA in 30% acetonitrile (ACN). The extracted peptides were pooled for nanoLC-ESI-CID/electron transfer dissociation (ETD)-MS/MS analysis.

### 2.4. Phosphatase treatment of spidroin-1

In order to verify the phosphorylation sites observed by MS/MS, the spots containing spidroin-1 were cut, destained and dried. Subsequently they were incubated in a solution containing 0.5 mL of calf intestine alkaline phosphatase (New England Biolabs, Ipswich, MA, USA) in 100 mM ammonium bicarbonate for 1 h at 37 °C. The spots were then washed with 100 mM ammonium bicarbonate, dehydrated in ACN and dried in a SpeedVac.

### 2.5. NanoLC-ESI-CID/ETD-MS/MS
The HPLC used was an Ultimate 3000 system (Dionex, Sunnyvale, CA, USA) equipped with a PepMap100 C-18 trap column (300 mm × 5 mm) and PepMap100 C-18 analytic column (75 mm × 150 mm). The gradient was (A — 0.1% FA in water, B — 0.08% FA in ACN) 4–30% B from 0 to 105 min, 80% B from 105 to 110 min, 4% B from 110 to 125 min. An HCT ultra ETD II (Bruker Daltonics, Bremen, Germany) was used to record peptide spectra over the mass range of m/z 350–1500, and MS/MS spectra in information-dependent data acquisition over the mass range of m/z 100–2800. Repeatedly, MS spectra were recorded followed by three data-dependent CID MS/MS spectra and three ETD MS/MS spectra generated from three highest intensity precursor ions. An active exclusion of 0.4 min after two spectra was used to detect peptides of low abundance. The voltage between ion spray tip and spray shield was set to 1500 V. Drying nitrogen gas was heated to 150 °C and the flow rate was 10 L/min. The collision energy was set automatically according to the mass and charge state of the peptides chosen for fragmentation. Multiple charged peptides were chosen for MS/MS experiments due to their good fragmentation characteristics. MS/MS spectra were interpreted and peak lists were generated by DataAnalysis 4.0 (Bruker Daltonics) as described previously by Bae et al. [26].

2.6. Protein identification and post-translational modifications

MASCOT searches were done by using the MASCOT 2.2.06 (Matrix Science, London, UK) against latest NCBI database for protein identification. Searching parameters were set as follows: taxonomy was limited to other metazoa, enzyme selected as trypsin (or corresponding enzymes), 2 maximum missing cleavage sites allowed, peptide mass tolerance was 0.2 Da for MS and 0.2 Da for MS/MS spectra, carbamidomethyl (C), methionine oxidation and phosphorylation (Y, T, S) were specified in MASCOT as fixed and variable modifications, respectively. Positive protein identifications were based on a significant MOWSE score and after protein identification, an error-tolerant search was done to detect unspecific cleavage and unassigned modifications. Protein identification and modification information returned were manually inspected and filtered to obtain confirmed protein identification and modification lists. PTM searches were done using Modiro® software with following parameters: enzyme selected as trypsin (or corresponding enzymes), 2 maximum missing cleavage sites allowed, peptide mass tolerance was 0.2 Da for MS and 0.2 Da for MS/MS spectra, carbamidomethyl (C) and methionine oxidation were specified as modification 1 and modification 2, respectively. Searches for unknown mass shifts, for amino acid substitution and calculation of significance were selected on advanced PTM-explorer search strategies. A list of 172 common modifications including phosphorylation was selected and added to virtually cleaved and fragmented peptides searched against experimentally obtained MS/MS spectra. Positive protein identification was first of all listed by spectra view and subsequently each identified peptide was considered significant based on the ion-charge status of peptide, b- and y-ion fragmentation quality, ion score (> 200) and significance scores (> 80). Protein identification and modification information returned were manually inspected and filtered to obtain confirmed protein identification and modification lists. The gel-based proteomic approach was based on a previous publication by Kang et al. [27].

2.7. Scanning electron microscopy

In order to demonstrate the dissolution of the spider silk fibers, a short length (ca. 5 mm) was put into a saturated solution of lithium thiocyanate. Then, aliquots were taken from the solution, placed on scanning electron microscopy stubs and dried on a heating plate for
several hours at 70 °C. Subsequently, stubs were coated with gold in a Cressington sputter coater using a planetary drive. Stubs were then studied in a Vega II scanning electron microscope (Tascan, Brno, Czech Republic) at 7 kV and a working distance of about 8 mm.

2.8. Analysis of o-tyrosine, 3,4-dihydroxyphenylalanine and dityrosine in hydrolysates of spider silk by HPLC applying column switching

Spider silk (1.0–1.4 mg) was hydrolyzed in 6N hydrochloric acid at 90 °C for 18 h. The volatiles were removed by rotary evaporation at 30 mbar and 60 °C and the residue was reconstituted in 400 μL of 0.1 M ammonium acetate, pH 4.8 for analysis. A chromatographic system comprising pump 1 and 2: Merck/Hitachi L-6300 (Darmstadt, Germany), autosampler: Merck/Hitachi AS-2000A, column thermostat: thermotech (Langenzersdorf, Austria), switching valve: Merck ELV 7000-2 and a detector: Merck/Hitachi fluorescence detector F-1080 were used. The reference compounds used were L-tyrosine p.a. (Sigma, Austria), o-tyrosine p.a. (Sigma, Austria), 3,4-dihydroxyphenylalanine (l-DOPA) p.a. (Serva, Austria) and dityrosine was synthesized as described by Dong-Ik et al. [28]. For analysis of o-tyrosine and 3,4-dihydroxyphenylalanine the following procedure was used: First dimension — column Purospher Star RP18, 5 μm; 250 × 4 mm i.d.; eluent A — 0,1% HCOOH, eluent B — methanol; linear gradient from 0 to 20 min, running from 95% A + 5%B to 75%A + 25%B; flow 0.8 mL/min; injection volume 40 μL; Second dimension — column Nucleosil strong acid cation exchange SA, 125 × 4 mm i.d., 10 μm particle size; eluent A — 0,1% HCOOH, eluent B — methanol; A + B = 93 + 7 (v/v); flow 1.0 mL/min; detection fluorescence 285/410 nm. For analysis of dityrosine the following procedure was used: First dimension — column Purospher Star RP18, 5 μm; 250 × 4 mm i.d.; eluent A — 0.1% HCOOH + NH₃, pH 3.0, eluent B — methanol; linear gradient from 0 to 20 min, running from 95% A + 5%B to 75%A + 25%B; flow 0.8 mL/min; injection volume 40 μL; switching interval 7.7 min–9.1 min; retention time dityrosine 8.4 min. Second dimension — column Nucleosil strong acid cation exchange SA, 125 × 4 mm i.d., 10 μm particle size; eluent A — 0.1% HCOOH, eluent B — 0.1 M HCOOH + NH₃, pH 3.7; A + B = 60 + 40 (v/v); flow 1.0 mL/min; detection fluorescence 285/410 nm.

3. Results and discussion

An experimental approach for the study of spidroin-1 was developed combining 2-DE with multiple rounds of proteolytic in-gel digestion, followed by mass spectrometry analysis using collision-induced dissociation (CID) and electron-transfer dissociation (ETD) for fragmentation.

The multiple proteolytic in-gel protein digestions followed by MS identification using two fragmentation principles allowed the generation of high-sequence coverage of spidroin-1 from the major ampullate silk produced by three species of spiders, which showed high identity among their sequences. Solubilization of silk in LiSCN represented a key step for dissolving the silk proteins and results from scanning electron microscopy analysis of major ampullate silk from *N. clavipes* revealed the ultrastructure of the silk during the dissolution process in LiSCN (Fig. 1). The complete dissolution after 60 min demonstrates the homogeneity of the sample used for proteomic analysis. Thus, solubilized major ampullate silk protein extracted from the three species of orb-web spiders *N. clavipes*, *N. edulis* and *N. madagascariensis* were processed prior to 2-DE.
Scanning electron microscopy analysis of major ampullate silk produced by *N. clavipes* spiders. A–D show the dissolution pattern of silk in LiSCN. The complete dissolution at 60 min confirmed the homogeneity of the sample.

Representative 2-DE gels of the major ampullate silk from the three species of spiders are shown in Fig. 2. Fig. 2A shows the 2-DE gel of the *N. clavipes* major ampullate silk proteins, which revealed 4 spots; Fig. 2B shows the 2-DE gel of *N. edulis* major ampullate silk proteins, which revealed 3 spots; and Fig. 2C shows the 2-DE gel of *N. madagascariensis* major ampullate silk proteins, which also revealed 3 spots. In the three gels, all spots demonstrated apparent molecular weights above 250 kDa. Protein identification by mass spectrometry was performed on all of the spots observed in the three gels. Supplementary Table S1 shows the identification of all protein spots observed in Fig. 2. The identified proteins in the *N. clavipes* gel were: spidroin-1 (spots 1, 2, 3 and 4 in Fig. 2A), GenBank ID access code P19837 and spidroin-2 (spots 3 and 4 in Fig. 2A), GenBank ID access code P46804; in the *N. edulis* gel were: spidroin-1 (spots 1, 2 and 3 in Fig. 2B), GenBank ID access code P19837 and spidroin-2 (spots 1, 2 and 3 in Fig. 2B), GenBank ID access code P46804; and in the *N. madagascariensis* gel were: spidroin-1 (spots 1, 2 and 3 in Fig. 2C), GenBank ID access code Q8WSW4 and spidroin-2 (spot 1 in Fig. 2C), GenBank ID access code Q2VLH2.
Representative 2-DE profiles of the major ampullate silk of three species of orb web producing spiders. A — *N. clavipes*, B — *N. edulis* and C — *N. madagascariensis*, stained with Coomassie Colloidal Blue.

**Figure options**

Results from 2-DE in-gel digestion using different enzymes such as trypsin, chymotrypsin, pepsin, proteinase 10 and subtilisin followed by MS identification showed high sequence coverage of spidroin-1 from the three species of spiders. **Table 1** shows the efficiency of the proteases against each spidroin-1 with a total sequence coverage of 98.06% for *N. clavipes*, 95.05% for *N. edulis*, and 98.37% for *N. madagascariensis*. Theoretical molecular masses of 61,210 Da, 56,770 Da, and 51,760 Da were determined for the spidroin-1 from *N. clavipes*,...
N. edulis, and N. madagascariensis, respectively; the higher values observed for the apparent molecular weight shown in Fig. 2 and Supplementary Table S1 may be due to cross-links or to glycosylation, known to reduce the mobility of proteins in the gel. All sequences were identified from partial sequence data in protein databanks (DBs). At present, partial sequences are available for 21 spidroins expressed in the major ampullate glands of 10 species of spiders from six different genera. The complete list of all peptide sequences assigned to spidroin-1 from the three species of spiders is provided in the supplementary information: Tables S2, S3, S4, S5, S6 and S7.

Table 1.

Sequence coverage of spidroin-1 from the major ampullate silk produced by N. clavipes, N. edulis and N. madagascariensis spiders by in-gel protein digestion using different proteolytic enzymes.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>N. clavipes</th>
<th>N. edulis</th>
<th>N. madagascariensis</th>
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<tr>
<td>Trypsin (%)</td>
<td>46.39</td>
<td>42.70</td>
<td>56.22</td>
</tr>
<tr>
<td>Chymotrypsin (%)</td>
<td>89.37</td>
<td>66.26</td>
<td>87.26</td>
</tr>
<tr>
<td>Pepsin (%)</td>
<td>41.91</td>
<td>53.25</td>
<td>35.90</td>
</tr>
<tr>
<td>Subtilisin (%)</td>
<td>Not identified</td>
<td>34.67</td>
<td>Not identified</td>
</tr>
<tr>
<td>Proteinase 10 (%)</td>
<td>Not used</td>
<td>32.53</td>
<td>Not used</td>
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<tr>
<td>Total coverage (%)</td>
<td>98.06</td>
<td>95.05</td>
<td>98.37</td>
</tr>
</tbody>
</table>

The enzyme efficiency was evaluated by combined sequence coverage obtained from all spots individually.

Table options

The sequences of spidroin-1 from the three species of spiders were aligned with each other as shown in Fig. 3. Identity values among primary sequences of these proteins were 95% and 99%, respectively, between N. clavipes and N. edulis; 89% and 95%, respectively, between N. clavipes and N. madagascariensis; and 88% and 93%, respectively, between N. edulis and N. madagascariensis. It is important to emphasize that the sequences of N. clavipes and N. edulis were identified with the same access code P19837, while the sequence of N. madagascariensis was identified with the access code Q8WSW4.
Multiple alignment among the primary sequence of the spidroin-1 from the major amullate silk of three species of orb web producing spiders *N. clavipes* (N.c.), *N. edulis* (N.e.) and *N. madagascariensis* (N.m.). The regions of identity are marked in dark blue, highlighting the highly conserved residues in three sequences; in light blue are marked only conserved residues in two sequences; and non-conserved residues in any of the three sequences are marked in white. The residues marked with an asterisk and in red color show the identified phosphorylation sites in each one of the sequences.

A series of amino acid substitutions were observed in the sequence of spidroin-1 of *N. clavipes* based upon sequences deposited in databases: G11C; Q41R; A67P; L92H; S94G; A110T; Q120K; G130R; Q136L; L164R; G165R; G170E; L187P; Q238L; R265S; E268K; G278E; L329E; G333E; Q336R; L366H; A373S; N422S; A439E; G465E; A583P; G610S; V613A; A649S; G657L; S663A; S664A; T736A and V743I. These amino acid substitutions may be due to mutations, single nucleotide polymorphism or simply reflect the change in the amino acid composition of the proteins in response to prey variation. Studies show that the
expression of spider silk proteins is altered due to the interaction between the diet, location and method of fiber collection [29] and [30], and moreover that these substitutions may alter the protein conformation and potentially even the mechanical properties of the silk fibers produced from them. Although a series of amino acid substitutions were observed, these are clearly still compatible with spider silk protein self-assembly and functional web/dragline formation.

High-sequence coverage of spidroin-1 proteins was revealed and a series of PTMs in their sequences were observed in the individual spidroins from the three spiders. Table 2 shows PTMs revealed by MASCOT and Modiro® identified on spidroin-1 from the major ampullate silk of N.clavipes, N. edulis and N. madagascariensis. A series of PTMs were observed such as phosphorylation, deamidation, dihydroxylation, methylation and oxidation. Several modifications including oxidations, deamidations and methylations may represent artifacts from sample preparation or analytical procedures. Deamidations are also known to be PTMs, and the presence of deamidases and deiminases leading to this PTM has been reported [31]. Deamidations therefore, may play a role in the spider silk storage/assembly process. Likewise, citrullination (the deamidation product of arginine) may be seen as a PTM or an artifact, and would be expected to contribute to the structure and function of the proteins [32] and [33]. Citrullination of a chemokine induced by peptidylarginine deiminase, led to changes of binding properties i.e. impaired protein–protein interactions [34]. Chen et al. [24] also identified several citrullinations in the light chain fibroin which is an important component of Bombyx mori silkworm silk; the mechanisms and functional consequences for deamidations of structural silk proteins are as yet unknown.

Table 2.

<table>
<thead>
<tr>
<th>Spot</th>
<th>Access code</th>
<th>PTMs by MASCOT</th>
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<tr>
<td>1</td>
<td>P19837</td>
<td>Phosphorylation (Y89, S260); hydroxymethyl (N128); deamidation (R161); methylation + deamidation (Q314, Q326, Q332, Q336)</td>
<td>Methylation (Q37, Q41, Q282, Q291, Q295,S742); deamidation (Q181, R585); dimethylation (R308)</td>
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<tr>
<td>2</td>
<td>P19837</td>
<td>Methylation (Q37, Q41, Q129, Q136); phosphorylation (Y59, Y151, Y353, Y387, Y478)</td>
<td>Methylation (Q37, Q41, R69, Q72, Q282, Q291, Q295) deamidation (Q181, Q252, Q611, Q621, Q633); Methylation + deamidation (Q504, Q514)</td>
</tr>
<tr>
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<td>P19837</td>
<td>Methylation (Q37, Q41); phosphorylation (S64, Y151, S260)</td>
<td>Deamidation (Q30, Q136, Q148, R161, Q181, Q336, Q350); methylation (Q95, Q282, Q291, Q295)</td>
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<tr>
<td>Spot</td>
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<td>PTMs by Modiro®</td>
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<tr>
<td>4</td>
<td>P19837</td>
<td>Deamidation (Q56, Q129, Q148, Q336, Q350, Q464); phosphorylation (Y151, S156, Y184, S462); dimethylation (R161)</td>
<td>Methylation (Q37, Q41, Q332, Q336, S742); deamidation (R69, Q72, R161, Q181, R585, Q588)</td>
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**Nephila edulis**

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<th>Spot</th>
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<th>PTMs by Modiro®</th>
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<tr>
<td>1</td>
<td>P19837</td>
<td>Deamidation (Q15, Q28, Q37, Q65, Q299, Q336, Q413, Q524, Q620, Q657); phosphorylation (Y18, Y31, Y59, S64, S156, Y184, Y285, S358, S523, S526)</td>
<td>Deamidation (Q238, Q326, R661); dihydroxylation (S641); methylation (Q41, N456, Q464, Q621)</td>
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<td>2</td>
<td>P19837</td>
<td>Deamidation (Q72, Q157, Q422, Q463, Q620)</td>
<td>Deamidation (R661); methylation (Q181, Q190, Q194); methylation + deamidation (Q37, Q41)</td>
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<td>3</td>
<td>P19837</td>
<td>Deamidation (Q15, Q167, Q399, Q524, Q620, Q656); phosphorylation (S523, S526)</td>
<td>Deamidation (Q336, R661); methylation (Q37, Q41); methylation + deamidation (Q37, Q56); trimethylation (R461)</td>
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**Nephila madagascariensis**

<table>
<thead>
<tr>
<th>Spot</th>
<th>Access code</th>
<th>PTMs by MASCOT</th>
<th>PTMs by Modiro®</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Q8WSW4</td>
<td>Oxidation (M1); phosphorylation (S21, S215); methylation + deamidation (Q346), deamidation (Q398, R557)</td>
<td>Oxidation (M1); dihydroxylation (Y134); phosphorylation (S215, Y235); methylation (Q346); dimethylation (R345); deamidation (Q83, Q181, Q319, Q384, Q507, Q517, R524)</td>
</tr>
<tr>
<td>2</td>
<td>Q8WSW4</td>
<td>Phosphorylation (S21, S51, S207, S215; S239, S373); methylation (Q346, Q353); deamidation (Q507, Q517)</td>
<td>Oxidation (M1); methylation (Q29, Q484, R26, R481); methylation + deamidation (Q43, Q407, Q411, Q498); deamidation (Q77, R557); dihydroxylation (R350)</td>
</tr>
<tr>
<td>3</td>
<td>Q8WSW4</td>
<td>Phosphorylation (S207, S215, S239, S373, Y401)</td>
<td>Oxidation (M1); phosphorylation (S21, S215); methylation (R26, Q29, Q137, R481, Q484); methylation + deamidation (Q150, Q241, Q251, Q288); deamidation (Q279, Q365, R378, Q411, Q507, Q517)</td>
</tr>
</tbody>
</table>

**Table options**

Hydroxylations of arginine on the spidroin-1 of *N. madagascariensis* (R350) leading to hydroxyarginine may contribute to protein stability in analogy to the hydroxylation of
arginine in polyphenolic adhesive proteins of the marine mussel *Mytilus edulis* [35]. Dihydroxylation on S641 in *N. edulis* spidroin-1 may in turn be responsible for preservation and protein stability of the silk protein as proposed for styelin D, an antimicrobial peptide from *Ascidian* hemocytes [36]. The underlying mechanism for hydroxylations may be hydroxylases/oxidases or hydroxyl-radical attack of the spider web in the environment in which it is produced (e.g. via exposure to UV radiation) [37] and [38].

Results from analysis of o-tyrosine, 3,4-dihydroxyphenylalanine (L-Dopa) and dityrosine in hydrolysates of spidroin-1 produced by *N. clavipes* and *N. madagascariensis* by HPLC applying column switching demonstrated that o-tyrosine was not detected in both samples (supplementary information: Fig. S4); while L-DOPA (supplementary information: Fig. S5) and dityrosine (supplementary information: Fig. S6) were detected in both samples. The relative amount of dityrosine in the spidroin-1 of *N. clavipes* was about 5 folds higher than in *N. madagascariensis*. Dityrosine as well as 3,4-dihydroxyphenylalanine observed in *N. clavipes* and *N. edulis* spidroin-1 samples may represent oxidation processes as well, although it remains unknown if the oxidation was introduced upon exposure to peroxidases at some point during its production/storage in the major ampullate silk gland or post fiber formation and exposure to UV radiation [39]. If dityrosine formation in spidroins reflects genetically predetermined cross-linking also remains to be elucidated. So far no dityrosine cross-links have been reported [5] and [40].

A large series of phosphorylation sites were detected and verified by phosphatase treatment. Fig. 3 and Table 3 show the mapping of the phosphorylation sites observed on spidroin-1 from the three species of spiders highlighting the position of these modifications in each sequence. A total of 11 phosphorylation sites at Y59, S64, Y89, Y151, S156, Y184, S260, Y353, Y387, S462 and Y478 were detected on spidroin-1 produced by *N. clavipes* spiders; a total of 10 phosphorylation sites at Y18, Y31, Y59, S64, S156, Y184, Y285, S358, S523 and S526 were detected on the spidroin-1 produced by *N. edulis* spiders; and a total of 8 phosphorylation sites at S21, S51, S207, S215, Y235, S239, S373 and Y401 were detected on the spidroin-1 produced by *N. madagascariensis* spiders. Considering the detection of PTM's in a single peptide by both, CID and ETD or in different peptides from digestion with different enzymes as shown in Table 3, out of 11 phosphorylation sites observed on spidroin-1 from *N. clavipes* spider, 8 phosphorylation sites were considered reliable at Y59, S64, Y89, Y151, Y353, Y387, S462 and Y478; out of 10 phosphorylation sites observed on spidroin-1 from *N. edulis* spider, 2 phosphorylation sites were considered reliable at Y59 and S64; and out of 8 phosphorylation sites observed on spidroin-1 from *N. madagascariensis* spider, 4 phosphorylation sites were considered reliable at S21, S215, S239 and S373. In addition, eleven peptides from different spidroin-1 spots produced by three species of *Nephila* spiders were repeats showing phosphorylation sites at Y59, S64, Y151, S156, Y184, S215, S239, S260, S373, S523 and S526.

Table 3.

Mapping of phosphorylation sites observed on spidroin-1 (major ampullate silk protein) produced by three species of *Nephila* spiders, showing the position of these modifications in each sequence.
<table>
<thead>
<tr>
<th>Phosphorylation sites</th>
<th><em>Nephila</em> sp. <em>a</em></th>
<th>Enzyme</th>
<th>Fragmentation method</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>Tryp.</td>
</tr>
<tr>
<td>Y18</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
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<td>Y31</td>
<td>x</td>
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<td>S51</td>
<td>x</td>
<td>x</td>
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<td>Y59</td>
<td>x</td>
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<td>x</td>
<td>x</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S64 (S21)</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
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<td>Y89</td>
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<td>S239</td>
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<tr>
<td>S260 (S207)</td>
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<td>x</td>
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<td>x</td>
</tr>
</tbody>
</table>

*a*
See Fig. 3 for multiple alignment among the primary sequence of the spidroin-1 from the major ampullate silk of three species of orb web producing spiders (A) *N. clavipes*, (B) *N. madagascariensis* and (C) *N. edulis*; sometimes the residue position is different from that observed for *N. clavipes*.

**Table options**

Representative CID/ETD-MS/MS spectra of spidroin-1 from the three species of spiders are given in Fig. 4. **Fig. 4A** shows a phosphorylation site at Y151 in the CID MS/MS spectrum of tryptic peptide GGQGAAAAAGGAGQGGY*GGLGSQGAGR* (134–161) of spidroin-1 from *N. clavipes* spider, selecting the m/z 811.080 [M + 3H]^{3+} as precursor ion; **Fig. 4B** shows Y151 dephosphorylation in the same tryptic peptide of spidroin-1 from *N. clavipes* spider after phosphatase treatment, selecting the m/z 784.420 [M + 3H]^{3+} as precursor ion; **Fig. 4C** shows a phosphorylation site at Y59, Y151, Y353, Y387 and Y478 in the ETD MS/MS spectrum of tryptic peptide AAAAAGGAGQGGY*GGLGSQGAGR* (47–69, 139–161, 341–363, 375–397, 466–488) of spidroin-1 from *N. clavipes* spider, selecting the m/z 1067.439 [M + 2H]^{2+} as precursor ion; and **Fig. 4D** demonstrates the presence of a phosphorylation site at S239 in the CID MS/MS spectrum of tryptic peptide GGQGAGAAAAAGGAGQGGY*GGLGSQGAGR* (216–244) of spidroin-1 from *N. madagascariensis* spider, selecting the m/z 805.370 [M + 3H]^{3+} as precursor ion. All results about phosphorylation sites detected on spidroin-1 from the three species of spiders are given in the supplementary information: Figs. S1, S2 and S3.

**Fig. 4.**

Representative CID/ETD-MS/MS spectra of spidroin-1. A — CID MS/MS spectrum of tryptic peptide GGQGAAAAAGGAGQGGY*GGLGSQGAGR* (134–161), selecting the m/z 811.080 [M + 3H]^{3+} as precursor ion, and showing the Y151 phosphorylation site observed on the spidroin-1 that was secreted by the major ampullate gland and identified in the silk produced by *N. clavipes* spiders. B — CID MS/MS spectrum of tryptic peptide GGQGAAAAAGGAGQGGY*GGLGSQGAGR* (134–161), selecting the m/z 784.420 [M + 3H]^{3+} as precursor ion, and showing the
Y151 dephosphorylation site observed on the spidroin-1 from the major ampullate silk produced by *N. clavipes* spiders after phosphatase treatment. C — ETD MS/MS spectrum of tryptic peptide AAAAGGAGQGGY*GGLGSQGAGR* (47–69, 139–161, 341–363, 375–397, 466–488) selecting the m/z 1067.439 [M + 2H]^{2+} as precursor ion, and showing the Y59, Y151, Y353, Y387 and Y478 phosphorylation sites observed on the spidroin-1 that was secreted by the major ampullate gland and identified in the silk produced by *N. clavipes* spiders. D — CID MS/MS spectrum of tryptic peptide GGQGAGAAAAAGGAGQGGYGGLGS*QGAGR* (216–244), selecting the m/z 805.370 [M + 3H]^{3+} as precursor ion, and showing the S239 phosphorylation site observed on the spidroin-1 that was secreted by the major ampullate gland and identified in the silk produced by *N. madagascariensis* spiders.

No threonine phosphorylations were observed, however, serine and tyrosine phosphorylations were the major PTMs detected in all the spidroin-1 proteins of the current study. In general, the introduction of one or more phosphate groups onto the backbone of proteins is known to induce significant conformational changes, and consequently profoundly affects protein activity and protein–protein interactions [41]. Importantly, proteomic studies revealed the presence of such PTMs on all of the proteins (i.e. heavy chain fibroin, light chain fibroins and the P25 protein) comprising *B. mori* silkworm fibroin [24] and [42], and we postulate that the phosphate residues may play important roles in the storage of the silk proteins at high concentrations in the silk gland and in the self-assembly of the proteins during the fiber spinning process [43] and [44]. It is particularly noteworthy that in vivo the transition from soluble protein to solid fibers involves a combination of chemical and mechanical stimuli (such as ion exchange, extraction of water and shear forces) [13], [45] and [46], and moreover that phosphate anions are known to promote hydrophobic interactions in other systems [40] and [46]. Michal et al. [43] reported evidence of the presence of phosphorus in the major ampullate silk fibers of *N. clavipes* obtained via 31P NMR spectroscopy of acid hydrolyzed fibers, indicating probable phosphorylation of tyrosine and serine residues of some of the proteins within the fibers, although it was not demonstrated that these modifications were specifically to spidroin-1. Likewise, Stewart et al. [47] and Addison et al. [48] have reported evidence of the phosphorylation of the serine residues of aquatic silk fibroin produced by caddisfly larvae. Interestingly, Winkler et al. [49] demonstrated that it is possible to control β-sheet assembly via enzymatic phosphorylation/dephosphorylation of short (ca. 25 kDa) recombinantly spidroin-inspired proteins produced by *N. clavipes*. The biological significance of the many phosphorylations observed on the spidroin-1 produced by these orb web producing spiders remains to be elucidated, however, in agreement with the data described in other studies, we believe that their presence may play an important role in the conformation of the proteins (particularly in their stability during storage in the silk gland), and potentially promoting interactions between the silk proteins during the natural fiber spinning process.

The unique mechanical properties of the natural spidroins rely on the alignment of some amino acid sequences (structural motifs), which form stacking of beta-sheets in an amorphous biological material, resulting in the formation of protein nanocrystals, which in turn apparently are the structural elements responsible for a significant part the mechanical properties of these proteins [19] and [50]. The sequence of spidroin-1 has GGX, (GA)\textsubscript{n}, and (A)\textsubscript{n} motifs, which in turn form the structural elements that result in ‘β-sheet stacking’; the GGX motif may be responsible the elasticity properties, while (GA)\textsubscript{n} and (A)\textsubscript{n} motifs are
related to the tensile strength of spidroin-1 [10] and [15]. A careful observation of the localization of the PTMs of spidroin-1 reveals that all the PTMs reported in the present investigation are localized exactly within these structural motifs, specially the phosphorylation and deamidation, which mostly occur at the GGX motifs. The PTMs certainly will affect the spidroin-1 conformation, and therefore the physical properties of this protein. Possibly, we can say that these PTMs may be acting in a synergistic manner in the characterization of these properties.

4. Conclusions

In this study, a diversity of PTMs present on the spidroin-1 proteins of the silk fibers were observed. Spider silk is a natural material that outperforms almost any synthetic material in its combination of strength and elastic properties. Thus, these findings for major ampullate silk spidroin-1 from Nephila spiders will address new requirements for the synthetic/recombinant production of novel spider web-based polymers. The results are also important for the design of antibodies against spidroin-1 and provide the basis for biophysical and conformational investigations of silk for biotechnological and biomedical potential applications.

Conflict of interests

The authors declare no conflict of interests.

Acknowledgments

This work was supported partially by grants from FAPESP (Proc. 2010/19051-6 and Proc. 2011/51684-1), the CNPq and the Gert Lubec Proteomics Laboratory at the University of Vienna. M.S.P. is a researcher from the National Research Council of Brazil—CNPq, provided N. clavipes samples and participated in the original design of the study. J.R.A.S.P. is a PhD student fellow from FAPESP who carried out all analytical work in the Gert Lubec Proteomics Laboratory at the University of Vienna and participated in writing the manuscript. W.Q.C. and S.H. assisted in carrying out analytical work. J.G.H. initiated the collaboration between the Scheibel and Lubec groups, sourced N. edulis and N. madagascariensis samples, assisted in writing the manuscript, and thanks the Alexander Von Humboldt Foundation for a Postdoctoral Research Fellowship. H.P. carried out scanning microscopical analyses. T.R.S. approved the manuscript and thanks both the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG SCHE 603/4-3) and the German Federal Ministry of Education and Research (Bundesministerium für Bildung und Forschung, BMBF 13 N9736) for financial support. G. Lamprecht analyzed tyrosine adducts by HPLC. G. Lubec had the project idea, designed and supervised the study and wrote the manuscript.

Appendix A. Supplementary data

Supplementary tables.
Table S1. Protein identification within the 2-DE gels from the major ampullate silk produced by *N. clavipes*, *N. edulis* and *N. madagascariensis* spiders.

Table S2. Amino acid sequence of spidroin-1 from the major ampullate silk produced by *N. clavipes* spiders by in-gel protein digestion using different proteolytic enzymes. Spot number, protein name, enzyme used, start to end, observed m/z, experimental mass, theoretical mass, delta between experimental mass and theoretical mass, number of missed cleavage sites, peptide sequences, PTMs, amino acid substitutions, fragmentation method and MASCOT ion scores were listed for all identified peptide.

Table S3. The characterization of spidroin-1 from the major ampullate silk produced by *N. clavipes* spiders by Modiro®. Spot number, protein name, enzyme used, start to end, observed m/z, theoretical m/z, delta between observed m/z and theoretical m/z, number of missed cleavage sites, peptide sequences, PTMs, amino acid substitutions, fragmentation method, Modiro® ion scores and significant scores were listed for all identified peptide.

Table S4. Amino acid sequence of spidroin-1 from the major ampullate silk produced by *N. madagascariensis* spiders by in-gel protein digestion using different proteolytic enzymes. Spot number, protein name, enzyme used, start to end, observed m/z, experimental mass, theoretical mass, delta between experimental mass and theoretical mass, number of missed cleavage sites, peptide sequences, PTMs, fragmentation method and MASCOT ion scores were listed for all identified peptide.

Table S5. The characterization of spidroin-1 from the major ampullate silk produced by *N. madagascariensis* spiders by Modiro®. Spot number, protein name, enzyme used, start to end, observed m/z, theoretical m/z, delta between observed m/z and theoretical m/z, number of missed cleavage sites, peptide sequences, PTMs, fragmentation method, Modiro® ion scores and significant scores were listed for all identified peptide.

Table S6. Amino acid sequence of spidroin-1 from the major ampullate silk produced by *N. edulis* spiders by in-gel protein digestion using different proteolytic enzymes. Spot number, protein name, enzyme used, start to end, observed m/z, experimental mass, theoretical mass, delta between experimental mass and theoretical mass, number of missed cleavage sites, peptide sequences, PTMs, fragmentation method and MASCOT ion scores were listed for all identified peptide.

Table S7. The characterization of spidroin-1 from the major ampullate silk produced by *N. edulis* spiders by Modiro®. Spot number, protein name, enzyme used, start to end, observed m/z, theoretical m/z, delta between observed m/z and theoretical m/z, number of missed cleavage sites, peptide sequences, PTMs, fragmentation method, Modiro® ion scores and significant scores were listed for all identified peptide.

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Controlling beta-sheet assembly in genetically engineered silk by enzymatic phosphorylation/dephosphorylation


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Nanoconfinement controls stiffness, strength and mechanical toughness of β-sheet crystals in silk


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