

Molecular studies of a novel dragline silk from a nursery web spider, *Euprostheno* sp. (Pisauridae)[☆]

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Abstract

Various spider species produce dragline silks with different mechanical properties. The primary structure of silk proteins is thought to contribute to the elasticity and strength of the fibres. Previously published work has demonstrated that the dragline silk of *Euprostheno* sp. is stiffer than comparable silk of *Nephila edulis*, *Araneus diadematus* and *Latrodectus mactans*. Our studies of *Euprostheno* dragline silk at the molecular level have revealed that nursery web spider fibroin has the highest polyalanine content among previously characterised silks and this is likely to contribute to the superior qualities of pisaurid dragline.

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1. Introduction

Mechanical studies of the dragline silk, reeled from four web building spiders—*Euprostheno* sp. (Pisauridae), *Latrodectus mactans* (Theridiidae), *Araneus diadematus* (Araneidae) and *Nephila edulis* (Tetragnathidae), have shown that different spider species produce draglines with rather different mechanical properties (Madsen et al., 1999; Vollrath and Knight, 2001). The silk of *Euprostheno* sp., a nursery web spider found in tropical dry habitats, is stiffer than the comparable silks of *Nephila*, *Araneus* and *Latrodectus* and requires more force to break it.

Thus far, dragline silk proteins of *Nephila*, *Araneus* and *Latrodectus* have been partially characterised at the molecular level (Xu and Lewis, 1990; Guerette et al., 1996; Gatesy et al., 2001). All silk proteins are encoded by the fibroin gene family and are composed of long series of peptide repeat motifs. Consensus ensemble repeat motifs of these proteins comprise a number of segments and contain polyalanine regions linked by glycine-rich seg-

ments. Glycine-rich regions are thought to contribute to the elasticity of the silk fibre, whereas polyalanine regions adopt a β -sheet like conformation and are believed to endow the silks with strength and stiffness (Guerette et al., 1996; Jelinski et al., 1999; Van Beek et al., 2002; Kishore et al., 2002).

Comparative Raman spectroscopy studies have indicated that the stiffer *Euprostheno* sp. silk appears to have a greater proportion of crystalline β -conformations than those of *A. diadematus* (Shao et al., 1999). The present study of *Euprostheno* sp. dragline silk at the molecular level supports this notion and reveals that pisaurid fibroin has the highest polyalanine content among all species so far subjected to analysis.

2. Materials and methods

2.1. Spiders

Euprostheno specimens were collected from the wild in Kenya, and the species name is not known. *Nephila senegalensis* spiders were reared in an environmental green house facility at the Department of Zoology, University of Oxford, UK.

All spiders were maintained under laboratory conditions in clear Perspex boxes (fed flies *Musca domestica* and

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sprayed with tap water as required). Single adult female spiders of both species were used for genomic DNA and RNA isolation. Spiders were not stimulated to spin prior to silk gland dissection.

2.2. Genomic DNA isolation and cloning of the non-repetitive 3'-end of *Euprostheno*s dragline fibroin

Genomic DNA was isolated from cephalothorax that consists of the head and the thorax combined using GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma) following the manufacturer's instructions. A polymerase chain reaction (PCR) was performed with primers based on known 3'-end sequence for *Nephila clavipes* spidroin 1 gene (S1R and S1L; Xu and Lewis, 1990). A total of 10 µl of PCR reaction mix was run on 1% agarose gel; a single band of about 400 bp was isolated from the gel, subcloned into Invitrogen pCR2.1-TOPO vector and sequenced at MWG sequencing facility, Germany.

2.3. *Euprostheno*s cDNA synthesis

Major ampullate glands for RNA preparation were dissected in ice-cold dissection buffer (100 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 10 mM Tris–pH 6.9, 10 mM EDTA and 5 U/ml RNasin Inhibitor from Promega).

Total RNA was isolated using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma) following the manufacturer's instructions. First-strand cDNA synthesis reaction was performed according to Clontech SMART™ Library Construction protocol (<http://www.clontech.com/smart/index.shtml>). A specific 3'-primer S1R (Xu and Lewis, 1990) was used instead of an oligo-dT primer in the first-strand synthesis reaction driven by Invitrogen ThermoScript RNase H⁻ reverse transcriptase. It was followed by conventional second-strand synthesis procedure.

2.4. Cloning of a partial cDNA sequence encoding the repetitive region of *Euprostheno*s major ampullate gland fibroin

Second-strand cDNA synthesis mix served as a template and Invitrogen ThermalAce Polymerase was used in subsequent PCR reactions. Primer compositions were as follows: 5'ALA-5'-GCTGCCG CAGCAGCT-3' (current study) and S1R (Xu and Lewis, 1990) served as a 3' primer. A total of 10 µl of PCR reaction mix was separated on 1% agarose gel, various size PCR products ranging from 500 to 1000 bp were isolated from the gel, subcloned into Invitrogen pCR2.1-TOPO vector and sequenced at MWG sequencing facility, Germany.

2.5. Northern blot analysis

Total RNA was isolated from major ampullate glands and cephalothorax of *Euprostheno*s and *N. senegalensis* spiders using GenElute™ Mammalian Total RNA Miniprep Kit

(Sigma) following the manufacturer's instructions. About 10 µg of total RNA was used for each loading. RNA was separated on formaldehyde gel, transferred to a Hybond-N membrane according to standard procedure (<http://www.MolecularCloning.com>) and hybridised with the following ³²P-labelled RNA template. Linearised pCR II-TOPO vector (Invitrogen) containing about 600-bp partial cDNA sequence encoding the repetitive region of *Euprostheno*s major ampullate gland fibroin served as a template for the riboprobe synthesis. The probe was generated using Promega Riboprobe® in-vitro transcription system following the manufacturer's instructions.

Hybridisation and washing conditions were as follows: hybridisation overnight at 42 °C with consequent washes in: 2 × SSC at 42 °C for 15 min, 0.5 × SSC/0.1% SDS at 60 °C for 10 min and 0.2 × SSC/0.1% SDS at 60 °C for 15 min.

3. Results and discussion

To expand the database of known spider silk sequences, we obtained a number of partial cDNA clones encoding the repetitive region of *Euprostheno*s sp. major ampullate gland fibroin, as well as a genomic clone encoding its C-terminal. Like previously published fibroin sequences from different spiders (Xu and Lewis, 1990; Guerette et al., 1996; Gatesy et al., 2001), our sequences encode a repetitive alanine- and glycine-rich protein (MaSp1-like; Xu and Lewis, 1990) (Fig. 1A). From the data gathered, the ensemble *Euprostheno*s sp. repeats are not rigidly conserved. Alanine-rich regions are sometimes interrupted by substitutions from alanine to threonine (Fig. 1A), an amino acid, which is rare in araneoid fibroins (Gatesy et al., 2001), although the transition of alanine (GCX) to threonine (ACX) requires only a single nucleotide substitution. Single or coupled alanine to valine, glycine (Fig. 1A) or serine (data not shown) changes are also present within some polyalanine regions.

Fig. 1B shows an alignment of individual repeats within the sequence given, where all polyalanine blocks terminate distinctly with a GRG or GQG triplet (in grey, Fig. 1B). Interestingly, GAGSS motif (in bold black) occurs frequently at the amino-end of every other poly-A block. It is known that two different types of the polyalanine motif exist, the (A)_n and (GA)_n types. GA repeat, with occasional S exchanged for A, is the signature of β-sheet crystals in *Bombyx mori* silk and it is also a feature of the crystal repeats inferred for spider minor ampullate fibroins (Hayashi et al., 1999; Gosline et al., 1999). Therefore, GAGSS motif found in *Euprostheno*s silk might be a strong candidate for contributing to the crystal formation. Such an addition to the poly-A stretch would produce just over 50% of crystal-forming sequence of the pisaurid repeat.

In order to generate a consensus ensemble repeat for *Euprostheno*s sp. silk, a number of partial cDNA sequences

silk suggested that repeat type II-long rather than repeat type I-short (Fig. 1C) is a more accurate reflection of its organisation.

Comparison of the ensemble repeats (Fig. 1C) reveals some other interesting characteristics. Notably, dragline silks of *Latrodectus* and pisaurid nursery spider *Euprostheno*ps can be distinguished from those of *Nephila* and *Araneus* by the presence of greater amounts of alanine with polyalanine regions of the *Euprostheno*ps being the most extensive. Long polyalanine repeats were also found in silk proteins made by lepidopteran larvae (caterpillars). For instance, the tussah silk of *Antheraea pernyi* is characterised by the presence of numerous stretches of polyalanine with 12–14 alanines in each (Sehnal and Zurovec, 2004). Interestingly, in the context of the current paper, tussah silk has been shown to be stiffer than that of the wax moth *Galleria mellonella*, which is made from a protein lacking such repeats (Fedic et al., 2003).

In case of *Euprostheno*ps silk, alanine content in polyalanine stretches of its ensemble repeat unit stands at about 42% in repeat type II (~36% in repeat type I). This could be increased even further, if GAGSS motifs were to contribute to the crystal-forming sequence. Alanine content in *L. geometricus* repeat stands at 28% in repeat type II (~24% in repeat type I), followed by *N. clavipes* at about 21% and *A. diadematus* at approximately 14%. Polyalanine regions are hypothesised to be critical for the high tensile strength of spider silk. The presence of longer polyalanine stretches in *Euprostheno*ps sp. silk might lead to higher levels of β -conformations and the highest degree of crystallinity. This is consistent with earlier mechanical studies (Madsen et al., 1999; Vollrath and Knight, 2001), where authors compared the stress–strain relationships of the dragline silks in a range of spider species. Such stress–strain curves were shown to vary greatly between dragline silks from different spiders. For instance, the estimated initial modulus (stiffness) of *Euprostheno*ps sp. dragline was three times greater than that of *N. edulis* and *A. diadematus*. There was even a larger difference in the yield stress, i.e. the stress level at which modulus falls from its high, initial value. Based on the data presented by Vollrath and Knight (2001), we estimate that the yield stress for *Euprostheno*ps dragline was about 1.2 GPa, whereas the yield stress for *Latrodectus* measured at around 0.7 GPa and less than 0.2 GPa for *Nephila* and *Araneus*. The extension to failure (failure strain) was about 0.17 (17% extension) for *Euprostheno*ps sp., whereas the failure strain of *L. mactans*, *N. edulis* and *A. diadematus* draglines was between 0.3 and 0.35. It seems likely that the greater stiffness and yield stress as well as the lower extensibility seen in *Euprostheno*ps silk compared to the other three species could be directly related to the relative proportions of β -sheet forming regions in the respective silk proteins.

Regarding the segments dominated by GGX that are implicated in the formation of elastic modules in the silk fibre (Hayashi et al., 1999), it is difficult to compare these

regions among different silks due to their variability. However, *Euprostheno*ps glycine-rich regions are comparable to glycine-rich regions of *Latrodectus*.

Previous studies have provided evidence for sequence conservation in the C-terminal region of spider silk proteins (Beckwitt and Arcidiacono, 1994). Cloning of the 3'-end of *Euprostheno*ps fibroin and the comparison of its predicted amino acid sequence with other major ampullate gland fibroins (*N. clavipes* (P19837), *A. diadematus* (AAC47010, fibroin-3) and *L. geometricus* (AAK30602)) has revealed that the C-terminal portion of all four proteins is highly conserved (Fig. 1D).

Northern blot analysis of total RNA isolated from cephalothorax (Cx) and major ampullate gland (MA) of an adult female *N. senegalensis* (Ns) and *Euprostheno*ps sp. (Es) spiders was performed in order to establish the size of the *Euprostheno*ps major ampullate gland fibroin transcript. Total RNA was hybridised with a probe generated from the repetitive region of *Euprostheno*ps fibroin (Fig. 2). It is difficult to comment on the exact size of *Euprostheno*ps dragline silk mRNA as a wide range of bands is apparent in the blot. Furthermore, it is known that spider dragline silk is composed of two fibroins that share some sequence similarity, especially in the C-terminal region (Guerette et al., 1996; Beckwitt and Arcidiacono, 1994; Hinman and Lewis, 1992). The top two bands in

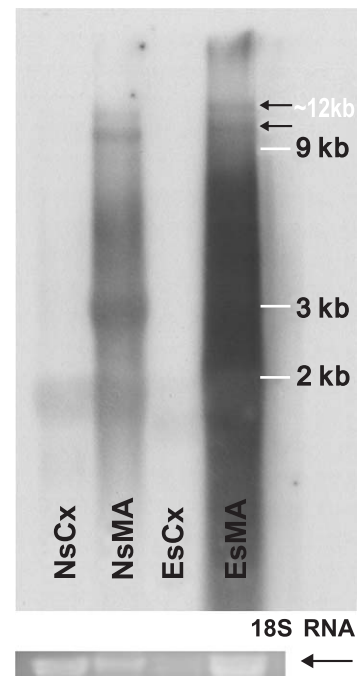


Fig. 2. Northern analysis of *Euprostheno*ps major ampullate gland fibroin. Each line contains approximately 10 μ g of total RNA isolated from cephalothorax (Cx) and major ampullate gland (MA) of an adult female *Nephila senegalensis* (Ns) and *Euprostheno*ps sp. (Es) spiders and hybridised with a probe generated from the repetitive region of *Euprostheno*ps major ampullate gland fibroin cDNA. Lower panel represents UV photograph of 18S RNA fraction and serves as a loading control.

Fig. 2, indicated with arrows, are most likely to represent full size MA fibroin transcripts. A similar pattern was observed, when the total RNA from *N. senegalensis* and *Euprosthenops* sp. spiders was hybridised with a probe corresponding to the highly conserved C-terminal portion of the gene (data not shown), indicating that the size of *Euprosthenops* fibroin mRNA is comparable to that of *Nephila*. Currently, there appears to be no consensus in the literature on the exact molecular size of two major protein components (MaSp1 and MaSp2) of *Nephila* spp. dragline silk. The latest records indicate that the size of *Nephila* spp. major ampullate gland spidroin 1 (MaSp1) mRNA is in the region of 12.0 kb and the size of MaSp2 transcript is estimated to be about 11.5 kb (Hinman and Lewis, 1992). Therefore, based on the comparative Northern blot analysis (Fig. 2), the size of the full mRNA transcripts for *Euprosthenops* sp. major ampullate gland fibroins also appears to be in the region of 12 kb.

We notice a number of other bands in the figure. One possible explanation could be the fibroin RNA degradation during isolation. Indeed, mRNAs of such a large size are typically more susceptible to damage during preparation. However, additional expression studies on *N. senegalensis* (Pouchkina et al., 2003), where the very same blot was hybridised with a number of other probes for *N. senegalensis* peroxidase (NsPox) and an ATPase, revealed no substantial degradation of the transcripts corresponding to these genes. An alternative explanation for this pattern is that targeted degradation of fibroins might be taking place in spider silk glands. It is known that when the reservoir of a silk gland becomes depleted, new synthesis of fibroin starts within minutes (Foelix, 1996). Obviously, when the reservoir is full there is no need in further accumulation of the silk transcripts, which could result in removal of such mRNAs by targeted RNA degradation. We note that the spiders in this study were not stimulated to spin silk prior to dissection and therefore the glands were not depleted of their contents. Further investigations are required in order to distinguish between isolation related degradation and targeted degradation of silk fibroins in vivo.

Molecular studies of a novel dragline silk fibroin from *Euprosthenops* sp. have further shown that a number of amino acid motifs are retained among araneoid and non-araneoid fibroins. This supports previously published data on the conservation and convergence of spider silk fibroin sequences, suggesting that the sequences themselves play a role in determining the elasticity and strength of different silk fibres (Gatesy et al., 2001). Indeed, several authors have suggested that the structure of silk protein largely determines the macroscopic mechanical properties of the silk fibre (Hinman and Lewis, 1992; Gosline et al., 1999; Hayashi and Lewis, 2000). On the other hand, the final fibre characteristics are also thought to be dependent on the silk spinning and drawing process (Calvert, 1998; Vollrath, 1999; Shao and Vollrath, 2002). For instance, it has been

shown that alterations in spinning conditions can have a direct effect on silk fibre formation and properties (Vollrath and Knight, 2001; Shao and Vollrath, 2002), though it is unclear whether the differences due to spinning conditions might also reflect differences in silk diameter. Although spinning and two-protein fibre formation of the dragline silk no doubt have an impact on the qualities of the dragline thread, our results suggest that differences in silk primary structure are likely to contribute to the mechanical properties of the silk fibre.

The four spiders compared in this analysis belong to different families and are genetically separated by many million years, but all use their major ampullate silks in web construction. The European garden cross spider (*Araneus*) and golden silk spider (*Nephila*) are both orb web weavers and build short-term (1–5 days) two-dimensional orb webs, whereas the North American black widow (*Latrodectus*) and African nursery spider (*Euprosthenops*) construct long-term (2–3 months) three-dimensional webs (Madsen et al., 1999).

Whilst short-lived orb weaver webs are specialised for prey capture incorporating highly elastic dragline silk in the struts and extremely sticky capture spiral silk to immobilise prey, the nursery web spider builds a long-lasting rigid web, senses the presence of prey through vibrations in the silk and captures the prey by running rapidly across the web. In addition, female *Euprosthenops* construct a protective nursery web around the egg case, which then is attached to vegetation with greater requirements for toughness than for elasticity. It seems likely that these functional requirements have selected for more elastic dragline silks in the orb weavers and more rigid silks in the nursery web spider.

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