Spider Minor Ampullate Silk Proteins Are Constituents of Prey Wrapping Silk in the Cob Weaver *Latrodectus hesperus*[†]

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ABSTRACT: Spiders spin high performance fibers with diverse biological functions and mechanical properties. Molecular and biochemical studies of spider prey wrapping silks have revealed the presence of the aciniform silk fibroin AcSp1-like. In our studies we demonstrate the presence of a second distinct polypeptide present within prey wrapping silk. Combining matrix-assisted laser desorption ionization tandem time-of-flight mass spectrometry and reverse genetics, we have isolated a novel gene called *MiSp1-like* and demonstrate that its protein product is a constituent of prey wrap silks from the black widow spider, *Latrodectus hesperus*. BLAST searches of the NCBInr protein database using the amino acid sequence of MiSp1-like revealed similarity to the conserved C-terminal domain of silk family members. In particular, MiSp1-like showed the highest degree of sequence similarity to the nonrepetitive C-termini of published orb-weaver minor ampullate fibroin molecules. Analysis of the internal amino acid sequence of the black widow MiSp1-like revealed polyalanine stretches interrupted by glycine residues and glycine-alanine couplets within MiSp1-like as well as repeats of the heptameric sequence AGGYGQG. Real-time quantitative PCR analysis demonstrates that the *MiSp1-like* gene displays a minor ampullate gland-restricted pattern of expression. Furthermore, amino acid composition analysis, coupled with scanning electron microscopy of raw wrapping silk, supports the assertion that minor ampullate silks are important constituents of black widow spider prey wrap silk. Collectively, our findings provide direct molecular evidence for the involvement of minor ampullate fibroins in swathing silks and suggest composite materials play an important role in the wrap attack process for cob-weavers.

The ability of spiders to spin multitask fibers with a wide range of mechanical features is a distinctive feature of the diverse order Araneae (>37,000 described species) (*1*). Araneoid spiders use specialized abdominal glands to manufacture distinct fibers types and glues (*2*). Spinning high performance spider threads with different mechanical properties facilitates locomotion, reproduction, protection of developing offspring, and prey capture (*3*).

Analyses of amino acid sequences of spider silk proteins have revealed common molecular architectures (*4, 5*). Repeats of four fundamental motifs have been identified in the spider silk proteins: (i) alternating glycine and alanine couplets [$(GA)_n$], (ii) polyalanine blocks of 6–9 residues [$(A)_n$], (iii) GGX (X = any amino acid) modules, and (iv) GPGGX repeats. Biophysical studies have demonstrated that the polyalanine or glycine-alanine couplet repeats form β-sheet structures in the solid fibers (*6–8*), whereas the GPGGX repeats have been hypothesized to form β-spirals that function to provide fiber elasticity (*9*). Protein structural modeling supports this assertion and implies that GPGGX repeats produce a series of consecutive β-turns that form winding, helical structures (*10*). More recently it was proposed that the repeats of GPGGX modules provide a proline-rich environment, which increases a fiber's sensitivity to water and promotes supercontraction of the major ampullate (MAA[1]) silks (*11*).

Several different fibroins that are selectively expressed in specialized abdominal glands of spiders have been elucidated. The most extensively studied spider silk is dragline silk (major ampullate silk) from the orb weaver *Nephila clavipes*. Minor ampullate (MIA) silks are related, but their molecular characterization has lagged behind that of MAA silks. From a mechanical property perspective, both major and minor silks have high tensile strength, but MIA silks irreversibly deform when stretched while MAA silks are more elastic (*1, 12*). Immunological data support the presence of the silk proteins

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[1] Abbreviations: CAD, collision activated dissociation; MIA silks, minor ampullate silks; MAA silks, major ampullate silks; ORF, open-reading frame; MS, mass spectrometry; GdnHCl, guanidium hydrochloride; MiSp1-like, minor ampullate silk-like spidroin; BLAST, basic local alignment and search tool; AcSp1-like, aciniform spidroin 1-like; MALDI, matrix-assisted laser desorption/ionization.

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MaSp1 and MaSp2 in orb-weaver dragline silks (*13*), and it has been postulated that the MiSp1 and MiSp2 fibroins are constituents of MIA silks (*14*); however, no direct experimental evidence has been published to demonstrate that MiSp1 or MiSp2 products are spun into MIA silks. In the silk community, it has been widely accepted that MIA silks are used for temporary spiral scaffolding (web building), but other ecological functions are unknown (*15, 16*).

Recently, some of the molecular constituents of tubuliform silks have been determined (*17–19*). For tubuliform silks, mass spectrometric analyses have demonstrated that one of the major constituents of the cob-weaver tubuliform silks is TuSp1 (*17*). MS/MS analyses of tryptic peptides obtained from egg case and prey wrapping silks have also revealed the presence of a common aciniform fibroin, AcSp1-like (*20*). Although it has been experimentally demonstrated that AcSp1-like is an important constituent of prey wrapping silk, the predicted amino acid composition of AcSp1-like does not closely align with the amino acid composition profile of raw wrapping silk (*20*). Chemical analyses of black widow spider prey wrapping silk reveals substantial percentages of glycine and alanine, two amino acids that are overrepresented in major and MIA silks. Since high amounts of glycine and alanine have been reported within prey wrapping silks from the black widow spider, we hypothesized that the major and/or minor ampullate fibers play an important role during the immobilization of prey for black widow spiders. Here, we demonstrate, by mass spectrometry and reverse genetics, the presence of a minor ampullate-restricted gene product named MiSp1-like that is assembled into prey-capture wrapping threads. These findings provide new insight into the molecular properties of MIA silks and reveal a novel role for this silk type that extends beyond the traditional application of MIA silks and their usage in web construction. Characterization of the molecular constituents of the different silk types and their functional applications will increase our understanding on the evolution of spider silks.

EXPERIMENTAL PROCEDURES

Tryptic Digests of Dissolved Silk Proteins Collected from Wrapped Cricket. Female black widow spiders were fed a single cricket each week. Silk fibers from freshly wrapped crickets (12 h after feeding) were collected using sterile scissors and forceps with the aid of a dissecting microscope (Leica MZ 16). Approximately 10 μg of wrapping silk was dissolved in 100 μL of 8 M GdnHCl. Spidroin solubilization was facilitated by vortexing for 20 min at room temperature, followed by heating at 95 °C for 10 min. Prior to trypsin digestion, the sample was neutralized with 50 mM NH_4HCO_3 (pH = 7.8). Thirty micrograms of trypsin (Trypsin Gold, Promega) was added, and the sample was incubated at 37 °C for 8 h. Peptides were extracted and desalted using a C18 Zip-Tip according to the manufacturer's instructions.

Mass Spectrometric Analysis. Conventional mass spectra were obtained with a MALDI/TOF/TOF mass spectrometer (4800 Proteomics Analyzer, Applied Biosystems, Foster City, CA) operated in reflector mode. MS/MS spectra were acquired by operating the 4800 mass spectrometer in MS/MS mode. Samples from the in-solution tryptic digest of wrapping silk were separated by HPLC (Magic C18 5u, 200A, 0.15 × 150 mm column, 5–35% acetonitrile in water in a 35 min gradient), and fractions were collected onto a MALDI plate, which was subsequently analyzed using the mass spectrometer. Both MS and MS/MS (where appropriate) spectra were collected for each spot. *De novo* peptide sequences were obtained by manual interpretation of the high energy CAD spectra.

Cloning of the MiSp1-like Gene. A partial nucleic acid sequence of MiSp1-like was obtained during a search for cDNAs that encode aqueous glue components; these components coat the web of black widow spiders (*21*). Briefly, using anchored PCR, the forward primer (5′-ATG TAT GCT CGA GTA TTG GTT TTC G-3′; encodes MYARVLVFV) together with the reverse primer from the pGAL4-AD library vector (5′-GAT CAG AGG TTA CAT GGC CAA GAT TGA AAC-3′) led to the amplification of a 849 bp fragment. Products were gel purified using the QIAquick gel extraction kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions, ligated into the pcDNA3.1/V5-His TOPO vector and then transformed into *Escherichia coli*. Transformants were grown overnight and plasmid DNA was retrieved and subject to DNA sequencing as previously described (*22*). Translation of the cDNA, followed by BLAST searches, revealed that this segment encoded the C-terminus of a silk fibroin. To obtain a larger cDNA fragment, the 849 bp piece of nucleic acid encoding MiSp1-like was removed from the pcDNA3.1/V5-His vector using the restriction enzymes *Eco*RV and *Bam*HI and used as a probe to screen our cDNA library by conventional nucleic acid−nucleic acid hybridization. The cDNA library, which is a composite library, was constructed using silk-producing glands from the major ampullate, minor ampullate, tubuliform, flagelliform, aciniform and pyriform glands (*23*).

Real-Time PCR Analysis of MiSp1-like in Silk-Producing Glands of the Black Widow Spider. Reverse transcription reactions were performed as previously described (*24*). Real-time PCR fluorescence detection was monitored using an Opticon II instrument (MJ Research Inc.). Amplification products were monitored with SYBR Green (Bio-Rad) detection and routinely checked using dissociation curve software and agarose gel electrophoresis. Primers used for the analysis of MiSp1-like expression levels were the forward and reverse oligonucleotides 5′-TGG AGC AGG AGG TTA TGG-3′ and 5′-TCC TAA AGC TGC CCC AAG TGA-3′, respectively. To avoid the potential for amplification of other silk family members, these primers were designed to target regions away from the conserved, nonrepetitive C-terminus coding region. Oligonucleotides used for normalization were the forward and reverse β-actin primers 5′-CCC TGA GAG GAA GTA CTC CGT-3′ and the 5′-ATC CAC ATC TGC TGG AAG GTG-3′, respectively.

Scanning Electron Microscopy. Wrapping silk was coated to a thickness of 14–30 nm with gold alloy in a Pelco SC-7 autosputter coater with an FTM-2 film thickness monitor. Several different crickets immobilized in swathing silk were analyzed. Strand diameters were measured on a Hitachi S-2600 SE operated with an accelerator voltage of 3 kV. Diameters of the strands were measured to the nearest 0.01 μm at three distant places in the sample. Experiments were conducted at ambient temperature and humidity, which were 25 °C and 30–36%, respectively.

Table 1: *De Novo* Sequences Obtained by Mass Spectrometry[b]

peptide mass (M^+H)	sequence	identity
1178.8	LLQALVPALLK	AcSp1-like
1206.6	AVHHYEVPVR	SCP-1
1295.7	SLASTLASSGVFR	AcSp1-like
1423.8	LASNLGLDYATASK	AcSp1-like
1462.7	(GAG/GQ)GAGAGAGAGAGAGGYGR	unknown
1555.8	TLFNQAADILDHVV	SCP-2
1593.7	SAGAGAGAGAASGAGAGGYGR	unknown
1668.9	VSTPVAVQLTDALVQK	AcSp1-like
1746.8	**AGGYGQGQGAGAAAGAAAGAGR**	**MiSp1-like[b]**
1788.9	AGGYGQGQVAGAAAGAAAGAGR	unknown
1872.9	ATQALSSVSADSDSTAYAK	AcSp1-like
2081.0	. . .MDDLSSLSDTLLSALER	unknown
2104.0	. . .**GSGSASVSTGGYGQSQVAR**	**MiSp1-like[b]**
2372.1	. . .GAGAAAAGAAGAGAGGYGQGAGGYGR	unknown
2434.1	. . .VWDSTATAEAFLGSFNS. . .	unknown
2751.3	. . .AGAGAGAGAAAGAAASGAGGYGQGAGGYGR	unknown
3332.5	**AGGYGQGAGGYGQGQGAGAAAGAAAGAGAGGYGQGAGGYGR**	**MiSp1-like[b]**

[a] MS/MS analyses of tryptic fragments generated from the in-solution digest of solubilized wrapping silk from *L. hesperus* support the presence of the AcSp1-like protein, SCPs, and other fibroins. Parentheses indicate residues that could not be conclusively determined by the MS/MS analysis. The N-terminus of peptides giving ions at *m/z* 2081.0, 2104.0, 2372.1, 2434.1, and 2751.3 could not be successfully determined by the MS/MS analysis. However, theoretical digestion of the predicted amino acid sequence of MiSp1-like generated a peptide with the same mass and sequence as the peptide giving the ion at *m/z* 2104.0. Sequences in bold case indicate peptides that were identical to those found in the translated sequence of MiSp1-like. AcSp1-like was previously reported (*20*) as well as SCP-1 and SCP-2 (*21*). [b] Sequences were later found to match regions within the predicted amino acid sequence of MiSp1-like.

Amino Acid Composition of Wrapping Silk, Major Ampullate Glands, and Minor Ampullate Glands. Wrapping silk fibers were subjected to amino acid analysis at the Protein Chemistry Laboratory of Texas A & M University as previously described (*25*). Wrapping silk and luminal fluids collected from the major and minor ampullate glands were hydrolyzed with 6 M HCl to produce the constituent amino acids. Luminal fluids were collected by treating the major and minor ampullate glands with 2% SDS, followed by boiling at 95 °C for 10 min. Proteins were precipitated using 2 volumes of acetonitrile. Residual traces of acetonitrile were removed by speed vacuum at medium heat setting for ~20 min. Amino acids were derivatized with *o*-phthalaldehyde and 9-fluoromethylchloroformate, followed by separation by reversed phase high performance liquid chromatography with UV detection.

RESULTS

Tryptic Digest of Solublized Wrapping Silk and Mass Spectrometric Sequence Analysis. To investigate whether major or minor ampullate fibroins were present within swathing silk, we dissolved wrapping silk in 8 M GdnHCl and digested the protein mixture with trypsin, and then used MALDI-MS analysis to examine the fingerprint of tryptic peptides. MS analysis of the tryptic products yielded a complex spectrum (data not shown). Samples from the in-solution digest of wrapping silk were further purified using HPLC, and 17 precursor ions were selected and sequenced using high energy CAD (Table 1). Peptide sequences of the peptides yielding precursor ions *m/z* 1178.8, 1295.7, 1423.8, 1668.9, and 1872.9 (MH^+, monoisotopic) were determined to be identical to regions found within the translated cDNA sequence of AcSp1-like after theoretical digestion with trypsin (Table 1), confirming a previous report that the AcSp1-like product is a constituent of wrapping silk (*20*). Additionally, MS/MS analysis of the two precursor ions of *m/z* 1206.6 and 1555.8 revealed that the corresponding peptides were derived from the aqueous glue coating proteins, SCP-1 and SCP-2, respectively (Table 1) (*21*). BLAST searches of the NCBInr protein database using the amino acid sequences of the peptides giving ions at *m/z* 1462.7, 1593.7, 1746.8, 1788.9, 2081.0, 2104.0, 2372.1, 2434.1, 2751.3 and 3332.5 revealed no significant similarities to other silk proteins in the database, including the full-length protein sequences of *L. hesperus* MaSp1 and MaSp2. The product ion spectra of two peptides, whose MH^+ ions were at *m/z* 1746.8 and 2104.0, are shown in Figure 1, A and B, respectively. Manual inspection of the sequences obtained by MS/MS showed that the peptides giving ions at *m/z* 1462.7, 1593.7, 1746.8, 1788.9, 2104.0, 2372.1, 2751.3 and 3332.5 were extremely rich in the amino acids glycine and alanine, whereas the peptides giving ions at *m/z* 2081.0 and 2434.1 contained significantly lower levels of glycine and alanine (Table 1).

Cloning of a Novel Minor Ampullate Fibroin cDNA. Since BLAST searches of the NCBInr protein database with the sequences of peptide ions at *m/z* 1462.7, 1593.7, 1746.8, 1788.9, 2104.0, 2372.1, 2751.3 and 3332.5 failed to match regions within the full-length sequences of *L. hesperus* MaSp1 and MaSp2 or the partial sequence of AcSp1-like, we hypothesized that these products could be derived from a distinct fibroin(s). On a previous search for cDNA sequences encoding aqueous glue proteins from *L. hesperus*, we fortuitously isolated a cDNA fragment that encoded a product with similarity to the nonrepetitive, conserved C-terminal region of the silk family. This sequence was distinct from the C-terminal regions of *L. hesperus* MaSp1, MaSp2 and AcSp1-like, and represented a potentially new fibroin (data not published). To obtain a larger fragment of cDNA, we screened our composite silk gland cDNA library based upon nucleic acid−nucleic acid hybridization. During this screen, we retrieved several positive clones that were subject to DNA sequence analysis. The largest clone carried a cDNA fragment sized at ~1.6 kb. Translation of the ~1.6 kb cDNA sequence indicated that this fragment contained a long open reading frame (ORF), encoding the C-terminal

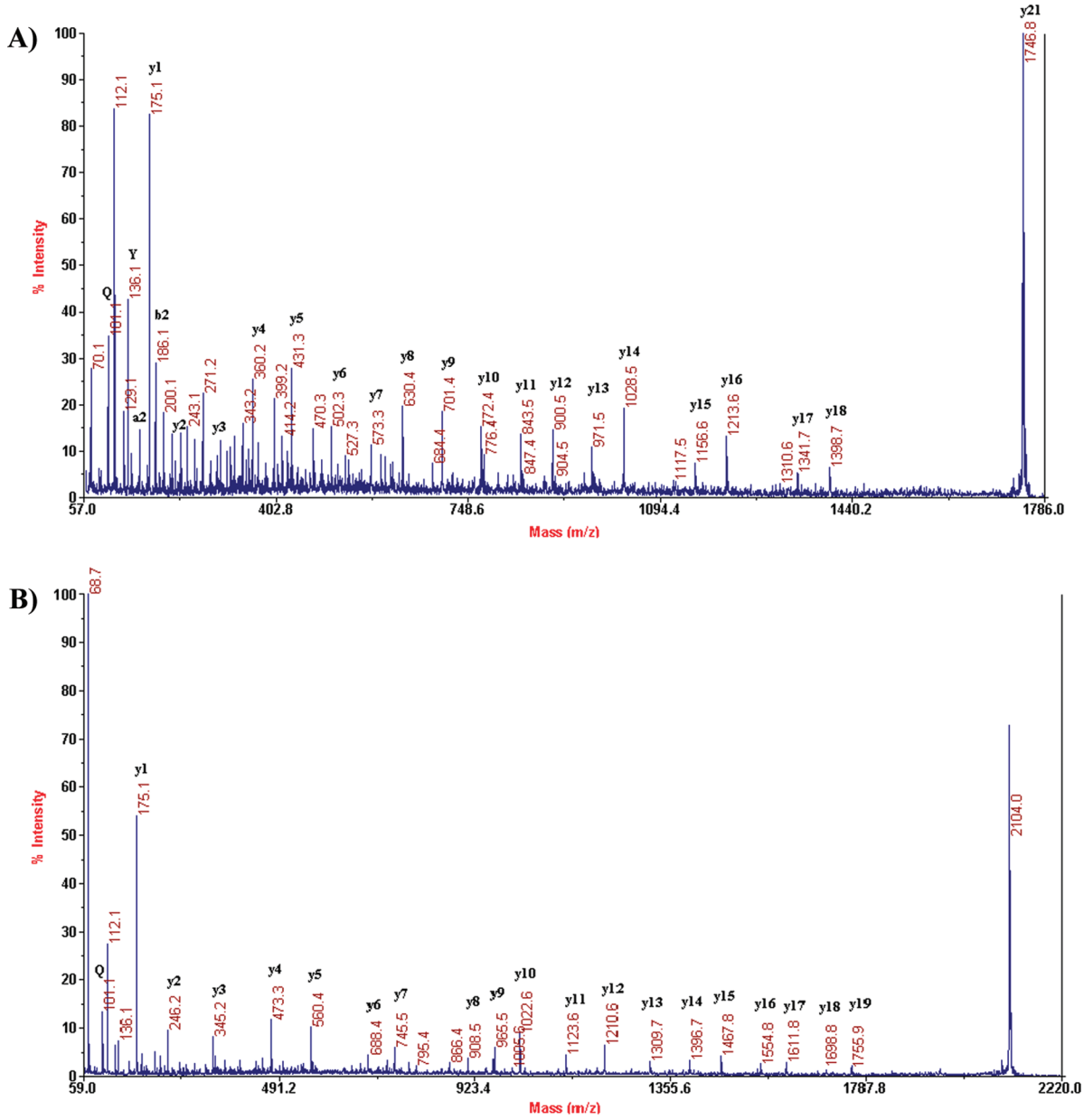

FIGURE 1: MALDI tandem TOF spectra of ions from tryptic peptides generated from the in-solution digestion of wrapping silk from *L. hesperus*. (A) High energy CAD (MS/MS) spectrum of the precursor ion with *m/z* 1746.8. (B) High energy CAD (MS/MS) spectrum of the precursor ion with *m/z* 2104.0.

479 amino acids of a spider fibroin (Figure 2A; GenBank accession number EU394445). This protein had a predicted molecular mass of ~40 kDa and a pI of 9.70. Several other clones carrying shorter cDNAs with nearly identical nucleotide sequences were also retrieved during the library screen (data not shown). Nucleic acid−nucleic acid BLAST searches against the ~1.6 kb nucleotide sequence revealed that the strongest match was to minor ampullate spidroin-like mRNA (*Nephilengys cruentata*, GenBank accession number EF638447; E value = 1.0×10^{-89} or 68% identical over 933 nucleotides). Based upon the sequence similarity and pattern of expression (Figure 3), we have named our product *Latrodectus hesperus* MiSp1-like, which is derived from the abbreviation minor ampullate spidroin 1-like. In protein–protein BLAST searches against the entire predicted amino acid sequence, the top matches corresponded to two published minor ampullate fibroins (*Nephila clavipes* MiSp, GenBank accession number AF027736; *Nephila antipodiana* MiSp1, GenBank accession number DQ338462). As expected for fibroin family members that share a nonrepetitive conserved C terminus, *L. hesperus* MiSp1-like was found to be 66% identical to MiSp1-like from *N. cruentata* (69/104) and 63% identical to MiSp1 from *N. antipodiana* (66/104 identities) and MiSp from *N. clavipes* (66/104 identities) (Figure 2B). *L. hesperus* MiSp1-like was found to contain alanine residues, but the characteristic alanine blocks (6–8 residues) embedded within the ensemble repeat sequences of major ampullate fibroins were absent (Figure 2A). GA couplets were very prominent, and the heptameric sequence AGGYGQG was found 13 times (Figure 2A). Analysis of the C-terminus revealed a serine-rich region as well as indicated that MiSp1-like lacked the conserved Cys residue found in many of the major ampullate silk fibroin family members (Figure 2A).

Two of the peptides sequenced by MS/MS (*m/z* 3332.5 and 1746.8) showed 100% identity to the translated region

A)

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PRAGGYGRGQ GAGAGVGAGA GAAAGAAAIA RAGGYGQGAG GYGQGQGAGA AAGAAAGAGA 60
GGYGQGAGGY GRGQGAGAGA GAGAGARGYG QGAGAGAAAG AAASAGAGGY GQGAGGYGQG 120
QGAGAAAGAA ASAGAGGYGQ GAGGYGQGQG AGAGAGAGAG AGGYGQGSGA GAAAGAAASA 180
GAGGYGQGAG GYGRGQGASA GAGAGAGTGG YGQGAGAGAA AGAAASAGAG GYGQGAGGYG 240
QGTGAGAGGY GQGTGAGAAA GAGASAGARG YGQRAGGYGQ GQGAGAAAGA AAGAGRYGQG 300
SGSASVSTGG YGQSQVARAS SSSAVGTSSS VSTSGSSGYS QVSGGYGQSS AVGQASAGYG 360
QMQSGVAVSG GSASATISSA ASRLSSPSSS SRISSAASSL ATGGVLNSAA LPSVVSNIMS 420
QVSASSPGMS SSEVVIQALL ELVSSLIHIL SSANIGQVDF NSVGNTAAVV GQSLGAALG  479
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B)

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N ant MiSp1          TVASTTSRLSTAEASSRISTAASTLVSGGYLNTAALPSVIADLFAQVGASSPGVSDSEVL
N cla MiSp           TVTSTTSRLSSAEACSRISAAASTLVSG-SLNTAALPSVISDLFAQVSASSPGVSGNEVL
N cru MiSp1-like     TVASTTSRLSSAQASSRISAAASTLISGGYLNTSALPSVISDLFAQVSASSPGVSDSEVL
L hes MiSp1-like     TISSAASRLSSPSSSSRISSAASSLATGGVLNSAALPSVVSNIMSQVSASSPGMSSSEVV
                     *::*::****:..:.****:***:* :*  **::*****::::::**.*****:*..**:

N ant MiSp1          IQVLLEIVSSLIHILSSSSVGQVDFSSVGSSAAAVGQSMQVVMG
N cla MiSp           IQVLLEIVSSLIHILSSSSVGQVDFSSVGSSAAAVGQSMQVVMG
N cru MiSp1-like     IQVLLEIVSSLIHILSSSSVGQVDFNSVGSSAAAVGQSMQVVMG
L hes MiSp1-like     IQALLELVSSLIHILSSANIGQVDFNSVGNTAAVVGQSLGAALG
                     **.***:**********:.:*****.***.:**.****: ..:*
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FIGURE 2: The amino acid sequence of MiSp1-like shows similarity to the spider silk family. (A) Translation of the nucleotide sequence from the MiSp1-like cDNA contains an open reading frame (ORF). The longest ORF encodes a protein 479 amino acids in length. Peptide sequences determined by MS/MS (in-solution digest products of wrapping silk) that are found within the ORF are indicated with the gray shading. The boxed region denotes the nonrepetitive C terminus. Underlined regions represent the heptameric sequence AGGYGQG. (B) Alignment of minor ampullate fibroin C-terminal sequences was performed using the computer algorithm CLUSTALW. Amino acids are represented by one-letter abbreviations; the hyphen indicates a gap. The sequences are identified as *N ant* (*Nephila antipodiana*) MiSp1 (GenBank accession number DQ338462), *N cla* (*Nephila clavipes*) MiSp (AF027736), *N cru (Nephilegys cruentata)* MiSp1-like (EF638447) and *L hes* (*Latrodectus hesperus*) MiSp1-like (EU394445). Identities are shown in shades of gray or asterisks; colons (:) represent side chain groups with similar polarity and size; periods (.) indicate residues with similar polarity or R-group size.

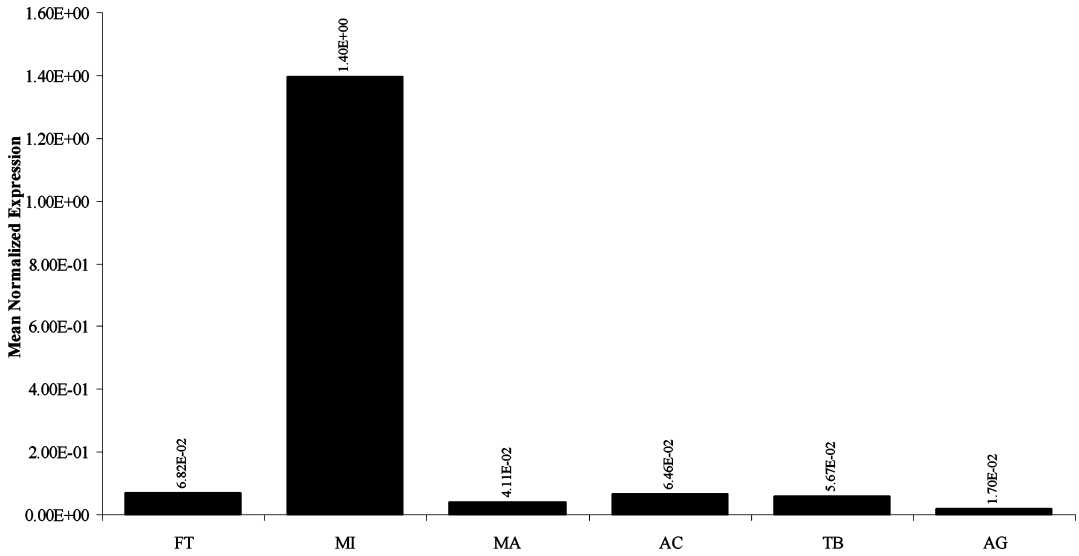

FIGURE 3: MiSp1-like mRNA is expressed in a tissue-restricted pattern. Real time quantitative PCR was used to determine the expression pattern of MiSp1-like in a variety of different tissues. Total RNA was isolated from fat (FT), the minor ampullate gland (MI), the major ampullate gland (MA), aciniform (AC), tubuliform (TB) and aggregate glands (AG). Equivalent quantities of total RNA were reverse-transcribed using Moloney murine leukemia virus and aliquots used for real time quantitative PCR. Samples were analyzed in triplicate and normalized internally using the black widow actin mRNA. Data are representative of experimental results obtained from two independent trials.

of the MiSp1-like cDNA (Table 1 and Figure 2A). Peptides giving ion masses at *m*/*z* 3332.5 and 1746.8 were 41 and 22 residues long, respectively. The peptide giving rise to *m*/*z* 2104.0, which could not be sequenced at its N-terminus by MS/MS, was found to match a peptide mass from the MiSp1-like protein after theoretical digest with trypsin (Table 1 and Figure 2A). Collectively, the presence of three distinct peptides in wrapping silk, all which have sequences identical to regions found within the translated MiSp1-like cDNA sequence, indicates that the MiSp1-like mRNA molecules are efficiently translated into fibroins that are assembled into wrapping silk.

Expression of MiSp1-like mRNA Is Restricted to the Minor Ampullate Gland. We performed real time quantitative PCR analysis to examine the mRNA expression profile of *L. hesperus* MiSp1-like in a variety of different silk-producing

Table 2: Predicted Amino Acid Composition of Raw Wrapping Silk (Untreated), Minor and Major Ampullate Luminal Contents, Aciniform Luminal Contents, and the Predicted Sequences of MiSp1-like, AcSp1-like, MaSp1, and MaSp2 of *L. hesperus*[a]

	Gly	Ala	Glx	Tyr	Pro	Arg	Asx	Ser	Leu	Ile	Thr	Val	Phe
raw wrapping silk of *L. hesperus* ($n = 4$)	31.2 ± 5.2	25.8 ± 4.9	10.4 ± 0.6	3.8 ± 0.7	4.0 ± 1.7	2.5 ± 0.5	3.1 ± 1.1	5.7 ± 1.4	1.9 ± 0.7	2.0 ± 1.0	2.8 ± 1.2	1.8 ± 0.7	1.0 ± 0.4
predicted MiSp1-like of *L. hesperus*	32.6	26.3	7.7	5.0	0.8	2.5	1.2	12.7	2.3	1.7	1.9	4.2	0.2
% deviation of raw wrapping silk and predicted MiSp1-like from *L. hesperus*	−1.4	−0.5	2.7	−1.2	3.2	0	1.9	−7.0	−0.4	0.3	0.9	−2.4	0.8
minor ampullate glands of *L. hesperus* ($n = 2$)	33.3 ± 1.0	28.2 ± 1.0	8.3 ± 0.1	5.7 ± 0.3	0.6 ± 0.2	3.0 ± 0.1	2.2 ± 0.4	7.4 ± 0.6	1.3 ± 0.4	1.5 ± 0.5	3.9 ± 0.2	2.2 ± 0.2	1.1 ± 0.5
major ampullate glands of *L. hesperus* ($n = 3$)	40.0 ± 0.1	28.6 ± 0.3	10.8 ± 0.5	5.2 ± 0.2	2.5 ± 0.4	2.4 ± 0.1	1.3 ± 0.4	3.7 ± 0.1	1.0 ± 0.2	0.9 ± 0.2	1.4 ± 0.3	0.8 ± 0.1	0.6 ± 0.1
predicted AcSp1-like of *L. hesperus*[b]	11.5	12.9	6.7	0	3.7	3.2	10.5	14.7	10.9	5.2	5.2	8.0	2.0
PLS and PMS aciniform silk glands of *L. hesperus* ($n = 1$)	11.6	12.8	6.8	1.9	4.5	3.5	9.8	13.7	10.1	5.2	5.9	6.8	2.6
predicted MaSp1 of *L. hesperus* (EF595246)[b]	42.3	32.7	11.5	4.9	0.4	1.6	1.0	2.0	0.7	0.6	0.8	0.6	0.4
predicted MaSp2 of *L. Hesperus* (EF595245)[b]	33.5	31.1	7.4	5.2	8.6	1.8	0.9	7.1	0.7	0.5	1.2	1.2	0.3

[a] The results are expressed as an average of multiple samples (n). Predicted values for *L. hesperus* MaSp1 and MaSp2 were calculated using the theoretical MaSp1 and MaSp2 amino acid sequences (GenBank Accession nos. EF595245 and EF595246, respectively). The values are expressed as mole percent per 100 residues. PMS and PLS denote the posterior medial spinnerets and posterior lateral spinnerets, respectively. Percent deviation between the raw wrapping silk and predicted amino acid composition of MiSp1-like from *L. hesperus* was calculated by subtracting the mean amino acid composition of wrapping silk from the theoretical amino acid composition of the MiSp1-like protein. [b] Data are derived from the predicted amino acid sequence of *L. hesperus* AcSp1-like (*20*), *L. hesperus* MaSp1 and MaSp2 (*27*), respectively, using the computer algorithm ProtParam (*30*).

glands. MiSp1-like mRNA levels were the highest in the minor ampullate gland, with lower levels detected in the major ampullate gland, aciniform, tubuliform, aggregate, and fat (Figure 3). Relative to the aciniform gland, the expression pattern of MiSp1-like mRNA was found to be >20-fold higher in the minor ampullate gland (Figure 3). The low levels of MiSp1-like mRNA detected in the fat, major ampullate gland, aciniform, tubuliform and aggregate likely reflect baseline levels of gene transcription. Taken together, these data support the minor ampullate gland as the chief tissue responsible for the expression of the MiSp1-like gene.

Amino Acid Composition of MiSp1-like, Minor and Major Ampullate Gland Luminal Fluids, and Raw Wrapping Silk. To determine whether MiSp1-like represented a major constituent stored within the minor ampullate gland, we examined the luminal contents of the minor ampullate gland. Amino acid composition profiles of the luminal contents of the minor ampullate glands from black widow spiders were remarkably similar to the amino acid composition predicted from the translated MiSp1-like cDNA sequence. Both were determined to contain glycine and alanine levels near 33% and 27%, respectively (Table 2). Other residue compositions were also similar; however, serine levels were slightly higher in the predicted sequence of MiSp1-like relative to the luminal contents. Amino acid compositions of the luminal contents of the minor ampullate gland and predicted MiSp1-like sequence were also strikingly close to raw wrapping silk compositions (Table 2). Relative to the amino acid composition profiles of the luminal contents collected from the major ampullate glands, as well as the predicted full-length sequences of MaSp1 and MaSp2, the alanine and glycine levels from the fluid of the minor ampullate glands were more closely aligned to wrapping silk (Table 2). Collectively, these findings support that MiSp1-like represents a major constituent stored in the minor ampullate gland as well as reveal that the amino acid composition of the minor ampullate gland luminal contents is strikingly similar to the amino acid composition of raw wrapping silk.

Physical Examination of Wrapping Silk Using Scanning Electron Microscopy. Since real-time quantitative PCR analysis confirmed high levels of MiSp1-like mRNA in the minor ampullate gland and mass spectrometry analysis demonstrated that the MiSp1-like product was assembled into wrapping silk, we hypothesized that minor ampullate fibers should be present within prey wrap. To further characterize the physical nature of wrapping silk, we examined swathing silk collected from crickets immobilized in silk filaments by microscopy (Figure 4A−D). Analysis of wrapping silk using scanning electron microscopy revealed the presence of at least three different size diameter fibers (Figure 4B−D). Based upon the close proximity of the multiple aciniform spigots, the bundled fibers likely represent materials extruded from the aciniform gland; the average diameter of an aciniform thread was 0.52 ± 0.03 μm (Figure 4D). These fibers contain the AcSp1-like fibroin. Medium size fibers, which displayed average diameters of 1.56 ± 0.04 μm, were consistent with threads spun from the minor ampullate gland (Figure 4D). The largest diameter fibers were determined to have average diameters of 3.56 ± 0.08 μm; this is closely aligned with previous measurements of major ampullate

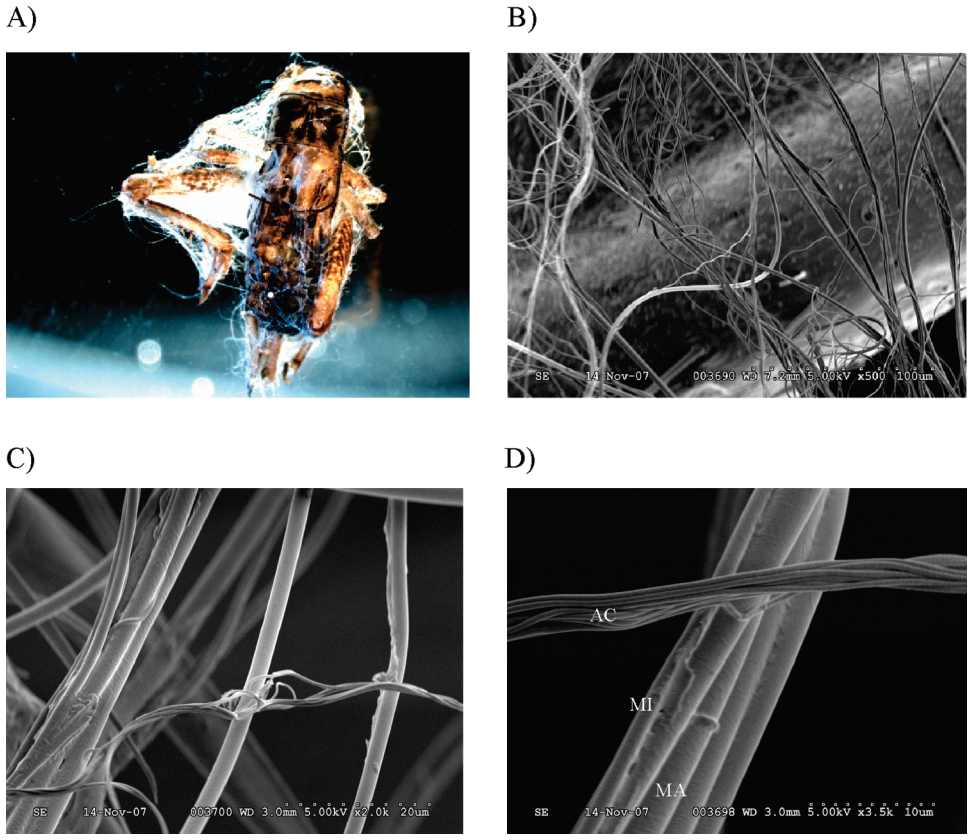

FIGURE 4: Analysis of black widow spider wrapping silk reveals a complex material with fibers containing different diameters. (A) Cricket wrapped in silk photographed on a Leica MZ16 dissecting microscope at 1X magnification. (B−D) Scanning electron microscopy of wrapping silk swathed around a cricket analyzed at magnifications of 500× (B), 2500× (C), and 4000× (D). The large structure in the background of 4B represents a cricket body part. The MI labeling indicates a minor ampullate fiber; the MA designation represents a major ampullate fiber; and the AC marking indicates aciniform bundles.

threads (*26*). Overall, these data support the presence of aciniform bundles, MIA and MAA threads within swathing silks.

DISCUSSION

Histochemical studies and detailed molecular analyses have implicated the aciniform gland and their silks in the prey wrapping process. In our studies we demonstrate that minor ampullate fibroins are also important constituents of wrapping silk for black widow spiders. Several lines of experimental evidence support this assertion: (1) MS/MS analyses have revealed the presence of MiSp1-like peptides in solubilized wrapping silk digested with trypsin; (2) amino acid composition studies of wrapping silk are similar to both the luminal contents of the minor ampullate gland and the translated MiSp1-like amino acid sequence; (3) SEM experiments demonstrate crickets wrapped in swathing silks contain fibers with diameters similar to reported MIA silks.

MS/MS analyses of tryptic digest products generated from wrapping silk reveal the presence of AcSp1-like, SCP-1, SCP-2 and MiSp1-like within the swathing material (Table 1). AcSp1-like is also found in aciniform fibers that are constituents of egg cases (*20*), whereas the SCPs, which are expressed by the aggregate gland, are peptides that coat spider silk threads (*21*). SDS−PAGE analysis of solubilized wrapping silk proteins stained with silver demonstrates the presence of several large proteins with molecular masses >200 kDa (data not shown), and we have previously demonstrated that one of these proteins, AcSp1-like, is also common to egg case materials (*20*).

Several of the wrapping silk peptides sequenced by our MS/MS analysis failed to match other proteins in the NCBInr database. In particular, peptides yielding ions with *m*/*z* 1462.7, 1593.7, 1788.9, 2372.1 and 2751.3, which contained sequences rich in alanine and glycine, did not match any published silk protein sequence in the NCBI database or correspond to any region of the translated sequence of *L. hesperus* MiSp1-like. Several different possibilities could explain the failure of these peptide sequences to match previously published silk fibroins and/or aqueous glue components. First, because our AcSp1-like and MiSp1-like cDNAs represent partial clones that lack the complete N-terminal information, these peptides could be derived from these missing sequence locations. Second, these peptides could be generated from novel fibroin genes whose products remain unknown. Since MAA and MIA silks are alanine and glycine rich, it would seem plausible that these peptides are generated from these silk types. In particular, our SEM data support the presence of both minor and major ampullate silks as constituents of the prey wrap (Figure 4D). Although the full-length genomic sequences for *L. hesperus* MaSp1 and MaSp2 have been published (dragline silk) (*27*), it remains somewhat unclear why our mass spectrometric analysis failed to detect peptides corresponding to the dragline MaSp1 and MaSp2 silk proteins. One possibility could be that the large diameter fibers observed in swathing silk contain products expressed from MaSp1 or MaSp2 gene variants that reside

in the genome. Recently, it has been reported that multiple copies of the MaSp1 gene exist in *L. hesperus* (*28*) and several MaSp1 gene variants exists in the nursery-web spider *Euprosthenops australis*, along with a new MaSp-like spidroin with distinct homogeneous submotifs within their respective Gly rich repeats (*29*). Alternatively, the inability to detect MaSp1 and MaSp2 peptides in wrapping silk could reflect their reduced solubility in 8 M GdnHCl, or perhaps their limited capacity to be digested by trypsin due to their relatively low levels of arginine and lysine, which could hinder their detection in the mass spectrometric analyses. In the future, we plan to investigate the presence of MaSp1 and MaSp2 proteins in wrapping silk using different solvents as well as other proteolytic enzymes.

Amino acid composition analyses of the wrapping silk collected from immobilized crickets deviated substantially from the predicted amino acid content of the translated AcSp1-like cDNA sequence and the luminal fluids of the aciniform glands; however, it was remarkably similar to the luminal contents of the minor ampullate gland as well as the predicted sequence of MiSp1-like (Table 2). In particular, the glycine and alanine levels for the prey wrap, minor ampullate glandular fluid, and the translated MiSp1-like amino acid sequence (glycine and alanine levels were ~32% and ~26%, respectively) were extremely similar. Relative to the prey wrap and minor ampullate luminal contents (serine levels were 5.7% and 7.4%, respectively), serine levels were elevated in the translated MiSp1-like amino acid sequence (serine amounts were 12.7%). The reason for this discrepancy likely reflects the fact that our translated MiSp1-like sequence contains the complete nonrepetitive C-terminus, which is serine-rich, but only a portion of the internal region (Figure 2A). Furthermore, the amino acid content of the luminal fluids of the major ampullate gland was also close to wrapping silk, but glycine levels were 40% compared to 31.2%, respectively (Table 2). Consistent with the SEM data, minor ampullate fibers, and what appear to be MAA silks, comprise a large amount of the fibrous material used to immobilize prey.

The presence of MIA silks within swathing silk raises several intriguing questions, in particular with respect to the biological function of MIA silks in prey capture. After feeding black widow spiders, we routinely observe that the spiders tie the back legs of the crickets ("hog-tie") very quickly. Because MIA silks have higher tensile strength relative to aciniform silks, it could be required to help prevent the fibers from breaking during the prey capture process. Therefore, we propose that in the cob weaver, *L. hesperus*, the MIA silks are used to immobilize prey, in particular, large prey, because this material is considerably stronger relative to the aciniform silks. It is intriguing to postulate that the aciniform silks function to hold the MIA silks together; however, further experimental work will be required before this hypothesis can be verified. Moreover, additional studies will be needed to determine whether orb weavers also use MIA silks to immobilize prey, in addition to their traditional function of web construction.

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