Aciniform Spidroin, a Constituent of Egg Case Sacs and Wrapping Silk Fibers from the Black Widow Spider Latrodectus hesperus*

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Spiders produce high performance fibers with diverse mechanical properties and biological functions. Molecular and biochemical studies of spider egg case silk have revealed that the main constituent of the large diameter fiber contains the fibroin TuSp1. Here we demonstrate by SDS-PAGE and protein silver staining the presence of a distinct ~300-kDa polypeptide that is found in solubilized egg case sacs. Combining matrix-assisted laser desorption ionization tandem time-of-flight mass spectrometry and reverse genetics, we have isolated a novel gene called AcSp1-like and demonstrate that its protein product is assembled into the small diameter fibers of egg case sacs and wrapping silks from the black widow spider, Latrodectus hesperus. BLAST searches of the NCBI protein data base using the amino acid sequence of AcSp1-like revealed similarity to AcSp1, an inferred protein proposed to be a component of wrapping silk. However, the AcSp1-like protein was found to display more nonuniformity in its internal iterated repeat modules than the putative AcSp1 fibroin. Real time quantitative PCR analysis demonstrates that the AcSp1-like gene displays an aciniform gland-restricted pattern of expression. The amino acid composition of the fibroins extracted from the luminal contents of the aciniform glands was remarkably similar to the predicted amino acid composition of the AcSp1-like protein, which supports the assertion that AcSp1-like protein represents the major constituent stored within the aciniform gland. Collectively, our findings provide the first direct molecular evidence for the involvement of the aciniform gland in the production of a common fibroin that is assembled into the small diameter threads of egg case and wrapping silk of cob weavers.

The ability to spin multiple task-specific silks is a defining feature of the diverse order Araneae (>37,000 described species). Araneoid spiders use specialized abdominal glands to manufacture up to seven different protein-based silks/glues that have diverse mechanical properties (1). Spinning high performance fibers with different mechanical properties enable spiders to perform a wide range of functions, including prey capture, locomotion, and protection of developing offspring (2).

Amino acid sequences of spider fibroins (spidroins) share a number of distinctive features. Repeats of four fundamental amino acid motifs characterize the majority of sequenced spider silks as follows: (i) alternating glycine and alanine ((GA)n), (ii) polyalanine (A)n, (iii) GGX (X = subset of residues), and (iv) GPGGX. Biochemical studies indicate that these motifs correspond to distinct structural modules, e.g. A, and (GA)n repeats form crystalline β-sheets, whereas β-spirals are generated from a series of concatenated β-turns from the repeat structure GPGGX (3). It has been proposed that these different structural modules contribute to the mechanics of the fibers. Combinations of these motifs form larger repetitive units termed ensemble repeats, which are organized in tandem copies throughout the silk sequence. In addition to assemble repeat architecture, all spidroins have nonrepetitive C and N termini that exhibit length and sequence conservation (4–8). The C terminus has been postulated to play a role in the assembly process of spider silks (9, 10).

Spiders construct egg cases to serve as protective cocoons for their offspring (2). Egg sacs must be designed to resist damage from predator/parasitoid invasion as well as temperature and humidity fluctuations (11–13). Analyses of the physical structure of egg sacs collected from the cob weaver Latrodectus hesperus, commonly referred to as the black widow spider, have demonstrated that their egg case sacs consist of two different diameter fibers (14, 15). MS/MS* analyses of black widow peptides generated from solubilized egg case materials digested with trypsin have shown that the large diameter core fibers contain the fibroin TuSp1, as well as the egg case proteins

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank**/EBI Data Bank with accession number(s) EU025854.

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4 The abbreviations used are: MS/MS, tandem mass spectrometry; MALDI, matrix-assisted laser desorption ionization; TOF, time of flight; CAD, collision-activated dissociation; BLAST, basic local alignment search tool; AcSp1-like, aciniform spidroin 1-like; GdnHCl, guanidine hydrochloride; GdnSCN, guanidine thiocyanate; ORF, open reading frame; HPLC, high performance liquid chromatography.
ECP-1 and ECP-2 (egg case protein 1 or 2), which form a trimeric complex in cob weavers (14, 16, 17). Real time quantitative PCR and Northern blot analyses have demonstrated tubuliform-restricted patterns of expression for these gene products (14, 17–19). Although it has been widely accepted by the silk community that the aciniform glands produce egg case silk constituents and wrapping silk, no molecular evidence has been reported to demonstrate that aciniform-specific fibroins are directly assembled into these diverse materials (1). Recently, it has been postulated that AcSp1, an inferred protein produced by the aciniform gland, represents a main constituent of the luminal fluid from aciniform glands; however, experimental evidence to support the hypothesis that this fibroin is synthesized and assembled into wrapping fibers of orb weavers was not presented (20). Because the aciniform glands have been implicated in the production of egg case silk and wrapping silk, we hypothesized that spiders spin common fibroin(s) into both egg case silk and wrapping threads. Here we demonstrate by mass spectrometry and reverse genetics, the presence of the first aciniform gland-restricted product, dubbed AcSp1-like, that is assembled into egg case silk fibers, as well as prey-capture wrapping threads. These findings provide new insight into the molecular constituents of aciniform silks and reveal that black widow spiders use a common fibroin for egg case sac production and prey-capture wrapping silk.

**EXPERIMENTAL PROCEDURES**

**Tryptic Digests of Wrapping Silk and the Egg Case Protein**—Sequencing grade trypsin (Trypsin Gold, Promega) was dissolved in 50 mM acetic acid at a concentration of 1 mg/ml. In-gel tryptic digestion of the ~300-kDa band was performed following a published protocol (21). Briefly, the ~300-kDa band in Fig. 1, lane 2 (asterisk), was excised from the gel. After destaining, the pieces were mined into fine particles with sealed pipette tips, washed with 25 mM NH4HCO3, 50% acetonitrile, and then dried in a vacuum centrifuge. The dried pieces were rehydrated and reduced with 10 mM dithiothreitol, alkylated with 55 mM iodoacetamide, dried again, and then rehydrated and digested for ~8 h with 250 ng of trypsin. Peptides were extracted with two changes of 30 μl of a 50% acetonitrile, 0.1% trifluoroacetic acid solution. Peptides were desalted with C18 Zip-Tips (Omix, Varian, Inc., Palo Alto, CA) according to the manufacturer’s instructions.

In-solution digests of the solubilized wrapping silk were performed as follows. Three micrograms of wrapping silk were dissolved in 100 μl of 8 M GdnHCl. Fibroin solubilization was facilitated by heating at 95 °C for 10 min. Prior to trypsin digestion, the sample was neutralized by the addition of 700 μl of 50 mM NH4HCO3 (pH 7.8). Ten micrograms of trypsin were added, and the sample was incubated at 37 °C overnight. Peptides were extracted and desalted with a C18 Zip-Tip as described above.

**Mass Spectrometric Analysis**—Conventional mass spectra were obtained with a MALDI/TOF/TOF mass spectrometer (4700 Proteomics Analyzer, Applied Biosystems, Foster City, CA) operated in reflector mode. MS/MS spectra were obtained by operating this same instrument in MS/MS mode. *De novo* peptide sequences were obtained by manual interpretation of the high energy CAD spectra. 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 that was subsequently introduced into the 4700 mass spectrometer. An equal volume of CHCA matrix, ~0.75 μl (10 g/liter in 80% aqueous acetonitrile, 0.1% trifluoroacetic acid), was mixed with each fraction as it eluted from the column. Both MS and MS/MS (where appropriate) spectra were acquired from each spot.

**Cloning of the AcSp1-like Gene**—The partial sequence from the peptide sequence QVDIITSTR was used to synthesize an oligonucleotide. Primer degeneracy was eliminated by placing an A or T at the wobble position. PCRs containing the forward primer 5'-CAA GTT GAT ACA TCT ACA CG-3' and the reverse anchor pGAL4-AD primer 5'-GCA CAG TTG AAG TGA ACT TGC-3' successfully amplified a 1,049-bp fragment of AcSp1-like from a cDNA library produced from black widow silk glands (corresponding to the boxed amino acids in Fig. 3A). The cDNA library represented a composite library constructed from several silk-producing glands, which included the major and minor ampullate glands, aciniform, tubuliform, pyriform, aggregate, and the flagelliform glands. The 1,049-bp fragment was sequenced as described previously (22). To obtain a larger nucleic acid fragment encoding AcSp1-like, we screened the cDNA library using conventional approaches with a nucleic acid-nucleic acid hybridization using the partial AcSp1-like cDNA as a probe.

**Real Time PCR Analysis**—Reverse transcription reactions were performed as described previously (23). 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. Oligonucleotides used for the analysis of AcSp1-like were the forward and reverse primers 5'-ACT CTC CAG ATG ACA ATA CC-3' and 5'-AGA TGC CAG TGT AGA TGC-3', respectively. Primers used for normalization were the forward and reverse β-actin oligonucleotides 5'-CCC TGA GAG GAA GTA CTC CGT-3' and 5'-ATC CAC ATC TGC TGG AAG GTG-3', respectively.

**Scanning Electron Microscopy**—Wrapping silk and egg case silk were coated to a thickness of 14–30 nm with gold alloy in a Pelco SC-7 auto-sputter coater with an FTM-2 film thickness monitor. Strand diameters were measured on a Hitachi S-2600 S.E. 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 was 25 °C and 30–36%, respectively.

**Amino Acid Composition of Wrapping Silk and Aciniform Gland Contents**—Wrapping silk fibers were subjected to amino acid analysis at the Protein Chemistry Laboratory of Texas A & M University as described previously (24). Wrapping silk and luminal fluids collected from the aciniform gland were hydrolyzed with 6 M HCl to generate the constituent amino acids. Luminal fluids were collected by treating aciniform glands with 2% SDS, followed by boiling at 95 °C for 10 min. Proteins were
precipitated using 2 volumes of acetone. 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 the ~300-kDa Protein and Mass Spectrometric Sequence Analysis—To identify major structural proteins in the small diameter silk fibers of egg case sacs, we treated the threads with 8 M GdnHCl to solubilize them. This treatment efficiently dissolves the small diameter fibers and leads to the partial digestion of the large diameter threads (16). SDS-PAGE analysis and silver staining of the solubilized egg case extract revealed the presence of 3–4 abundant proteins, with one having an apparent molecular mass of ~300 kDa (Fig. 1). Similar banding patterns were observed when the chaotropic salt GdnSCN was used to dissolve the egg case samples (data not shown). Previous studies have demonstrated that two of the abundant proteins represent egg case protein gene products (Fig. 1, lane 2) (14, 16). The ~300-kDa protein does not represent the major constituent of the large diameter fiber, TuSp1, which is not readily dissolved under these conditions and is missing from detection (Fig. 1, lane 2). Because fibroins are typically high molecular weight proteins, in-gel tryptic digestion was performed on the ~300-kDa protein (Fig. 1, asterisk). This digest was found to contain numerous peptides, whose masses were determined by MALDI-MS analysis (Fig. 2A). Six peptides were sequenced using high energy CAD, those with precursor ion masses (MH⁺, monoisotopic) of 1178.8, 1295.7, 1423.8, 1475.8, 1872.9, and 2443.2. The product ion spectra of
the peptides MHt 1178.8, 1295.7, and 1423.8 are shown in Fig. 2, B–D, respectively. The N terminus of 1475.8, as well as the N and C termini of 2443.2, could not be determined, but a significant amount of sequence information was derived (Table 1). The amino acid sequence of the remaining peptide ion 1872.9 was also determined (Table 1). Analysis of the deduced peptide sequences using the algorithm BLAST revealed no significant similarity to any polypeptides in the NCBI protein data base.

The amino acid sequence of the peptide ion 1872.9 could not be determined. The peptides MHt 1178.8, 1295.7, and 1423.8 were not retrieved by conventional screening of the cDNA library using the 1,049-bp cDNA sequence as a probe for nucleic acid-nucleic acid hybridization. Translation of the ~2-kb cDNA sequence indicated this fragment contained a long open reading frame (ORF), encoding the C-terminal 599 amino acids of a spider fibroin (Fig. 3A; GenBank access number EU025854). This protein had a predicted molecular mass of ~60 kDa and a pI of 5.41. Several other clones carrying shorter cDNAs with nearly identical nucleotide sequences were also retrieved during the library screen (data not shown). In protein-protein BLAST searches against the entire predicted amino acid sequence, the top matches corresponded to two putative fibroins (Argiope trifasciata AcSp1, GenBank accession number AY426339; Llaborous diversus AcSp1, GenBank accession number AY426339). Based upon this sequence similarity and pattern of expression, we have named this product AcSp1-like, which is derived from the abbreviation acini-fibroin 1-like. AcSp1-like was ~31% identical to the A. trifasciata and U. diversus AcSp1 amino acid sequences, with statistical E-values of 4 × 10−26 and 8 × 10−17, respectively. As expected for fibroin family members that share a nonrepetitive conserved C terminus, AcSp1-like was found to be ~44% identical to AcSp1 from A. trifasciata (48/108 identities) and U. diversus (37/84 identities) (Fig. 3B). AcSp1-like contained internal iterated modules that were ~187 amino acid residues in length (Fig. 3A). However, unlike the homogeneous internal modules found in the AcSp1 fibroin, which show nearly 100% identities across 200 amino acid block modules, AcSp1-like ensemble repeats had somewhat less sequence homogeneity (Fig. 3A). Pairwise alignment of the two ensemble repeats found within the amino acid sequence of AcSp1-like were determined to be only 45% identical, with 62% similarities. In addition, the traditional spidroin sub-repeats Aπ and Gα were poorly represented in the AcSp1-like sequence, but several GGX modules were present (Fig. 3A).

Four of the peptides sequenced by MS/MS showed 100% identity to translated regions of the AcSp1-like cDNA (m/z 1178.8, 1295.7, 1423.8, and 1872.9). The peptide with m/z 2443.2, which could not be sequenced at its N or C terminus by MS/MS, was found to match a peptide mass from the AcSp1-like protein after theoretical digest with trypsin (Fig. 3A). The partial sequence of the peptide QVDTSTR was retrieved by conventional screening of the cDNA library, was~20 kb, using the 1,049-bp cDNA sequence as a probe for nucleic acid-nucleic acid hybridization. Translation of the ~2-kb cDNA sequence indicated this fragment contained a long open reading frame (ORF), encoding the C-terminal 599 amino acids of a spider fibroin (Fig. 3A; GenBank accession number EU025854). This protein had a predicted molecular mass of ~60 kDa and a pI of 5.41. Several other clones carrying shorter cDNAs with nearly identical nucleotide sequences were also retrieved during the library screen (data not shown). In protein-protein BLAST searches against the entire predicted amino acid sequence, the top matches corresponded to two putative fibroins (Argiope trifasciata AcSp1, GenBank accession number AY426339; Llaborous diversus AcSp1, GenBank accession number AY426339). Based upon this sequence similarity and pattern of expression, we have named this product AcSp1-like, which is derived from the abbreviation acini-fibroin 1-like. AcSp1-like was ~31% identical to the A. trifasciata and U. diversus AcSp1 amino acid sequences, with statistical E-values of 4 × 10−26 and 8 × 10−17, respectively. As expected for fibroin family members that share a nonrepetitive conserved C terminus, AcSp1-like was found to be ~44% identical to AcSp1 from A. trifasciata (48/108 identities) and U. diversus (37/84 identities) (Fig. 3B). AcSp1-like contained internal iterated modules that were ~187 amino acid residues in length (Fig. 3A). However, unlike the homogeneous internal modules found in the AcSp1 fibroin, which show nearly 100% identities across 200 amino acid block modules, AcSp1-like ensemble repeats had somewhat less sequence homogeneity (Fig. 3A). Pairwise alignment of the two ensemble repeats found within the amino acid sequence of AcSp1-like were determined to be only 45% identical, with 62% similarities. In addition, the traditional spidroin sub-repeats Aπ and Gα were poorly represented in the AcSp1-like sequence, but several GGX modules were present (Fig. 3A).

The amino acid sequence of AcSp1-like shows similarity to spider fibroins. A translation of the nucleotide sequence from the AcSp1-like cDNA contains an open reading frame (ORF). The longest ORF encodes a protein 599 amino acids in length. Peptide sequences determined by MS/MS (in-gel tryptic digestion products from the excised peptide mass from the AcSp1-like cDNA) that are found within the ORF are indicated with an underline. Red and blue coloration denotes the ensemble repeats; the first blue block is only a partial ensemble repeat. The boxed region represents the nonrepetitive C terminus. Boldface characters represent the presence of GGX motifs. B alignment of araneoid aciniform silk fibroin C-terminal sequences was performed using the computer algorithm ClustalW. Amino acids are represented by one-letter abbreviations; gaps are indicated by dashes. The sequences are identified as A tr (A. trifasciata) AcSp1 (GenBank accession number AY426339), L hes (L. hesperus) AcSp1-like (EU025854), and U div (U. diversus) AcSp1 (DQ399333). Asterisks represent identical residues; colons represent side chain groups with similar polarity and size; periods indicate residues with similar polarity or R-group size.

### TABLE 1

| Peptide mass (M + H) | Sequence |
|---------------------|----------|
| 1178.8             | LIQALVPALFK |
| 1295.7             | SLASTASSVFR |
| 1423.8             | IAGNGLDVAATASK |
| 1475.8             | ...QVDTSTR (partial) |
| 1872.9             | ATQALSSVSADSSSTAVAK |
| 2443.2             | (NO) SIDDLYALA1SSSALAEVLS (SSGK) (partial) |

### FIGURE 3

The amino acid sequence of AcSp1-like shows similarity to spider fibroins. A translation of the nucleotide sequence from the AcSp1-like cDNA contains an open reading frame (ORF). The longest ORF encodes a protein 599 amino acids in length. Peptide sequences determined by MS/MS (in-gel tryptic digestion products from the excised peptide mass from the AcSp1-like cDNA) that are found within the ORF are indicated with an underline. Red and blue coloration denotes the ensemble repeats; the first blue block is only a partial ensemble repeat. The boxed region represents the nonrepetitive C terminus. Boldface characters represent the presence of GGX motifs. B alignment of araneoid aciniform silk fibroin C-terminal sequences was performed using the computer algorithm ClustalW. Amino acids are represented by one-letter abbreviations; gaps are indicated by dashes. The sequences are identified as A tr (A. trifasciata) AcSp1 (GenBank accession number AY426339), L hes (L. hesperus) AcSp1-like (EU025854), and U div (U. diversus) AcSp1 (DQ399333). Asterisks represent identical residues; colons represent side chain groups with similar polarity and size; periods indicate residues with similar polarity or R-group size.
ever, a theoretical digest of the predicted AcSp1-like amino acid sequence with trypsin did not yield the expected peptide ion mass. The inability to locate a peptide mass that precisely corresponds to m/z 1475.8 within our translated AcSp1-like cDNA sequence may indicate that this peptide resides in the uncharacterized N terminus of the AcSp1-like amino acid sequence.

Expression of AcSp1-like mRNA Is Restricted to the Aciniform Gland—Because our mass spectrometric data confirm that AcSp1-like is present within egg case silk fibers, we next investigated whether AcSp1-like mRNA was found within the aciniform gland. We performed real time quantitative PCR analysis to examine the mRNA expression profile of AcSp1-like in aciniform glands dissected from the black widow spider (Fig. 4A). AcSp1-like mRNA levels were by far the highest in the aciniform gland, with virtually no detection in the major and minor ampullate glands, tubuliform, aggregate, and flagelliform glands (Fig. 4B). The expression of AcSp1-like mRNA in the aciniform gland was found to be >1000-fold higher than the flagelliform gland or any other silk gland tissues (Fig. 4B).

The AcSp1-like Protein Is a Component of Wrapping Silk—One of the chief functions proposed for the aciniform gland is the production of wrapping silk for prey capture (1). To elucidate the physical structure of wrapping silk in L. hesperus, we examined wrapping silk collected from crickets immobilized in silk threads using scanning electron microscopy. Analysis of wrapping silk revealed the presence of two different size diameter fibers (Fig. 5A). The larger fibers were ~2.5 μm in diameter, whereas smaller fibers were ~500 nm in diameter (Fig. 5B). Bundles of the small diameter fibers were also very prominent in the microfilaments; often these threads contained 4–8 individual fibers grouped together (Fig. 5B). Based upon the diameters obtained from cross-sections of the aciniform spigots, the size of the small diameter fibers is consistent with their being produced by the aciniform silk-producing gland. Individual small diameter fibers observed in wrapping silk were of similar size to the egg case small diameter fibers, supporting the observation that the aciniform gland produces threads involved in wrapping silk and egg case sacs (Fig. 5, C and D).

To confirm that the AcSp1-like protein was also present in wrapping silk, we treated these threads with 8 M GdnHCl to facilitate solubilization. Digestion of the solubilized wrapping silk with trypsin revealed the presence of numerous peptides whose masses were determined by MALDI-MS analysis (Fig. 6A). The masses of two of the abundant peptides, 1178.8 and 1295.7 Da (MH+, monoisotopic), were identical to those observed from the AcSp1-like protein in egg case silk. The product ion spectra of these two peptides and thus their sequences were in fact found to be identical to those of peptides found in the small diameter egg case silk fibers (data not shown). Furthermore, the peptide ion mass 1783.1 Da (MH+, monoisotopic), which we also sequenced by MS/MS analysis, was identical to the sequence of peptide ion 1178.8, with the exception of an additional five residues at the C terminus because of a single missed tryptic cleavage event (Fig. 6B). All three peptide sequences for ions 1178.8, 1295.7, and 1783.1 were 100% identical to regions found within the translated AcSp1 cDNA sequence (Fig. 3A; Tables 1 and 2). Even though we were able to sequence three of the major peptides after tryptic digestion (Fig. 6A; m/z 1178.8, 1295.7 and 1783.1), we also analyzed the samples for some of the lower abundance peptides...
after enrichment using HPLC. To further substantiate that the egg case AcSp1-like protein was present within wrapping silk, we compared the theoretical tryptic peptide masses from the translated AcSp1-like cDNA sequence (Fig. 3A, zero or single missed tryptic cleavage events) within a mass range of a 700–2000 m/z ratio to the experimental masses obtained from wrapping silk after digestion with trypsin. After HPLC-MS analysis of the tryptic digest products generated from wrapping silk, several additional precursor ions were identified as candidates derived from the AcSp1-like protein, which included ion masses 743.5, 964.6, 1468.9, 1692.9, and 1872.9 Da (MH⁺, monoisotopic). These five peptides were further sequenced by MS/MS. All five peptides were found within the translated AcSp1-like cDNA sequence (Table 2). Peptide ion 743.5 showed a 100% sequence match to two distinct regions of the translated AcSp1-like cDNA sequence (Table 2; Fig. 3A). Peptide ions 1468.9 and 1692.9 were also determined to be identical to regions found within the translated AcSp1-like cDNA sequence, with each peptide ion containing a single missed tryptic cleavage site (Table 2; Fig. 3A). The peptide ion 964.6, which was determined to have the sequence (PS/AL)YKRGTR, contained two missed tryptic cleavage events, and its N-terminal region was determined by MS/MS analysis to represent either PS or AL (Table 2). Examination of the translated AcSp1-like cDNA sequence supported that these two residues most likely represented the residues AI (Table 2; Fig. 3A). It is sometimes difficult to distinguish Leu or Ile by MS/MS because they have identical masses. Peptide ion 1872.9, which yielded considerable sequence information after MS/MS analysis (...) ALSSVSADSDSTAYAK, could not be fully sequenced because of the relatively poor quality CAD MS/MS spectrum. Theoretical digestion of the translated AcSp1-like cDNA sequence with trypsin yielded a peptide that contained an identical mass (m/z 1872.9) and sequence, as well as revealing the missing N-terminal three residues (ATQ) that were not successfully retrieved from the de novo peptide sequencing by MS/MS (Fig. 3A and Table 2). Collectively, these studies demonstrate that in-solution tryptic digestion of wrapping silk, followed by MS/MS analysis, results in four peptides identical to those observed in the in-gel tryptic digestion of egg case AcSp1-like protein (m/z 1178.8, 1295.7, 1423.8 and 1872.9; see Table 1 and 2). Additionally, four other peptides derived from wrapping silk after trypsin digestion (m/z 743.5, 964.6, 1468.9, and 1783.1), which were also sequenced by MS/MS, also showed 100% identities to regions found within the translated AcSp1-like cDNA sequence. Thus, these data support that both wrapping silk and egg case threads contain the AcSp1-like fibroin.

**Amino Acid Composition of AcSp1-like, Aciniform Luminal Fluids, and Wrapping Silk**—Because spider silk glands are specialized structures designed to store large quantities of fibroins, we examined the luminal contents of aciniform glands for the presence of the AcSp1-like protein. The predicted amino acid composition of AcSp1-like was strikingly similar to the amino acid profile generated from the luminal fluids collected from the aciniform gland (Table 3), suggesting that AcSp1-like represents one of the major constituents synthesized by the aciniform glands. Analysis of the amino acid composition of the fibroins found in the wrapping silk, however, deviated signifi-
Significantly from the theoretical amino acid composition of AcSp1-like (Table 3). Substantially higher amounts of glycine and alanine were found in wrapping silk relative to the stored aciniform glandular fibroins (Table 3). In wrapping silk glycine and alanine levels were 33.5 and 24.9%, respectively, suggesting the presence of additional fiber types. The presence of additional fiber types in wrapping silk was consistent with the scanning electron micrograph data, which revealed the presence of both aciniform fibers as well as an unknown, larger diameter fiber (Fig. 5B).

**DISCUSSION**

Although it is widely accepted by the spider silk community that the aciniform glands function to produce silks that are assembled into egg case and wrapping threads, conclusive empirical molecular evidence to support this assertion has not
been presented. By combining proteomics and reverse genetics, we have isolated a novel cDNA sequence that encodes a member of the fibroin superfamily that belongs to the aciniform silks. Using quantitative real time PCR analysis, we also demonstrate that AcSp1-like mRNA levels are greater in the aciniform gland than in other silk-producing glands. MALDI MS/MS analyses of peptide fragments generated from egg case and wrapping silk fibers after tryptic digestion provide strong evidence for the presence of the AcSp1-like protein in both materials. This assertion is supported by the detection of the identical peptides (m/z 1178.8, 1295.7, 1423.8 (contains the same sequence as the partial tryptic cleavage ion 1692.9), and 1872.9) from the in-gel tryptic digestion of the egg case AcSp1-like protein, as well as the in-solution tryptic digest of wrapping silk.

**TABLE 3**

| Peptide mass (M + H) | Predicted AcSp1-like sequence | De novo sequence | Location (Fig. 3A) |
|---------------------|------------------------------|------------------|-------------------|
| 743.5               | LATGILR                      | LATGILR          | 30–36 and 406–412 |
| 964.6*              | A1YKROTR                     | (PS/AL) YERKROTR | 320–327           |
| 1178.8              | LLQALVPLLX                 | LLQALVPLLX       | 304–314           |
| 1295.7              | SLASTLASSGUVFR              | SLASTLASSGUVFR   | 120–132           |
| 1468.9*             | LATGIILGRSSAVPR            | LATGIILGRSSAVPR | 30–44 and 406–420 |
| 1692.9*             | IASNLGDYATASKLR            | IASNLGDYATASKLR | 155–170           |
| 1783.1*             | LLQALVPLLXGVFV             | LLQALVPLLXGDVFV | 304–319           |
| 1872.9              | ATOQALSSVSADSDSTAYAK       | ATOQALSSVSADSDSTAYAK | 360–378 |

* This peptide contains a single or double missed tryptic cleavage event.

**TABLE 2**

| Peptide | Gly | Ala | Glx | Tyr | Pro | Arg | Asx | Ser | Ile | Leu | Thr | Val | Phe |
|---------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| PLS and PMS aciniform silk glands of *L. hesperus* (n = 1) | 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 |
| Predicted AcSp1-like of *A. trifasciata* | 15.2 | 14.2 | 5.5 | 1.9 | 3.9 | 3.5 | 8.1 | 21.5 | 8.7 | 2.5 | 5.9 | 6.5 | 3.5 |
| Predicted AcSp1 of *L. hesperus* | 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 |
| Raw wrapping silk of *L. hesperus* (n = 2) | 33.5 ± 4.9 | 24.9 ± 3.8 | 10.6 ± 0.3 | 3.9 ± 0.4 | 3.8 ± 1.4 | 2.2 ± 0.2 | 2.9 ± 1.2 | 5.8 ± 1.3 | 1.6 ± 0.6 | 1.7 ± 1.0 | 2.5 ± 1.2 | 1.7 ± 0.8 | 0.8 ± 0.3 |
| Raw wrapping silk of *L. hesperus* (n = 3) | 7.3 ± 0.6 | 26.0 ± 0.2 | 10.8 ± 0.2 | 1.5 ± 0.1 | 1.6 ± 0.0 | 1.4 ± 0.2 | 4.4 ± 0.1 | 25.6 ± 0.7 | 5.0 ± 0.0 | 2.5 ± 0.0 | 5.1 ± 0.2 | 5.0 ± 0.1 | 4.1 ± 0.1 |
| Core egg case silk of *L. hesperus* (n = 3) | 7.7 ± 0.6 | 23.3 ± 0.6 | 11.0 ± 0.0 | 1.7 ± 0.6 | 2.0 ± 0.6 | 1.3 ± 0.6 | 6.3 ± 1.1 | 25.7 ± 0.6 | 5.0 ± 0.0 | 2.0 ± 0.0 | 5.0 ± 0.0 | NA | NA |

| Predicted AcSp1-like of *A. trifasciata* | 10.8 | 12.8 | 6.8 | 1.9 | 4.5 | 3.5 | 9.8 | 13.7 | 10.1 | 5.2 | 5.9 | 6.8 | 2.6 |

* Data are derived from the predicted amino acid sequence of *A. trifasciata* AcSp1 (20) using the computer algorithm ProtParam (29).

**Note:**

- Gly, Ala, Glx, Tyr, Pro, Arg, Asx, Ser, Ile, Leu, Thr, Val, and Phe are expressed as mol % per 100 residues.
- PMS and PLS denote the posterior medial spinnerets and posterior lateral spinnerets, respectively.
AcSp1 repeat modules (20), was completely absent from the amino acid sequence of AcSp1-like. The predicted amino acid composition profiles of *L. hesperus* AcSp1-like and *A. trifasciata* AcSp1, despite some similarities, showed notable differences in some residues, including lower levels of serine, threonine, and glycine in the AcSp1-like protein (Table 3). Consequently, we are reluctant to classify the cob-weaver AcSp1-like gene as the orb-weaver AcSp1 orthologue based upon similarities in amino acid compositions, also in part because the dragline silk fibroins MaSp1 and MaSp2, which have similar predicted amino acid profiles, have recently been shown to be encoded by distinct genes (25). Furthermore, BLAST searches with the AcSp1-like cDNA sequence against the NCBInr nucleotide data base showed no similarity to the *A. trifasciata* AcSp1 nucleic acid sequence. This is in marked contrast to the large diameter core fibroin TuSp1, which shows conservation at the nucleotide and amino acid level across species for the genera *Argiope* and *Latrodectus*. It is also worth noting that during our cDNA library screen for AcSp1-like, we failed to retrieve any clones that were similar to reported AcSp1 gene sequences. This is likely because of the lack of conservation of sequence at the nucleotide level between the AcSp1 and AcSp1-like genes. Therefore, to determine whether the cob-weaver AcSp1-like gene has diverged significantly from the orb-weaver AcSp1 gene, or whether AcSp1-like represents a second distinct gene, will require further characterization of the genomic DNA sequences from these spiders.

Because dragline silk fibers are composed of two distinct fibroins, containing the MaSp1 and MaSp2 proteins distributed in a heterogeneous fashion within major ampullate silks (26), it is interesting to hypothesize that aciniform threads may be spun from AcSp1 and AcSp1-like molecules. However, biochemical evidence to support this supposition is not consistent with our observed data. Theoretical digestion of the predicted amino acid sequence of *A. trifasciata* AcSp1 with trypsin failed to generate peptides that corresponded to ion masses found in enzymatically digested black widow wrapping silk after GdnHCl solubilization (Fig. 6A). The inability to find predicted *A. trifasciata* peptide ions masses in our solubilized wrapping silk samples digested with trypsin could be explained by the following: 1) the lack of AcSp1 in black widow spider fibers or 2) the divergence in the amino acid sequences of AcSp1 in *Argiope* and *Latrodectus*, which could shift the expected peptide ion masses in our MS spectrum.

Previous amino acid composition analysis of the aciniform glandular silk proteins of *A. trifasciata* indicates that this tissue has significantly lower levels of serine and threonine than to the predicted amino acid composition of the inferred AcSp1 protein (20). This discrepancy implies that the inferred AcSp1 protein, if translated, would be stored with other as yet uncharacterized fibroins. Interestingly, the amino acid composition of the AcSp1-like protein closely corresponds to the fibroin amino acid composition profile of the luminal contents extracted from the aciniform glands of *A. trifasciata* and *L. hesperus*. This correspondence supports the assertion that this fibroin is one of the major constituents stored in the aciniform glands, becoming extruded during the spinning process of aciniform silks in *L. hesperus*. Currently, it remains to be determined whether the AcSp1 mRNAs detected in the aciniform glands of *A. trifasciata* are translated into fibroins that are assembled into egg case and wrapping silks. Given the recent report of appreciable quantities of MaSp1 and MaSp2 mRNAs in the tubuliform gland (27), the inability to detect these fibroins in tubuliform silks by MS/MS analyses of tryptic peptides generated from solubilized egg cases (14, 16, 17) raises the question of whether certain fibroin mRNAs are translated at specific times during the life of the spider that serve other biological functions. Therefore, it remains to be established whether the reported AcSp1 mRNAs in orb weavers are translated and assembled into wrapping silk for prey capture, egg cases, or other silk structures.

Previous studies of black widow egg cases treated with GdnHCl have shown that the large diameter fibers consist of a trimeric complex of TuSp1, ECP-1, and ECP-2 (16). Egg case fibers are also coated with aqueous glue-coating materials, called SCP-1 and SCP-2, which have been detected in water-washed egg cases (28). The small diameter fibers of egg cases can be efficiently solubilized by limited GdnHCl treatment, whereas the large diameter fibers are intrinsically more resistant to GdnHCl solubilization (16), as well as GdnSCN treatment (data not shown). Several of the following observations support the assertion that the AcSp1-like protein is assembled into the small diameter fibers of egg cases. 1) The AcSp1-like protein can be detected in the solubilized fractions of egg case extracts following limited GdnHCl treatment, which preferentially solubilizes the small diameter fibers. 2) The diameter of the aciniform spigots corresponds to the diameter of the ∼500-nm fibers. 3) The AcSp1-like mRNA is localized virtually exclusively in the aciniform gland. Additionally, the presence of similar diameter fibers in wrapping silk is consistent with the AcSp1-like protein localizing to the small diameter fibers of prey-capture wrapping threads. Presently, the origin of the large diameter fibers observed in *L. hesperus* wrapping silk is unknown. However, given the similarity of the diameter size to that of major ampullate silks and the high glycine and alanine content of raw wrapping silk, one possibility is that these large diameter fibers are major ampullate silks, with some minor ampullate silk threads wrapped around these fibers. Analysis of additional peptides generated by in-solution tryptic digestion, followed by sequence analysis using MS/MS, should help to clarify whether other peptide ions observed in the solubilized wrapping silk are from peptides that are constituents of the major ampullate silk fibroins MaSp1, MaSp2, or other as yet uncharacterized minor ampullate fibroins.

One intriguing question regarding aciniform silks centers around why these fibers are spun into egg cases and wrapping silk, two markedly different structures serving distinct biological functions. Based upon our observations, we propose that cob-weaver aciniform silks serve to hold large diameter fibers together, which could explain their presence in both egg case and wrapping silks. Further characterization of the molecular mechanics and the constituents of aciniform silks should help provide insight into the overlapping presence of aciniform silks in these structures.

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