Analysis of Aqueous Glue Coating Proteins on the Silk Fibers of the Cob Weaver, *Latrodectus hesperus*†

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ABSTRACT: Elucidation of the molecular composition and physical properties of spider glue is necessary to understand its function in the mechanics of the web and prey capture. Previous reports have indicated that components of the adhesive coating contain inorganic molecules, phosphorylated glycoproteins, lipids, and organic low-molecular mass (LMM) compounds. Using a proteomic strategy, we have investigated the viscid, aqueous components that coat different silk fiber types from the black widow spider, *Latrodectus hesperus*. After in-solution tryptic digestion of the aqueous protein material extracted from egg case sacs, gumfooted lines, and the web scaffolding connection joints, followed by peptide analysis using MALDI tandem TOF mass spectrometry, we demonstrate that these fibers are coated with common peptides. Utilizing a reverse genetics approach, we have isolated the cDNAs encoding two distinct fiber coating products, which we have named spider coating peptide 1 and 2 (SCP-1 and SCP-2). Secreted forms of SCP-1 and SCP-2 contain 36 and 19 amino acids, respectively, and their primary sequences display no significant similarities to ensemble repeat units from traditional fibroins. Quantitative real-time reverse transcription PCR analyses show that these mRNAs are chiefly produced by the aggregate gland. Biochemical studies also demonstrate that the SCP-1 peptide has intrinsic metal binding properties, suggesting a role of peptide−metal ion interactions with the fiber constituents to enhance thread performance. Collectively, these investigations are the first to reveal a novel role for the aggregate gland in the production of peptides that coat spider silk threads.

Spiders produce a diverse number of silk fibers that have different mechanical properties that facilitate prey capture, reproduction, and survival (*1*, *2*). Silk fibers consist of proteins (spidroins) that are manufactured by specialized abdominal glands. For typical orb-weaving spiders, the abdominal glands include the major and minor ampullate, tubuliform, flagelliform, aggregate, piriform, and aciniform glands (*2*). Primary sequences for spidroins that represent the major proteins produced by many of these glands have been determined, with the exception of the aggregate and piriform glands (*3*−*8*). Analyses of the primary sequences of these fibroins reveal common architectures, which include ensemble repeats (iterations of amino acid sequence that are 50−200 residues long) and nonrepetitive N- and C-termini (*3*, *4*, *8*−*11*). The majority of the fibroins contain sub-repeat modules that are embedded within the ensemble repeats, which include Gly-Gly-X (GGX), Gly-Pro-Gly-X-X (GPGXX), and poly-Ala (A_n) or Gly-Ala couplets $(GA)_n$. Biophysical studies support the assertion that the GGX modules form a 3_{10} helix, whereas the A_n or GA couplets form beta-sheet structures that contribute to the crystalline regions of the fiber (*12*−*14*). GPGXX repeats have been proposed to form beta-turn structures that contribute to fiber elasticity (*15*).

Although much emphasis has been focused on the elucidation of the spidroins that comprise different silk fibers, less is known regarding the glue substances that coat the threads. Typically, orb-weaving spiders coat their capture core fibers as they emerge from their spigots with a layer of aqueous glue coating produced from the aggregate gland. Perhaps the most comprehensive analyses of adhesive materials have been derived from orb weaver spiral capture silk. These studies have revealed the presence of organic molecules in the aqueous components, which include GABamide (γ-aminobutyramide), *N*-acetyltaurine, choline, betaine, and isethionic acid (*16*, *17*). In addition, *N*-acetylputrescine, proline, alanine, glycine, potassium nitrate, and potassium dihydrogenphosphate have been reported (*17*−*20*). Fatty acids have also been discovered within the aqueous solution (*16*). Because these droplets have been demonstrated to contain substances related to neurotransmitters with hygroscopic properties, it has been proposed that following extrusion these molecules function to adsorb water from the

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atmosphere (*16*). Adsorption of atmospheric water results in swelling, leading to the formation of evenly spaced droplets that resemble beads on a string. Functionally, hydration of the capture threads serves to plasticize the filaments and provide additional elasticity to the fibers due to the surface tension of the liquid (*21*).

Although the identities of organic molecules, salts, and fatty acids have been identified in the aqueous materials, little is known regarding the molecular identities of the aqueous proteins in the glue droplets. In fact, no primary sequences have been published for any water soluble protein found within the glue droplets or connecting narrow liquid bridges. The only information that has been reported is that the glue material contains glycoproteins, with one of the carbohydrate modifications representing *N*-acetylgalactosamine (*22*, *23*). These glycoproteins have been described as residing within nodules along the core fibers of the capture spiral, within the viscid, aqueous droplets, which are often referred to as "droplets within droplets" (*22*). Because different fiber types in black widow spiders have adhesive substances associated with them, excluding dragline silk, we hypothesized that the glue materials found on the different fiber types share common aqueous proteins. To test this premise, the molecular identities of the aqueous glue proteins on black widow egg case sacs, gumfooted lines, and scaffolding silk web connection joints were investigated using proteomics and reverse genetics. Tryptic digestions of water-extracted materials collected from the different fiber types, followed by MS/MS analysis, revealed the presence of many peptides with identical or similar sequences. Two of the most prominent peptides were demonstrated to be encoded by distinct genes that display aggregate-gland-restricted patterns of expression, with one of the products shown to contain intrinsic metal binding activity. Taken together, our data suggest that these gene products represent major protein constituents of the black widow spider aqueous fiber coatings and support a role of peptide−metal ion interactions on the surface of silk fibroins.

EXPERIMENTAL PROCEDURES

Collection of the Aqueous Glue Materials from Black Widow Spider Fibers. Freshly spun egg cases from black widow spiders were collected and dissected using sterile scissors as previously described (*24*). In brief, the embryos were removed with care from each individual egg case sac. If any embryos were broken or damaged during the dissection, the egg case was completely discarded. To collect web scaffolding connection joints and gumfooted lines, the filaments were obtained by housing black widow spiders individually in 22 × 40 × 30 cm aquarium tanks with 14 × 18 × 25 cm wooden frames inserted to facilitate cobweb construction. Gumfooted lines and scaffolding web connection joints were carefully removed using a sterile, acid washed glass hook. To obtain pure dragline samples, the spiders were first rendered unconscious using 5 psi of CO_2 gas for 30 min. The spiders were then immobilized on a dissection station using cardboard and rubber bands. After the spiders were revived, pure dragline silk fibers were pulled at about 1 cm/s from the spigot onto a precleaned and sterile glass rod using a rotating motor.

Glue Component Extraction, In-Solution Tryptic Digestion, and Mass Spectrometric Analysis. Glue constituents were extracted by dissolving the silk fibers in distilled water (0.3 mL for egg cases, other silk samples were 0.1 mL), followed by mechanical agitation for 20 min. Aqueous glue extracts from egg case threads were digested with 1 μg of trypsin, whereas the other silk filaments were digested with 100 ng of trypsin (Trypsin Gold, Promega, Madison, WI). Digestions of the aqueous fractions were performed overnight at 37 °C. Negative control samples containing only trypsin and distilled water were employed to monitor for potential contamination during the preparation. After digestion, tryptic peptides were desalted and concentrated using Varian C18 Omix tips (Varian, Inc., Palo Alto, CA) and analyzed with a MALDI[1] tandem TOF mass spectrometer (4700 Proteomics Analyzer, Applied Biosystems, Foster City, CA). The matrix used was α-cyano-4-hydroxycinnamic acid in 50% (v/v) ACN at a concentration of 10 mg/mL. One-half microliter digests were mixed with an equal volume of matrix solution and spotted onto the MALDI target plate. The monoisotopic masses of the peptides generated by trypsin digestion were examined in positive full scan mode. Target peptides were selected to undergo high energy collision activated dissociation (CAD) to determine their primary amino acid sequences. The resulting MS/MS spectra were interpreted manually to obtain the *de novo* peptide sequences.

The LMM compounds in the droplets of the gumfooted lines were not found to interfere with the MS analysis. In addition, absolute quantities across different fiber preparations using MS analysis were difficult to compare due to the lack of common internal controls for each sample.

Isolation of the cDNAs That Encode Peptides Found within the Aqueous Glue Coatings. The peptide sequence AVH-HYEVPVR was used to design a gene-specific reverse oligonucleotide (underlined region corresponds to primer design area). PCR reactions containing the pGAL4-AD forward anchor primer (5′-CGA TGA TGA AGA TAC CCC ACC-3′), together with the reverse primer (5′-CGA ACT GGA ACT TCG TAA TG-3′), successfully amplified the SCP-1 cDNA sequence from the black widow cDNA library prepared from the silk glands (*24*). After DNA sequencing, the data were used to design a gene-specific forward primer for a secondary PCR reaction. 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 pGAL4-AD library vector (5′-GAT CAG AGG TTA CAT GGC CAA GAT TGA AAC-3′), effectively amplified the cDNA encoding the aqueous glue protein from our library. Products were gel purified using the QIAquick gel extraction kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions, ligated into pcDNA3.1/V5-His TOPO vector, and then transformed into *Escherichia coli*. Several different clones were selected and their recombinant plasmid DNA molecules were subject to DNA sequencing as previously described (*25*). After DNA sequencing, the retrieved sequences were translated into the primary sequences of SCP-1.

[1] Abbreviations: SCP-1, spider coating peptide 1; SCP-2, spider coating peptide 2; MALDI, matrix assisted laser desorption ionization; TOF, time of flight; CAD, collision activated dissociation; ACN, acetonitrile; BLAST, basic local alignment search tool; LMM, low molecular mass; HPLC, high performance liquid chromatography; SEM, scanning electron micrograph; LB, lactose broth; qRT-PCR, quantitative reverse transcription-polymerase chain reaction.

The peptide with the precursor ion *m*/*z* at 1555.8 was subject to MS/MS analysis and determined to have the sequence TLFNQAADLLDHVV. To isolate the cDNA that encoded this peptide segment, we amplified the corresponding nucleic acid sequence from our silk-gland-specific cDNA library using the forward gene-specific primer designed against the underlined peptide region (5′-TTT AAT CAA GCW GCW GAY-3′; W = A or T; Y = C or T) and the reverse pGAL4-AD library primer (5′- GAT CAG AGG TTA CAT GGC CAA GAT TGA AAC-3′; anchor oligonucleotide). After DNA sequencing, the gene-specific reverse primer (5′-AAT TAA TCT CAG TGT TCC TCA AGA GAT ATC AAT-3′) and the pGAL4-AD forward anchor primer were used to amplify the 5′ end of the cDNA. Following sequencing, both DNA products (initial and secondary PCR reactions) were overlapped and used to reconstruct the full-length 342 bp cDNA encoding SCP-2.

Reverse Transcription Quantitative PCR (RT-qPCR) Analyses. Reverse transcription reactions were carried out essentially as previously described (*24*). Briefly, 3 μL of a reverse transcription reaction was used for real-time PCR analysis using the BioRAD SYBR Green qPCR kit (BioRad Laboratories, Hercules, CA). Real-time PCR fluorescence detection was performed in 96-well plates using an Opticon II instrument. Amplification products were monitored by SYBR Green detection and consistently checked using dissociation curve software and 2% agarose gel electrophoresis. Oligonucleotides used for the amplification of the SCP-1 cDNA included the forward primer SCP-1 F1 (5′-GTA CAC CAT TAC GAG GTT CCA G-3′) and reverse primer SCP-1 R1 (5′-TCA TCT ATA CCT TGA TGC TTG TG-3′). Primers that were used for the quantification of SCP-2 levels used the forward oligonucleotide SCP-2 F1 (5′-GTG AAT TTG CAG TAT CGT AC-3′) and the reverse primer SCP-2 R1 (5′-CAC CCC TTA GAC TAC ATG ATC GAG GAT-3′). Normalization was performed using the beta-actin sense primer (5′-CCC TGA GAG GAA GTA CTC CGT-3′) and antisense primer (5′-ATC CAC ATC TGC TGG AAG GTG-3′).

Construction of SCP-1 Prokaryotic Expression Vectors, Production of Recombinant SCP-1, and Metal-Binding Assays. Full-length SCP-1 cDNA lacking the secretion sequence was amplified from our cDNA library using PCR with the primer set SCP-1 F2 and SCP-1 R2 (5′-CTC GAG GCA GTA CAC CAT TAC GAA G-3′ and 5′-GAA TTC AAG TAT GTC ATT TGC GAG GTG-3′, respectively). The amplified product was gel-purified and ligated into the prokaryotic expression vector pBAD using the TOPO TA Expression Kit (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's instructions to create pBAD-SCP1$_{(+\text{His}-\text{tag})}$. The restriction sites *Xho*I and *Eco*RI were added to the 5′ termini of the SCP-1 F2 and R2 primers, respectively, to facilitate the detection of the correct insert orientation. After the construction of pBAD-SCP1$_{(+\text{His}-\text{tag})}$, we used this prokaryotic expression plasmid to amplify the SCP1 cDNA and remove the 6x His-tag using the primers SCP-1 F2 and the pBAD R1 reverse primer (5′-GTC GAC TCA ACC GGT ACG CGT AGA ATC GAG-3′). The underlined region denotes the addition of a *Sal*-I restriction site and the addition of a stop codon. Amplified SCP-1 cDNAs that lacked the 6x His-tag were ligated into the pBAD cloning vector to create pBAD-SCP1$_{(-\text{His}-\text{tag})}$.

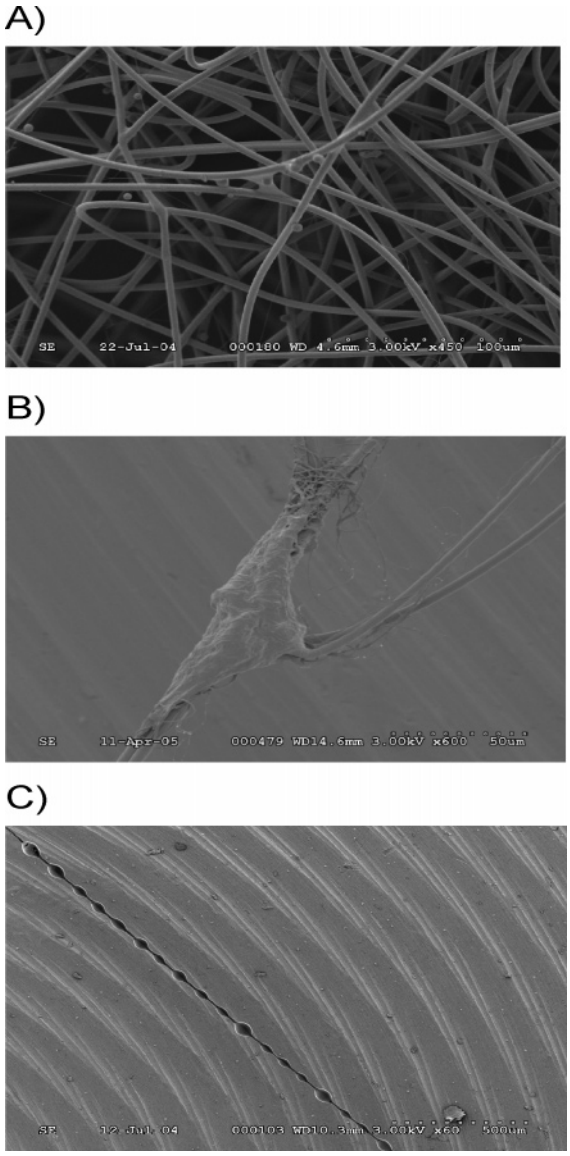

FIGURE 1: Glue coatings are found on egg case filaments, web scaffolding joints and gumfooted threads. (A) Scanning electron micrograph (SEM) of egg case silk at 450× magnification. (B) SEM of a scaffolding web joint at 600× magnification. (C) SEM of gumfooted silk at 60× magnification. Similar results of black widow coated gumfooted lines have been reported (*32*).

For recombinant protein expression, 20 mL of bacterial cultures were grown overnight to saturation. The following day, the saturated cultures were diluted with 80 mL of fresh LB and recombinant protein expression was induced with 0.02% arabinose. For negative controls, no arabinose was added to the cultures. Four hours after induction, the cells were pelleted at 4000*g*, washed with PBS, and then resuspended in 0.8 mL of 1X lysis buffer [50 mM NaH_2PO_4, 500 mM NaCl (pH 8.0)]. The resuspended pellet was then supplemented with lysozyme (1 mg/mL) and sonicated (3 consecutive 10 s pulses at maximum setting). The samples were then flash frozen in liquid nitrogen followed by incubation at 37 °C and sonication. Samples were clarified by centrifugation at 16000*g* for 30 min, and 0.4 mL was applied to a Ni-NTA agarose column. Proteins were eluted and used for Western blot analysis according to the manufacturer's protocol (Invitrogen Corporation, Carlsbad, CA).

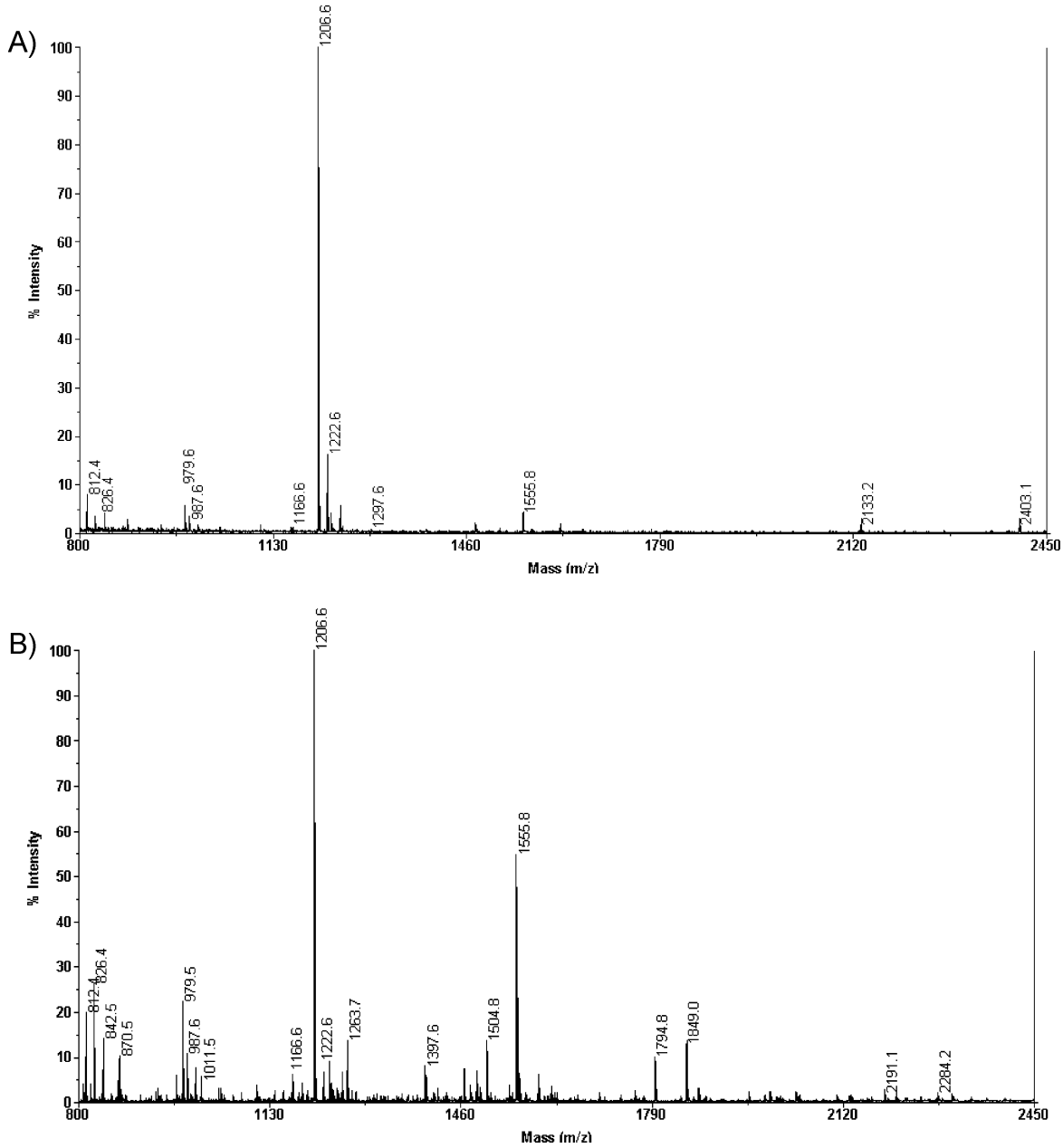

FIGURE 2: MALDI-TOF spectra of tryptic fragments generated from the aqueous glue coating components removed from egg cases and web scaffolding joints. (A) Peptide map of in-solution tryptic digest of the aqueous material extracted from egg cases. (B) Peptide map of in-solution digestion of the aqueous glue material removed from the web scaffolding connection joints.

RESULTS

Mass Spectrometric Analysis of Glue Components That Coat Different Silk Fiber Types Reveals Common Aqueous Protein Constituents. Glue coatings were identified on egg case threads, web scaffolding connection joints, and gumfooted lines (Figure 1A−C). To remove the water-soluble protein components on the silk threads, we extracted the coating substances with distilled water. After the glue coating materials were dissolved, the solubilized samples were individually subjected to in-solution tryptic digestion, followed by MS analysis. After digestion and mass measurement using MALDI-MS, numerous peptides were identified from the water-extracted egg case and scaffolding web connection joint samples (Figure 2A,B). Ten peptides were sequenced using high energy CAD, those with precursor ions of *m*/*z* 812.4, 826.4, 979.6, 987.6, 1011.5, 1166.6, 1206.6, 1222.6, 1297.6, and 1555.8 (Table 1). Peptides with *m*/*z* 812.4, 826.4, 979.6, 987.6, 1166.6, 1206.6, 1222.6, and 1555.8 were present in the water-extracted egg case and web scaffolding connection joint samples after tryptic digestion (Figure 2A,B). Similar results were obtained from water extracts collected from the gumfooted lines; however, none of these precursor ions were detected in water-extracted dragline silk samples (data not shown). The major peptide detected in the digestion of the aqueous extracts from all threads was observed as *m*/*z* 1206.6, which supports the idea that this peptide is derived from an abundant water-soluble glue component (Figure 2A,B). In addition, the species containing the peptide with experimental *m*/*z* 1555.8 was also relatively abundant in web scaffolding joint connection extracts, given the relatively high peak intensity in the peptide map (Figure 2B). The product ion spectra from the peptides

Table 1: Peptide Sequences Obtained from MALDI Tandem TOF Analyses of Precursor Ions That Show Overlapping Fingerprints in the Glue Coating Materials Removed from Egg Case Threads, Web Scaffolding Connection Joints, and Gumfooted Lines[a]

peptide mass (M^+H)	sequence	fiber found
812.4	TVHHYR	A, B, C
826.4	TIHHYR	A, B, C
979.5 (979.6)	HGLLNNVGR	A, B, C
987.6	LNIVYIPR	A, B, C
1011.5	LNIIYIPR	B
1166.6	EFFGGLGNIGR	A, B, C
1206.6	**AVHHYEVPVR**	**A, B, C**
1222.6	AVHHYEVLVR	A, B, C
1297.6	LGDDLFEYGLR	A, C
1555.8	**TLFNQAADLLDHVV**	**A, B, C**

[a] The boldface regions denote the peptides for which full-length cDNA sequences were retrieved by screening a cDNA library prepared from silk glands. These peptides were found to be present after translation of the isolated cDNA sequence. The letters A, B, and C indicate peptides observed in egg case, web scaffolding connection joints, and gumfooted lines, respectively.

of MH^+ 1206.6 and 1555.8 are shown in Figure 3A,B, respectively. Some peptides observed in the digests displayed strikingly similar amino acid sequence profiles, differing only by a single amino acid residue, e.g., AVHHYEVPVR and AVHHYEVLVR (underlined region denotes variation; Table 1). These sequences may represent different isomeric forms found in the spider population. Protein−protein BLAST (blastp) searches of the nonredundant NCBI database with these peptides revealed no significant matches with any published fibroins or other proteins deposited in the database (*E* values >0.12). These results support the assertion that the aqueous components deposited on egg case, scaffolding web connection joints, and the gumfooted lines contain a common group of proteins that represent novel coating constituents.

Even though we were able to sequence many of the major peptides after tryptic digestion (Table 1), we also analyzed the samples for some of the lower abundance peptides after enrichment using high performance liquid chromatography (HPLC). HPLC−MALDI TOF MS analysis of the water extracts from egg cases revealed the presence of additional ions at *m*/*z* 1615.8, 1619.8, 1682.8, 1734.9, 1774.9, 2038.1, and 2040.1. When these peptides were analyzed using MS/MS, the loss of 203 Da was observed. These mass differences correspond to the masses of GlcNAc or GalNAc, which supports the presence of glycoproteins with post-translational modifications (PTMs). Because of the relatively poor quality CAD MS/MS spectrum of the peptide at *m*/*z* 1734.9, we were only able to determine a partial sequence (...SDGGSNVGGNEYR). Collectively, these results support the existence of glycoproteins in egg case components and reinforce the fact that it is difficult to solubilize them in distilled water (data not shown).

Isolation of the Genes Encoding the Major Constituents in Aqueous Glue Fiber Coatings Reveals the Presence of Peptides. To isolate the nucleic acid sequence that encoded the aqueous coating component corresponding to the peptide at *m*/*z* 1206.6, we screened a cDNA library prepared from black widow spider silk glands using anchored PCR. The forward pGAL4.2 anchor primer and the reverse gene-specific oligonucleotide, which was designed against the underlined region of the peptide sequence AVHHYEVPVR, successfully led to the amplification of a single 139 bp cDNA fragment. Using this nucleotide sequence data, we amplified the remaining cDNA by applying a modified form of 3′ rapid amplification of cDNA ends (3′ RACE). One overlapping cDNA fragment obtained from 3′ RACE was used to reconstruct a 411 bp cDNA fragment that encoded an aqueous coating component, which we have named spider coating peptide 1 (SCP-1; GenBank Accession Number EF153411). Inspection of the cDNA sequence revealed the presence of 5′ and 3′ UTRs, along with a polyadenylation signal (data not shown). Translation of the cDNA revealed the presence of an ORF containing the AVHHYEVPVR sequence (Figure 4A). The predicted molecular mass of SCP-1 is ∼3.8 kDa, with a p*I* of 5.11. Protein−protein BLAST searches using the entire sequence of SCP-1 resulted in no strong matches with proteins deposited in the database. Analysis of the predicted amino acid content demonstrated that ∼50% of the peptide consisted of four residues: alanine, aspartate, valine, and histidine. Of particular note was the presence of 4 histidine residues, in two pairs separated by 11 amino acids (Figure 4A). Examination of the SCP-1 primary sequence using the SignalIP 3.0 algorithm revealed a potential signal cleavage site between amino acid residues 21 and 22 (Figure 4A). This cleavage site was confirmed by mass spectrometric analysis and explains the presence of the AVHHYEVPVR peptide, which resulted from the combination of a signal peptide cleavage event and tryptic digestion (Figure 4A). In addition, during the screening of the cDNA library for the nucleic acid sequence of SCP-1, we retrieved several different clones for DNA sequence analysis. Examination of these cDNA sequences after translation revealed the presence of different SCP-1 isoforms (Figure 4C).

Because the predicted full-length sequence of SCP-1 lacked the peptide sequence TLFNQAADLLDHVV, which corresponded to the peak at *m*/*z* 1555.8, we hypothesized that this stretch of residues must be derived from a second distinct gene. In order to obtain the cDNA sequence, we used an anchored PCR strategy that was similar to that used to isolate SCP-1. Using partially degenerate oligonucleotides (forward primer that corresponded to residues FNQAAD) and a reverse anchor primer, we successfully amplified two distinct DNA fragments from our silk-gland-restricted cDNA library. One fragment was ∼1.3 kb, while the other piece was ∼250 base pairs. Both fragments were subject to DNA sequencing, and their sequences were analyzed using translational programs and protein−protein BLAST searches of the nonredundant NCBI database. The larger fragment was determined to represent a false positive, encoding the enzyme histidine decarboxylase, and inspection of the primary sequence demonstrated that it lacked the FNQAAD sequence (data not shown). However, translation of the noncoding DNA strand from the smaller fragment predicted the amino acid sequence FNQAAD as well as continued with the ILDHVV sequence before reaching a stop codon (Figure 4B). The amplified cDNA also contained a 3′ UTR and a putative polyadenylation signal, suggesting that the TLFNQAADILDHVV peptide was derived from the C-terminus of the aqueous coating component. To further confirm this observation, we designed a gene-specific primer to the 3′ UTR region and performed 5′ RACE with a forward primer (anchor library oligonucleotide). Following amplification, we obtained a

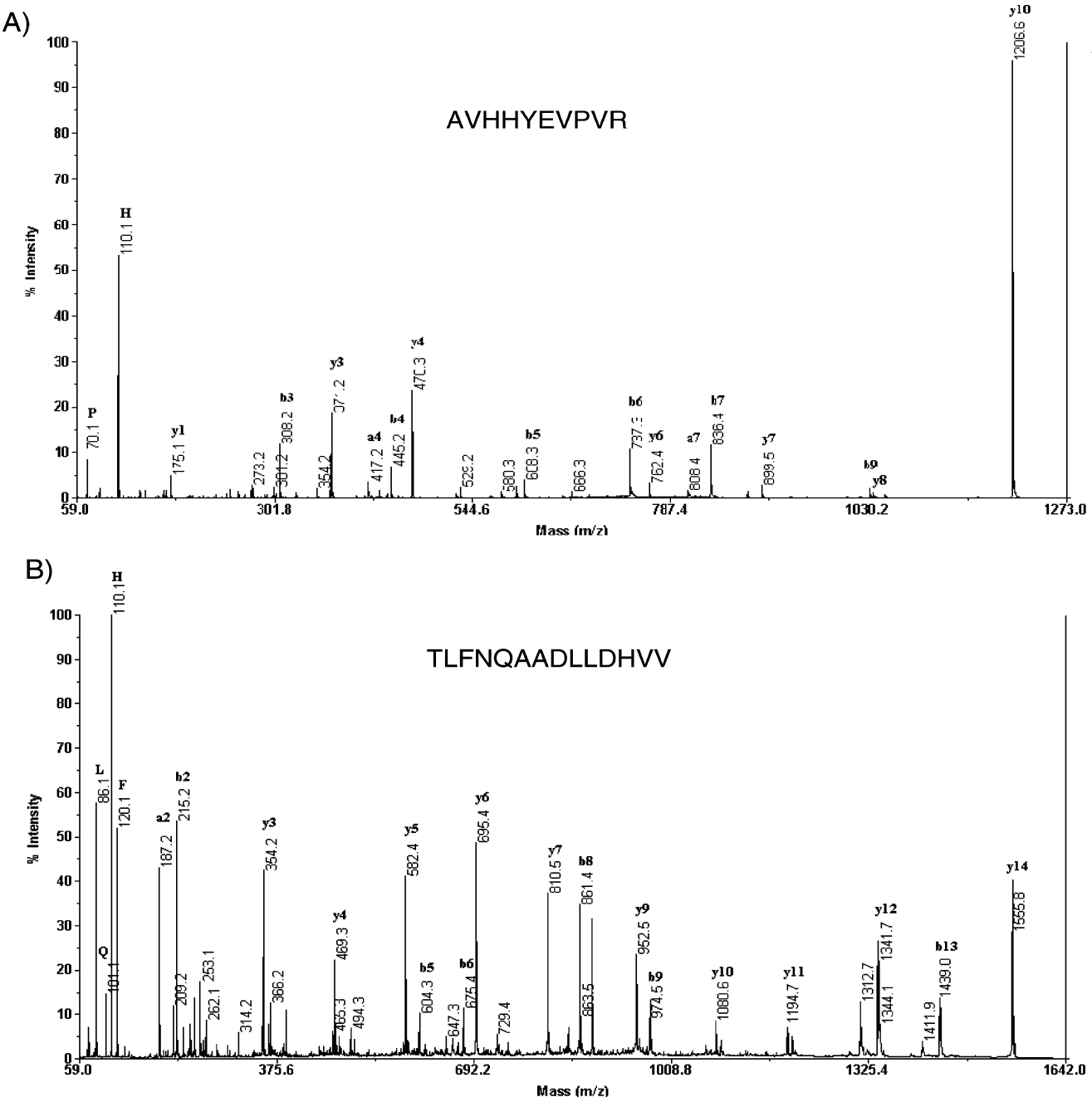

FIGURE 3: Mass spectra of common peptide fragments observed in the aqueous glue coating components of the different fiber types. (A) High energy CAD MS/MS spectrum of fragments of the precursor ion of *m*/*z* 1206.6. The sequence of this peptide was determined to be AVHHYEVPVR. (B) High energy CAD MS/MS spectrum of fragments of the peptide at *m*/*z* 1555.8. The peptide sequence was determined to be TLFNQAADLLDHVV. The second L in the sequence was later found to be I from the predicted primary sequence of the retrieved cDNA sequence (Figure 4B).

single fragment that could be overlapped with the initial PCR product, leading to the construction of a full-length cDNA sequence that corresponded to 342 bp (GenBank Accession Number EF153412). Analysis of the cDNA sequence demonstrated the presence of an ORF that encoded a protein with 41 amino acid residues prior to removal of the signal peptide (Figure 4B). We have dubbed this product spider coating peptide 2 (SCP-2). Inspection of the SCP-2 primary sequence with the SignalP 3.0 algorithm (www.cbs.dtu.dk/services/SignalP/) predicted a cleavage site between residues 21 and 22 (*26*); however, experimentally our data supported a cleavage event following residue 27 (Table 1; Figure 4B). After the removal of the secretion signal at this site, the predicted molecular weight of SCP-2 is ~2 kDa and the p*I* is 3.84. Analysis of the predicted amino acid composition profile demonstrated that ~60% of the peptide consisted of 5 residues: alanine, aspartate, leucine, proline, and valine.

Aggregate Glands Express the Aqueous Glue Coating Peptides in High Levels. Biochemical evidence supports the role of the aggregate gland as the chief tissue responsible for the production of spider glue droplet components; however, no genes that encode aqueous droplet proteins have been demonstrated to be exclusively expressed in this tissue. To examine the expression patterns of SCP-1 and SCP-2, we isolated total RNA from a variety of different tissues from the black widow spider for quantitative reverse

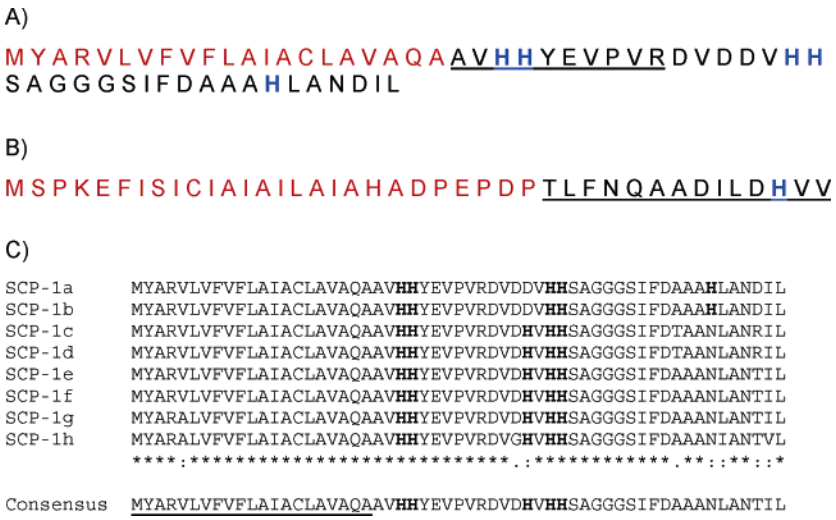

FIGURE 4: Primary sequences of SCP-1 and SCP-2. Amino acids are indicated with one-letter abbreviations, and the red color denotes the experimentally determined secretion signal that is removed after processing. Boldface dark blue letters indicate conserved histidine residues. (A) Translation of the SCP-1 nucleotide sequence predicts a protein with 36 amino acids after the removal of the secretion signal. (B) Translation of the SCP-2 cDNA sequence reveals a peptide that is 19 residues after signal peptidase cleavage. (C) Alignment of different SCP-1 isoforms demonstrates the presence of two duplicate histidine residue sets that are separated by 11 residues. Colons indicate residues with related side chain properties, whereas periods indicate dissimilar R group side chains.

transcription real-time PCR analysis (qRT-PCR). Equivalent amounts of total RNA were reversed transcribed from each tissue and used for qRT-PCR. Both SCP-1 and SCP-2 displayed aggregate gland restricted patterns of expression (Figure 5A,B). Relative to other silk producing glands and control tissues, SCP-1 and SCP-2 mRNA levels were ~150-fold and ~600-fold higher, respectively (Figure 5A,B). Lower levels of SCP mRNAs detected in the nonaggregate tissue samples likely represent basal transcription levels (Figure 5A,B).

SCP-1 Has Intrinsic Metal Binding Properties. During the molecular cloning of the SCP-1 cDNA sequence, we observed the presence of different isomeric forms of SCP-1 (Figure 4C). In particular, most of the differences in the amino acid residues appeared localized toward the C-terminus. In contrast, the N-terminus showed little variation; in particular, 4 histidine residues were found to be invariant in the SCP-1 isoforms (Figure 4C). Based upon this conservation and the role of histidine in metal ion coordination, we hypothesized that these residues may participate in the binding of metal ions. To test this premise, we overexpressed SCP-1 6x histidine-tagged (6xH-SCP1) and non-histidine-tagged (SCP1) fusion proteins in bacteria and tested their metal-binding activity. Both 6xH-SCP1 and SCP1 were designed to contain the V5 epitope tag to facilitate protein detection using an anti-V5 monoclonal antibody. Crude lysates containing bacterial expressed 6xH-SCP1 and SCP1 were applied to a Ni-resin column and tested for their ability to bind Ni ions by Western blot analysis. As expected, the 6xH-SCP1 protein was capable of binding the resin, being readily detected in the imidazole-eluted fraction (Figure 6). Strikingly, SCP1, which lacked the 6x His-tag, was also capable of binding the Ni resin as efficiently as the 6xH-SCP1 recombinant proteins (Figure 6). Collectively, these data imply that SCP-1 has intrinsic metal binding activity.

DISCUSSION

This study has led to the identification of small peptides that could be efficiently stripped from the coatings of different black widow silk fiber types after water treatment. Applying mass spectrometry, combined with reverse genetics, we have cloned the cDNAs encoding two peptides that are found in the sticky materials collected from egg cases, gumfooted lines, and web scaffolding connection joints. The observation that these peptides are absent from water washed dragline fibers, which is well established to represent a dry, non-glue coated thread, supports the assertion that these products can be classified as glue coating substances. Consistent with glue materials being manufactured by the aggregate gland, SCP-1 and SCP-2 mRNAs are shown to be highly expressed in the aggregate tissue. These molecules represent the first aggregate-derived peptides to be identified at the molecular level that display high levels of expression. Although we performed a fairly comprehensive tissue analysis for the expression of the SCP genes, additional studies will need to be performed to determine whether expression extends into other, nontested silk-producing glands. In particular, it would be interesting to determine if the piriform gland, which is responsible for producing web scaffolding connection joints, also displays elevated SCP-1 and -2 expression levels. Furthermore, given the complex nature of egg sac constituents, which reportedly contain products from the aciniform gland, piriform gland, and tubuliform gland in orb weavers (*27*), it is difficult to confirm, at this time, whether the coating materials are being removed from the smaller and/or larger diameter fibers observed in egg sacs (*24*, *27*). Moreover, it is also possible that they could be constituents of the piriform-derived attachment discs that help fasten the egg case to its substratum. If so, the piriform gland could be responsible for the extrusion of the SCPs during egg sac formation as well as the web scaffolding connection joints. Given the low levels of SCP-1 and SCP-2 mRNA detected in the tubuliform gland by the qRT-PCR analysis, it would seem logical to conclude that this tissue is not responsible for the abundant nature of the peptides detected by the mass spectrometry

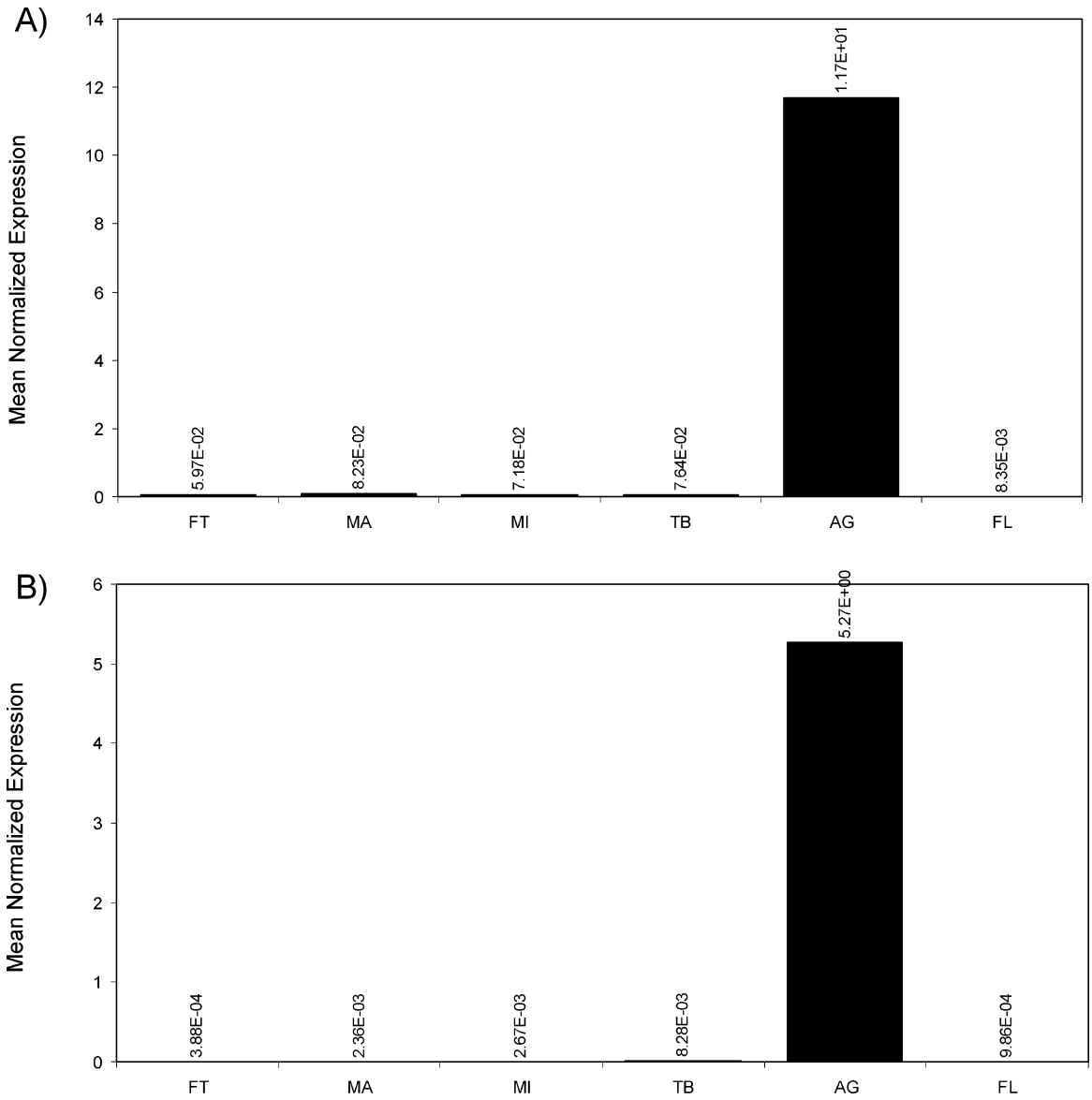

FIGURE 5: SCP-1 and SCP-2 mRNAs are predominantly expressed in the aggregate gland. qRT-PCR was used to determine the expression pattern of SCP-1 and SCP-2. Total RNA was isolated from the fat (FT), major ampullate (MA), minor ampullate (MI), tubuliform (TB), aggregate (AG), and the flagelliform (FL) glands as previously described (*33*). (A) qRT-PCR analysis of SCP-1 mRNA levels. (B) qRT-PCR analysis of SCP-2 transcript levels. Equivalent amounts of total RNA were reverse-transcribed using avian myeloblastosis virus (AMV) and aliquots used for qRT-PCR. Experiments were performed in triplicate and normalized internally using the housekeeping actin gene. Data are representative of experimental results obtained from two independent trials.

analysis of water washed egg case sacs. Thus, its origin would seem likely to be derived from the aggregate and/or piriform gland.

Previous studies performed with orb weavers have demonstrated that the adhesive droplets on the capture silk contain an aqueous solution consisting of organic LMM compounds; however, our studies are the first to demonstrate the presence of common coating peptides on different fiber types, which has led to the identification of these constituents and their primary sequences. It has been suggested that some of the LMM organic compounds serve a hygroscopic role, which allows for the condensation of water from the atmosphere to enhance fiber adhesiveness and extensibility (*21*). It has also been observed that the concentrations of specific LMM organic compounds change depending on the spider's environment and dietary conditions (*19*). Presently, the precise biological relationship between the SCPs and the LMM compounds is unclear. It is also unknown whether SCP-1 and SCP-2 physically interact with the nodular glycoprotein(s) in the droplets on gumfooted lines and/or the core fibroins to influence their macromolecular structure on the surface of the fibers. Although our preliminary studies indicate that bacterially expressed SCP-1 can bind nickel ions in vitro, further studies will need to be performed to establish whether SCP-1 binds metal ions in the web. Additional experiments will also be directed at testing whether the SCPs physically interact with the core fibroins, such as the egg case spidroin TuSp1 and the egg case proteins, ECP-1 and ECP-2 (*24*, *28*).

One intriguing question that arises from our studies centers on the function of these small peptides, in particular with respect to the web mechanics, prey capture, or reproduction. Since it has been well-established that the mechanical and functional properties of different fiber types are largely

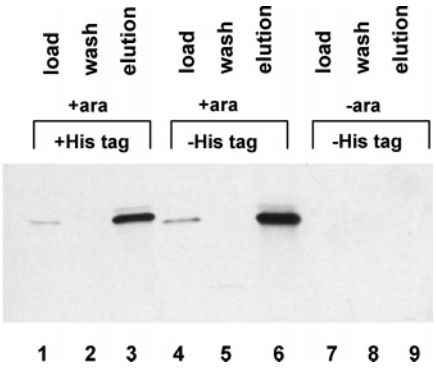

FIGURE 6: SCP-1 has intrinsic metal binding activity. SCP-1 (−His tag) and 6xH-SCP1 (+6x His tag) recombinant proteins were produced in bacteria and tested for their ability to bind a Ni-NTA agarose resin by Western blot analysis. "Load" represents the input of the crude lysate prior to purification, whereas "wash" indicates the proteins that fail to bind to the Ni-NTA agarose resin. "Eluted" indicates proteins removed by the addition of imidazole. In all cases, equivalent volumes of the samples were analyzed. Ara refers to cells treated with arabinose.

attributable to distinctions in the primary sequences of their core fibroins, coating the surfaces of different threads with common peptides would seem to imply that these molecules do not give rise to the differences in the mechanical properties of the fibers. Although it remains to be determined whether SCP-2 can bind metal ions, the ability of SCP-1 to bind a Ni-agarose resin, combined with its presence on different fiber types, suggests that metal ion−peptide interactions on the surface of the threads could serve an important biological function for the spider. One interesting possibility is that SCP-1 functions as a peptide chelator, which releases metal ions in response to pH changes to inhibit microbial growth. Inhibition of microbial activity could serve to protect the web or prevent damage of the developing eggs, which could be endangered by prey, predators, or airborne materials that introduce microbes into the fibers. If this hypothesis is correct, these molecules would represent the first class of natural antimicrobial peptides that prolong web longevity and durability. Consistent with this proposal is the observation that *Bombyx mori* fibers artificially coated with metal ions exhibit significant antibacterial activity relative to non-metal coated fibers (*29*). Because cob weavers spin webs that must survive in harsh environments for prolonged periods of time, it would seem logical that some provision would be made to help protect the web or enclosed eggs from microbial damage. Since dragline threads are chiefly used as temporary locomotive fibers, it would also seem reasonable from an energy management standpoint to leave these fibers in an uncoated state.

Although the potential for the SCPs to serve as antimicrobial agents is interesting, they could serve other biological functions. For example, they could take part in some unidentified oxidation−reduction reaction with other materials secreted by the aggregate gland on the exterior of the core fibroins. Although certain metal ions have been reported to facilitate conformational transitions of silk core fibroins during the extrusion process, it would seem unlikely that SCP-1 participates in this process because gluey substrates secreted from the aggregate gland are added after core fibroin extrusion (*30*, *31*). However, it is quite plausible that SCP-1 influences the conformational state of the large, adhesive glycoprotein(s) in the droplets on the gumfooted lines because they could be coextruded from the aggregate gland. Thus, SCP-1 could be important to the conformation of the nodular glycoproteins in the droplets on gumfooted lines. Future experiments will be directed at the examination of the secondary structure of SCP-1 in the presence of different metal ions such as Zn^{2+}, Cu^{2+}, Fe^{2+}, and Co^{2+}, the potential function of SCPs as antimicrobial agents, as well as the examination of in vivo metal-binding activity of the SCPs.

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