Molecular cloning and expression of the C-terminus of spider flagelliform silk protein from *Araneus ventricosus*

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A cDNA coding for the C-terminus of spider flagelliform silk protein (AvFlag) was cloned from *Araneus ventricosus*. Analysis of the cDNA sequence shows that the C-terminus of AvFlag consists of 167 amino acids of a repetitive region and 87 amino acids of a C-terminal non-repetitive region. The peptide motifs found in spider flagelliform silk proteins, GPGGX and GGX, were conserved in the repetitive region of AvFlag. Phylogenetic analysis further confirmed that AvFlag belongs to the spider flagelliform silk proteins. The AvFlag cDNA was expressed as a 28 kDa polypeptide in baculovirus-infected insect cells. As a new expression approach for spider silk protein, the combination of polyhedrin and AvFlag creates a polyhedrin AvFlag fusion protein (61 kDa) that is produced as recombinant polyhedra; this provides a basis for the source of spider silk proteins for various applications.

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1. Introduction

Spider silks are attractive biomaterials that are of particular biotechnological interest for industrial and medical purposes because of their unique physical and mechanical properties (Scheibel 2004). A range of material applications in biomedicine, textile technology and personal care products such as cosmetics are being targeted for these proteins. For instance, spider silks are being considered in the field of biopolymers, contact lens material, surgical threads, biomaterial membranes, scaffolds and tissue engineering (Foo and Kaplan 2002; Scheibel 2004). Spider silks are classified according to the gland in which they are produced by the spinning apparatus of the spider. Among the spider silks, the major ampullate silk, which forms the primary dragline, has an extremely high tensile strength. Flagelliform silk shows the highest elasticity (more than 200%). All spider silks have small peptide motifs that can be grouped into four major categories: GPGXX, alanine-rich stretches [A_n or $(GA)_n$], GGX and spacers (Scheibel 2004).

Despite their promising properties, spider silks cannot be obtained in large quantities from spiders, because spiders cannot be reared on a large scale due to their highly territorial and cannibalistic nature. Several attempts to produce recombinant spider silks or spider silk mimics have been reported in various expression hosts (Arcidiacono *et al* 1998; Scheller *et al* 2001; Lazaris *et al* 2002; Huemmerich *et al* 2004). The recombinant silk proteins were mainly produced in microbes or in cell culture.

Among the types of spider silks, draglines from *Nephila clavipes* and *Araneus diadematus* have been the most intensely studied. However, the entirety of a spider silk gene has not been cloned yet, and sequence data from

Keywords. *Araneus ventricosus*; baculovirus expression vector; cDNA cloning; flagelliform silk protein; spider.

Abbreviations used: BSA, bovine serum albumin; EST, expressed sequence tag; AvFlag, flagelliform silk protein from *A. ventricosus*; HRP, horseradish peroxidase; MOI, multiplicity of infection; PAUP, Phylogenetic Analysis Using Parsimony; PFU, plaque-forming units; PAGE, polyacrylamide gel electrophoresis

the only the 3′ ends of partial cDNA clones of spider silk genes have been reported (Xu and Lewis 1990; Hinman and Lewis 1992; Beckwitt and Arcidiacono 1994; Guerette *et al* 1996; Hayashi and Lewis 1998). Previous studies used partial cDNA constructs of dragline silk genes to produce recombinant spider silk proteins in *Escherichia coli* (Arcidiacono *et al* 1998), in mammalian cells (Lazaris *et al* 2002), or in insect cells (Huemmerich *et al* 2004).

Our objective in initiating this study is to illustrate the structure of the spider silk gene from *Araneus ventricosus*. This paper describes the cloning and expression of a partial cDNA encoding the C-terminus of flagelliform silk protein from *A. ventricosus* (AvFlag). We cloned and sequenced the partial cDNA encoding the C-terminus of AvFlag and expressed the recombinant AvFlag in baculovirus-infected insect cells. The recombinant AvFlag was produced as a 28 kDa polypeptide in large amounts and as a polyhedrin–AvFlag fusion protein (61 kDa) in large-shaped recombinant polyhedra.

2. Materials and methods

2.1 *cDNA library screening, nucleotide sequencing and data analysis*

The clone containing the cDNA insert was selected from the expressed sequence tags (ESTs), which were generated from a cDNA library constructed using whole bodies of *A. ventricosus* (Lee *et al* 2003). The plasmid DNA was extracted using a Wizard mini-preparation kit (Promega, Madison, WI, USA) and sequenced using an ABI 310 automated DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The sequences were compared using the DNASIS and BLAST programs provided by the NCBI (*http://www.ncbi.nlm.nih.gov/BLAST*). MacVector (version 6.5, Oxford Molecular Ltd., Oxford, UK) was used to align the amino acid sequences of the spider silk genes. With the eight GenBank-registered spider silk protein sequences, phylogenetic analysis was performed using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0 (Swofford 2000). The accession numbers of the spider silk protein sequences in GenBank are as follows: *Araneus ventricosus* flagelliform (EF025541; this study), *Argiope trifasciata* flagelliform (AF350264), *Nephila clavipes* flagelliform (AF027973), *Latrodectus hesperus* major ampullate spidroin 1 (DQ409057), *Latrodectus hesperus* major ampullate spidroin 2 (DQ409058), *Nephila clavipes* dragline (M37137), *Nephila clavipes* spidroin 1 (U20329), *Nephila clavipes* spidroin 2 (M92913), and *Bombyx mori* fibroin heavy chain (AF226688). The hydrophobicity of amino acid sequences was predicted using Kyte and Doolittle mean hydrophobicity profiles (Kyte and Doolittle 1982).

2.2 *Expression of recombinant protein*

A baculovirus expression system (Je *et al* 2001) that used the *Autographa californica* nucleopolyhedrovirus (AcNPV) and an insect cell line (Sf9) was employed for the production of recombinant AvFlag protein. The partial cDNA encoding the C-terminus of AvFlag was excised from pBlueScript–*AvFlag* by digestion with *Xho*I and *Eco*RI and inserted into the same sites of the transfer vector, pBacPAK9 (Clontech, Palo Alto, CA, USA), to express AvFlag under the control of the AcNPV polyhedrin promoter. The resulting plasmid was designated pBacPAK9–*AvFlag*. In addition, the partial cDNA encoding the C-terminus of AvFlag from pBlueScript–*AvFlag* was subcloned into the 3′ end of the structural sequences of AcNPV polyhedrin to yield the transfer vector pBacPAK9–*Polh–AvFlag*. Five hundred nanograms of the construct (pBacPAK9–*AvFlag* or pBacPAK9–*Polh–AvFlag*) and 100 ng of the AcNPV viral DNA (bAcGOZA) (Je *et al* 2001) were co-transfected into 1.0–1.5 × 10^6 Sf9 cells for 5 h using lipofectin reagent (Gibco BRL, Gaithersburg, MD, USA). The transfected cells were cultivated in TC100 medium (Gibco BRL) at 27°C for 5 days. The recombinant AcNPV was propagated in Sf9 cells, and the titre was expressed as plaque-forming units (PFU) per millilitre as per the standard methods (O'Reilly *et al* 1992).

2.3 *SDS–polyacrylamide gel electrophoresis*

Insect Sf9 cells were mock-infected or infected with the wild-type AcNPV and recombinant AcNPV in a 35 mm diameter dish (1 × 10^6 cells) at a multiplicity of infection (MOI) of 5 PFU per cell. After incubation at 27°C, cells were harvested at 3 days post-infection (p.i.). For SDS–PAGE (Laemmli 1970) of cell lysates, uninfected Sf9 cells and cells infected with virus were washed twice with PBS, mixed with protein sample buffer (10% 2 mercaptoethanol, 4% SDS, 10% sucrose, 0.004% bromophenol blue, 0.12 M Tris HCl, pH 6.8), and boiled for 3 min. The total cellular lysates were subjected to 10% SDS–PAGE. After electrophoresis, gels were fixed and stained with 0.1% Coomassie Brilliant Blue R-250.

2.4 *Production of antibody*

Recombinant AvFlag (~5 mg) was electroeluted from the SDS-PAGE gel, mixed with an equal volume of Freund complete adjuvant (a total of 200 ml, Sigma) and injected into Balb/c mice (Lee *et al* 2005). Two successive injections of antigens mixed with equal volumes of Freund incomplete adjuvant (a total of 200 ml) were administered at 1-week intervals beginning one week after the first injection. Blood was collected 3 days after the last injection of antigens only and centrifuged at 13,000 rpm for 5 min.

The supernatant containing the antibodies was stored at –70°C until use.

2.5 *Western blot analysis*

Western blot analysis was carried out using an ECL Western blotting analysis system (Amersham Biosciences, Arlington Heights, IL, USA). The protein samples were mixed with the sample buffer, boiled for 5 min, and loaded onto a 10% SDS–PAGE gel. The proteins were blotted onto a sheet of nitrocellulose transfer membrane (Schleicher & Schuell, Dassel, Germany). After blotting, the membrane was blocked by incubation in a 1% bovine serum albumin (BSA) solution, incubated with antiserum solution (1:1000 v/v) at room temperature for 1 h, and washed in TBST [10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.05% (v/v) Tween-20]. The membrane was then incubated with anti-mouse IgG–horseradish peroxidase (HRP) conjugate and HRP–streptavidin complex. After repeated washing, the membrane was incubated with enhanced chemiluminescent (ECL) detection reagents (Amersham Biosciences) and exposed to autoradiography film.

3. Results and discussion

3.1 *Cloning, sequencing and analysis of AvFlag cDNA*

A search of *A. ventricosus* ESTs identified a cDNA clone that had high homology to previously reported spider flagelliform silk genes. The cDNA clone, including the 3′ end of the spider flagelliform silk gene of *A. ventricosus*

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  1 GGC GGA GGT CCA GGA GGA CCA GGC GGT GCA GCC GGA GGA GCA GGA GCT GGT GGT GTC GGA
  1 G   G   G   P   G   G   P   G   G   A   A   G   G   A   G   A   G   G   V   G
 61 CCT GGA GGA GGA TCC TCT GGA GGT GCT GGA GGA GCA GGT CCG GTA TCT GTT TCT TCT TCT
 21 P   G   G   G   S   S   G   G   A   G   G   A   G   P   V   S   V   S   S   S
121 GTA GTT GTC AGT GGC GCT GGA GGA CCC AGT GGA CCA GGA GCT GGT GGT GTT GGA CCT GGA
 41 V   V   V   S   G   A   G   G   P   S   G   P   G   A   G   G   V   G   P   G
181 GGT GCT GGA CCA GGA GGT ATT TAC GGA CCA GGA GGA GCT GGC GGA CTT TAC GGA CCG GGC
 61 G   A   G   P   G   G   I   Y   G   P   G   G   A   G   G   L   Y   G   P   G
241 GCA GGT GGA TCT TTC GGA CCA GGA GGT GGA GCT GGT GGT CCA GGA GGA CCT GGA GGT GCT
 81 A   G   G   S   F   G   P   G   G   G   A   G   G   P   G   G   P   G   G   A
301 GGT GGA CTC GGC GGC CCA GGT GGT CCA GGA GGA GCT GGA GTA AGC GGT GGT GTT GTA CCT
101 G   G   L   G   G   P   G   G   P   G   G   A   G   V   S   G   G   V   V   P
361 GTA GCT GGT GGT GCT GCA CCA GGA GCT GGT GGT TCA GGC CCG ACA ACT GTC ACA GAA GTT
121 V   A   G   G   A   A   P   G   A   G   G   S   G   P   T   T   V   T   Q   V
421 GTG TCT ATA ACT GTC AGT GGT GGC CAG CCT TCT GGT GGT GTT CTC CCT GGA GGT AGT TAT
141 V   S   I   T   V   S   G   G   Q   P   S   G   G   V   L   P   G   G   S   Y
481 ACC CCT GCA GCC GGT GGA AGT TCA AGA TTA CCG TCT CTA ATT AAC GGA ATT ATG AGT TCG
161 T   P   A   A   G   G   S   S   R   L   P   S   L   I   N   G   I   M   S   S
541 ATG CAG GGA GGT GGA TTC AAT TAC CAG AAT TTC GGA AAC GTT CTC TCC CAG TTT GCT ACC
181 M   Q   G   G   G   F   N   Y   Q   N   F   G   N   V   L   S   Q   F   A   T
601 GGA ACC GGA ACT TGC AAC AGC AAC GAT TTG AAT CTC TTG ATG GAC GCC CTC TTA TCT GCC
201 G   T   G   T   C   N   S   N   D   L   N   L   L   M   D   A   L   L   S   A
661 CTC CAC ACC CTG AGC TAC CAA GGA ATG GGC ACT GTT CCG TCA TAT CCC TCG CCA TCC GCT
221 L   H   T   L   S   Y   Q   G   M   G   T   V   P   S   Y   P   S   P   S   A
721 ATG TCA GCT TAT TCT CAA TCC GTC CGA AGA TGC TTT GGA TAT TGA ATGAGTTTTCATGAC
241 M   S   A   Y   S   Q   S   V   R   R   C   F   G   Y   *
781 CTTTGGATTCGTCTTTAATTTTTATTGCGAAATTTCAAATATAAGTGTTTTGAGAATGAA
841 TTGTATTTCACATTCTAAAAAATATCTATATTGATAATAATAAATTACCATTATGCAAGC
901 AAAAAAAAAAAAAAAAAAAA
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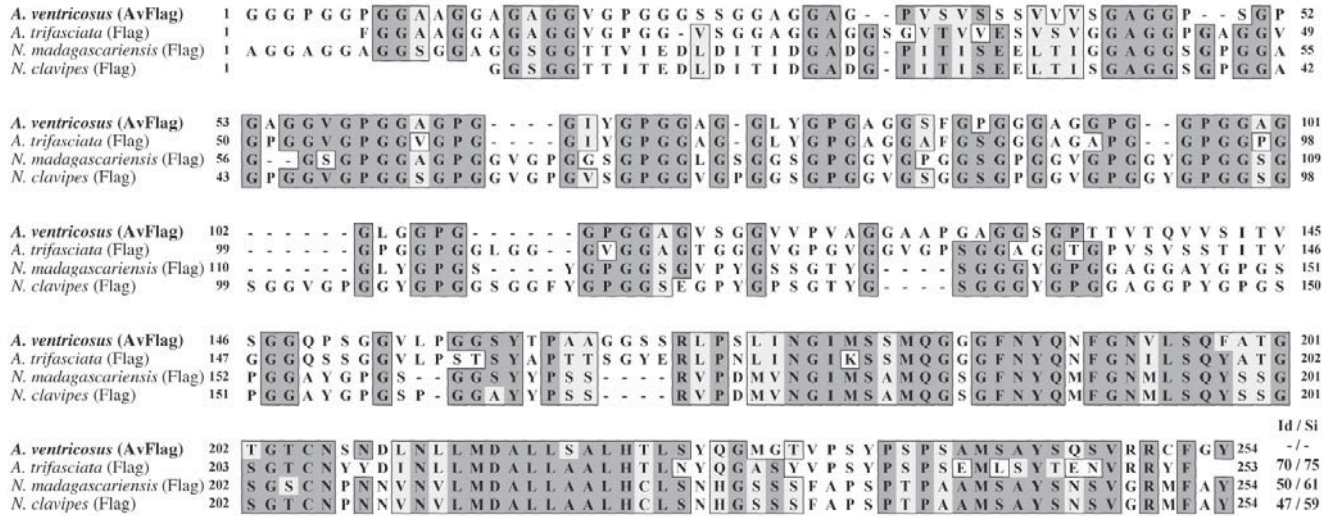
Figure 1. The nucleotide and deduced amino acid sequences of *AvFlag* cDNA. The termination codon (TGA) is indicated by an asterisk. The polyadenylation signal (AATAAA) is underlined. The GenBank accession number is EF025541.

(AvFlag), was sequenced and characterized (figure 1). The partial AvFlag cDNA is 921 bp long and contains the 3′ end of the spider flagelliform silk gene, and is capable of encoding a 254 amino acid polypeptide. The partial cDNA has both a stop codon (TGA) and putative polyadenylation signal (AATAAA). The AvFlag cDNA sequence has been deposited in GenBank under the accession number EF025541.



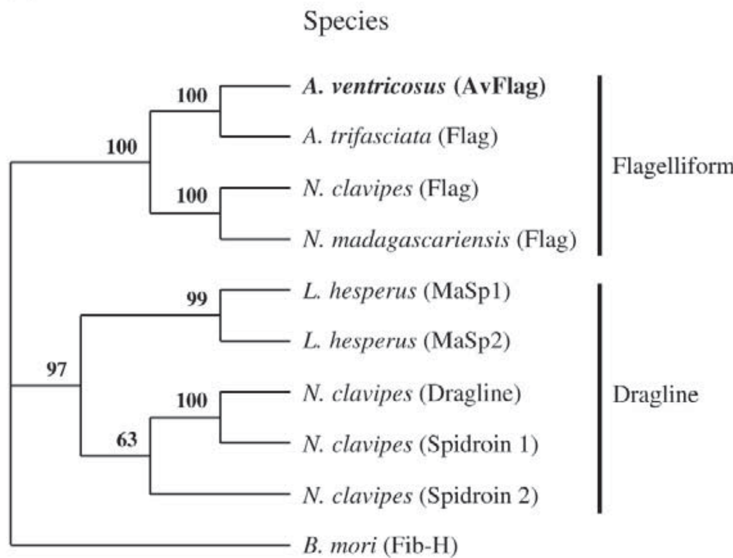


Figure 2. Comparison of the protein sequence of AvFlag with that of other spider silk proteins. (**A**) Alignment of the deduced amino acid sequence of AvFlag with other spider flagelliform silks. Residues are numbered according to the aligned flagelliform silk protein sequences, and invariant residues are shaded black. Dots represent gaps introduced to improve alignment. Identical residues are shown in solid boxes. The sequence sources are described in Materials and methods. The AvFlag sequence was used as a reference for the identity/similarity (Id/Si) values. (**B**) A maximum parsimony analysis for the amino acid sequences of AvFlag and known spider silk proteins. The sequence sources are described in Materials and methods. The tree was obtained by bootstrap analysis with the option of heuristic search and the numbers on the branches represent bootstrap values for 1,000 replicates. *B. mori* fibroin heavy chain was utilized as an outgroup.

Multiple sequence alignment of the deduced protein sequence of *AvFlag* cDNA with available spider flagelliform silk sequences is shown in figure 2A. Alignment with the deduced amino acid sequence of *AvFlag* cDNA indicated that the AvFlag sequence was closely related to the spider flagelliform silks from other species. AvFlag showed the closest amino acid identity with the flagelliform silk protein of the spider *Argiope trifasciata* (70% identity). A phylogenetic analysis using the deduced amino acid sequences of known spider silk genes yielded two separate groups (figure 2B). AvFlag and *A. trifasciata* flagelliform silk showed a close relationship, forming a subgroup. Based on sequence similarity and phylogenetic analysis, AvFlag is assumed to be a spider flagelliform silk.

The alignment analysis of protein sequences shows that the C-terminus of AvFlag consists of 167 amino acids of a repetitive region and 87 amino acids of a C-terminal non-repetitive region (figure 3A). The peptide motifs, GPGGX and GGX, which are commonly present in spider flagelliform silk proteins, were conserved in the repetitive region of AvFlag, which is composed of 8 amino acid motifs of GPGGX and 19 of GGX. A hydrophobicity profile of AvFlag indicates that a region of high hydrophobicity is found in the repetitive region of AvFlag (figure 3B).

The peptide motifs in spider silk proteins have been assigned structural roles (Scheibel 2004). The GPGGX and GGX motifs have been suggested to be involved in a β-turn spiral, which provides the elasticity of the fibre (Dong *et al* 1991; Hutchinson and Thornton 1994; Lewis *et al* 1996; Van Dijk *et al* 1997; Van Beek *et al* 2002). Flagelliform silks, which show the highest elasticity (more than 200%), consist of contiguous repeats of these motifs

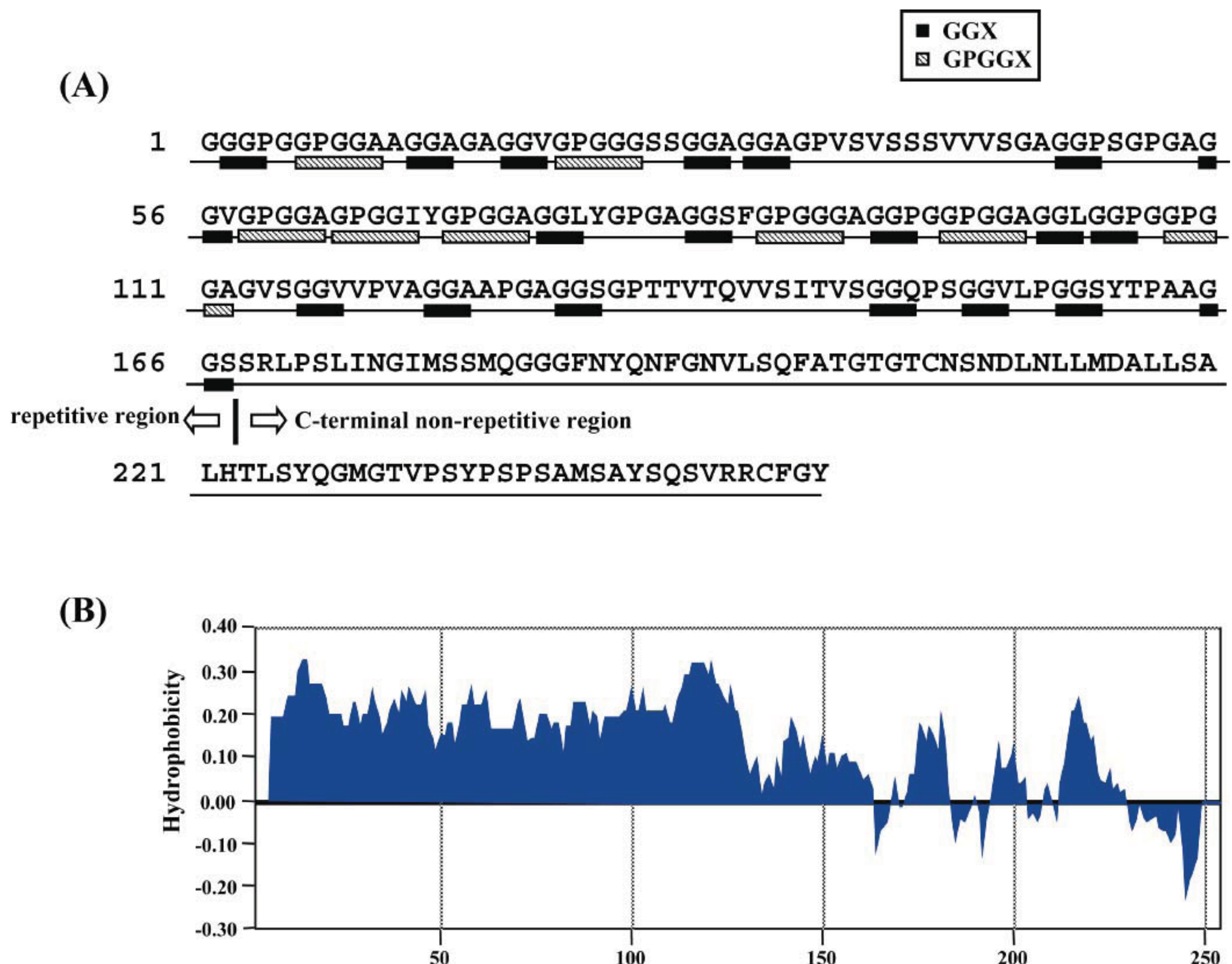


Figure 3. Composition (**A**) and hydrophobicity profile (**B**) of AvFlag. The highly repetitive region and C-terminal non-repetitive region are indicated. The GPGGX and GGX structural motifs in the repetitive region are indicated by shaded boxes and solid boxes, respectively, under the corresponding sequences.

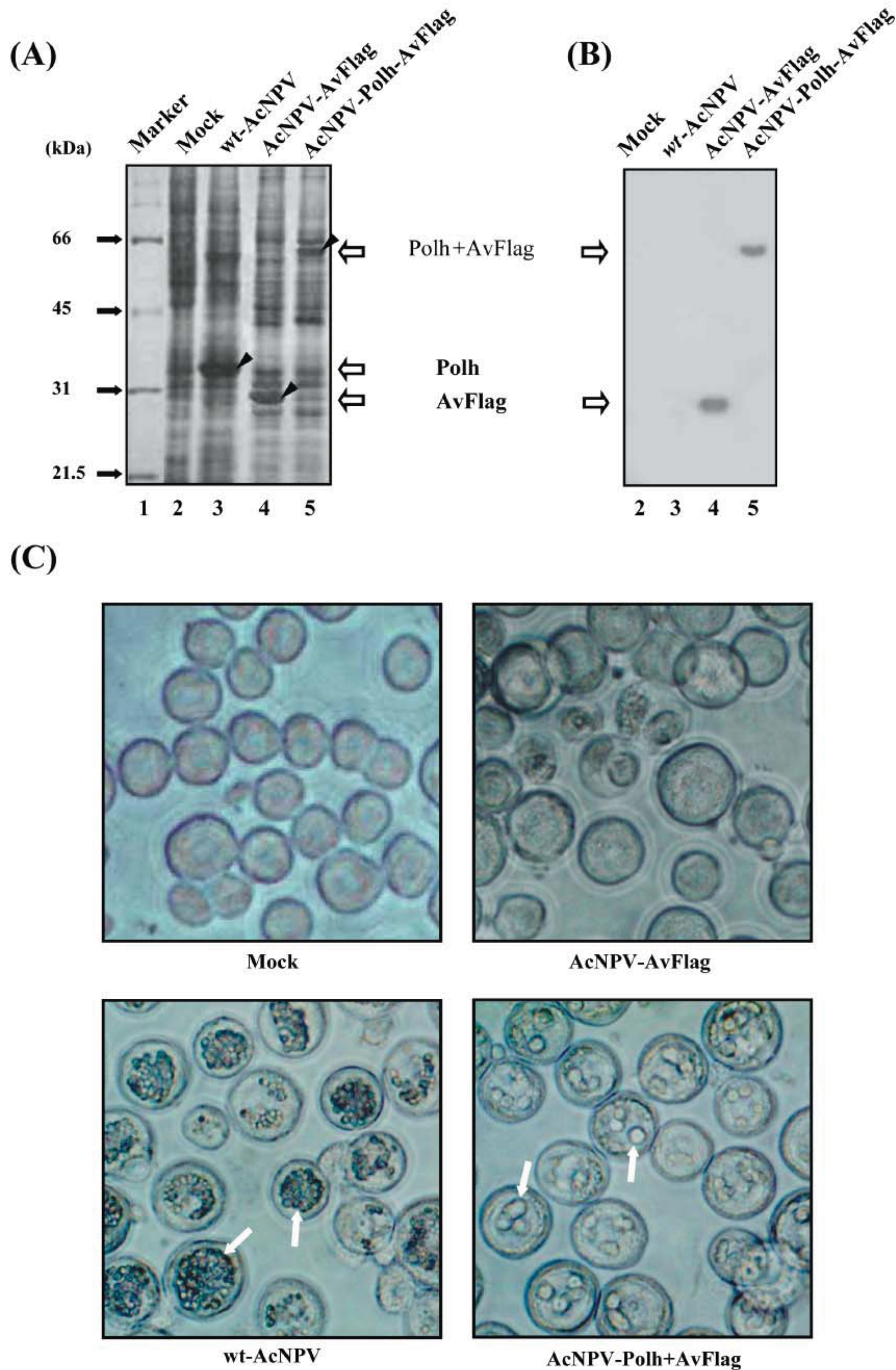


Figure 4. Expression of recombinant AvFlag in baculovirus-infected insect cells. Sf9 cells were mock-infected (lane 2) or infected with wild-type AcNPV (lane 3) and recombinant AcNPVs, AcNPV-AvFlag (lane 4), or AcNPV-Polh-AvFlag (lane 5), at an MOI of 5 PFU per cell. Cells were collected at 3 days p.i. Total cellular lysates were subjected to 10% SDS–PAGE (**A**), electroblotted, and incubated with recombinant AvFlag antibody (**B**). Molecular weight standards were used as a size marker (lane 1). The recombinant AvFlag (28 kDa), polyhedrin–AvFlag fusion protein (61 kDa) and polyhedrin (33 kDa) are indicated by an arrow on the side of the panel. (**C**) Production of recombinant polyhedra in insect Sf9 cells. Insect Sf9 cells infected with virus were photographed at 3 days p.i. (400×). The polyhedra are indicated by arrows.

(Scheibel 2004). Silk proteins typically consist of a non-repetitive N-terminus, a highly repetitive region and a non-repetitive C-terminus. The high hydrophobicity found in the repetitive region and the non-repetitive C-terminus of spider silks shows a high degree of conservation in terms of primary amino acid sequence (Challis *et al* 2006). All these observations together suggest that *AvFlag* is a novel spider silk gene, belonging to the spider flagelliform silk proteins.

3.2 *Expression of AvFlag in baculovirus-infected insect cells*

To assess *AvFlag* cDNA, the cDNA was inserted into a baculovirus transfer vector. The baculovirus transfer vector was used to generate a recombinant virus expressing AvFlag. The transfer vector, pBacPAK9–*AvFlag*, was constructed by insertion of *AvFlag* cDNA under control of the AcNPV polyhedrin promoter of pBacPAK9. Recombinant AcNPV, which we have termed AcNPV–AvFlag, was produced in insect Sf9 cells by co-transfection with wild-type AcNPV DNA and the transfer vector. Recombinant AvFlag protein synthesis in Sf9 cells infected with the recombinant virus AcNPV–AvFlag was assessed by SDS–PAGE (figure 4A). The recombinant AvFlag protein was present as a 28 kDa polypeptide in cells infected with the recombinant virus, but not in cells infected with the wild-type AcNPV or mock-infected cells. In order to further characterize the expression of AvFlag, the recombinant AvFlag protein was electroeluted from the SDS–PAGE gel and used to produce a mouse antibody. The expression of the recombinant AvFlag in baculovirus-infected insect cells was confirmed by Western blotting using the antibody against recombinant AvFlag (figure 4B).

Next, the expression of recombinant AvFlag was further investigated by recombinant polyhedra technology (Je *et al* 2003). As a new approach for spider silk protein expression, we tried to combine polyhedrin with AvFlag. The transfer vector, pBacPAK9–*Polh–AvFlag*, was constructed by insertion of *AvFlag* into the 3′ end of structural sequences of AcNPV polyhedrin. Recombinant AcNPV, which we have termed AcNPV–Polh–AvFlag, was produced in insect Sf9 cells. Recombinant Polh–AvFlag fusion protein synthesis in Sf9 cells infected with the recombinant virus AcNPV–Polh–AvFlag was assessed by SDS–PAGE and Western blot analysis (figures 4A and 4B). The result shows that the combination of polyhedrin and AvFlag was expressed as a 61 kDa polyhedrin–AvFlag fusion protein. As expected, insect cells infected with the recombinant virus AcNPV–Polh–AvFlag produced the recombinant polyhedra (figure 4C), which suggests that the large morphology of recombinant polyhedra could be due to the combination of polyhedrin and AvFlag.

Compared to the expression level of polyhedrin, which accumulates up to 50% of the total cellular protein in baculovirus-infected insect cells (O'Reilly *et al* 1992), the recombinant AvFlag was produced in large amounts (as shown in lane 4 of figure 4A). A previous study has shown that the most promising expression system seems to be the baculovirus system, since it was possible to efficiently produce dragline silk components at a high yield (Huemmerich *et al* 2004). The combination of polyhedrin and AvFlag resulted in the production of recombinant polyhedra with AvFlag. The technique is an alternative method of expressing recombinant spider silk protein, providing a basis for a source of spider silk proteins for various applications. In conclusion, the production of spider silk proteins in large quantities would be very useful for biomaterial applications of spider silks. The biochemical and structural characteristics of expressed spider silk proteins of AvFlag should be investigated further.

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