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Transient Expression of a Major Ampullate Spidroin 1 Gene Fragment from *Euprosthenops* sp. in Mammalian Cells

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Abstract. *Spider silk possesses extraordinary and unsurpassed mechanical properties and several attempts have been made to artificially produce spider silk in order to manufacture strong and light engineering composites. In the field of oncology, recombinant spider silk has the potential to be used as a biomaterial for bone replacement after tumour surgery. In this study, a 636-base pair gene fragment, coding for a part of major ampullate spidroin 1 from the African spider, Euprosthenops sp., was cloned into the expression vector pSecTag2/Hygro A, designed for the production of protein in mammalian cells. COS-1 cells were subsequently transfected with the recombinant plasmids and transient expression of low amounts of the corresponding silk protein fragment was obtained. The expressed fragment contained repetitive sequences associated with intrinsic biomechanical properties and has potential as a starting material for designed biopolymers.*

Primary bone tumours or metastatic bone disease often require surgery, not only to cure but also to treat impending or existing pathological fractures, as well as to alleviate pain. Depending on the position of the neoplastic lesion, the techniques available for reconstruction include arthrodesis, prosthesis with or without allografts, inverted prostheses or massive allografts (1). Bone cancer surgery presents a number of problems. Surgery often has to be radical, which leads to major defects (1, 2). In addition, with larger implant mass, the proportion of the implant relative to the remaining bone increases, generating a biomechanical disadvantage to the limb (3). Finally, grafted bone does not always succeed in replacing the deficient bone mass (3). Most attempts to replace bone have been based on transplantation of homologous bone tissue. However, the use of allograft bone has a number of potential risks, *e.g.,* the transfer of viruses or prions. Even though autologous bone grafts, fresh-frozen allografts, freeze-dried allografts and demineralised bone matrix are still frequently used, ceramics and new biomaterials are playing an increasing role as bone replacement tissue (4).

In this context, our attention was drawn to spider silks due to their extraordinary and unsurpassed biomechanical properties. Besides being strong and elastic, it is also biocompatible (5). Spider silks are composed of proteins with a predominance of alanine, glycine and serine. These proteins are synthesised in specialised epithelial cells and are subsequently secreted into the glandular lumen where they are stored until silk formation (6). The liquid crystalline protein solution is finally extruded and irreversibly converted to insoluble fibres. Unlike silkworms, which have one set of paired glands, spiders can have up to 7 silk-producing glands that generate different types of silk, each with unique mechanical properties (6).

Over the last decade, interest has been focused on dragline silk, mainly because it has the most desirable properties to be copied for bioengineering applications. Spider dragline silk is produced by the major ampullate gland and possesses a rare combination of biomechanical properties. It is of low density, high strength and displays a considerable elongation before breaking. Taken together, this gives a toughness (defined as energy stored before fracture per unit mass) superior to the best synthetic fibres (7). Molecular studies have determined partial DNA sequences for dragline silk proteins from different spider

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species (8-13). These proteins consist mainly of repetitive sequences with poly-alanine regions, which vary between 4 and 14 alanine residues, sandwiched between sequences rich in glycine. Furthermore, the proteins contain non-repetitive, highly conserved C-terminal domains. Recent studies have also revealed a non-repetitive and well-conserved N-terminal sequence (14). Structural studies have shown that the protein fibre consists predominantly of crystals formed by stacked poly-alanine regions in β-sheet conformation, interspersed with glycine-rich regions forming an amorphous matrix (15-19). Recently, sequence studies have also been conducted on other spider silks, such as flagelliform, tubuliform, aciniform and minor ampullate silk (20-28). Thus, a substantial data set has been collected on the structural motifs of spider silk proteins. These data are being supplemented by a plethora of physical techniques, *e.g.,* tensile testing, Raman spectroscopy, X-ray diffraction, circular dichroism and NMR studies.

The expression of recombinant partial spider silk genes in bacterial and yeast systems has been reported to be inefficient and truncated synthesis is a limiting factor when expressing silks of high molecular weight in *Escherichia coli* (29-34). The highly repetitive and GC-rich sequences may cause unusual mRNA secondary structures, with concomitant inefficient translation. Moreover, these genes contain codons that are not efficiently translated in microbial expression systems. Mammalian cell systems have proved to efficiently overcome many of these limitations (35). In this study, we succeeded in developing a system that enables the transient expression of a fragment of the major ampullate spidroin 1 (MaSp1) gene from the African spider *Euprosthenops* sp.

Materials and Methods

Materials. E. coli strain Stbl 2-competent cells (Invitrogen), Stockholm, Sweden, were used for cloning.

The plasmid used for cloning of the construct pIgkappa1-14 was pSecTag2/HygroA (Invitrogen). Two additional plasmids were used: pSecTag2/Hygro/PSA (Invitrogen) as a positive control for expression and pCMVβ (Clontech, Mountain View, CA, USA) to optimise the transfection conditions.

Cell culture. COS-1 cells (ATCC, Manassas, VA, USA) were grown with DMEM medium (Sigma, Stockholm, Sweden) containing 10% heat-inactivated foetal bovine serum (Invitrogen), 2 mM L-glutamine (Invitrogen) and 100 μg/ml gentamycin (Invitrogen). The cells were maintained with 5% CO_2 at 37˚C.

Vector constructions. The clone used in this study (called pER1-14), encoding a partial MaSp1 protein, was described previously and originates from an *Euprosthenops* spider of unknown species (12), (GenBank entry CQ816656). pER1-14 contains a 636-bp cDNA encoding repetitive parts of MaSp1 (Figure 1).

The 636-bp cDNA insert of pER1-14 was released with *Eco*RI, gel-purified using QIAquick gel extraction kit (Qiagen, West Sussex, UK) and subsequently treated with the Klenow fragment of DNA polymerase I (Invitrogen). The mammalian expression vector pSecTag2/Hygro A (Invitrogen) was digested with *Bam*HI and *Eco*RV and gel-purified. The plasmid ends were polished with the Klenow fragment, dephosphorylated with Shrimp alkaline phosphatase (Promega, Mannheim, Germany) and, thereafter, the ER1-14 fragment was ligated into the vector. The resulting vector was named pIgkappa1-14. The sequences of positive clones, obtained after *E. coli* transformation, were verified by DNA sequencing using a MegaBase 1000 instrument (GE Healthcare, Uppsala, Sweden). Plasmids were prepared using the EndoFree plasmid maxi kit (Qiagen).

Protein expression. To optimise transfection conditions, transfection with a reporter vector (pCMVβ, Clontech) and staining of the cells expressing the bacterial enzyme β-galactosidase were performed with 4 different N/P ratios (the relation of nitrogen residues of transfection reagent per DNA phosphate). The COS-1 cells were split the day before transfection and seeded in 10-cm diameter plates. Two h before transfection, the 50-70% confluent cells were incubated in serum-free DMEM media. Thereafter, the cells were transfected with 25 μg pIgkappa1-14 using a linear polyethylenimine reagent, ExGen 500 (Fermentas, Helsingborg, Sweden), according to the manufacturer's instructions. After 72 h, the cells were washed twice with PBS and gently detached with a cell scraper. The cells were briefly centrifuged, and, thereafter, lysed in a total of 560 μl of Laemmli sample buffer at 95˚C for 5 min.

Western blot analysis. The protein samples were electrophoretically separated using 15% polyacrylamide gels and were subsequently transferred to a polyvinylidene difluoride membrane. The His-tagged spider fusion protein was detected using a Penta-His HRP conjugate kit (Qiagen) and the ECL Western blotting system (GE Healthcare), according to the manufacturer's instructions.

Results

The vector pSecTag2/Hygro A, which places the current gene fragment under the control of the human cytomegalovirus (CMV) immediate-early promoter, was chosen for expression. The proteins expressed from this vector are fused at the N-terminus to the Ig kappa chain leader sequence for protein secretion and at the C-terminus to a polyhistidine tag that can be used for detection and affinity purification. The transfection conditions were optimised with respect to cytotoxicity and transfection frequency, using a reporter vector (pCMVβ) and yielded a transfection success rate of 54% (data not shown).

The results of the analysis of the COS-1 cells transfected with different constructs are provided in Figure 2. The cells transfected with 2 different clones of the pIgkappa1-14 construct gave rise to 2 unique bands with estimated sizes of about 25 kDa and 22 kDa, respectively (Figure 2, lanes 4 and 5), which agree with the calculated sizes of 24.9 and 22.5 for MaSp1 protein fragments, with and without leader peptide, respectively. These bands were absent in untransfected cells, in cells transfected with an empty

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          10          20          30          40          50          60
GAA TTC GCC CTT GCT GCC GCA GCA GCA GCT GGA CAG GGC GGT CAA GGT GGA TAT GGT GGA
 E   F   A   L   A   A   A   A   A   A   G   Q   G   G   Q   G   G   Y   G   G>

          70          80          90          100         110         120
CTA GGT CAA GGA GGA TAT GGA CAA GGT ACA GGA AGT TCT ACA GCC GCC GCC GCC GCA GCC
 L   G   Q   G   G   Y   G   Q   G   T   G   S   S   T   A   A   A   A   A   A>

          130         140         150         160         170         180
GCC GCC GCC GCC GCA GGT GGA CAA GGT GGA CAA GGT CAA GGA GGA TAT GGA CAA GGT GCA
 A   A   A   A   A   G   G   Q   G   G   Q   G   Q   G   G   Y   G   Q   G   A>

          190         200         210         220         230         240
GGA AGT TCT GCA GCC GCC GCC GCT GCA GCA GCA GCA GCA GCC GCA GCA GCT GGA CGA GGT
 G   S   S   A   A   A   A   A   A   A   A   A   A   A   A   A   G   R   G   G>

          250         260         270         280         290         300
CAA GGA GGA TAT GGT CAA GGT TCT GGA GGT AAT GCC GCT GCA GCA GCC GCT GCA GCT GCA
 Q   G   G   Y   G   Q   G   S   G   G   N   A   A   A   A   A   A   A   A   A>

          310         320         330         340         350         360
GCA GCA GCA GCA TCT GGA CAA GGA GGA CAA GGT GGA CAA GGT CAA GGT GGA TAT GGA CAA
 A   A   A   A   S   G   Q   G   G   Q   G   G   Q   G   Q   G   G   Y   G   Q>

          370         380         390         400         410         420
GGT GCA GGA ATT TCT GCA GCC GCC GCC GCC GCC GCT GCA GCA GCA GCC GGC GCA GCA GCT
 G   A   G   I   S   A   A   A   A   A   A   A   A   A   A   G   A   A   A   A>

          430         440         450         460         470         480
GGA CGA GGT CAA GGA GGA TAT GGT CAA GGT GCT GGA GGT AAT GCC GCT GCA GCA GCC GCA
 G   R   G   Q   G   G   Y   G   Q   G   A   G   G   N   A   A   A   A   A   A>

          490         500         510         520         530         540
GCA GCT GCC GCC GCC GCC GCA GCT GGA CAG GGC GGT CAA GGT GGA TAT GGT GGA CAA GGT
 A   A   A   A   A   A   A   A   G   Q   G   G   Q   G   G   Y   G   G   Q   G>

          550         560         570         580         590         600
CTA GGA GGA TAT GGA CAA GGT GCA GGA AGT TCT GCA GCC GCT GCC GCA GCA GCA GCT GGA
 L   G   G   Y   G   Q   G   A   G   S   S   A   A   A   A   A   A   A   A   G>

          610         620         630
CGA GGT CAA TCA GTT TAT CAA GCC CTA GGT GAA TTC
 R   G   Q   S   V   Y   Q   A   L   G   E   F>
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Figure 1. *cDNA sequence of the gene fragment expressed (ER1-14, 636 bp) from Euprosthenops MaSpI and its deduced amino acid sequence. The first and last 6 nucleotides (GAATTC) of the sequence are not part of the spidroin gene itself, but represent the EcoRI restriction sites from the donor vector.*

expression vector and in cells transfected with pSecTag2/Hygro/PSA. The cloned and expressed MaSp1 gene sequence from *Euprosthenops* sp. codes for 7 larger poly-alanine stretches, containing 6-14 alanine residues each, with glycine-rich segments in between (Figure 1). Even though this fragment only represents a small part of the entire protein, it should be sufficiently large to allow structural studies, *e.g.,* by circular dichroism spectroscopy.

Discussion

The expression levels obtained with pIgkappa1-14 were low, as can be variously explained. Although mammalian cells (35) and other eukaryotic systems (36) seem to be more suitable for the expression of spidroin genes than their microbial counterparts, they still suffer from the complications associated with transcribing and translating repetitive sequences. Low copy numbers of transfected constructs or limitations of the cell translational machinery could also be contributing factors. The spider produces gland-specific pools of tRNAs for glycine and alanine to meet the demands associated with the highly repetitive silk protein sequences (37). It is possible that the Gly- and Ala-tRNA pools of the COS-1 cells are insufficient to allow high-level expression. Aggregation of recombinant spider silk proteins is known to occur (36) and is ascribable to the hydrophobic nature of the alanine repeats. This may contribute to the low expression levels now observed. Some authors have suggested that the

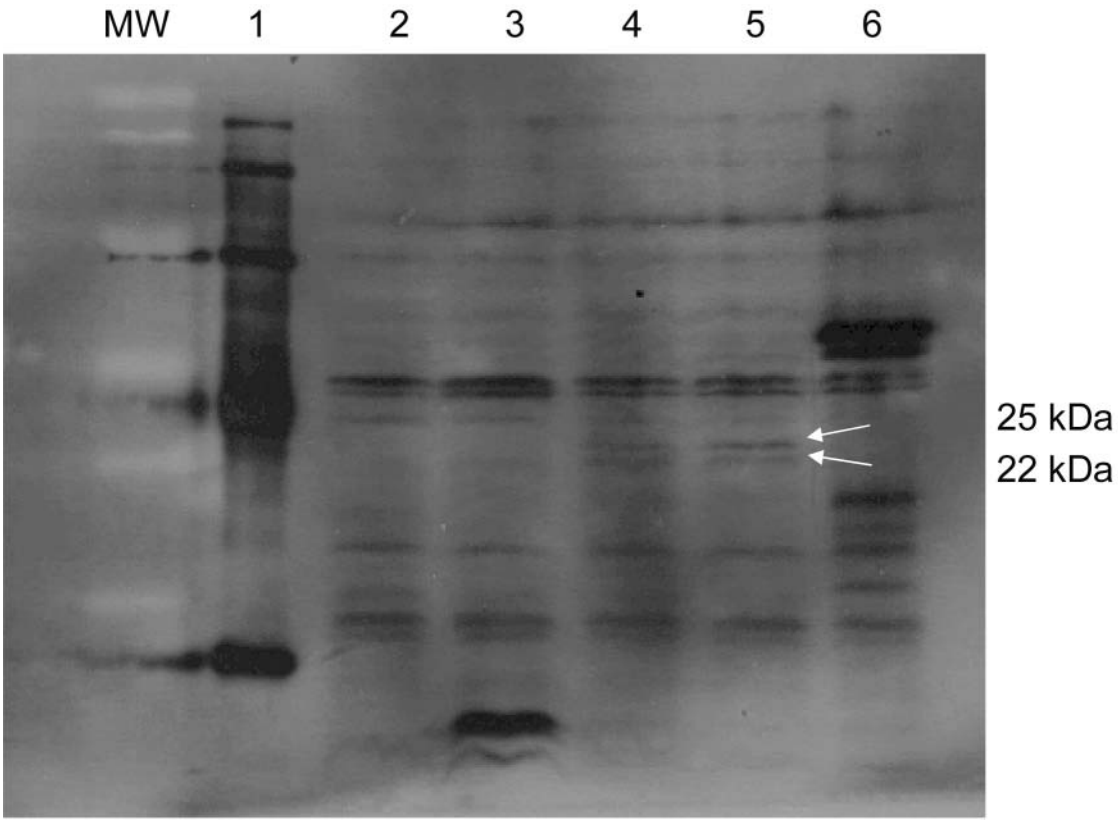

Figure 2. *Analysis of the proteins in untransfected and transfected COS-1 cells by Western blotting using an anti-polyhistidine antibody. MW: molecular weight marker; lane 1: 6X His protein ladder; lane 2: untransfected cells; lane 3: cells transfected with pSecTag2/HygroA; lane 4: cells transfected with pIgkappa1-14; lane 5: cells transfected with pIgkappa1-14; lane 6: cells transfected with pSecTag2/HygroA/PSA. Arrows indicate recombinant spidroin proteins.*

non-repetitive C-terminal domain could contribute to the solubility of spider silk proteins (35). Fusing the repetitive fragment expressed in this study with the corresponding C-terminal domain (12) might increase the expression levels and recovery of soluble protein.

The reported biocompatibility of spider silk with mammalian cells (38) and tissue (5) makes this material an interesting candidate for large-scale production. There are innumerable clinical applications – not only for bone replacement during cancer surgery, but also for the development of other orthopaedic devices. The demonstration of the successful transient expression of a repetitive fragment of a spider silk gene is a valuable contribution towards a protocol for production of recombinant spider silk protein.

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