Spider silks from plants – a challenge to create native-sized spidroins

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Abbreviations

MaSp, major ampullate spidroin; **FLAG,** flagellliform silk; **C-terminal,** carboxyterminal; **N-terminal,** aminoterminal**; kDa,** kilodalton; **ELP,** elastin like peptides; **ER,** endoplasmatic reticulum

Abstract

Silk threads from spiders exhibit extraordinary mechanical properties as superior toughness and elasticity. All these silks consist of different high-sized repetitive proteins as basic materials responsible for such outstanding features. The production of spider silk protein variants in plants opens up new horizons for production as well as for functional investigation to allow the use in nanotechnology and biomedicine in future. This review summarizes and discusses this topic in terms of plant expressions systems, purification strategies and characteristics of spider silk variants. Furthermore the challenge of production is outlined for native-sized spidroins *in planta*. Three different strategies as dimerization by non-repetitive C-terminal domains, intein-based multimerization and multimerization by transglutaminase are presented.

1. Introduction

1.1. Spider Silks

Spiders are fascinating animals able to cope with several environmental challenges by the development of useful mechanical tools as multiple types of protein-loaded silk fibers, that are tailored to specific functions as catching and wrapping prey, escaping predators or protection of their offspring (for review see [1-3]). They exhibit extraordinary properties in terms of toughness and elasticity superior to synthetic high-tech fibers. Dragline silk of orb web spiders used in frame threads or even in draglines has extraordinary mechanical properties: its five times stronger by weight than steel and three times tougher than the p-aramid, one of the strongest known man-made fibers [4, 5]. Dragline silk consists of two different spider silk fibroins, termed spidroins: major ampullate spidroin 1 (MaSp1) and major ampullate spidroin 2 (MaSp2) [6, 7]. Prey-catching flagelliform silk does not show such extraordinary hardness, but could be extended to several times of its length before breaking. This type of silk exhibits very high elasticity and ductility [8-10]. Therefore, flagelliform silk is able to absorb the kinetic energy of flying prey in an ideal manner and would be a suitable material for vibration dampening. Flagelliform silk consists of a single protein, called FLAG [11]. In general, the molecular structure of spider silk proteins is characterized by crystalline areas interrupted by relatively unorganized peptide chains [12]. Secondary structures are defined by different repetitive primary structure modules, so-called ensemble repeats. The properties of these secondary structures are related to different spider silks [10, 13]. Crystalline ß-sheets especially contribute to the high stiffness of spider silk fibers. ß-Sheets are built by interactions between amino acids of multiple repeats of alanine, glycine-alanine or glycine-alanine-serine. Typically, they are present in MaSp1 and MaSp2 from *Nephila clavipes* as well as in fibroin 3 and fibroin 4 of *Araneus diadematus* [6, 7, 14]. Non-crystalline regions are either GPGXX-repeats (ß-spirals)

or helical GGX repeats [12]. These structures are thought to cause elasticity and are common in the protein FLAG of flagelliform silk of *N. clavipes* [15]. Non-repetitive terminal domains have been found at N- and C-terminal ends of spider silk proteins and are thought to have essential functions for the protein assembly [16]. Actual research activities focus to define their role during chain assembly in dependence on ion content, on pH change during passage of the spinning gland and on mechanical forces [17-19]. Two models have been set up to describe the mechanism of fiber assembly: liquid-crystalline behavior of the spinning dope or, alternatively, development of micelles and formation of globules that are transformed into fibers by shearing forces [10]. An additional point of investigations is the enormous molecular weight of spider silk proteins from at least 250 kDa to several hundreds of kDa. All spider silk proteins described so far showed such a high molecular weight. For this reason it was conducted, that this extended size is a key factor for superior toughness and tensile strength [20-22]. Several repeated motifs of large silk proteins facilitate multiple intra- and intermolecular interactions. The use of large proteins for spinning could avoid chain end caused gaps [22]. Therefore, we discuss three different possibilities to produce native-sized spider silk proteins in plants.

1.2. Molecular Farming

Molecular Farming means large-scale production of proteins and other compounds for pharmaceutical and technical use in plants or in plant-based expression systems. This field of biotechnology topic started to develop in 1989 with the successful expression of antibodies in plants followed by the first reports of plant-based vaccine production [23, 24]. Since these reports an extended number of different antibodies, antibody variants of several types, vaccines and other therapeutic proteins for medical and veterinary purposes have been produced in plants, for reviews see [25-32]. Plant-based expression systems are able to provide complex proteins in a correctly folded and posttranslationally modified form [26]. Plants have distinct advantages in comparison to conventional eukaryotic and prokaryotic expression systems as cost, safety and scalability [33]. Here it is important to point out, that the cost of downstream processing steps as protein extractions, protein recovery and protein purification are generally similar in all recombinant production systems. They can cover more than 80% of the overall processing costs [34]. Such costs are mainly attributed to chromatography, labor and capital equipment [33]. Major benefits of plant-based expression systems are savings in the upstream components. Expensive fermenters are not necessary and costs for specific media and skilled workers are also lower. Where costs of goods sold from mammalian cell culture are estimated to be $ 300 per g of a potential therapeutic protein, the raw material costs are between $ 0.1 and 1 per g recombinant protein from plants [35]. There is of course a clear dependence on expression level. In chapter 2 we will discuss, why plant-based production

provides a suitable tool for spider silks. High costs and inefficiency of downstream processing including purification of recombinant proteins from plants form the main technical bottleneck for a broader use of this technology [36]. In the last years, new strategies as ELPylation or induction of protein bodies have been develop to circumvent this bottleneck [37, 38]. The use of this and other methods optimizing spider silk purification from plant material will be the matter of discussion in the second chapter, too. Once expression and purification strategies were available, spider silk-based materials came in the focus of possible biomedical applications, which will be discussed in chapter 4.

2. Why choosing plants as production platforms?

2.1. Plant expression systems

In general, three basic methods are available to set up plant production systems for spider silk proteins: transgenic plants, transient expression and production in plant single cell systems (Figure 1). Transgenic plants have been introduced as a tool for research as well as for plant-based production in 1983 by Mary-Dell Chilton and Marc van Montagu and Jeff Schell [39, 40]. Ever since a whole orchestra of tools as promoters for high and ubiquitous expression, for organ-specific expression, for inducible expression, for compartment-specific expression as well as different transformation systems for many different plant species including crops has been developed; for reviews see [25, 41]. Plant expression of spider silk proteins has been reported for the first time by Scheller and co-workers [42]. Different sized derivatives of MaSp1, called SO1, have been expressed in tobacco and potato leaves using ER retention, which has also successfully been proven for expression of MaSp1 and MaSp2 derivatives in tobacco in a greenhouse as well as in field trials [43]. Stable spider silk proteins until molecular weights of more than 100 kDa have been produced in tobacco as well as in potato leaves. The CaMV 35S promoter used in these studies was also active in potato tubers and provided expression of recombinant antibodies as well as storage stability at low temperatures [44]. Comparable experiments have been performed with transgenic potato tubers containing spider silk proteins. These proteins have not been stable in tubers, neither during low temperatures storage after harvest nor even in tubers in the soil as detected during a field trial (Münnich, Rakhimova and Conrad, unpublished). Field studies with transgenic plants have been reported by Menassa and co-workers [43]. Barr and collegues designed two plant-optimized spider silk-like proteins mimicking spidroin 1 (MaSp1) coding for 64 kDa and 127 kDa, respectively. These proteins have been expressed in *Arabidopsis* leaves and seeds as well as in soybean embryos [45]. Yang and co-workers showed in a targeting study, that the combination of seed-specific expression and ER retention provided the highest accumulation levels for spider silk proteins

in transgenic seeds [46]. Comparable results have been achieved for production of recombinant immunoglobulins in seeds [47]. Principially, enrichment of recombinant spidroins in protein-rich legume seeds seems to be a favorable tool for plant based spider silk protein production, but has not been demonstrated to our knowledge so far. Fusion proteins provide a further tool for optimizing expression levels of selected proteins in transgenic plants. Here, fusion to ELP has been used for optimized spider silk expression. Indeed, the expression level of recombinant proteins was essentially improved [37, 48, 49]. In addition, ELP exhibits structural homologies to the spider silk protein FLAG. Whereas the production of different spider silk proteins (MaSp1, MaSp2 and their derivatives, FLAG) in tobacco and partially in potato leaves and tubers has been generally shown [42, 43, 45, 50] and plant-specific purification strategies have been proposed, storage trials and purification protocols for seed-produced spider silk proteins have not been provided, so far. Transient expression in tobacco leaves has been used [51], but general studies using several effective transient systems as i.e. provided for antibodies are currently not available. Successful strategies to achieve native-sized spider silk proteins will be discussed in chapter 3.

2.2. Purification strategies

In early published purification reports *Escherichia coli* (*E. coli*) expression systems were preferred to enrich heterologous spider silk proteins, which were further purified by affinity chromatography due to a His-tag, a technology that relies on reversible immobilization of the target protein [52-55]; for review see [56]. Affinity chromatography is connected with high costs and difficulties to scale up. The high expression level in *E.coli* allowed the successful development of scalable purification methods based on acid [57] and salt precipitation [22] as well as enormous heat stability of spider silk proteins [58, 59]. A similar approach (heat stability, stability under acidic conditions and ammonium sulfate precipitation) has been applied for enrichment of plant produced spider silk derivatives [60]. ELPylation technology, based on C-terminal fusion of elastin like polypeptides (ELPs) with specific target proteins, was used as a powerful tool to purify efficiently recombinant spider silk proteins from plants [37, 48, 49]. ELPs exhibit a highly useful property called thermally responsive reversible phase transition [61, 62]. The ELP fusion proteins inherit this useful characteristic, too. The temperature-dependent, reversible self aggregation/precipitation behavior provides a simple purification method using heat treatment for soluble fusion proteins [63] and has been successfully extended to plant cells [37, 48, 64]. The ‘inverse transition cycling’ (ITC) method is not only a simple, but also less expensive and readily upgradable to a preparative scale. Laboratory scale extraction of 1 kg tobacco leaf material led to a yield of 80 mg pure spider silk elastin protein [48]. However, application of other progressive high-efficient purification strategies for recombinant spider silk proteins from plants like the membrane-based inverse transition cycling method [64, 65] or a

combination of ELPylation technology with intein technology, designed to induce self-cleavage to remove intein-ELP from the target protein [66-68] are attractive future perspectives for production of also native-sized recombinant spider silk proteins in a large scale.

3. How could native –sized spider silk proteins be produced in plants?

3.1. Dimerization by non-repetitive C-termini

Spider silk proteins as major and minor ampullate spidroins from major and minor ampullate glands as well as FLAG from flagelliform glands contain non-repetitive C-termini [6, 7, 69-71]. These C-terminal sequences in ampullate spidroins as well as in flagelliform protein are highly conserved [71]. They appear to be the only conserved sequences among spider silk proteins. Between the most divergent pairs by grouping C-terminal spider silk sequences still 45% amino acid identity was identified [72]. The presence of C-terminal peptides in the spinning dope and in mature spidroins has been demonstrated. The C-termini of major ampullate gland produced proteins as well as FLAG protein contain one or two cysteines ([73, 74], (Figure 2c). These cysteines are thought to serve to cross-link the spidroin molecules by disulfide bridge formation. High conservation and the presence in spinning dope and fiber filament are used as arguments to demonstrate the crucial role of non-repetitive C-termini for formation and structure of the spider silk filaments [74]. We intended to use these C-terminal ends to produce spider silk protein dimers *in planta.*

Partial dimerization in dependence on present C-terminal non-repetitive sequences has been demonstrated. The c-myc-tag did not influence this procedure occurring in the ER. Interestingly, under reducing conditions (applying ß-mercaptoethanol or dithiothreitol), the dimerization pattern was unchanged (data not shown). This prompted us to hypothesize, that hydrophobic interactions are responsible for the partial dimerization due to the non-repetitive C-terminal domain. Experimental expression of spidroins with conserved non-repetitive C-terminal domains in insect cells showed, that the C-terminal domain of dragline silk is important for the correct structure of nanofibers [16]. Nevertheless, the results presented in Figure 2 are critical, because on the one hand half of the FLAG proteins seem to be dimerized. On the other hand the hydrophobic domains are at least more than 30 amino acids upstream from the C-terminal end. Thus the resulting structure is not an end-by-end fusion and how this would influence the proposed functions of the C-terminal domains for nanofiber assembly remains uncertain [16].

3.2. Intein-mediated multimerization

Inteins are characterized as selfish protein elements that excise themselves from a precursor protein in an autocatalytic reaction and join the flanking extein segments with a new polypeptide bond in a process called protein splicing [75]. Today more than 500 inteins in all three domains of life are known (see InBase, [76]). These protein splicing elements offer a full suite of advanced features for use in molecular biology and biotechnology and several research groups engineered this great proteinaceous tool for particular applications. Here most commonly are protein purification systems that make use of an intein fused to a target protein and an affinity tag allowing chromatography [77-79]. In addition intein-facilitated protein purification approaches without chromatographic tags were performed (for review see [80]). Fong et al. 2010 [81] described the expression and purification via inverse transition cycling of self-cleaving ELP-intein-tagged proteins yielded in highly purified target proteins. Furthermore intein-based systems are applied for a selective labeling of proteins to enable NMR analysis [82], for a controlled expression of toxic proteins [83, 84] or to establish stable phenotypic traits in transgenic wheat [85]. Intein-based systems have also been used to ligate proteins [86]. The first successful fusion of a high repetitive synthetic spider silk protein with inteins was reported by Hauptmann and co-workers [51]. Here, the natural splicing activity of the C- and N-terminal intein sequences from *Synechocystis* sp. gene *DnaB* were used for multimerization of a synthetic FLAG avoiding time-consuming classical cloning procedures and the need for highly repetitive transgenes. Intein-based fusion leads to an end-by-end connection avoiding intercrossings. Preliminary data suggested, that cyclization did not occur to a higher extend [51]. The specificity of the intein-based protein fusion process was verified by a genetic proof. Key amino acids were mutagenized to prevent multimerization of spider silk protein monomers in different independent transgenic tobacco lines. The intein-based multimerization has also been shown for 60xELP. In general, we believe, that this approach opened up a new avenue not only for the production of high molecular weight spider silk proteins larger than 250 kDa, but also for the production of a broad range of different repetitive proteins *in planta*. Repetitive proteins, such as spider silk proteins and ELPs, but also other proteins like monocot glutelins [87] offer a plethora of possibilities to form nanospheres, hydrogels or different fibers, as has been discussed in detail for spider silk derivatives [10, 88, 89]. Intercrossing of pairs of distinct transgenic lines offers a further plant-specific way for production of composite materials. The intein-mediated multimeric structures form microfibers which demonstrate the potential as a biomaterial. But these properties need to be verified by extended investigations in terms of determination of nanomechanical properties and properties according use in tissue engineering as well.

3.3. Crosslinking by transglutaminase

Transglutaminases are catalytic enzymes that form covalent bonds between a free amine group (a peptide- or protein bound lysine) and the γ–carboxamide group of a peptide or protein-bound glutamine. Transglutaminases originated from *Streptoverticillium mobaraensis* are commercially available. Recombinant transglutaminases could be produced in *E. coli* as pro-enzyme [90] and purified after activation by proteinase K by His-tag-based affinity chromatography [91]. Transglutaminases have been used to produce extracellular matrix mimicking material. For this purpose, crosslinking sites, glutamine and lysine, have been introduced into recombinant human elastin like peptides by genetic engineering [92]. These matrices have been used for biopolymer gel encapsulation of mammalian cells [93]. In a similar approach, spider silk proteins were engineered in fusing glutamine- or lysine-tags to the N-terminal end of the spider silk coding gene [94]. Here, it should be taken into account, that spider silk proteins like MaSp1 and FLAG contain several glutamines [6, 15]. In addition, in transgenic plants, ER retention by C-terminal KDEL has been proven as the method of choice for high level expression of spider silk proteins [42]. This lysine at the C-terminal region and the internal glutamines could cause several cross bonds leading to non-linear connections and intercrossing of peptide chains, thus providing non-linear multimers. Purification methods described above (chapter 2.2) could be used to remove the transglutaminase from multimerized spider silk proteins.

4. What could be done with spider silk proteins in nanotechnology and medicine?

Spider silk combines outstanding mechanical properties with biocompatibility [95, 96], a low inflammatory potential, antimicrobial activity [97] and slow biodegradation [98], thus enabling new options for a growing number of nanotechnological and biomedical applications.

In recent years impressive experiments in connection with spider silk were performed in the field of regenerative medicine. A very promising method to replace a two cm deficit of the sciatic nerve in rats was described by Allmeling and co-workers [99]. After implantation of an artificial nerve construct consisting of acellularized veins, dragline silk fibers, Schwann cells and Matrigel, the regeneration of peripheral nerves with high functionality was obtained. In comparison a control construct consisting of only a vein and Matrigel resulted in sparely myelinated nerve fibers and distinctive muscle degeneration. Similar results were obtained in sheep where the injury of the tibial nerve resulting in functional recovery after integration of nerve constructs consisting of decellularized vein grafts filled with spider silk fibers [100]. Former experiments showed the ability for adherence and elongation of human primary Schwann cells along native dragline silk fibers *in vitro* [101]. The supporting effects of spider silk on cell growth and differentiation were also well studied in other *in vitro* systems, for

example with human mesenchymal stem cells [102] or human primary chondrocytes [48]. MaSp1 derived synthetic spider silk protein SO1 has been fused to 100xELP and produced in stable transformed tobacco plants. Spider silk-ELP fusion proteins were purified *via* ITC. Cell culture plates were coated with spider silk-ELP proteins. The growth of chondrocytes was enhanced and shown to be comparable to the growth support by collagen. The viability of the chondrocytes was improved whereas dedifferentiation was inhibited. Going further, applications for bone tissue engineering are described including sponges [103, 104], films [105, 106], hydrogels [107] and non-woven silk mats [108]. Since ancient times it is known, that the placement of a spider web would promote wound healing [109, 110]. Today, medical uses in the field of dermatology expand into the area of skin repair and regeneration. Fibroblast and keratinocyte cell lines were able to adhere and proliferate to native spider dragline silk which can be used as a biological matrix for skin cell culturing [111]. Further medical applications in form of films exploit the antithrombic nature of the material [112]. For all reported and also further applications we have to keep in mind that spiders are territorial and produce only low amounts of silk. They cannot be employed for large-scale silk production which would be necessary for all demands. Therefore the need for high quality recombinant spider silk, especially for native-sized proteins, is remained indispensable. Recombinant expression in plants could open up new horizons for the production on a larger scale to cope the growing requirements.

5. Concluding remarks

In this review we summarized the actual knowledge about plant-made spider silk proteins. Molecular Farming and the appropriate background as plant expression systems and purification strategies were also discussed. The manufacturing of native-sized recombinant spider silk proteins is an essential presumption for full exploration of the outstanding mechanical properties of spider silk proteinaceous materials. Spidroins are highly repetitive proteins and their expression causes problems in terms of genetic instability. Here, we demonstrated and discussed three different methods of posttranslational multimerization to bear down this hurdle. Preliminary data about fusion by non-repetitive C-terminal domains were presented and posttranslational multimerization *via* inteins or transglutaminase were discussed, respectively. Biomedical applications are promising tools for regenerative medicine, as mentioned in the final part of this review. A future challenge will be to extend this field of possible applications of recombinant spider silk proteins and to establish this kind of plant-made spider silk-derived pharmaceutical proteins as important and accepted tools in biomedicine.

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7. Conflict-of-interest statement

The authors declare no commercial of financial conflict of interest.

8. References

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9. Figure legends

Figure 1: Strategies for production of native-sized spider silk proteins in plants. Spider silk coding genes and their derivatives (based on genetic information for dragline silk as well as for flagelliform silk) can be successfully expressed in transiently or stably transformed plants. Different methods could be applied for multimerization of such highly repetitive proteins, either by fusion of protein monomers *in vivo* or by crosslinking of purified proteins *in vitro* resulting in native-sized spider silk proteins.

Figure 2: Dimerization of spider silk protein FLAG derivatives by non-repetitive C-terminal domains *in planta.* FLAG derivatives have been expressed in transgenic tobacco plants under control of the ubiquitous CaMV 35S promoter. ER retention has been chosen as compartmentalization for optimized expression [113]. Standard procedures have been used for production, propagation, selection and characterization of transgenic plants [47, 114]. (A): FLAG coding gene design: Sequences have been taken from Hayashi and co-workers.[15]. In principle plant expression vectors have been described by Scheller and Conrad [60]. Construct designations are given as numbers in A and B. (B): Detection of FLAG protein expression in tobacco leaf extracts by non-reducing SDS-PAGE on 10 % polyacrylamide gels combined with immunoblotting on nitrocellulose membranes. The immunological detection was performed with an anti–N-peptide serum from rabbits, followed by an anti-rabbit immunoglobulin G-peroxidase and ECL visualization. The rabbit sera have been raised against a peptide (produced in *E.coli*) containing 143 amino acids (~15 kDa) spanning from the initial methionine to the carboxyterminal end of the N-terminal non-repetitive sequence [15, 50]. 10µg of leaf extract (TSP) have been applied to each slot. The FLAG N-terminal peptide (FNT) has been used as a standard for estimation of the protein expression level. WT: wildtype tobacco (C): Amino acid sequence of the C-terminal non-repetitive domain of FLAG. The cysteine-

containing region was enlarged, two cysteines are labeled by arrows. Hydrophobicity plot was prepared by Protean 3D software (Lasergene® 10.0.1 software suite, DNASTAR Inc., USA) according Kyte and Doolittle [115].