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Production of an active recombinant thrombomodulin derivative in transgenic tobacco plants and suspension cells

Helga Schinkel[1], Andreas Schiermeyer[1], Raphael Soeur[1], Rainer Fischer[1,2] & Stefan Schillberg[1,*]

[1]*Fraunhofer-Institut für Molekularbiologie und Angewandte Oekologie, IME, Worringerweg 1, 52074 Aachen, Germany*

[2]*Institut für Molekulare Biotechnologie (Biologie VII), RWTH Aachen, Worringerweg 1, 52074 Aachen, Germany*

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Abstract

Thrombomodulin is a membrane-bound protein that plays an active role in the blood coagulation system by binding thrombin and initiating the protein C anticoagulant pathway. Solulin™ is a recombinant soluble derivative of human thrombomodulin. It is used for the treatment of thrombotic disorders. To evaluate the production of this pharmaceutical protein in plants, expression vectors were generated using four different N-terminal signal peptides. Immunoblot analysis of transiently transformed tobacco leaves showed that intact Solulin™ could be detected using three of these signal peptides. Furthermore transgenic tobacco plants and BY2 cells producing Solulin™ were generated. Immunoblot experiments showed that Solulin™ accumulated to maximum levels of 115 and 27 μg g^{-1} plant material in tobacco plants and BY2 cells, respectively. Activity tests performed on the culture supernatant of transformed BY2 cells showed that the secreted Solulin™ was functional. In contrast, thrombomodulin activity was not detected in total soluble protein extracts from BY2 cells, probably due to inhibitory effects of substances in the cell extract. N-terminal sequencing was carried out on partially purified Solulin™ from the BY2 culture supernatant. The sequence was identical to that of Solulin™ produced in Chinese hamster ovary cells, confirming correct processing of the N-terminal signal peptide. We have demonstrated that plants and plant cell cultures can be used as alternative systems for the production of an active recombinant thrombomodulin derivative.

Abbreviations: CHO – Chinese hamster ovary; ELISA – enzyme–linked immunosorbent assay; TSP – total soluble protein

Introduction

Thrombomodulin is an endothelial, cell-surface glycoprotein that binds thrombin and accelerates both the thrombin-dependent activation of protein C and the inhibition of antithrombin III (Esmon et al., 1982). Thrombomodulin consists of a lectin-like domain, six epidermal growth factor-like domains, an O-linked glycosylation site, a transmembrane region and a cytosolic domain (Suzuki et al., 1987). Solulin™, a soluble derivative of human thrombomodulin, lacks the transmembrane and cytosolic domains and has

*Author for correspondence
E-mail: schillberg@ime.fraunhofer.de

six mutations that improve protein stability (Weisel et al., 1996). Solulin™ is a glycoprotein containing an 18-amino-acid N-terminal signal peptide, which promotes co-translational insertion into the secretory pathway in mammalian cells (Glaser et al., 1991). The mature glycosylated protein is 487 amino acids in length and has an apparent molecular mass of ≈75 kDa as determined by SDS-PAGE under reducing conditions. Thrombotic disorders are caused by clot formation in response to a stimulus like a damaged vessel wall. This triggers the coagulation cascade generating thrombin, which is converting fibrinogen to fibrin, the matrix of the clot. The systemic administration of soluble, oxidation-resistant thrombomodulin analogues such as Solulin™ inhibits the procoagulant activity of thrombin by building a thrombin–thrombomodulin complex and acting as an anticoagulant by activation of protein C, which in turn attenuates the clotting cascade by inactivation of several activated cofactors (Esmon, 1989). This mechanism can be used to treat diseases in which thrombus formation plays a significant etiological role, e.g. myocardial infarction, disseminated intravascular coagulation (Gonda et al., 1993), deep vein thrombosis, pulmonary embolism, septic shock, acute respiratory distress syndrome, unstable angina and other arterial and venous occlusive conditions (Glaser et al., 1991; Aoki et al., 1994; Solis et al., 1994).

To date, recombinant thrombomodulin is produced in mammalian cell culture such as Chinese hamster ovary (CHO) cells (Lin et al., 1994). However, the increasing demand on this protein for the development of various applications in the treatment of thrombotic and vascular disease therapies requires a safe and cost-effective large-scale production system. A number of expression platforms based on plants have been developed recently, and these offer several advantages in terms of production scales, safety and economy for the production of pharmaceutical proteins (Twyman et al., 2003). We therefore assessed the feasibility of producing recombinant soluble thrombomodulin in tobacco plants and BY2 cell suspension cultures. To evaluate the influence of the signal peptide on the accumulation and integrity of Solulin™, the recombinant protein was fused to four different signal peptides, of which one was the original signal peptide of human thrombomodulin. The highest yields in transgenic plants and BY2 cells were achieved using two non-innate signal peptides. Active Solulin™ was produced in plant cells showing correct processing of the signal peptide demonstrating the potential of plants as an alternative production platform.

Materials and methods

Bacteria and plants

Escherichia coli strain DH5α (Ausubel et al., 1994) was used for general cloning. *Agrobacterium tumefaciens* strain GV3101 (pMP90RK, Gmr Kmr), RifR (Koncz & Schell, 1986) was used for agroinfiltration and stable transformation. Tobacco plants *Nicotiana tabacum* cv. Petite Havana SR1 and cv. Maryland Mammoth were used for agroinfiltration, and cv. Maryland Mammoth was also used for stable transformation. Suspension cells originated from *N. tabacum* L. cv. bright yellow 2 (BY2).

Plant expression vector design and construction

The cDNA encoding the thrombomodulin derivative (Solulin™) including the original signal peptide (ORI) was amplified by PCR from the source plasmid pTHR525 (provided by PAION GmbH, Aachen, Germany) using the primer set Solulin™-for (signal) 5′-TTT ATA AAA AAA AAA AAA ACA ATG CTT GGG GTC CTG GTC-3′ and Solulin™-back (stop) 5′-GCG CGA AGC TTC TCG AGT TTA CGG AGG AGT CAA GGT AGA CCC-3′. The primary PCR product was then used as the template in a second PCR with primers Solulin™-for (5′UT) 5′-CGC GAA TTC ACA ACA CAA ATC AGA TTT ATA GAG AGA TTT ATA AAA AAA AAA AAA AC-3′ and Solulin™-back (stop) to fuse the *Petroselinum crispum* chalcone synthase 5′ untranslated region to the 5′ end of the cDNA. The product was ligated into the vector pTRAkc (a derivative of pPAM, GenBank accession number AY027531), using the *Eco*RI and *Xho*I sites. This vector contained the double-enhanced CaMV 35S promoter (Kay et al., 1987) and the CaMV termination sequence, preceded by a polyadenylation site (Figure 1). The resulting construct was named Solulin™-ORI.

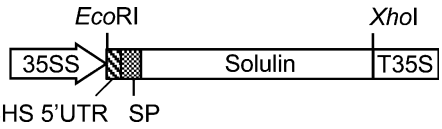

Figure 1. Schematic presentation of plant expression constructs. 35SS, CaMV 35S promoter with duplicated 35S enhancer region; CHS 5′UTR, Chalcone synthase 5′ untranslated region; SP, signal peptide; Solulin™, coding sequence for Solulin™; T35S, CaMV 35S terminator sequence.

To direct Solulin™ to the apoplast, the cDNA of mature Solulin™ without the original signal peptide was fused to the sequence of the N-terminal signal peptide of a murine antibody heavy chain (Voss et al., 1995; Vaquero et al., 1999). The DNA sequence encoding the last three amino acids of this signal peptide was removed since the algorithm SignalP (Nielsen et al., 1997; http://www.cbs.dtu.dk/services/SignalP) predicted that such action was necessary to achieve correct processing. The resulting APO^{-3} sequence was amplified by PCR using the forward primer 35Sseq2 5′-ATC CTT CGC AAG ACC CTT CC-3′ and the reverse primer Apo-back 5′-GCC ACC CGG CTG CGG CTC ACC TGC AGT GCC GCT G-3′. In parallel, the Solulin™ cDNA was amplified from plasmid pTHR525 using the primers Solulin™-Apo 5'-CAG CGG CAC TGC AGG TGA GCC GCA GCC GGG TGG CAG CCA G-3′ and Solulin™-back. The two PCR fragments were then assembled in an SOE-PCR using the primers 35Sseq2 and Solulin™-back. The product of the SOE-PCR was ligated into pTRAkc using *Eco*RI and *Xho*I restriction sites, resulting in the final construct Solulin™-APO^{-3}.

To construct Solulin™-VTS^{-4}, the Solulin™ cDNA without the sequence encoding the original signal peptide was combined with the N-terminal signal peptide of *Catharanthus roseus* strictosidine synthase (VTS; McKnight et al., 1990). The DNA encoding the last four amino acids was omitted to ensure proper signal peptide cleavage, based on predictions by the SignalP algorithm (see above). This truncation was achieved by cutting the VTS sequence from the template vector using *Asc*I and *Sap*I. The Solulin™ cDNA was amplified by PCR from the source plasmid pTHR525 using primers Solulin™-VTS 5'-CTA GCT CTT CAA GCG GAG CCG CAG CCG GGT GGC AGC-3' and Solulin™-back. The PCR product was digested with *Xho*I and *Sap*I, mixed with the *Asc*I and *Sap*I- restricted VTS^{-4} sequence, and introduced into the pTRAkc vector (prepared by digestion with *Asc*I and *Xho*I) in a triple ligation, resulting in the final construct Solulin™-VTS^{-4}.

To construct Solulin™-PbTS, Solulin™ cDNA without the sequence encoding the original signal peptide was fused to the signal peptide of *Pisum sativum* legumin A2 (PbTS; Rerie et al., 1990). PbTS was amplified from a template vector with the primers 35Sseq2 and PbTS-back 5′-GCC ACC CGG CTG CGG TCG GCA AAG CAG CCT CCC-3′. In addition, the Solulin™ cDNA was amplified from plasmid pTHR525 using the primers Solulin™-PbTS 5′-GGG AGG CTG CTT TGC CGA GCC GCA GCC GGG TGG-3′ and Solulin™-back. The two PCR fragments were then assembled by SOE-PCR using the primers 35Sseq2 and Solulin™-back. The product of the SOE-PCR was ligated into pTRAkc using *Eco*RI and *Xho*I, resulting in the final construct Solulin™-PbTS.

Transformation of tobacco plants and suspension cells

The plant expression vectors described above were introduced into *A. tumefaciens* using a Gene Pulser II electroporation system (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Transient expression in *N. tabacum* cv. Petite Havana SR1 or cv. Maryland Mammoth leaves was carried out by vacuum infiltration as previously described (Kapila et al., 1997; Vaquero et al., 1999). Transgenic *N. tabacum* cv. Maryland Mammoth plants were generated by the leaf disc transformation method and transgenic plants were regenerated from transformed callus (Horsch et al., 1985). Plants were grown in a greenhouse under a 16-h natural daylight photoperiod and a 25°C/day, and 22°C/night temperature regime.

Stably transformed *N. tabacum* cv. BY2 cells were generated by co-cultivation with recombinant *A. tumefaciens* (An, 1985). BY2 cells were maintained in Murashige & Skoog (1962) medium supplemented with minimal organics [BY2 medium: 0.47% (w/v) Murashige and Skoog salts, 0.15 μg mL^{-1} thiamine, 0.02 μg mL^{-1} KH_2PO_4 and 3% (w/v) sucrose, pH 5.8] in an orbital shaker (New Brunswick Scientific, Edison,

USA) at 180 rpm and 26°C in the dark. Cultures were subcultered every week with a 5% (v/v) inoculum.

Protein extraction and analysis

The total soluble protein (TSP) from tobacco leaves was extracted as described by Fischer et al. (1999) using a 1:2 (w/v) ratio of plant material and extraction buffer I [200 mM Tris–HCl, pH 7.4, 5 mM EDTA, 5 mM DTT, 0.1% (v/v) Tween 20] or extraction buffer II [150 mM Na_2CO_3, pH 9.5, 500 mM NaCl, 1% (v/v) DMSO]. Protein extraction from cell cultures was carried out 4–6 days after reseeding, when the packed cell volume was 25–40%. Cells were centrifuged ($500 \times g$, 5 min, room temperature) and the packed cells were sonicated (UW2070 with SH70G, MS-72 probe tip, $9 \times 10\%$, 20%, 1 min; Bandelin, Berlin, Germany) in a 1:2 (w/v) ratio of BY2 cells and extraction buffer I. The extracts were clarified by centrifugation prior to immunoblot analysis. Cell culture supernatants were used without further treatment.

TSP concentrations were determined according to Bradford (1976) (Roti-Quant, Roth, Karlsruhe, Germany) using bovine serum albumin (BSA) as a standard. For immunoblot analysis, TSP extracted from leaves or cells was resolved by SDS-polyacrylamide gel electrophoresis under reducing conditions and blotted onto PVDF membrane. Blotted Solulin™ was detected using the monoclonal anti-Solulin™ primary antibody 1043 (kindly provided by PAION GmbH, Aachen, Germany) diluted either 1:1000 in PBS with 0.05% (v/v) Tween 20 (PBS-T) or 1:1500 in PBS-T with 1% (w/v) skimmed milk powder. Binding of the primary antibody was detected using a goat-anti-mouse secondary antibody conjugated to alkaline phosphatase (Jackson Immuno Research Laboratories, West Grove, USA) diluted 1:5000 in PBS-T, or a goat-anti-mouse secondary antibody conjugated to horseradish peroxidase (Jackson Immuno Research Laboratories) diluted 1:8000 in PBS-T with 1% (w/v) skimmed milk powder. For alkaline phosphatase, the signal was developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate solution (NBT/BCIP, Roth). For horseradish peroxidase, the signal was developed using the ECL-advance kit (Amersham Biosciences, Freiburg, Germany). Solulin™ levels were quantified using an LAS-1000 luminescence image analyzer (Fuji, Duesseldorf, Germany) and Aida program version 2.31 (Raytest GmbH, Straubenhardt, Germany) with CHO-produced Solulin™ as the standard.

Protein purification and N-terminal protein sequencing

Recombinant soluble thrombomodulin was purified from suspension cell cultures by ammonium sulphate precipitation and anion exchange chromatography. The supernatant from a 300-mL culture was retrieved by vacuum filtration and was buffered with 8 mM Tris–HCl (final pH 8.5). $MgSO_4$ was added to a final concentration of 2 mM. To remove DNA, 500 units of Benzonase (Merck, Darmstadt, Germany) was added and the solution was incubated for 1.5 h at room temperature, followed by centrifugation ($27{,}138 \times g$, 20 min, 4 °C). A 50% ammonium sulphate precipitation was performed, followed by a centrifugation as above to collect the precipitate. The pellet was redissolved in dialysis buffer (30 mM ammonium acetate, pH 6) and dialysed against the same buffer.

The desalted sample was processed by anion exchange chromatography using a 1-mL Sepharose Q column (Amersham Biosciences) equilibrated with dialysis buffer. The column was then washed with dialysis buffer containing 100 mM NaCl, and proteins were eluted with dialysis buffer containing 300 mM NaCl. The elution fraction was concentrated in centrifugal concentration devices (Pall Filtron, East Hills, USA) and separated by SDS-PAGE (Lämmli 1970) under reducing conditions prior to transfer onto PVDF membrane using CAPS-buffer [10 mM CAPS, pH 11, 10% (v/v) methanol] in a semi-dry blotting apparatus (Bio-Rad). The blot was stained with amido black, and the band corresponding to the recombinant thrombomodulin was cut out and used for N-terminal protein sequencing performed by TopLab (Martinsried, Germany).

Thrombomodulin activity test

All measurements were done in a volume of 25 μL containing 5 μL sample and 20 μL assay

buffer (20 mM Tris–HCl, pH 7.4, 100 mM NaCl, 2.5 mM $CaCl_2$, 0.15 mg mL^{-1} BSA). The assays were performed in 96-well microtitre plates (Greiner, Solingen, Germany). Briefly, the 25 μL assay aliquots were incubated with 25 μL thrombin (3 nM; Haemochrom, Essen, Germany) and 25 μL protein C (0.5 μM; Merck) for 1 h at 37°C. Four units of hirudin (Roche, Mannheim, Germany) was then added, bringing the total volume to 100 μL, followed by incubation at 37°C for 15 min. Subsequently, 100 μL of the chromogenic substrate S-2366 (1.4 mM; Haemochrom) was added. The reaction was incubated at room temperature for 2 min and measured for 15 min at 405 nm using a kinetic protocol. Solulin™ standards (0, 2, 4, 6, 10 and 20 ng) derived from CHO cells were included with each test. The standards were kindly provided by PAION GmbH. Measurements of samples and standards were normalized by subtracting the values of the blanks containing 5 μL of culture supernatant of a wild type BY2 cell line.

Results

Construction of the Solulin™ expression cassettes

To evaluate the influence of the N-terminal signal peptide on Solulin™ accumulation and integrity, the cDNA of the mature Solulin™ was fused to four different N-terminal signal peptide sequences (APO^{-3}, ORI, PbTS, VTS^{-4}; Table 1) to enable transport of the Solulin™ to the secretory pathway (Figure 1). Importantly, the plant-derived Solulin™ should be identical in terms of amino acid sequence to the Solulin™ produced in CHO cells. To evaluate the possibility of improper signal cleavage, which would result in truncation or the presence of additional N-terminal residues, the nucleotide sequences of Solulin™ joined to the three non-innate signal peptide sequences were analysed by the SignalP server, which predicts cleavage sites using a combination of several artificial neural networks (Nielsen et al., 1997). The analysis showed that the *P. sativum* legumin A2 signal peptide PbTS (Rerie et al., 1990) could be used without any changes. However, the last four internal amino acids of the signal peptide from *C. roseus* strictosidine synthase (VTS^{-4}, McKnight et al., 1990), and the last three internal amino acids of the signal peptide from the murine mAb24 heavy chain (APO^{-3}; Vaquero et al., 1999) had to be removed to guarantee accurate cleavage of these signal peptides.

Transient and stable expression of recombinant soluble thrombomodulin in tobacco leaves and plants

All four Solulin™ constructs were transiently expressed in detached tobacco leaves of cultivars Petite Havana SR1 or Maryland Mammoth by agroinfiltration. Immunoblot analysis using a Solulin™-specific monoclonal antibody demonstrated that recombinant Solulin™ was produced in transiently transformed tobacco leaves having a size of ≈75 kDa corresponding to that of the CHO cell-produced standard (Figure 2). However, some minor degradation of the recombinant protein was detectable. No signal was detected in non-infiltrated leaves. Three of the constructs gave rise to strong Solulin™ bands, but Solulin™-VTS^{-4} could not be detected (Figure 2, lane 4). Therefore, further experiments with the VTS^{-4} construct were abandoned. Solulin™ accumulation levels were similar using the signal peptides APO^{-3} or PbTS, but variations were seen

Table 1. Sequence and origin of signal peptides used for inserting Solulin™ into the secretory pathway. The one letter code for amino acids was used

Signal peptide[a]	Origin	Number of amino acids	Amino acid sequence
APO^{-3}	*M. musculus*	16	MEWSWIFLFLLSGTAG
PbTS	*P. sativum*	22	MATKLLALSLSFCFLLLGGCFA
VTS^{-4}	*C. roseus*	28	MANFSESKSMMAVFFMFFLLLLSSSSSS
ORI	*H. sapiens*	18	MLGVLVLGALALAGLVFP

[a]The original signal peptides APO and VTS were shortened by the indicated number of amino acids at the C-terminus to achieve correct cleavage of the signal peptide from Solulin™.

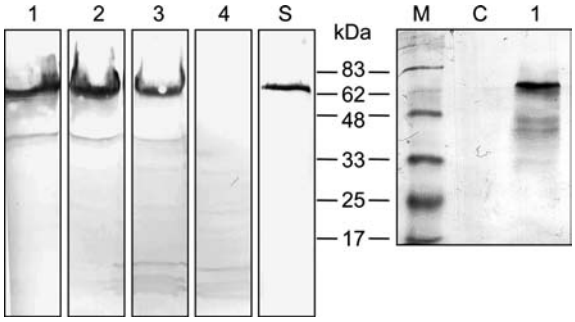

Figure 2. Immunoblot analysis of Solulin™ constructs transiently expressed in *Nicotiana tabacum* cv. Petite Havana SR1. Total soluble proteins were separated by 12% SDS-PAGE and transferred onto a PVDF membrane. Solulin™ was detected using a monoclonal mouse anti-Solulin™ antibody as primary antibody and a goat-anti mouse secondary antibody conjugated to alkaline phosphatase, followed by NBT/BCIP staining. Size standards are indicated. (1) Solulin™-ORI, (2) Solulin™-APO^{-3}, (3) Solulin™-PbTS, (4) Solulin™-VTS^{-4}. S, 200 ng purified Solulin™ standard from CHO cells; M, prestained protein marker; C, total soluble protein from non-infiltrated leaf.

between different infiltrations. However, higher yields of the protein were achieved using the Maryland Mammoth cultivar compared to Petite Havana SR1 (data not shown), so the former was chosen for stable transformation experiments.

The Solulin™-APO^{-3}, Solulin™-PbTS and Solulin™-ORI constructs were introduced into tobacco leaf discs. Transgenic plants were recovered and tested for recombinant Solulin™ accumulation by immunoblot (Table 2). The maximum yields of Solulin™ differed according to which signal peptide was used with Solulin™-APO^{-3} showing the highest accumulation (115 μg g^{-1} fresh weight), followed by Solulin™-PbTS (38 μg g^{-1} fresh weight) and Solulin™-ORI (27 μg g^{-1} fresh weight).

Table 2. Solulin™ yields in transgenic tobacco plants

Construct	Number of analysed plants	Number of plants producing Solulin™[a]	Maximum Solulin™ accumulation (μg g^{-1} FW)[b]
Solulin™-APO^{-3}	40	33	115
Solulin™-PbTS	65	55	38
Solulin™-ORI	68	64	27

Recombinant Solulin™ levels in protein extracts from transgenic tobacco plants were determined by immunoblot analysis using a Solulin™-specific monoclonal antibody and an alkaline phosphatase-conjugated secondary antibody followed by NBT/BCIP staining[a] or a horseradish peroxidase-conjugated secondary antibody followed by chemiluminescence based-detection[b]. FW, fresh weight.

Stable expression of Solulin^{TM} in BY2 cells

Transgenic BY2 calli were tested for the presence of recombinant Solulin™ by immunoblot and those with the highest levels of expression were used to establish cell suspension cultures. High levels of Solulin™ were detected in BY2 cell extracts (Table 3), although intact Solulin™ was also secreted and accumulated in the culture supernatant. The highest accumulation levels of Solulin™ were observed in BY2 cells producing Solulin™-PbTS (27 μg g^{-1} fresh weight), followed by Solulin™-ORI (18.2 μg g^{-1} fresh weight) and Solulin™-APO^{-3} (6.6 μg g^{-1} fresh weight).

Activity of plant cell-produced Solulin™

We tested the activity of Solulin™ in BY2 cell extracts and culture medium. No activity was detected in cell extracts, suggesting the presence of inhibiting compounds released upon cell disruption. This was confirmed in spiking experiments by the addition of functional, CHO-derived Solulin™, whose activity was also destroyed (data not shown).

In contrast, strong and reproducible thrombomodulin activity was detected in BY2 culture

Table 3. Solulin™ yields in transgenic BY2 cell cultures

Construct	Kanamycin-resistant calli[a]	Established cell cultures	Solulin™ accumulation in elite cell line (ng mL^{-1} M or ng g^{-1} C)[b]	
Solulin™-APO^{-3}	23	6	M	940
			C	6.570
Solulin™-PbTS	27	5	M	500
			C	27.000
Solulin™-ORI	30	6	M	70
			C	18.200

Recombinant Solulin™ levels in BY2 cell extracts and culture medium were determined by immunoblot analysis using a Solulin™-specific monoclonal antibody and an alkaline phosphatase-conjugated secondary antibody followed by NBT/BCIP staining[a] or a horseradish peroxidase-conjugated secondary antibody followed by chemiluminescence-based detection[b]. M, culture medium; C, cell fresh weight.

supernatants. Indeed, Solulin™ levels calculated on the basis of activity were higher than those determined by immunoblot (see Tables 3 and 4) suggesting that only a proportion of the recombinant protein was transferred to the membrane in immunoblot experiments. Therefore, we assume that the yields of recombinant proteins expressed in plants may be underestimated when measured by immunoblot analysis alone.

The culture medium contains 50–100 times less total soluble protein (40–75 μg mL^{-1}) than the total cell extract, resulting in a favourable recovery of active Solulin™ when expressed as percentage TSP (0.8–3.3%). This is a good starting point for the purification of Solulin™, since the amount of contaminating proteins is low.

Purification and N-terminal protein sequencing

Solulin™ was purified from the BY2 culture medium. The crucial steps prior to anion exchange chromatography were DNAse treatment and ammonium sulphate precipitation with subsequent dialysis to remove DNA and other compounds interfering with the chromatography process. Although this procedure resulted in only partial purification of Solulin™-APO^{-3} and Solulin™-PbTS (Figure 3), the yield and purity was sufficient to perform N-terminal protein sequencing. The results of these experiments revealed that the five N-terminal amino acids (Glu-Pro-Gln-Pro-Gly) were identical to those of the mature protein derived from CHO cells, demonstrating that the two signal peptides are processed correctly in plant cells.

Discussion

We have demonstrated that plant cells can produce intact and functional Solulin™, with maximum yields of 27 μg g^{-1} fresh weight in BY2 cells and 115 μg g^{-1} in transgenic tobacco leaves (corresponding to 0.4 and 1.5% TSP respectively). The levels obtained in transgenic plants were in a comparable order of magnitude as those obtained in CHO cells. However, the overall space time yield of Solulin™ in mammalian cell systems is much higher since production levels of up to several hundred μg per mL culture medium can be achieved and harvested daily. Nevertheless, the yield of recombinant-soluble thrombomodulin in plants is high when compared with other thrombolytic or blood proteins. For example, recombinant hirudin was produced in transgenic canola seeds (*Brassica napus* cv. Westar) as a fusion protein with oleosin. The expression was restricted to seeds and accumulation levels of approx. 1% of total soluble seed protein were reported (Parmenter et al., 1995). In transgenic tobacco, human protein C reached only 0.002% of TSP (Cramer et al., 1996). To optimize Solulin™ accumulation, we compared several signal peptides (Table 1). Although the signal from *C. roseus* has been used successfully for the production of various recombinant proteins (unpublished results), Solulin™-VTS^{-4} could not be detected in transient transformation experiments. The highest Solulin™ levels were obtained using the murine APO^{-3} or plant PbTS signal peptides. Correct cleavage of the sequences

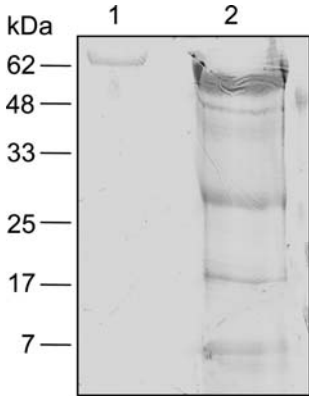

Figure 3. SDS-PAGE analysis of partially purified Solulin™-PbTS. Solulin™-PbTS was purified from 300 mL of BY2 suspension culture (4 days after seeding) by precipitation and anion exchange chromatography. The elution fraction was concentrated 20-fold and 16 μL was separated by 10% SDS-PAGE. After electrophoresis, the gel was stained with Coomassie brilliant blue. Size standards are indicated to the left. Lane 1: 800 ng purified Solulin™ standard from CHO cells. Lane 2: partially purified Solulin™-PbTS.

Table 4. Average Solulin™ activities in BY2 cell culture supernatants

Construct	Active solulin™ (ng mL^{-1})	TSP (μg mL^{-1})
Solulin™-APO^{-3}	1.300	40
Solulin™-PbTS	2.100	75
Solulin™-ORI	400	50

TSP, total soluble protein.

was confirmed by N-terminal sequencing, validating the *in silico* cleavage prediction carried out to optimize construct design. Both of these signal peptides have been used previously and successfully for recombinant protein production in plants (Vaquero et al., 1999). This supports the notion that well established heterologous signal peptides are preferable to endogenous signals that may already be present on the target protein. However, even though the original Solulin™-ORI signal peptide did not perform as well as the APO^{-3} and PbTS signals, it did nevertheless facilitate the accumulation of Solulin™ in plants and suspension cells (Tables 2 and 3).

The Solulin™ produced in transgenic plants and suspension cells had an apparent molecular mass of ≈ 75 kDa, which is the same size as the Solulin™ produced in CHO cells but 25 kDa larger than expected from the amino acid sequence. This deviation can be attributed to glycosylation. It is well known that N-glycan structures slightly differ between mammalian and plant cells (Bardor et al., 1999). However, functional tests showed that thrombomodulin activity was not impaired by the presence of plant-derived N-glycans. Active Solulin™ was recovered from the BY2 culture supernatant but not from total BY2 cell extracts. Our suspicion that the missing activity in cell extracts reflected the presence of inhibitory compounds was confirmed when the extracts were spiked with active Solulin™ from CHO cells, and still lacked any sign of thrombomodulin activity. The levels of recombinant protein secreted to the culture medium were low compared to amounts in cells, suggesting that Solulin™ is either inefficiently secreted due to its large size and size exclusion limit of the cell wall (Carpita et al., 1979; Tepfer & Taylor, 1981) or degraded in the culture medium. Both of these phenomena have been reported in previous studies involving the secretion of other recombinant proteins from plant suspension cells (Titel & Ehwald, 1999; Sharp & Doran, 2001). However, secretion of large proteins and protein complexes through the plant cell wall has been described (Sharp and Doran, 2001; Drake et al., 2003) supporting the assumption that Solulin™ was not stable in the culture medium. Nevertheless, a two-step purification scheme involving salt precipitation and anion exchange chromatography was sufficient to isolate partially purified intact Solulin™ from the culture supernatant. We conclude that the production of intact and active Solulin™ is feasible both in tobacco plants and suspension cell cultures. Solulin™ yields are likely to be increased further by selfing and backcrossing with elite plant lines (Hood et al., 2002) and by optimizing medium and culture conditions of BY2 cells (James et al., 2000). Presuming such increased yields and improved purification processes, the production of Solulin™ in plants could become economically feasible, providing a new source to meet the high demands for this therapeutic protein.

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