Purification of a miniature recombinant spidroin protein expressed in *E. coli* using ÄKTA pure

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Application note 29-0211-98 AA Chromatography systems

Purification of a miniature recombinant spidroin protein expressed in *E. coli* using ÄKTA™ pure

Recombinant miniature spidroin, RepCT, derived from the nursery web spider, *Euprosthenops australis*, was purified using protocols developed for ÄKTA pure chromatography system. To facilitate chromatographic purification, the miniature spidroin was fused to a solubility protein and histidine tag resulting in HisSolRepCT. After induced protein expression in *Escherichia coli* (*E. coli*), harvesting using centrifugation, cell lysis, and clarification, the clarified lysate was loaded onto a HiTrap™ IMAC HP column, captured, and eluted using step gradients with increasing concentrations of imidazole. Proteolytic on-column tag cleavage was simplified by the predefined affinity chromatography method in UNICORN™ v6.3 software. After desalting, the miniature spidroin, RepCT, was subjected to a polishing step consisting of anion exchange chromatography or gel filtration. This purification strategy resulted in a purity of RepCT exceeding 95% and a recovery of 85%. Identification of RepCT was confirmed by nanoscale LC-MS/MS and biological activity of the miniature spidroin was confirmed by the ability to assemble into macroscopic fibers.

Introduction

Spider dragline silk, used by spiders as safety line and web frame, is an unusually strong fiber that on a per weight basis outperforms man-made material such as high-tensile steel and Kevlar™. These properties are attractive for the development of new materials for technical and medical applications. Spider silk consists of two similar proteins, major ampullate spidroin (MaSp) 1 and MaSp 2 with molecular masses (M_r) in the range of 200 000 to 720 000.

Spidroins are mainly composed of hundreds of repeat blocks consisting of 30 to 40 amino acid residues rich in alanine and glycine. The repeats are flanked by nonrepetitive C- and N-terminal domains consisting of about 100 to 150 amino acids, which are conserved among different spider species. The C-terminal domain can form disulfide-mediated dimers and is important for fiber formation (1). The N-terminal domain is highly soluble and has been shown to accelerate self-assembly at pH values below 6.4 but it delays aggregation above pH 7 (2). Recombinant dragline silk proteins have been expressed in different organisms (3) but several factors complicate the cloning and expression processes. Alanine and glycine repeats are coded from repetitive and GC-rich gene sequences, which can lead to genetic instability and formation of undesirable mRNA secondary structures. These structures can result in truncations, rearrangements, and translation pauses. Moreover, in the spider, tRNA pools for alanine and glycine are unusually large while these pools are easily depleted during recombinant expression, resulting in low yields. Recombinant expression is further complicated by the fact that spidroins are very prone to assemble. However, the spider manages to keep the proteins in soluble conformation at high concentrations (30 to 50%). Upon self-assembly, the spidroins secondary structure converts from random/helical into mainly β-sheets (1), a process that involves shear forces, drop in pH, and changes in ion composition.

Here we describe the development of automated chromatographic purification methods for a miniature spidroin derived from the African nursery web spider (*Euprosthenops australis*) expressed in *E. coli* (1, 4). A schematic view of the recombinant expression of histidine-tagged miniature spidroin (HisSolRepCT) is shown in Figure 1.

Fig 1. Schematic view of the expression of HisSolRepCT, the histidine-tagged miniature spidroin, which also contains a solubility tag (Sol tag) and a protease cleavage site prior to the RepCT spidroin.

Materials and methods

Disruption of bacterial cells

The cell pellet expressing the histidine-tagged miniature spidroin, HisSolRepCT, was dissolved in 20 mM Tris-HCl pH 8.0 followed by 50 mg/ml lysozyme, 25 units DNAse I, and 1 M $MgCl_2$. After careful stirring, lysis took place on ice for 1 h and the crude extract was clarified by centrifugation at 40 000 × g, at 4ºC for 30 min. The supernatant was collected and 4 M NaCl was added to a concentration of 500 mM followed by addition of 2 M imidazole to a final concentration of 30 mM. The lysates were kept on ice as short time as possible before immobilized metal ion affinity chromatography (IMAC).

Chromatography and media

ÄKTA pure chromatography system controlled by UNICORN v6.3 was used for protein purification and peak evaluation. Fractions were collected in the external fraction collector. Samples were loaded from a 50 ml Superloop™, capillary loops, or for sample volumes larger than 50 ml from system pump A. All purification steps were performed in a cold room at 4ºC.

Analytical runs for assessing purity and cleavage control was performed using ÄKTAmicro™ chromatography system controlled by UNICORN v5.12.

Prepacked chromatography columns were used in all experiments. HiTrap IMAC HP columns (1 or 5 ml) were charged with Zn^{2+} according to instructions from GE Healthcare. In brief, the columns were washed with three column volumes (CV) of water, 0.5 CV of 200 mM $ZnCl_2$ was applied followed by a wash with 3 CV distilled water.

The conditions for immobilized metal ion chromatography (IMAC), anion exchange chromatography (AIEX), gel filtration (GF), and on-column tag removal were as described in the Figure captions for the respective technology.

SDS-PAGE

SDS-PAGE analysis was performed according to instructions for ExcelGel™ SDS. Samples were mixed 3:1 with 4×, nonreducing sample buffer (NSB) containing 100 mM Tris acetate, pH 7.5, 4% SDS, and 0.02% BFB. For reduced conditions, Bond-Breaker™ TCEP solution was added to 40 mM and the samples were heated for 5 min at 95˚C. Aliquots of 15 µl of each sample were applied to IEF sample application pieces on an ExcelGel SDS gradient 8-18 gel. Limiting settings on the power supply were: 600 V, 50 mA, 30 W.

The gels were stained using Deep Purple™ Total Protein Stain according to recommended instructions and scanned using Ettan™ DIGE Imager fluorescence scanner and evaluated with the image software, ImageQuant™ TL, for relative yield and purity.

PhastSystem™ and PhastGel™ Gradient 8-25 were used for rapid electrophoretic examination of chromatographic fractions. The samples were mixed 3:1 with 4× Loading buffer (40 mM Tris-HCl pH 8.0, 4 mM EDTA, 10% SDS, 0.04% BFB). For reducing conditions, DTT was added to 100 mM and the samples were heated for 5 min at 95ºC. The gels were loaded with 1 or 3 µl of each sample and electrophoresis was performed during 60 Vh. Protein bands were visualized by staining with PhastGel Blue R-350.

Tag cleavage

The miniature spidroin, RepCT, was released from its fusion partner by proteolytic cleavage using a recombinant protease (Spiber Technologies AB). The cleavage reaction was performed at 4ºC for at least 15 h. To remove NaCl and imidazole, the sample was desalted using HiPrep™ 26/10 Desalting column. On-column tag removal was performed using the method template provided in UNICORN v6.3.

Identification of RepCT using nanoscale LC-MS/MS

RepCT from the final AIEX step was reduced and trypsin digested prior to separation by nanoRPC using Ettan MDLC coupled to an LTQ™ linear ion trap mass spectrometer. 1 to 2 µg of digested RepCT was loaded onto a trap column (Zorbax™, 0.3 × 5 mm) and RPC separation was performed on a 0.075 × 150 mm Zorbax column. A linear gradient from 0% to 60% B (A: 0.1% formic acid and B: 84% acetonitrile and 0.1% formic acid) for 60 min was used. The MS method consisted of a scan cycle combining one full scan mass spectrum followed by three MS/MS events. Peptides were identified using the information in the MS/MS spectra and TurboSEQUEST™. The peptide matches were filtered based on cross-correlation scores (Xcorr) of 1.5, 2, and 2.5 for charge states 1^+, 2^+ and 3^+, respectively

Results and discussion

Optimization of growth conditions for increased protein expression

To optimize soluble expression of the fusion protein, HisSolRepCT, a screening experiment was performed by growing small flask cultures of the *E. coli* strain expressing HisSolRepCT at different growth conditions. After incubation the cultures were put on ice and cells were collected by centrifugation and lysed as described in methods. The clarified lysates were loaded onto HiTrap IMAC HP 1 ml charged with Zn^{2+} and the content of HisSolRepCT from each growth condition was analyzed by peak integration and SDS-PAGE. Typical examples resulting in different levels of soluble gene expression are shown in Figure 2.

The amount of HisSolRepCT obtained during the condition screening (7 points, double samples ± 15%) were subjected to multiple linear regression analysis, which resulted in a two-dimensional contour plot (Fig 3) and a clear model indicating optimal expression. A 40-fold variation in the soluble expression of HisSolRepCT was obtained within the range of conditions in the screen.

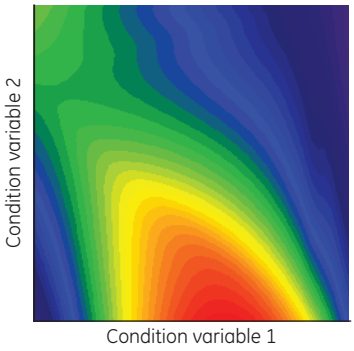

Fig 3. Contour plot derived from values obtained from the screening of expression conditions. The warmer the color, the closer the optimal expression condition. Condition variables are intentionally not disclosed.

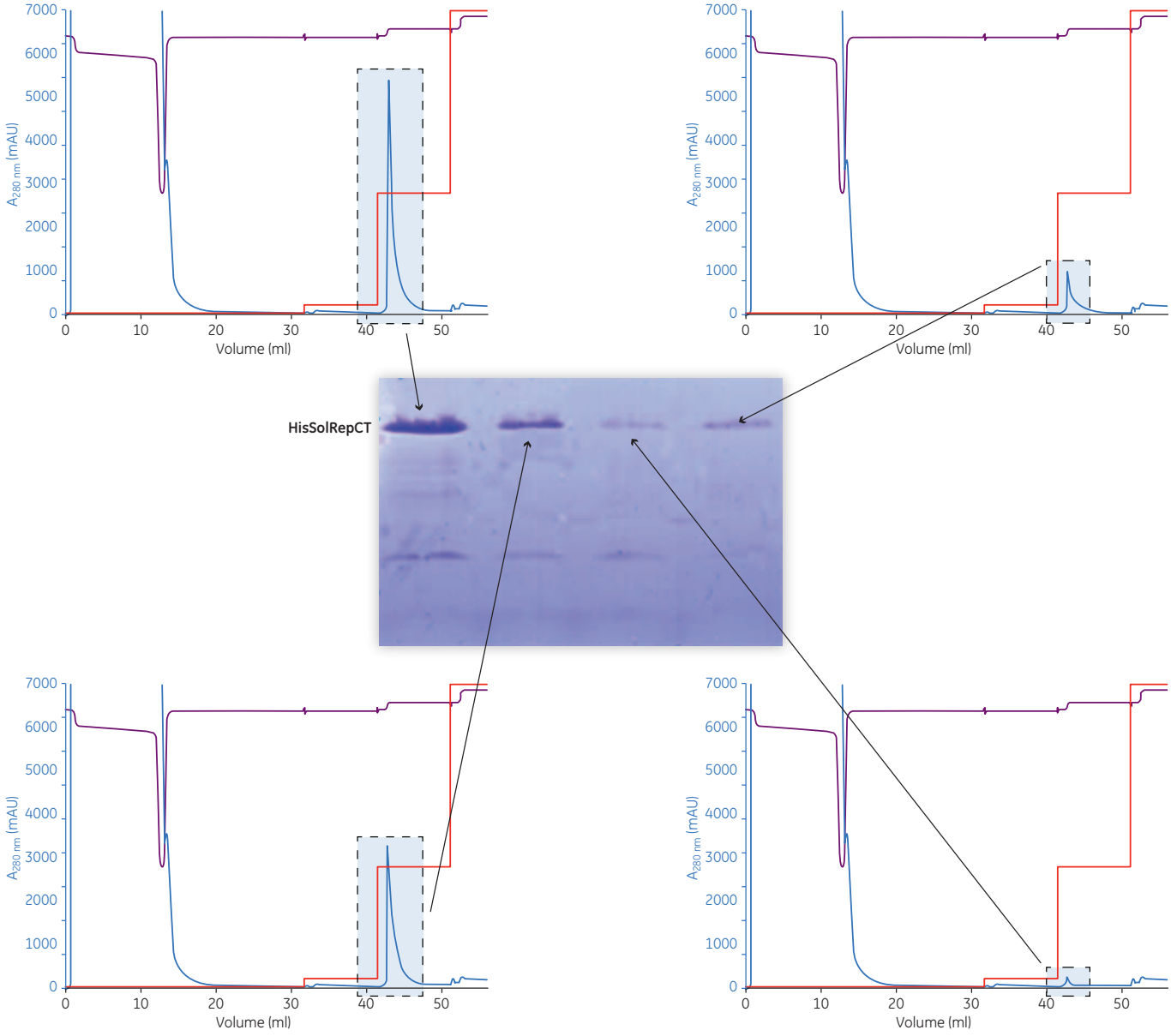

Fig 2. Chromatograms after capture of HisSolRepCT on HiTrap IMAC HP from lysates prepared after growth using different conditions. SDS-PAGE analysis shows pooled fractions containing the target protein (within the dashed lines).

Scaled-up capture of HisSolRepCT

The conditions resulting in maximal soluble protein expression were used in a scale-up experiment where the cell content was about 50-fold higher compared with the screening experiments. The clarified lysate (250 ml, 500 mM NaCl, 30 mM imidazole) was loaded onto a HiTrap IMAC HP 5 ml column freshly charged with Zn^{2+} using system pump A of ÄKTA pure. The column was washed with 20 column volumes (CV) of Eluent A and a second wash consisting of 4% Eluent B for 10 CV (Fig 4). Finally the material bound to the column was eluted using a step gradient consisting of 40% B for 10 CV, which was followed by a washing step of 100% B. The resulting chromatogram together with part of a Deep Purple stained SDS-PAGE gel is shown in Figure 4. Purity of the captured HisSolRepCT was determined by image analysis using ImageQuant TL, resulting in a purity of 70% for the fusion protein.

Column:	HiTrap IMAC HP charged with Zn^{2+}
Sample:	250 ml of HisSolRepCT lysate
Eluent A:	20 mM Tris-HCl pH 8.0, 30 mM imidazole, 500 mM NaCl
Eluent B:	20 mM Tris-HCl pH 8.0, 300 mM imidazole, 500 mM NaCl
Flow rate:	10 ml/min
Elution:	Step elution
Fraction size:	3 ml
Detection:	UV 280 nm
System:	ÄKTA pure

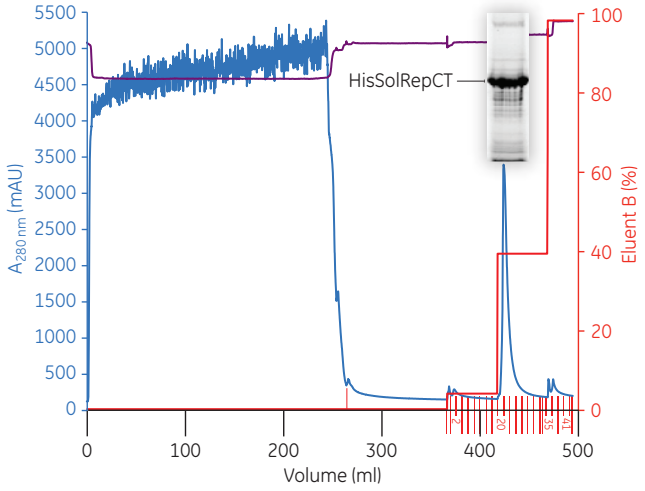

Fig 4. Capture step on HiTrap IMAC HP 5 ml charged with Zn^{2+} using elution by a step gradient. Fractions containing the fusion protein were pooled and analyzed by SDS-PAGE under reducing conditions and stained with Deep Purple. Image analysis indicated a purity of 70% for HisSolRepCT.

Characterization of HisSolRepCT using analytical GF

The purity and size homogeneity of the captured fusion protein was also assessed by analytical GF using Superdex 200 PC 3.2/30 column and ÄKTAmicro system. A small aliquot (10 µl) of the captured HisSolRepCT was applied to the column and separation was done using a flow rate of 50 µl/ min during 1.5 CV (3.6 ml). The resulting chromatogram is shown in Figure 5. Integration of the protein containing peaks showed a purity of 75%. This is in relative good agreement with results from ImageQuant TL analysis.

Column:	Superdex™ 200 PC 3.2/30
Sample:	10 µl of RepCT from Zn-IMAC
Eluent:	20 mM Tris-HCl pH 8.0, 150 mM NaCl
Flow rate:	50 µl/min
Detection:	UV 214 nm
System:	ÄKTAmicro

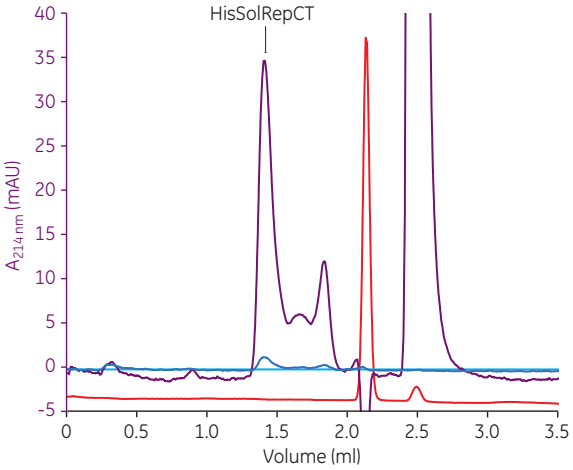

Fig 5. Purity check of 10 µl of the pooled fractions from the capture step containing HisSolRepCT using Superdex 200 PC 3.2/30. The purple and blue lines correspond to absorbance at 214 nm and 280 nm, respectively. The conductivity signal (red) demonstrates that the NaCl in the sample is eluting after 2.1 ml. The large peak eluting around 2.5 ml likely corresponds to an impurity present in the imidazole used for elution in the capturing step and was therefore not investigated further. Integration of the protein-containing peaks revealed a purity of 75% for HisSolRepCT.

Tag cleavage and polishing using GF

After capture of HisSolRepCT, the tag was cleaved off the RepCT moiety using a protease. The fusion protein was mixed with protease and the cleavage mix was incubated at 4°C for 15 h. Since GF is a separation technique that requires little or no optimization, this was first tried as a polishing step for purification of RepCT. After cleavage, the extent of cleavage was checked using PhastSystem and PhastGel Gradient 8-25 under denaturing conditions, which demonstrated that the completeness of cleavage was acceptable. The cleavage mixture was loaded onto a Superdex 75 10/300 GL column and separated at 0.5 ml/min using ÄKTA pure (Fig 6).

The use of GF enabled convenient purification of RepCT from the cleavage mixture since the sample could be directly loaded and no sample conditioning was necessary prior to loading onto the column. The drawback with the method is that it is not suited for large volumes and is thus not suitable for scale-up.

Column: Superdex 75 10/300 GL
Sample: 0.6 ml of protease-digested HisSolRepCT
Eluent: 20 mM Tris-HCl pH 8.0, 150 mM NaCl
Flow rate: 0.5 ml/min
Fraction size: 0.5 ml
Detection: UV 280 nm, 10 mm UV-cell
System: ÄKTA pure

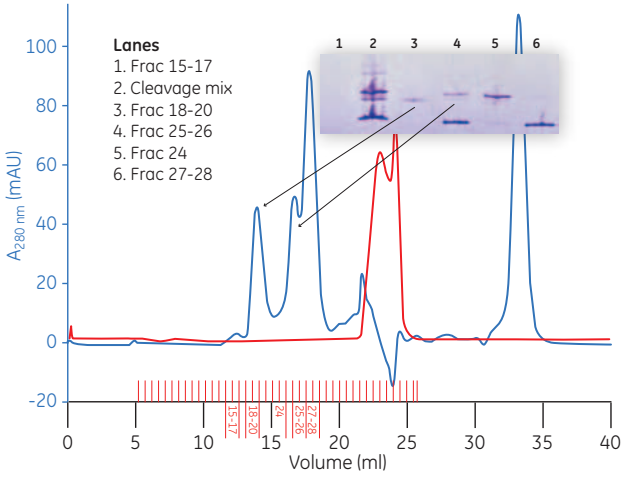

Fig 6. Semipreparative GF using Superdex 75 10/300 GL and ÄKTA pure. SDS-PAGE analysis of different fractions demonstrated that the purified miniature spidroin was of high purity. The peak eluting at 34 ml is discussed in Figure 5, where it elutes at 2.5 ml.

Column: HiPrep 26/10 Desalting (two columns coupled in series)
Sample: 24 ml of protease-digested HisSolRepCT
Eluent: 20 mM Tris-HCl pH 8.0
Flow rate: 10 ml/min
Fraction size: 5 ml
Detection: UV 280 nm, 10 mm UV-cell
System: ÄKTA pure

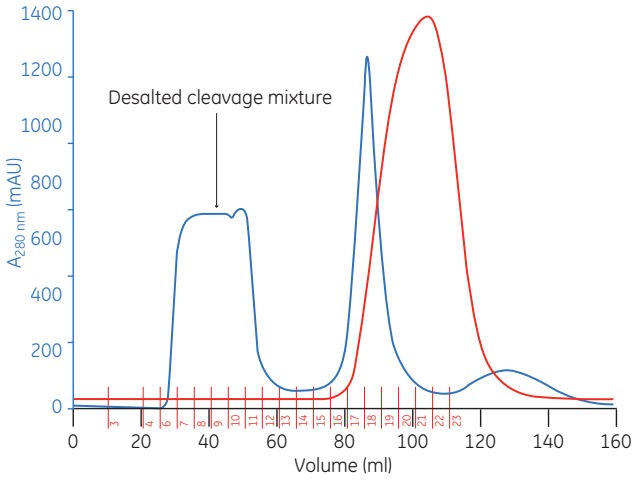

Fig 7. Desalting of 24 ml of protease cleaved HisSolRepCT. The fractions (6 to 12) containing desalted material were pooled resulting in a total volume of 35 ml. The red trace is the conductivity signal. The peak eluting at 87 ml is discussed in Figure 5, where it elutes at 2.5 ml.

Tag cleavage and polishing using AIEX

To develop an alternative method for purification of RepCT that is suited to larger scale chromatography, theoretical titration curves for the three main components in the cleavage mixture were examined. At a pH of 8.0, the protease and the tag should be negatively charged and bind to an AIEX column while RepCT should be positively charged and should pass straight through; this assumption is, however, only valid provided that the ionic strength is close to zero. This was far from the case when the cleavage reaction was performed directly on HisSolRepCT containing fractions from the capture step (500 mM NaCl, 120 mM imidazole). A desalting (DS) step was thus required before AIEX purification of RepCT from the cleavage mix could be performed. An example of desalting of a 24 ml cleavage mixture using HiPrep 26/10 Desalting and ÄKTA pure is shown in Figure 7.

Since the maximum sample volume is 15 ml for HiPrep 26/10 Desalting, two columns were coupled in series to accommodate the volume of the cleavage mixture. The desalted cleavage mixture was loaded onto an AIEX column (HiTrap Q HP 5 ml) at a flow rate of 5 ml/min (Fig 8). The flowthrough contains pure RepCT, while tag, protease, and minor impurities eluted at 100% of Eluent B, which was 500 mM NaCl. The separation pattern was thus as predicted from the theoretical titration curves.

Column: HiTrap Q HP 5 ml
Sample: 33 ml of desalted cleavage mix
Eluent A: 20 mM Tris-HCl, pH 8.0
Eluent B: 20 mM Tris-HCl pH 8.0, 500 mM NaCl
Flow rate: 5 ml/min
Elution: Step gradient
Fraction size: 3 ml
Detection: UV 280 nm, 10 mm UV-cell
System: ÄKTA pure

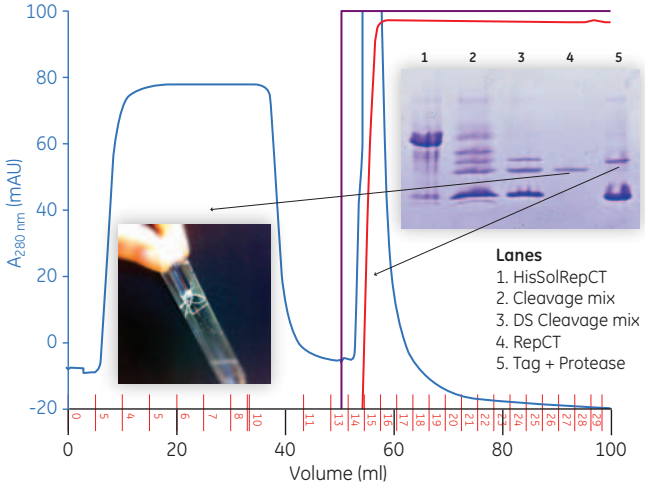

Fig 8. Noncapturing AIEX on HiTrap Q HP 5 ml column and ÄKTA pure of protease-digested and desalted HisSolRepCT. The flowthrough material, fractions 2 to 11 (33 ml) were collected. SDS-PAGE analysis shows different steps of the purification of the miniature spidroin. Biological activity of the miniature spidroin was confirmed by the ability to assemble into macroscopic fibers (inset image).

On-column tag removal

One advantage of ÄKTA pure is the possibility to use a predefined affinity chromatography method with included on-column tag removal. In brief, the method consists of applying the sample containing the histidine-tagged protein followed by a wash step. An optional wash step can then be performed to condition the sample bound to the affinity column in the preferable buffer. The protease is added through a capillary loop and incubation is performed, and finally elution of the untagged protein is accomplished. This method was tested by preparing a HisSolRepCT containing lysate and applying it to HiTrap IMAC HP 1 ml charged with Zn^{2+} using system pump A of ÄKTA pure (Fig 9). A wash step of 10 CV eluent A was performed followed by an additional wash step using 5 CV of 20 mM Tris-HCl pH 8.0. Next, protease solution was applied using a capillary loop onto the column, and the system was set on hold for 15 h. After incubation, the sample was eluted using 5 CV of 20 mM Tris-HCl, pH 8.0, followed by 5 CV of 40% Eluent C, and a step consisting of 100% C.

SDS-PAGE analysis showed that most of the miniature spidroin eluted in fractions 5 to 13 using 20 mM Tris-HCl, pH 8.0 upon restart of the flow after the incubation. This material was thus released from the column because of loss of an affinity tag due to proteolytic cleavage. These fractions also contain weak bands on the SDS-PAGE that could be the tag and uncleaved HisSolRepCT. The major parts of the protease and the tag eluted at the first step using 40% C in fractions 37 to 47. Because of the small amount of impurities in the RepCT containing fractions, a polishing step using AIEX was applied (Fig 10) resulting in RepCT of high purity.

Column: HiTrap IMAC HP 1 ml charged with Zn^{2+}
Sample: 50 ml of HisSolRepCT lysate
Eluent A: 20 mM Tris-HCl pH 8.0, 20 mM imidazole, 500 mM NaCl
Eluent B: 20 mM Tris-HCl, pH 8.0
Eluent C: 20 mM Tris-HCl pH 8.0, 300 mM imidazole, 500 mM NaCl
Flow rate: 1 ml/min
Elution: Step elution
Detection: UV 280 nm, 10 mm UV-cell
Fraction size: 0.2 ml
Detection: UV 280 nm
System: ÄKTA pure
Temp: 4 °C

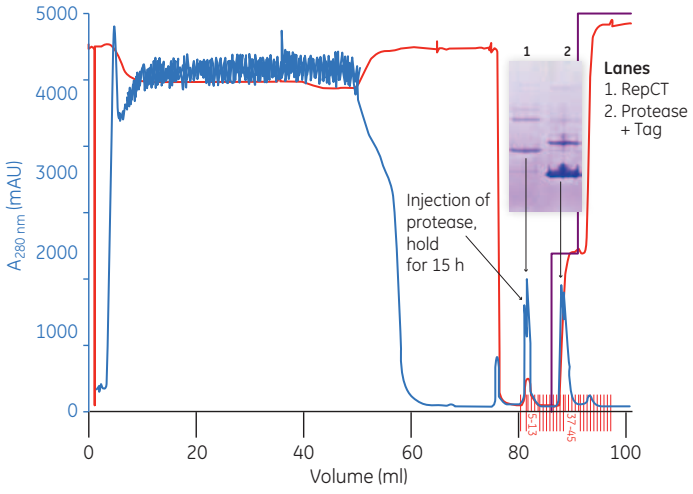

Fig 9. On-column tag removal of HisSolRepCT using HiTap IMAC HP 1 ml charged with Zn^{2+}.

Column: HiTrap Q HP 1 ml
Sample: Fractions 5 to 13 from on-column tag removal
Eluent A: 20 mM Tris-HCl pH 8.0
Eluent B: 20 mM Tris-HCl pH 8.0, 500 mM NaCl
Flow rate: 1 ml/min
Fraction size: 1 ml
Detection: UV 280 nm, 10 mm UV-cell
System: ÄKTA pure

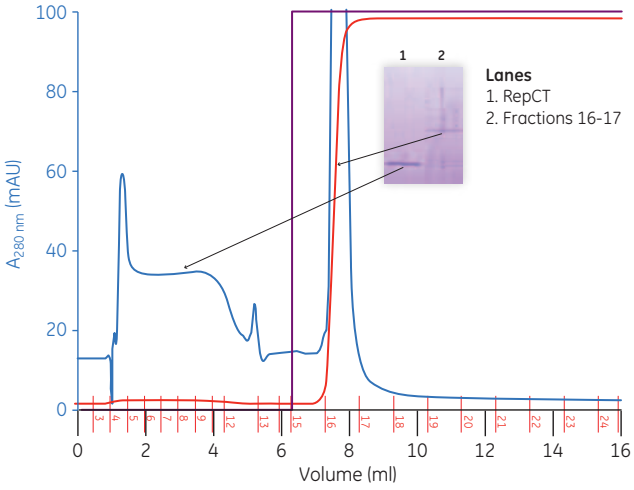

Fig 10. Noncapture AIEX of RepCT from fractions 5 to 13 of the on-column tag removal experiment.

Purity check of RepCT

After the final polishing step of RepCT assessment of purity was performed using ÄKTAmicro and Superdex 200 PC 3.2/30 as shown in Figure 11.

Column: Superdex 200 PC 3.2/30
Sample: 5 µl of RepCT
Eluent: 20 mM Tris-HCl pH 8.0, 150 mM NaCl
Flow rate: 50 µl/min
Detection: UV 214 nm
System: ÄKTAmicro

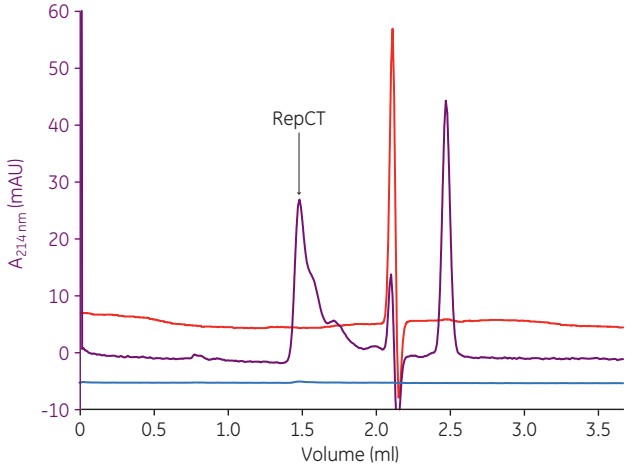

Fig 11. Example data for purity check on Superdex 200 PC 3.2/30 of 5 µl of the pooled fractions from the AIEX polishing step (after tag removal in vial) of RepCT. The purple and blue line corresponds to absorbance at 214 nm and 280 nm, respectively. The low signal at 280 nm is due to the low content of amino acid residues containing aromatic rings in RepCT. Integration of the protein containing peaks revealed a purity of RepCT exceeding 95%. The peak eluting at 25 ml is discussed in Figure 5, where it elutes at 2.5 ml.

The purification results from capture using HiTrap IMAC HP 5 ml charged with Zn^{2+}, tag cleavage, desalting using HiPrep 26/10 Desalting, and polishing using noncapturing AIEX on HiTrap Q HP 5 ml are shown in Table 1. Capture by IMAC and polishing using GF resulted in yield of 82% to 88% RepCT for IMAC-GF and IMAC-AIEX, respectively. Purity of RepCT was 90% and 95% for IMAC-GF and IMAC-AIEX, respectively. On-column tag removal in combination with an AIEX polishing step using HiTrap Q HP 1 ml resulted in somewhat lower yield and purity (70% and 85%, respectively) indicating an optimization potential for on-column cleavage conditions. Otherwise, this was the most convenient of the methods tested for purification of RepCT after tag cleavage.

Table 1. Purification results for IMAC, as well as polishing by GF and AIEX

Purification scheme	[1]Amount RepCT (mg)	Yield (%)	Purity (%)
IMAC-GF			
Start material (clarified lysate)	1.1	100	N.D.
IMAC	1.0	95	70
GF	0.9	82	90
IMAC-AIEX			
Start material (clarified lysate)	5.4	100	N.D.
IMAC	5.1	95	70
AIEX	4.7	88	95
IMAC (OCTR[2])-AIEX			
Start material (clarified lysate)	1.2	100	N.D.
IMAC (OCTR)	1.1	95	N.D.
AIEX	0.8	70	85

[1] The RepCT moiety constitutes 60% of HisSolRepCT
[2] On-column tag removal

Identification of RepCT by LC-MS/MS

RepCT was identified in the AIEX fractions in the polishing step after tag cleavage in vial by trypsin digestion followed by nanoscale LC-MS/MS analysis. Figure 12 shows MS/MS data of one tryptic peptide with the sequence STVANSVSR. In total, a sequence coverage of 80% of the RepCT sequence was obtained resulting in an unambiguous identification.

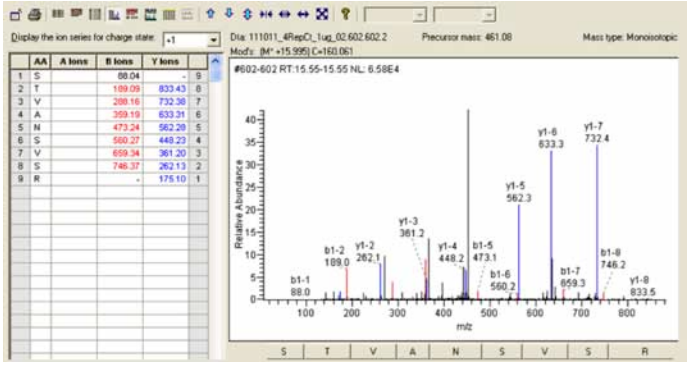

Fig 12. MS/MS spectrum of one peptide from trypsin digested RepCT from the final AIEX step. Matching b- and y-ions are almost complete resulting in an unambiguous identification of a peptide with the sequence STVANSVSR of RepCT. A total sequence coverage of 80% of the RepCT sequence was obtained.

Conclusions

HisSolRepCT was expressed under optimal conditions in *E. coli* and captured using Zn^{2+}-charged IMAC on ÄKTA pure. On-column cleavage and removal of the tag was simplified by the predefined affinity chromatography method with tag removal provided in the UNICORN v6.3 software. After proteolytic cleavage using protease, AIEX and GF polishing steps performed on ÄKTA pure were compared and gave similar yield (~ 88%) and purity (95%) of the target protein. Identification of RepCT was confirmed by nanoscale LC-MS/MS. A total sequence coverage of 80% was achieved. Biological activity of the miniature spidroin was confirmed by the induced ability to assemble into macroscopic fibers.

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Ordering information

Product	Code number
ÄKTA pure M1	29-0182-27
Mixer valve kit V9-M	29-0113-54
ÄKTAmicro	28-9483-03
HiTrap IMAC HP	17-0920-03
HiPrep 26/10 Desalting	17-5087-01
HiTrap Q HP	17-1154-01
Superdex 200 PC 3.2/30	17-1089-01
Superdex 75 10/300 GL	17-5174-01
Dithiothreitol, DTT	17-1318-02
Tris	17-1321-01
Sodium dodecyl sulfate (SDS)	17-1313-01
Glycerol	17-1325-01
ExcelGel SDS Buffer Strips	17-1342-01
ExcelGel SDS Gradient 8-18	80-1255-53
IEF Sample application pieces	18-1129-46
PhastGel Gradient 8-25	17-0542-01
PhastGel SDS Buffer Strips	17-0516-01
PhastGel Blue R-350 Tablets	17-0518-01
Deep Purple Total Protein Stain	RPN6305
LMW-SDS Marker Kit	17-0446-01

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