A systematic and methodical approach for the efficient purification of recombinant protein from silkworm larval hemolymph

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Short running title: Purification of recombinant protein expressed in silkworm

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Abstract

The silkworm, *Bombyx mori*, is a promising expression system for the production of recombinant proteins, but the purification of these proteins is not easy because of the large amount of host proteins present. To investigate purity, recovery and scale-up ability of the purification of recombinant proteins expressed in silkworm larval hemolymph without any affinity tags, we used mCherry, a red fluorescence protein, as a model. The host cell proteins could be greatly reduced using a three-step chromatography protocol consisting of hydrophobic interaction chromatography (HIC), size exclusion chromatography (SEC) and heparin chromatography after heat pretreatment. The thermal treatment had the greatest impact on the removal of host cell extracellular proteins and increasing purity. There were still some minor traces of host cell proteins in the purified sample, which showed that the purification of recombinant proteins from the silkworm hemolymph was still challenging. The proposed protocol and affinity tag purification reduced the overall protein content by 99.84% and 99.95%, respectively, while the amount of DNA was reduced by 98.41% and 99.53%, respectively. Purities of our proposed protocol based on SDS-PAGE and capillary electrophoresis (CE) analyses were 85.45% and 43.60%, respectively, while those of Strep-tag affinity purification were 100% or 63.69%, respectively. Using densitometry, the overall recovery was calculated was 5.78%, which was higher than 4.09% using Strep-tag affinity purification. This proposed protocol, mainly based on thermal treatment, HIC, SEC and HiTrap Heparin HP column chromatography, is applicable to an upscalable purification for the silkworm expression system without employing affinity tag chromatography process.

Keywords: Hemolymph, Heat treatment, Chromatography, mCherry, Recombinant protein, Silkworm.

1. Introduction

Different expression systems are used for protein production. The *Escherichia coli* system is the most common because of its high productivity. However, it suffers from low protein quality (e.g., the presence of inclusion bodies), poor protein folding, endotoxin and a lack of posttranslational modifications for proteins of eukaryotic origin. In contrast, the yeast-based protein production system does not present the drawbacks of the *E. coli* system but can exhibit lower productivity, inappropriate glycosylation patterns and a lack of chaperonins for proper folding [1]. The baculovirus expression system using a Bac-to-Bac shuttle vector derived from a baculovirus is also popular for the production of proteins, mainly in *Spodoptera frugiperda* ovarian cell lines (Sf9 cell lines) because these cells are robust and able to perform most posttranslational modifications. The gold standard for the production of complex proteins and posttranslational modifications is still mammalian cell lines such as the HEK293, CHO or HeLa cell lines, but these systems are more expensive than production in other expression systems and more complex to handle [1,2]. Compared to these approaches, the use of the Bac-to-Bac system in insect larvae, such as silkworm (*Bombyx mori*) larvae, provides a comparable cheaper production method that produces eukaryotic proteins with the same posttranslational modifications as the insect cell lines do and shows even higher protein yields than the abovementioned systems [1,3–7]. Under this approach, the cells of the larvae are infected with the recombinant virus shuttle and express the target proteins. The proteins can be secreted into the hemolymph or remain inside the cells, depending on the expressed recombinant protein [3].

However, a drawback of the silkworm expression system is that the purification of the target protein from silkworm larvae is extremely challenging because of the presence of abundant host proteins [1]. This may also be true for the other systems, but in the silkworm system, the amount of host proteins and lipids is particularly high [8,9]. In most studies,

protein tags are used for purification via affinity chromatography [5,6,10], but there is always a possibility that these additional amino acid sequences may interfere with the assembly and structure of the proteins, for example, through steric hindrance. In addition, if the intended recipient is the human body, it is desirable to minimize the presence of foreign proteins as much as possible because the protein tags could alter the response to the desired antigen [11] or elicit an immune response [12], making it impossible to use the proteins even after optimization. The affinity purification method and the often-used sucrose density gradient centrifugation approach are not suitable for industrial use [8], and therefore, in this study, we aimed to establish an upscalable purification protocol. Additionally, these purification procedures are usually not explored for use in optimization [13,14], particularly for the silkworms larval hemolymph [15]. There is currently no systematic methodical approach for the purification of proteins of interest from silkworm larval hemolymph. This report provides details of the first steps towards a systematic purification approach for upscalable purification process.

In the beginning of the purification procedure, it is important to remove cell debris and clarify the hemolymph sample [16]. For this purpose, centrifugation, precipitation, and filtration are normally sufficient. We explored a broad range of centrifugation setups including low to high forces and times, that should be practical on a laboratory scale. For the precipitation experiments, well-established methods were chosen, such as the classical ammonium sulfate precipitation method [17,18]. Polyethylene glycol (PEG) precipitation was also used, which is an alternative method that, unlike ethanol precipitation, shows only a slight tendency of protein interaction [19], because we could not find any study in which this method was applied for purification from silkworm compartments. This polymer is positively charged at a neutral pH and therefore binds to negatively charged molecules such as nucleic acids or acidic proteins. The amount of bound acidic proteins depends on the salt concentration and the acidic strength of the protein [18]. Furthermore, it has been shown that

heat treatment at up to 80°C followed by centrifugation can reduce the amount of host cell proteins significantly if the target protein is heat stable [20,21]; therefore, this approach was also investigated in this study, since the aforementioned methods and chromatography strategies were not sufficient to reduce the host protein content, as we will discuss later.

After reducing the amount of host cell proteins, fast performance liquid chromatography (FPLC) is the method of choice for efficient purification. The FPLC is an established and reliable method but requires product-specific method development and careful selection of separation strategies. In this study, a modified form of mCherry was used as a model protein, which has a molecular mass approximately 34.5 kDa and displayed a strong red fluorescence (Ex. 540-590 nm Em. 550-650 nm) [22], which made it easily traceable during the purification process as a reporter protein.

The purification protocol involved a centrifugation step and a precipitation step as pretreatments, followed by necessary chromatography steps, which should be as few as possible. In the end, we report the first possibility for an upscalable classical purification protocol for silkworm hemolymph designed for industrial use, which involves centrifugation and polyethylene glycol precipitation, followed by hydrophobic interaction, size exclusion and heparin chromatography steps, and we describe the behavior of the host impurities during each purification step. The protocol was further improved by introducing a thermal precipitation step during pretreatment, which increased the purity and recovery of protein. For comparison, the strep tag affinity chromatography was performed.

2. Materials and Methods

2.1. Recombinant Bombyx mori nucleopolyhedrovirus (BmNPV) bacmid preparation

The DNA fragment of mCherry was subcloned from the commercial plasmid pmCherry (Catalog # 632522, Takara Bio, Japan) using specific primers (mCherryFw: 5'-GTGAGCAAGGGCGAGGAGGAT-3', mCherryRv: 5'-CTACTTGTACAGCTCGTCCATG-3'). A DNA sequence encoding secreted poly-tags (30K-Flag-Strep-SpyTag, 30K-FSS, MRLTLFAFVLAVCALASNADYKDDDDKGGGSAWSHPQFEKGGGSENLYFQGSQPV PTIVMVDAYKRYKGSSGSGGSG) was designed and synthesized by GeneWiz (Suzhou, China). The encoded sequence consisted of the signal peptides (SPs) from silkworm 30K proteins for secretion, followed by a Flag-tag, StrepTag II, tobacco etch virus (TEV) protease cleavage site and a Spy-tag. The resulting plasmid for expressing the secreted mCherry protein (approximately 34.5 kDa) was designated pFastBac-30K-FSS-mCherry. It was constructed and then utilized to generate a recombinant BmNPV bacmid. Subsequently, the recombinant baculovirus was generated in cultured silkworm Bm5 cells according to our previous report [23]. The cell culture supernatant was collected and used for serial infections to obtain high-titer virus stocks, which were employed to infect silkworm larvae.

2.2. Silkworms

Fifth-instar larvae of the silkworm (Ehime Sansyu, Ehime, Japan) were reared on an artificial diet (Silkmate S2, Nosan, Yokohama, Japan) in a rearing chamber (MLR-351H, Sanyo, Moriguchi, Japan) at 25°C. On the second day of the 5^{th} instar, the silkworm larvae were injected with 10 µL of a PBS solution containing 250 µL/mL recombinant baculovirus using a 1 mL syringe (26G × 1/2, 0.45 × 13 mm). Five days postinjection (dpi), the hemolymph of

the larvae was collected with 20 µL of 200 mmol/L 1-phenyl-2-thiourea per silkworm as a preservative and stored at –80°C until use.

2.3. Centrifugation

Centrifugation was performed using a Kubota 3700 centrifuge (Kubota, Japan, Tokyo) equipped with an AF-2724A rotor. The tested setups were grouped into high-, intermediate- and low-centrifugal force groups. The high centrifugation forces were as follows: 20,400 × g, 60 min; 17,800 × g, 10 min and 10,000 × g, 30 min. The intermediate centrifugation forces were as follows: 6000 × g, 30 min; 5000 × g, 15 min; 3000 × g, 5 min and 2000 × g, 10 min. The low centrifugation forces were as follows: 1000 × g, 5 min; 500 × g, 5 min; 100 × g, 20 min and 100 × g, 5 min.

2.4. Precipitation

The setups were grouped into 3 main precipitation protocols as follows. If necessary, ultrapure water (Puric-ZII, Organo, Tokyo, Japan) was added for concentration adjustment. $(NH_4)_2SO_4$ precipitation was performed with a concentration of 0.34 mol/L, 1 mol/L, 2 mol/L, 3 mol/L, 4 mol/L or 5 mol/L. Incubation was performed for 30 min on ice, except when a concentration of 0.34 mol/L was used, when incubation was performed for 4 h. Thereafter, the samples were centrifuged at 5000 × g for 20 min. PEG 6000 was added at concentrations of 2.5%, 5% and 10% (w/v), followed by incubation on ice for 60 min. Centrifugation was performed at 4200 × g for 20 min. Polyethyleneimine (PEI) was added at a final concentration of 0.1%, 0.3% or 0.5% (w/v), together with NaCl at a concentration of 0.1 mol/L or 0.5 mol/L. The solution was mixed for 5 min and then incubated on ice for 30 min. Centrifugation was performed at 2300 × g for 5 min.

2.5. Thermal treatment of hemolymph

Samples were treated at 50°C, 60°C or 70°C for 30 min or at 70°C for 20 min. After thermal treatment, the solution was centrifuged again at 17,800 × g for 10 min.

2.6. Chromatography

Chromatography was carried out using the BioLogic DualFlow system (Bio-Rad, Hercules, CA, USA) with the associated software BioLogic DualFlow V. 5.30 Build 6 (Bio-Rad), and the instrument was equipped with a BioFrac Fraction collector (Bio-Rad) and an Auto-Injector Valve AVR7-3 (Bio-Rad). The following columns were used: HiTrap DEAE Sepharose 5 mL (GE Healthcare, Tokyo, Japan), HiTrap Butyl FF 5 mL (GE Healthcare), Bio-Scale Mini CHT Type II Cartridge (Bio-Rad), HisTrap HP 5 mL (GE Healthcare), Superdex Sephacryl S-200 120 mL (GE Healthcare), HiTrap Heparin HP 5 ml (GE Healthcare) and Strep-Tactin Superflow Plus Cartridge (Qiagen, Tokyo, Japan). The chromatography protocol varied, and step or linear gradients were applied. Fractions were collected at volumes ranging from 1 to 5 mL. Generally, after the column was equilibrated with the starting buffer, the sample was loaded and washed with at least 5 column volumes (CVs) of the buffer. For linear gradient elution, elution was performed with at least 10 CVs with increasing proportions of elution buffer. Before reequilibration, the column was washed with at least 5 CVs of elution buffer. Each step of the step gradients involved at least 5 CVs and was individually determined. The standard flow rate was 2 mL/min, and that for size exclusion chromatography (SEC) was 0.5 mL/min if not indicated otherwise. The samples were filtered through at least 0.8 µm filters and, if necessary, were adjusted to the binding buffer before loading onto the column. If necessary, samples from a previous chromatography

step could also be adjusted to the next binding buffer and filtered at 0.45 µm. The individual protocols are indicated in the corresponding figure legends.

In contrast, purification using the Strep-Tactin column was performed using a manual peristaltic pump at a low flow rate (0.5 mL/min). The sample was centrifuged at 17,800 × g for 5 min at 4°C and filtered through a 0.8 µm filter before chromatography (**Supplementary material Fig. S1**). The elution fraction with a reddish/purple color was collected separately. All fractions were examined to determine if they still emitted red fluorescence.

2.7. Final purification protocol

For this procedure, the hemolymph was centrifuged at 17,800 × g for 10 min, followed by a 2.5% PEG 6000 precipitation, with centrifugation at 4,200 × g for 20 min. The supernatant was then subjected to a 70°C heat treatment for 20 min and centrifuged at 17,800 × g for 10 min. The supernatant was filtered with a 0.45 µm filter and used in further experiments. For chromatography, a HiTrap Butyl FF column, 5 mL (GE Healthcare), was used in the indicated system. Elution was performed with a two-step gradient in which the first step contained 100% binding buffer (10 mmol/L Tris-HCl, 3 mol/L NaCl, pH 8) and the second step 0%. The sample was adjusted with an additional 3 mol/L NaCl. The program was as follows: 2 mL binding buffer, 5.2 mL sample load with binding buffer, 7 CVs binding buffer and 5 CVs elution buffer. The flow rate was 2 mL/min, while the loading rate was 1 mL/min. Fractions were collected at volumes of up to 2 mL, with a threshold of 0.075 AU. The elution fractions were pooled and concentrated via ultrafiltration with a cut off of 3 kDa. The resulting sample was loaded onto a Superdex Sephacryl S-200 column, 120 mL, with buffer consisting of 10 mmol/L Tris and 150 mmol/L NaCl, pH 8. The program was as follows: 1 mL elution buffer, 5.2 mL sample load with elution buffer, and 121 mL elution buffer. The flow rate was 0.5 mL/min. Fractions were collected at volumes of up to 3 mL with a threshold of 0.075 AU.

The elution fraction with the target protein was again concentrated via ultrafiltration with a cut off of 3 kDa. As the last chromatography step, a HiTrap Heparin HP column, 5 mL, with 10 mmol/L NaH_2PO_4, pH 7 was used. Elution was performed with 2 mol/L NaCl. The program was as follows: 5.2 mL sample load with binding buffer, 1 mL binding buffer, 5.2 mL sample load with binding buffer, 4 CVs binding buffer, 5 CVs 100% elution buffer and 2 CVs binding buffer again. The flow rate was 1.5 mL/min, while the loading rate was 1 mL/min. Fractions were collected at volumes of up to 2 mL with a threshold of 0.003. The elution fraction was pooled and concentrated via ultrafiltration with a cut off of 3 kDa.

2.8. Sodium dodecyl sulfate-capillary electrophoresis (SDS-CE)

For the CE experiments, the following equipment and reagents were used: ultrapure water (arium pro VF system, Sartorius, Goettingen, Germany), HEPES (Carl Roth, Karlsruhe, Germany), β-mercaptoethanol (BME) (Carl Roth, Karlsruhe, Germany), Rotilabo CME 0.22 µm syringe filter (Carl Roth), Amicon Ultracell-0.5 mL 10k (Merck KGaA, Darmstadt, Germany), sodium hydroxide (Merck KGaA), 5417C centrifuge (Eppendorf, Hamburg, Germany), VV3 vortex (VWR, Leuven, Belgium), Maurice (ProteinSimple, San Jose, USA), Maurice CE-SDS Application Kit (ProteinSimple) and Maurice CE-SDS Molecular Weight Markers (ProteinSimple).

The lyophilized samples were dissolved in ultrapure water to a final protein concentration of approximately 1 mg/mL. For desalting, the sample solutions were transferred to the Amicon Ultracell filter devices and centrifuged for 10 min at 14 000 × g. Thereafter, 200 µl HEPES buffer (10 mmol/L, pH 7.5) was added, followed by careful mixing (20 × aspiration and dispensing with a pipette), and the filter devices were centrifuged with the same parameters used before. In total, buffer addition and centrifugation were repeated four times. Deviating from the previous parameters, the last centrifugation step was performed at 14,000

× g for 20 min. Thereafter, the samples were weighed, and buffer was added as previously described until the starting weight was reached. The filter devices were inverted and centrifuged for 2 min at 1020 × g into collection tubes. To 25 µL of each sample, 25 µL of the 1 × sample buffer, 2 µL of the internal standard and 2.5 µL BME were added. The mixtures were vortexed three times for 5 s each and subsequently centrifuged at 1000 × g for 5 min. Next, they were heated at 70°C for 10 min and cooled on ice for 5 min immediately thereafter. Finally, the samples were vortexed and centrifuged as described in the preceding step and then transferred to a 96-well plate for measurements on the Maurice instrument (ProteinSimple). The measurements were performed using the CE-SDS Size Application Kit, the Maurice CE-SDS IgG Standard and Maurice CE-SDS Molecular Weight Markers. The CE-SDS cartridges contained a fused-silica capillary with a 15 cm effective length and a 50 µm internal diameter. The sample tray was tempered at 10°C, and the samples were loaded electrokinetically at 4600 V for 20 s and separated at 5750 V for 35 min. For detection, UV absorption at 220 nm was measured. Compass for iCE version 2.0.10 was used for instrument control and integration.

2.9. SDS-PAGE

Samples were diluted with an equal amount of sample buffer (0.125 mol/L Tris-HCl, 4% SDS, 20% Glycerol, 0.01% mercaptoethanol, 0.15 mmol/L bromophenol blue), mixed and heated at 95°C for 5 min if the gel was to be subjected to Coomassie blue (CBB) staining. Size classification was performed with the PM1700 ExcelBand standard (Smobio, Hsinchu City, Taiwan). The 10% acrylamide gels were analyzed via fluorescence imaging or Coomassie staining. The constant voltage was set at 90 V for the stacking gel and 120 V for the running gel. Samples were analyzed at the following dilutions: 10 µL sample to 22 µL or 8 µL sample to 24 µL for Coomassie brilliant blue (CBB) staining; 12 µL sample to 24 µL or

10 µL sample to 24 µL for fluorescence imaging. Each time, 15 µL of the dilution was loaded in the gel lane. For fluorescence imaging, the samples were not heat denatured before SDS-PAGE, and images were taken with a Pharos FX Plus system (Bio-Rad). mCherry was detected using the Texas red set up (Ex. 532 nm, Em. 640 nm).

For the determination of the relative recovery of mCherry, ImageJ 1.51j8 (Wayne Rasband, National Institutes of Health, USA) was used. First, the program plotted the intensity of the SDS-PAGE images automatically, and the integration of the area under the curve of the signals was then performed manually. Calibration was performed with samples with known mCherry concentrations (127.2, 63.6 and 31.8 µg/mL), This approach was used to obtain a more objective measure for the comparison of recovery during the purification protocol compared with observation by the naked eye.

2.10. Protein assay for purification improvement

For the measurement of the overall protein concentration, the Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, USA) was used. The microplate assay was performed according to manufacturer's manual. The absorbance was measured at 570 nm. The analysis was performed in duplicate.

2.11. DNA assay for purification improvement

For the measurement of the DNA concentration, the Qubit dsDNA BR Assay Kit (Thermo Scientific) was used. The assay was performed on a 96-well microtiter plate, and the fluorescence was measured on the microplate reader Infinite M Plex (Tecan, Kawasaki, Japan) at an excitation of 485 nm and an emission of 530 nm. The analysis was performed once or twice.

3. Results and discussion

3.1. Centrifugation

On a small scale, we investigated the behavior of mCherry and hemolymph proteins subjected to low, intermediate and high centrifugation forces to evaluate the ability to separate cell debris and host cell proteins. In addition to its red fluorescence, mCherry has an easy to observe red color, which also makes its detection and traceability simple. Despite the high centrifugation forces applied, mCherry was present only in low concentrations in the pellet; this low mCherry loss can be explained by mCherry that was not yet secreted from the cells into the hemolymph and was spun down together with the cells during centrifugation. There were no significant differences between the different forces according to the CBB staining and fluorescence images; therefore, 17,800 × g for 10 min was selected as the best option because of the short time and high centrifugation force required to separate the cell debris and host cell proteins into the pellet (data not shown). The effect for the host cell proteins was difficult to evaluate because of the high quantity present. The important point here is that there was no loss of mCherry at this force, but it can be assumed that more host cells were precipitated at this force than at lower forces.

3.2. Precipitation

$(NH_4)_2SO_4$, PEG 6000 and polyethylene (PEI) precipitations were investigated on a small scale. Treatment with 2 mol/L $(NH_4)_2SO_4$ and 5% PEG resulted in no mCherry loss into the precipitate, and these conditions were therefore investigated for larger-scale precipitation. During this experiment, 2 mol/L $(NH_4)_2SO_4$ and 5% PEG resulted in higher loss of mCherry compared to the small-scale experiment (**Supplementary material Fig. S2**). For this reason,

the use of 2.5% PEG was additionally reinvestigated for the larger-scale and showed comparable results to small-scale precipitation with 2 mol/L $(NH_4)_2SO_4$; therefore, 2.5% PEG precipitation was chosen, and the supernatants were used for chromatography. These results indicate the challenging nature of the goal of this study. This kind of screening experiment is usually performed on a small scale with the expectation that no differences will arise during upscaling to the industrial scale apart from some minor changes. If such a problem arises now during the screening of hemolymph purification protocols, it will almost certainly occur a larger scale, meaning that all screening experiments would need to be repeated.

3.3. Chromatography matrixes as the 1st or 2nd purification step

Using a DEAE column following the anion exchange (AIEX) strategy, mCherry was bound together with most of the host cell proteins in the column and could not be separated from them during the linear gradient procedure (**Supplementary material Fig. S3**). Since the Phenyl column showed high adsorption and low recovery, only the Butyl FF column was investigated, which utilizes the hydrophobic interaction chromatography (HIC) strategy. Using 1.2 mol/L $(NH_4)_2SO_4$ as binding buffer, mCherry was bound mostly to the column and was mainly eluted in the middle of the linear gradient (**Supplementary material Fig. S4**), which reduced the amount of host cell proteins. Therefore, the protocol was optimized to a two-step gradient with 66% and 0% binding buffer (**Supplementary material Fig. S5**), but opposite to our expectation, mCherry and most of the proteins were detected in the flow-through fraction. One possible explanation might be that the ionic strength of the 66% binding buffer was insufficient. This would mean that the elution of mCherry previously was delayed and that elution began at a reduced ionic strength.

The Butyl FF column was also investigated using 3 mol/L NaCl to promote hydrophobic interaction (**Supplementary material Fig. S6**). In this case, the host cell proteins could be

strongly reduced because the majority of the host proteins were in the flow-through fraction, while mCherry was present in the elution fractions.

Using metal affinity combined with a cation exchange strategy, ceramic hydroxyapatite (CHT) did not achieve the desired separation effect (**Supplementary material Fig. S7**). mCherry was present in the same fractions as the main impurities. Moreover, the elution behavior of the main impurities changed compared to the behavior associated with a GFP-fusion protein, in which the impurities were eluted during the linear gradient (data not published), whereas they were present in the flow-through fraction in the current experiment. This finding raises questions because the known differences between these two runs do not seem relevant. For the GFP-fusion protein purification experiment, the sample had a slightly higher ionic strength, but for elution in the CHT column, the phosphate concentration was the relevant factor. If the reason for this effect is also the cause of the joint elution of the target protein and impurities, it seems worthy of further investigation.

Another tested approach was to use a HisTrap column to separate the remaining storage proteins of approximately 70 kDa in the hemolymph because these proteins may have an affinity for a nickel column because of their composition. However, the proteins did not bind to the column and were present in the flow-through. Therefore, this approach was not further employed.

Using the elution fraction from HIC NaCl purification (as already shown in **Supplementary material Fig. S6**), the separation effect of SEC was investigated with a Superdex Sephacryl S-200 column with a length of 60 cm. As shown in **Fig. 1,** all except for small traces of the main impurities at 60–80 kDa could be separated from the mCherry. The only challenging issues were the proteins with a similar size to the target protein and that the sample resulting from the SEC diluted during elution. Separation might be significantly improved by a longer separation distance or with a more suitable matrix. From these

preliminary investigations, it was concluded that an HIC step followed by an SEC step resulted in the best outcome, and this approach was therefore repeated and further investigated.

3.4. Further HIC and SEC investigations

To combine all steps of the protocol, two runs of 17,800 × g, 10 min centrifugation of the hemolymph; 2 mol/L $(NH_4)_2SO_4$ precipitation; HIC with NaCl and SEC were performed. For HIC, NaCl was chosen for setting the ionic strength, and the protocol was changed to use a two-step gradient in which the first step involved 100% and the last step 0% binding buffer. The elution fraction, which was purple and emitted red fluorescence, was combined with one part of the flow-through fraction, which was red and emitted purple fluorescence. This pool was then loaded for SEC. In both runs, the same elution behavior was achieved (**Supplementary material Figs. S8**). AIEX chromatography with a DEAE column previously showed good results in the capture of mCherry, although it was unable to separate mCherry from the impurities; therefore, this approach was tested to concentrate one of the pooled SEC elution fraction, but failed. It is likely that because of the 150 mmol/L NaCl in the SEC elution buffer, the ionic strength was already strong enough to disturb the interaction with the column matrix. The mCherry was distributed in all AIEX fractions according to its fluorescence. Nevertheless, prior to DEAE chromatography, the amount of host proteins could be reduced, even if the protein concentration of the SEC fraction was slightly diluted due to the high volume. These SEC results were repeated and concentrated via ultrafiltration (**Supplementary material Fig. S9**). After concentration, it was again shown that in the elution fraction of mCherry contained, protein impurities with a similar size as mCherry (**Supplementary material Fig. S9**).

3.5. Fine tuning of the first two chromatography steps and addition of a 3rd step

As an adjustment of the protocol, only the elution fraction with red fluorescence from the HIC step was used for the next step. It was concluded that the red part of the flow-through fraction with purple fluorescence emission contained denatured protein. This conclusion is supported by the fact that the reference mCherry sample purified by strep-tag affinity (**Supplementary material Fig. S1**, **A-3 of Supplementary material Figs. S10**) did not exhibit this fluorescence emission, and the purple fluorescence (**Supplementary material Figs. S10C and D**) was only observed in the flow-through during the affinity chromatography procedure. Moreover, as already mentioned in Section 3.2, 2 mol/L $(NH_4)_2SO_4$ precipitation was switched to 2.5% PEG precipitation. With these changes, the protocol was performed without freezing the samples to determine whether the freeze/thaw cycle has an influence on the purification and recovery of the mCherry. In spite of the aforementioned changes and the lack of freezing interruptions, the chromatograms were as expected and the same as the previous results (**Fig. 2A and B**). CBB staining showed that the purity was also improved, after concentration of the SEC elution fraction with ultrafiltration (**Fig. 2C and D**). Moreover, the HIC flow-through fraction with purple fluorescence emission contained more host proteins than the elution fraction with red fluorescence and showed a lower fluorescence response at the expected protein size (**Lanes HIC FT and HIC Elu of Fig. 2C and D**). This result could be repeated in two runs in which the samples were frozen between the chromatography steps (**Supplementary material Figs. S11**). This finding was also clear according to CBB staining and fluorescence imaging (**Supplementary material Fig. S12**).

To address the issue of the main remaining impurities with sizes of approximately 45 kDa and 25 kDa (**Fig. 3**), the strong AIEX Q HP, CHT and HiTrap Heparin HP column approaches were reinvestigated as polishing step, but neither Q HP nor CHT achieved

separation, and both approaches resulted in high loss of the mCherry. On the other hand, heparin shows a high affinity for plasma proteins; therefore, we concluded that our mCherry would most likely not bind and that there would be a greater possibility of binding of the host proteins. As expected, mCherry did not bind to the column, and only one of the remaining host proteins bound to the column (**Supplementary material Fig. S13**). Hence, we successfully repeated this 3-step chromatography protocol and modified the HiTrap Heparin elution procedure from a linear gradient to a one-step gradient and used Strep-tag purification as purification method for comparison based on CBB staining (**Fig. 3A and B, Supplementary material Fig. S1**). **Supplementary material Table S1** shows the results of the densitometry, protein and DNA assay for each step. The final protein and DNA reductions achieved were 99.58 and 99.29%, respectively, but the mCherry recovery was very low, at 1.57%.

3.6. Improved purification protocol with additional thermal treatment

The thermal treatment approach was investigated to further reduce the amount of protein in the chromatography loading because the results of the three-step protocol were not satisfactory compared to those of affinity chromatography. Prior experiments showed that 30 min at 30°C or 40°C was not enough for protein denaturation (data not shown), but at higher temperature, more proteins were denatured and could be removed. During screening with 1 mL of hemolymph, no denatured mCherry could be observed even at 70°C, but during scaling up with 5 mL of hemolymph, the pellet showed a weak pink color; thus, the incubation time was decreased to 20 min at 70°C, which significantly reduced mCherry denaturation. This thermal treatment was incorporated in the previous protocol (**Fig. 4**) and resulted in a significant improvement in purity. Moreover, it was found that the PEG precipitation step could be omitted because there was only a very weak pellet during this step, and the CBB

staining results also showed no visible differences **(Lane P of Fig. 5)**. A similar conclusion was reached regarding the heparin column chromatography step, where the small benefit after the SEC purification for the final purity was not necessary (**Lane He of Fig. 5**). The Butyl column removed the majority of proteins, and SEC served as a polishing step. Utilizing densitometry on the respectively 35.4 kDa protein band, we could roughly calculate the mCherry recovery at each step (**Table 1**). Three different dilutions of mCherry purified with the StrepTactin column were used as standards for mCherry quantification. With our improved 3-step protocol, we achieved an overall recovery of 5.78%, and with Strep-tag purification, recovery of 4.09% was achieved, as based on the CBB band intensity at 35 kDa from the crude sample as base (**Lane H of Fig. 5**). Additionally, it seemed that the 3-step protocol recovery was quite high because the recovery of the previous protocol with one fewer step was 1.57%, and that from affinity purification was only 4.09%. In **Table 1,** we summarize the purification results. Moreover, we suspect that the HIC step can still be further improved regarding the sample loading amount and, thus, the recovery. We hypothesize that the column was overloaded with mCherry because mCherry was still present in the flow-through fraction, but we decided not to reduce the loading amount to ensure that there was a relatively high concentration in the elution fraction for the following steps. As a side note, with our 3-step protocol, we achieved a similar DNA reduction to the control method, at over 99%, and the protein content was reduced by over 99.98% and rather 99.95% (**Table 1**).

3.7. Purity comparison using capillary electrophoresis

Using capillary sodium dodecyl sulfate-electrophoresis (SDS-CE), we analyzed the purity of the purified samples. The achieved purity under the protocol without thermal treatment was 11% with densitometry and 14.86% with CE, which was very low (**Supplementary material Fig. S14**). Affinity tag purification achieved a purity of 100% with densitometry, and 63.69%

with CE (**Fig. 6A**). In this study, the thermal treatment protocol resulted in a purity of approximately 85.45% and 43.60% when using densitometry and CE, respectively (**Fig. 6B**). This clearly shows the benefit of the thermal treatment and the superiority of CE for purity analysis. An internal standard was used to indicate molecular weight; the CE resulted in a method depending different molecular mass than the theoretical mass of approximately 34.5 kDa, which was also determined by SDS-PAGE. Therefore, the (apparent) molecular weight for affinity purification was determined to be 40.8 kDa, and for our protocol, it was determined to be 43 kDa. This size difference is currently the subject of further investigations.

4. Conclusion and outlook

We significantly reduced the host cell proteins during the purification of mCherry from silkworm larval hemolymph and successfully purified mCherry with a upscalable protocol, which was successfully repeated. Because of the low molecular mass of the protein, we could apply strong centrifugation forces and achieve good separation from cell debris and a decrease in high molecular weight proteins. The 70°C thermal treatment had the greatest impact on reducing the host cell proteins without noticeable mCherry loss. Following HIC and SEC for further separation, we were able to purify the target recombinant protein to a high degree, even if some minor traces of host proteins remained. One of these protein groups could be further removed by using a heparin column in flow-through mode. During the chromatography experiments, it was obvious that the separation of the host proteins was difficult. In particular, the main impurities observed at 60–80 kDa seem to show ambivalent elution behavior. They bound to the column during HIC but were also present in the flow-through. Moreover, the clear binding behavior could be reversed when a different recombinant protein was purified (data not published; data not shown). During the purification process, the hemolymph batch also played a crucial role, especially regarding the

amount of preservative used. When a greater amount of preservative was used, more proteins were visibly denatured as was the target protein, which affected its recovery very negatively.

In conclusion, several different pretreatments and chromatography strategies were investigated, and only some of these approaches had an acceptable impact on the purification of our target protein (**Supplementary material Fig. S15**). Through heat treatment, the host cell proteins were significantly reduced. Furthermore, the use of 2 – 3 SEC columns in a row or a better SEC column in the 2nd chromatography step, should significantly improve the target protein separation and resolution. We had to introduce heat treatment after purification with our columns, and the precipitation methods did not achieve a sufficient reduction in the unwanted proteins. This led to a significant protein reduction but also limits the use of this protocol to partially heat-stable proteins. On the other hand, we showed that a temperature of 50°C is sufficient to denature large amounts of silkworm proteins. In conclusion, we demonstrated the possibility of effectively purifying thermally stable proteins from the silkworm larval hemolymph using methods that can easily be upscaled for industrial purpose.

Declaration of Interest

All authors declare that they have no competing interests.

Acknowledgements

This work was financially supported by the Japan Society for the Promotion of Science (JSPS) (KAKENHI A, 16H02544). Maurice and the required reagents for the CE-SDS analysis were kindly provided by ProteinSimple.

Appendix A. Supplementary material

Supplementary data for this article can be found online at https://doi.org/ .

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Table 1. Summary of purification with and without thermal treatment compared to affinity tag purification.

Sample	Volume [ml]	mCherry conc [mg/ml]	mCherry amount [mg]	mCherry recovery [%]	Protein amount [mg]	Protein reduction [%]	DNA amount [mg]	DNA reduction [%]
Affinity chromatography								
Hemolymph	5	0.90	4.5	---	395.7	---	411.7	---
Loading	4.67	0.86	4.0	89.62	313.0	20.91	304.8	25.96
Elution	1.5	0.12	0.2	4.09	0.2	99.95	2.0	99.52
Three-step purification								
Hemolymph	6	0.54	3.3	---	434.8	---	147.8	---
Centrifugation supernatant	5.77	0.44	2.5	78.04	375.2	13.69	127.9	13.46
70°C supernatant	5	0.39	2.0	60.26	225.5	48.13	88.2	31.04
PEG precipitation supernatant	5.41	0.35	1.9	58.59	223.4	48.63	97.6	23.69
0.45 µm Filtered	4.8	0.35	1.7	52.20	166.4	61.74	105.6	17.49
Butyl elution	6.1	0.06	0.4	11.46	13.7	96.86	10.4	91.85
SEC elution conc.	2.75	0.08	0.2	6.75	1.1	99.75	3.8	97.02
Heparin Flow-through conc.	1.5	0.13	0.2	5.78	0.7	99.84	2.0	98.41
Heparin elution fr. 7 conc.	0.98	Not possible	Not possible	Not possible	0.1	99.98	1.2	99.03
Three-step purification without heat treatment								
Heparin flow-through conc.	1.7	0.04	0.07	1.57	1.4	99.58	3.3	99.29

Figure legends

Fig. 1. Size exclusion of mCherry in a Superdex Sephacryl S-200 column with 10 mmol/L Tris, 150 mmol/L NaCl, pH 8. Chromatogram (A), Coomassie blue-stained SDS-PAGE gel (B) of the fractions in comparison with the fluorescent image (C). In each lane, 15 µL was loaded. For SDS-PAGE, 10 µL of sample was diluted to 22 µL, and for the fluorescence image, 12 µL was diluted to 24 µL. The program was as follows: 1 mL elution buffer, 5.2 mL loading buffer, and 121 mL elution buffer. The flow rate was 0.5 mL/min. The number indicates the fraction number, which had a volume of up to up to 3 mL, and the collection threshold was 0.075 AU; M; marker; 0.45: 0.45 µm filtration of the pooled HisTrap flow-through pool; black boxes indicate mCherry fluorescence.

Fig. 2. Results of the two-step chromatography protocol for mCherry purification, involving 2 continuous (A) HIC steps for mCherry on a Butyl FF column with 10 mmol/L Tris, pH 8 + 3 mol/L NaCl. Elution was performed with a two-step gradient, where the first step involved 100% and the second step 0% binding buffer. The sample was adjusted with an additional 3 mol/L NaCl. The program was as follows: 2 mL binding buffer, 5.2 mL sample load with binding buffer, 7 CVs binding buffer and 5 CVs elution buffer. The flow rate was 2 mL/min, and the loading rate was 1 mL/min. The fractions were collected at volumes up to 2 mL with a threshold of 0.075 AU. (B) Size exclusion of mCherry on a Superdex Sephacryl S-200 column with 10 mmol/L Tris, 150 mmol/L NaCl, pH 8. The program was as follows: 1 mL elution buffer, 5.2 mL sample load with binding buffer, and 121 mL elution buffer. The flow rate was 0.5 mL/min. Fractions were collected at volumes of up to 3 mL with a threshold of 0.075 AU. The elution fraction from HIC chromatography was loaded. (C) Coomassie blue-stained SDS-PAGE gel of the fractions in comparison with (D) a fluorescence image. In each lane, 10 µL was loaded. For SDS-PAGE, 8 µL of the sample was diluted to 22 µL, and for the fluorescence image, 10 µL was diluted to 24 µL. Hc: crude hemolymph; Cs: 17,800 × g, 10

min centrifugation of the supernatant; Pp: 2.5% PEG precipitation pellet; M: marker; 0.8: 0.8 µm filtration of the precipitation supernatant; HIC FT: HIC red flow-through fraction; HIC Elu: HIC elution fraction with red fluorescence; SEC conc.: SEC pool concentrated with Amicon 3K; Ref: reference mCherry purified with affinity chromatography and SEC; arrows indicate mCherry; black box indicates mCherry fluorescence.

Fig. 3. Coomassie blue-stained SDS-PAGE gel of mCherry purified using Strep-Tactin affinity (A) and mCherry purified using a 3-step protocol (B). Both gels also contain three standards used for the calculation of recovery via densitometry. An 8 µL sample was diluted to 22 µL. In each lane, 15 µL was loaded. Lane S1: Standard with no dilution (same as aE); Lane S2: standard at a 1:1 dilution; S3: standard at a 1:4 dilution; Lane aH: hemolymph used for affinity purification, 1:20; Lane aL: sample loaded onto the affinity column after centrifugation at 17,800 g, 5 min, 1:20; Lane aE: elution fraction of the Strep-tag affinity purification; Lane H: hemolymph, 1:20; Lane C: centrifugation supernatant, 1:20; Lane P: supernatant from PEG precipitation, 1:20; Lane F: 0.8 µm filtration, 1:20, sample loaded for HIC; Lane Sl: sample loaded for SEC; Lane Hl: sample loaded onto the heparin column; He: Amicon 3K-concentrated heparin elution pool; M: marker; black arrows indicate the target protein; black boxes indicate major remaining impurities or their reduction.

Fig. 4. A short summary of the final 3-step chromatography purification protocol including the pretreatment. The PEG 6000 precipitation step can be omitted, and if it is not necessary, also the refinement step with the heparin column.

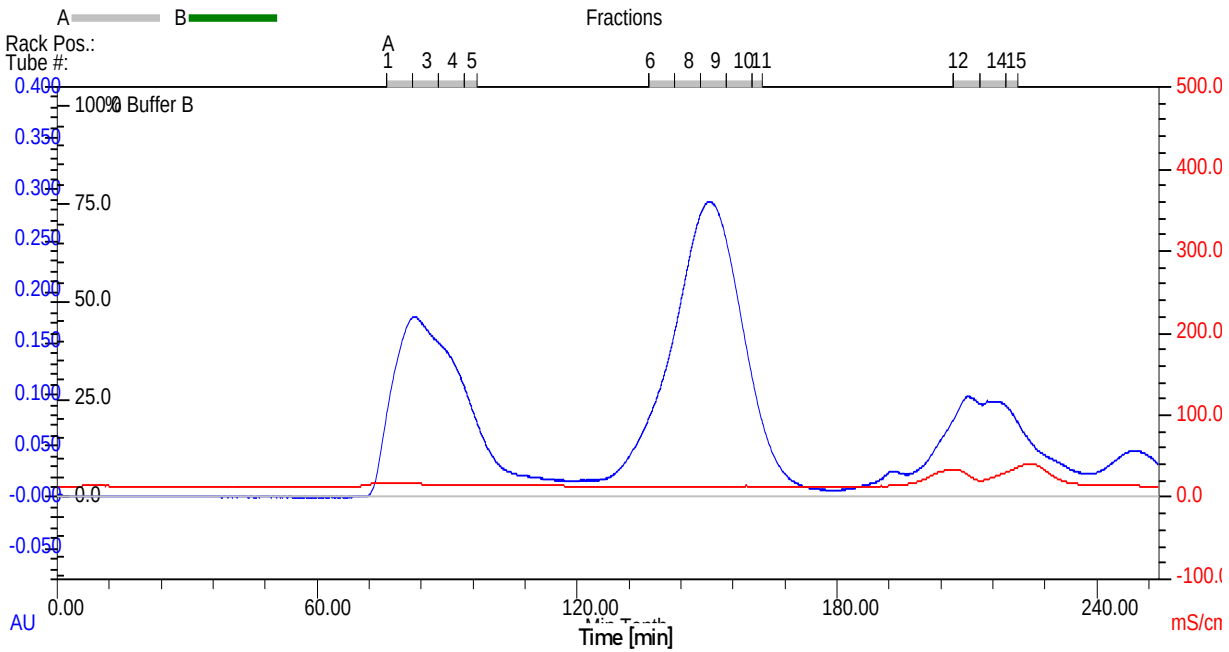
Fig. 5. Coomassie blue-stained SDS-PAGE gel image of mCherry purified using a 3-step protocol with heat precipitation. Each lane shows the effect of the previous purification step and is itself the loading fraction for the next step. The gel also contains three standards used for the calculation of recovery via densitometry. An 8 µL sample was diluted to 22 µL. In each lane, 15 µL was loaded. Fluorescence color development during purification is shown:

Hemolymph (H); filtrate (F); final heparin flow-through fraction (He). Lane S1: standard with no dilution (same as aE); Lane S2: standard in a 1:1 dilution; S3: standard in a 1:4 dilution; Lane H: hemolymph, 1:20; Lane C: centrifugation supernatant, 1:20; Lane 70: supernatant after 70°C treatment followed by centrifugation, 1:20; Lane P: supernatant from PEG precipitation, 1:20; Lane F: 0.8 μm filtration, 1:20, sample loaded for HIC; Lane Sl: sample loaded for SEC; Lane Hl: sample loaded onto the heparin column; He: Concentrated heparin elution pool with Amicon 3K; M: marker; Black arrow indicate the target protein; heparin FT (He) is also more highly concentrated than the SEC fraction (Hl).

Fig. 6. Results of CE-SDS analysis. Overlay of the three electropherograms from the purification protocols. (A) Purification with the Strep-tag affinity column. (B) Purification using our proposed protocol with thermal treatment. The target protein mCherry is located at the 40.8 or 43 relative migration time and is indicated by the arrow. IS = internal standard at a relative migration time of approximately 9.98.

Fig. 1, Minkner et al.

A



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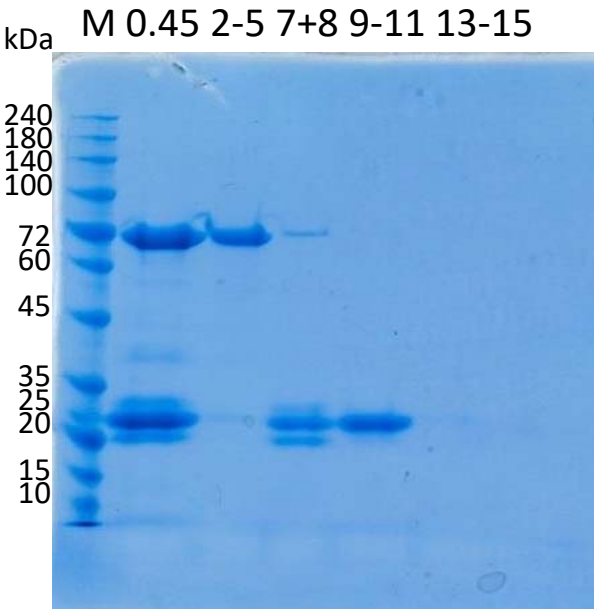

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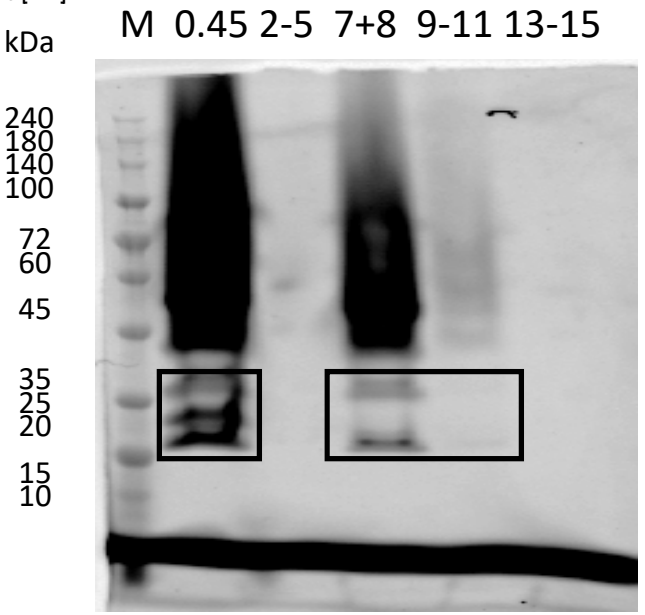
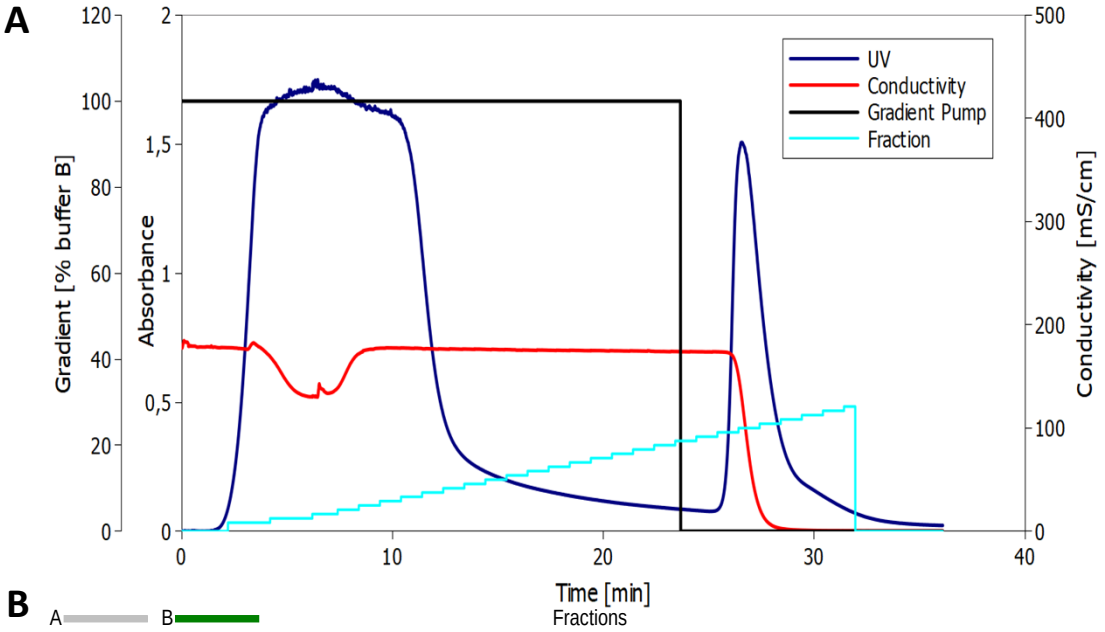
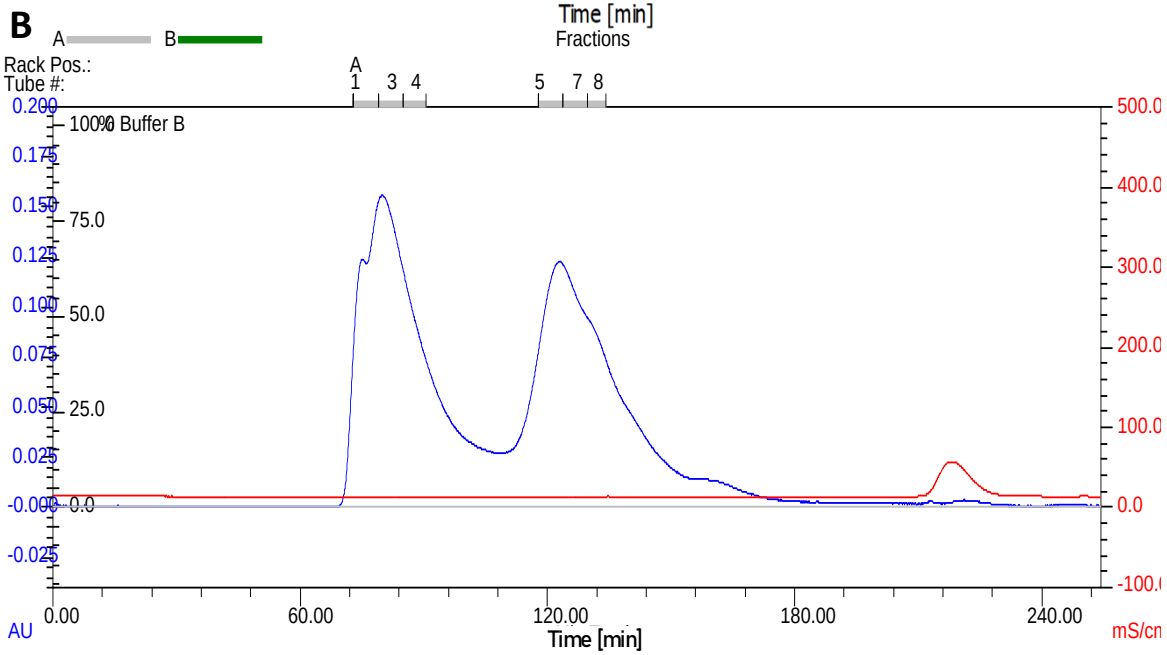
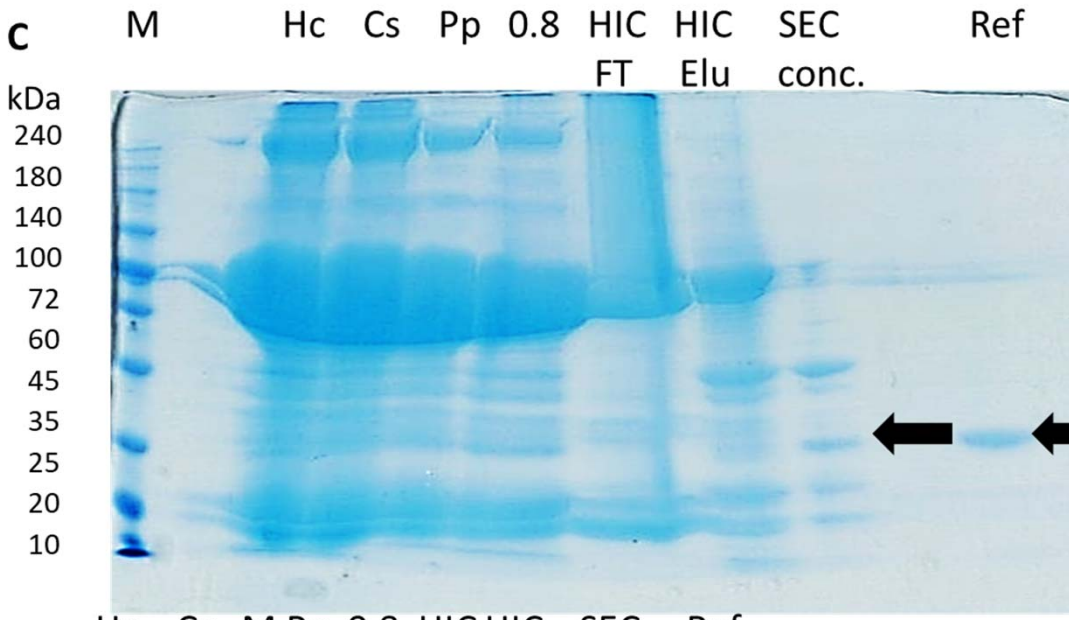


Fig. 2, Minkner et al.







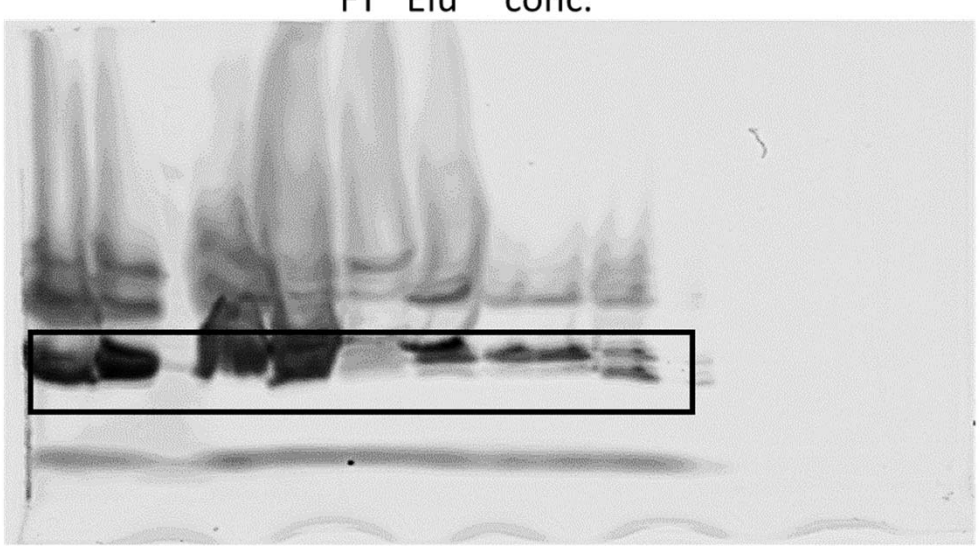


Fig. 3: Minkner et al.

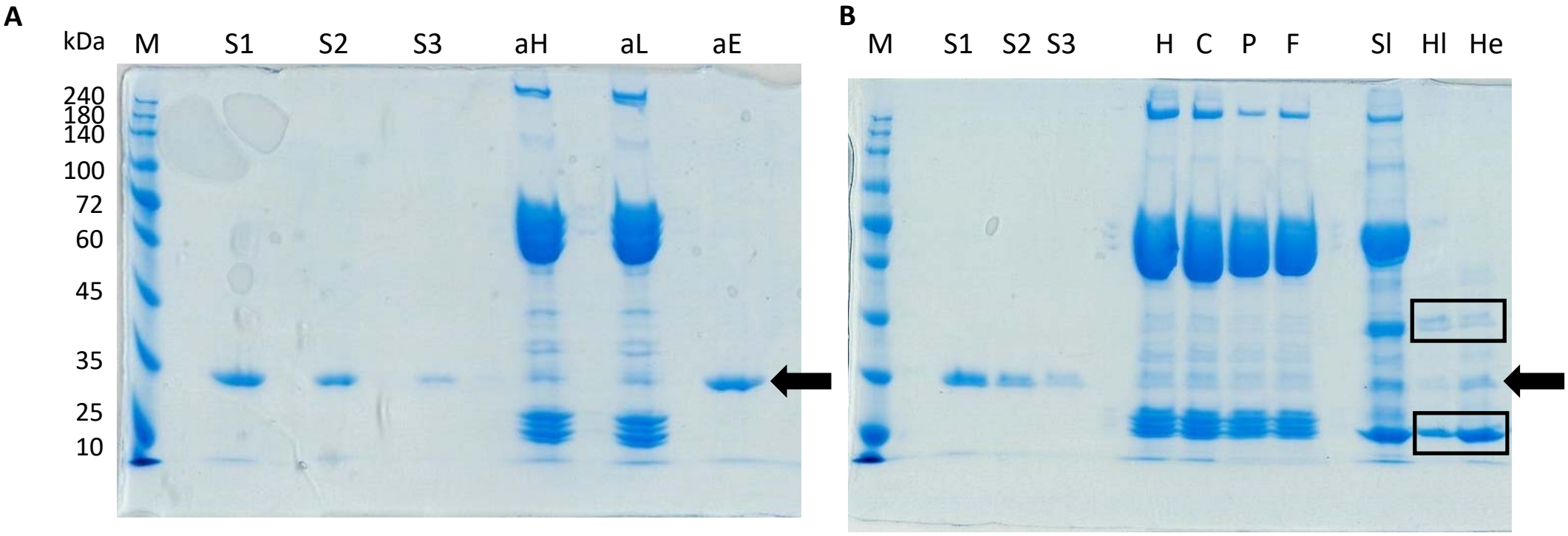
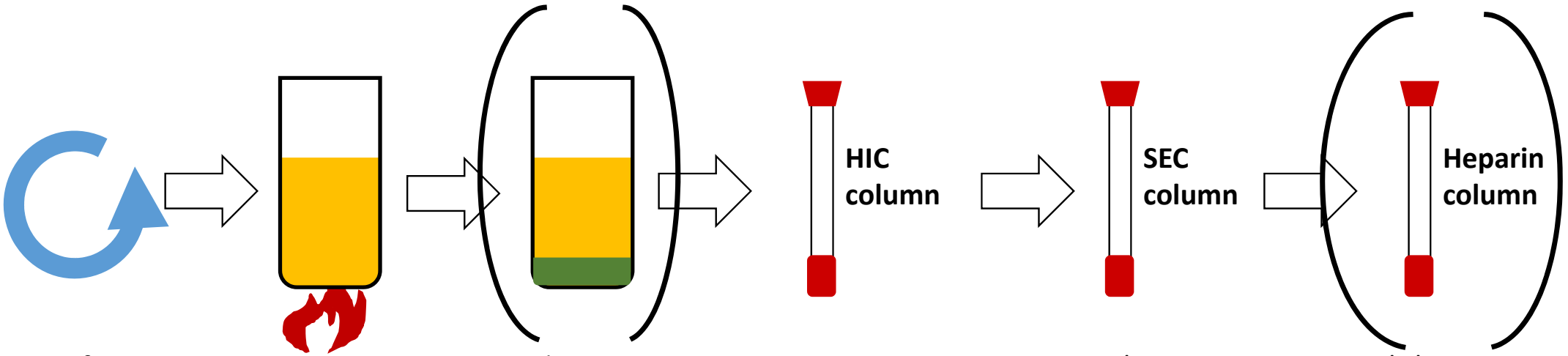


Fig. 4: Minkner et al.



Centrifugation
17800 x g, 10 min

Heat treatment
70°C, 20 min

2.5 % PEG 6000
precipitation

Capturing step
HiTrap Butyl FF
2 step gradient
with 3 mol/L NaCl

Intermediate step
Superdex Sephacryl S-200
120 ml

Polishing step
HiTrap Heparin HP
Flow-through mode

Fig. 5: Minkner et al.

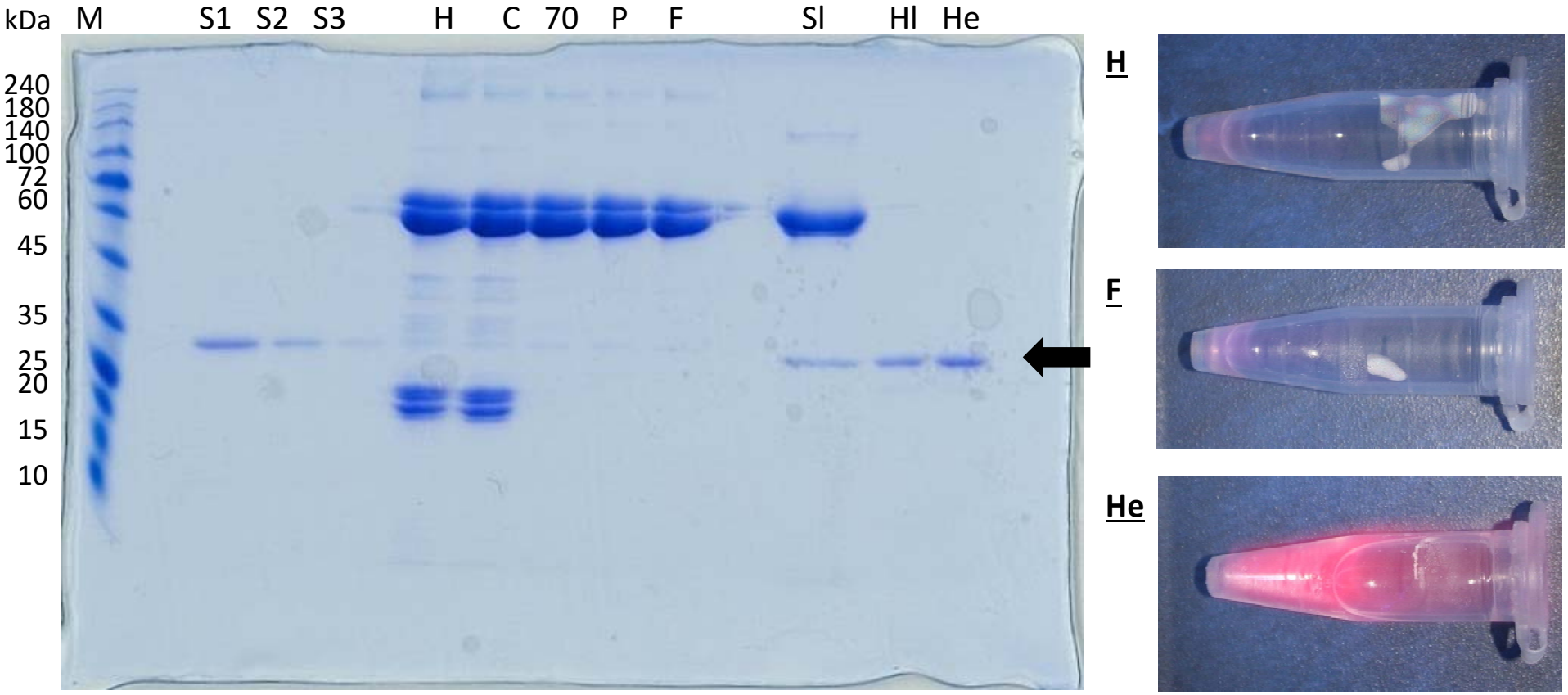
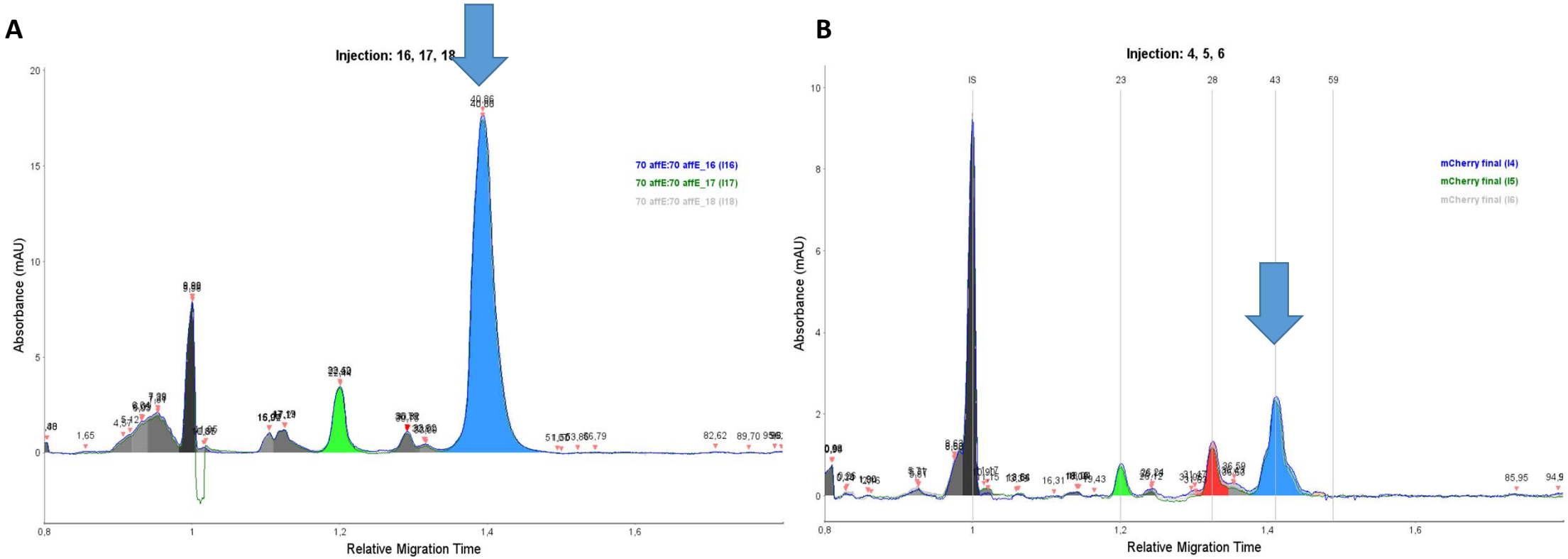


Fig. 6: Minkner et al.



Supplementary file for

A systematic and methodical approach for the efficient purification of recombinant protein from silkworm larval hemolymph

Short running title: Purification of recombinant protein expressed in silkworm

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Table S1: This table shows the results of the mCherry protocol without the thermal treatment

Sample	Volume [ml]	mCherry conc [mg/ml]	mCherry amount [mg]	mCherry recovery [%]	Protein amount [mg]	Protein reduction [%]	DNA amount [mg]	DNA reduction [%]
Affinity chromatography								
Affinity Haemolymph	5	0.90	4.49	---	395.7	---	411.7	---
Affinity Load	4.67	0.86	4.02	89.62	313.0	20.91	304.8	25.96
Affinity Elution	1.5	0.12	0.18	4.09	0.2	99.95	2.0	99.52
Three-step protocol								
Haemolymph	5	0.88	4.42	---	329.9	---	463.6	---
Centrifugation supern.	4.25	0.77	3.27	74.09	283.5	14.06	325.1	29.88
PEG preci supernatant	4.82	0.49	2.38	53.88	260.5	21.03	250.7	45.93
Filtered (0.8 μm)	4	0.56	2.25	51.03	195.3	40.79	252.2	45.61
SEC load	3.2	0.10	0.32	7.25	9.0	97.28	20.3	95.63
Heparin load	9	0.02	0.14	3.10	2.8	99.16	11.2	97.58
Heparin elution (FT) conc.	1.7	0.04	0.07	1.57	1.4	99.58	3.3	99.29

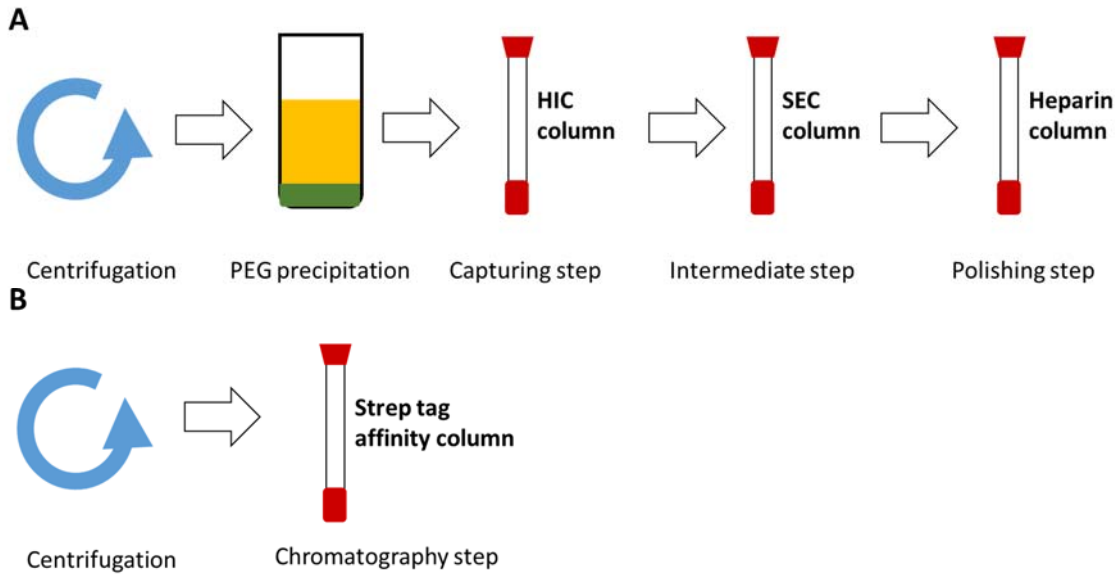

Fig. S1: A short summary of the intended purification protocols. **(A)** The original protocol without thermal heat treatment. **(B)** The Strep-tag affinity purification which was used as comparison method.

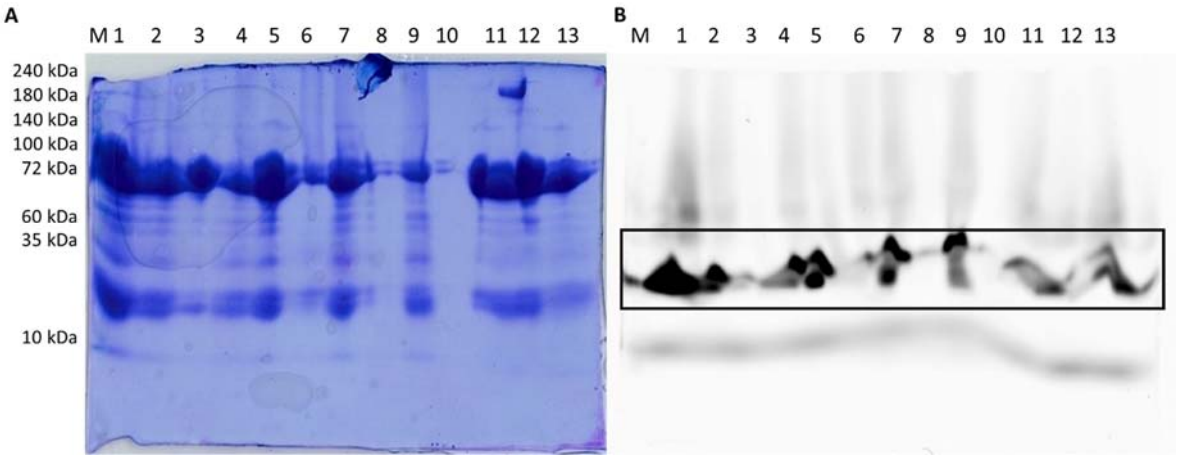

Fig. S2: Pre-treatment precipitations 1. Coomassie blue staining SDS-gel **(A)** with mCherry precipitations in comparison with fluorescence Image **(B)**. On each line 15 µl was loaded. On the SDS-PAGE it was 10 µl sample to 22 µl dilution and on the Fluorescence image it was 12 µl to 24 µl dilution. Lane 1: centrifugation supernatant 17800 g, 10 min; Lane 2: failed precipitation 1 M $(NH_4)_2SO_4$; Lane 3 and 4: pellet and supernatant 2 mol/l $(NH_4)_2SO_4$; Lane 5 and 6: pellet and supernatant 3 mol/l $(NH_4)_2SO_4$; Lane 7 and 8: pellet and supernatant 4 mol/l $(NH_4)_2SO_4$; Lane 9 and 10: pellet and supernatant 5 mol/l $(NH_4)_2SO_4$; Lane 11: failed 2.5 % PEG precipitation; Lane 12 and 13: pellet and supernatant 5 % PEG; M is Marker; black box indicates mCherry fluorescence.

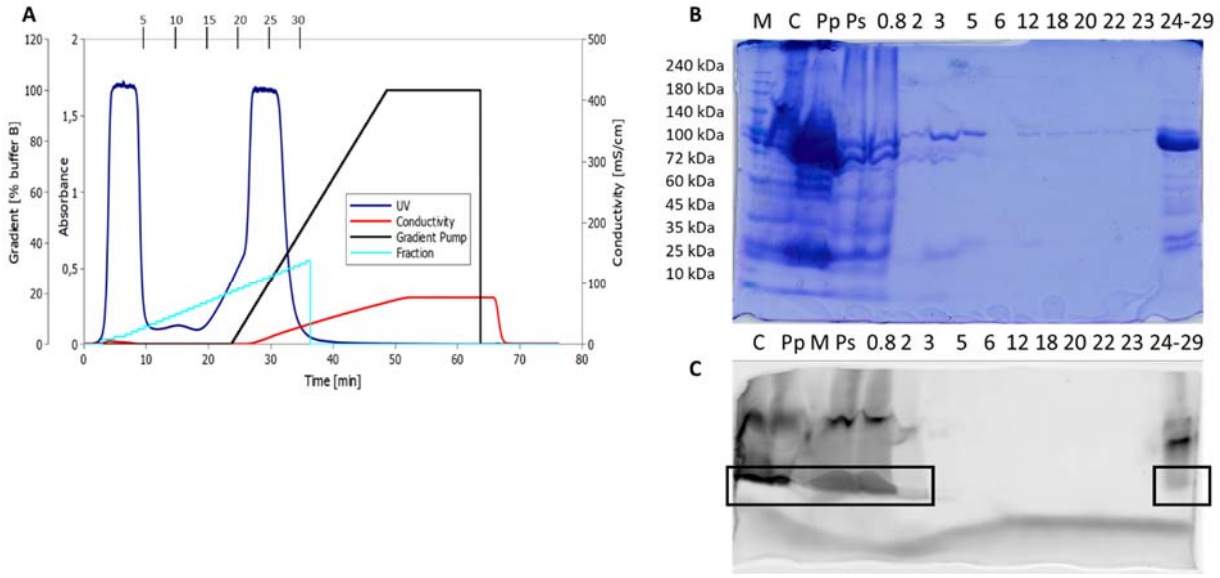

Fig. S3: AIEX of mCherry on DEAE with 10 mM Tris, pH 8. Elution was done with up to 1 mol/l NaCl **(A)**. Coomassie blue staining SDS-gel **(B)** of the fractions in comparison with fluorescence Image **(C)**. On each line 15 µl was loaded. On the SDS-PAGE it was 10 µl sample to 22 µl dilution and on the Fluorescence image it was 12 µl to 24 µl dilution. The program was 2 ml binding buffer, 5.2 ml load, 7 CV binding buffer, 10 CV linear gradient up to 100 % elution buffer, 6 CV elution buffer and 5 CV binding buffer. Flow rate was 2 ml/min and for load 1 ml/min. The number indicates fraction number, which is up to 2 ml and collection threshold was 0.05 AU; M is Marker; C: supernatant of centrifugation; Pp: 5 % PEG precipitation; Ps: precipitation supernatant; 0.8: 0.8 µm filtration of supernatant; black boxes indicates mCherry fluorescence.

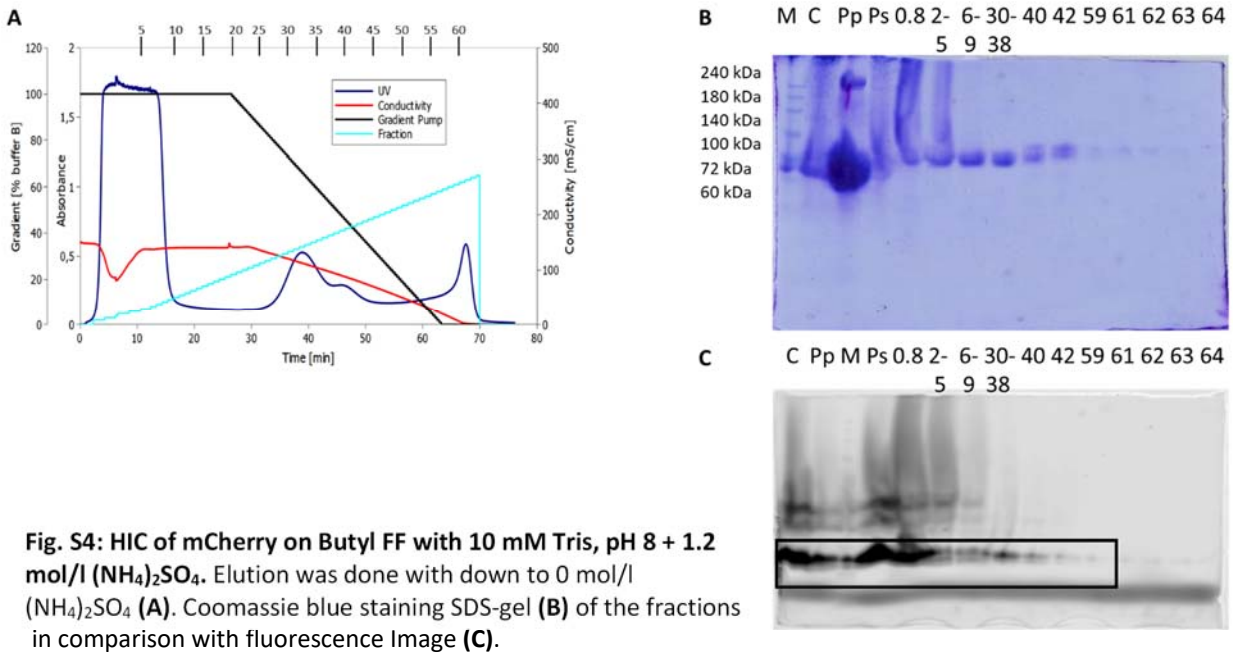

Fig. S4: HIC of mCherry on Butyl FF with 10 mM Tris, pH 8 + 1.2 mol/l $(NH_4)_2SO_4$. Elution was done with down to 0 mol/l $(NH_4)_2SO_4$ **(A)**. Coomassie blue staining SDS-gel **(B)** of the fractions in comparison with fluorescence Image **(C)**.
On each line 15 µl was loaded. On the SDS-PAGE it was 10 µl sample to 22 µl dilution and on the Fluorescence image it was 12 µl to 24 µl dilution. The sample was adjusted with additional 1.2 mol/l $(NH_4)_2SO_4$. The program was 2 ml binding buffer, 5.2 ml load, 7 CV binding buffer, 15 CV linear gradient up to 100 % elution buffer, 5 CV elution buffer. Flow rate was 2 ml/min and for load 1 ml/min. The number indicates fraction number, which is up to 2 ml and collection threshold was 0.05 AU; M is Marker; C: supernatant of centrifugation; Pp: 2.5 % PEG precipitation; Ps: precipitation supernatant; 0.8: 0.8 µm filtration of supernatant; black box indicates mCherry fluorescence.

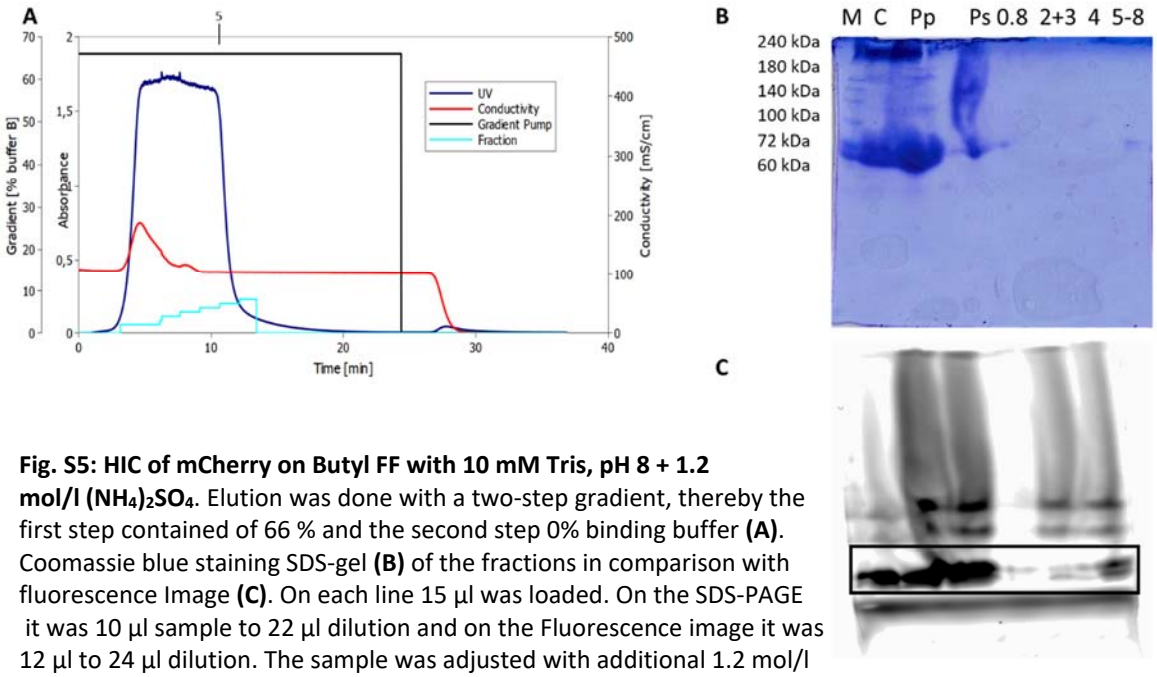

Fig. S5: HIC of mCherry on Butyl FF with 10 mM Tris, pH 8 + 1.2 mol/l $(NH_4)_2SO_4$. Elution was done with a two-step gradient, thereby the first step contained of 66 % and the second step 0% binding buffer **(A)**. Coomassie blue staining SDS-gel **(B)** of the fractions in comparison with fluorescence Image **(C)**. On each line 15 µl was loaded. On the SDS-PAGE it was 10 µl sample to 22 µl dilution and on the Fluorescence image it was 12 µl to 24 µl dilution. The sample was adjusted with additional 1.2 mol/l $(NH_4)_2SO_4$. The program was 2 ml 66 % binding buffer, 5.2 ml load, 7 CV 66 % binding buffer and 5 CV elution buffer. Flow rate was 2 ml/min and for load 1 ml/min. The number indicates fraction number, which is up to 3 ml and collection threshold was 0.1 AU; M is Marker; C: supernatant of centrifugation; Pp: 2 mol/l $(NH_4)_2SO_4$ precipitation; Ps: precipitation supernatant; 0.8: 0.8 µm filtration of supernatant; black box indicates mCherry fluorescence.

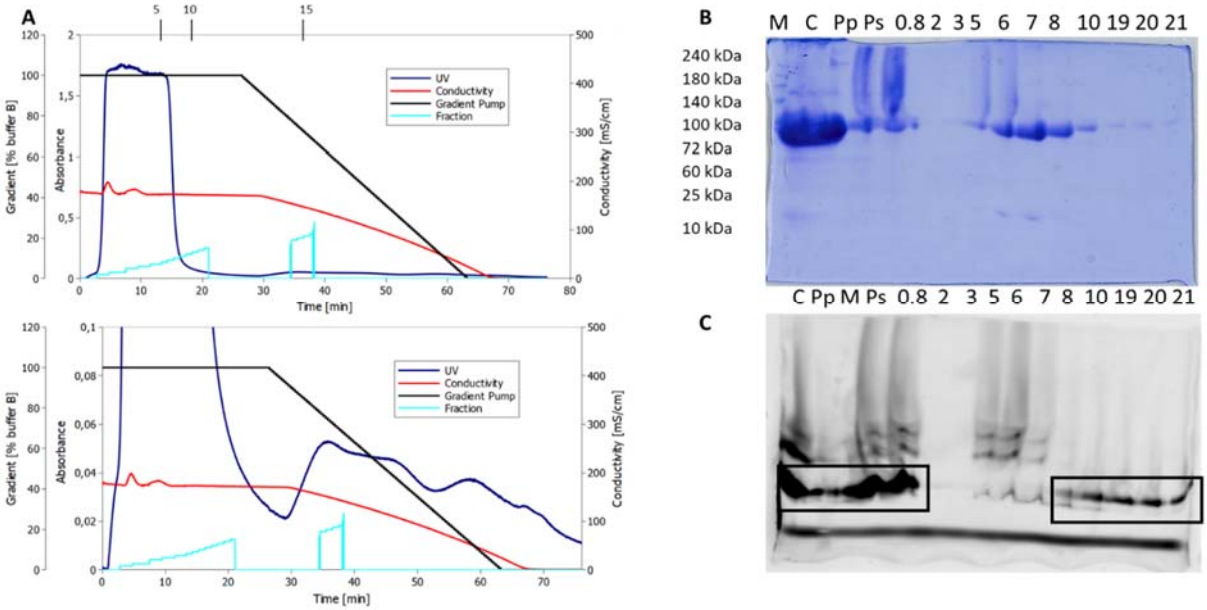

Fig. S6: HIC of mCherry on Butyl FF with 10 mM Tris, pH 8 + 3 mol/l NaCl. Elution was done with down to 0 mol/l NaCl. A zoomed in version for the elution fraction is also shown **(A)**. Coomassie blue staining SDS-gel **(B)** of the fractions in comparison with fluorescence Image **(C)**. On each line 15 µl was loaded. On the SDS-PAGE it was 10 µl sample to 22 µl dilution and on the Fluorescence image it was 12 µl to 24 µl dilution. The sample was adjusted with additional 3 mol/l NaCl. The program was 2 ml binding buffer, 5.2 ml load, 7 CV binding buffer, 15 CV linear gradient up to 100 % elution buffer, 5 CV elution buffer. Flow rate was 2 ml/min and for load 1 ml/min. The number indicates fraction number, which is up to 2 ml and collection threshold was 0.05 AU; M is Marker; C: supernatant of centrifugation; Pp: 2 mol/l $(NH_4)_2SO_4$ precipitation; Ps: precipitation supernatant; 0.8: 0.8 µm filtration of supernatant; black boxes indicates mCherry fluorescence.

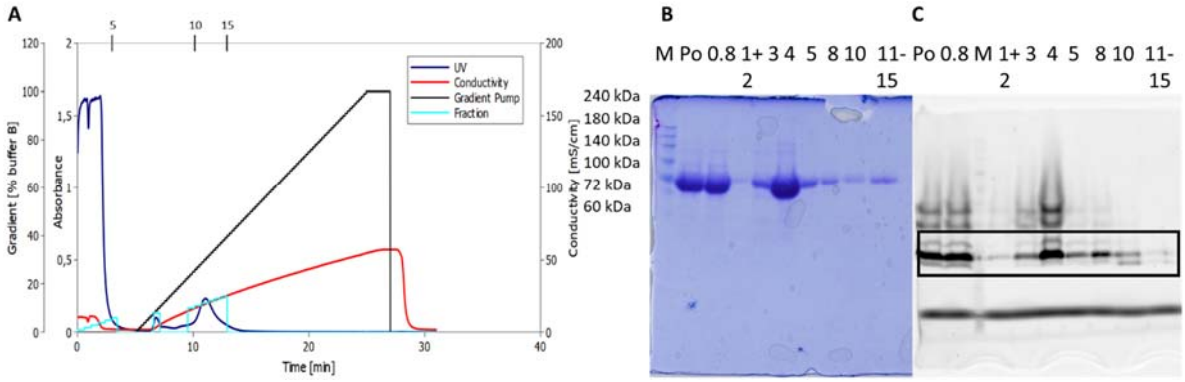

Fig. S7: CHT chromatography of mCherry with 8 mmol/l KH_2PO_4, pH 8. Elution was done with up to 600 mmol/l KH_2PO_4, pH 8 **(A)**. Coomassie blue staining SDS-gel **(B)** of the fractions in comparison with fluorescence Image **(C)**. On each line 15 µl was loaded. On the SDS-PAGE it was 10 µl sample to 22 µl dilution and on the Fluorescence image it was 12 µl to 24 µl dilution. The program was 5 ml load, 4 CV binding buffer, 20 CV linear gradient up to 100 % elution buffer, 2 CV elution buffer. Flow rate was 5 ml/min. The number indicates fraction number, which is up to 3 ml and collection threshold was 0.05 AU; M is Marker; Po: DEAE pool for load; 0.8: 0.8 µm filtration; black box indicates mCherry fluorescence.

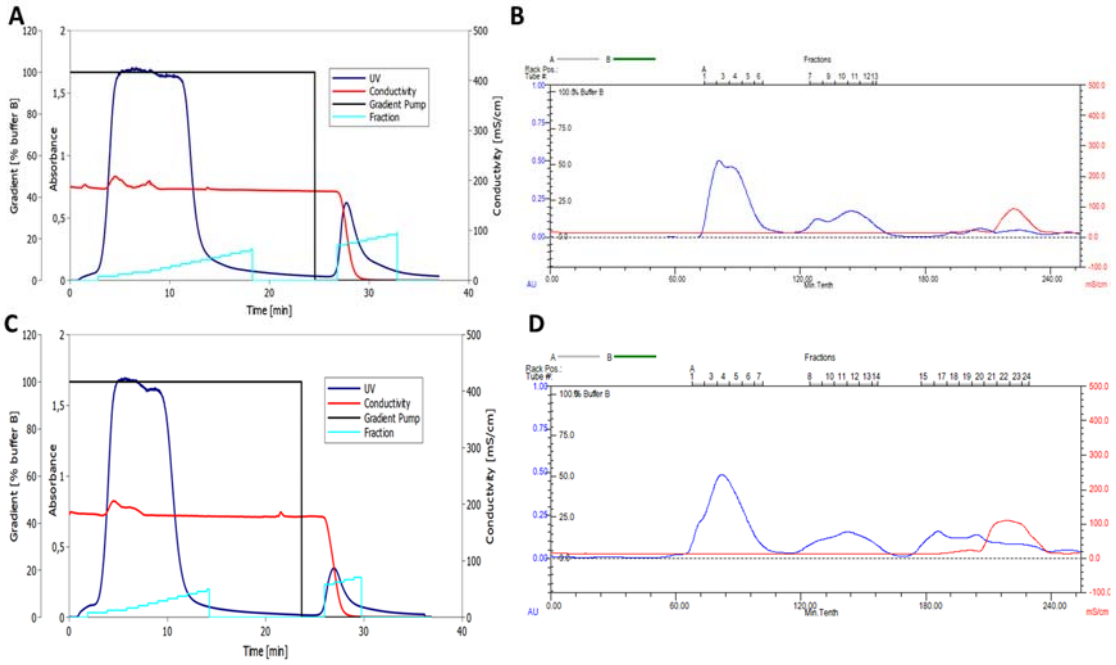

Fig. S8: Two step chromatography protocol for mCherry purification (A+C) HIC of mCherry on Butyl FF with 10 mM Tris, pH 8 + 3 mol/l NaCl. Elution was done with a two-step gradient, thereby the first step contained of 100 % and the second step 0% binding buffer. The sample was adjusted with additional 3 mol/l NaCl. The program was 2 ml binding buffer, 5.2 ml load, 7 CV binding buffer and 5 CV elution buffer. Flow rate was 2 ml/min and for load 1 ml/min. Fraction were collected up to 2 ml with a threshold of 0.075 AU **(B+D)** Size exclusion of mCherry on Superdex Sephacryl S-200 with 10 mmol/l Tris, 150 mmol/l NaCl, pH 8. The program was 1 ml elution buffer, 5.2 ml load and 121 ml elution buffer. Flow rate was 0.5 ml/min. Fraction were collected up to 3 ml with a threshold of 0.075 AU. Loaded was the elution fraction of the HIC chromatography pooled together with one flow through fraction which had a strong red colour. **(A+B)** are Run 1 and **(C+D)** are Run 2.

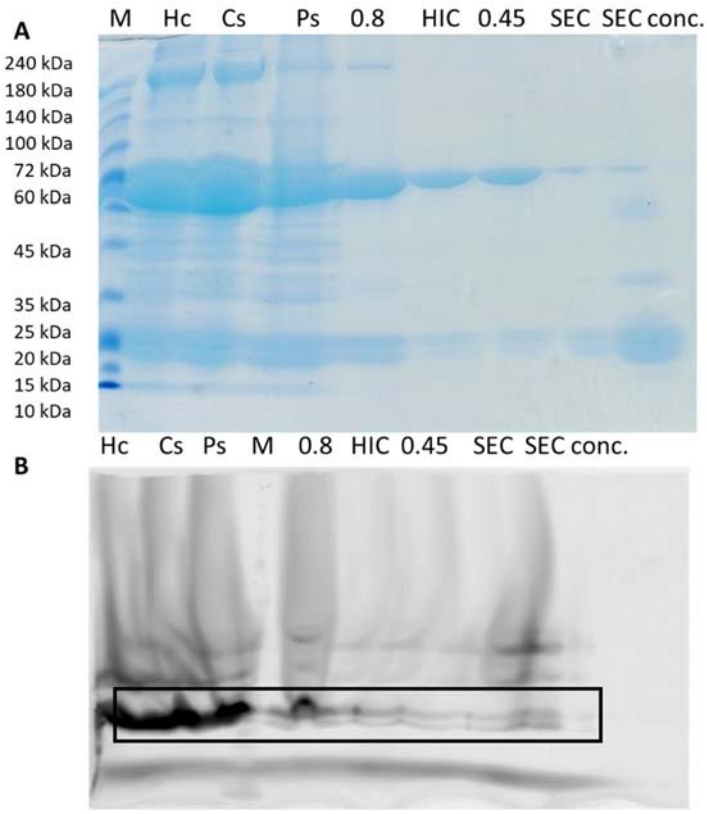

Fig. S9: Results of the two-step chromatography protocol for mCherry purification concentrated with ultracentrifugation. Coomassie blue staining SDS-gel **(A)** of the fractions in comparison with fluorescence Image **(B)**. On each line 15 µl was loaded. On the SDS-PAGE it was up to '0.45' 2 µl sample was diluted to 22 µl, after this 8 µl to 22 µl. On the Fluorescence image it was 12 µl to 24 µl dilution. Hc: crude haemolymph; Cs: 17800 g, 10 min centrifugation supernatant; Ps: 2 mol/l $(NH_4)_2SO_4$ precipitation supernatant; M is Marker; 0.8: 0.8 µm filtration of supernatant; HIC: pooled HIC fraction; 0.45: 0.45 µm filtration; SEC: SEC pool; SEC conc.: SEC pool concentrated with Amicon 30K; black box indicates

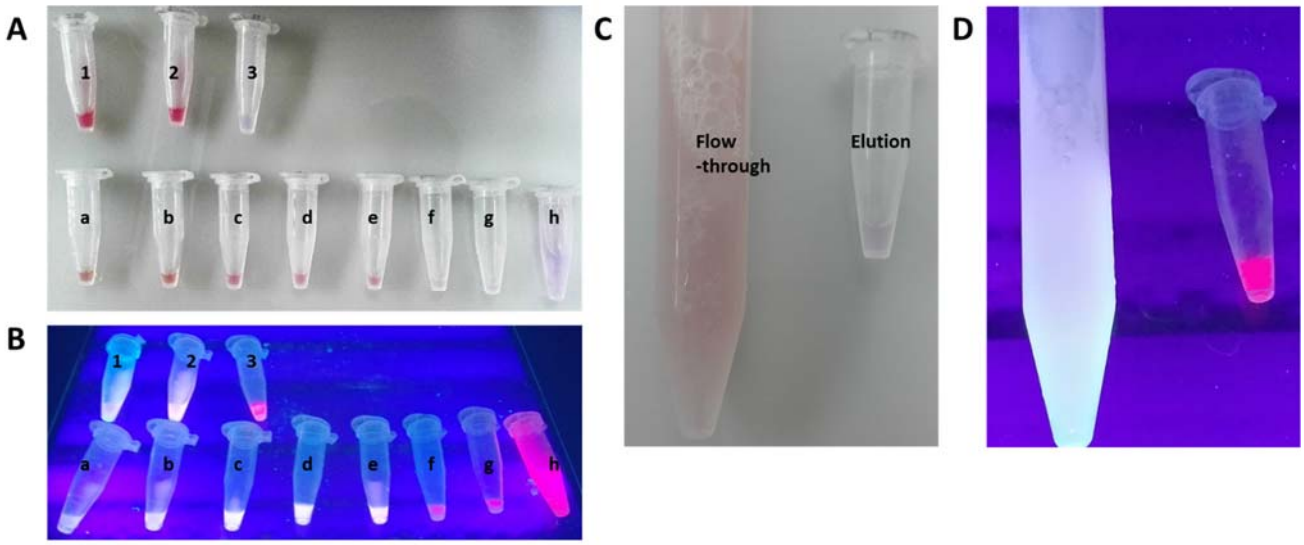

Fig. S10: Oversight about the colour and fluorescence changes during the Strep-ag purification and the heat treatment protocol. (A) shows the colour of each purification step and **(B)** the corresponding fluorescence. It's becomes clear that the red colour is associated with the purple fluorescence and the purple colour with the typical red mCherry fluorescence. 1: haemolymph; 2: Load; 3: Strep-tag affinity elution; a: haemolymph; b: centrifugation supernatant; c: 70°C supernatant; d: PEG precipitation supernatant; e: filtration; f: Butyl elution fraction; g: SEC elution fraction concentrated; h: Heparin elution fraction concentrated. **(C)** is the direct colour comparison the flow-through and the elution of the Strep-tag affinity purification, whereby **(D)** is the direct fluorescence comparison and this also shows that the purple colour is associated with the red fluorescence and the red colour with a weak purple fluorescence.

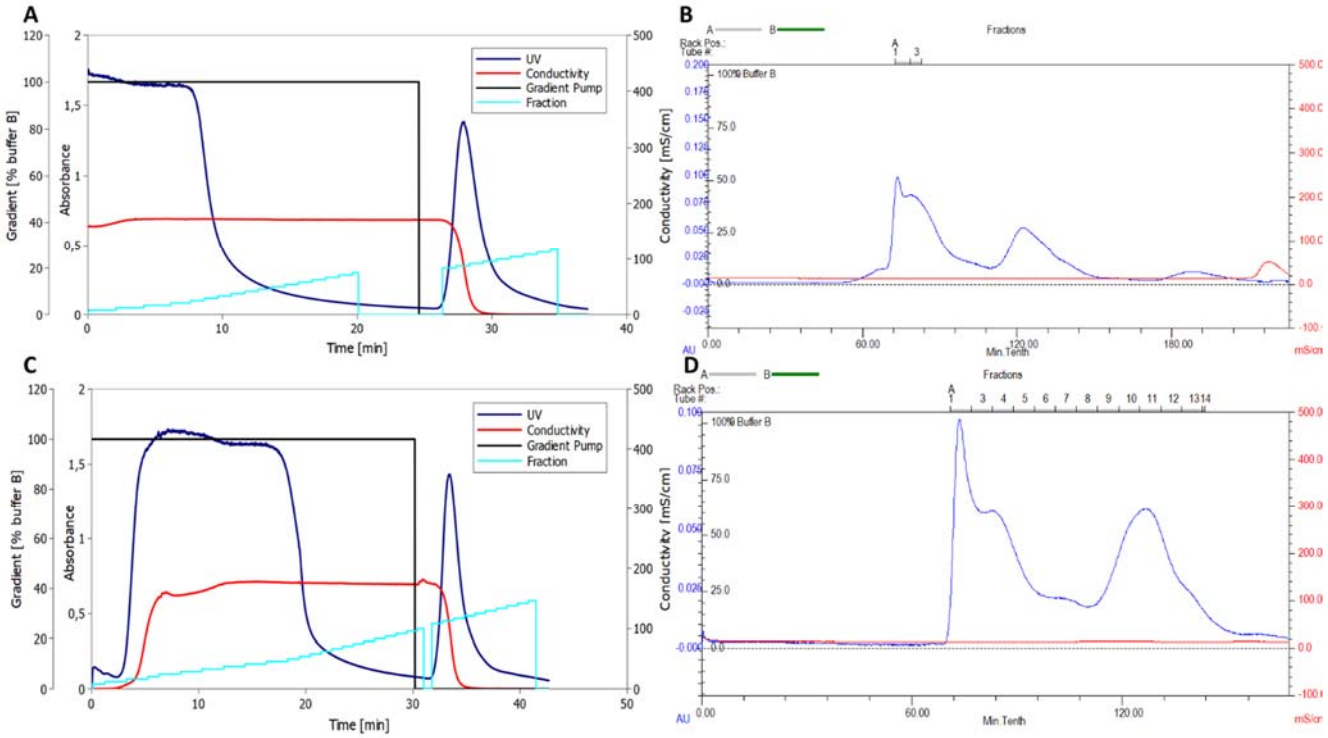

Fig. S11: Reproducibility of the two-step chromatography protocol for mCherry purification (A+C) HIC of mCherry on Butyl FF with 10 mM Tris, pH 8 + 3 mol/l NaCl. Elution was done with a two-step gradient, thereby the first step contained of 100 % and the second step 0% binding buffer. The sample was adjusted with additional 3.5 mol/l NaCl. The program was 2 ml binding buffer, 5.2 ml load, 7 CV binding buffer and 5 CV elution buffer. Flow rate was 2 ml/min and for load 1 ml/min. Fraction were collected up to 2 ml with a threshold of 0.075 AU **(B+D)** Size exclusion of mCherry on Superdex Sephacryl S-200 with 10 mmol/l Tris, 150 mmol/l NaCl, pH 8. The program was 1 ml elution buffer, 5.2 ml load and 121 ml elution buffer. Flow rate was 0.5 ml/min. Fraction were collected up to 3 ml with a threshold of 0.075 AU. Loaded was the elution fraction of the HIC chromatography. Divergent: **(B)** The threshold for the SEC was too low, therefore, the target elution fraction wasn't collected. **(D)** The SEC run was abducted after elution of the target fraction.

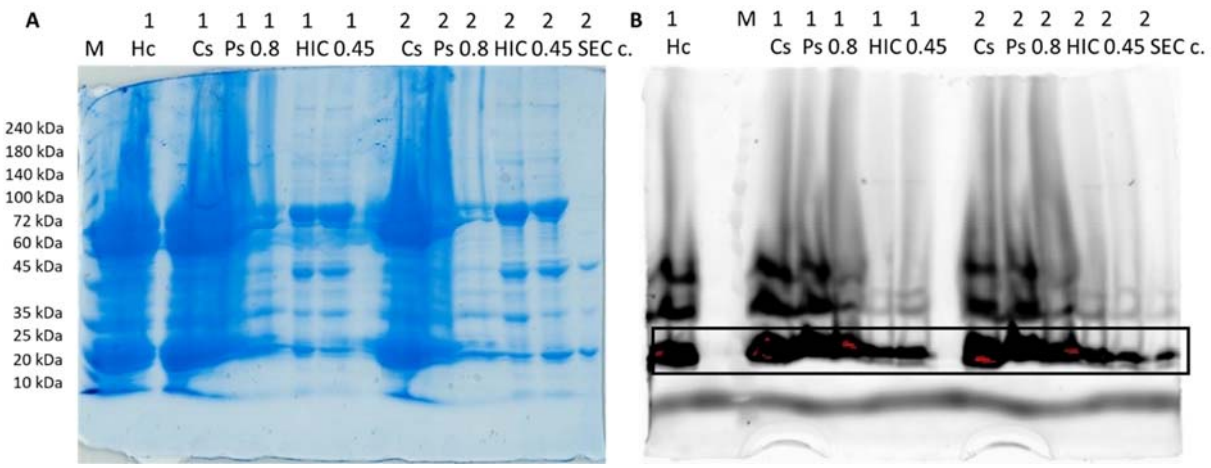

Fig. S12: Results of the two-step chromatography protocol for mCherry purification run 1 and run 2 Coomassie blue staining SDS-gel **(A)** of the fractions in comparison with fluorescence Image **(B)**. On each line 10 µl was loaded. On the SDS-PAGE it was 8 µl sample to 22 µl dilution and on the Fluorescence image it was 10 µl to 24 µl dilution. Hc: crude haemolymph; Cs: 17800 g, 10 min centrifugation supernatant; Ps: 2.5 % PEG precipitation supernatant; M is Marker; 0.8: 0.8 µm filtration of supernatant; HIC: HIC elution fraction with red fluorescence; SEC c.: SEC pool concentrated with Amicon 3K; The numbers 1 and 2 indicate if the sample belongs to run 1 or 2; black boxes indicate mCherry fluorescence

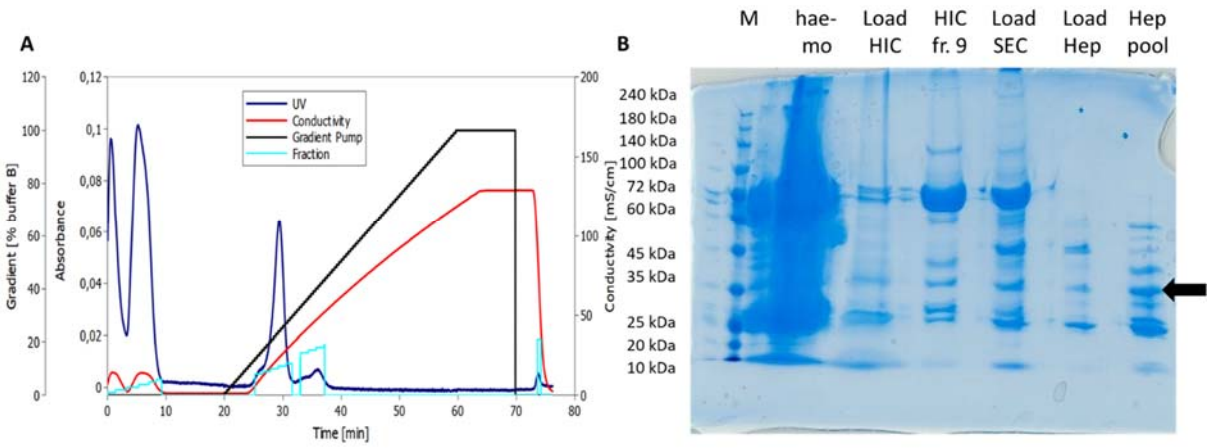

Fig. S13: Results of the heparin chromatography step (A) mCherry on HiTrap Heparin HP with 10 mM NaH_2PO_4, pH 7. Elution was done with a linear gradient up to 2 mol/ NaCl. The program was 2 ml binding buffer, 5.2 ml load, 4 CV binding buffer and 12 CV linear gradient up to 100 % elution buffer. After this, 3 CV 100 % elution buffer, followed from 2 CV binding buffer. Flow rate was 1.5 ml/min and for load 1 ml/min. Fraction were collected up to 2 ml with a threshold of 0.003. **(B)** CBB staining of the purification protocol. On each line 15 µl was loaded. On the SDS-PAGE it was 8 µl sample to 22 µl dilution. M is Marker; haemo: crude haemolymph; Load HIC: Sample after pre-treatment; HIC fr. 9: Flow through fraction 9 of the HIC; Load SEC: Pooled HIC pool; Load Hep: Pooled SEC elution pool; Hep pool: With Amicon 3K concentrated Heparin flow through pool; black arrow indicates mCherry.

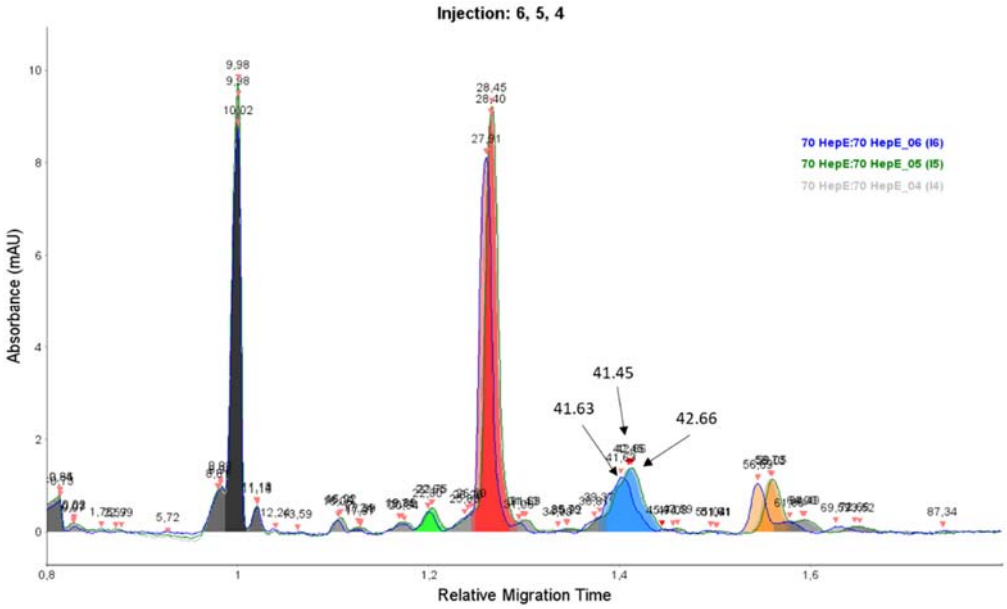

Fig. S14: CED-SDS of the proposed purification protocol without heat-treatment. This is the overlay of the three electropherograms from the purification protocol without heat treatment. Here the molecular weight of the mCherry is around 42 kDa.

Fig. S15: Investigated chromatography methods. Here are all used chromatography methods listed with were investigated for the purification of mCherry. The left column names the used principle, the middle column for what purpose and the last column summarize very shortly the outcome. AIEX: Anion exchange chromatography; HIC: Hydrophobic interaction chromatography; CHT: Ceramic Hydroxyapatite; SEC: Size exclusion chromatography.