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Expression, purification and crystallization of Leader peptidase I from *Escherichia coli*

Rebecca Nyman

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Author

Rebecca Nyman

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Abstract

The secretory pathway, in which proteins are exported from the cytoplasm into the periplasm, is essential for the viability of bacteria. One component in the pathway is leader peptidase I. In this project a truncated leader peptidase I, LepB_V77, from *Escherichia coli*, *E. coli*, has been expressed and purified. Activity assays have been performed as well as crystallization screens. The protein was purified using immobilised metal affinity chromatography, IMAC. Purification resulted in a pure protein solution with *Ec*LepB_V77 but low yield. Crystals were obtained in several conditions. The activity assay did not result in any reliable data.

Keywords

Leader peptidase I, LepB, *Escherichia coli*, protein purification, immobilized metal affinity chromatography, crystallization

Supervisors

Sherry Mowbray, Lu Lu
Uppsala University

Scientific reviewer

Mikael Widersten
Uppsala University

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Biology Education Centre Biomedical Center Husargatan 3, Uppsala
Box 592, S-751 24 Uppsala Tel +46 (0)18 4710000 Fax +46 (0)18 471 4687

Expression, purification and crystallization of Leader peptidase I from *Escherichia coli*

Rebecca Nyman

Populärvetenskaplig sammanfattning

Bakterier som till exempel *Eschericha coli* har olika system för att transportera proteiner genom t.ex. cellmembran. Ett av dessa system, det såkallade sekretionssystemet är livsviktigt för att bakterien ska överleva.

För att protein ska kunna transporteras genom detta system så måste de klippas och bli kortare. Ett enzym som heter *leader peptidase I* fungerar som saxen som klipper dessa proteiner. Utan denna sax i cellen så skulle cellen inte överleva.

För att studera och lära sig mer om detta livsviktiga enzym så kan man behöva rena ur det från bakterien. Det är intressant att studera detta enzym då det skulle kunna vara ett potentiellt mål för ett läkemedel som hämmar enzymets funktion vid infektioner som är orsakade av *E. coli*. För att underlätta rening av enzymet är det konstruerat så att det är kortare än det enzym som vanligtvis finns i membranet hos *E. coli*. Proteinet saknar den del av enzymet som vanligen korsar membranet. Arbetet har handlat om att rena enzymet på ett sådant sätt att det urskiljs från alla andra proteiner och enzymer i bakterien. Försök har också gjorts för att undika att enzym går till spillo under reningens gång.

När enzymet väl har renats ut så har försök gjorts för att få enzymet att ordna sig i kristaller. Detta har gjorts för att genom röntgenkristallografi få en tredimensionell bild av enzymet.

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Background

The secretory pathway, in which proteins are secreted from the cytoplasm into the periplasm, is a pathway essential for bacteria to maintain growth and to survive (1).

The preprotein with a leader peptide at the amino-terminal end is carried to the membrane. The preprotein is then transferred through the SecYEG channel. After translocation of the preprotein, leader peptidase I cleaves the preprotein. The mature protein can then proceed to its final destination (2). A simplified figure of the components in the secretory pathway is shown in Figure 1.

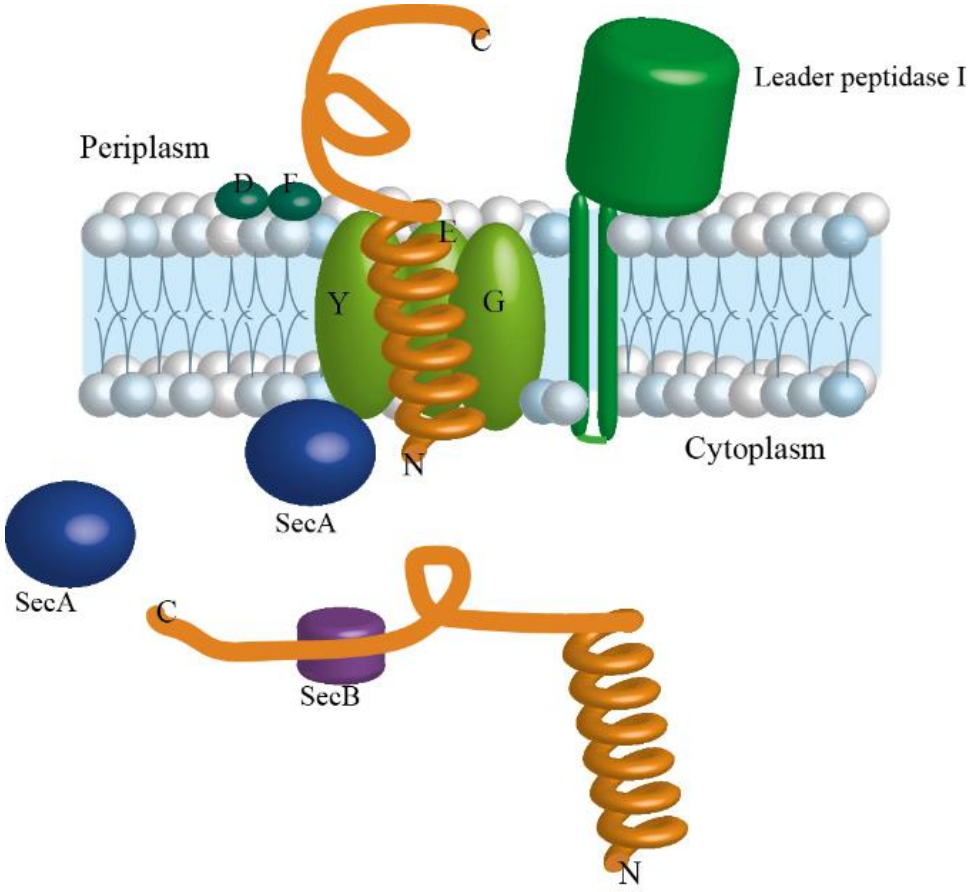

Figure 1. The secretory pathway in which proteins are secreted from the cytoplasm into the periplasm. Figure modified from Paetzel, M., Dalbey, R. E. & Strynadka, N. C. The structure and mechanism of bacterial type I signal peptidases: a novel antibiotic target. Pharmacol. Ther. ***87,*** *27–49 (2000).*

The leader peptidase I, LepB, in *E. coli* is attached to the membrane through two N-terminal segments. The catalytic domain of the protein is located in the periplasm, close to the translocation channel. LepB is a serine protease that utilizes a serine/lysine dyad at positions 91 and 146 of the protein (3). The catalytic dyad of LepB cleaves the preprotein after a sequence of Ala-X-Ala, where X can be any amino acid (4).

Inhibition of leader peptidase I results in accumulation of preprotein in the membrane, due to non-cleaved preprotein. This accumulation results in cell death. Because of this phenomenon, it is interesting to study LepB, primarily with the purpose of developing inhibiting antibiotics (5).

Leader signal peptidase I from *E. coli* consists of 324 amino acids and has a molecular weight of 36 kDa (UniProt Accession number: P00803). The protein has a tertiary structure including both alpha helices and beta strands.

The protein that has been studied in this project, is truncated between amino acid 76 and 77, with addition of methionine at position 76. This truncation results in a version of the protein that does not have the two transmembrane segments. The truncated Leader peptidase I, *Ec*LepB_V77, consists of 249 amino acids with a molecular weight of 28 kDa (ProtParam tool http://web.expasy.org/protparam/).

Kuo *et al.* denatured *Ec*LepB_V77 in 4M guanidine hydrochloride and purified the protein using size exclusion chromatography. The refolding was carried out by dialysis against a buffer containing 0.5% Triton X-100. The detergent was removed by the use off an Extracti-Gel D column which contain a detergent removing gel (6).

Paetzel *et al.* denatured the protein in 6M guanidine hydrochloride. The refolding was made by dialysis towards a buffer with 0.5% Triton X-100. The purification was performed with an anion exchange column. Triton X-100 was removed by wash with buffer while the protein was bound to the column. A second dialysis was performed to change the elution buffer. Crystals were obtained using 1.0M ammonium dihydrogen phosphate and 0.1M sodium citrate at pH 5.6 in the reservoir. Larger crystals were achieved by the use of 0.7M ammonium dihydrogen phosphate, 0.1M sodium citrate and 5% 2-methyl pentane-2,4-diol, pH 5.6(3).

The goal with this project was to express, purify and crystalize LepB_V77 from *E. coli*.

Material and methods

Cloning

The *Ec*LepB_V77_his gene was created using the polymerase chain reaction, PCR. The *Ec*LepB wild type gene was used as template and primer `atggtgcgttcgtttatttatgaa` as the forward primer and primer `ttaatgatgatgatgatggatgccgccaatgcgacttaagcg` as the reverse primer.

The truncated *Ec*LepB_V77 has the following amino acid sequence:

```
MVRSFIYEPFQIPSGSMMPTLLIGDFILVEKFAYGIKDPIYQKTLIETGHPKRGDIVVFKYP
EDPKLDYIKRAVGLPGDKVTYDPVSKELTIQPGCSSGQACENALPVTYSNVEPSDFVQTFSR
RNGGEATSGFFEVPKNETKENGIRLSERKETLGDVTHRILTVPIAQDQVGMYYQQPGQQLAT
WIVPPGQYFMMGDNRDNSADSRYWGFVPEANLVGRATAIWMSFDKQEGEWPTGLRLSRIGGI
H
```

The PCR was carried out in a solution of 25µl phusion buffer (Agilent), 1µl of forward primer (20µM, Invitrogen), 1µl of reverse primer (20µM, Invitrogen), 1µl of *Ec*LepB wild type plasmid (169ng/µl), 22µl of dH_2O. The following procedure was used: initiation step for 2 min at 95°C, denaturation for 1 min at 95°C, annealing for 1 min at 59°C, elongation for 2 min at 72°C, in 30 cycles, ending with final elongation for 10 min at 72°C.

The PCR products were run on a 1% agarose gel with SYBR Safe DNA Gel Stain, the band at 747 base pairs was cut out of the gel and centrifuged through a filter for 2min at 16.2 x1000g in a HERAEUS PICO21 centrifuge from Thermo Scientific.

17µl of the flow through was added to a solution of 2µl 10X Taq buffer (Bio labs), 0.5µl dNTP (Invitrogen), 0.2µl Taq polymerase and incubated for 10 min at 72°C to add dNTA to

the 3’ end of the PCR product. After incubation, 1µl of the sample was added to a mix of 0.5µl salt solution (1.2M NaCl and 0.06M $MgCl_2$ from Invitrogen), 1µl H_2O and 0.5µl pEXP5/CT-TOPO vector (Invitrogen), and incubated at 20 min in room temperature, to ligate the his-tagged gene into the vector.

His_*Ec*LepB_V77 was created by Viktor Jakobsson, a project student working in the lab earlier.

The plasmids with His_*Ec*LepB_V77 and *Ec*LepB_V77_his were transformed into TOP10 competent cells. The plasmids were added to TOP10 competent cells and put on ice for 3 min followed by 30 s at 42°C, ending with 2.5 min on ice. The cells were then spread on agar plates (containing ampicillin (50µg/ml) as selection media due to the ampicillin resistance gene in the pEXP5/CT-TOPO vector) and incubated overnight at 37°C.

One colony for each construct were picked from the plates and added to LB with 50µg/ml and incubated at 37°C. The cells were incubated at 37°C until the cultures were dense. Plasmid purification was carried out on the cell cultures containing the *Ec*LepB_V77_his strain. The plasmid purification was carried out with NucleoSpin Plasmid EasyPure from Macherey-Nagel using their standard protocol.

An analytical PCR was performed to determine the direction of the gene inserted in the plasmid. Analytical PCR was made with colonies expressing His_*Ec*LepB_V77, while the *Ec*LepB_V77_his from the plasmid purification was used. The PCR was performed in a solution of 18.3 µl of dH_2O, 2.5 µl 10XTaq buffer (Bio labs), 1 µl DMSO, 0.5 µl dNTP (Invitrogen), 1 µl T7 forward primer (20 µM, Invitrogen), 1 µl reverse primer (20 µM, Invitrogen), 0.5 µl template from plasmid purification (91.1 ng/µl of His_*Ec*LepB_V77 and 72.2 ng/µl of *Ec*LepB_V77_his), 0.2 µl Taq enzyme (Bio labs). The following PCR protocol was used: initiation step at 95°C for 5 min, denaturation at 95 °C for 1 min, annealing at 57 °C for 1 min, elongation at 72 °C for 2 min, ending with final elongation at 72 °C for 10 min. The denaturation, annealing and elongation cycle was repeated 25 times. The PCR product was run on a 1% agarose gel with SYBR Safe DNA Gel Stain and the band at around 750 bp was analysed to confirm the direction of the gene in the plasmid.

His_*Ec*LepB_V77 that was used in the transformation to TOP10 (Invitrogen) was sent for sequencing along with *Ec*LepB_V77_his from the plasmid purification.

The plasmids together with primers were sent to the Uppsala Genome Center Sequencing Service for sequencing. The results were analysed in 4Peaks (http://nucleobytes.com/index.php/4peaks). The sequence was then aligned against the *Ec*LepB_V77 gene sequence with the pairwise sequence alignment tool, EMBOSS Needle (http://www.ebi.ac.uk/Tools/psa/emboss_needle/) showing 100% identity.

Expression

The plasmid was transformed into *E. coli* C43 (DE3) competent cells (provided by Lu Lu and Sanjeewani Sooriyaarachchi). C43 (DE3) was used because they are efficient in expressing membrane proteins. 0.5µl of plasmid (91.1ng/µl off His_*Ec*LepB_V77 and 72.2ng/µl of *Ec*LepB_V77_his) were added to 100µl of C43 (DE3) and put on ice for 3 minutes. The sample was put at 42°C for 40 s and again put on ice for 2.5 minutes. The cells were then spread on plates containing 50µg/ml of ampicillin. The plate was incubated at 37°C overnight.

Colonies were picked from the plate and transferred to 2ml of LB containing 50µg/ml of ampicillin and incubated in 37°C. When the cells had grown dense, the culture was again transferred to 50ml of LB with 50 µg/ml of ampicillin and incubated at 37°C. When the cells had grown dense the culture was transferred to 1000ml of LB with 50 µg/ml of Ampicillin. When the OD_{600} was approximately 0.6 the cells were induced with 0.1mM IPTG. The cells were incubated at room temperature overnight and harvested in the morning. During harvest the culture was centrifuged at 13261xg by Avanti J-26SXP from Beckman Coulter for 10min. The pellet was suspended in a solution containing 0.15M NaCl and 0.01 $NaHPO_4$ at pH 7.4. The sample was centrifuged at 3813xg from Heraeus multifuge 3SR for 20 minutes and supernatant discarded. The pellet was stored at -20°C.

Transformation was also performed using BL21 (DE3) competent cells with the above described protocol.

Expression of protein was performed at room temperature as default but the expression was also carried out at 15°C, 10°C and 4°C.

One sample of culture before induction was centrifuged at 16200xg in a HERAEUS PICO21 centrifuge from Thermo scientific. 5x SDS loading dye was then added to the pellet.The same procedure was performed after induction and expression.The samples were heated and loaded to Phastgel Homogenous with 12.5% acrylamide from GE Healthcare and run with the homogenous protocol of the PhastSystem from Pharmacia. The gel was stained in Instant Blue (Expedeon).

Purification

The original purification protocol was inherited from Viktor Jakobsson, who was an earlier project student working in the lab. During the work, the purification protocol has been modified with the aim to give a high yield of the purified sample. The purification protocols used in the project can be found in Appendix I.

The original purification protocol

The cells were suspended in 6ml/g_{pellet} of buffer A. DNase was added to sample before lysis. The cells were lysed by cell disruptor two times at 2 bar. The sample was centrifuged at 17210xg by Sorvall RC6 for 10 minutes. The protein in the pellet was denatured in buffer B for two hours at room temperature. The sample was then centrifuged at 43150xg by Sorvall RC6 for 20 minutes. The supernatant after centrifugation was added to a column with 1 ml nickel charged resin (Sepharose 6 fast flow from GE Healthcare), and incubated at 4°C for one hour. After incubation, the column was washed with buffer B. To perform the refolding of the protein, Buffer C was added to the column and incubated at 4°C for one hour. The column was then washed with buffer C. The protein was eluted with buffer D.

Buffer A
5mM $MgCl_2$ (203.30g/mol, Merck)
20mM Tris (121.14g/mol, Merck)
7mM β- mercaptoethanol added fresh (14M stock solution, Sigma Aldrich)
pH 7.4

Buffer B
6M guanidine hydrochloride (95.53g/mol, Sigma Aldrich)
in buffer A
pH 7.5

Buffer C
0.5% Triton ™X-100(100%, Sigma Aldrich)
in buffer A

Buffer D
250mM imidazole
in buffer C

The imidazole was removed from the sample by the use of a 10DG desalting column from Bio-rad where the protein was eluted with buffer C.

The samples from each step in the purification was added to 5X SDS loading dye and heated. In 45µl of loading dye, 5µl of β-mercaptoethanol was added to make sure that all the disulfide bonds were reduced. The samples were loaded to a Phastgel Homogenous with 12.5% acrylamide from GE Healthcare and run with the homogenous protocol of PhastSystem from Pharmacia. Along with the samples, a low molecular weight ladder was used as a reference. The gel was stained with Instant Blue (Expedeon).

Purification of His_*Ec*LepB_V77

The original purification protocol

His_*Ec*LepB_V77 was initially purified with the original purification protocol.

Purification protocol version two

For the next purification, the incubation time when protein can bind to the column was extended while the denaturation time was shortened. Purification protocol version two can be found in the appendix.

The sample from the elution in purification protocol version two were run on a Phastgel Homogenous 12.5. The gel was sent to the mass spectrometry facility at SciLifeLab. The analysis was made to investigate the content of two prominent bands in the elution analysed by SDS-PAGE.

Purification protocol version three

To improve binding of protein to the resin, 300mM NaCl was added to buffer A, which can be seen in purification protocol version three.

Protease inhibitor cocktail tablets (Roche) were added during lysis to prevent cleavage of the protein during purification.

Purification protocol version four

In purification protocol version four, 5% v/v glycerol(92.09g/mol, VWR chemicals) was added to buffer A. This change was made to hopefully improve the stability of the protein. The thought about the addition of 5% glycerol came after reading the article *Expression, purification and crystallization of a membrane-associated, catalytically active type I signal peptidase from Staphylococcus aureus* of *Yi Tian Ting et al.* (7). Glycerol could hopefully stabilize the protein.

10mM β-mercaptoethanol was used in buffers according to a standard protocol from a colleague in the lab.

8M urea (60.06g/mol, VWR chemicals) was used in the denaturation buffer instead of 6M guanidine hydrochloride. The change was made to simplify the interpretation of the Sodium dodecyl sulfate- polyacrylamide gel electrophoresis, SDS-PAGE, results after purification. Guanidine precipitates in SDS loading dye which results in undefinable bands in SDS-PAGE analysis.

Earlier the incubation of protein to the resin was made at 4°C, when using urea the incubation was done at room temperature to prevent urea from crystallizing.

Purification protocol version five
In purification protocol version five the refolding with 0.5% triton X-100 was changed to a stepwise wash with a urea gradient. This change was made with the hope to give a higher activity and to be more cautious with the protein.

In the original purification protocol to purification protocol version four the refolding of the protein started when 0.5% Triton X-100 buffer was added to the column followed by incubation. The change in denaturant concentration happened fast compared to the refolding with dialysis made by Kuo *et al.* and Paetzel *et al* (3,6). The drop in denaturant concentration could result in that the protein becomes aggregated and/or misfolded.

The change in refolding procedure was also made because of a solution with 0.5% Triton X-100 is hard to work with. In earlier articles 0.1% Triton X-100 was used in activity assays and crystal set up (3,8). One aspect was that when Triton X-100 once is added to solution, it is difficult to remove the detergent. Earlier work suggest that Triton X-100 could at least partially be removed by Extracti-Gel D. The amount of Triton X-100 in the elution and final buffer would, however, be difficult to estimate and therefore need to be measured.

When eluting protein from PD-10 desalting column from GE Healthcare, the elution was performed with buffer A instead of buffer C. The change was made with the idea that the protein needs to be concentrated before setting up crystals. The concentration of protein would also lead to concentration of Triton X-100. The Triton X-100 micelles have an average molecular weight of 80 kDa (product specification).

Purification protocol version five was performed both with 5g and 10g of cells as starting material of purification. When using 10g of cells as starting material 1.5ml of resin was used. Because of this the protein was eluted with 3x2.5ml to ensure that all of the protein was eluted.

Purification protocol version six
Purification protocol version six was performed using 10g of cells as starting material, which is double the amount compared to the standard in the other purifications.

0.5% Triton X-100 was used in lysis and therefore no washing step of the pellet after lysis was performed.

The amount of nickel charged resin was the same as when purification was made with 5g of cells as starting material, because the Sepharose 6 fast flow from GE Healthcare has a binding capacity of 40mg of his-tagged protein per ml of resin, which is more than the amount of protein eluted from these purifications.

At this stage of the purification optimization it became clear that even if the double amount of cells were used in purification, the amount of protein in the end of the purification was the same as when the single amount of cells was used. This loss of protein throughout the purification could be explained by the loss of protein in flow through. There seems to be a problem with protein not binding to the nickel charged resin.

The importance of achieving a larger amount of protein in purification is for the crystal set up. Paetzel *et al.* used a protein concentration of 10mg/ml in the crystallization set up of *Ec*LepB_V77 (3). Even though the protein can be concentrated, the volume off protein

solution needs to be sufficient for the screening. It is not recommended to pool protein from different purifications (9).

Western blot

Due to loss of His_*Ec*LepB_V77 in flow-through during purification a Western blot was made, using detection with an anti-his-tag antibody with samples of supernatant after the last centrifugation, pellet after the last centrifugation, flow through and elution. The samples used were generated through protocol version six.

It was suspected that the loss of protein in flow through could be due to a missing his-tag in the protein. A Western blot was performed to analyse the presence of a his-tag in *Ec*LepB_V77.

The western blot started by doing an SDS-PAGE. The samples were loaded to a Phastgel Homogenous 12.5 from GE Healthcare and run with the homogenous protocol of PhastSystem from Pharmacia. Nitrocellulose and filter papers was cut in pieces of the same size as the Phastgel. The pieces were soaked in transfer buffer containing 39mM glycine, 48mM Tris, 20% methanol, 0.037% SDS pH 8.3. A Sandwich was made with filter paper, nitrocellulose and the Phastgel. The sandwich was put into the PhastSystem and the blotting program was run. When the program had ended, the nitrocellulose was soaked in Tris buffered saline with tween 20 (TBST) + 5% w/v non-fat dry milk, containing 20mM Tris, 500mM NaCl, 0.05% Tween 20, 5% w/v milk powder, for 10 min. The nitrocellulose was then washed with TBST containing 20mM Tris, 500mM NaCl and 0.05% Tween 20. Tris-buffered saline with monoclonal anti-polyHistidine (Sigma-Aldrich) containing 20mM Tris, 500mM NaCl and monoclonal anti-polyHistidine was added to the membrane and incubated in room temperature overnight. The membrane was then washed 3X 5 min with TBST. The nitrocellulose was soaked in Anti-Mouse igG peroxidase (Sigma Aldrich) in TBST for 60min. A second wash 3X 5min with TBST was made. The membrane was then treated with western blotting detecting reagents (Pharmacia).

Purification protocol version seven

In purification protocol version seven, Triton X-100 was not used in the steps before protein was added to resin. The change was made because of loss of His_*Ec*LepB_V77 in the flow through due to that the protein did not bind to the resin.

The idea is that Triton X-100 creates micelles that enfold and hide the tag and therefore prevent His_*Ec*LepB_V77 from binding to the resin. 0.1% Triton X-100 was used in the imidazole wash, elution and buffer used to elute the protein from the PD-10 column. The addition of Triton X-100 was made to solubilize the protein and possibly increase the shelf life.

Purification protocol version eight

The change made in purification protocol version eight was that the protein was incubated with the resin overnight. This change in increasing incubation time was made to investigate if protein bound to the resin would increase, which would show up as an increase of protein in elution.

Purification of *Ec*LepB_V77_his

Purification protocol version five

*Ec*LepB_V77_his that had been expressed at 15°C instead of room temperature was purified according to protocol version five. This purification was made to study the difference in purity and activity between the construct with different positions of the his-tag.

The change in incubation temperature was made to study the impact of temperature on inclusion body formation. The thought was that the reduced expression temperature could affect the aggregation in a positive manner and reduce the inclusion body formation.

*Ec*LepB_V77_his expressed at 10°C was also purified according to protocol version five with the same thoughts about the expression temperature.

Purification protocol version nine

In purification protocol version nine guanidine hydrochloride was used for denaturation and refolding instead of urea. The change in denaturant was made to analyse if the denaturant did influenced the binding to the column. For this purification the plasmid had been transformed into BL21 (DE3) competent cells according to the protocol below the "expression heading". BL21 (DE3) was tried because they were used in transformation when Kuo *et al.* expressed and purified *Ec*LepB_V77 (6). The impact of the competent cells on the purification was investigated.

Purification protocol version ten

The denaturant was then changed back to urea. In purification protocol version ten 6M urea was used instead of 8M urea. The urea concentration was lowered to investigate if the high urea concentration could affect the binding of protein to the resin. The maximum urea concentration compatible with Sepharose 6 fast flow used in the purification is 8M and therefore the concentration used was lowered. BL21 (DE3) competent cells were used in transformation and expression.

Purification protocol version eleven

In purification protocol eleven cobalt charged resin from TALON was used instead of nickel charged resin from GE Healthcare. The switch of resin was made to investigate the impact off the resin in terms of binding the protein. Could another resin stop the loss of protein in flow through, and thereby improve the amount of protein in the elution? *Ec*LepB_V77_his expressed at 4°C was the starting material in the purification.

Purification protocol version twelve

Osmotic shock

After expression of *Ec*LepB_V77_his at room temperature, osmotic shock was performed. This additional method was made based on the theory that *E. coli* can produce metal chelators associated with the periplasm during stress. Cells harvested after expression were resuspended in $5ml/g_{pellet}$ osmotic shock buffer containing, 50mM HEPES, 1mM EDTA and 20% sucrose, followed by centrifugation at 7000xg for 30 minutes. The pellet after centrifugation was resuspended in $MgSO_4$ buffer and put on ice for 10 minutes. The cells were then centrifuged at 4000rpm for 20 minutes. Supernatant was discarded and pellet stored at -20°C (10).

The cells exposed to osmotic shock were purified according to purification protocol version twelve.

Activity assay

The activity of the protein was analysed in a fluorescence resonance energy transfer assay, FRET assay, using a substrate with the specific Ala-X-Ala sequence. The substrate has Dabcyl and Edans at the two ends. Edans get excited by light at 336 nm and emits light at 472 nm. Dabcyl absorbs light between 400 nm and 600 nm with a peak at 490 nm.

The filters used in the Envision 2104 multilabel reader from Perkin Elmer are excitation filter at 340 nm and emission filter at 535 nm. When the substrate is intact, Dabcyl and Edans are close to each other and the emission of Edans is quenched by Dabcyl through FRET. This result in no signal to detect. When the substrate gets cleaved the distance between Edans and Dabcyl is larger. Dabcyl can no longer absorb the emission of Edans and a signal can be detected. The signal was measured once every minute for one hour.

The assay contained 27 µl of protein in 3 µl of 100 µM substrate. The protein used was protein stored at -20°C in a range of 50 to 500nM and protein stored at 4°C in a range of 50nM to 50µM. The substrate had a final concentration of 10µM.

*Ec*LepB wild type was used as positive control while inactive *Ec*LepB with a mutation in serine at position 91 was used as negative control.

The derivative of the activity of the first 30 minutes was calculated. The activity with regard to amount of protein was calculated to make it possible to compare the activity between the samples and also to analyse the stability of the method. The difference between values of activity that are standardized in regard to the amount of protein should be small.

The activity of *Ec*LepB_V77 was compared to the positive and negative control.

Crystal set up

Four crystallization screens were used in the search for finding the conditions resulting in crystals. The screens used were Structure screen, JCSG plus and Morpheus from molecular dimensions together with JBScreen solubility from Jena Bioscience. The method used was sitting drop vapour diffusion. Before crystal set up His_*Ec*LepB_V77 was concentrated to a range of 3.4mg/ml to 5mg/ml. The plate used for the screening contained three sub-wells and one reservoir for each condition. In one sub-well, the protein was added directly after concentration. In another sub well 0.1% Triton X-100 was added to the sample before adding it to the sub well. The third sub-well was left empty. The protein solution and reservoir were added to the sub-wells using a Mosquito nanolitre liquid handler from ttplabtech. The sitting drop contained 150nl of protein solution together with 150nl of reservoir.

Crystals obtained were mounted into loops and analysed at the European Synchrotron Radiation Facility, ESRF, in Grenoble.

Results and discussion

Cloning

The result of the analytical PCR and sequencing of the constructs were analysed.

The result showed that both His_*Ec*LepB_V77 and *Ec*LepB_V77_his genes were inserted in the right direction in the vector.

The sequencing of the two constructs showed 100% identity to the desired design.

Purification

Purification of His_*Ec*LepB_V77

The original purification protocol

The original purification protocol gave an elution of 1.4mg/ml corresponding to $4mg_{protein}/L_{culture}$. The concentration of protein after buffer exchange was 1.1mg/ml corresponding to 4mg of protein. The SDS-PAGE analysis of the purification can be seen in figure 2.

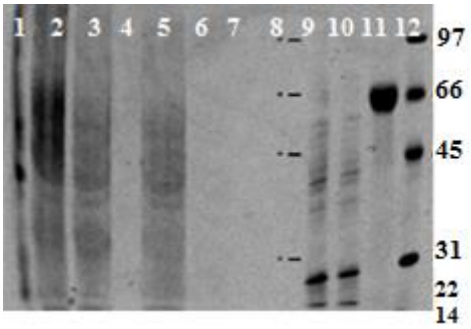

Figure 2. SDS-PAGE showing samples from the original purification protocol. 1, Supernatant after lysis; 2, pellet after lysis, 3, supernatant after denaturation; 4, pellet after denaturation; 5, flow through after incubation with the resin; 6, wash with 6M guanidine hydrochloride; 7, flow through after refolding; 8, wash with 0.5% Triton X-100; 9, elution; 10, buffer exchange; 11, BSA 0.7mg/ml; 12, LMW.

This purification resulted in impure elution and thus buffer exchange. The first lanes are unclear with blurry bands which makes the result difficult to interpret.

Purification protocol version two

Purification according to protocol version two resulted in a protein concentration in elution of 1.1mg/ml corresponding to $3mg_{protein}/L_{culture}$. The protein concentration after buffer exchange was 0.8mg/ml corresponding to $3mg_{protein}/L_{culture}$. The SDS-PAGE analysis of the purification can be seen in figure 3.

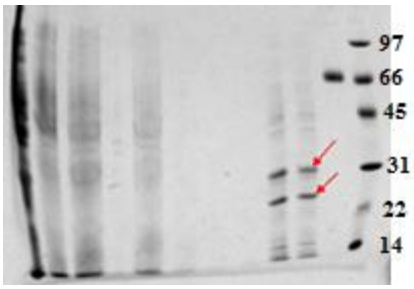

Figure 3. SDS-PAGE showing samples from protein purification version two. 1, Supernatant after lysis; 2, pellet after lysis; 3, supernatant after denaturation; 4, pellet after denaturation; 5, flow through after incubation with the resin; 6, wash with 6M guanidine hydrochloride; 7, flow through after refolding; 8, wash with 0.5% Triton X-100 and 20mM imidazole; 9, elution; 10, buffer exchange; 11, BSA 0.7mg/ml; 12, LMW. The topmost arrow indicates His_EcLepB_V77, whereas the bottom arrow indicates superoxide dismutase.

The second purification protocol resulted in impure elution and buffer exchange. In figure 3, two prominent bands are shown at 31kDa and 22kDa in lane nine and ten. The elution with

two prominent bands were sent for Liquid chromatography mass spectrometry, LC-MS. The analysis showed that the band around 31kDa was Leader peptidase I whereas the band around 22kDa was manganese binding Superoxide dismutase. The Superoxide dismutase can bind to nickel instead of manganese which can explain the prominent band in elution and buffer exchange.

Purification protocol version three

Purification according to protocol version three resulted in a protein concentration in elution of 0.5mg/ml corresponding to 1.5$mg_{protein}/L_{culture}$. The protein concentration after buffer exchange was 0.6mg/ml corresponding to 2.5$mg_{protein}/L_{culture}$. The SDS-PAGE analysis of the purification can be seen in figure 4.

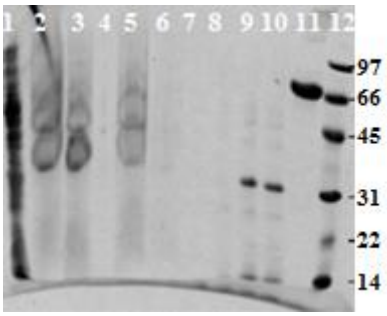

Figure 4. SDS-PAGE showing samples from protein purification version three. 1, Supernatant after lysis; 2, pellet after lysis; 3, supernatant after denaturation; 4, pellet after denaturation; 5, flow through after incubation with the resin; 6, wash with 6M guanidine hydrochloride; 7, flow through after refolding; 8, wash with 0.5% Triton X-100 and 20mM imidazole; 9, elution; 10, buffer exchange; 11, BSA 0.7mg/ml; 12, LMW.

In purification number three, 300mM NaCl was added in buffer A to improve binding of protein to the resin. The amount of protein in the elution was not higher compared to earlier purifications but the elution was more pure from unwanted proteins.

A protease inhibitor cocktail tablet was added to the lysis buffer to prevent cleavage and degradation of *Ec*LepB_V77 during purification.

The result of protein concentration measurements revealed that the amount of protein after buffer exchange was higher than the amount of protein after elution from IMAC. This is not possible.

Purification protocol version four

Purification according to protocol version four resulted in a protein concentration in elution of 0.3mg/ml corresponding to 0.9$mg_{protein}/L_{culture}$. The protein concentration after buffer exchange was 0.3mg/ml corresponding to 0.9$mg_{protein}/L_{culture}$. The SDS-PAGE analysis of the purification can be seen in figure 5.

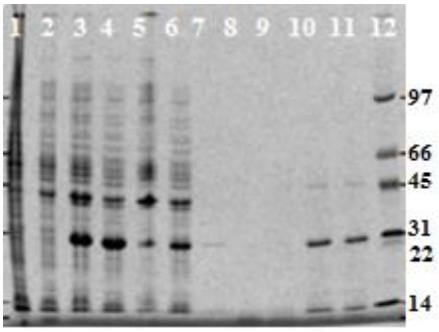

Figure 5. SDS-PAGE showing samples from protein purification version four. 1, Supernatant after lysis; 2, wash of pellet after lysis; 3, pellet after wash; 4, supernatant after denaturation; 5, pellet after denaturation; 6, flow through after incubation to the resin; 7, wash with 8M urea; 8, flow through after refolding; 9, wash with 0.5% Triton X-100 and 20mM imidazole; 10, elution; 11, buffer exchange; 12, LMW.

In the fourth protocol the concentration of β- mercaptoethanol was increased. Glycerol was also added to stabilize the protein. It is difficult from these result to say if the glycerol did improved the stability of the protein. What can be said is that no loss of protein during buffer exchange is done, which could indicate that the protein is happy.

In this purification, a washing step of the pellet after lysis was performed. As figure 5 shows, a lot of proteins were washed away without losing a lot of His_*Ec*LepB_V77. 8M urea was used as denaturant which made the interpretations of proteins in SDS-PAGE easier than before. In lane four it can be seen that His_*Ec*LepB_V77 was successfully denatured and that a small amount of His_*Ec*LepB_V77 was lost in lane five which represent the pellet after denaturation and centrifugation.

Purification protocol version five

Purification according to protocol version five, when purifying 5g of cells resulted in a protein concentration in elution of 1.1mg/ml corresponding to 3.3$mg_{protein}/L_{culture}$. The protein concentration after buffer exchange was 0.03mg/ml corresponding to 0.1$mg_{protein}/L_{culture}$. The SDS-PAGE analysis of the purification can be seen in figures 6 and 7.

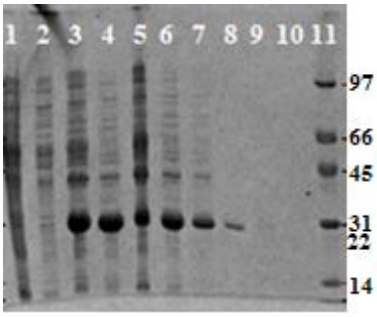

Figure 6. SDS-PAGE showing samples from protein purification version five. 1, supernatant after lysis; 2, wash of pellet after lysis; 3, pellet after wash; 4, supernatant after denaturation; 5, pellet after denaturation; 6, flow through after incubation to the resin; 7, wash with 8M urea; 8, wash with 6M urea; 9, wash with 4M urea; 10, wash with 3M urea; 12, LMW.

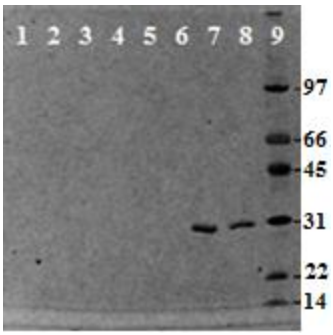

Figure 7. SDS-PAGE showing samples from protein purification version five. 1, wash with 2M urea; 2, wash with 1.5M urea; 3, wash with 1M urea; 4, wash with 0.5M urea; 5, wash with 0M urea; 6, wash with 20mM imidazole; 7, elution; 8, buffer exchange; 9, LMW.

In purification protocol version five the refolding with incubation of 0.5% Triton X-10 was exchanged to refolding with the assistance of a stepwise wash with a urea gradient. The protocol gives about the same protein concentration in elution and buffer exchange compared to earlier purifications. The elution looks pure. The loss of protein during buffer exchange was due to usage of a desalting column from another brand, without changing the protocol for loading of protein and elution of protein.

Purification protocol version five was also used when purifying protein with 10g of cells as starting material. The purification resulted in 0.5mg/ml of protein, corresponding to $1.75mg_{protein}/L_{culture}$. After buffer exchange the protein concentration was 0.3mg/ml corresponding to $1.5mg_{protein}/L_{culture}$. Purifying the double amount of cells did thus not result in the double amount of protein. The SDS-PAGE analysis of the purification can be seen in figures 8 and 9.

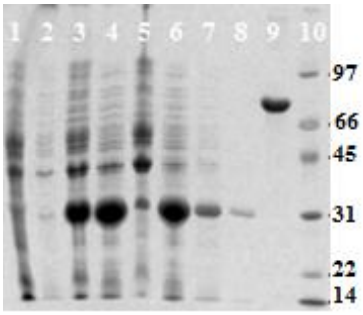

Figure 8. SDS-PAGE showing samples from protein purification version five. 1, supernatant after lysis; 2, washed pellet after lysis; 3, pellet after wash; 4, supernatant after denaturation; 5, pellet after denaturation; 6, flow through; 7, wash with 8M urea; 8, wash with 6M urea; 9, BSA 0.7mg/ml; 10 LMW.

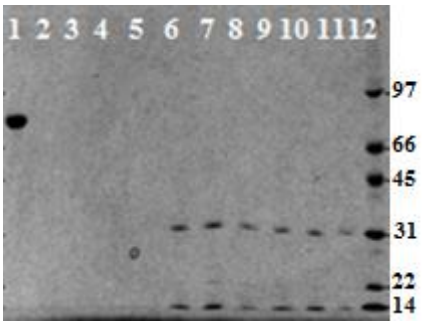

Figure 9. SDS-PAGE showing samples from protein purification version five. 1, BSA 0.7mg/ml; 2, wash with 1M urea; 3, wash with 0.5M urea; 4, wash with 0M urea; 5, wash with 20mM imidazole; 6, elution; 7, buffer exchange; 8, elution; 9, buffer exchange; 10, elution; 11, buffer exchange; 12, LMW.

Purification protocol version six

Purification according to protocol version six resulted in a protein concentration in elution of 0.1mg/ml corresponding to 0.15$mg_{protein}/L_{culture}$. The protein concentration after buffer exchange was 0.1mg/ml corresponding to 0.2$mg_{protein}/L_{culture}$. The SDS-PAGE analysis of the purification can be seen in figures 10 and 11.

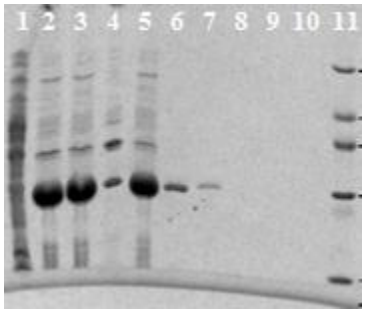

Figure 10. SDS-PAGE showing samples from protein purification version six. 1, supernatant after lysis; 2, pellet after lysis; 3, supernatant after denaturation; 4, pellet after denaturation; 5, flow through after incubation to the resin; 6, wash with 8M urea; 7, wash 6M urea; 8, wash with 4M urea; 9, wash with 3M urea; 10, wash with 2M urea; 11, LMW.

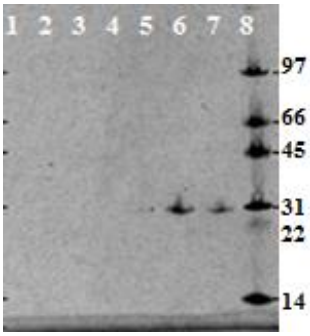

Figure 11. SDS-PAGE showing samples from protein purification version six. 1, wash with 1.5M urea; 2, wash with 1M urea; 3, wash with 0.5M urea; 4, wash with 0M urea; 5, wash with 20mM imidazole; 6, elution; 7, buffer exchange; 8, LMW.

The proteins concentration measurements indicated that the sample in the buffer exchange contains more protein that the elution. This is unreasonable.

The purification was made using 10g of cells as starting material, however it did not result in a larger amount of protein in elution.

Western blot

The western blot in figure 12 shows that the protein in supernatant after lysis, the pellet after lysis and flow through has the his-tag. There is no band in the sample of the elution, this can be explained by the low concentration of protein in the sample. NDH2 was used as positive control. *Ec*LepB wild type had earlier been used in western blot without showing the existing his-tag. Therefore, it was used as negative control. Here we can see that EcLepB wild type has the his-tag.

Protein that do not bind to the resin during purification, can according to the western blot not be explained by that the protein does not have a his-tag. *Ec*LepB_V77 in the flow through has a his-tag.

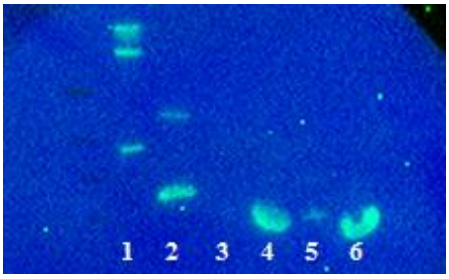

Figure 12. Western blot showing: 1, NDH2; 2, EcLepB wild type; 3, elution; 4, flow through; 5, pellet after lysis; 6, supernatant after lysis.

Purification protocol version seven

Purification according to protocol version seven resulted in a protein concentration in elution of 0.1mg/ml corresponding to 0.3$mg_{protein}/L_{culture}$. The protein concentration after buffer exchange was 0.1mg/ml corresponding to 0.4$mg_{protein}/L_{culture}$. The SDS-PAGE analysis of the purification can be seen in figures 13 and 14.

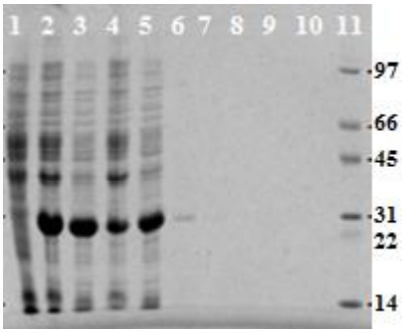

Figure 13. SDS-PAGE showing samples from protein purification version seven. 1, supernatant after lysis; 2, pellet after lysis; 3, supernatant after denaturation; 4, pellet after denaturation; 5, flow through after incubation to the resin; 6, wash with 8M urea; 7, wash with 6M urea; 8, wash with 4M urea; 9, wash with 3M urea; 10, wash with 2M urea; 12, LMW.

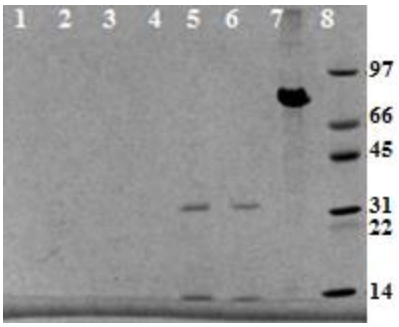

Figure 14. SDS-PAGE showing samples from protein purification version seven. 1, wash with 1.5M urea; 2, wash with 1M urea; 3, wash with 0.5M urea; 4, wash with 20mM imidazole; 5, elution; 6, buffer exchange; 7, BSA 0.7mg/ml; 8, LMW.

The protein concentration measurements indicates larger amount of protein after buffer exchange compared to elution of IMAC. This is as well impossible.

Purification protocol version seven was made to investigate if Triton X-100 has an impact on that the protein do not bind to the resin. In lane five in picture 13 it can be seen that there is a

prominent band at 31kDa which indicates that not all of his_*Ec*LepB_V77 has bound to the resin. Triton X-100 should therefore not influence the protein binding to the resin.

Purification protocol version eight

Purification according to protocol version eight resulted in a protein concentration in elution of 0.1mg/ml corresponding to 0.3$mg_{protein}/L_{culture}$. The protein concentration after buffer exchange was 0.04mg/ml corresponding to 0.1$mg_{protein}/L_{culture}$. The SDS-PAGE analysis of the purification can be seen in figures 15 and 16.

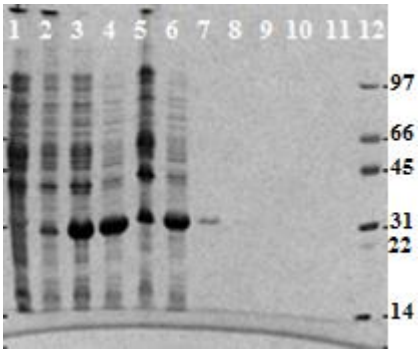

Figure 15. SDS-PAGE showing samples from protein purification version eight. 1, supernatant after lysis; 2, wash of pellet after lysis; 3, pellet dissolved in 8M urea; 4, supernatant after last centrifugation; 5, pellet after centrifugation; 6, wash with 8M urea; 7, wash with 6M urea; 8, wash with 4M urea; 9, wash with 3M urea; 10, wash with 2M urea; 11, wash 1.5M urea; 12, LMW.

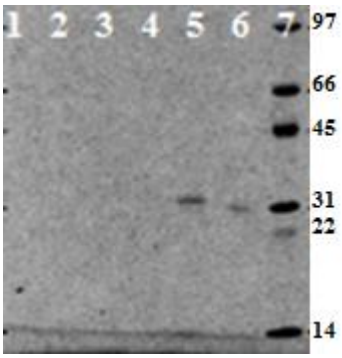

Figure 16. SDS-PAGE showing samples from protein purification version eight showing: 1, wash with 1M urea; 2, wash with 0.5M urea; 3, wash with 0M urea; 4, wash with 20mM imidazole; 5, elution; 6, buffer exchange; 7, LMW.

Incubation of the supernatant overnight after the last centrifugation to the resin, did not improve the concentration of protein in elution. It can also be seen from lane six in figure 15 that protocol version eight, did not result in less loss of his_*Ec*LepB_V77 in the flow through.

Purification of *Ec*LepB_V77_his

Purification protocol version five

Purification of *Ec*LepB_V77_his expressed at 15°C, purified according to protocol version five, resulted in a protein concentration in elution of 0.52mg/ml corresponding to 1.4$mg_{protein}/L_{culture}$. The protein concentration after buffer exchange was 0.32mg/ml corresponding to 1.1$mg_{protein}/L_{culture}$. The SDS-PAGE analysis of the purification can be seen in figures 17 and 18.

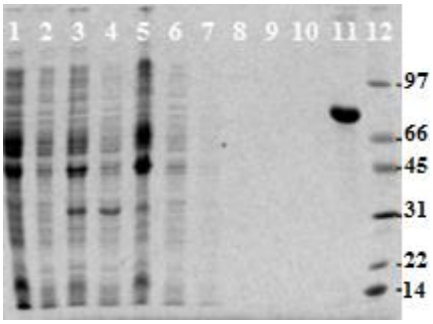

Figure 17. SDS-PAGE showing samples from protein purification version five. 1, supernatant after lysis; 2, wash of pellet after lysis; 3, pellet after wash; 4, supernatant after denaturation; 5, pellet after denaturation; 6, flow through; 7, wash with 8M urea; 8, wash with 6M urea; 9, wash with 4M urea; 10, wash with 3M urea; 11, BSA 0.7mg/ml; 12, LMW.

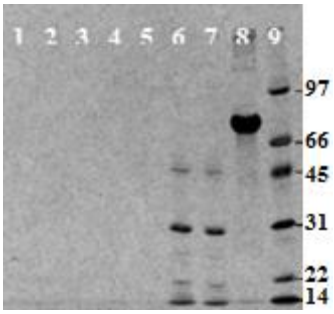

Figure 18. SDS-PAGE showing samples from protein purification version five. 1, wash with 2M urea; 2, wash with 1.5M urea; 3, wash with 1M urea; 4, wash with 0.5M urea; 5, wash with 20mM imidazole; 6, elution; 7, buffer exchange; 8, BSA 0.7mg/ml; 9, LMW.

The purification of *Ec*LepB_V77_his according to protocol version five, did not improve the protein concentration. The elution also seemed to be a bit more impure. The expression of the protein was made in 15°C to investigate the impact on inclusion body formation. From figures 17 and 18 it does not look like the protein expression temperature influences the formation of inclusion bodies. *Ec*LEpB_V77_his is still in inclusion bodies which can be seen in lane three in figure 17.

In lane six in figure 17 it does not look like as much *Ec*LepB_V77_his is lost in the flow through compared to earlier purifications.

When comparing *Ec*LepB_V77_his and his_*Ec*LepB_V77 purified according to protocol version five it looks like the elution is more pure when purifying his_*Ec*LepB_V77. This could be explained by that *Ec*LepB_V77_his is expressed at lower temperature which gives a lower amount of protein. This gives more binding possibilities in the resin for other unwanted proteins and can thus give an impure protein elution.

Purification of 5g of cells with *Ec*LepB_V77_his expressed at 10°C, purified according to protocol version five resulted in a protein concentration in elution of 0.2mg/ml corresponding to 0.4$mg_{protein}/L_{culture}$. The protein concentration after buffer exchange was 0.1mg/ml corresponding to 0.3$mg_{protein}/L_{culture}$. The SDS-PAGE analysis of the purification can be seen in figure 19.

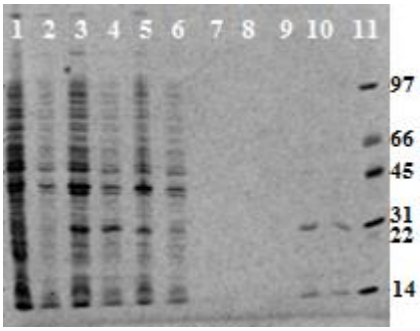

Figure 19. SDS-PAGE showing samples from protein purification version five. 1, supernatant after lysis; 2, wash of pellet after lysis; 3, pellet after wash; 4, supernatant after denaturation; 5, pellet after denaturation; 6, wash with 8M urea; 7, wash with 6M urea; 8, wash with 4M urea; 9, elution; 10, buffer exchange; 11, LMW.

Purification of 10g of cells with *Ec*LepB_V77_his expressed at 10°C, purified according to protocol version five resulted in a protein concentration in elution of 0.15mg/ml corresponding to 0.2$mg_{protein}/L_{culture}$. The expression at 10°C was made to study the impact of expression temperature on inclusion body formation and the impact of the purification. The SDS-PAGE analysis of the purification can be seen in figure 20.

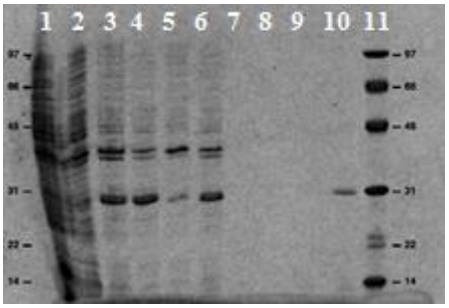

Figure 20. SDS-PAGE showing samples from protein purification version five. 1, supernatant after lysis; 2, wash of pellet after lysis; 3, pellet after wash; 4, supernatant after denaturation; 5, pellet after denaturation; 6, flow through; 7, wash with 8M urea; 8, wash with 6M urea; 9, wash with 20mM imidazole; 10, elution; 11, LMW.

Expressing *Ec*LepB_V77_his at different temperatures did not have an impact on the protein concentration after purification. It also did not have an impact on the loss of *Ec*LepB_V77_his in the flow through.

Purification protocol version nine

Purification of *Ec*LepB_V77_his expressed in BL21 (DE3), purified according to protocol version nine resulted in a protein concentration in elution of 0.04mg/ml corresponding to 0.1$mg_{protein}/L_{culture}$. The protein concentration after buffer exchange was 0.04mg/ml corresponding to 0.1$mg_{protein}/L_{culture}$. The SDS-PAGE analysis of the purification can be seen in figure 21.

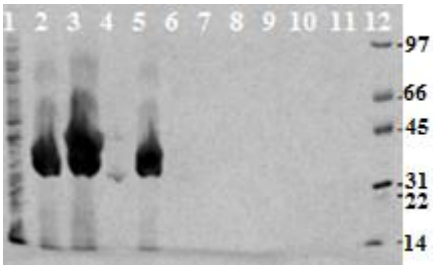

Figure 21. SDS-PAGE showing samples from protein purification version nine. 1, supernatant after lysis; 2, pellet after lysis; 3, supernatant after denaturation; 4, pellet after denaturation; 5, flow through; 6, wash with 6M guanidine hydrochloride; 7, wash with 4M guanidine hydrochloride; 8, wash with 3M guanidine hydrochloride; 9, wash with 20mM imidazole; 10, elution; 11, buffer exchange; 12, LMW.

Changing to guanidine hydrochloride as the denaturant did not improve the amount of protein in the elution. The result of the SDS-PAGE is difficult to interpret but it is probably *Ec*LepB_V77_his that can be seen as the blurry spot in the flow through in lane five.

Purification protocol version ten

Purification of *Ec*LepB_V77_his expressed in BL21 (DE3), purified according to protocol version ten resulted in a protein concentration in elution of 0.6mg/ml corresponding to 1.5$mg_{protein}/L_{culture}$. The protein concentration after buffer exchange was 0.1mg/ml corresponding to 0.3$mg_{protein}/L_{culture}$. The SDS-PAGE analysis of the purification can be seen in figure 22.

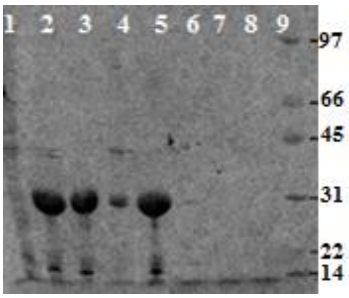

Figure 22. SDS-PAGE showing samples from protein purification version ten. 1, supernatant after lysis; 2, pellet after lysis; 3, supernatant after denaturation; 4, pellet after denaturation; 5, flow through; 6, wash with 6M urea; 7, wash with 20mM imidazole; 8, elution; 9, LMW.

The 8M urea concentration was not the feature that prevented the protein from binding to the resin, since the reduction in urea concentration did not improve the binding of protein to the resin.

The band in lane three in figure 22 indicates that the protein is denatured even though a lower concentration of urea was used. The bands in lane four which represent the non-denatured protein also indicated that a small portion of *Ec*LepB_V77_his was non-denatured.

Purification protocol version eleven

Purification of 10g of cells with *Ec*LepB_V77_his expressed at 4°C, purified according to protocol version eleven resulted in a protein concentration in elution of 0.3mg/ml corresponding to 0.5$mg_{protein}/L_{culture}$. The protein concentration after buffer exchange was

0.1mg/ml corresponding to 0.8$mg_{protein}/L_{culture}$. The SDS-PAGE analysis of the purification can be seen in figure 23.

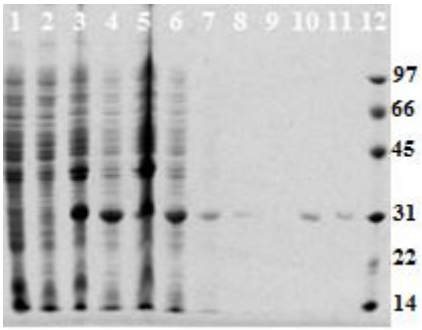

Figure 23. SDS-PAGE showing samples from protein purification version eleven. 1, supernatant after lysis; 2, wash of pellet after lysis; 3, pellet after wash; 4, supernatant after denaturation; 5, pellet after denaturation; 6, flow through; 7, wash with 8M urea; 8, wash with 6M urea; 9, wash with 5mM imidazole; 10, elution; 11, buffer exchange; 12, LMW.

The use of cobalt charged resin instead of nickel charged resin did not improve the protein concentration. Thus it did not have an impact on the purity.

Purification protocol version twelve

Purification of 5g of cells with *Ec*LepB_V77_his, purified according to protocol version twelve resulted in a protein concentration in elution of 0.1mg/ml corresponding to 0.3$mg_{protein}/L_{culture}$. The protein concentration after buffer exchange was 0.1mg/ml corresponding to 0.3$mg_{protein}/L_{culture}$. The SDS-PAGE analysis of the purification can be seen in figure 24.

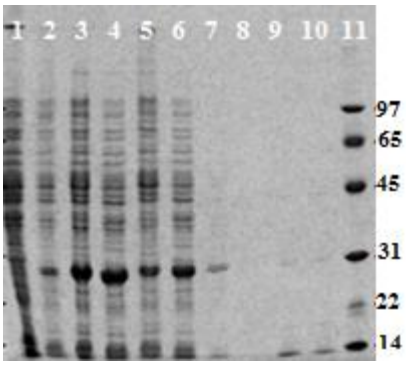

Figure 24. SDS-PAGE showing samples from protein purification version twelve. 1, supernatant after lysis; 2, wash of pellet; 3, pellet after wash; 4, supernatant after denaturation; 5, pellet after denaturation; 6, flow through; 7, wash with 8M urea; 8, wash with 20mM imidazole; 9, elution; 10, buffer exchange; 11, LMW.

Purification of 10g of cells with *Ec*LepB_V77_his, purified according to protocol version twelve resulted in a protein concentration in elution of 0.1mg/ml corresponding to 0.15$mg_{protein}/L_{culture}$. The protein concentration after buffer exchange was 0.06mg/ml corresponding to 0.1$mg_{protein}/L_{culture}$. The SDS-PAGE analysis of the purification can be seen in figure 25.

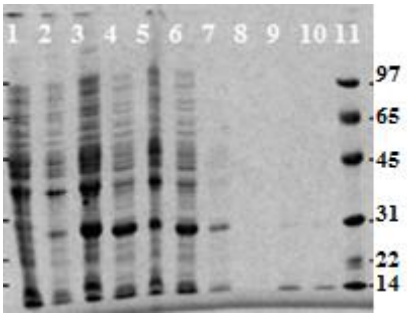

Figure 25. SDS-PAGE showing samples from protein purification version twelve. 1 ,supernatant after lysis; 2, wash of pellet after lysis; 3, pellet after wash; 4, supernatant after denaturation; 5, pellet after denaturation; 6, flow through; 7, wash with 8M urea; 8, wash with 20mM imidazole; 9, elution; 10, buffer exchange; 11, LMW.

The osmotic shock after harvest did not improve the amount of *Ec*LepB_V77_his in the flow through or in the elution. The loss of flow through cannot be explained by the formation of metal chelators. With the assumption that the osmotic shock was successful.

Conclusion concerning the purification optimization
Non off the attempts to increase the amount of protein purified did result in a significant larger amount of protein in the elution. Even though the starting material for the purification was increased it did not affect the amount of purified protein.

Several properties like buffer components and resin used in the purification protocols were changed to study the impact on the purification. The optimization experiments did not result in any improved yield. However, the purification protocol that resulted in the highest amount of protein in elution and still maintained a pure elution was protocol version five.

When comparing the two constructs purified with protocol version five the results show a more pure elution with higher amount of protein, when purifying *Ec*LepB_V77 with an N-terminal his-tag. Something to have in mind when comparing these purifications is that *Ec*LepB_V77_his was expressed at lower temperature. This could affect the amount of protein in the elution.

One possible strategy to purify EcLepB_V77 could be to perform an ion exchange chromatography and refolding on the column. Another potential strategy could be to purify the protein with size exclusion chromatography and refolding with dialysis. The purification would in that case be similar to what has been done earlier (3,6).

Another approach could be to modify the expression to obtain a soluble protein without the need of refolding. It is however unclear how the expression would affect the yield of purification, it needs to be determined empirically.

Activity assay

The samples showed some activity but the triplicates of each sample was uneven and not trustworthy. Comparing the activity between the samples, when the value of the activity was standardized, showed a large variation which also made the result unreliable. The time point for each measurements was also unsteady, which partially could be explained by the low DMSO concentration in the experiment set up. The DMSO in the experiment had its origin

from the substrate stock solution. The low DMSO concentration resulted in an unstable substrate which precipitated during the assay.

What needs to be done to optimize the assay is to do a DMSO tolerance test on *Ec*LepB_V77 to investigate how much DMSO can be used in the assay. This should be made to prevent the substrate from precipitation and get steadier time points. The optimal protein concentration for the assay should also be studied. The protein concentration used should give a linear slope of the activity.

Crystal set up

Crystals were obtained under several conditions. A list of the conditions generating crystals can be seen in table 1.

Table 1. Table showing the conditions resulting in crystals of his_EcLepB_V77.

0.2M Magnesium chloride **0.1M Sodium HEPES** **30% 2-Propanol** **pH 7.5**
0.1M Sodium PIPES **pH 7**
0.1M CHES(2-(Cyclohexylamino)ethanesulfonic acid) **5% Glycerol** **pH 9**
1M Sodium acetate **0.1M imidazole** **pH 6.5**
0.2M Sodium chloride **0.1M Sodium acetate** **30% 2 Methyl 2.4-pentanediol** **pH 4.6**
1M di-ammonium hydrogen phosphate **0.1M Sodium acetate** **pH 4.5**
0.2M ammonium dihydrogen phophate **0.1M Tris** **50%2 Methyl 2.4-pentanediol** **pH 8.5**

At the ESRF, some of the loops were empty. The remaining crystals did not diffract. Because of no diffraction it is difficult to determine if the crystal is made of salt or protein. Experiments using the conditions listed should therefore be tested again.

Acknowledgements

First and foremost I want to give gratitude to Sherry Mowbray for being my supervisor, thanks for all the advice and guidance. I am thankful for all the help from Lu Lu, who have been my preceptor in the laboratory, thank you for teaching me a lot. Thanks to Alwyn Jones for all the good advice in the meetings. I want to thank Sanjeewani Sooriyaarachchi and Annette Roos for always being helpful and answering my questions. Thanks to Mikael

Widersten for being my scientific reviewer. Last but not least, thanks to my boyfriend and family for always being supportive.

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Appendix I

Expression

The expression of his_*Ec*LepB_V77 expressed in C43 (DE3) competent cells is shown in figure 26.

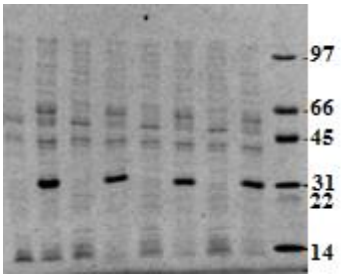

Figure 26. Expression of his_EcLepB_V77 in C43 (DE3) competent cells in four 1 liter cultures. First lane is before induction and second line is after induction, for all pairs of lanes corresponding to a 1 liter culture. The last lane is LMW.

The expression of *Ec*LEpB_V77_his expressed in C43 (DE3) competent cells is shown in figure 27.

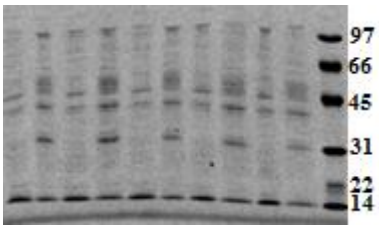

Figure 27. Expression of EcLepB_V77_his in C43 (DE3) competent cells in five 1 liter cultures. First lane is before induction and second line is after induction induction, for all pairs of lanes corresponding to a 1 liter culture. The last lane is LMW.

The expression of *Ec*LepB_V77_his expressed in C43 (DE3) competent cells at 15°C is shown in figure 28.

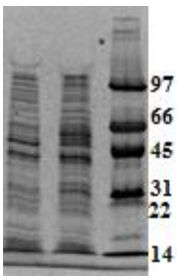

Figure 28. Expression of EcLepB_V77_his in C43 (DE3) competent cells in a 1 l culture at 15°C. First lane is before induction and second line is after induction. The third lane is LMW.

The expression of *Ec*LepB_V77_his expressed in C43 (DE3) and BL21 (DE3) competent cells and his_*Ec*LepB_V77 expressed in BL21 (DE3) is shown in figure 29.

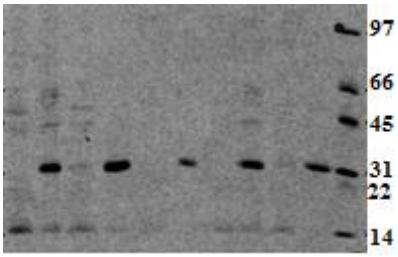

Figure 29. Expression of EcLepB_V77_his in C43 (DE3), EcLepB_V77_his in BL21 (DE3) and his_EcLepB_V77 in BL21 (DE3). 1 & 2 and 7 & 8 EcLepB_V77_his in C43 before and after induction, 3 &4 and 5& 6EcLepB_V77_his in BL21 (DE3) before and after induction, 9 & 10 His_EcLepB_V77 in BL21 (DE3) before and after induction,11 LMW.

The expression of *Ec*LepB_V77_his expressed in C43 (DE3) competent cells at 10°C is shown in figure 30.

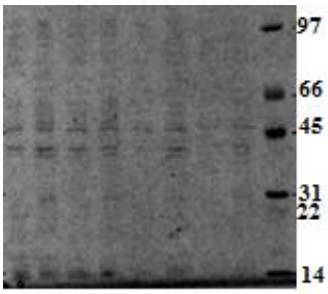

Figure 30. Expression of EcLepB_V77_his in C43 (DE3) competent cells in four 1 liter cultures at 10°C. First lane is before induction and second line is after induction, for all pairs of lanes corresponding to a 1 liter culture. The last lane is LMW.

The expression of *Ec*LepB_V77_his expressed in C43 (DE3) competent cells at 4°C is shown in figure 31.

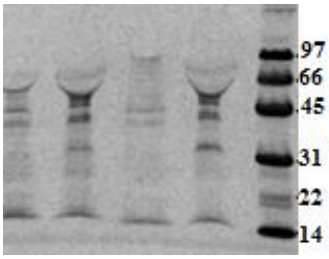

Figure 31. Expression of EcLepB_V77_his in C43 (DE3) competent cells in two 1 liter cultures at 4°C. First lane is before induction and second line is after induction, for all pairs of lanes corresponding to a 1 liter culture. The last lane is LMW.

Purification protocols

The original purification protocol

The cells were resuspended in 6ml/g_{pellet} of buffer A. DNase was added to the sample before lysis. The cells were lysed by cell disruptor two times at 2 bar. The sample was centrifugated at 17210xg in Sorvall RC6 for 10 minutes. The protein in pellet was denatured in buffer B for two hours at room temperature. The sample was then centrifugated at 43150xg in Sorvall RC6 for 20 minutes. The supernatant after centrifugation was added to the column with 1 ml nickel charged resin, and incubated in 4°C for one hour. After incubation, the column was washed with buffer B. To perform the refolding of protein, Buffer C was added to the column and incubated at 4°C for one hour. The column was then washed with buffer C. The protein was eluted with buffer D.

Buffer A
5mM $MgCl_2$ (203.30g/mol, Merck)
20mM Tris-HCl (121.14g/mol, Merck)
7mM β- mercaptoethanol added fresh (14M stock solution, Sigma Aldrich)
pH 7.4

Buffer B
6M guanidine hydrochloride (95.53g/mol, Sigma Aldrich)
in buffer A
pH 7.5

Buffer C
0.5% Triton ™X-100(100%, Sigma Aldrich)
in buffer A

Buffer D
250mM imidazole (68.08g/mol, Merck)
in buffer C

The imidazole was removed from the sample by the use of a 10DG desalting column from Biorad, where the protein was eluted with buffer C.

Purification protocol version two

The cells were resuspended in 6ml/g_{pellet} of buffer A. DNase was added to the sample before lysis. The cells were lysed by cell disruptor two times at 2 bar. The sample was centrifugated at 17210xg in Sorvall RC6 for 10 minutes. The protein in pellet was denatured in buffer B for one hour at room temperature. The sample was then centrifugated at 43150xg in Sorvall RC6 for 20 minutes. The supernatant after centrifugation was added to the column with 1 ml nickel charged resin, and incubated in 4°C for two hour. After incubation, the column was washed with buffer B. To perform the refolding of protein, Buffer C was added to the column and incubated at 4°C for one hour. The column was then washed with buffer D. The protein was eluted with buffer E.

The imidazole was removed from the sample by the use of a 10DG desalting column from Biorad, where the protein was eluted with buffer C.

Buffer A
5mM $MgCl_2$ (203.30g/mol, Merck)
20mM Tris-HCl (121.14g/mol, Merck)
7mM β- mercaptoethanol added fresh (14M stock solution, Sigma Aldrich)
pH 7.4

Buffer B
6M guanidine hydrochloride (95.53g/mol, Sigma Aldrich)
in buffer A
pH 7.5

Buffer C
0.5% Triton ™X-100 (100%, Sigma Aldrich)
in buffer A

Buffer D
20mM imidazole (68.08g/mol, Merck)
in buffer C

Buffer E
250mM imidazole (68.08g/mol, Merck)
in buffer C

Purification protocol version three

The cells were resuspended in 6ml/g_{pellet} of buffer A together with protease inhibitor cocktail. DNase was added to the sample before lysis. The cells were lysed by cell disruptor two times at 2 bar. The sample was centrifugated at 17210xg in Sorvall RC6 for 10 minutes. The protein in pellet was denatured in buffer B for one hours at room temperature. The sample was then centrifugated at 43150xg in Sorvall RC6 for 20 minutes. The supernatant after centrifugation was added to a column with 1 ml nickel charged resin, and incubated in 4°C for two hour. After incubation, the column was washed with buffer B. To perform the refolding of protein, Buffer C was added to the column and incubated at 4°C for one hour. The column was then washed with buffer D. The protein was eluted with buffer E.

The imidazole was removed from the sample by the use of a 10DG desalting column from Biorad, where the protein was eluted with buffer C.

Buffer A
5mM $MgCl_2$ (203.30g/mol, Merck)
20mM Tris-HCl (121.14g/mol, Merck)
300mM NaCl (58.44g/mol, VWR chemicals)
7mM β- mercaptoethanol added fresh (14 stock solution, Sigma Aldrich)
pH 7.4

Buffer B
6M guanidine hydrochloride (95.53g/mol, Sigma Aldrich)
in buffer A
pH 7.4

Buffer C
0.5% Triton ™X-100 (100%, Sigma Aldrich)
in buffer A

Buffer D
20mM imidazole (68.08g/mol, Merck)
in buffer C

Buffer E
250mM imidazole (68.08g/mol, Merck)
in buffer C

Purification protocol version four

The cells were resuspended in 6ml/g_{pellet} of buffer A together with protease inhibitor cocktail. DNase was added to the sample before lysis. The cells were lysed by cell disruptor two times at 2 bar. The sample was centrifugated at 17210xg in Sorvall RC6 for 10 minutes. The pellet after lysis was resuspended in buffer C followed by centrifugation at 17210xg in Sorvall RC6 in 10 minutes. The protein in pellet was denatured in buffer B for one hours at room temperature. The sample was then centrifugated at 43150xg in Sorvall RC6 for 20 minutes. The supernatant after centrifugation was added to a column with 1 ml nickel charged resin, and incubated in room temperature for two hour. After incubation, the column was washed with buffer B. To perform the refolding of protein, Buffer C was added to the column and incubated at 4°C one hour. The column was then washed with buffer D. The protein was eluted with buffer E.

The imidazole was removed from the sample by the use of a a 10DG desalting column from Biorad, where the protein was eluted with buffer C.

Buffer A
5mM $MgCl_2$ (203.30g/mol, Merck)
20mM Tris-HCl (121.14g/mol, Merck)
300mM NaCl (58.44g/mol, VWR chemicals)
5% glycerol
10mM β- mercaptoethanol added fresh (14M stock solution, Sigma Aldrich)
pH 7.4

Buffer B
8M Urea (60.06g/mol, Merck)
in buffer A
pH 7.5

Buffer C
0.5% Triton ™X-100 (100%, Sigma Aldrich)
in buffer A

Buffer D
20mM imidazole (68.08g/mol, Merck)
in buffer C

Buffer E
250mM imidazole (68.08g/mol)
in buffer C

Purification protocol version five

The cells were resuspended in 6ml/g_{pellet} of buffer A together with protease inhibitor cocktail. DNase was added to the sample before lysis. The cells were lysed by cell disruptor two times at 2 bar. The sample was centrifugated at 17210xg in Sorvall RC6 for 10 minutes. The pellet after lysis was resuspended in buffer C followed by centrifugation at 17210xg in Sorvall RC6 in for 10 minutes. The protein in pellet was denatured in buffer B for one hours at room temperature. The sample was then centrifugated at 43150xg in Sorvall RC6 for 20 minutes. The supernatant after centrifugation was added to a column with 1 ml nickel charged resin, and incubated in room temperature for one hour. After incubation, the column was washed with buffer B. To perform the refolding of protein, a stepwise wash with urea gradient was used. The urea grdient was 8M, 6M, 4M, 3M, 2M, 1.5M, 1M, 0.5M, 0M urea. The column was then washed with buffer D. The protein was eluted with buffer E.

Buffer A
5mM $MgCl_2$(203.30g/mol, Merck)
20mM Tris-HCl (121.14g/mol, Merck)
300mM NaCl (58.44g/mol, VWR chemicals)
5% v/v glycerol (92.02g/mol, VWR chemicals)
10mM β- mercaptoethanol added fresh (14M stock solution, Sigma Aldrich)
pH 7.4

Buffer B
8M urea (60.06g/mol, VWR chemicals)
in buffer A
pH 7.5

Buffer C
0.5% Triton ™X-100(100%, Sigma Aldrich)
in buffer A

Buffer D
20mM imidazole (68.08g/mol, Merck)
in buffer A

Buffer E
250mM imidazole (68.08g/mol, Merck)
in buffer A

The imidazole was removed from the sample by the use of a PD-10 desalting column from GE Healthcare, where the protein was eluted with buffer A.

Purification protocol version six

The cells were resuspended in 6ml/g_{pellet} of buffer C together with protease inhibitor cocktail. DNase was added to the sample before lysis. The cells were lysed by cell disruptor two times at 2 bar. The sample was centrifugated at 17210xg in Sorvall RC6 for 10 minutes. The protein in pellet was denatured in buffer B for one hours at room temperature. The sample was then centrifugated at 43150xg in Sorvall RC6 for 20 minutes. The supernatant after centrifugation was added to a column with 1 ml nickel charged resin, and incubated in room temperature for one hour. After incubation, the column was washed with buffer B. To perform the refolding of protein, a stepwise wash with urea gradient was used. The urea grdient was 8M, 6M, 4M, 3M, 2M, 1.5M, 1M, 0.5M, 0M urea. The column was then washed with buffer D. The protein was eluted with buffer E.

The imidazole was removed from the sample by the use of a PD-10 desalting column from GE Healthcare, where the protein was eluted with buffer A.

Buffer A
5mM $MgCl_2$(203.30g/mol, Merck)
20mM Tris-HCl (121.14g/mol, Merck)
300mM NaCl (58.44g/mol, VWR chemicals)
5% v/v glycerol (92.02g/mol, VWR chemicals)
10mM β- mercaptoethanol added fresh (14M stock solution, Sigma Aldrich)
pH 7.4

Buffer B
8M urea (60.06g/mol, VWR chemicals)
in buffer A
pH 7.5

Buffer C
0.5% Triton ™X-100(100%, Sigma Aldrich)
in buffer A

Buffer D
20mM imidazole (68.08g/mol, Merck)
in buffer A

Buffer E
250mM imidazole (68.08g/mol, Merck)
in buffer A

Purification protocol version seven

The cells were resuspended in 6ml/g_{pellet} of buffer A together with protease inhibitor cocktail. DNase was added to the sample before lysis. The cells were lysed by cell disruptor two times at 2 bar. The sample was centrifugated at 17210xg in Sorvall RC6 for 10 minutes. The protein in pellet was denatured in buffer B for one hours at room temperature. The sample was then centrifugated at 43150xg in Sorvall RC6 for 20 minutes. The supernatant after centrifugation was added to a column with 1 ml nickel charged resin, and incubated in room temperature for one hour. After incubation, the column was washed with buffer B. To perform the refolding of protein, a stepwise wash with urea gradient was used. The urea grdient was 8M, 6M, 4M, 3M, 2M, 1.5M, 1M, 0.5M, 0M Urea. The column was then washed with buffer C. The protein was eluted with buffer D.

The imidazole was removed from the sample by the use of a PD-10 desalting column from GE Healthcare, where the protein was eluted with buffer A.

Buffer A
5mM $MgCl_2$ (203.30g/mol, Merck)
20mM Tris-HCl (121.14g/mol, Merck)
300mM NaCl (58.44g/mol, VWR chemicals)
5% glycerol (92.09g/mol, VWR chemicals)
10mM β- mercaptoethanol added fresh (14M stock solution, Sigma Aldrich)
pH 7.4

Buffer B
8M urea (60.06g/mol, VWR chemicals)
in buffer A
pH 7.5

Buffer C
20mM imidazole (68.08g/mol, Merck)
0.1% triton (100%, Sigma Aldrich)
in buffer A

Buffer D
250mM imidazole (68.08g/mol, Merck)
0.1% triton (100%, Sigma Aldrich)
in buffer A

Buffer E
0.1% triton (100%, Sigma Aldrich)
in buffer A

Purification protocol version eight

The cells were suspended in 6ml/g_{pellet} of buffer A together with protease inhibitor cocktail. DNase was added to the sample before lysis. The cells were lysed by cell disruptor two times at 2 bar. The sample was centrifuged at 17210xg in Sorvall RC6 for 10 minutes. The pellet after lysis was resuspended in buffer C followed by centrifugation 17210xg in Sorvall RC6 in 10 minutes. The protein in pellet was denatured in buffer B for one hours at room temperature. The sample was then centrifuged at 43150xg in Sorvall RC6 for 20 minutes. The supernatant after centrifugation was added to a column with 1 ml nickel charged resin, and incubated in room temperature overnight. After incubation, the column was washed with buffer B. To perform the refolding of protein, a stepwise wash with urea gradient was used. The urea gradient was 8M, 6M, 4M, 3M, 2M, 1.5M, 1M, 0.5M, 0M urea. The column was then washed with buffer D. The protein was eluted with buffer E.

The imidazole was removed from the sample by the use of a PD-10 desalting column from GE Healthcare, where the protein was eluted with buffer F.

Buffer A
5mM $MgCl_2$ (203.30g/mol, Merck)
20mM Tris-HCl (121.14g/mol, Merck)
300mM NaCl (58.44g/mol, VWR chemicals)
5% glycerol (92.09g/mol, VWR chemicals)
10mM β- mercaptoethanol added fresh (14M stock solution, Sigma Aldrich)
pH 7.4

Buffer B
8M urea (60.06g/mol, VWR chemicals)
in buffer A
pH 7.5

Buffer C
0.5% triton (100%, Sigma Aldrich)
in buffer A

Buffer D
20mM imidazole (68.08g/mol, Merck)
0.1% triton (100%, Sigma Aldrich)
in buffer A

Buffer E
250mM imidazole (68.08g/mol, Sigma Aldrich)
0.1% triton (100%, Sigma Aldrich)
in buffer A

Buffer F
0,1% triton
in buffer A

Purification protocol version nine

The cells were resuspended in 6ml/g_{pellet} of buffer C together with protease inhibitor cocktail. DNase was added to the sample before lysis. The cells were lysed by cell disruptor two times at 2 bar. The sample was centrifugated at 17210xg in Sorvall RC6 for 10 minutes. The pellet after lysis was resuspended in buffer C followed by centrifugation 17210xg in Sorvall in 10 minutes. The protein in pellet was denatured in buffer B for one hours at room temperature. The sample was then centrifugated at 43150xg in Sorvall RC6 for 20 minutes. The supernatant after centrifugation was added to a column with 0.5 ml nickel charged resin, and incubated in room temperature one hour. After incubation, the column was washed with buffer B. To perform the refolding of protein, a stepwise wash with urea gradient was used. The guanidine hydrochloride gradient was 6M, 4M, 3M, 2M, 1.5M, 1M, 0.5M, 0M guanidine hydrochloride. The column was then washed with buffer D. The protein was eluted with buffer E.

The imidazole was removed from the sample by the use of a PD-10 desalting column from GE Healthcare, where the protein was eluted with buffer F.

Buffer A
5mM $MgCl_2$ (203.30g/mol, Merck)
20mM Tris-HCl (121.14g/mol, Merck)
300mM NaCl (58.44g/mol, VWR chemicals)
5% glycerol (92.09g/mol, VWR chemicals)
10mM β- mercaptoethanol added fresh (14M stock solution, Sigma Aldrich)
pH 7.4

Buffer B
6M guanidine hydrochloride (95.53g/mol, Sigma Aldrich)
in buffer A
pH 7.5

Buffer C
0.5% Triton ™X-100 (100%, Sigma Aldrich)
in buffer A

Buffer D
20mM imidazole (68.08g/mol, Merck)
0.1% triton (100%, Sigma Aldrich)
in buffer A

Buffer E
250mM imidazole (68.08g/mol, Merck)
0.1% triton (100%, Sigma Aldrich)
in buffer A

Buffer F
0.1% triton
in buffer A

Purification protocol version ten

The cells were resuspended in 6ml/g_{pellet} of buffer C together with protease inhibitor cocktail. DNase was added to the sample before lysis. The cells were lysed by cell disruptor two times at 2 bar. The sample was centrifugated at 17210xg in Sorvall RC6 for 10 minutes. The protein in pellet was denatured in buffer B for one hours at room temperature. The sample was then centrifugated at 43150xg in Sorvall RC6 for 20 minutes. The supernatant after centrifugation was added to a column with 0.5 ml nickel charged resin, and incubated in room temperature one hour. After incubation, the column was washed with buffer B. To perform the refolding of protein, a stepwise wash with urea gradient was used. The urea gradient was 6M, 4M, 3M, 2M, 1.5M, 1M, 0.5M, 0M urea. The column was then washed with buffer D. The protein was eluted with buffer E.

The imidazole was removed from the sample by the use of a a PD-10 desalting column from GE Healthcare, where the protein was eluted with buffer F.

Buffer A
5mM $MgCl_2$ (203.30g/mol, Merck)
20mM Tris-HCl (121.14g/mol, Merck)
300mM NaCl (58.44g/mol, VWR chemicals)
5% glycerol (92.09g/mol, VWR chemicals)
10mM β- mercaptoethanol added fresh (14M stock solution, Sigma Aldrich)
pH 7.4

Buffer B
6M urea (60.06g/mol, VWR chemicals)
in buffer A
pH 7.4

Buffer C
0.5% Triton ™X-100 (100%, Sigma Aldrich)
in buffer A

Buffer D
20mM imidazole (68.08g/mol, Merck)
0.1% triton (100%, Sigma Aldrich)
in buffer A

Buffer E
250mM imidazole (68.08g/mol, Sigma Aldrich
0.1% triton (100%, Sigma Aldrich)
in buffer A

Buffer F
0.1% triton (100%, Sigma Aldrich)
in buffer A

Purification protocol version eleven

The cells were resuspended in 6ml/g_{pellet} of buffer A together with protease inhibitor cocktail. DNase was added to the sample before lysis. The cells were lysed by cell disruptor two times at 2 bar. The sample was centrifugated at 17210xg in Sorvall RC6 for 10 minutes. The pellet after lysis was resuspended in buffer C followed by centrifugation 17210xg in Sorvall RC6 in 10 minutes. The protein in pellet was denatured in buffer B for one hours at room temperature. The sample was then centrifugated at 43150xg in Sorvall RC6 for 20 minutes. The supernatant after centrifugation was added to a column with 0.5 ml cobolt charged resin, and incubated in room temperature one hour. After incubation, the column was washed with buffer B. To perform the refolding of protein, a stepwise wash with urea gradient was used. The urea gradient was 8M, 6M, 4M, 3M, 2M, 1.5M, 1M, 0.5M, 0M urea. The column was then washed with buffer D. The protein was eluted with buffer E.

The imidazole was removed from the sample by the use of a PD-10 desalting column from GE Healthcare, where the protein was eluted with buffer F.

Buffer A
5mM $MgCl_2$ (203.30g/mol, Merck)
20mM Tris-HCl (121.14g/mol, Merck)
300mM NaCl (58.44g/mol, VWR chemicals)
5% glycerol (92.09g/mol, VWR chemicals)
5mM β- mercaptoethanol added fresh (14M stock solution, Sigma Aldrich)
pH 7.4

Buffer B
6M urea (60.06g/mol, VWR chemicals)
in buffer A
pH 7.4

Buffer C
0.5% Triton ™X-100 (100%, Sigma Aldrich)
in buffer A

Buffer D
5mM imidazole (68.08g/mol, Merck)
0.1% triton (100%, Sigma Aldrich)
in buffer A

Buffer E
200mM imidazole (68.08g/mol, Sigma Aldrich
0.1% triton (100%, Sigma Aldrich)
in buffer A

Buffer F
0.1% triton (100%, Sigma Aldrich)
in buffer A

Purification protocol version twelve

The cells were resuspended in 6ml/g_{pellet} of buffer A together with protease inhibitor cocktail. DNase was added to the sample before lysis. The cells were lysed by cell disruptor two times at 2 bar. The sample was centrifugated at 17210xg in Sorvall RC6 for 10 minutes. The pellet after lysis was resuspended in buffer C followed by centrifugation 17210xg in Sorvall RC6 in 10 minutes. The protein in pellet was denatured in buffer B for one hours at room temperature. The sample was then centrifugated at 43150xg in Sorvall RC6 for 20 minutes. The supernatant after centrifugation was added to a column with 0.5 ml nickel charged resin, and incubated in room temperature one hour. After incubation, the column was washed with buffer B. To perform the refolding of protein, a stepwise wash with urea gradient was used. The urea gradient was 8M, 6M, 4M, 3M, 2M, 1.5M, 1M, 0.5M, 0M urea. The column was then washed with buffer D. The protein was eluted with buffer E.

The imidazole was removed from the sample by the use of a PD-10 desalting column from GE Healthcare, where the protein was eluted with buffer F.

Buffer A
5mM $MgCl_2$ (203.30g/mol, Merck)
20mM Tris-HCl (121.14g/mol, Merck)
300mM NaCl (58.44g/mol, VWR chemicals)
5% glycerol (92.09g/mol, VWR chemicals)
10mM β- mercaptoethanol added fresh (14M stock solution, Sigma Aldrich)
pH 7.4

Buffer B
8M urea (60.06g/mol, VWR chemicals)
in buffer A
pH 7.5

Buffer C
0.5% triton (100%, Sigma Aldrich)
in buffer A

Buffer D
20mM imidazole (68.08g/mol, Merck)
0.1% triton (100%, Sigma Aldrich)
in buffer A

Buffer E
250mM imidazole (68.08g/mol, Sigma Aldrich)
0.1% triton (100%, Sigma Aldrich)
in buffer A

Buffer F
0.1% triton
in buffer A