Purification of the recombinant SAD-C protein from Pisum sativum (pea)

Author: Johanna Mattsson
Supervisors: Irina Kalbin and Elin Grahn
Examiner: Åke Strid
Abstract

*SAD-C*, a gene belonging to the small short-chain alcohol dehydrogenase-like protein (SAD) gene family, is up-regulated in *Pisum sativum* (pea) when the plant is exposed to UV-B (280-320 nm) radiation. SAD-C has a molecular weight of about 28 kDa and adopts a tetrameric structure. The aim of this work was to purify the protein SAD-C from *Pisum sativum* when overexpressed in *E. coli* strain BL21 Star™ (DE3) One Shot®. The purification was facilitated by the presence of a His-tag consisting of six histidine residues at the C-terminal end of the protein. The purification trials of SAD-C were faced with problems since the sample fractions contained several other proteins as well. Several purification steps seem to be necessary for future trials. A crystallization trial was still set up and crystals were formed, but the crystals formed were probably not of SAD-C.
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Introduction

An increase in solar ultraviolet-B (UV-B; 280 -320 nm) radiation is a consequence of the increasing depletion of the stratospheric ozone shield caused by artificial air pollutants [1]. This increase is potentially harmful to all living organisms, but especially to plants because they are in constant need of sunlight in order to survive.

As a result of a low intensity UV-B treatment, as well as other abiotic stresses, the small short-chain alcohol dehydrogenase-like protein (SAD) gene family is up-regulated in pea (Pisum sativum) [2,3]. This family consist of at least three members; SAD-A, SAD-B and SAD-C. SAD-A and SAD-C are strongly up-regulated, while SAD-B increases to a smaller extent when exposed to UV-B radiation [2]. If these genes are compared with other UV-B regulated genes, such as for example the genes coding for chalcone synthase (CHS) and ammonia lyase (PAL), the SAD genes are activated at considerably lower UV-B levels (0,08 kJ m⁻²). This implies a role for these proteins at an early stage in stress responses in plants [2]. This indicates as well that the SAD promoter is the place where several signaling pathways are integrated to generate a response to stress stimuli [4]. The SAD sequences have a high degree of homology in common and they contain an amount of cis-elements, which may be associated to their function. An 11-bp GC-rich binding site for regulatory proteins has been detected in the promoters for SAD-A and SAD-C genes. This region has features in common with other known classes of cis-elements involved in stress-correlated regulation of gene expression in plants, and may explain the responsiveness of the SAD genes to multiple stresses [4].

Coiling of pea tendrils is an effect seen when the plants are exposed to UV-B radiation [5], however the SAD protein content in tendrils does not increase [3]. UV-B radiation does on the other hand increase the SAD protein content in pea epidermal and sub-epidermal cells in leaves and stems. This indicates that increased expression of SAD genes is restricted to the exposed surface [3].

The three SAD proteins belong to the large short-chain dehydrogenase/reductase superfamily (SDR) [7]. The SDR-enzymes have about 250 residue subunits and are catalyzing NAD(P)(H)-dependent oxidation/reduction reactions [8]. The N-terminal region binds the coenzymes NAD(H) or NADP(H), while the C-terminal region binds the substrate.

At present the SDR superfamily includes about 3000 proteins in sequence databases, and the corresponding genomes represent all forms of life, from bacteria to humans [9]. The substrates are ranging from alcohols, steroids, aromatic compounds and sugars to xenobiotics [8]. The SDRs have been divided into five families; classical, extended, intermediate, divergent and complex, where the classical and extended families are the largest.

The SAD proteins are found in stems, flowers, leaves, tendrils, roots, and pea seeds. The wide distribution of SAD in pea also indicates a second possible role for the proteins; involvement in plant development [3].

The pea SAD proteins are very similar to each other [2]. The SAD-C contains the same number of amino acids (268) as SAD-A, and 265 of these are identical. SAD-B has larger differences compared to these two isoforms. A deletion of 36 bases removes one-half of the nucleotide binding site in the N-terminal region of the protein. This region is probably encoding an α-helix [2].
SAD-C has a molecular weight of $28170\pm1$ Da and adopts a tetrameric structure when recombinant [3]. SAD-C purified after overexpression in *E. coli* forms stable homodimers [2]. SAD-C has a quinone-reducing activity where the predominant cofactor is NADH, but the true substrate is still to be identified [3].

The protein structure of SAD-C has not yet been determined, neither its correct substrate [3]. Because of this the correct function for SAD-C is still unknown. Previous attempts to produce recombinant SAD proteins have among other things resulted in formation of inclusion bodies [3].

When the protein was expressed from the pET8c cloning vector during a previous trial to crystallize the protein, six histidines were added to the N-terminal end of translated recombinant proteins. Later crystals were formed but the result gave too low resolution. The His-tag on the N-terminal end was suggested to be the reason why the crystallization failed. Prior work has transformed the cloned vector pET101/D-TOPO® into One Shot® TOP10 Chemically Competent *E. Coli* strain BL21 Star™ (DE3). This strain contains the lambda DE3 lysogen which carries the gene for T7 RNA polymerase. When the inductor isopropyl β-D-1-thiogalactopyranoside (IPTG) is added, expression of T7 RNA polymerase from the promoter lacUV5 is started. The T7 RNA polymerase has an extremely high activity, and some basal level expression of the gene of interest will occur [11].

The cloning of the TOPO vector was accomplished and two fusion markers at the C-terminal end of the SAD-C protein was added; a V5-tag and His-tag consisting of six histidines. The vector also contained a carbenicillin resistance gene.

The aims of this work were to purify the protein SAD-C from *Pisum sativum* when overexpressed in chemically competent *E. coli* strain BL21 Star™ (DE3) One Shot® and to attempt to crystallize it. By crystallizing the protein the structure can be determined. The long term aim is to find the correct function for SAD-C and its regulation mechanisms.
Materials and methods

Bacteria: Chemically competent *E. coli* strain BL21 Star<sup>TM</sup> (DE3) One Shot<sup>®</sup> (Invitrogen, Carlsbad, CA, USA) already containing the vector pET101/D-TOPO<sup>®</sup> (Invitrogen, Carlsbad, CA, USA) with the *SAD*-C gene were used. Two fusion markers at the C-terminal end of the SAD-C protein had been added; a V5-tag and His-tag consisting of six histidines. The vector also contained a carbenicillin resistance gene. The plasmid had been transformed and sequenced by scientists at Örebro University. The molecular weight of the protein is increased by 3 kDa when the two tags are linked to the protein.

Antibodies: *SAD*-C specific primary antibodies produced by Davids Biotechnologie (Regensburg, Germany) and secondary antibodies anti-rabbit IgG Alkaline Phosphatase from Sigma were used.

The purification of SAD-C was performed several times with small changes during the different trials to optimize the purification method.

Trial 1

**Protein purification**

Disruption of the cells:

Purification of the recombinant SAD-C protein was faciliated by the presence of a polyhistidine tag consisting of six histidine residues at the C-terminal end of the protein. The bacteria used had previously been overexpressed and harvested by scientist at Örebro University and were stored in -20°C. The cells were broken using an X-press (AB Biox, Göteborg, Sweden). The X-press is an instrument for disintegrating tissue and cells. By disrupting the walls of micro-organisms and other cells makes it possible to release and disperse the cellular contents and to separate soluble materials from particulate matter. The disrupted cells were mixed with 24 ml equilibrium buffer (20 mM Tris-HCl, 500 mM sodium chloride, 5 mM L-Histidin, 1 mM PMSF, [pH 8.0]) and 600 μl 40 mM PMSF. PMSF (phenylmethylsulphonyl fluoride) is a protease inhibitor, which prevents the protein from being degraded. PMSF is degraded rapidly, and can only be added to the solutions just prior to use. DNase (deoxyribonuclease) (DNase I from bovine pancreas Grade II) was also added to the cells. DNase is an enzyme that degrades DNA. The cells were then sonicated (Bandelin Sonoplus Ultrasonic Homogenizers) in three cycles with power 70 % until the solution was smooth. Each cycle lasted for two minutes. Sonication disrupts the cells and homogenizes mixtures. Then the cells were centrifuged in an ultracentrifuge to separate cytoplasmatic and membrane fractions and to remove unbroken cells. (Beckman Optima<sup>™</sup> L-90 K Ultracentrifuge, Ti 70, 45 000 rpm, 45 min, 4ºC).

Incubation with Ni-NTA:

The supernatant from the ultracentrifuge was then mixed with HIS-Select Nickel Affinity Gel (Sigma, St. Louis, USA) (Ni-NTA). The Ni-NTA had previously been placed in six eppendorf-tubes, each containing 2 ml of Ni-NTA and the tubes were then centrifuged for 1 min at 5000xg. The supernatant was then removed and 1 ml equilibrium buffer was added to each tube, and the content in the tubes were mixed and centrifuged for 1 min at 5000xg. This
The supernatant from the ultracentrifuge of the cells was incubated with Ni-NTA and kept on a roller at 4°C over night. During the incubation the SAD-C proteins with six histidine residues at the C-terminal attached to the nickel ions in the HIS-Select Nickel Affinity Gel.

**Chromatography:**
The incubated solution containing Ni-NTA was transferred to a chromatography column with a filter to keep the gel with the nickel particles in place. The chromatography was performed at 4°C. The column was washed two times with 20 ml wash buffer (20 mM Tris-HCl, 500 mM sodium chloride, 5 mM L-Histidine, 1 mM PMSF, [pH 8.0]), and then the proteins were eluted with 20 ml elution buffer (20 mM Tris-HCl, 500 mM sodium chloride, 150 mM L-Histidine, [pH 8.0]) and collected in four 5 ml fractions. The flow through and wash fractions were collected as well and the purity of the proteins were studied by performing a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on the different fractions.

**SDS-PAGE:**
The different protein samples were dissolved in 10 μl 2x loading buffer SDS-PAGE (50 mM Tris, [pH 6.8], 2% SDS, 20% Glycerol, 0.02% Bromophenol Blue and 100 mM DTT and 2 μl 1 M DTT. DTT (Dithiothreitol) is used to reduce the disulfide bonds between proteins and to prevent intramolecular and intermolecular disulfide bonds from forming between cysteine residues of proteins. The samples were then boiled for 5 minutes. The samples were separated on a 15 % Tris-HCl gel (Ready-Gel, Bio-Rad, CA) for 60 minutes at 150 V. As a size marker 10 μl Prestained Protein Ladder Plus from Fermentas Life Sciences PageRuler™ was used.

SDS-PAGE maintains polypeptides in a denatured state. This allows separation of proteins by their molecular weight. The proteins become covered in the negatively charged SDS and move to the positively charged electrode through the acrylamide gel. Smaller proteins migrate faster through the gel and the proteins are thus separated according to size.

When the electrophoresis was performed 1xSDS-PAGE (24.8 mM Tris, 86.2 mM Glycine and 3.4 mM SDS) buffer was used. After the electrophoresis the gel was stained with Coomassie Brilliant Blue for 1 hour, and the gel was then washed with a solution containing 30% methanol, 10% acetic acid and water to remove surplus stain. The wash was accomplished three times, each time lasting for 30 minutes.

**Concentrating the protein samples**

The protein samples were concentrated by using Amicon-Ultra Centrifugal Filtering Device (Millipore, Billerica, MA, USA) which retains proteins with a size larger than 10 000 Da. Because SAD-C has a size of approximately 31 kDa when the two tags are included, the proteins will stay above the filter. The filtering tubes were first washed to purify the filters. This was accomplished by centrifuging them with water for 15 minutes at 4000xg and 4°C. After the purification the second wash (W2) was poured in one of the Amicon-Ultra tubes and the four elution fractions were poured in the other Amicon-Ultra tube, and the filtering device were centrifuged at 4000xg for 15 minutes at 4°C. After the concentration, there was 0.6 ml solution left above the filter in each Amicon-Ultra tube. The solution that went through the filter, e.g. the proteins with a size smaller than 10 000 Da, was discarded.
SDS-PAGE:
To check the purity of the protein an new SDS-PAGE was performed. The solution in the Amicon-Ultra tube which contained W2 was divided into two eppendorf tubes and diluted in two different ways; 1:9 and 3:7. To these two mixtures 10 μl 2x loading buffer SDS-PAGE and 2 μl 1 M DTT were added.
The solution in the Amicon-Ultra tube which contained the elution fractions were divided in the same way. The SDS-PAGE was performed as previously described.

Trial 2

Overexpression of SAD-C in BL21 Star™ (DE3) One Shot® E. coli

Two smaller pre-cultures were at first grown to ascertain what time for IPTG induction that was most favorable for the bacteria. IPTG (isopropyl β-D-1-thiogalactopyranoside) activates the T7 RNA polymerase gene in BL21(DE3), which induces transcription of the T7 promotor in the pET101 plasmid that contains the SAD-C insert.
E.coli strain BL21 Star containing SAD-C was grown in two different Falcon tubes containing 5 ml LB-medium with carbenicillin at a final concentration of 50 μl/ml. The bacteria were grown overnight at 37°C and 200 rpm.
Each overnight culture was added to 50 ml LB-medium with 50 μl/ml carbenecillin and grown at 37°C. The absorbance at 600 nm was measured and the culture was grown until the OD₆₀₀ value was 0.9-1.0. IPTG was added to one of the cultures with a final concentration of 0.1 mM to induce protein expression. Samples were taken from both cultures at 1 hour (0, 1, 2, 3 and 4) intervals for later investigation to see which induction time that is the most favorable for the protein.
To confirm the overexpression of the recombinant protein, an SDS-PAGE was performed on the samples. All samples were boiled at 99°C for 6 minutes and then centrifuged at 13 000 rpm for 5 min to obtain a pellet. Then the pellet was mixed with 10 μl 2x loading buffer SDS-PAGE and 2 μl 1 M DTT. The SDS-PAGE was then performed as previously explained with the exception that a 12% Tris-HCl polyacrylamide gel was used. As a size marker 5 μl Prestaind Protein Ladder Plus from Fermentas Life Sciences PageRuler™ was used.

To scale up the overexpression of the recombinant SAD-C protein for purification, the transformed BL21 Star™ (DE3) One Shot® E. coli was grown in six different Falcon tubes containing 20 ml LB-medium with 50 μl/ml carbenicillin at 37°C and 200 rpm over night. Each overnight culture was added to 1 liter LB-medium containing 50 μl/ml carbenecillin and grown at 37°C. The absorbance at 600 nm was measured and the culture was grown until the OD₆₀₀ value was 0.9-1.0. IPTG was added to each culture with a final concentration of 0.1 mM to induce protein expression. Three hours after induction at 37°C and 200 rpm the cultures were harvested by centrifugation for 15 minutes at 6000xg at 4°C. (Beckman Coulter, Avanti™ J-20XP Centrifuge, JLP 8.1000). The supernatant was discarded and the bacterial pellets were frozen in liquid nitrogen and stored at -20°C until required.

Protein purification

Disruption of the cells:
The stored cells were broken in the X-press, but the X-press was not properly screwed together which resulted in that some of the bacteria were not appropriately broken. The partly
disrupted cells were collected in two tubes, and 24 ml equilibrium buffer (50 mM NaPi, [pH 8.0], 0.3 M sodium chloride) and 600 μl 40 mM PMSF were added. The cells were sonicated in six cycles with power 70 % until the solution was smooth. Each cycle lasted for two minutes. After the sonication some DNase (DNase I from bovine pancreas Grade II) was added to the mixture to break down DNA. Then the cells were centrifuged in an ultracentrifuge to separate cytoplasmatic and membrane fractions and to remove unbroken cells. (Beckman Optima™ L-90 K Ultracentrifuge, Ti 70, 45 000 rpm, 45 min, 4°C).

Incubation with Ni-NTA:
The pH of the supernatant from the ultracentrifuge was adjusted to 8.0 with 1 M NaOH and then mixed with HIS-Select Nickel Affinity Gel. HIS-Select Nickel Affinity Gel had previously been placed in two 50 ml Falcon tubes containing 2.5 ml each. The tubes were held on ice, and the supernatants were discarded. Equilibrium buffer was added to each tube, and the content in the tubes were mixed for 15 minutes at 4°C on a roller. The tubes were then held on ice, and again the supernatants were discarded, and the HIS-Select Nickel Affinity Gel in each Falcon tube was mixed with 35 ml of the cytosolic fraction from the ultracentrifuge and kept on a roller at 4°C over night.

Chromatography:
The incubated solution containing Ni-NTA was transferred to a chromatography column with two thin filters to keep the gel with the nickel particles in place. The speed at which the flow through would have eluted was very low, so the column and the incubated protein and Ni-NTA mixture were centrifuged for three minutes at 4000xg and 4°C. The supernatant was removed, but saved, and the agarose left was mixed with approximately 25 ml wash buffer (50 mM NaPi, [pH 8.0], 0.3 M sodium chloride) and poured into a new column, now with only one filter. This counts as the first wash. No second wash was performed. Then the proteins were eluted with 20 ml elution buffer (50 mM NaPi, [pH 8.0], 0.3 M NaCl, 250 mM L-Histidine) and collected in four 5 ml fractions.

SDS-PAGE:
The purity of the proteins was studied by performing an SDS- PAGE. The SDS-PAGE was performed as previous times. As a size marker 5 μl Prestaind Protein Ladder Plus from Fermentas Life Sciences PageRuler™ was used.

Trial 3

Overexpression of SAD-C in BL21 Star™ (DE3) One Shot® E. coli

The overexpression of SAD-C was performed as in trial 2.

Protein purification

Disruption of the cells:
The disruption was performed as in trial 2, the only exception was that RNase also was added to the disrupted cells before the soncation was started.

Small-scale test:
A small-scale test was performed according to instructions regarding the HIS-Select Nickel Affinity Gel. The small-scale test was accomplished before attempting a large-scale
purification to determine if the standard operation conditions were well suited for SAD-C. Every step was performed at room temperature.

50 μl His-Select Nickel Affinity Gel Suspension was added to an eppendorf tube and centrifuged at 5000xg for 30 seconds. The supernatant was discarded. 200 μl equilibrium buffer (50 mM NaPi, [pH 8.0], 0.3 M sodium chloride) was then added and mixed. The mixture was centrifuged for 30 seconds at 5000xg, and the supernatant was removed and discarded. Then, 100 μl ultracentrifuged protein solution was added and carefully mixed for 1 minute. The mixture was again centrifuged for 30 seconds at 5000xg. The supernatant was saved for later use (FT). To the affinity gel 500 μl wash buffer (50 mM NaPi, [pH 8.0], 0.3 M sodium chloride) was added and mixed carefully and then centrifuged for 30 seconds at 5000xg. The supernatant was saved for later use (W1). The affinity gel was washed once more in the same way, and the supernatant was again saved for later use (W2). SAD-C was eluted two times with 50 μl elution buffer (50 mM NaPi, [pH 8.0], 0.3 M sodium chloride, 250 mM L-Histidine). The buffer was added to the affinity gel and carefully mixed, and then centrifuged for 30 seconds at 5000xg. The supernatant was saved for later use (E1 and E2). Most of the protein will be eluted in the first fraction, but some residual protein may be eluted the second time.

SDS-PAGE:
The fractions were analyzed by SDS-PAGE to determine if SAD-C had bound to the affinity gel and had been eluted. The SDS-PAGE was performed as previously described. As a size marker 5 μl Prestaind Protein Ladder Plus from Fermentas Life Sciences PageRuler™ was used.

Western Blotting:
A Western blot was performed to determine where the SAD-C was fractionated during the small-scale purification trial.

10 μl of the different protein samples were dissolved in 10 μl 2x loading buffer SDS-PAGE and 2 μl 1 M DTT. The samples were boiled for 5 minutes. Samples were separated on a 15 % Tris-HCl gel (Ready-Gel, Bio-Rad, CA) for 60 minutes at 150 V. As a size marker 5 μl Prestaind Protein Ladder Plus from Fermentas Life Sciences PageRuler™ was used.

In order to make the proteins accessible to antibody detection, they were moved from within the gel onto a membrane of nitrocellulose (Hybond™-ECL™, Amersham Biosciences, UK). The gel is placed on a fiber plate and two filter papers. The membrane is placed on the gel, and on top of the membrane two filter papers and one fiber plate are placed. The entire stack is placed in a 1x blotting buffer (25 mM Tris, 192 mM Glycine, 20 % v/v methanol) and the proteins are transferred by using an electric current (40 V for 90 minutes) to pull proteins from the gel into the nitrocellulose membrane The proteins are maintaining the organization they had within the gel.

Blocking non-specific binding interactions between the membrane and the antibody is prevented by placing the membrane in a solution of 5 ml TBST (0.02 M Tris base, 0.15 M sodium chloride, 0.05% Tween 20, [pH 7.5]) and 25 g dry milk (Semper). The proteins in the solution attaches to the membrane in all places where the target proteins have not attached. Thus, when the primary antibody is added, there is no room on the membrane for it to attach to other than on the binding sites of the specific target protein. This gives a Western blot with clearer results.
After the blocking the membrane was washed with TBST to remove the milk. Then the membrane was incubated with 5 ml TBST and 2 μl primary antibodies (SAD-C specific antibody from rabbit; Davids Biotechnologie (Regensburg, Germany)) for 30 minutes (1:2500 v/v). After the incubation the membrane was washed with TBST 1x10 minutes and 2x5 minutes to remove unbound primary antibodies. Then the membrane was incubated with 7.5 ml TBST and 1.5 μl secondary antibodies (anti-rabbit IgG AP, Sigma®) for 1 hour. The secondary antibodies will bind to the primary antibodies and enhance the signal. After the incubation the membrane was washed with TBST 1x10 minutes and 2x5 minutes to remove unbound secondary antibodies and finally the membrane was washed with AP-buffer (100 mM Tris, 100 mM sodium chloride, 5 mM magnesium chloride, [pH 9.5]). The membrane was developed in 10 ml AP-buffer, 66 μl NBT (nitro blue tetrazolium) and 33 μl BCIP (5-bromo-4-chloro-3-indolyl-phosphate) until a clear picture appeared on the membrane. (BCIP/NBT Color Development Substrate: Promega, USA).

Small-scale test:
The small-scale test was performed on two additional sets of buffer solutions to see which was best suited for SAD-C and to further optimize the purification method.
The small-scale test was performed on both sets of buffer solutions as previously described.

Set 1:
Equilibrium- and wash buffer
- 50 mM NaPi, pH=8.0
- 0.3 M NaCl
- 10 mM L-Histidine

Elution buffer
- 50 mM NaPi, pH=8.0
- 0.3 M NaCl
- 250 mM L-Histidine

Set 2:
Equilibrium- and wash buffer
- 50 mM NaPi, pH=8.0
- 200 mM NaCl
- 20 mM L-Histidine
- 0.05% Tween 20
- 1 mM PMSF

Elution buffer
- 50 mM NaPi, pH=8.0
- 200 mM L-Histidin
- 20 % glycerol

SDS-PAGE:
The fractions were then analyzed by SDS-PAGE as previously described.

Incubation with Ni-NTA:
The incubation was performed as described in trial 2.
Chromatography:
The incubated solution containing Ni-NTA was transferred to a chromatography column with two thin filters to keep the gel with the nickel particles in place. The elution was performed with the set of buffers that gave the best result in the small-scale test. The column was washed two times with 20 ml wash buffer (50 mM NaPi, [pH 8.00], 0.3 M NaCl, 10 mM L-Histidin), and then the proteins were eluted with 20 ml elution buffer (50 mM NaPi, [pH 8.00], 0.3 M NaCl, 250 mM L-Histidin) and collected in four 5 ml fractions.

SDS-PAGE:
The fractions were analyzed by SDS-PAGE as earlier explained.

Trial 4

Overexpression of SAD-C in BL21 Star™ (DE3) One Shot® E. coli

The overexpression was performed as in trial 2 with the only exception that the LB-media contained 2.5 mM betain. The betain is increasing the protein solubility and is preventing inclusion-bodies to form.

Protein purification

Disruption of the cells:
The disruption of the cells was performed a previously described, the only differences was that the cells were sonicated in four cycles instead of six.

Incubation with Ni-NTA:
Half the supernatant was used for incubation with Ni-NTA this time. The Ni-NTA were placed in two 15 ml Falcon tubes each containing 1.25 ml HIS-Select Nickel Affinity Gel. The tubes were held on ice, and the supernatant was discarded. 12 ml equilibrium buffer (50 mM NaPi, [pH 8.0], 0.3 M NaCl, 10 mM L-Histidine) was added to each tube, and the content in the tubes was mixed for 15 minutes at 4°C on rotating rolls. The tubes were then held on ice, and again the supernatant was discarded, and the HIS-Select Nickel Affinity Gel was mixed with 35 ml of the cytosolic fraction from the ultracentrifuge in each tube and kept on rotating rolls at 4°C over night.
During the incubation the SAD-C proteins with six histidine residues at the C-terminal end attached to the nickel particles in the HIS-Select Nickel Affinity Gel.

Chromatography:
The incubated solution containing Ni-NTA was transferred to a chromatography column containing one thick filter at the bottom to keep the gel with the nickel ions in place. The column was washed two times with 20 ml wash buffer (50 mM NaPi, [pH 8.0], 0.3 M sodium chloride, 10 mM L-Histidine), and then the proteins were eluted with 20 ml elution buffer (50 mM NaPi, [pH 8.0], 0.3 M sodium chloride, 250 mM L-Histidine) and collected in four 5 ml fractions. 50 mM PMSF was added to the wash buffer during the chromatography. The fractions were saved for later use.

SDS-PAGE:
An SDS-PAGE and a western blot were performed on the fractions as previously described.
Concentration of the protein samples

Concentration of the samples was performed as in trial 1.

Silver staining:
A silver staining was performed on the concentrated protein samples. The silver staining was performed because it is more sensitive than regular Coomassie staining.
The samples were loaded on the gel in different dilutions; 1:5, 1:2 and 1:1. Each sample was mixed with 10 μl 2xloadingbuffer SDS-PAGE and 2 μl 1 M DTT.

All samples were boiled for 5 minutes and then separated on a 12 % Tris-HCl gel (Ready-Gel, Bio-Rad, CA) for 60 minutes at 150 V. As a size marker 5 μl Prestained Protein Ladder Plus from Fermentas Life Sciences PageRuler™ was used.

After the electrophoresis the gel was incubated in a fixing solution that contained 40% ethanol, 10% acetic acid and 50% water. The gel was washed for one hour in water and then sensitized for 1 min in a sensitizer solution (0.02% Na₂S₂O₃). Then the gel was washed for 3 x 20 seconds and then incubated in a silver nitrate solution (0.1% AgNO₃, 0.02% formaldehyde) for 20 minutes at 4°C. Again, the gel was washed for 3 x 20 seconds, and then, for 1 minute. The gel was developed in 3% Na₂CO₃ and 0.05% formaldehyde. The gel was then washed in H₂O for 20 seconds and the staining was terminated with the incubation in 5% acetic acid.

Western blotting:
Western blotting was performed on the concentrated protein samples as well. The samples were loaded on the gel in different dilutions; 1:5, 1:2 and 1:1. Each sample was mixed with 10 μl 2xloadingbuffer SDS-PAGE and 2 μl 1 M DTT.
The rest of the western blotting was performed according to previous descriptions.

SDS-PAGE:
An SDS-PAGE with a Coomassie Brilliant Blue staining was performed on the concentrated samples. The samples were loaded on the gel in different dilutions; 1:5, 1:2 and 1:1. Each sample was mixed with 10 μl 2xloadingbuffer SDS-PAGE and 2 μl 1 M DTT.
The rest of the SDS-PAGE was performed according to previous descriptions.

Crystallization
To perform crystallization, the concentration of the protein must be known and a standard curve has to be constructed.

Standard curve for protein concentration:

<table>
<thead>
<tr>
<th>Standard concentration (mg/ml)</th>
<th>Vol. BSA (μl)</th>
<th>Vol. distilled H₂O (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>0.2</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>0.4</td>
<td>20</td>
<td>80</td>
</tr>
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</tr>
<tr>
<td>0.8</td>
<td>40</td>
<td>60</td>
</tr>
</tbody>
</table>
5 μl of the BSA (Bovine serum albumin) of different concentrations was mixed with 795 μl water. Then, 200 μl BioRad Protein Assay Reagent was added and after 15 minutes the absorbance was measured.

For measurement of the protein samples, 5 μl of the concentrated wash sample and eluate were mixed with 795 μl H₂O and 200 μl BioRad Protein Assay Reagent and the absorbance was measured at 595 nm after 15 minutes. Diluted samples were measured as well; 2.5 μl of the two different samples were mixed with 797.5 μl H₂O and 200 μl BioRad Protein Assay Reagent and the absorbance was measured at 595 nm after 15 minutes.

For crystallizing the protein, the hanging drop vapour diffusion method was used applying a commercial crystal screening kit (Hampton Research). The drops were a mix of 1.5 μl protein sample and 1.5 μl well solution and were left to equilibrate over the well solution.
Results and discussion

Trial 1

Recombinant SAD-C protein in *E. coli* strain BL21 Star™ (DE3) One Shot® was overexpressed and purified by the presence of a His-tag consisting of six histidine residues at the C-terminal end of the protein. The different fractions from the protein purification were separated and analyzed by SDS-PAGE using a 15% Tris-HCl polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue, see Figure 1.

![Figure 1](image)

Pale bands are showing on the gel in wash 2 (W2) and in eluates 2 and 3 (E2 and E3) at the correct size of SAD-C, e.g. approximately 31 kDa. A concentration of wash 2 and the four elute fractions pooled together was performed by using Amicon-Ultra Centrifugal Filtering Device with a cut-off at 10 kDa, and a new SDS-PAGE with a Coomassie staining was accomplished on the two fractions.

The resulting bands on the gel were not at the correct size for SAD-C, but instead quite strong bands were showing at approximately twice the size of the protein (figure not shown). This implies that the protein had formed dimers. According to earlier research, SAD-C can form dimers when purified after overexpression in *E. coli*.

An explanation to why SAD-C was not seen on the gel could be that SAD-C was caught in the filter of the Amicon-Ultra tube during the concentration step.

The bands on the gel were anyway too pale for a crystallization trail, and a new batch of bacteria was grown.
Trial 2

Overexpression of SAD-C in BL21 Star\textsuperscript{TM} (DE3) One Shot\textsuperscript{®} E. coli

The recombinant SAD-C in E. coli strain BL21 Star\textsuperscript{TM} (DE3) One Shot\textsuperscript{®} was overexpressed. To verify that overexpression of SAD-C had occurred, an SDS-PAGE was performed, see Figure 2. The two cultures, one induced and one non-induced were analyzed every hour (0, 1, 2, 3 and 4).

![Figure 2](image)

**Figure 2.** To confirm that overexpression of the SAD-C protein had occurred, an SDS-PAGE was performed. The two cultures, one induced and one non-induced, were analyzed every hour (0, 1, 2, 3 and 4). The induced bacteria are marked with an (I). The molecular weight marker (MW) was Prestained Protein Ladder Plus from Fermentas Life Sciences PageRuler\textsuperscript{TM}.

The results from the SDS-PAGE confirmed that the overexpression of the SAD-C protein had occurred. Other bands are stronger than that for SAD-C, but the conclusion can be drawn that SAD-C at least is present in the samples. Little difference is shown between the induced and non-induced samples, which mean that SAD-C is expressed without IPTG as well. A larger amount of IPTG could have been used, which perhaps could have resulted in a larger difference between the induced and non-induced samples. Induction time at three hours where chosen for future IPTG-inductions.

Protein purification

During trial 1, the HIS-Select Nickel Affinity Gel lost some of its blue color. When the blue color faded away it implied that the nickel ions had disappeared. Because of this, new buffer solutions were made according to the instructions for the HIS-Select Nickel Affinity Gel.

Recombinant SAD-C protein was purified. The different fractions from the protein purification were separated on a 15% Tris-HCl agarose gel and stained with Coomassie Brilliant Blue, see Figure 3.
The first eluate showed many bands, which implies that it contained many proteins. The eluates ought to contain only SAD-C. The other proteins are supposed to be eluted with the flow through and wash since SAD-C with its His-tag should be the only protein that could bind to the nickel ions. One explanation for this could be that the concentration of SAD-C was too small and the amount of HIS-Select Nickel Affinity Gel was too large, and unspecific binding occurred between other proteins containing histidine residues. Another explanation could be that the protein supernatant was incubated with the Ni-NTA for too long and unspecific binding occurred because of this.

In the second eluate, a very pale band can be distinguished at the correct size for SAD-C, but even stronger bands are showing at approximately twice the size of the protein. The two remaining eluates did only contain bands of the double size. This implies that SAD-C probably formed a dimer this time as well.

A new batch of bacteria was grown.
Trial 3

Recombinant SAD-C protein in *E. coli* strain BL21 Star™ (DE3) One Shot® was overexpressed. A small-scale purification test was accomplished before attempting a large-scale purification to determine if the standard operation conditions and the buffer solutions were well suited for SAD-C. The small-scale test was performed using equilibrium and wash buffers containing 50 mM NaPi, [pH 8.0] and 0.3 M sodium chloride and elution buffer containing 50 mM NaPi, [pH 8.0], 0.3 M sodium chloride and 250 mM L-Histidine. The samples from the purification step were analyzed by SDS-PAGE on a 15% Tris-HCl polyacrylamide gel, see Figure 4. The concentrated samples from trial 1 were also included on the gel.

![Figure 4](image)

**Figure 4.** Visualization of the different sample fractions collected from the small-scale purification test. The flow through is here marked with (FT), the wash fractions are marked with (W) and the eluate fractions are marked with (E). The concentrated protein samples from the first trial are also included. The concentrated second wash is marked (A1) and the concentrated four elute fractions are called (A2). The molecular weight marker (MW) used was Prestaind Protein Ladder Plus from Fermentas Life Sciences PageRuler™.

A strong band a bit below the size of SAD-C is seen in lane E1, and only a very pale band can be distinguished at the correct size, e.g. 31 kDa. A strong band at about the double size is seen as well, which indicates a formation of a dimer. In lane E2, a pale band at the size of SAD-C can be seen, but an even stronger band at twice the size. There are also many other proteins. In lanes A1 and A2 very pale bands are seen a bit below the correct size of SAD-C, but an even stronger band is showing at approximately twice the size of the protein in lane A2. Again this indicates a formation of dimers.

To see whether SAD-C was included in the samples a Western Blot was performed as well. With the Western Blot it is easier to decide if the correct protein is present, because the antibodies used were specific for denaturated SAD-C, see Figure 5.

The two concentrated protein samples from trial 1 were included this time as well.
Figure 5. The different sample fractions collected from the small-scale purification test are shown on a nitrocellulose membrane from the Western blot. The flow through is here marked with (FT), the wash fractions are marked with (W) and the eluate fractions are marked with (E). The concentrated protein samples from the first trial are also included. The concentrated second wash was marked (A1) and the concentrated four elute fractions were called (A2). The second elute from trial 2 was also included and is here marked with (FE2). The molecular weight marker (MW) was Prestained Protein Ladder Plus from Fermentas Life Sciences PageRuler™.

The arrows to the right indicate where the protein parts from a possible degradation of the protein can be seen.

The membrane showed strong bands at the size of SAD-C, but even stronger bands at twice the size, at approximately 55 kDa. The antibodies were specific for denaturated SAD-C and should only bind to the correct protein. The development of several bands on the gel when specific antibodies were used implies that SAD-C has formed dimers and maybe parts of the protein had been degraded resulting in bands on the gel at different sizes; see the arrows to the right.

The small-scale purification test was performed on two additional sets of buffer solutions to see which was best suited for SAD-C and further optimize the purification method, see Figure 6.

The elute fractions from previous small-scale test were also included on the gel.
Figure 6. Visualization of the different sample fractions collected from the small-scale purification test performed with two different sets of buffer solutions. FE1 and FE2 are the eluate fractions from the previous small-scale test. The wash fractions are marked with (W) and the eluate fractions are marked with (E). The buffer solutions from set one is marked with (1) and the buffer solutions from set two are marked with (2). The molecular weight marker (MW) was Prestained Protein Ladder Plus from Fermentas Life Sciences PageRuler™.

The gel shows that that the bands in the eluate with set 1 of buffer solutions gave the strongest bands. This indicates that the big scale purification should be performed with set 1 of buffer solutions.

According to the molecular weight marker, the strongest band in the eluate fractions seems to be approximately 25 kDa, i.e. slightly smaller than what is expected for SAD-C. One possible explanation is that the band is not SAD-C, but another protein or that the proteins migrate differently than the molecular weight standards.

A large scale purification was performed with set 1 of the buffer solutions and the fractions were analyzed by SDS-PAGE, see Figure 7.
<table>
<thead>
<tr>
<th>FT</th>
<th>FT</th>
<th>W1</th>
<th>W2</th>
<th>E1</th>
<th>E2</th>
<th>E3</th>
<th>E4</th>
<th>_</th>
<th>MW</th>
</tr>
</thead>
</table>

**Figure 7.** Visualization of the different sample fractions collected from the purification with set 1 of the buffer solutions performed with two different sets of buffer solutions. The flow through are marked with (FT), the wash fractions are marked with (W) and the elute fractions are marked with (E). The molecular weight marker (MW) was Prestained Protein Ladder Plus from Fermentas Life Sciences PageRuler™.

The bands at 31 kDa in the eluates are very pale, and they seem stronger in the washes. The eluates also contain many other bands. This implies that most of SAD-C was eluted with the washes and that the concentration of the protein in the elute fractions is very small.
Trial 4

During this trial the bacteria were grown in LB-medium containing 2.5 mM betain. The betain is increasing the protein solubility and is preventing inclusion-bodies to form. The SAD-C protein was overexpressed in *E. coli* and purified by the presence of six histidines at the C-terminal end of the protein. The different fractions from the protein purification were separated by SDS-PAGE on a 12% Tris-HCl agarose gel and was stained with Coomassie Brilliant Blue, see Figure 8.

![Image of SDS-PAGE gel with bands at 55, 35, and 25 kDa](FT W1 W2 W3 E1 E2 E3 E4 MW)

**Figure 8.** Purification of the recombinant SAD-C expressed in *E. coli* strain BL21 Star™ (DE3) One Shot® was facilitated by the presence of a His-tag at the C-terminal end of the protein. The flow through is marked (FT), the different wash fractions are marked (W) and the eluate fractions are marked (E). The molecular weight marker (MW) was Prestained Protein Ladder Plus from Fermentas Life Sciences PageRuler™.

The gel shows many bands in the flow through and the two washes, but no bands are seen in neither of the four elute fractions. To see if the different fractions contain SAD-C a Western blot using specific SAD-C antibodies was performed as well, see Figure 9. A 12% Tris-HCl gel was used during the Western blot.
Figure 9. The figure shows the membrane from the western blot performed on the different fractions from the purification during trial 4. The flow through is marked (FT), the different wash fractions are marked (W) and the eluate fractions are marked (E). The molecular weight marker (MW) was Prestained Protein Ladder Plus from Fermentas Life Sciences PageRuler™.

W3 and the four elute fractions contained the protein SAD-C.

The protein samples were concentrated and a silver staining was performed because of the higher sensitivity to stain proteins compared to a regular SDS-PAGE with Coomassie Brilliant Blue staining.

The samples were loaded on the gel in different concentrations, see Figure 10.
Bands appeared on the gel, but the most dominating bands lie below the correct size for SAD-C. Pale bands of the correct size are also showing, but to a much lesser extent. One explanation to why SAD-C is not the dominating band may be that this protein is not stained by silver. To be sure of whether SAD-C is present in the samples or not, a Western blot was performed on the concentrated samples as well. The samples were loaded on the gel in different concentrations, see Figure 11.
Figure 11. Concentrated and purified protein fractions from expression in *E. coli* were analyzed with Western blotting using a 15% polyacrylamid gel and SAD-C specific primary antibodies to detect the SAD-C protein. The wash fractions are marked with (W) and the elute fractions are marked with (E). The samples are of different concentrations (diluted 1:5, 1:2 and 1:1). The molecular weight marker (MW) was Prestaind Protein Ladder Plus from Fermentas Life Sciences PageRuler™.

The membrane showed strong bands at the correct size. The membrane ought to agree with the gel that was silver stained, but it does not. This suggests that SAD-C is not the dominant protein in the sample solutions. But, as mentioned earlier, the silver may not stain SAD-C, and an SDS-PAGE with a Coomassie Brilliant Blue Protein Staining was performed as well. See Figure 12.

Figure 12. The gel is showing the concentrated wash- and elute fractions at different dilutions (1:5, 1:2 and 1:1), stained with Coomassie Brilliant Blue. The wash fractions are marked (W) and the elute fractions are marked (E). The molecular weight marker (MW) was Prestaind Protein Ladder Plus from Fermentas Life Sciences PageRuler™.
The gel had the strongest band a bit below 25 kDa, which also were the strongest bands on the gel that were silver stained. This indicates that the protein of correct size, approximately 31 kDa, is not the dominating protein in the samples. A crystallization trial was still performed, because if crystals will form, it can be a purification step in it self if the crystals are removed and dissolved in a new solution. However, the probability that crystals of the correct protein and not the unwanted proteins will from is small.

To perform a crystallization, the concentration of the protein sample must be known. To determine the protein concentration, a standard curve was constructed. A concentration gradient between 0.2 mg/ml and 0.8 mg/ml was prepared and the absorbance at 595 nm was measured three times at each concentration. The mean value was used to construct the curve, see Figure 13.

For calculations of mean values at each concentration, see Appendix 2.

Then the absorbance of the samples was measured.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Absorbance (595 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W1:1</td>
<td>0.1312</td>
</tr>
<tr>
<td>E1:1</td>
<td>0.0436</td>
</tr>
<tr>
<td>W1:2</td>
<td>0.0925</td>
</tr>
<tr>
<td>E1:2</td>
<td>0.0909</td>
</tr>
</tbody>
</table>

![Standard curve, determination of the protein concentration](image)

**Figure 13.** The mean value of the three absorbances at each concentration was used to construct a standard curve.

The SAD-C protein from the wash diluted 1:1 had an $A_{595\ \text{nm}}$ of 0.1312. This corresponds to a protein concentration of 0.5 mg/ml according to the standard curve, see Figure 13. The concentration in the eluted sample (E1:1) is hard to tell because it lies outside the curve.
according to its absorbance of 0.0436, but when trying to extrapolate from the curve the concentration seems to be about 0.15 mg/ml. When a crystallization is performed the concentration should preferably be 10 mg/ml. This means that the concentrated samples have to be further concentrated. 800 μl wash sample was concentrated to approximately 100 μl, and 800 μl elute sample was concentrated to approximately 100 μl as well. This means the samples have been concentrated eight times, from 0.5 mg/ml and ~0.15 mg/ml to 4 mg/ml and ~1.2 mg/ml.

**Crystallization**

Out of 50 different conditions for crystallization of purified recombinant SAD-C protein, using a commercial screening kit from Hampton Research, four conditions gave crystals. The first condition was 0.02 M calcium chloride dehydrate, 0.1 M sodium acetate trihydrate, pH 4.6 and 30% v/v 2-methyl -2,4-pentanediol. The second condition was 0.2 M calcium chloride dehydrate, 0.1 M sodium acetate trihydrate, pH 4.6 and 20% v/v isopropanol. The third condition was 0.1 M imidazole, pH 6.5 and 1.0 M sodium acetate trihydrate. The fourth and final condition that gave crystals was 0.05 M potassium dihydrogen phosphate and 20% v/v polyethylene glycol 8000. The crystals were shaped like flat plates ordered like a bouquet.

The probability that the crystals formed are of SAD-C is small only, instead the dominating protein shown in the gels that were Coomassie Brilliant Blue- and silver-stained may have formed crystals during the crystallization trial.

SAD-C seems to have problem binding to the HIS-Select Nickel Affinity Gel according to the results, since a big part of the protein probably is eluted as early as during the flow through and wash fractions and almost nothing is left in the elute fractions. If the amount of HIS-Select Nickel Affinity Gel was decreased maybe unspecific binding could be prevented because SAD-C containing six histidine residues would be the only protein that could bind to the nickel ions. If the resin volume is decreased, a higher competition is allowed between fusion proteins and contaminants for the same sites on the HIS-Select Affinity Gel. One other possible explanation to unspecific binding could be that the protein supernatant from the ultracentrifugation is incubated with the HIS-Select Nickel Affinity Gel for too long, allowing other proteins to bind to the nickel ions.

When Western Blot was performed only SAD-C specific denaturated antibodies were used. A trial could have been performed, where antibodies specific for His-tags were tested to see if other proteins containing histidine residues were present in the samples. Antibodies specific for proteins containing a V5-tag could also have been tested because SAD-C should be the only protein containing that tag.

One other aspect to consider is the amount L-Histidine present in the wash buffer. L-Histidine is added to the wash buffer to prevent unspecific binding of proteins, but perhaps the L-Histidine is contributing to the fact that SAD-C is eluted earlier than expected or not even binding at all. Perhaps the wash buffer should not contain any L-Histidine at all. However, during the small-scale test the set of buffers where the wash buffer was containing 10 mM L-Histidine were best suited for the purification. If a higher amount of L-Histidine was used in
the wash buffer binding of contaminants containing His-residues could perhaps be prevented, because L-Histidine would compete with contaminants to bind to the nickel-particles in the Ni-NTA. But a higher amount of L-Histidine could as well contribute to an early elution of SAD-C instead, due to the fact that L-Histidine could have a higher affinity to the nickel-particles than SAD-C. Instead of L-Histidine imidazole could be used to see if it will affect the purification. Imidazole has the same function as L-Histidine.

One other possibility regarding why SAD-C is present at such low amount may be that there are problems with the expression of the protein. If twice as much bacteria were grown and all the lysates were used at the same time, maybe the concentration of the protein would increase with a large amount. Also the concentration of IPTG used for induction could be too low. Increasing IPTG by one order of magnitude from 0.1 mM to 1.0 mM should be tested. This concentration is recommended by Invitrogen Life Technologies.

Since the protein successfully has been purified with the His-Tag at the N-terminal end, the problems in the present study maybe are due to the His-tag on the C-terminal end. The His-tag may be preventing correct folding of the protein.
References

9. www.invitrogen.com
### Appendix 1

<table>
<thead>
<tr>
<th>Concentration (mg/ml)</th>
<th>Absorbance (595 nm)</th>
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</thead>
<tbody>
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<tr>
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</tr>
<tr>
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</tr>
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</tr>
<tr>
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</tr>
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<td>0.2683</td>
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<td>0.2080</td>
</tr>
<tr>
<td>0.8</td>
<td>0.1721</td>
</tr>
</tbody>
</table>

**Calculations**

Mean value of the absorbances measured at 0.2 mg/ml:

\[
0.0943 + 0.0504 = 0.1447 \\
0.1447/2 = 0.0724
\]

Mean value of the absorbances measured at 0.4 mg/ml:

\[
0.0807 + 0.0536 + 0.0905 = 0.2248 \\
0.2248/3 = 0.0749
\]

Mean value of the absorbances measured at 0.6 mg/ml:

\[
0.1650 + 0.1664 + 0.1266 = 0.458 \\
0.458/3 = 0.1527
\]

Mean value of the absorbances measured at 0.8 mg/ml:

\[
0.2683 + 0.2080 + 0.1721 = 0.6484 \\
0.6484/3 = 0.2161
\]