Expression, purification and characterization of Violaxanthin De-Epoxidase from Spinach

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Abstract

Plants require sunlight to be able to perform photosynthesis but an excess of intense sunlight is harmful to the organism and therefore needs a method to protect itself. Violaxanthin De-Epoxidase (VDE) is located inside the thylakoid lumen and catalyzes the de-epoxidation of violaxanthin to zeaxanthin. Zeaxanthin absorbs the harmful light and dissipates the energy by emitting heat. How this enzymatic reaction is done by VDE is unknown as well as the structure of the full enzyme. The low natural abundance of this protein makes it hard to isolate from its original host and it is therefore overexpressed in *Escherichia coli*. Isolation and purification of VDE using the soluble proteins fraction resulted in low amounts of VDE with low specific activity but solubilization and refolding of the insoluble protein fraction resulted in high amounts of active VDE. Further purification steps including two-step precipitation and gel-filtration that resulted in monomeric, active VDE (determined to 99 % of protein composition with SDS-PAGE. Verification of expressed protein was made by SDS-PAGE and mass spectroscopy. Protein characterization by Circular Dichcroism (CD), Differential Scanning Fluorimetry (DSF), Dynamic Light Scattering (DLS) and metal analysis was preformed. Crystallization screens JCSG+ and PACT Premier were used to acquire protein crystals but was however not successful. Investigation of enzymatic activity by inhibition with low pH and DTT showed that both these factors can inhibit VDE separately and that the DTT inhibition is reversible.

Popular scientific summary

Photosynthesis is the reaction where the energy from the sunlight is collected and stored in chemical bonds. The sunlight is however also damaging the photosynthetic machinery. When exposed to intense light the photosynthesis not will be able to keep up, which will result in more damage to the photosynthetic machinery. The organism therefore needs some way of regulating how much light that reaches this machinery. Plants that are unable to cover themselves or move to a shadow have another way of regulating the amount of light that reaches the photosynthetic machinery. This is done by having light absorbing compounds that can dissipate the energy obtained from excessive light by emitting heat. One protein that is involved in this regulation is violaxanthin de-epoxidase (VDE). To learn more of this regulation it is important to study the properties of VDE. The main problem with investigating VDE is to obtain reasonable amounts. It is very low abundant in plants and results in low yields when overexpressed in other organisms. Therefore this project's aim was to find a method to obtain pure VDE in a high concentration, which will further characterization of the protein.

The method that was used in this project to obtain pure VDE in a high concentration was to isolate the large amount of inactive protein that was obtained after expressing VDE in *E. coli*. The inactive VDE could then be refolded back to its active state. This step was followed by purification and then different characterization methods that gave hints about the structure and function of VDE.

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1. Introduction

Plants use the photosynthesis to convert carbon dioxide to organic compounds. This process requires light, which the plant receives from the sun. An excess of sunlight is however harmful to the plant and therefore the plant needs someway to protect itself from this light. Unable to cover itself or move to a shadow the plant instead shields itself by a chemical conversion, called the Xanthophyll cycle.

Xanthophylls are yellow pigments from the cartenoid group. The Xanthophyll cycle is the transition between violaxanthin, antheraxanthin and zeaxanthin (fig. 1). These molecules are located in the thylakoid membrane. When exposed to intense light violaxanthin is de-epoxidised to zeaxanthin, which can absorb the excessive sunlight and dissipate the energy as heat. This de-epoxidation is catalyzed by Violaxanthin De-Epoxidase (VDE) [1]. The opposite reaction, epoxidation of zeaxanthin to violaxanthin is catalysed by Zeaxanthin Epoxidase (ZE). Antheraxanthin is the intermediate with only one side of the molecule converted.

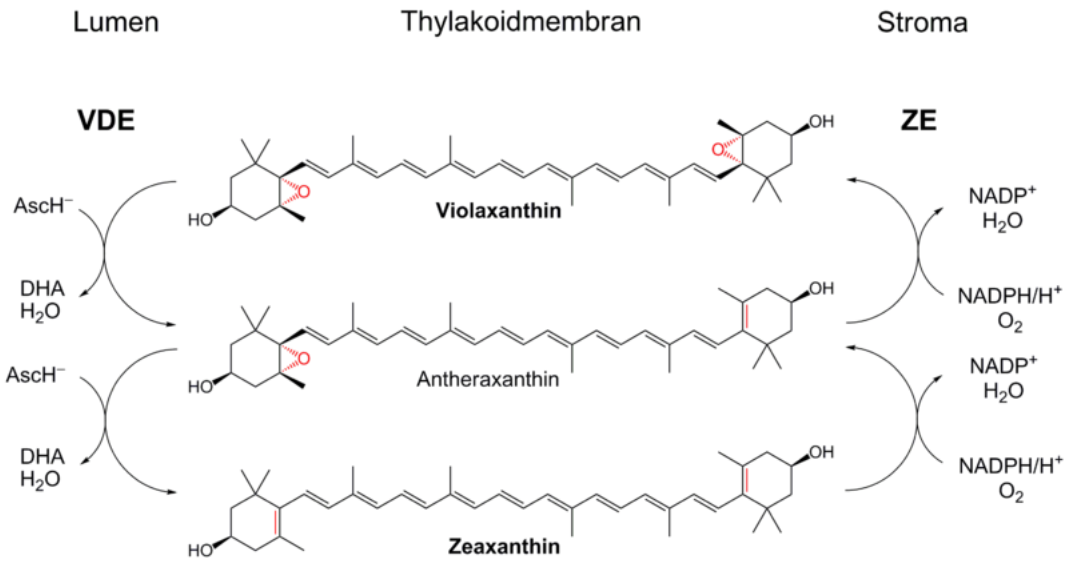

Fig. 1. Schematic overview of the Xanthophyll cycle. Picture from Wikipedia.

VDE is an enzyme located inside the thylakoid on the lumen side. To target this location VDE have two N-terminal transit peptides. The first part targets the inside of the chloroplast and a second transit peptide targets the inside of the thylakoids. These transit peptides have been cleaved off in the mature version of VDE [2].

The mature sequence of VDE could be divided into three domains (fig. 3). The N-terminal contains 11 of the mature protein's 13 cysteines and is therefore called the cysteine rich domain (fig. 3). The C-terminal domain is negatively charged containing 25 of the proteins total 37 glutamates. The central part of the sequence has a lipocalin fold consisting of eight antiparallel β-strands forming a β-barrel. This lipocalin domain structure has been confirmed by X-ray crystallography [3]. Lipocalins are often involved in transport of small hydrophobic molecules and this domain in VDE is therefore suggested to bind violaxanthin. Predictions with docking software have been made to dock both violaxanthin and ascorbate inside the lipocalin domain, where the reaction is suggested to occur [4]. Ascorbate is a cosubstrate for the de-epoxidation.

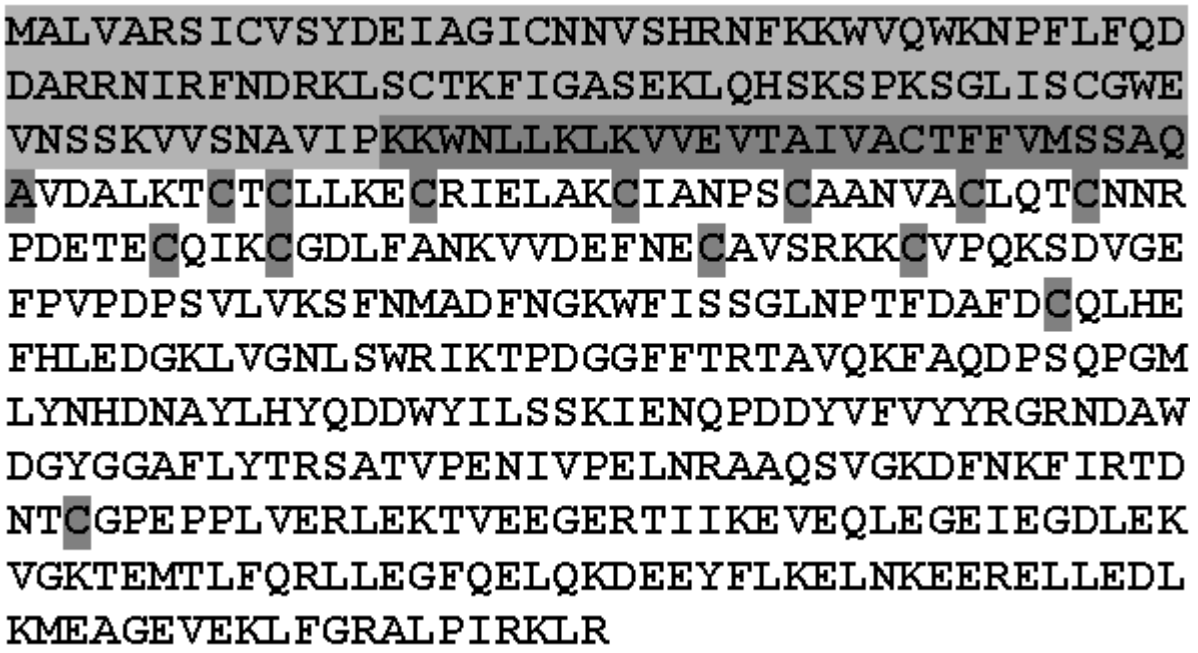

Fig. 2. The full sequence of VDE in spinach. Light gray marked sequence is predicted to be the first part of the transit peptide that targets the chloroplast stroma. The sequence marked dark gray is predicted to be the second part of the transit peptide that targets the thylakoid lumen. Cysteines are also highlighted.

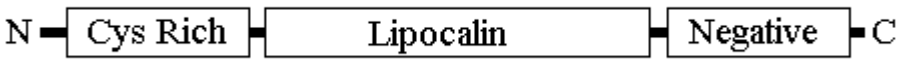

Fig. 3. Overview of the three domains of VDE.

The mechanism for the de-epoxidation of violaxanthin by VDE is not known. VDE is both a soluble protein and bound to the membrane depending on the pH. The transition occurs at pH 6.7 for VDE from spinach [5]. This suggests that VDE change its conformation at this pH. That the change occurs around pH 6 indicates that a histidine is involved. VDE have four histidines, all located in the lipocalin domain and by directed site mutagenesis been shown to be important for the enzymatic activity [4, 6]. The optimal enzymatic activity is observed at pH 5.2. That the active state is found in lower pH is a way of regulating the activity of VDE [5]. When the plant is exposed to intense light the photosynthesis is more active, something that lowers the pH inside the thylakoid lumen if the protons are not exported quick enough. The low pH could activate VDE and thereby increase the concentration of zeaxanthin, which in turn participate in the dissipation of excess energy. Photosystem I and II will then be exposed to less light, which will reduce the risk of photoinhibition. How this activation of VDE and membrane binding occurs is unknown.

Expression of truncated VDE from *Arabidopsis thaliana* have shown that removing 71 amino acids of the negatively charged C-terminal did not affect the VDE activity, indicating that this domain is not important for the enzymatic activity [7]. But the assay used to measure the enzymatic activity in this experiment does not necessarily require the protein to bind to thylakoid membranes. The C-terminal domain could have a more important roll in membrane binding. Incubation of the truncated VDE with micelles consisting of MGDG and DGDG at pH 5 resulted in that fully active VDE with 71 amino acids of the C-terminal removed did not bind to the micelles as well as VDE with the full C-terminal [7]. Removing only four of the conserved N-terminal residues resulted in total loss of VDE activity, which suggests that the cysteine rich domain is crucial for the enzymatic function [7].

The activity of VDE has been shown to be inhibited by the presence of dithiothreitol (DTT) [8]. This is probably an effect of reduction of disulphide bridges. The removal of the DTT restores the activity [9], which could mean that these disulphide bridges are reoxidized quickly. Another explanation could be that the DTT just disturbs the assay by for example binding to the substrate. An experiment that further indicates that the loss of activity is depending of lost disulphide bridges is that when VDE was incubated with DTT and iodoacetamide followed by washing away these compounds the VDE activity was not regained [9]. If VDE, incubated with DTT for a long time have lower activity than VDE that have only been incubated a short time with DTT suggests that there is a reaction between VDE and DTT causing inhibition.

Inactivation of VDE by DTT and iodoacetamide was merely detectable at pH 7.2 but was extensive at pH 5.1 [9]. This was explained by that the active state of VDE at pH 5.1 have the disulphide bridges necessary for the enzymatic reaction exposed enough to be reduced by DTT. One thing that not has been tested is if exposure to pH 5 alone reduces the VDE activity. Indication of this has been observed when VDE from spinach was incubated in pH 5.2 [10]. This incubation resulted in a loss of 80 % of the VDE activity but was somehow partially regained in later purification steps.

The properties of VDE have mostly been investigated with only partially pure protein, which could have an effect on the result. To further confirm some results of previous experiment a higher grade of purity is required. If also a higher concentration of VDE could be acquired more studies of the protein structure could be made. The central lipocalin domain has been crystallized and its structure resolved [3]. But the structure and function of the cysteine rich N-terminal domain, important for the enzymatic reaction is still unknown. The mechanism for this enzymatic reaction and if there is any prosthetic group involved in the electron transfer is also unknown. The structure and function of the negatively charged C-terminal, which not seems to have an important roll in the enzymatic reaction but may be involved in activity regulation or the membrane binding, is also unknown. The rapid reactivation after removal of DTT could be due to oxidation by oxygen, which in that case could be measured with an oxygen electrode if a high enough concentration of VDE could be acquired.

A large problem when working with VDE is the difficulty to obtain large amounts. It is very low abundant in plants and overexpression of VDE in various organisms has resulted in very low yields. Overexpression of VDE in *E. coli* with the second transit peptide (KKW - AQA) has shown to yield more VDE activity [6], which could mean that the transmembrane transportation works in *E. coli* and that the maturation of VDE may require this transport, which could explain the low yield of active VDE. Something that also supports that the transport across the membrane works in *E. coli* is that most of the VDE activity is found in the periplasm fraction [11]. If the transit peptide is cleaved off during this transport has not been confirmed. However, expressing VDE with an N-terminal His-tag attached to VDE before the second transit peptide resulted in that the flow-through of Ni-NTA chromatography contained all VDE activity [11]. This could be due to that the transit peptide along with the His-tag has been cleaved off. A construct with a C-terminal His-tag would not lose its His-tag if the transit peptide is cleaved off and may therefore have a better chance to bind to the column.

The aim of this project was to obtain pure VDE in high concentration to allow further characterization studies. This was done by solubilization of VDE inclusion bodies and refolding through dialysis. The pure VDE in high concentration allowed studies of metal presence, stability investigation with DSF, secondary structure analysis with CD-spectroscopy, protein size determination with DLS, crystallization trials, enzymatic studies regarding the inhibition of VDE by incubation in low pH, the inactivation of VDE by reduction with DTT and the reoxidation recovery.

2. Material & Methods

2.1. Plasmid construction

Plasmid0: The *vde* gene from spinach encoding proposed second transit peptide starting with KKWN inserted in pET16b between restriction site *Nde*I and *Bam*HI. When expressed gives protein VDE0 consisting of N-terminal His-tag followed by the secondary transit peptide and VDE from spinach.

Plasmid1: The mutated *vde* gene from spinach with proposed second transit peptide starting with KKWN inserted in pET22b+ between restriction site *Nde*I and *Xho*I. When expressed gives protein VDE1 consisting of the secondary transit peptide, the mutated VDE (Y227C) from spinach and a C-terminal His-tag.

Plasmid2: The mature part of the *vde* gene from spinach starting at VDAL inserted in pET16b between restriction site *Nde*I and *Bam*HI. When expressed gives protein VDE2 consisting of N-terminal His-tag followed by VDE from spinach.

Plasmid3: The mature part of the *vde* gene from spinach starting at VDAL inserted in pET22b+ between restriction site *Nde*1 and *Bam*H1. When expressed gives protein VDE3 only consisting of VDE from spinach.

Plasmid0 and Plasmid2 were constructed in an earlier project.

Plasmid1 was constructed by PCR amplification of the VDE gene from plasmid0 and addition of restriction sites for Nde1 and BamH1 with primers below, from Invitrogen.
Forward: 5' GTACATATGAAGAAATGGAATCTGTTGAA 3' 29 bp
Reverse: 5' ATCTCGAGCCGAAGCTTTCTTATAGGTA 3' 28 bp
The amplified and cut gene was then inserted in pET22b+.

Plasmid3 was constructed by cutting out the VDE gene with its stop codon from Plasmid2 and inserting it in pET22b+.

Material used for plasmid construction

Lysogeny Broth (10 g Bacto-tryptone, 10 g NaCl and 5 g Yeast extract per liter) for liquid culture growth. And addition of 15g Agar per liter for agarplates.
GeneJET Plasmid Purification Kit from Fermentas.
GeneJET PCR Purification Kit from Fermentas.
GeneJET Gel Extraction Kit from Fermentas.
*Nde*I, Fast *Nde*I, *Bam*HI, Fast *Bam*HI, *Xho*I, Shrimp Alkaline Phosphatase, T4 DNA Ligase and DreamTaq from Fermentas.
Agarose gel (1% Agarose in TAE buffer).
Plasmids were amplified in *E. coli* strain Top10 from Invitrogen.
Nucleotide concentrations determined with NanoDrop ND-1000 from Saveen Werner.
Sequencing of plasmids with T7 primers were done by Eurofins MWG Operons.

2.2. Expression

The plasmids where transformed into *E. coli* strain BL21(DE3) using chemical transformation. After confirmation of the plasmid sequence, one colony was picked and grown overnight in Lysogeny Broth (LB) with ampicillin (100 µg/ml). This liquid culture was used to inoculate 750 ml of LB with ampicillin (100 µg/ml) in a baffled flask, that where incubated at 37°C with shaking. After the cultures reached an OD600 of 0.5 – 0.6 they were induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). The cells where harvested by centrifugation at 6 000 g for 10 min at 4°C and washed once with 50 mM phosphate buffer. The cell pellet was either stored at -20°C overnight or the proteins were isolated directly.

2.3. Purification and refolding of VDE from inclusion bodies

Six frozen cell pellets (each prepared as above) were thawed and resuspended in 60 ml phosphate buffer (50 mM, pH 7.5) and 1 % (v/v) Triton X-100. The cells where lysed with a French press (Sim-Aminco) and centrifugated at 15 000 g for 15 min at 4°C. The pellet was washed once with 60 ml phosphate buffer (50 mM, pH 7.5) and 1 % (v/v) Triton X-100 then once with 60 ml phosphate buffer (50 mM, pH 7.5). The final pellet was dissolved in 60 ml phosphate buffer (50 mM, pH 7.5) and 8 M Urea and centrifugated at 15 000 g for 15 min at 4°C. The supernatant was diluted 5 fold (20 ml to 100 ml) with phosphate buffer (50 mM, pH 7.5) and 8 M Urea and was dialyzed (Spectra/Por 4 Dialyze Membrane, 12k - 14k MWCO) against 10 L phosphate buffer (50 mM, pH 7.5) over night at room temperature. The dialyzed solution was concentrated with two-step precipitation using ammonium sulphate at 20 % and 60 % saturation (incubation at 4°C for 30 min and centrifugation at 15 000 g for 30 min at 4°C). The pellet after 60 % ammonium sulphate saturation was dissolved with 2.5 ml phosphate buffer (50 mM, pH 7.5) and 2 ml was loaded on a gel-filtration column (HiPrep 26/60 Sephacryl S-200), equilibrated with phosphate buffer (50 mM, pH 7.5), flow rate 1.0 ml/min. Absorbance at 280 nm was measured and fractions containing highest specific activity where pooled and concentrated with a 30 kDa cut-off spin column (Amicon Ultra 4, Ultracel 30k MWCO, Millipore).

2.4. Isolation of periplasmic proteins

An induced *E. coli* culture (prepared as above) was centrifuged 5 min at 8 000 g at 4°C. The pellet was washed in 2x300 ml Tris-HCl pH 7.3 (10 mM), NaCl (30 mM) and centrifuged 5 min at 8 000 g at 4°C, then resuspended in 200 ml Tris-HCl pH 7.3 (33 mM), EDTA (0.1 mM), sucrose (0.5 M) and incubated in room temperature for 10 min to be centrifuged 5 min at 8 000 g at 4°C. The received pellet was resuspended in 100 ml ice cold $MgCl_2$ (0.5 mM), incubated on ice for 30 min and centrifuged 10 min at 10 000 g at 4°C. The supernatant was kept as isolated periplasmic proteins.

2.5. VDE activity measurement

VDE activity was measured according to [12] with a Shimadzu UV-3000 Dual-wavelength/double beam recording spectrophotometer. The assay consisted of citrate-phosphate buffer (50 mM citrate, 110 mM phosphate, pH 5.2), monogalactosyldiacylglyceride (MGDG, 9.0 μM), ascorbate (30 mM) and violaxanthin (0.33 μM) that was purified from spinach thylakoids using HPLC. The amount of active VDE was estimated from activity using kcat = 256 μmol/s [13].

2.6. Protein concentration determination

Total protein concentration was measured with Quick Start Bradford Dye Reagent (Bio-Rad) and a Shimadzu UV-160A UV-visible Recording Spectrophotometer at 595 nm. A calibration curve was made with known concentrations of BSA.

2.7. SDS-PAGE

NuPAGE Sample Buffer 4x (Invitrogen) and NuPAGE Reducing Buffer 10x (Invitrogen) was used on NuPAGE Novex 4-12 % Bis-Tris Midi Gels from Invitrogen and with Bio Rad Power Pac 300 at 200 mV for 40 min. Gels where stained in a solution of Commassie Brilliant Blue R 250 (0.05 %), methanol (50 % v/v) and acetic acid (7 % v/v) over night and destained with methanol (50 % v/v) and acetic acid (7 % v/v) for ~5 h. Protein standard used was PageRuler Prestained Protein Ladder #SM0671 (Fermentas).

2.8. Ni-NTA Chromatography

Chelating Sepharose Fast Flow (GE Healthcare, 1 ml) loaded with Ni(II)sulphate (0.2 ml 0.2 M), equilibrated and washed with phosphate buffer (50 mM, pH 7.0), NaCl (0.5 - 1.0 M) and various amounts of imidazole. Different amounts of cell lysate or isolated periplasmic proteins loaded and eluted with imidazole (0.5 - 1.0 M).

2.9. Mass Spectroscopy (MS-MALDI/TOF/TOF)

Protein bands were cut from the SDS-PAGE gel and destained with 75 µl of NH_4HCO_3 (50 mM) in ethanol (50 %) for 60 min two times, dehydrated with 75 µl ethanol (100 %) for 5 min, reduction of disulphide bonds with 20 µl DTT (10 mM) in 50 mM NH_4HCO_3 for 30 min at 37°C, dehydration with 75 µl ethanol (100 %) for 5 min, alkylation with 20 µl iodoacetamide (55 mM) in 50 mM NH_4HCO_3 for 30 min in darkness, washing with 75 µl of NH_4HCO_3 (50 mM) and ethanol (50 %) for 5 min, dehydration with 75 µl ethanol (100 %) for 5 min, evaporation of remaining ethanol in fume hood for 30 min, digestion of protein with 20 µl sequencing-grade trypsin (Promega, Madison, WI) (10 ng/µl) in 50 mM NH_4HCO_3 for 15 min on ice, removal of liquid and addition of 10 µl 50 mM NH_4HCO_3 followed by incubation over night in 37°C. The liquid was transferred to a new tube and an addition of 10 µl TFA (0.5 %) in ethanol (50 %) to the gel pieces were made and incubated for 30 min, followed by transfer of the liquid to the new tube.

The sample (0.5 µl) was spotted on the target plate and left to dry before addition of matrix solution (0.5 µl) containing acetonitrile/TFA/citric acid (50 %, 0.1 %, 50 mM) and α-cyano-4-hydroxycinnamic acid (10 mg/ml). Every sample was spotted twice, one spot contained calibration peptides for internal calibration, and the other did not contain calibration peptides to get higher detection level if the concentration of peptides were low. These spots were calibrated externally with the same calibration peptides in other spots.

MS and MS/MS spectra were recorded using a 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA) mass spectrometer in positive reflector mode.

2.10. Differential Scanning Fluorimetry (DSF)

DSF screen of different buffers (Appendix 1) was done on purified VDE2 (0.1 mg/ml) with SYPRO orange at Cassiopeia Crystallization Facility at Maxlab according to [14].

2.11. Circular Dichroism (CD)

CD spectra were obtained with a Jasco J-815 CD-Spectrometer in the range 190-260 nm with 0.2 mg/ml VDE2 in 50 mM phosphate buffer pH 7.5.

2.12. Dynamic Light Scattering (DLS)

Particle size of protein solutions were measured with a Zetasizer Nano-S (Malvern) at 20°C.

2.13. Crystallization

Pre-Crystallization Test from Hampton Research was used to determine if a protein solution is acceptable for crystallization.

Crystallization was preformed using sitting-drop vapor diffusion with crystallization screens PACT Premier and JCSG+ from Molecular Dimensions at Cassiopeia Crystallization Facility at Maxlab. Each crystallization condition had two drops. One consisting of 100 nl reservoir and 100 nl protein solution, and another one with twice the amount of protein solution to concentrate the protein to twice its concentration.

2.14. Metal analysis

Concentration of metals in solution of purified VDE2 (0.1 mg/ml, 2 % active) and VDE3 (0.14 mg/ml, 30 % active) was measured with ICP-AES (OPTIMA 3000DV from Perkin Elmer). Metals analyzed were Al, As, B, Be, Ca, Cd, Co, Cr, Cu, Fe, Hg, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, S, Sb, Se, Si, Sr, Ti, V, Zn, Zr. The detection limit was 3 ppb and accuracy of 5 %. Samples were measured three times.

2.15. Inactivation of VDE

All inactivation and reactivation experiments were done with purified VDE3.

2.15.1. With low pH

VDE3 (20 µg/ml) was incubated in citrate-phosphate buffer (25 mM citrate, 50 mM phosphate) with different pH for 2.5 to 4.5 hours at room temperature before VDE activity was measured. The pH in the activity assay was 5.2 as usual and was not changed more than 0.1 units by the incubation buffer. The pH in the storage buffer of the protein did also not change the pH in the incubation buffer more than 0.1 units.

2.15.2. With DTT

VDE (20 µg/ml) was incubated with 0, 1, 5 and 10 mM DTT for 2 to 5 hours at room temperature for inactivation at pH 7.5. Short inactivation with same DTT concentrations at pH 5.2 were done by adding DTT to the activity measurement assay buffer. The pH was not altered by the addition of DTT.

2.16. Reactivation of VDE

2.16.1. By dilution

VDE samples treated with 1, 5 and 10 mM DTT and incubated for 2 to 5 hours at room temperature where diluted and directly followed by activity measurement. Final DTT concentration at activity measurement was <0.1 mM.

2.16.2. By desalting with PD-10 column

Inactivation of VDE (0.3 mg/ml) with 10 mM DTT was done at 4°C for 18 hours. This solution (1 ml) was desalted with a PD-10 column (GE Healthcare) and incubated at room temperature during activity measurements.

2.17. Oxygen consumption

The concentration of oxygen in a sample consisting of VDE2 (0.4 mg/ml) and DTT (5 mM) in either Hepes buffer (10 mM, pH 7.5) or Citrate-phosphate buffer (50 mM citrate, 110 mM phosphate, pH 5.2) was measured with a Clark-type electrode (Hansatech).

3. Results & Discussion

3.1. Plasmid construction

Plasmid1 and Plasmid3 were constructed successfully and their sequence determined. The reason why purification experiments were preformed on mutated VDE (VDE1) is that the results of the sequencing of Plasmid1 was delayed and since the expressed VDE1 had VDE activity some purification experiments were done during this period. The mutation was probably created in the PCR or that the DNA template (Plasmid0) had this mutation.

3.2. Isolation of proteins

Harvesting proteins with French press resulted in VDE activity for all constructs. Expression of VDE1 with the N-terminal transit peptide was also harvested by osmotic shock which also resulted in VDE activity. Harvesting by osmotic shock gave both a higher specific activity and a higher total amount of active VDE but in a more dilute solution compared with harvesting protein with French press. When using osmotic shock, the same amount of VDE activity that the final isolation fraction had could be found in the sucrose fraction, which suggests that the outer membrane of half of the cells break in that step and therefore half of the amount of expressed active VDE is lost. No activity could be found in the growth media or other steps of the osmotic shock procedure.

Using French press to isolate cytoplasmic proteins causes the protein to shift from an reducing environment to an oxidizing, that could cause reduced cysteines to form unspecific disulphide bridges. Therefore DTT was sometimes added in the buffers to maintain the cysteines in reduced form. This did however not noticeable improve the activity of the final purified protein, which suggests that the disulphide bridges forms correctly anyway or that the wrongly formed disulphide bridges does not disturbs the activity of the protein.

During isolation of expressed proteins no protease inhibitor was used. Incubation of purified VDE2 one hour in room temperature with 1 mM Phenylmethanesulfonyl fluoride (PMSF) reduced the activity of VDE to half. Ethylenediaminetetraacetic acid (EDTA) also showed indications of reducing the activity but was not fully confirmed.

3.3. Expression

Due to low activity of isolated VDE fractions variations in expression of VDE1 was done to increase the amount of expressed active VDE. Longer time of induction increased the amount of VDE activity of both the periplasm and cytoplasm fractions. Induction time of one day resulted in about ten times more VDE activity than one to four hours of incubation. More than one day of induction resulted in less VDE activity. But using five times higher ampicilin concentration the obtained VDE activity was twice as high after three days of incubation compared to one day of induction.

Induction over night at temperature of 37°C or 30°C resulted in about the same amount of VDE activity, but induction in room temperature the obtained VDE activity was 30% less.
When expressed VDE1 without IPTG induction the same amount of VDE activity was obtained as if induced with IPTG, which suggests that the rate of processing VDE to its mature, active form is less or equal to the rate of VDE expressed by the vector without IPTG presence. But when analyzing the soluble and insoluble fraction after harvest with French press with SDS-PAGE (fig. 4) there is a dominating protein band at 50 kDa in the insoluble fraction, which is only seen with IPTG induction. This protein band was analyzed by mass spectroscopy and turned out to be VDE1 with the transit peptide intact. The soluble fraction showed no visible protein band that could be identified as VDE by mass spectroscopy. The insoluble protein fraction showed no VDE activity.

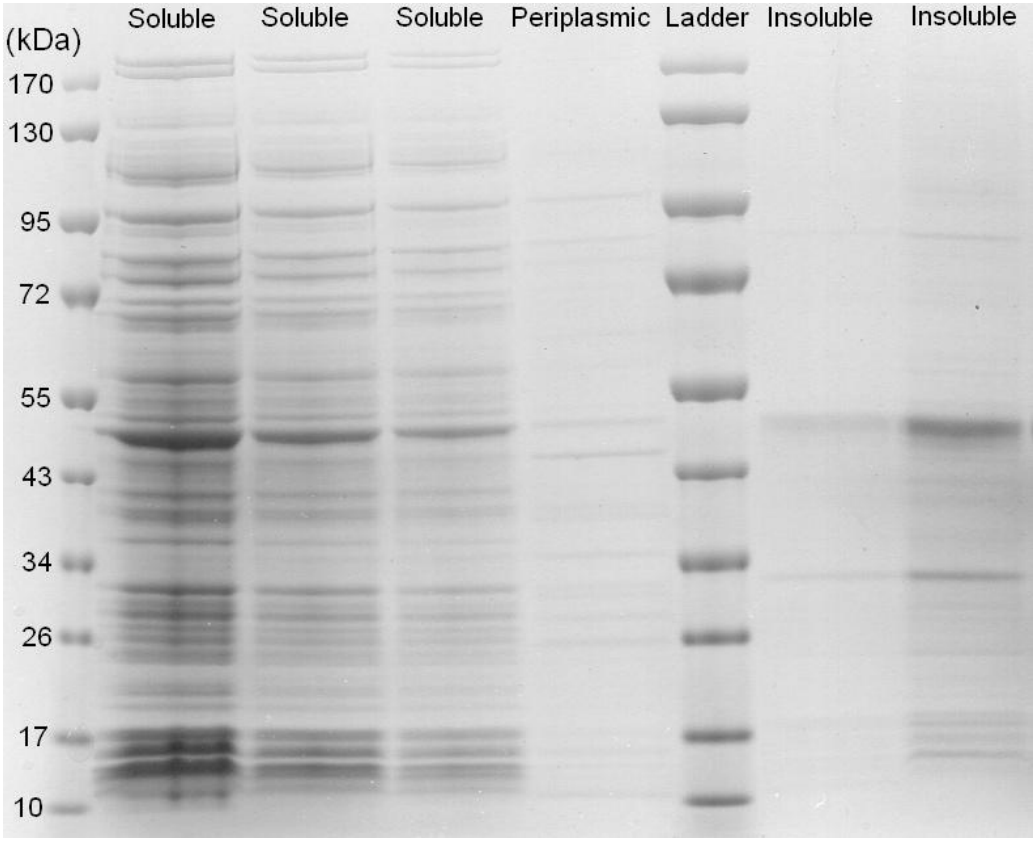

Fig. 4. SDS-PAGE with isolated proteins after expressing VDE1. Soluble proteins fraction was the supernatant obtained after French press and centrifugation. The pellet consists of insoluble proteins. Isolated periplasmic proteins were isolated by osmotic shock. Dominating band at 50 kDa in soluble proteins fraction is Elongation factor Tu (EF-Tu) and in the insoluble protein fraction was VDE1 with transit peptide and His-tag (determined by MS). Different amounts of soluble and insoluble proteins were loaded in different wells.

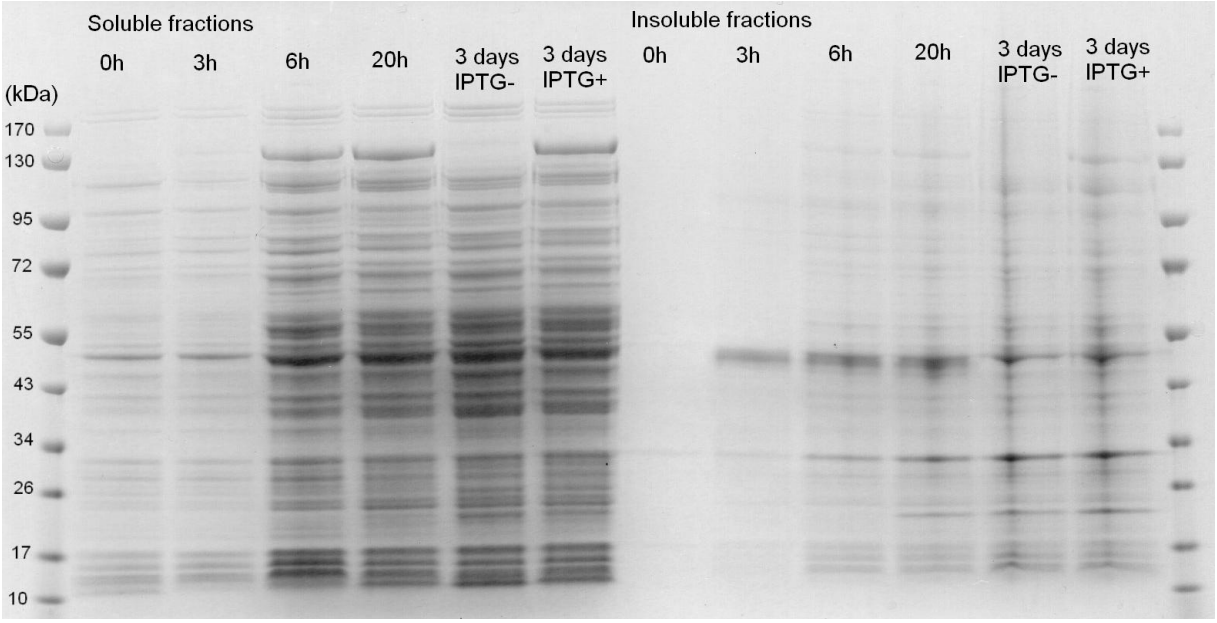

Fig. 5. SDS-PAGE with isolated proteins after expressing VDE1 with different induction length. The 130 kDa band in the well for soluble proteins induced three days with IPTG was β-galactosidase (determined by MS). The 20 kDa band in the well for soluble proteins induced three days without IPTG was DNA protection during starvation protein (determined by MS). The strong 50 kDa band in the well for soluble proteins induced three days with IPTG was Elongation factor Tu. The 50 kDa band in the well for insoluble proteins was absent without induction and does not get much more intense with longer induction than three hours. This band was VDE1 with transit peptide and His-tag (determined by MS).

One reason to why VDE1 forms inclusion bodies could be the transit peptide. To avoid the formation if inclusion bodies VDE was expressed without the transit peptide as VDE2. This resulted in approximately the same amount of VDE activity in the soluble protein fraction using French press after inducing one to four hours as VDE1. Lowering the temperature at induction to 30°C or the concentration of IPTG from 1.0 mM to 0.5 mM and 0.1 mM did not noticeable change the ratio of expressed VDE to other *E. coli* proteins in the insoluble fraction when looking at SDS-PAGE. These expression variations did not increase the amount of VDE activity in the soluble protein fraction.

To increase the purity and concentration of VDE1 and VDE2 affinity chromatography with Ni-NTA was applied.

3.4. Ni-NTA Affinity Chromatography

The isolated periplasmic proteins from expression of VDE1 were applied to a Ni-NTA column to bind the His-tagged VDE1 but with 0.65 M NaCl present all the VDE activity was found in the flow-through. With 1.0 M NaCl present no VDE activity was found in the flow-through, and could be eluted from the column with 0.5-1.0 M imidazol. But according to SDS-PAGE all isolated periplasmic protein was bound to the column at 1.0 M NaCl. Washing the bound proteins with 10 mM imidazol started to elute the VDE activity from the column and according to SDS-PAGE the eluted fraction still contained many contaminating proteins. One protein band (~50 kDa) was getting particularly stronger than other bands with more periplasmic proteins were loaded on the column. This band was Elongation Factor TU (EFTU) according to mass spectoscopy. EF-Tu naturally has 11 histidines and is therefore probably binding well to the Ni-NTA column. When trying to purify VDE1 from the soluble fraction with the Ni-NTA column under denaturing conditions with 8 M Urea present only one band was visible on SDS-PAGE in the eluted fractions, EF-Tu according to mass spectroscopy.

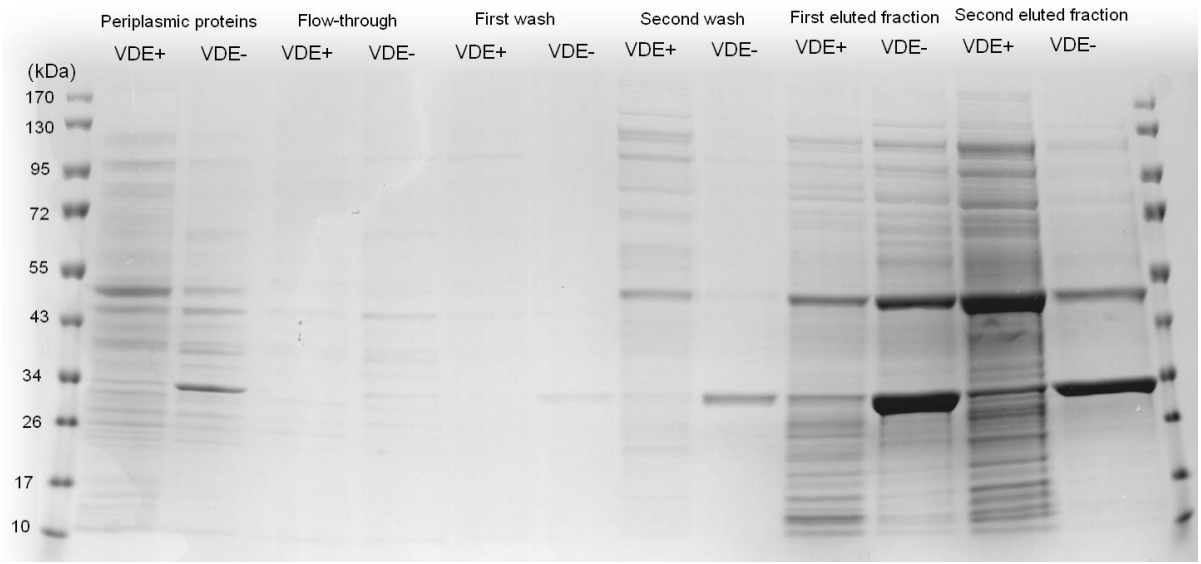

Fig. 6. Fractions from purification of VDE1 isolated with periplasmic proteins by Ni-NTA chromatography. VDE+ is isolated periplasm after expression of VDE1. VDE- is isolated periplasm after expression of empty pET22b+ vector. Expression of empty vector caused better growth and resulted in more *E. coli*. The quicker reach of the stationary phase may alter the ratio of other *E. coli* proteins. But the dominating band (50 kDa) concentrated during the purification was present in the control as well. The strong protein band at ~30 kDa is beleved to represent β-lactamase that is expressed in both VDE+ and VDE-.

Purification of VDE1 from the insoluble protein fraction, solubilized in 8 M urea, with Ni-NTA affinity chromatography resulted in VDE1 in flow-through, washing (10 mM Imidazol) and elution fractions along with other contaminating proteins, according to SDS-PAGE.

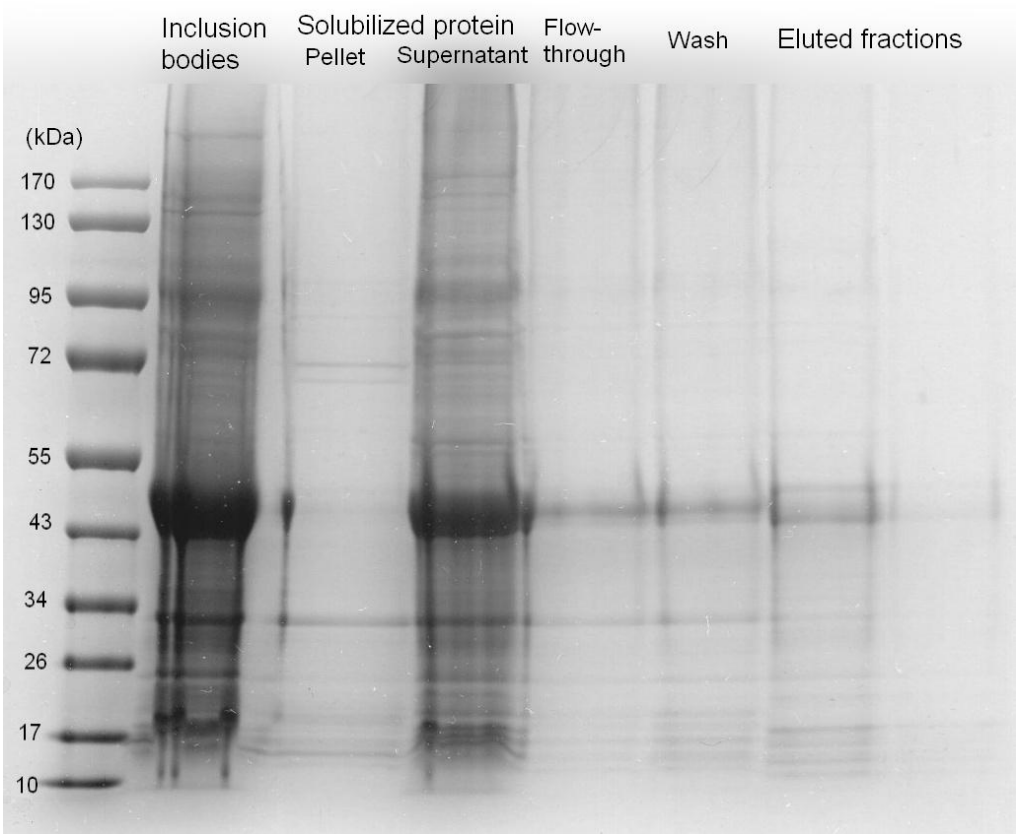

Fig. 7. Fractions from purification of VDE1, isolated with insoluble proteins, by Ni-NTA chromatography. VDE1 in flow-through and wash indicates that VDE1 binds poorly to the Ni-NTA column.

Repeating Ni-NTA purification with VDE2 from the insoluble protein fraction dissolved in 8 M urea showed that VDE2 did not elute from the column until the presence of 60 mM imidazol. With 40 mM imidazol present in wash no protein band was seen in the wash fraction on SDS-PAGE. This stronger binding could be due to that VDE2 have a longer His tag, 10xHis compared to VDE1 that have 6xHis. Or that the C-terminal tag of VDE1 is not as exposed as the N-terminal tag of VDE2.

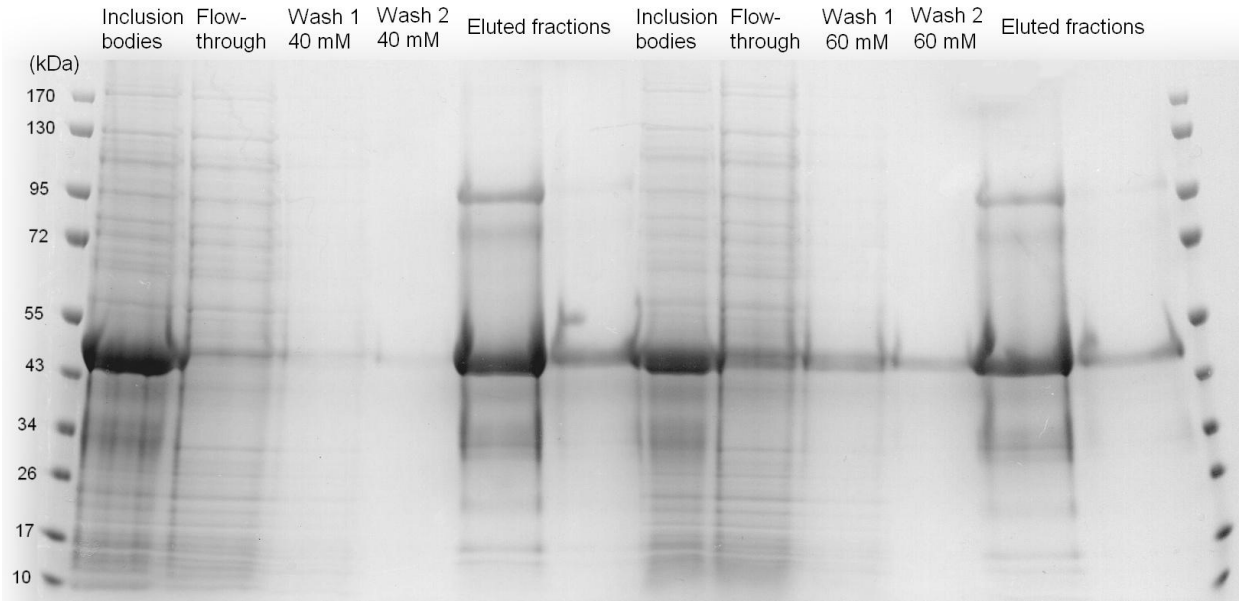

Fig. 8. Fractions from purification of VDE2, isolated with insoluble proteins, by Ni-NTA chromatography. The presence of VDE2 in the wash and flow-through fraction with 60 mM imidazole and absence with 40 mM imidazole indicates that it is in this range where VDE2 begins to be released from the column.

Ni-NTA affinity chromatography was not successful when purifying active VDE from the soluble proteins fraction. Probably due to that the initial VDE concentration after expression is to low compared to the other *E. coli* proteins. VDE2 from the insoluble protein fraction could be purified with this method but did not show any VDE activity. It could however be possible to refold the insoluble VDE to its active state.

3.5. Refolding

The refolding of VDE was done by lowering the concentration of urea in the protein solution, either by diluting the sample, dialysis or on-column washing.

3.5.1. By dilution

Isolated cytoplasmic and periplasmic proteins containing active VDE1 was treated with 8 M urea followed by dilution to 0.5 M urea and two hours of incubation in pH 7.7 and 5.4 at room temperature resulted in no regained VDE activity. The sample incubated at pH 5.4 got turbid directly after the dilution and after two hours the sample incubated at pH 7.7 also had become turbid. The VDE activity of the undiluted sample containing 8 M urea was measured, which includes dilution to 0.1 M urea and three minutes of incubation at pH 5.2. This showed an activity of 30 % compared to the untreated isolated cytoplasmic and periplasmic proteins containing active VDE1. This activity could either mean that VDE1 regain the active fold within three minutes at pH 5.2 or that it never fully lost the active fold when incubating with 8 M urea. This also means that the incubation was harmful for the VDE activity. The low concentration of active VDE compared to the concentration of other cell material could affect the denaturation and refolding process. To study the refolding of VDE, pure VDE would be preferable.

The reason that proteins form inclusion bodies is that either the mature fold or an intermediate is causing the protein to aggregate [15]. The overexpression of a protein in *E. coli* causes a high concentration of that protein, and a also a high concentration of protein that is folding at the same time. If the folding process has an intermediate that are prone to aggregate it would require a more dilute solution to reduce aggregation and increase the amount of correctly folded protein. But to dilute the protein so it can fold correctly would also result in a large volume of diluted protein. One way to concentrate this diluted protein solution is to bind the protein to an ion exchange column and elute in a smaller volume. This was tested on VDE2, as inclusion bodies from the insoluble protein fraction that were solubilized in 8 M urea and diluted 100 fold with strong stirring. DTT (5 mM) was present in the solution to maintain the cystines in reduced form. After two hours of incubation in room temperature the solution was loaded on a DEAE Sephacel (GE Healthcare) column and eluted with a NaCl gradient from 0 to 2 M without any DTT. The eluted fraction showed low VDE activity compared to refolding by dialysis, which could be due to that VDE did not had the time to oxidize its disulphide bridges. But later experiments with VDE3 showed that the majority of the VDE activity was regained within one hour. And if the N-terminal His-tag does not disturb the reoxidation there must be another explanation. During the loading there was a constant leaking of protein according to the detector, which could be due to high flow rate (10 ml/min) or that VDE2 binds poorly under those conditions. Another reason to the low activity could be because the dilution from 8 M urea to 80 mM urea was too rapid for optimal folding to take place.

VDE3, as inclusion bodies from the insoluble protein fraction was solubilized in 8 M urea and diluted to lower the concentration of urea to 0.8 M and 80 mM and incubated 2.5 hours. Activity measurement of these two dilutions showed that the one diluted 10 fold had 10 fold more activity than the sample diluted 100 fold, not shown in figure. This suggests that 0.8 M urea is low enough to allow VDE3 to fold. Unless the three minutes of incubation in the activity measurement at lower concentration of urea (5 mM) caused most of the refolding and that 0.8 M and 80 mM urea is too high to refold to the active state. Stepwise dilution by doubling the volume six times with one minute incubation time did not result in higher activity compared to the same dilution in one step. This means that either the folding process is rapid enough to be executed in the short time of a single dilution or the initial dilution needs to be slower for optimal folding. Slower lowering of the concentration could be done with dialysis.

3.5.2. By dialysis

Lowering the concentration of urea by dialysis was the method that gave the highest VDE activity, 1 000 to 5 000 fold higher than dilution. This indicates that a slower method of lowering the concentration of urea than dilution improved the refolding.

The previously discussed problem with formation of unspecific disulphide bridges when lysing the cells was furthermore investigated by treating VDE2 inclusion bodies from the insoluble protein fraction solubilized in 8 M urea with DTT (5 mM) and follow the refolding during dialysis by measuring VDE activity. The addition of DTT caused the reactivation of VDE2 to be delayed, probably due to the requirement for oxidation of cysteines. After 24 hours of dialysis the sample without initial DTT had 30 % less activity compared to the sample without initial DTT. After two days of dialysis the activity of the sample without initial DTT had decreased to the same level of activity as the sample with initial DTT. On the third day both samples showed the same activity as the day before. Storing protein samples several days in room temperature could however cause bacterial growth, which would have altered the result greatly. The fact that VDE2 without DTT treatment did not finally show more activity than untreated VDE2 once more suggests that either the disulphide bridges forms correctly anyway or that the wrongly formed disulphide bridges does not disturbs the activity of the protein.

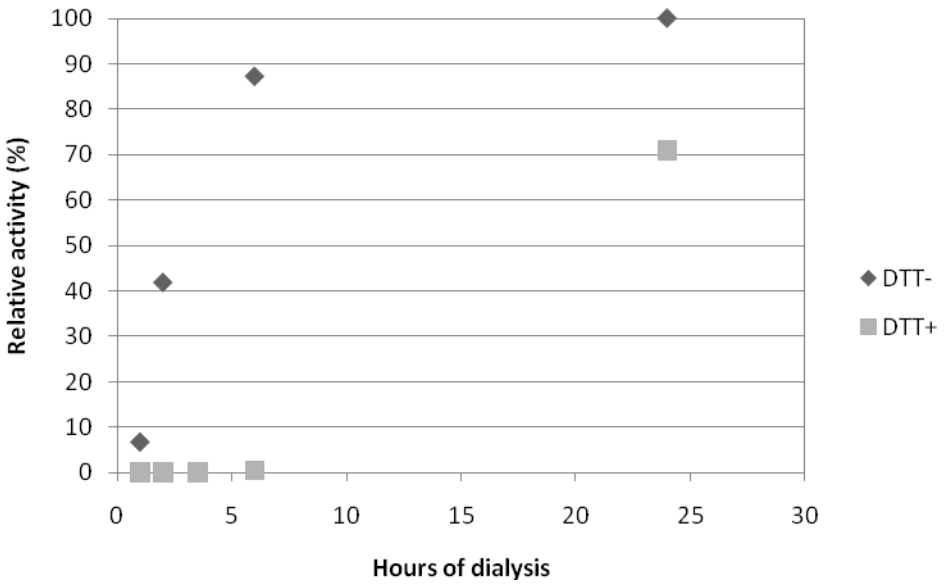

Fig. 9. The relative VDE activity of VDE2 samples solubilized in 8 M urea ± DTT as function of time of dialysis.

The previously mentioned issue with refolding at a high concentration of unfolded protein causing aggregation was investigated by dialyzing undiluted VDE2 inclusion bodies from the insoluble protein fraction dissolved in 8 M urea and ten fold dilution of the same solution, still in 8 M urea. Dialysed solutions were loaded on a gel-filtration column to se the ratio between the maximum intensity of the active VDE peak and the peak of aggregated proteins. The maximum intensity of the active VDE peak was 3 % compared to the maximum intensity of the void volume for the undiluted sample and 56 % for the diluted. This shows that higher concentration of unfolded VDE in the dialysis results in more aggregation.

To get less aggregation the protein concentration had to be lowered, which could be solved by dilution. Dialyzing diluted proteins will however result in lower concentration of active VDE. The refolding could be improved by changing the conditions of the refolding [15]. The presence of salt could reduce protein-protein interactions but adding 50, 100 or 200 mM of NaCl did not affect the amount of obtained active VDE during dialysis. Dialysis in 4°C resulted in half of VDE activity compared to dialysis in room temperature (data not shown). VDE from spinach is believed to shift between two conformations at pH 6.7 [5]. Refolding at pH 6 was therefore tested to utilize the extra mobility of the protein while shifting between two conformations. This did however only result in 1 % of VDE activity compared to refolding at pH 7.5. This could be the effect of decreased stability at low pH, discussed later.

The risk for aggregation at higher concentration of unfolded protein may be avoided by adjusting the rate of removal of urea. In dialysis the initial rate of decreasing the concentration of urea is quick and get slower over time. To slow down the initial rate of decreasing the urea concentration could be done by adjusting the concentration of urea in the dialysis reservoir, gradually from of 8 to 0 M urea. A quicker way to control the concentration of urea around the protein is to bind the protein to a column and wash away the urea.

3.6. On-column refolding

Removal of urea was tested by binding solubilized VDE2 inclusion bodies to a Ni-NTA column followed by washing without urea and eluting with imidazole. The eluted fraction was analyzed with SDS-PAGE and contained only VDE in high concentration but had almost no VDE activity. The removal of urea could have been too quick or the imidazole might have disturbed the refolding. To remove the urea, without the need to elute with either imidazole or NaCl, the solubilized inclusion bodies could be desalted with a PD-10 column.

VDE2, as inclusion bodies from the insoluble protein fraction, was solubilized in 8 M urea and desalted with a PD-10 column. The VDE activity directly after elution was approximately the same as the activity gained from one hour of dialysis. After two hours of incubation in room temperature the activity had increased 10 fold, suggesting that all unfolded VDE molecules were not folded at the same time the urea was removed and therefore needs longer incubation for proper folding. After two hours of dialysis the VDE activity was almost 10 fold higher than the activity of the desalted sample, incubated for two hours. This suggests that desalting with a PD-10 column removed the urea to quick for optimal refolding.

To slow down the desalting the solubilized inclusion bodies were loaded on the gel-filtration column equilibrated without urea. The unfolded protein would slowly be separated from the urea depending on the flow rate and be eluted without urea. This was tested once (1 ml/min) on solubilized VDE2 inclusion bodies and resulted in about the same level of VDE activity as if desalted with a PD-10 column. The gel-filtration took three hours compared to desalting with PD-10 that takes about three minutes. The slower rate of desalting did in this case not improve the refolding. Activity of eluted fractions was not measured later to see if it was increasing. This method could be tested more with slower flow rate to optimize the ratio between active VDE and aggregated VDE. The disadvantage with this method is that the volume size is limited by the column. And a higher concentration of the solubilized inclusion bodies would probably increase the amount of aggregation.

3.7. Two-step precipitation

Dialysis experiment showed that a diluted sample resulted in less aggregation during the refolding. The disadvantage to refolding a dilute sample is that the volume is larger, as long as the amount of protein is the same. To concentrate the sample, for further purification with gel-filtration, a two-step precipitation with ammonium sulphate was done. Precipitating dialyzed VDE with different levels of ammonium sulphate and measuring the VDE activity and protein concentration in both pellet and supernatant showed that two-step precipitation between 20 % and 60 % saturation increases the specific activity the most. This method was however not enough to remove all aggregates formed in the dialysis. This was instead done with gel-filtration.

3.8. Gel-filtration

The refolding methods could not be optimized enough to avoid the formation of aggregates but resulted in enough amount of active VDE to continue with characterization of the protein. The aggregated proteins were however first removed from the solution. This was done by gel-filtration. Previous experiment showed that Ovalbumin, that have the same mass as mature VDE from spinach (43 kDa) was eluted after 145 ml of eluting at 1 ml/min. Void volume at 88 ml and solvent volume at 165 ml. Gel-filtrations of dialyzed material usually resulted in three peaks (fig. 10). Largest particle at void volume (90 - 100 ml) followed by inactive oligomer (110 - 120 ml) and finally active monomer of VDE (140 - 150 ml).

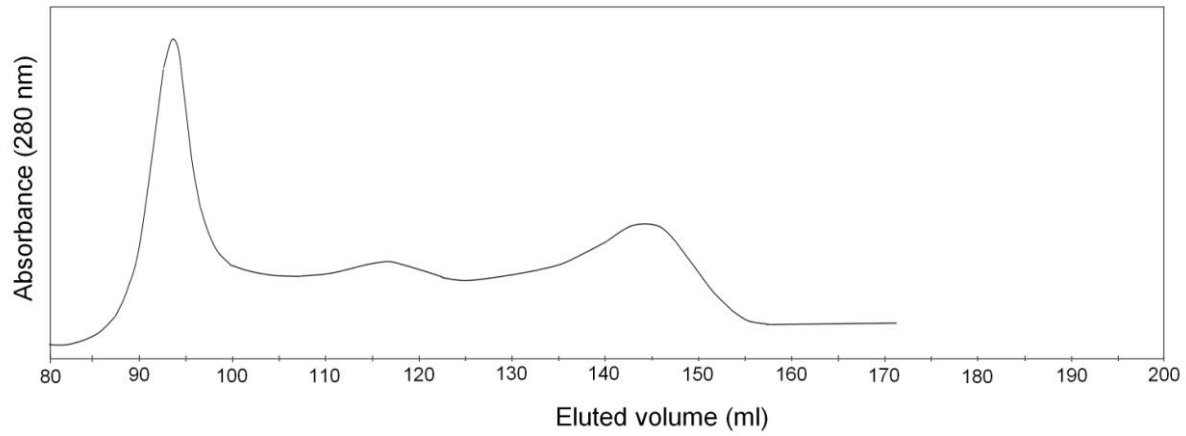

Fig. 10. Chromatogram from gel-filtration of solubilized and refolded VDE2. The void volume (fraction 90-100ml) showed a relative VDE activity of 0.2 % compared to the activity found in the highest fraction. The fraction containing inactive oligomers (fraction 110-120ml) showed a relative VDE activity of 0.4 % compared to the activity found in the highest fraction. The fraction containing monomeric VDE (fraction 140-150ml) showed a relative VDE activity of 100 % compared to the activity found in the highest fraction.

The buffer used in gel-filtration in fig. 10 was phosphate buffer (50 mM, pH 7.5). Using Hepes buffer (10 mM, pH 7.5) resulted in more formation of inactive oligomer, maybe due to lower ionic strength and increased protein-protein interactions. This increased formation of inactive dimer was also observed when loading higher concentrations of protein on the column. If the protein concentration is close to its maximum before precipitation it would cause the protein to intermittently interact with another protein and result in smaller proteins eluting earlier. This will result in more active monomer in the inactive oligomer fraction, which will show more VDE activity. A gel-filtration with low protein concentration resulted in 250 fold higher activity in the monomer fraction than the oligomer fraction, and with a high protein concentration resulted in 30 fold higher activity in the monomer fraction compared to the oligomer fraction. Gel-filtrations with high protein concentration loaded did not give a 280 nm absorption peak where the monomer should be eluted, but was rather detected as the tail of the peak of inactive oligomer.

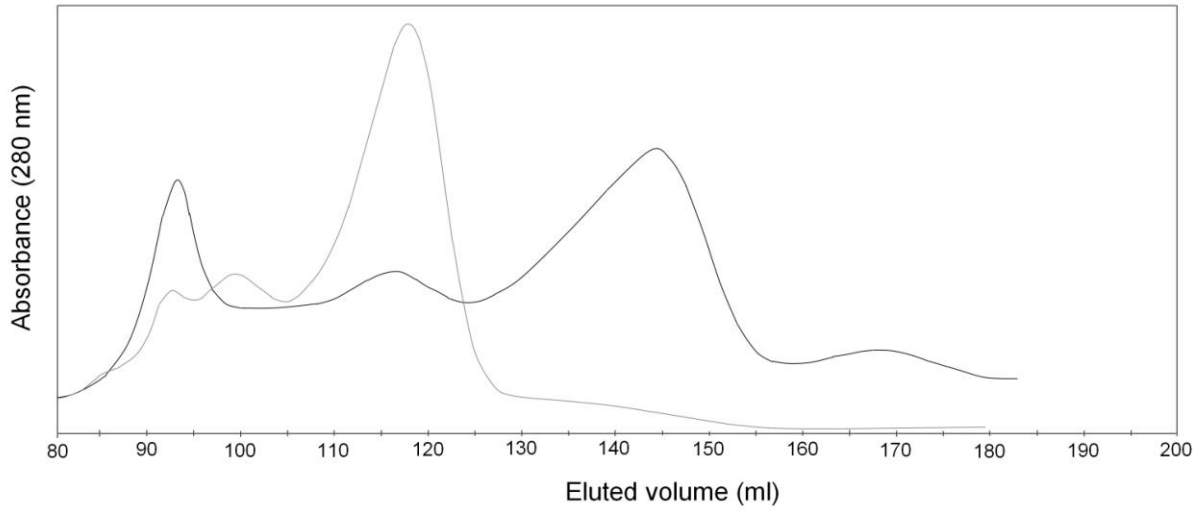

Fig. 11. Comparison of gel-filtration chromatogram of VDE2 inclusion bodies solubilized, refolded and purified in high (gray) or low (black) protein concentration. The chromatogram representing a high protein concentration has been scaled down.

That high protein concentration in gel-filtration is the reason to increased formation of inactive oligomer could be verified if both protein samples were prepared with the same method. There was however two differences when preparing the samples for gel-filtration. Firstly were experiments where a higher protein concentration loaded on the gel-filtration column refolded at a higher protein concentration, which could explain the increased formation of aggregates and oligomers. And secondly were the samples with high protein concentrations prepared with one additional step. This step was washing of the insoluble protein fraction after French press with Triton X-100, according to the method given in the methods section, to remove membrane fragments. Without this step the membrane fragments might be solubilized in the next step when treated with 8 M urea, and would therefore be present during refolding of VDE. The binding of VDE to thylakoid membranes may require hydrophobic parts to be exposed. But without a membrane to bind to and in a high protein concentration could cause VDE to associate to another VDE molecule, leading to oligomerization and aggregation. The presence of the lipids from solubilized membranes could improve the refolding by preventing this oligomerization. This is further discussed when reviewing the results from pH incubations of active VDE.

When loading solubilized inclusion bodies of VDE2 on the gel-filtration column, allowing the protein to refold during the separation the chromatogram showed one more absorption peak at the solvent volume. This component was not visible on the SDS-PAGE gel (fig. 13) and could therefore not be a protein. This could be that the high concentration of urea cause changes in the diffraction index or due to a low molecular component that were pelleted during centrifugation without urea and lost during dialysis. This component was not visible on the SDS-PAGE gel (fig. 13).

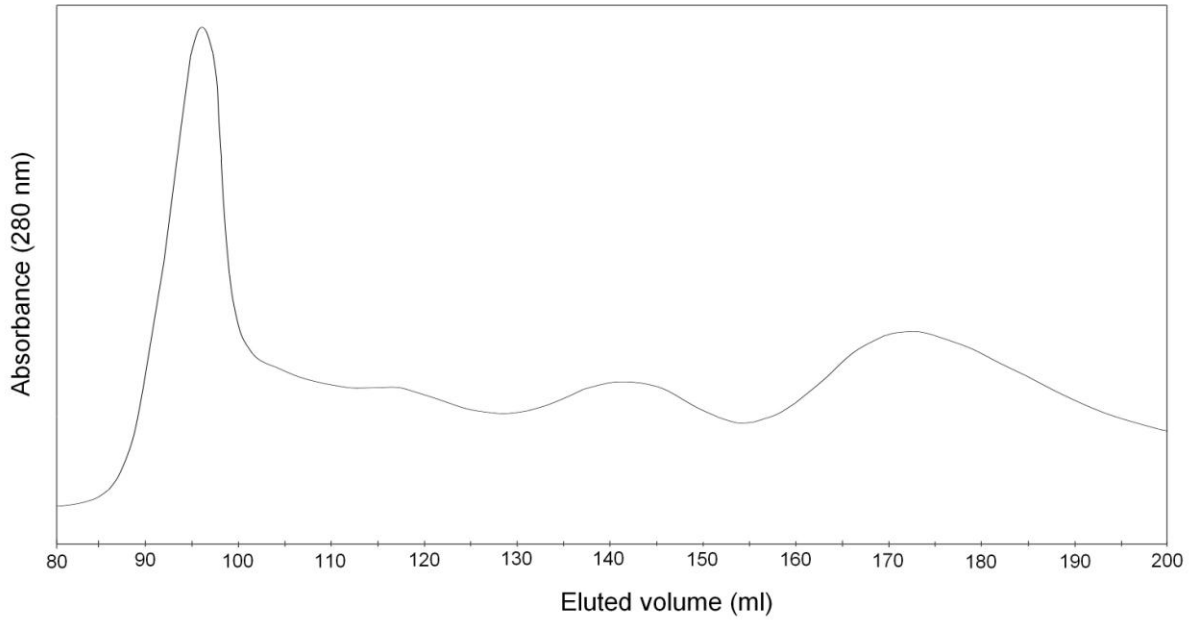

Fig. 12. Gel-filtration chromatogram for loaded solubilized VDE2 inclusion bodies with refolding during separation.

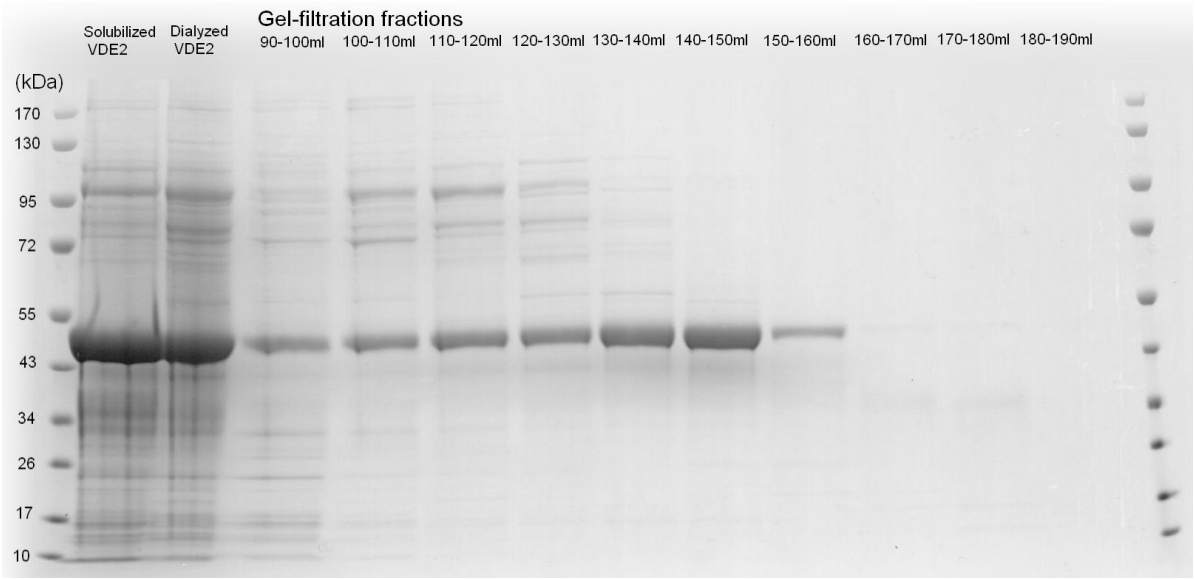

Fig. 13. SDS-PAGE with fractions from gel-filtration in fig. 12.

The eluted fractions from the gel-filtration, with solubilized inclusion bodies of VDE2, were analyzed with SDS-PAGE and revealed that VDE was the dominating band in most fractions except for the fraction containing the void volume that also contained other, probably aggregated proteins. When working with refolded VDE2 there was some signs of proteins with lower mass than VDE. These proteins could be degradation products but could be removed by gel-filtration. SDS-PAGE analysis of gel-filtration fractions with refolded VDE3 (fig. 15) showed stronger protein bands with lower mass than VDE suggesting probable degradation. One reason to why VDE2 could resist degradation better than VDE3 could be due to the N-terminal His-tag.

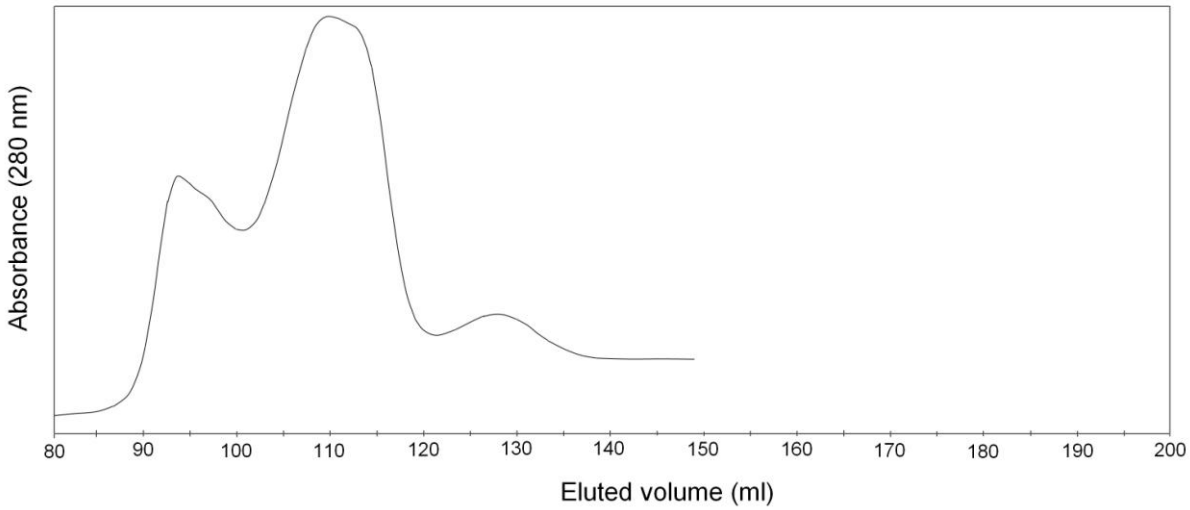

Fig. 14. Gel-filtration chromatogram for loaded solubilized and refolded VDE3 with high protein concentration. The peak at 110 ml is not homogeneous, which could be due to degradation.

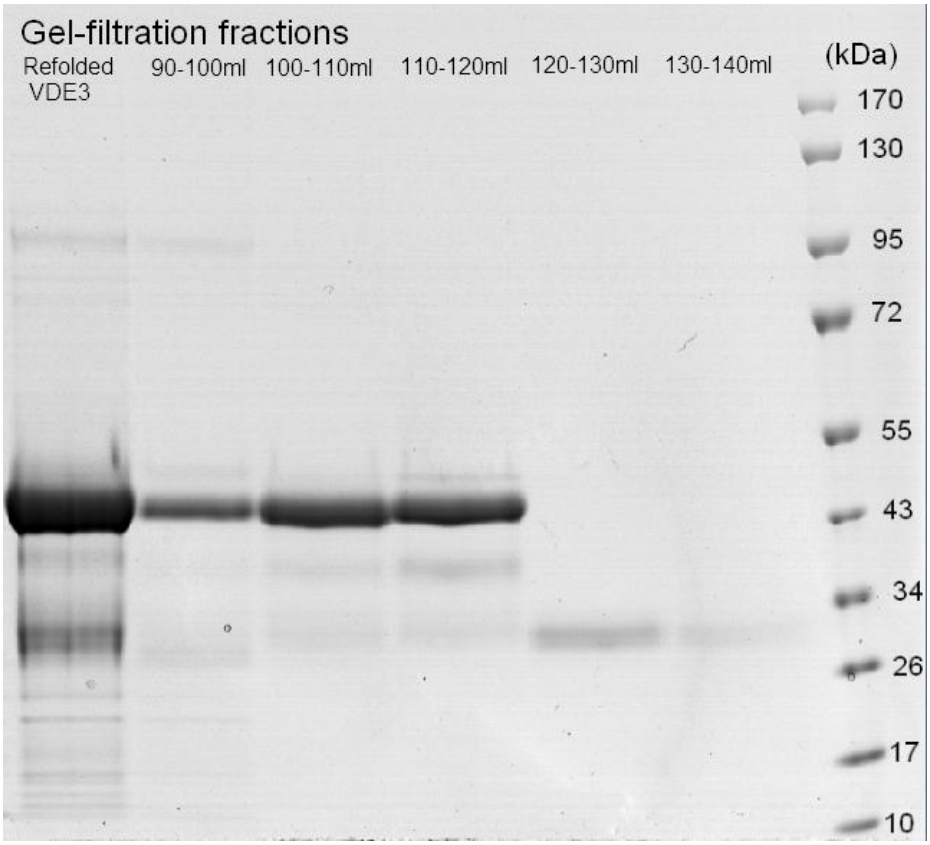

Fig. 15. SDS-PAGE with fractions from gel-filtration in fig. 14. Proteins with lower mass than VDE3, present in the loaded material as well, could be degraded VDE3.

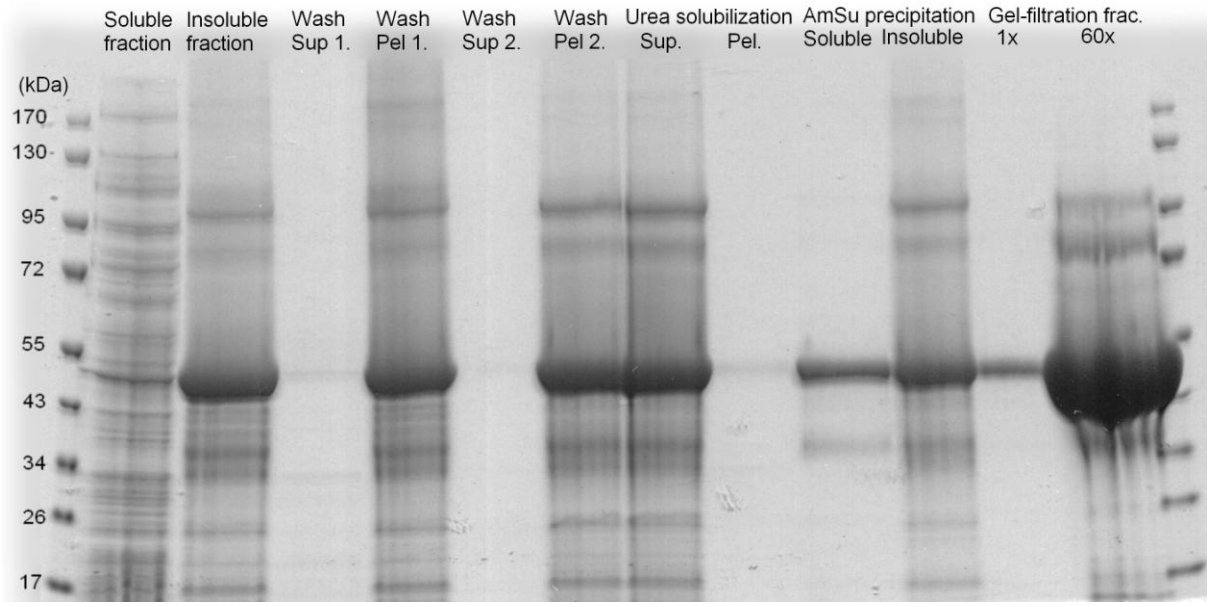

Fig. 16. SDS-PAGE with samples from different steps in the purification of VDE2, as described in methods section. Ammonium sulphate precipitation was done with 80 % saturation followed by resuspension of the pellet in solution without ammonium sulphate. The sample was centrifugated once more and the pellet was called insoluble fraction and the supernatant was called soluble fraction. The amount of loaded material is relative in all wells except the last where the volume of the final purified protein was overloaded 60 fold. No signs of degradation in the final purified protein well loaded 1x.

According to SDS-PAGE (fig. 16) the only protein present after gel-filtration is VDE as one band when not overloaded. When measuring the particle size distribution in the protein solution there was one sharp peak at 2.8 nm radius representing monomeric VDE and a less intense, wide peak around 100 to 1 000 nm, probably consisting of aggregated protein. The concentration of these aggregates was probably very low due to that larger particles spread light more than small particles and therefore were overrepresented. To continue to crystallization trials it would be preferable to get the concentration of aggregates as low as possible. The aggregates could either form after the gel-filtration, which means that VDE slowly aggregates in phosphate buffer (50 mM, pH 7.5) or Hepes buffer (10 mM, pH 7.5) at either 4°C or in room temperature during the time the protein were eluted. Or the aggregates originate from an earlier step and were not separated from monomeric VDE in the gel-filtration. This suggests that these aggregates could be removed from the active monomer with better separation in the gel-filtration. To improve the separation of active monomer from aggregates the flow rate was reduced from 1.0 ml/min to 0.5 and 0.1 ml/min but did not result in sharper peaks. It was however possible to remove the aggregates by ultracentrifugation with an AirFuge (Beckman) at 100 000 g for 30 minutes.

After purification of VDE with gel-filtration, the sample was regarded pure enough to proceed with characterization studies.

3.9. Mass spectroscopy

Analysis with mass spectroscopy was done to determine that the correct protein had been purified and to see if parts of the protein were missing.

After expressing VDE1 and separating the insoluble protein fraction by SDS-PAGE the dominating band was cut out, trypsinated and analyzed with MALDI/TOF.

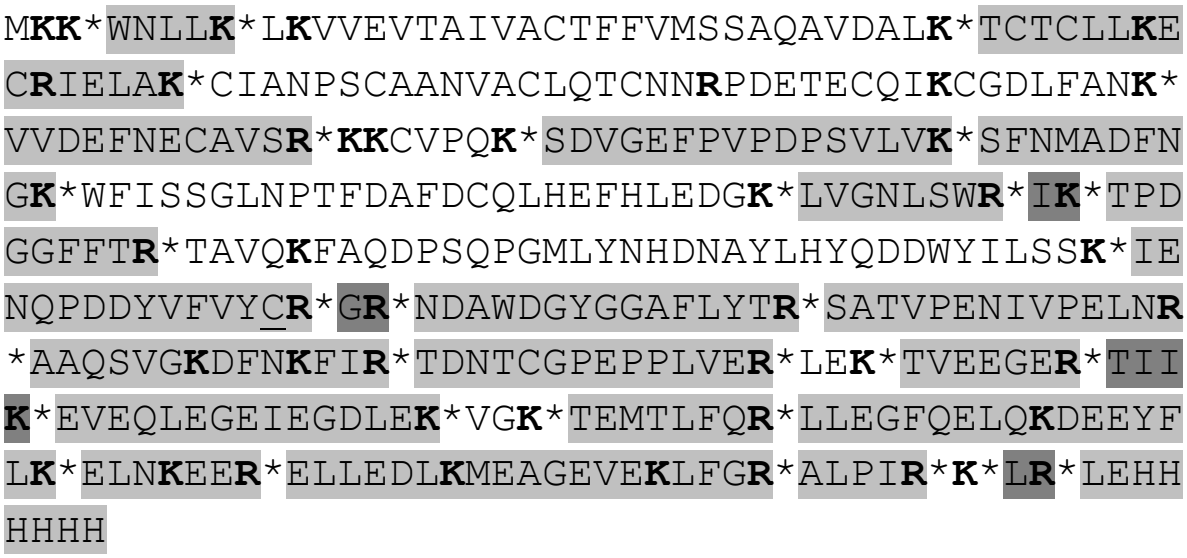

Fig. 17. Peptides of VDE1 found by MALDI/TOF are marked in light gray. Peptides marked with dark gray were found attached to the peptide to the right. Mutated residue Y227C is underlined. The presence of the transit peptide shows that most of the protein was not processed. Both the presence of the His-tag and the mutation were confirmed. The sequence coverage was 62 %.

Expressed and refolded VDE2 that was purified with gel-filtration and loaded on a SDS-PAGE gel. The single protein band was cut out, trypsinated and analyzed with MALDI/TOF.

MGHHHHHHHHHHSSGHIEG**R**HMVDAL**K**TC
TCLL**K**EC**R**IELA**K**CIANPSCAANVACLQTCNN**R**PDETECQI**K**CGDL
FAN**K***VVDEFNECAVS**R***KK**CVPQ**K***SDVGEFPVPDPSVLV**K***SFN
MADFNG**K***WFISSGLNPTFDAFDCQLHEFHLEDG**K***LVGNLSW**R***I
K*TPDGGFFT**R***TAVQ**K**FAQDPSQPGMLYNHDNAYLHYQDDWYILS
S**K***IENQPDDYVFVYY**R***G**R***NDAWDGYGGAFLYT**R***SATVPENIV
PELN**R***AAQSVG**K**DFN**K**FI**R***TDNTCGPEPPLVE**R***LE**K***TVEEGE
R*TII**K***EVEQLEGEIEGDLE**K***VG**K***TEMTLFQ**R***LLEGFQELQ**K**
DEEYFL**K***ELN**K**EE**R***ELLEDL**K**MEAGEVE**K***LFG**R***ALPI**R***K*L
R

Fig. 18. Peptides of VDE2 found by MALDI/TOF are marked in light gray. Peptides marked with dark gray were found attached to the peptide to the right. Neither the Cys-rich N-terminal nor the His-tag could be found. The sequence coverage was 63 %.

Expressed and refolded VDE3 that was purified with gel-filtration and loaded on a SDS-PAGE gel. The single protein band was cut out, trypsinated and analyzed with MALDI/TOF.

MVDAL**K**TC
TCLL**K**EC**R**IELA**K**CIANPSCAANVACLQTCNN**R**PDETECQI**K**CGDL
FAN**K***VVDEFNECAVS**R***KK**CVPQ**K***SDVGEFPVPDPSVLV**K***SFN
MADFNG**K***WFISSGLNPTFDAFDCQLHEFHLEDG**K***LVGNLSW**R***I
K*TPDGGFFT**R***TAVQ**K**FAQDPSQPGMLYNHDNAYLHYQDDWYILS
S**K***IENQPDDYVFVYY**R***G**R***NDAWDGYGGAFLYT**R***SATVPENIV
PELN**R***AAQSVG**K**DFN**K**FI**R***TDNTCGPEPPLVE**R***LE**K***TVEEGE
R*TII**K***EVEQLEGEIEGDLE**K***VG**K***TEMTLFQ**R***LLEGFQELQ**K**
DEEYFL**K***ELN**K**EE**R***ELLEDL**K**MEAGEVE**K***LFG**R***ALPI**R***K**L**R**

Fig. 19. Peptides of VDE3 found by MALDI/TOF marked in light gray. Peptides marked with dark gray were found attached to the peptide to the right. The Cys-rich N-terminal could not be found. The sequence coverage was 66 %.

Most of the VDE peptides could be found using MALDI/TOF except peptides that was too short or too long to be detected properly. The Cys-rich N-terminal for VDE2 and VDE3 was also not found, which could mean that it maybe was lost in degradation, something that VDE1 with the transit peptide could resist better. Or because VDE1 was not purified and the degradation occurs during the purification. It could also be that these peptides not easily get excited in the MALDI/TOF.

3.10. Differential Scanning Fluorimetry (DSF)

To investigate the stability of VDE in different conditions DSF was done on VDE2 (0.1 mg/ml) but resulted in a too high initial fluorescence signal. Dilution to half of the protein concentration did also give a high initial fluorescence signal. The fluorescence was measured every degree Celsius incremented until 95°C. The fluorescence did, however, not increase during the measurement. This result could indicate that either there is much denatured protein present or that VDE2 has hydrophobic parts already exposed. The lipocalin domain consists of a hydrophobic β-barrel [3], in which the fluorescence dye maybe can bind in the proteins active fold.

3.11. Circular Dichroism (CD)

The recorded CD-spectrum of VDE2 (0.3 mg/ml) in phosphate buffer (10 mM, pH 7.5) was analyzed by fitting CD-spectrum for pure α, β and random coil to match the recorded VDE2 CD-spectrum. The best fit resulted in 45 % α, 16 % β and 40 % random coil. This method is a very approximate way to calculate the component ratio of the secondary structure, due to that the pure form of one secondary structure type does not contribute exactly the same spectroscopic features when it is a part of a larger structure. The theoretical spectrum (fig. 21) is not overlapping the experimental spectrum perfectly but the calculated ratio of secondary structure from the experimental data is however matching the predicted secondary structure done by Jpred [16] (table 1).

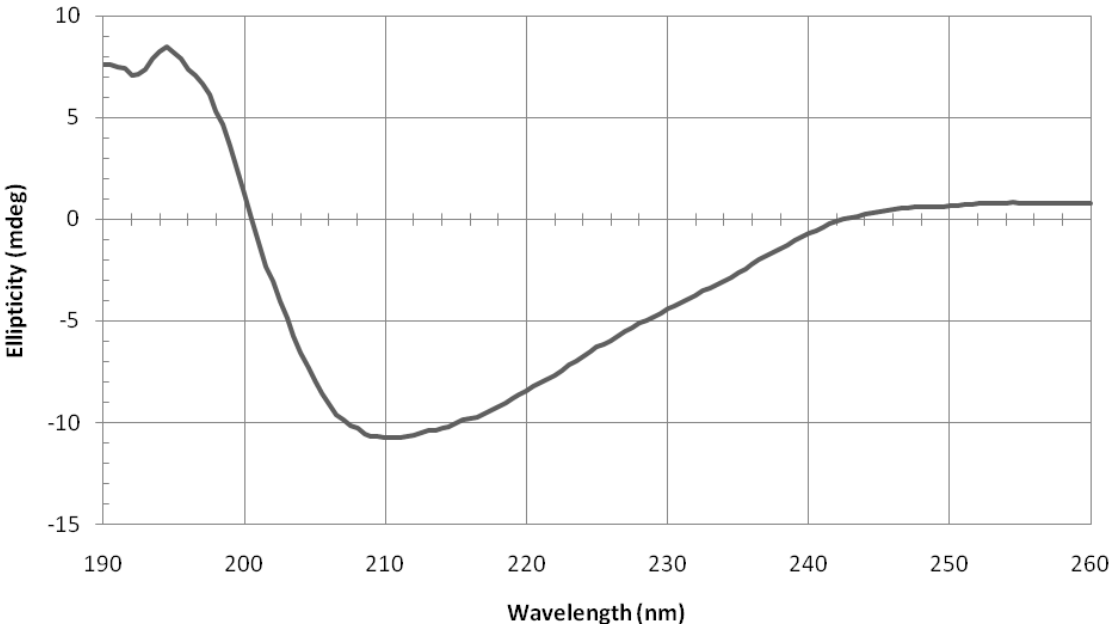

Fig. 20. CD-spectrum of purified VDE2 in 50 mM phosphate buffer pH 7.5 at 20°C.

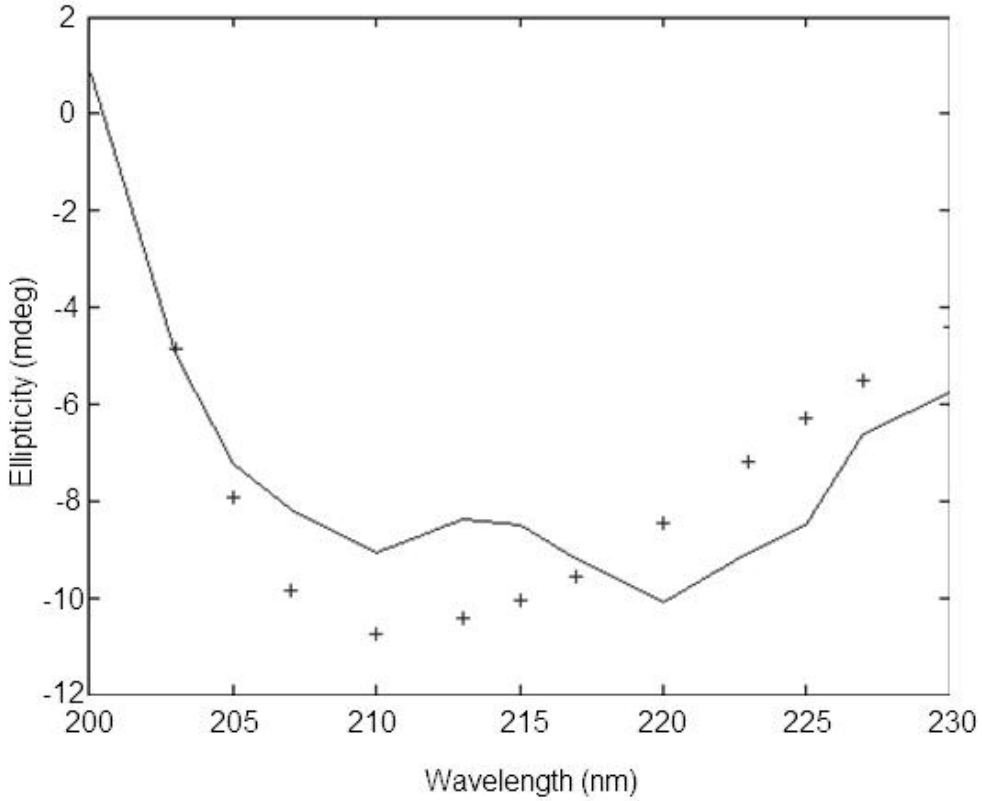

Fig. 21. Plotted values represent the actual CD-spectrum and the line represents the theoretical CD-spectrum of the calculated ratio of secondary structure.

Table 1. Values for secondary structure composition of VDE2 from CD-data and sequence prediction by Jpred.

Structure	Composition based on CD-data (%)	Prediction based on Jpred (%)	Difference
α	45	37	**7**
β	16	17	**1**
Random coil	40	45	**5**

The only part of the VDE structure that was confirmed was the lipocalin domain that is dominated by β-structure. And if the secondary structure prediction and CD analysis is correct the rest of VDE must mainly consist of α-structure. These results should however not be considered as exact but rather as a hint of the secondary structure composition.

3.12. Dynamic Light Scattering (DLS)

The size distribution of particles in the VDE2 sample after gel-filtration was measured with DLS and resulted in one dominating peak at 2.8 nm radius (fig. 22), which was calculated by the machine to correspond to a protein with the approximate mass of 49 kDa. This value varies with the form of the protein due to that DLS only calculate the hydrodynamic radius and should therefore not considered to be very accurate. Eluted fractions from the gel-filtration also contained another particle with a radius between 100 and 1 000 nm which probably is aggregate. The intensity of this peak was mostly about 10 to 30 % but due to that the DLS method is more sensitive to larger particles because these particles scatter light more these values can be recalculated to match the distribution of mass instead. The peak representing aggregates was not visible when viewing particle distribution based on mass suggesting that the concentration of aggregates was very low.

If the particles are big enough they can be pelleted using centrifugation. This was however not enough to remove all of the aggregates. But ultracentrifugation using an Airfuge was able to remove this peak in the DLS measurement.

The samples shown in (fig. 22) may contain high concentration of inactive oligomer due to refolding and purification in high protein concentration.

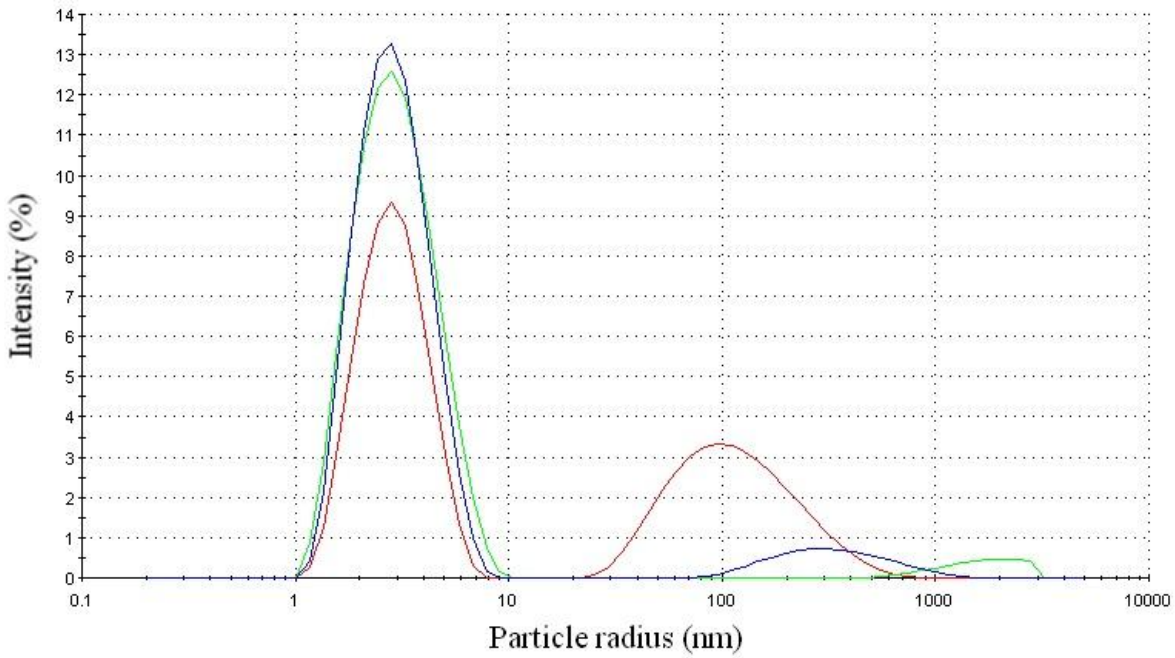

Fig. 22. Size distribution of particles in sample of purified VDE2. Red graph (pdi 0.5) represents sample that have been centrifugated 10 minutes in a tabletop centrifuge at 12 000 g. Green graph (pdi 0.20) represents sample ultracentrifugated in AirFuge for 30 minutes at 100 000 g. Blue graph (pdi 0.23) represents ultracentrifugated sample stored in 20°C for 40 minutes.

3.13. Crystallization

Pre-crystallization test was done to all protein solutions prior to the crystallization screen to ensure acceptable protein concentration. The first crystallization screen (PACT Premier and JCSG+) was done with purified VDE2 (1.3 mg/ml) in phosphate buffer (50 mM, pH 7.5). Nearly all drops was clear after incubation in 20°C up to two month, which indicates that the protein concentration was to low, but in drops where Ca^{2+} or Zn^{2+} was present crystals formed within some days. This suggests that the phosphate buffer formed salt crystals. Some of these conditions were repeated and crystals were dyed with Izit Crystal Dye (Hampton Research) without success. Repeated crystallization conditions without protein present still formed crystals. To avoid salt crystals the protein buffer was changed to Hepes (10 mM, pH 7.5).

The second crystallization screen (PACT Premier and JCSG+) was done on purified VDE2 from the gel-filtration fraction former called inactive oligomer but due to high protein concentration in the gel-filtration now probably a mixture of a small part of active monomer and a large part of inactive oligomer. Protein concentration was now 6.1 mg/ml. Both PACT Premier and JCSG+ screens were done twice, one stored in room temperature and one in 4°C. These screens resulted in more precipitation but the majority of drops were still clear after four month of incubation. The conditions that caused precipitation were mostly where the pH was five or lower, which could be explained by that the isoelectric point of VDE is 4.8. The presence of Zn^{2+} also caused precipitation. The crystallization drops stored in 4°C showed similar result as those in 20°C except that the precipitation took longer time. The high number of clear drops still indicates that the protein concentration was to low. Another reason to that the protein does not crystallize could be the mobile His-tag.

The third crystallization screen (PACT Premier and JCSG+) was done with VDE3 purified with the same method as that for VDE2 in the second crystallization, meaning that there probably was much inactive oligomer present. The protein was now concentrated to 15 mg/ml. This time the majority of the drops contained precipitation, suggesting that the protein concentration was high enough. The conditions causing quick and heavy precipitation was again low pH and the presence of Zn^{2+}. After one week also other conditions, mainly around pH 7 started to show precipitation, and days later formed droplets by phase separation. During following days and weeks these droplets grew slightly but no crystals formed.

The reason to why no crystals were formed could be due to that the negatively charged C-terminal domain is predicted to have a coiled coil region. These structures are mostly flexible and would disturb the crystallization. Another reason could be that VDE is prone to aggregate easily and therefore cause random protein-protein interactions instead of nucleate in an ordered way.

3.14. Metal Analysis

To analyze the possible presence of prosthetic groups metal analysis was done on VDE that was purified with gel-filtration, which means that a higher metal concentration than the buffer probably means that it was bound to the protein.

Table 2. Results for metal analysis of VDE2 (0.1 mg/ml, 2 % active) in Hepes buffer (10 mM, pH 7.5). Relative molar amount of 100 % equals 1:1 ratio.

Element	Concentration in protein sample (mg/l)	Concentration in buffer (mg/l)	Difference (mg/l)	Relative molar amount towards VDE (%)
Al	0.060	0.0049	0.055	82
Cu	0.017	0.0047	0.013	8
Mo	0.016	0.0038	0.012	5
K	1.1	0.15	0.91	930
Na	110	110	-2.4	-
S	270	260	7.8	7000
Si	0.59	0.73	-0.14	-

Table 3. Results for metal analysis of VDE3 (0.14 mg/ml, 30 % active) in phosphate buffer (50 mM, pH 7.5). Relative molar amount of 100 % equals 1:1 ratio.

Element	Concentration in protein sample (mg/l)	Concentration in buffer (mg/l)	Difference (mg/l)	Relative molar amount towards VDE (%)
Cu	0.029	0.024	0.005	2
Fe	0.39	0.18	0.21	107
K	0.60	0.55	0.05	37
Mo	0.015	0.0045	0.011	3
Na	2000	2000	-	-
P	1300	1300	-	-
S	1.6	0	1.6	1400
Si	1.4	0.61	0.80	810
Ti	0.023	0.005	0.018	11
Zn	0.005	0.006	-0.001	-

The presence of Al could be explained by that during autoclaving, growth and expression of VDE2 the growth flasks was covered with aluminum foil. The Hepes buffer also contained a low amount of aluminum, which also could be the source. After searching the literature no protein where Al is important for the activity could be found. Aluminum was more common to inhibit protein activities. Only two protein structures were found with Al^{3+} bound. D-Xylose isomeras (1XLM, 1XLG, 1XLH), that could bind Al^{3+} and Fe^{3+} instead of binding a divalent cation, for example Mg^{2+}, Co^{2+}, Mn^{2+}, necessary for activity. Binding Al^{3+} or Fe^{3+} inhibits the enzymatic activity of D-Xylose isomeras [17]. The second protein structure with aluminum bound was Transferrin (2D3I), which transports Fe^{3+} and was found to also bind Al^{3+} [18].

The expression of VDE3 was without aluminum foil and with purification without Hepes buffer. This sample did not contain any aluminum nor did the buffer. The phosphate buffer did however contain more iron than Hepes buffer and the VDE3 sample contained twice as much as its buffer. One explanation could be that it is Fe^{3+} that binds instead of Al^{3+} just like in D-Xylose isomeras and Transferrin. The VDE2 sample also had three times more Cu than its buffer. Cu should not be essential to the activity due to that the VDE3 sample did not contain much more Cu than its buffer. It could be the His-tag of VDE2 that causes the affinity for Cu. The His-tag could also explain the affinity for Al^{3+}.

The only metal that is of higher concentration in the protein sample than in its buffer in both VDE2 and VDE3 is Mo. The concentration of this element was however very low, 5 % for VDE2 and 3 % in VDE3. If Mo (3-5 %) is essential for the enzyme activity, the concentration of active VDE is lower than what could be calculated from the provided kcat value (2-30 %). Si was found in both the VDE2 sample and its buffer in approximately the same concentration. The source of this silica is probably glassware. The Si concentration for the VDE3 sample was however twice as high as its buffer, which could be explained if VDE3 have slightly different fold that has more affinity for Si than VDE2 where the only difference is the N-terminal His-tag.

Other components such as K, Na and S are part of the Hepes buffer and K, Na, Zn and P is part of the phosphate buffer. The concentration of K was however seven times higher in the VDE2 sample than in its buffer and would mean that every VDE2 molecule is binding nine K ions. The higher concentration of inactive VDE2 could maybe have a higher affinity for K or that the His-tag is increasing the potassium affinity.

The last component is Ti that was more common in the VDE3 sample than in its buffer. The Ti could come from the phosphate buffer and was therefore not seen in the VDE2 sample, where Hepes buffer was used instead. Ti could also come from plastic containers.

The concentration of sulphur could be used to calculate the concentration of VDE3 in the VDE3 sample, where sulphur was not a component in the buffer. This gives a VDE3 concentration of 0.1 mg/ml.

To improve these measurements a higher concentration of VDE should be analyzed, with a ratio of active VDE as high as possible. The low ratio of active VDE is maybe due to improper folding or lack of the correct metal ion or cofactor during expression or folding. Adding a mixture of suspected metal ions during expression or refolding, then obtain a higher ratio of active VDE and finally confirming the presence of the metal ion would improve the credibility of this experiment. The low ratio of active VDE could also be due to that the expressed VDE is less active than VDE isolated from plants. Posttranslational modifications or chaperones may in the original host increase the activity of VDE.

3.15. Inactivation of VDE

Inactivation and reactivation studies were done with purified VDE3.

3.15.1. By low pH

Incubation of VDE in pH 7.0 to 8.0 caused a decrease in activity of about 0 - 10 %. Incubation at lower pH (5.5 - 6.5) lowered the observed VDE activity noticeably in a linear way with maximal loss of 40 % of the activity. Incubation at even lower pH, 5.0 to 4.0 resulted in a loss of 90 % of the activity. Only minimal differences in activity when incubating 2.5, 3.5 and 4.5 hours were detected, which means that the activity loss took less than 2.5 hours and almost twice as long incubation time did not affect the activity. After 24 additional hours of incubation the VDE activity for sample incubated in pH 7 had decreased from 95 % to 65 %, suggesting that VDE is not stable in room temperature under these conditions. And the sample incubated in pH 5 had decreased from 10 % to 4 %. If the decrease of activity is approximated to be half of what it was 24 hours earlier, it could mean that the activity loss due to pH 5 was done within 2.5 hours and further activity losses was due to instability in room temperature and not pH dependant.

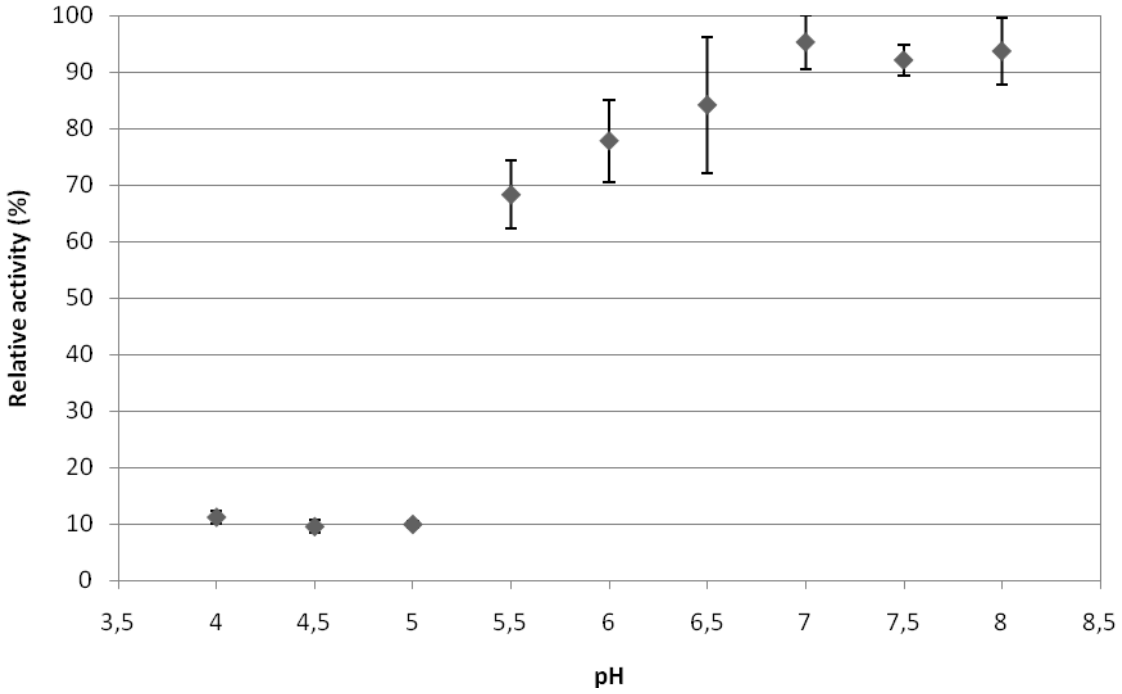

Fig. 23. Incubation of purified VDE3 in different pH for 2.5 - 4.5 hours. Error bars show the standard deviation for the three sample series made.

VDE is believed to shift conformation from an inactive state to an active state around pH 6.7, where VDE should be able to bind to the thylakoid membrane. The experiment in fig. 23 shows that low pH is harmful for VDE under these conditions. The pH where maximal activity was obtained is around pH 5, where also 90 % of the activity was lost after 2.5 hours of incubation in room temperature under these conditions. That the low pH is required for the activity and at the same time is destructive for the protein is maybe another way to regulate the xanthophyll cycle to avoid the conversion of too many violaxanthin to zeaxanthin. Or that this activity loss of incubation in low pH is an artifact due to unnatural environment for the protein. If low pH forces VDE to shift conformation to a state that have more affinity for the thylakoid membrane by exposing more hydrophobic parts to the surface of the protein, and there is no thylakoid membrane to bind to, this may force VDE to instead bind to another VDE molecule causing inactivation by aggregation. An unnaturally high concentration of VDE in this active state could also enhance the aggregation. The steep activity loss between pH 5.5 and 5.0 could suggest that it is in this range the majority of conformation change takes place. Or that the protein is close to its isoelectrical point at 4.8 and therefore precipitates easier.

Previously done experiments with inactivation of VDE by reduction with DTT states that due to low inactivation at pH 7 and high inactivation at pH 5 the active state exposes one or more disulphide bridges that are important for the activity and are reduced by DTT [9]. The pH incubation experiment in this report shows that incubation in low pH can lower the activity alone without the presence of DTT. The conditions for the DTT incubation experiment in different pH may prevent the inactivation of merely low pH. Those experiments were done in a cruder mixture with whole thylakoids and all of their content and could therefore contain components that prevented inactivation at low pH. To see if DTT inactivates VDE more effectively at low pH and that this effect not only was a summation of the DTT inactivation and the low pH inactivation, either prove that VDE in the crude mixture not was affected by the low pH, or compare the inactivation by low pH and by DTT to see if the combination causes more inactivation than the sum of each property itself. If components in the crude mixture prevented the inactivation at low pH the addition of a detergent may help by reduce aggregation, if that is the cause of inactivation at low pH.

3.15.2. By DTT

The increasing inactivation of VDE over time suggests that there is a reaction taking place between VDE and DTT and that the presence of DTT is not only disturbing the assay by for example associating to a substrate to prevent conversion.

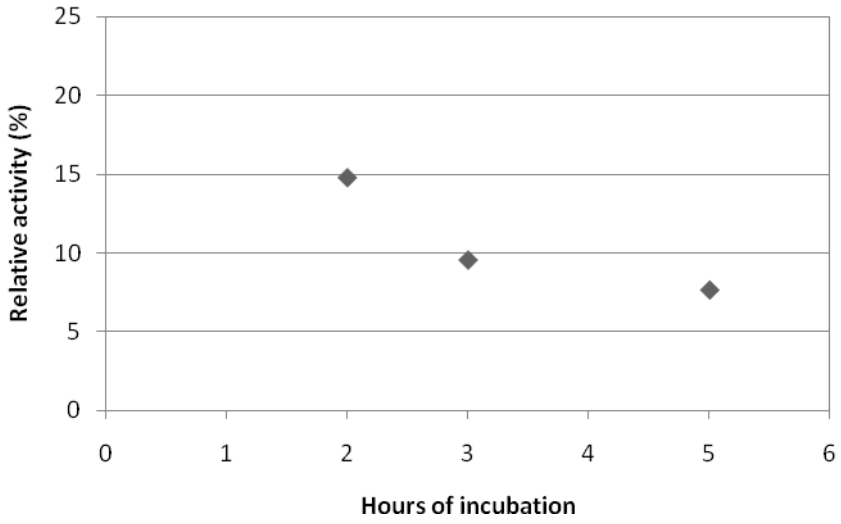

Fig. 24. Relative activity of VDE3 samples as a function of time at 1 mM DTT and pH 7.5.

The inactivation of VDE by short incubation with 1 mM DTT at pH 5.2 resulted in a loss of 79 % of the activity and 97 % when incubating with 10 mM DTT.

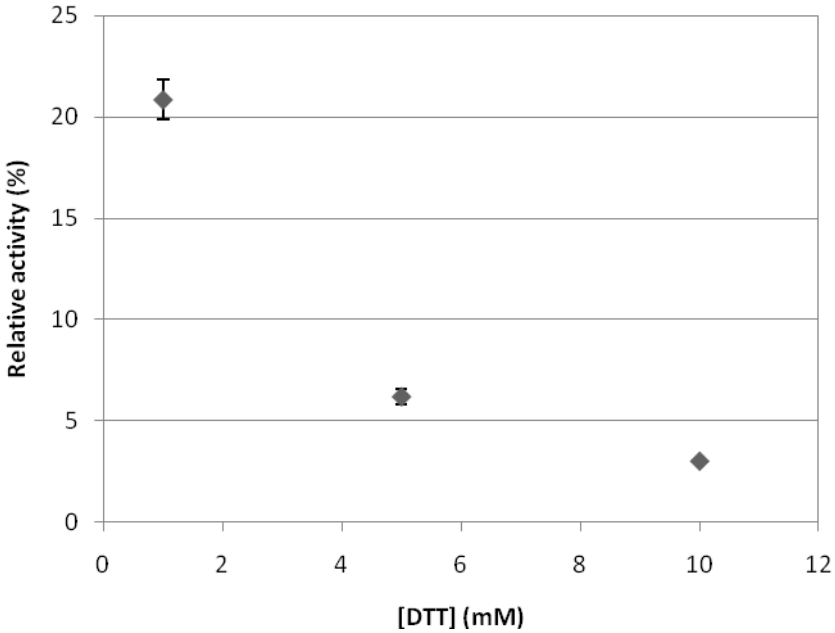

Fig. 25. Relative activity of VDE3 with different amounts of DTT present in assay during activity measurement. Error bars show the standard deviation for the three sample series made.

The inactivation of VDE by long incubation with 1 mM DTT at pH 7.5 and short incubation with 1 mM DTT at pH 5.2 resulted in a loss of 85 % of the activity and 100 % when incubating with 10 mM DTT.

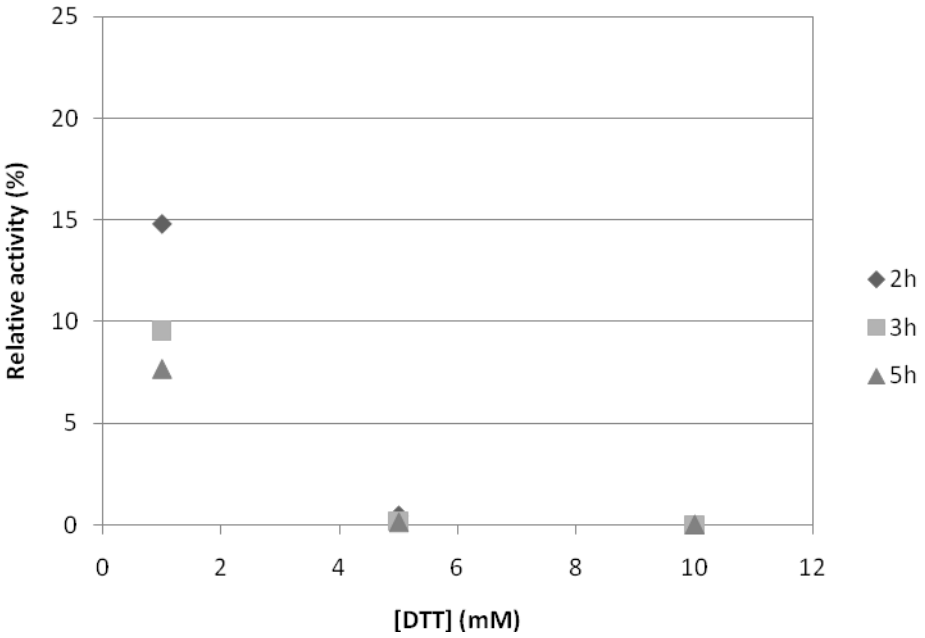

Fig. 26. Relative activity of VDE3 incubated with different amounts of DTT at pH 7.5 and the same concentration of DTT present in assay during activity measurement.

The low additional inactivation by incubating with DTT at pH 7.5 for several hours suggests that either the inactivation at pH 7.5 is low or that the final amount of active VDE for some reason does not get inactivated as easily. For example could a low concentration of contaminating lipid or detergent protect VDE in its active state from aggregation.

Comparing the inactivation of DTT in pH 5.2 and inactivation in pH 5.2 and pH 7.5 by dividing the value of activity after additional inactivation in pH 7.5 with the value of activity for inactivation in only pH 5.2 will theoretically result in the percentage of active VDE after DTT inactivation in pH 7.5 only.

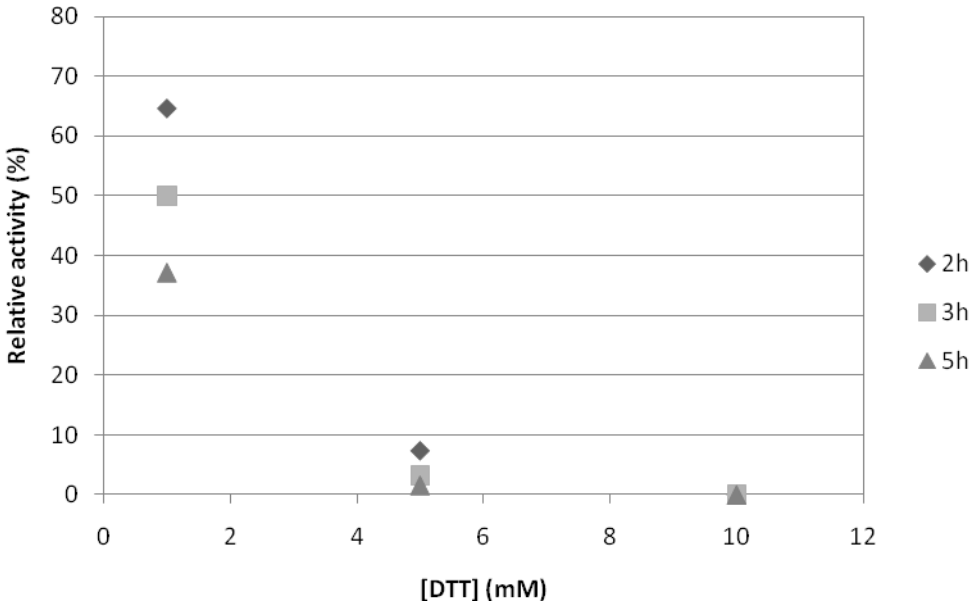

Fig. 27. The activity of VDE3 after different incubation periods with different concentrations of DTT at pH 7.5 relative to the activity of VDE3 with DTT present in the assay during activity measurement.

This diagram indicates that a large part of the inactivation by DTT was obtained in the short incubation in pH 5.2 but also reveals that longer incubation in pH 7.5 have a big effect on the activity at higher concentration of DTT.

Another way to test the effect of long incubation in pH 7.5 without inactivation at pH 5.2 was to dilute the DTT treated VDE to lower the concentration of DTT. This resulted in a diagram (fig. 28) similar to fig. 27, which confirms the idea that the majority of inactivation of VDE in 1 mM DTT was obtained at pH 5.2.

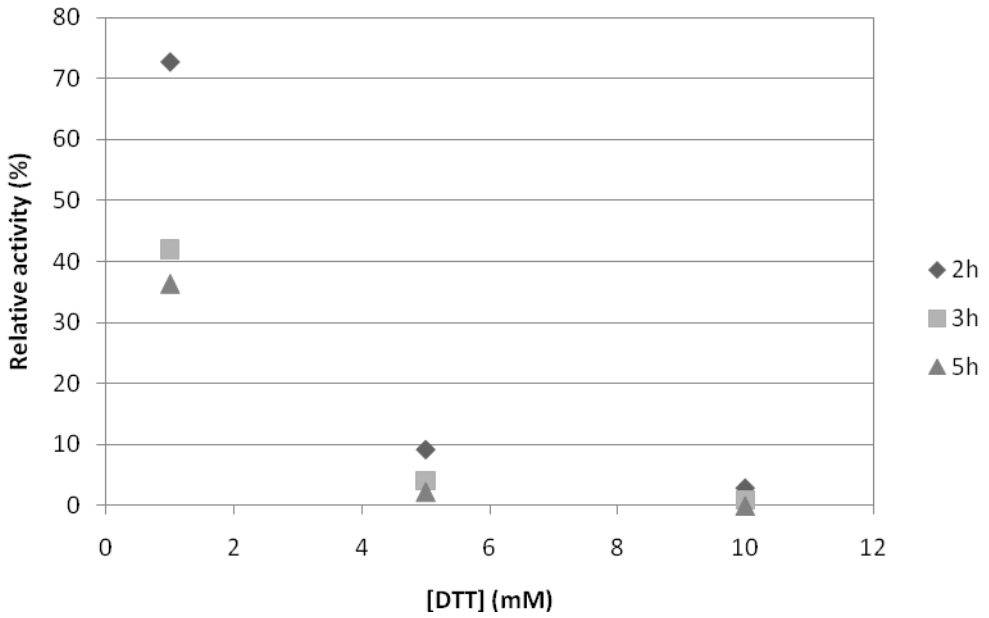

Fig. 28. The relative activity of VDE3 incubated with different amounts of DTT at pH 7.5 and diluted to <0.1 mM DTT in the activity measurement.

The reason why the VDE activity was higher when diluting the DTT concentration after hours of incubation in pH 7.5 could also be that the reduced disulphide bridges were oxidized during the activity measurement and therefore regain the active state. The differences in activity between Fig. 27 and Fig. 28 could be the reason that the diluted VDE samples still contained a small amount of DTT that disturbed the measurement or that the dilution caused oxidation of disulphide bridges that could increase the activity or a combination of both.

3.16. Reactivation

Reactivation of VDE was done by lowering or removing the DTT concentration to allow the cysteines to oxidize.

The experiment represented in fig. 28 with dilution of the VDE samples incubated with DTT showed that this diluted samples had more VDE activity than if the DTT concentration was the same during the activity measurement. This could either mean as previously stated that the cysteines have been allowed to oxidize or that they were not reduced and that the inactivation for samples incubated in pH 7.5 were done at pH 5.2 in the activity measurement. To ensure that VDE have regained its activity it must be certain that it first have lost it. Incubation at pH 7.5 with 10 mM DTT over night reduced the activity to 0.1 % with 10 mM DTT present in the activity measurement and 1.9 % when DTT concentration was diluted down to 0.17 mM. The DTT was removed by desalting the sample with a PD-10 column. The recovery of the activity over time is shown in fig. 29.

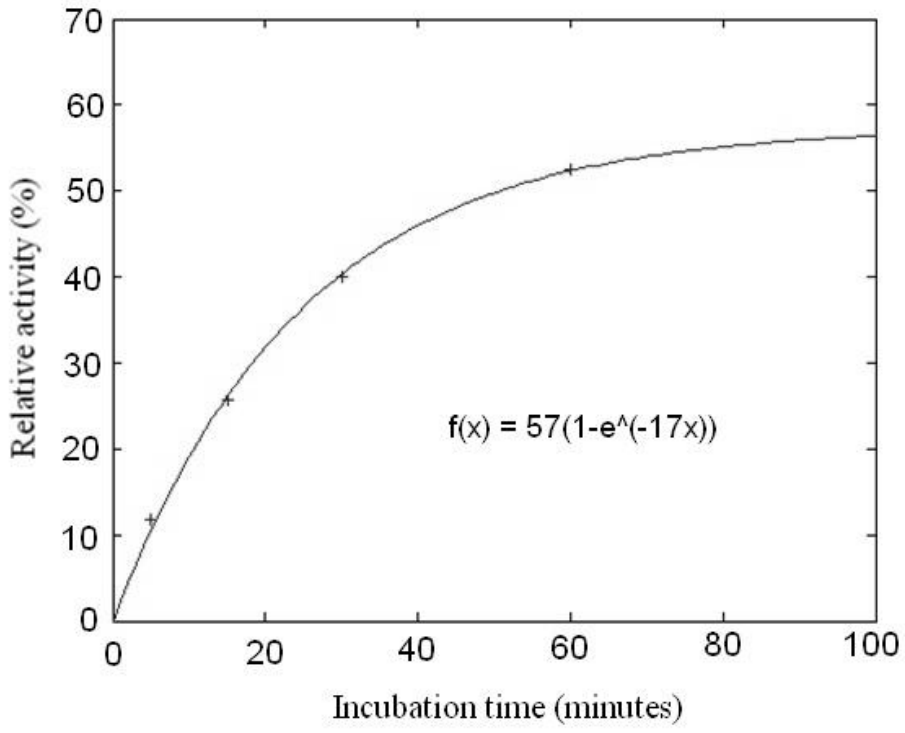

Fig. 29. The increase of activity of VDE3 over time after removal of DTT through desalting with PD-10 column. The maximum amount of relative activity was calculated to 57 % and the doubling time of the activity was calculated to 17 minutes.

The trend line for the plot in fig. 29 propose that the doubling time of recovered VDE activity is 17 minutes and that the maximum recovered activity will be 57 %. This value is calculated with the approximation that 100 % of the VDE that is added to the PD-10 column is eluted in the collected fraction. The maximum recovered activity value should therefore probably be higher, but a reason that not all the activity was recovered is if the wrong disulphide bridges are created, for example between two VDE molecules. This may happen if the shift to an oxidizing environment is to quick. To calculate the amount of VDE that is lost from desalting, a sample without DTT treatment could be desalted to measure the amount of recovered activity in the collected fraction.

The rate of reoxidation of VDE was slower than predicted. Previously done experiment have suggested that the VDE activity was fully recovered as soon as the DTT was removed [9]. These experiments were however done with lower concentration of VDE and in a more impure solution along with other thylakoid components that could disturb the experiment.

If the regained activity was due to that the cysteines were oxidized there must have been some component present that becomes reduced. The sample containing VDE was believed to be pure enough to not contain anything else but the protein and the buffer. Oxygen is therefore the suspected oxidant.

3.17. Consumption of oxygen

If oxygen is consumed to oxidize one or more cysteines to regain VDE activity there will be a decrease in the oxygen concentration, if the system is closed. This was measured with a Clark-type oxygen electrode. The decrease of oxygen concentration was however not detected when analyzing VDE2. The auto oxidation of DTT resulted in a mild slope that was not affected by the addition of VDE2. After calculation of the expected consumption of oxygen by using the doubling time for VDE activity recovery after DTT inactivation and desalting with PD-10 suggests that the slope for this concentration of VDE will be below the detection limit. To get a consumption of oxygen that will be measurable 10 times higher concentration of VDE is required.

4. Summary

Plasmid1 and Plasmid3 were constructed and confirmed by sequencing.

The overexpression of all of the plasmid constructs tested in this report resulted in a majority of VDE becoming insoluble. It was however possible to solubilize this insoluble protein in 8 M urea and refold it by dialysis to an active fold. This method resulted in high amounts of active VDE and after purification using two step precipitation and gel-filtration the active monomer could be separated from inactive oligomers and other contaminating *E. coli* proteins.

Protein characterization with CD-spectroscopy resulted in a ratio of secondary structure matching the predicted secondary structure result by Jpred. The result of DSF suggested that the protein sample was denatured or contained much hydrophobic residues exposed to the surface. The approximate radius of expressed VDE was determined by DLS. Metal analysis did not give a clear result if a metal is present in VDE but it may contain copper or molybdenum.

Inhibition of VDE by incubation in different pH showed that the pH for optimal enzymatic activity was harmful for the protein in the experimental conditions. Incubation with DTT was also inhibiting the enzymatic activity and has shown to have a stronger effect at low pH than neutral pH. This inactivation was however reversible. The VDE activity was fully or partially regained within few hours after desalting the sample with a PD-10 column followed by incubation in room temperature. A consumption of oxygen during DTT reduction could not be detected in the concentration of protein that was tested.

5. Future work

The method for obtaining active VDE from the insoluble protein fraction resulted in that much protein was lost during refolding due to formation of aggregates. To increase the yield more refolding conditions needs to be tested. The rate of urea removal could be varied if the protein was immobilized on a column or by varying the urea concentration in the reservoir during dialysis. Addition of additives could also affect the refolding and could be screened for using refolding kits. If assuming that aggregation causes loss of VDE activity during incubation in lower pH due to exposure of hydrophobic residues. This feature could then also cause aggregation during refolding. MGDG present during the refolding could allow VDE to bind to that instead of aggregating. A detergent present during refolding could also reduce the aggregation. Tween 20 has previously been used to prevent the interaction between VDE and column material at pH 5.2 [10]. It was also shown that Tween 20 inhibits the activity of VDE. A detergent present in the VDE activity assay could distort the packing of MGDG and affect the de-epoxidation. The detergent n-octyl-β-D-glucopyranoside has been used to solubilize precipitated VDE [19] and could also be added to VDE solutions to lower the formation of aggregates.

If one or several prosthetic groups are present in the mature version of VDE these, if known, could be added during the refolding or expression. Metal analysis would be a good tool to find out if there is, and in that case which metal ion that could be found in active VDE. To ensure the quality of the result a higher concentration of active VDE should be analyzed.

A thorough investigation of the stability of VDE in different conditions would simplify purification and further characterization experiments. This could be done by measuring the decrease of VDE activity during long incubations and repeated freezing and thawing steps. If VDE activity is lost due to aggregation and precipitation the decrease of protein concentration could also be measured after centrifugation. If these aggregates are not large enough to precipitate the size distribution could be monitored with DLS during the long incubations.

After finding a stable storage condition for VDE and ensuring that the active fold is dominating in the solution, further crystallization screens could be preformed. Prediction of chance for successful crystallization was very low according to XtalPred [20]. The reason to this is that the negatively charged C-terminal is predicted to be a coiled-coil region, which often does not crystallize due to flexibility. Expressing VDE without this region could improve the chance of crystallization. It has been shown that this region is not important for the enzymatic activity and the rest of VDE could therefore still be active.

With the truncated VDE without the C-terminal domain assays for evaluating the membrane binding capability compared to untruncated VDE could be done. This could be tested by lowering the pH in the presence of thylakoid membranes followed by centrifugation and activity measurement to see which fraction that shows the highest VDE activity, similar as the experiment done in [7]. One problem with this experiment is that if the C-terminal causes aggregation and precipitation at low pH this would be interpreted as it would have bound to the thylakoid membranes, if the activity is regained after resuspension in neutral pH. A control without thylakoid membranes should therefore also be tested. The experiment done in [7] was done with unpurified VDE which mean that other components present could have affected the membrane binding or precipitation. Removal of the C-terminal also increases the isoelectrical point which could affect membrane binding or precipitation. Another way to test the thylakoid membrane binding capability is to use violaxanthin containing thylakoids in the enzymatic activity assay to see if the truncated VDE is able to convert violaxanthin inside a membrane as well as full length VDE. An alternative is to use artificial violaxanthin containing liposomes to avoid contamination of other thylakoid content.

If the activity loss after incubation of VDE in low pH is due to aggregation could be investigated by measuring the size distribution with DLS. If this aggregation also causes precipitation these aggregates could be pelleted by centrifugation and resuspended in neural pH to see if the inactivation of low pH in reversible.

The buffers used when incubating VDE in different pH was made by mixing different ratio of citrate and phosphate. To make sure that not the higher concentration of citrate was inhibiting VDE different buffers or different buffer concentration could be used to confirm the pH inactivation.

That the VDE activity is reduced by the presence of DTT have been showed but to further confirm that this is due to reduction of cysteines other reductants used for reducing cysteines could be tested, for example β-mercaptoethanol and tris(2-carboxyethyl)phosphine (TCEP).

6. Acknowledgements

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7. References

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8. Appendix 1

DSF conditions

No.	**Buffer (100mM)**	**pH**	Other additives
1	Na acetate	5.0	0.15 M NaCl
2	MES	6.0	0.15 M NaCl
3	ammonium acetate	6.0	0.15 M NaCl
4	BisTris (73 mM)	6.5	0.15 M NaCl
5	Na cacodylate	6.5	0.15 M NaCl
6	imidazole	7.0	0.15 M NaCl
7	MOPS	7.2	0.15 M NaCl
8	PBS (12 mM)	7.3	0.15 M NaCl
9	HEPES / NaOH	7.4	0.15 M NaCl
10	Ammonium acetate	7.5	0.15 M NaCl
11	HEPES / acetate	7.5	0.15 M NaCl
12	HEPES / NaOH	8.0	0.15 M NaCl
13	Tris / HCl	8.0	0.15 M NaCl
14	Bicin	8.3	0.15 M NaCl
15	Glycine / NaOH	9.5	0.15 M NaCl
16	Na/K phosphate	10.0	0.15 M NaCl
17	HEPES / NaOH	7.4	
18	HEPES / NaOH	7.4	0.05 M NaCl
191	HEPES / NaOH	7.4	0.15 M NaCl
201	HEPES / NaOH	7.4	0.15 M NaCl
21	HEPES / NaOH	7.4	0.3 M NaCl
22	HEPES / NaOH	7.4	5% glycerol, 0.15 M NaCl
23	HEPES / NaOH	7.4	10% glycerol, 0.15 M NaCl
24	HEPES / NaOH	7.4	5% glycerol, 0.3 M NaCl