Purification of Violaxanthin De-epoxidase expressed in E. coli and identification of disulfide bonds using mass spectroscopy

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ABSTRACT

Plants need light to convert carbon dioxide to organic compounds, but when exposed to too much light the photosynthetic machinery takes damage. This is prevented by conversion of violaxanthin to zeaxanthin, which participate in a process that converts the excess light into heat. The conversion of violaxanthin to zeaxanthin is done by the enzyme violaxanthin de-epoxidase.

VDE from spinach have been sequenced and expressed in E. coli. After harvesting of periplasmic proteins VDE has been purified using DEAE column, two-step precipitation and lipid affinity precipitation with MGDG.

By not reducing VDE while running it on a SDS-PAGE gel and avoiding reducing substances in purification methods and sample preparation for mass spectroscopy the disulfide bonds should be intact. When analyzing non-reduced VDE with MS new mass peaks should appear, representing masses of two peptides linked together with a disulfide bond.

These peaks were found in the spectrum using Peptidemap. Then these peaks were further analyzed with MS/MS and data interpreted with xQuest. Four disulfide bonds could be confirmed with MS/MS according to xQuest.

Populärvetenskaplig sammanfattning

Rening av violaxanthin de-epoxidas uttryckt i E. coli och identifiering av disulfidbryggor genom mass spektroskopisk analys

Växter behöver ljus för att omvandla koldioxid till organiska föreningar, men när de utsätts för för mycket ljus blir komponenterna i fotosyntesen skadade. Detta kan förhindras genom att omvandla en molekyl i tylakoidmembran kallad violaxanthin till zeaxanthin, som deltar i en process som omvandlar denna energi till värme. Omvandlingen från violaxanthin till zeaxanthin görs av ett enzym som heter violaxanthin de-epoxidas.

VDE från spenat har sekvenserats och uttryckts i E. coli via en plasmid. Dessa bakterier har sedan odlats och skördats. VDE har därefter renats från andra proteiner från bakterien genom jonbytarkromatografi med DEAE, två-stegs utfällning med ammonium sulfat och lipid affinitets utfällning med MGDG.

Genom att inte reducera VDE varken vid uppreningen, elektroforesen eller vid provförberedningen till MS kommer disulfidbryggorna i VDE fortfarande vara intakta. Dessa peptider som sitter ihop med en disulfidbrygga borde ge en massa i mass spektrumet som indikerar vilka peptider som sitter ihop och på så sätt berätta vilka cysteiner som sitter ihop i VDE.

De peptider som var funna i mass spektrumet som skulle kunna vara flera peptider som sitter ihop via disulfidbryggor var också analyserade med MS/MS. Resultaten från MS/MS var sedan analyserat med programet xQuest, som kunde bekräfta fyra disulfidbryggor.

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1. INTRODUCTION

Photosynthesis is the process that plants uses to converts carbon dioxide to organic compounds using sunlight as energy source. But to much sunlight damages the plant by causing photoinhibation. This reduces the plant's capacity for photosynthesis, when excess light damages either the oxidizing or reducing side of PSII, damaging the water oxidizing complex on the donor side or blocking the flow of electrons to plastoquinone on the acceptor side [1].
Instead of moving to a shadow when exposed to intense light like animals do, plants defend them selves with a light absorbing compound, zeaxanthin, that converts the excess light to heat.

1.1. The Xanthophyll cycle

The Xantophyll cycle is located inside the chloroplast on both sides of the thylakoid membrane and consist of the epoxidation of zeaxanthin to violaxanthin and de-epoxidation of violaxanthin to zeaxanthin with the intermediat antheraxanthin.

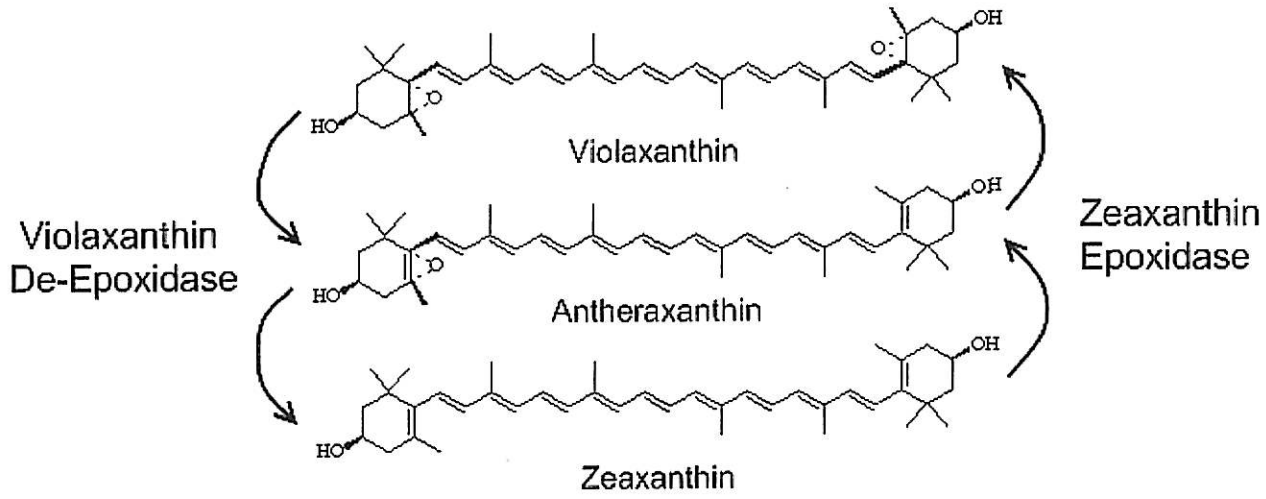

Fig. 1. The Xanthophyll cycle.

These cartenoids are located inside the thylakoid membrane and are epoxidised by the enzyme zeaxanthin epoxidase from the stroma side of the thylakoid membrane. The de-epoxidation is done by another enzyme on the lumenal side of the membrane, violaxanthin de-epoxidase.

1.2. Violaxanthin de-epoxidase (VDE)

The conversion of violaxanthin to zeaxanthin via anteraxanthin is done by the lumenal enzyme violaxanthin de-epoxidase and uses ascorbat as subsrate. Unprocessed VDE has a molecular weight of 53.7 kDa and mature VDE 39.7 kDa. Two signal peptides are removed from the N-terminal when VDE is transported through the membranes. (MALVA - AVIP) is removed when VDE passes through the chloroplast membrane to the stroma and proceeds later through the thylakoid membrane via the SEC-system [2] and removes (KKWN - SAQA).

At pH 7.2 VDE is water soluble and becomes membrane bound at pH 6.0 and lower [3]. The calculated isoeletrical point for VDE is 4.83 [4] and VDE has a pH optimum at pH 5.2 [5].

MALVARSICVSYDEIAGICNNVSHRNFKKWVQWKNPFLFQD
DARRNIRFNDRKLSCTKFIGASEKLQHSKSPKSGLISCGWE
VNSSKVVSNAVIPKKWNLLKLKVVEVTAIVACTFFVMSSAQ
AVDALKTCTCLLKECRIELAKCIANPSCAANVACLQTCNNR
PDETECQIKCGDLFANKVVDEFNECAVSRKKCVPQKSDVGE
FPVPDPSVLVKSFNMADFNGKWFISSGLNPTFDAFDCQLHE
FHLEDGKLVGNLSWRIKTPDGGFFTRTAVQKFAQDPSQPGM
LYNHDNAYLHYQDDWYILSSKIENQPDDYVFVYYRGRNDAW
DGYGGAFLYTRSATVPENIVPELNRAAQSVGKDFNKFIRTD
NTCGPEPPLVERLEKTVEEGERTIIKEVEQLEGEIEGDLEK
VGKTEMTLFQRLLEGFQELQKDEEYFLKELNKEERELLEDL
KMEAGEVEKLFGRALPIRKLR

Fig. 2. Sequence of unprocessed VDE in spinach [9]. Signal peptides and cysteines are highlighted.

The sequence of VDE could be devided into three different domains, the first domain at the N-terminal is a highly conserved cystein rich domain containing 13 cysteins. A second middle domain is not conserved but resembles a lipocalin protein, an eight stranded antiparallel β-barrel and a loop that works as a lid on top of the binding pocket [6]. Lipocalins are often used to transport small and hydrofobic molecyles. This domain have been crystallized and seems to be attach to another unit resulting in a structure of two β-barrels with the distance as the length of violaxanthin [7]. Two cysteins is located in the lipocalin domain and seems close enough to attach to each other. A highly negativily charged C-terminal is the last domain.

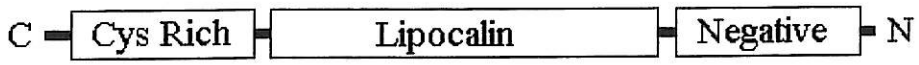

Fig. 3. Domains in mature VDE.

1.3. Previous purification methods

Former purification of VDE from spinach have been involving sonication of isolated thylakoids, first at low pH where VDE are bound to the membrane, then at higer pH where VDE becomes water soluble [5]. Further purification have consisted of ammonium sulphate precipitation to increcse the total protein concentration [3]. Gelfiltration, wich indicates that VDE is a monomer [11]. Anion exchange column, should be a useful purification method, when the isoeletrical point of VDE is low (4.83 [4]). A problem with HPLC equipment is that the volume of the protein sample must be filled in a "loop" which makes it harder to load larger volumes compared to a DEAE column.

Usage of Mono Q column showed that eluted fractions with NaCl gradient contained low activity [2]. The reason for this could be that VDE was not uniformly released resulting in many fractions containing VDE with low activity, or that the high number of positive charges in Mono Q denatures VDE, wich have a high number of negative charges at the C-terminal. It could also be that VDE activity reduces when NaCl is present. Loading large sample volumes would increse the concentration of eluted VDE, but also other proteins.

A successfull method used in [5] was lipid-affinity precipitation, where MGDG was incubated with VDE at low pH, ultracentrifugated and disolving of pellet with 0.5 % n-octyl-beta-D-glucopyranoside, which resulted in a 2D-gel with one clear spot. Much activity was lost in this step but removed other proteins well.

1.4. Expression in E. coli

The gene code for VDE has been indentified [9] and inserted in a plasmid (pET16b) so that the expressed protein contains a His-tag at the N-terminal followed by the signalpeptide that allows transport to lumen. When expressed in E.Coli this signalpeptide allows VDE to be transported through the cellmembrane via the SecYEG-system to end up in the periplasm [2]. This makes the purification proccess easier when all the cytoplasmic protein are still left in the cytosol. The downside to this is that the His-tag also could be removed so that Ni^{3+} affinity column can not be used for purification.

MGHHHHHHH
HHHSSGHIEGRHMKKWNLLKLKVVEVTAIVACTFFVMSSAQ
AVDALKTCTCLLKECRIELAKCIANPSCAANVACLQTCNNR
PDETECQIKCGDLFANKVVDEFNECAVSRKKCVPQKSDVGE
FPVPDPSVLVKSFNMADFNGKWFISSGLNPTFDAFDCQLHE
FHLEDGKLVGNLSWRIKTPDGGFFTRTAVQKFAQDPSQPGM
LYNHDNAYLHYQDDWYILSSKIENQPDDYVFVYYRGRNDAW
DGYGGAFLYTRSATVPENIVPELNRAAQSVGKDFNKFIRTD
NTCGPEPPLVERLEKTVEEGERTIIKEVEQLEGEIEGDLEK
VGKTEMTLFQRLLEGFQELQKDEEYFLKELNKEERELLEDL
KMEAGEVEKLFGRALPIRKLR

Fig. 4. Sequence of unprocessed VDE expressed in E. coli. Signal peptide, His-tag and cysteines are highlighted.

1.5. Identification of disulfide bonds using mass spectroscopy

When the protein is pure and in a high enough concentration to get a clear band on a SDS-PAGE gel, it should be possible to identify which cysteins that are connected to each other using mass spectroscopy, by analyzing the masses given when the disulfide bonds remain.

There are a number of programs calculating these bonds by masses, e.g xQuest, xBobcat, Prowl's Peptidemap and more. If treating the non-reduced protein with iodoacetamid it also should be possible to identify cysteins that do not connect to another cystein, which should be at least one due to the odd number of cysteins in VDE.

2. MATERIALS AND METHODS

All chemicals, except violaxanthin, was bought from Merck, Sigma-Aldrich and J.T Baker.

2.1. Obtaining VDE

All VDE was obtained from harvesting E. coli with the VDE gene inserted in pET16b.

2.1.1. Transformation, growth and induction of E.coli

pET16b vector containing the VDE gene and ampicillin resistance was transformed into E.coli strain BL21(DE3)pLysS. Then grown colonies on an agar plate with ampicillin was picked to a 10 ml Lauryl Broth solution and incubated over night in 37°C. The following day 100 µl of the graft was spread on another ampicillin containing agar plate and was incubated in 37°C over night. LB (3 ml) was spread on the plate and scrubbed, then transfered to 1 L LB with 100 µg/ml ampicillin in a incubation flask, which was incubated in an incubator 37°C untill OD_{600} = 0.5, ~ 4 h. Induction was done by addition of IPTG (0.5 mM) and further incubation in the incubator (30°C) for 4 h.

2.1.2. Isolation of periplasmic proteins

The grown and induced E.coli culture was centrufuged 15 min at 8 000 g. The pellet was washed in 2x300 ml Tris-HCl pH 7.3 (10 mM), NaCl (30 mM) and centrifuged 15 min at 8 000 g, 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 30 min to be centrifuged 15 min at 8 000 g. The recived pellet was resuspended in 100 ml ice cold $MgCl_2$ (0.5 mM), incubated on ice for 30 min and centrifuged 20 min at 10 000 g. The aquired supernatant was kept as isolated periplasmic proteins.

2.2. Purification of VDE

All purification methods were applied to VDE expressed in E. coli.

2.2.1. DEAE

The DEAE Sephacel was from GE Healthcare. All work was done in room temperature (25°C).

2.2.1.1. In batch

To investigate how VDE reacts with DEAE, isolated periplasmic protein, phosphate buffer and different concentrations of NaCl were mixed, incubated for ca 30 min and centrifuged 10 min at 13 400 rpm. Activities were later measured on the supernatants.

2.2.1.2. In column

The setup consisted of a peristaltic pump connected to the DEAE column followed by a absorbance detector (Bio Rad Econo UV monitor) working at 280 nm and a fraction collector. Sample was loaded directly on the column without using a "loop". The column was equilibrated with a solution containing phosphate buffer (50 mM, pH 7.1), $MgCl_2$ (0.5 mM) and various concentrations of NaCl, at least 5x the volume between each run. Standard flow rate was 0.5 ml/min.

2.2.2. Ni^{3+} Column

A sepharose-gel based column loaded with Ni^{3+} ions was used to separate VDE with its possible His-tag from the rest of the periplasmic proteins. Periplasmic proteins (2.5 ml, 4.8 µg VDE) was loaded on the column, washed with 50 mM imidazol and eluted with 1.0 M imidazole. Fractions of one ml was collected and run on a SDS-PAGE gel.

2.2.3. Ammonium sulfate precipitation

After establishing a precipitation curve a two-step precipitation was done using ammonium sulfate between 30 and 50 % saturation, incubation time was 40 min and centrifugation was at 13 000 rpm for 30 min and disolving the pellet in 80 µl water.

2.2.4. Lipid affinity precipitation with MGDG

Sample containing VDE (1 ml) was diluted with 1 ml 400 mM citrate pH 5.2, incubated with MGDG (0.11 mM) and violaxanthin (3.0 µM) for 40 min at 4°C and centrifugated at 83 000 g for 2.5 h at 4°C. Pellet was diluted with 100 µl n-octyl-β-D-glucopyranoside (0.5 %)

2.3. Activity measurements

VDE activity measurment was done according to [10] with a Shimadzu UV-3000 Dual-wavelength/double beam recording spectrophotometer. Assay consisted of citrat-phosphate buffer (50 mM citrat, 0.11 M phosphate, pH 5.2), MGDG (9.0 µM), ascorbate (30 mM) and violaxanthin (0.33 µM) that was purified from spinach thylakoids using HPLC. Amount of VDE was calculated by conversion of activity using kcat = 256 µmol/s [3].

2.4. Protein concentration measurements

Total protein concentration was measured using a Shimadzu UV-160A UV-visable Recording Spectrophotometer at 595 nm with Quick Start Bradford Dye Reagent 1x, diluted 5x, and a calibration curve ($R^2 > 0.99$) from known concentrations of BSA.

2.5. SDS-PAGE

NuPAGE Sample Buffer 4x (Invitrogen) was used without reducing agent on NuPAGE Novex 4-12 % Bis-Tris Midi Gels from Invitrogen and run with Bio Rad Power Pac 300 at 200 mV for ca 40 min. Stained in a solution of Commassive Brilliant Blue R 250 (0.05 %), methanol (50 %) and acetic acid (7 %) over night. Destained with methanol (50 %) and acetic acid (7 %) for ca 5 h. Protein standards used was Prestained Protein Molecular Weight #SM0441 (Fermentas), PageRuler Unstained Protein Ladder #SM0661 (Fermentas) and PageRuler Prestained Protein Ladder #SM0671 (Fermentas).

2.6. Mass spectroscopy

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

2.6.1. Sample preperation

Samples was 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 disulfide bonds with 20 µl DTT (10 mM) in 50 mM NH_4HCO_3 for 30 min in 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 remaning ethanol in fumehood 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 transfered 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 others 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.

If the sample had a low concentration of peptides it was concentrated 10 - 20 fold by using a poros R2 microcolumn with C18 HPLC chromatography beads from C18 Empore discs applied in a small pipette tip, that was first washed with 10 µl 60 % acetonitrile 0.1 % TFA, equillibrated with 10 µl 0.1 % TFA, sample applied, washed twice with 10 µl 0.1 % TFA, eluted with 1 µl 60 % acetonitrile 0.1 % TFA on the target plate and left to dry before addition of 0.5 µl matrix solution. The sample/matrix mix was left to dry over night.

2.6.2. Interpretation of MS and MS/MS data

Peptides was indentified by using GPS Explorer and Matrix Science - Mascot to identify proteins from the gel. Prowl - Peptidemap was used to identify peptides from given protein sequence.
Standard settings used in these programs was searching through all taxonomy in the SwissProt database with one misscleavage allowed, 50 ppm mass error, fixed modification of carbamidomethyl on cysteins and variable modification of methionine oxidation.

2.6.2.1. Identification of disulfide bonds

Possible mass peaks from fragments containing an intact disulfide bond was identified with MS analys by Peptidemap. These peaks was analysed with MS/MS and the resulting fragmentation was interpreted by xQuest. If xQuest gave the same hit as Peptide map the disulfide bond was considered confirmed by MS/MS.

Standard settings for Peptidemap was one missed cleavage, iodoacetamide (C) and oxidation (M) as variable modifications, disulfide as cross-link and 50 ppm mass tolerance.
Standard settings for xQuest was disulfide-bridge as cross-link, MS1 mass tolerance 50 ppm, MS2 mass tolerance 0.2 Da.

3. RESULTS

3.1. Obtaining VDE

In the first cultivation something grew in the graft but did not survive in the incubation flask containing ampicillin. The second cultivation was incubated first in a small falcon tube over night, then spread on a agar plate containing ampicillin as discribed in methods. OD_{600} was reached within 4 h and induced for 3.5 h in 37°C. Periplasmic proteins were then isolated but showed no VDE activity. This experiment was repeated once more with induction over night in 25°C but still showed no VDE activity. The size of the E.coli pellets were about half the size of what they use to be when VDE activity is present [8].

The thickest bands from gel with these cultivations was analyzed with MS and all identified as E. coli proteins except the most intense band (40 kDa) that was identified as ferrochelatase from B. subtilis with Mascot.

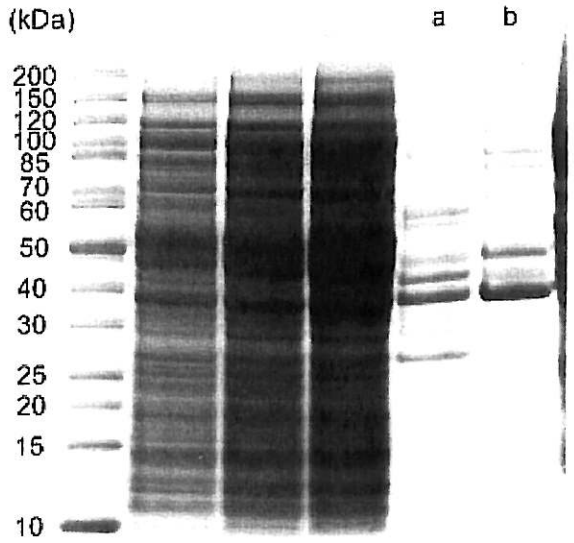

Fig. 5. Periplasmic proteins from E. coli incubated (a) over night in 25°C, (b) 3.5 h in 37°C.

The method of isolating periplasmic proteins was used on an old E.coli pellet containing VDE, which resulted in periplasmic proteins with VDE activity (2.6 µg VDE/ml). The most intense band (40 kDa) of the unpurified periplasmic proteins (fig. 6) was identified as ferrochelatase from B. subtilis by GPS - Explorer. Other bands were identified as E. coli proteins except the band below the ferrochelatase band, which could not be identified by GPS – Explorer or Mascot.

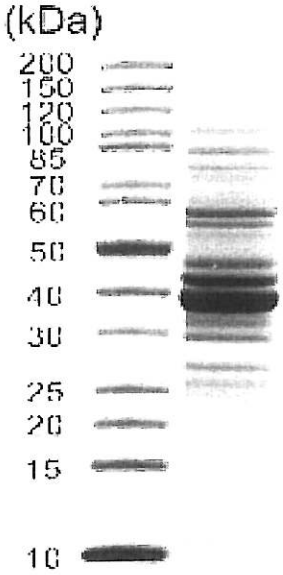

Fig. 6. SDS-PAGE gel for isolated unpurified periplasmic proteins, 57 ng VDE loaded.

The remaining pellet after removal of periplasmic proteins was french pressed, which also gave VDE activity (0.26 µg VDE/ml). All bands between 38 kDa and 57 kDa on the SDS-PAGE gel with cytoplasm without periplasm was identified with MS and all identified as E. coli proteins.

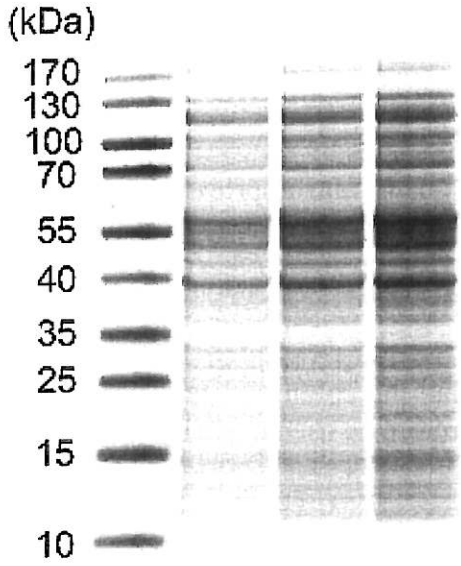

Fig. 7. SDS-PAGE for cytoplasmic proteins without periplasmic proteins at different concentrations, 1.3 ng, 2.6 ng and 6.5 ng VDE loaded.

There were no detectable activity in the sucrose supernatant in the step before the destruction of the cell wall when harvesting periplasmic proteins from my cultivations.

3.2. Purification of VDE

Summary of the purification methods used is shown if Fig. 8.

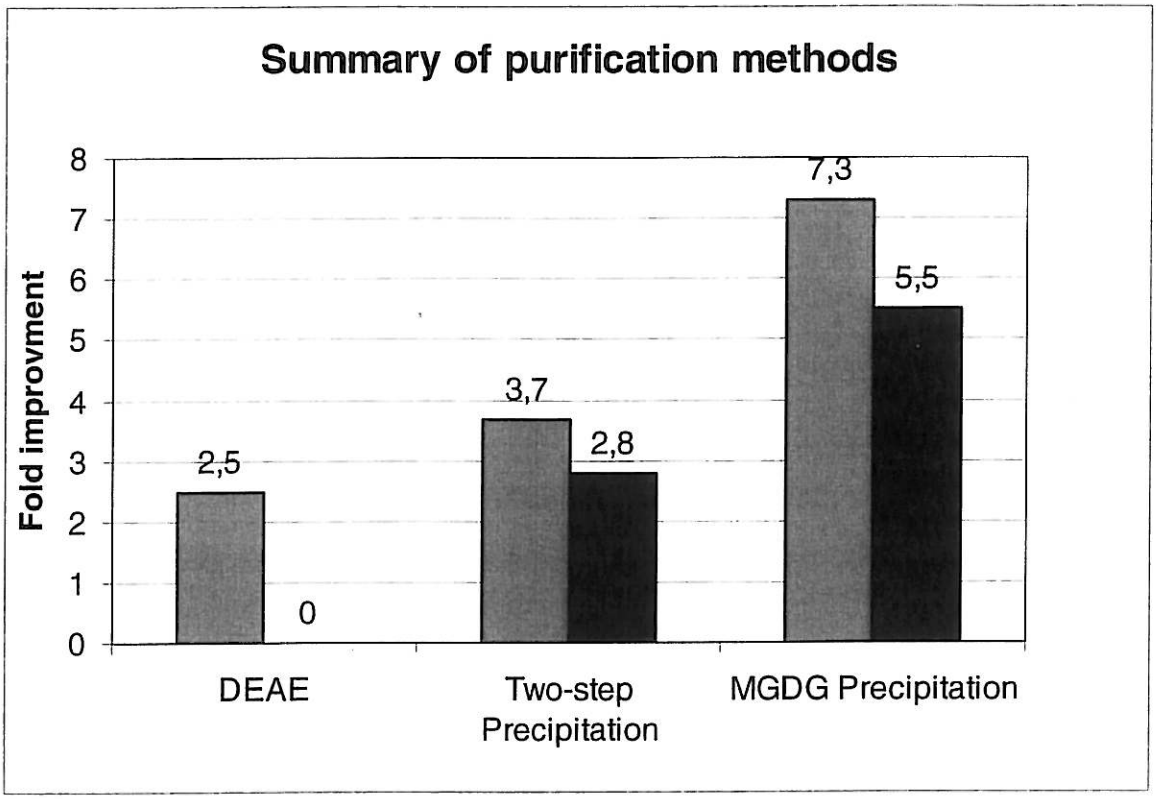

Fig. 8. Summary of purification methods by presenting the improvement in specific activity of used purification methods. Dark column is fold improvement in specific activity when combine used purification method with purification with DEAE column.

3.2.1. DEAE

Experiment with DEAE show that the column material does not denature VDE when the protein binds to the column, and can therefore be used to purify VDE from other proteins.

3.2.1.1. In batches

The experiments with DEAE and periplasmic proteins incubated in eppendorftubes with different concentrations of NaCl showed that VDE is released from the DEAE when NaCl concentrations is between 100 and 200 mM NaCl (fig. 9).

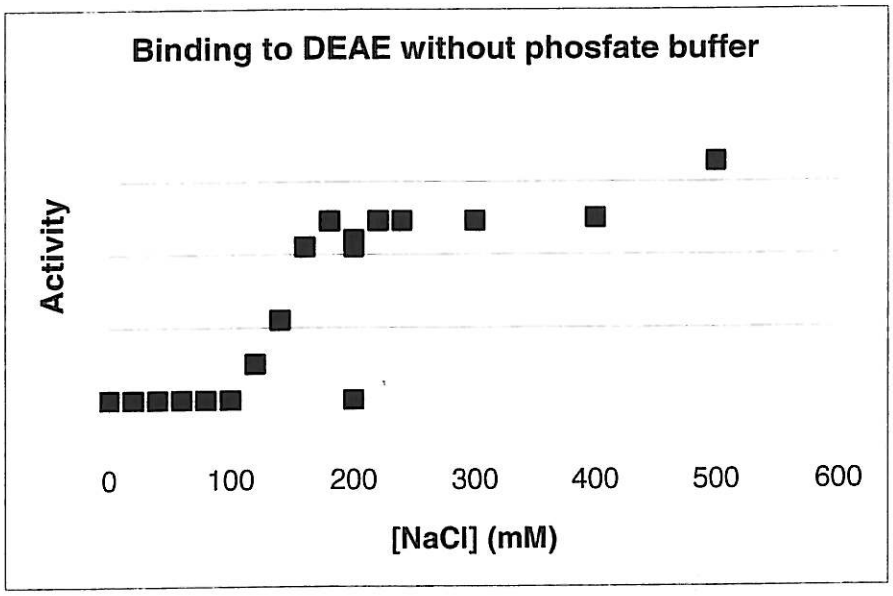

Fig. 9. DEAE, periplasmic proteins and different concentrarions of NaCl was incubated in ependorf tubes for 20 min, followed by centrifugation 13 000 rpm for 10 min. Activity was measured on the supernatant.

When phosphate buffer (50 mM, pH 7.1) is present VDE is released earlier, between 0 and 200 mM NaCl (fig. 10).

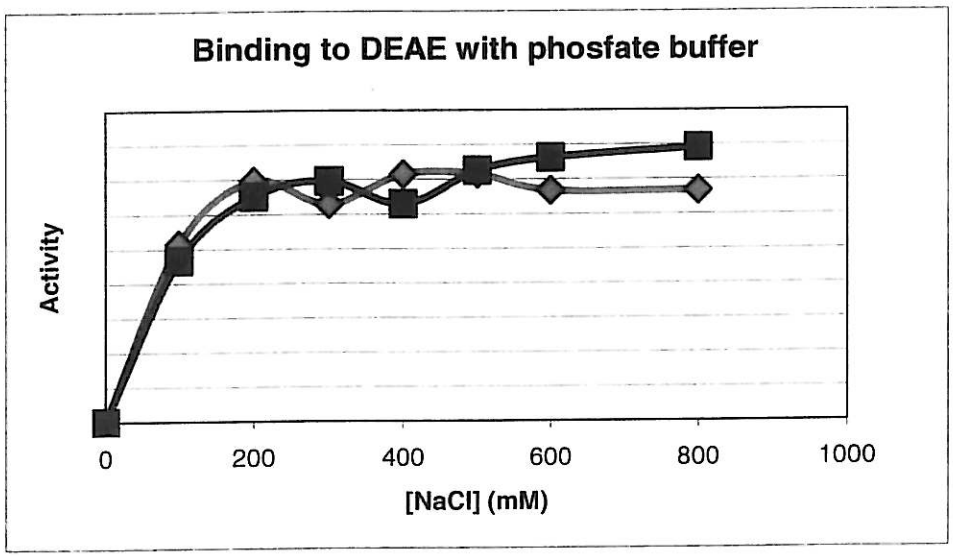

Fig. 10. DEAE, periplasmic proteins, phosphate buffer (50 mM, pH 7.1) and different concentrarions of NaCl was incubated in ependorftubes for 20 min, followed by centrifugation 13 400 rpm for 10 min. Activity was measured on the supernatant. The dark curve represent incubation of DEAE, phosphate buffer and periplasmic proteins first followed addition of NaCl and incubation for additional 20 min. The brighter curve represent incubation of DEAE, phosphate buffer and NaCl first followed addition of periplasmic proteins and incubation for additional 20 min.

3.2.1.2. In column

Nearly all VDE was released from the column between 50 and 200 mM NaCl. To collect all VDE, all fractions in this region had to be collected. The best way to do this was to equillibrate the column with 50 mM NaCl, wash it with 50 mM NaCl after applying the sample, and elute with 200 mM NaCl. Only one peak was detected when eluating with 200 mM NaCl, and contained many proteins according to the SDS-PAGE gel (fig. 12). Activity in these collected fractions increases rapidly at start and decreases more slowly after peak activity was reached.

The first fractions with lower activity had only a fifth of the peak activity, but the specific activity was three times higher. When analyzing the marked area in fig. 12. Mascot suggests significantly a mixure of two E. coli proteins (GCST_ECO24 and AHPC_ECO57). When the mass peaks from these proteins were removed from the spectrum VDE from spinach becomes the only significant hit left.

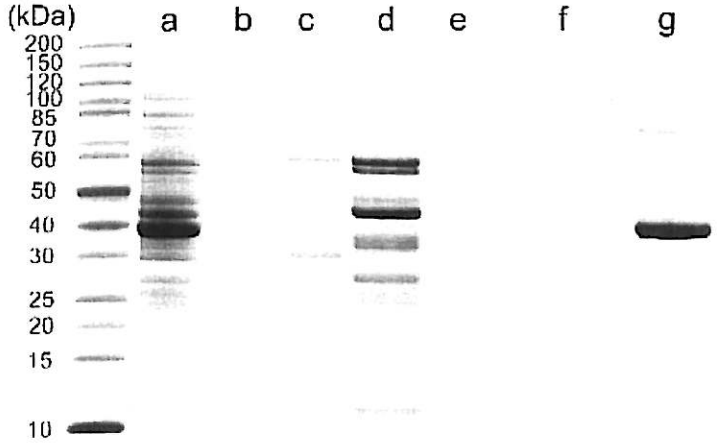

Fig. 11. SDS-PAGE gel for fractions collected after loading of 8.5 ml periplasmic protein (16 µg VDE), washing with 100 mM NaCl and eluting with 200 mM NaCl. (a) Periplasmic proteins, 57 ng VDE loaded (b-e) fractions collected before eluating with 200 mM NaCl, (e) contains 14 ng VDE, (f) fractions with highest activity, 26 ng VDE loaded, (g) fraction collected when eluating with 1.0 M NaCl, contained no detectable activity. Thick band (40 kDa) in (g) was identified as ferrochelatase from B. subtilis with MS.

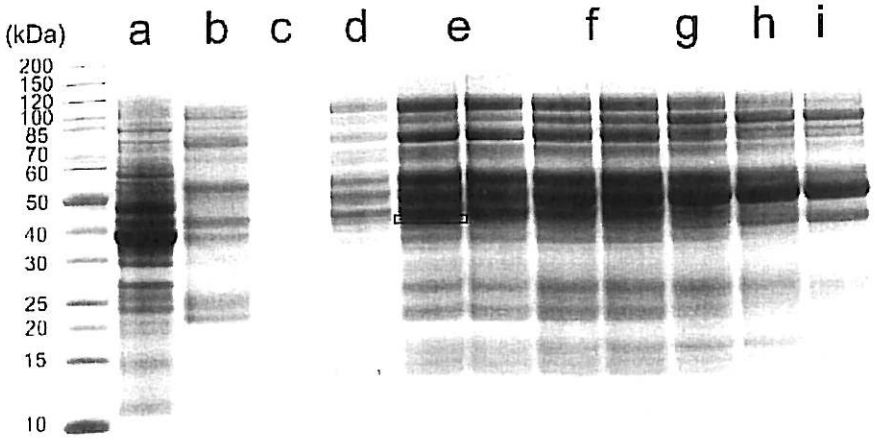

Fig. 12. SDS-PAGE for (a) Unpurified periplasmic proteins, 65 ng VDE loaded, (b) fraction from DEAE column concentrated with cut-off centrifugation tube (30 kDa), 45 ng VDE loaded, (c) early eluted fraction with high specific activity, 90 ng VDE loaded, (d) fraction after c, 200 ng VDE loaded, (e) fraction after d, 450 ng VDE loaded, (f) fraction after e, 390 ng VDE loaded, (g) fraction after e, 230 ng VDE loaded, (h) fraction after g, 130 ng VDE loaded, (i) fraction after h, 57 ng VDE loaded. Marked area is where VDE was identified with MS.

Using a NaCl gradien was not as succesfull as incresing NaCl concentration stepwise when high concentration of VDE was required. The first gradient used was 50 ml (0.0 - 1.0 M NaCl) on a 4 ml column loaded with (200 µl periplasmic proteins, 0.38 µg VDE), which gave too diluted fractions to obtain a detectable activity. The second gradient was 5 ml (0.0 - 1.0 M NaCl) on a 4 ml column loaded with (2 ml periplasmic proteins, 3.8 µg VDE), which resulted in that nearly all proteins eluted at the same time. The third gradient was 30 ml (50 - 250 mM NaCl) on a 2.4 ml column loaded with (10 – 20 ml periplasmic proteins, 19 - 38 µg VDE), which resulted in activity in all the fractions of the first 30 ml. These fractions (1.5 ml) was concentrated with ammonium sulfate precipitation at 50 % saturation and disolved in 50 µl water (fig. 13 and fig. 14).

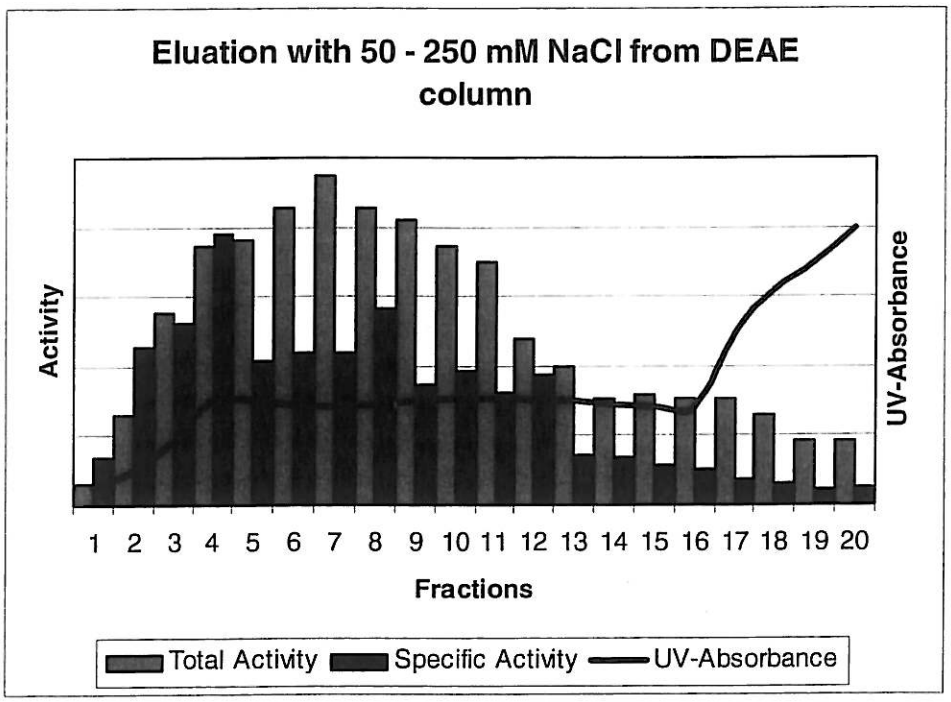

Fig. 13. Total activity, specific activity and UV absorbance for fractions collected (1.5 ml) after loading of approx. 15 ml periplasmic protein (approx. 29 µg VDE), washing with NaCl (50 mM), eluating with a linear NaCl gradient (50 - 250 mM), precipitated at 50 % ammonium sulfate saturation and disolved in 50 µl water.

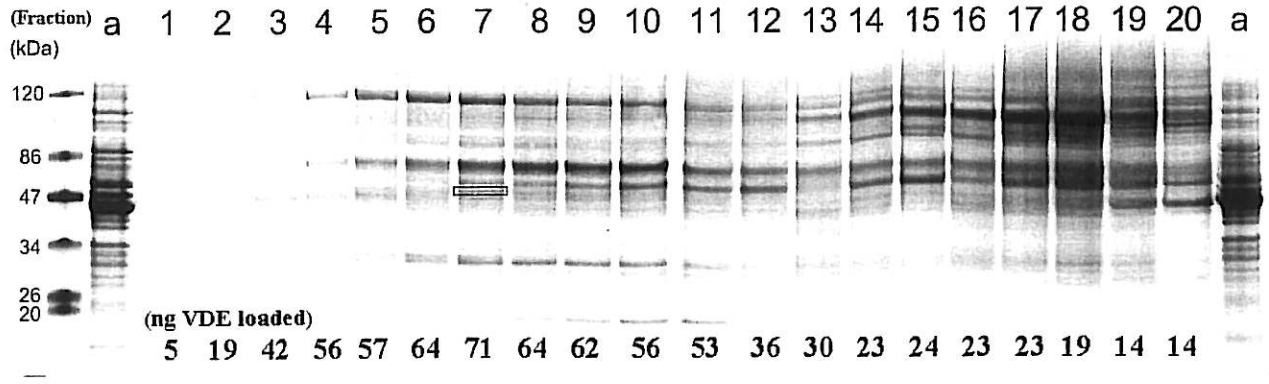

Fig. 14. SDS-PAGE for fractions collected after loading the DEAE column with 10 – 20 ml periplasmic protein (19 – 38 µg VDE), washing with NaCl (50 mM), eluating with a linear NaCl gradient (50 - 250 mM), precipitated at 50 % ammonium sulfate saturation and disolved in 50 µl water. Marked area is where VDE was believed to be.

When eluting with higher concentrations of NaCl after eluating with 200 mM NaCl no detectable activity was found without ammonium sulfate precipitation. A thick band at 40 kDa elutes after 200 mM NaCl, (g) in fig. 11.

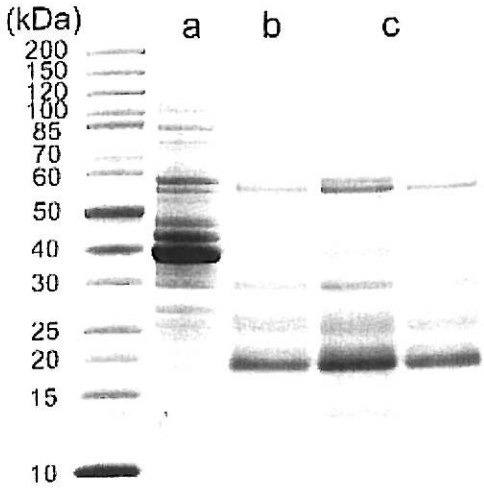

Fig. 15. SDS-PAGE for VDE purified from spinach by sonication at high pH. (a) Unpurified periplasmic proteins, (b) VDE from spinach with reducing agent, (c) VDE from spinach without reducing agent.

3.2.2. Ammonium sulfate precipitation

This purification method increased the specific activity 3.7 fold when precipitationg between 30 and 50 % saturation on unpurified periplasmic proteins. A 1.1 fold increase in specific activity was obtained when fractions from the DEAE column was used instead (fig. 8).

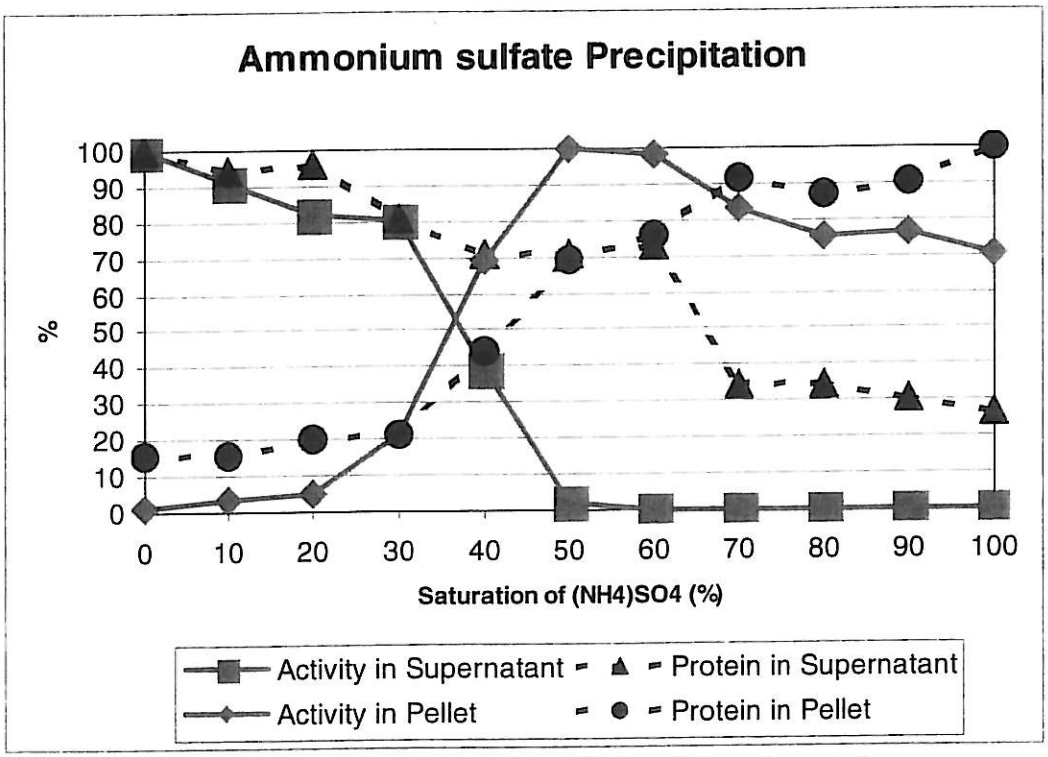

Fig. 16. Ammonium sulfate precipitation on unpurified periplasmic proteins.

When analyzing the marked area on Fig. 17. Mascot suggests two alternatives, highest significant score is a mixure of E. coli proteins (SUCC_ECO24, AHPC_ECO57 and GCST_ECO24), and second highest significant score was a mixure of SUCC_SHIDS, GCST_ECO24 and VDE from spinach.

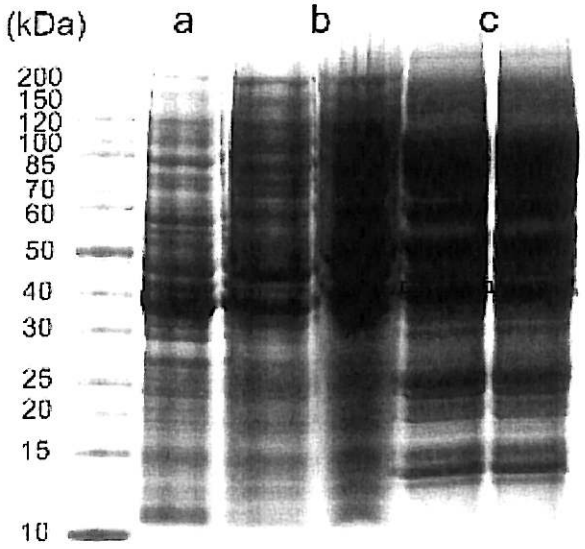

Fig. 17. SDS-PAGE for (a) unpurified periplasmic proteins, (b) two-step precipitation on periplasmic proteins, (c) two-step precipitation on fractions from DEAE column. Marked area are where VDE was found using MS.

3.2.3. Lipid affinity precipitation with MGDG

The specific activity of VDE was incresed 7.3 fold when applying this method to unpurified periplasmic proteins, and a loss of 56 % of the activity. All of the lost activity was found in the supernatant though. When applying on fractions from the DEAE column with 5.8 fold higher total activity and 2.5 fold higher specific activity than the unpurified periplasmic proteins 95 % of the activity was lost. But all of the lost activity was not found in the supernatant. 85 % of the VDE activity was lost after using this method on these fractions. The specific activity was incresed 5.5 fold.

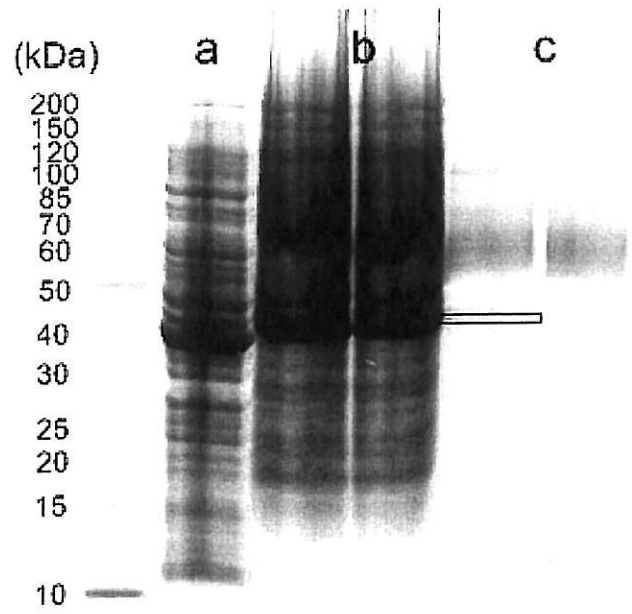

Fig. 18. SDS-PAGE for (a) unpurified periplasmic proteins, (b) lipid affinity precipitation on periplasmic proteins, (c) lipid affinity precipitation on fractions from DEAE column, marked area is where VDE was identified by MS.

When analysing the marked area in fig. 18 Mascot suggests VDE from spinach as the only significant protein. This area was also used to identify disulfide bridges by not reducing the protein in MS preparation.

3.2.4. Ni^{3+} column

The washing fraction contained the majority of VDE activity. The first eluted fraction showed minimal activity and the fraction after had lesser activity. No fraction after that had activity. The SDS-PAGE gel showed a 40 kDa band that was thicker in the second and third fraction when eluating with 1 M imidazol than the first fraction. This band was present in all fractions after. When analysing this band with MS GPS - Explorer suggests that this was ferrochelatase from B. subtilis as a significant hit.

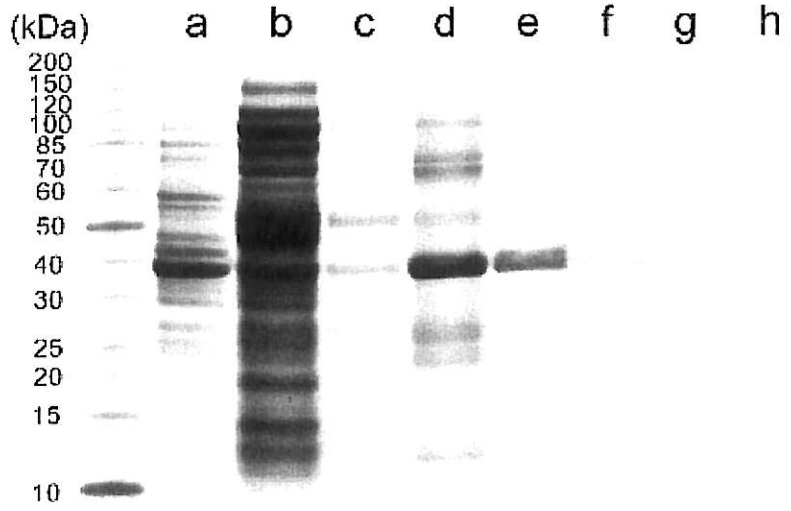

Fig. 19. SDS-PAGE for (a) unpurified periplasmic proteins, (b) washing fraction, 24 ng VDE loaded, (c) first ml eluted from the column (d) second, (e) third, (f) fourth, (g) fifth, (h) sixth.

3.3. Identification of VDE and disulfide bonds

The following identification of peptides from VDE and identification of peptides containing a disulfide bond was analysed with the gel piece cut from the marked area in fig. 18.

3.3.1. Identified VDE peptides

The peptides in fig. 20 are those peptides found from VDE that was confirmed with MS/MS by analysing marked area in fig. 18.

VDALKTCTCLLKECRIELAKCIANPSCAANVACLQTCNNRP
DETECQIKCGDLFANKVVDEFNECAVSRKKCVPQKSDVGEF
PVPDPSVLVKSFNMADFNGKWFISSGLNPTFDAFDCQLHEF
HLEDGKLVGNLSWRIKTPDGGFFTRTAVQKFAQDPSQPGML
YNHDNAYLHYQDDWYILSSKIENQPDDYVFVYYRGRNDAWD
GYGGAFLYTRSATVPENIVPELNRAAQSVGKDFNKFIRTDN
TCGPEPPLVERLEKTVEEGERTIIKEVEQLEGEIEGDLEKV
GKTEMTLFQRLLEGFQELQKDEEYFLKELNKEERELLEDLK
MEAGEVEKLFGRALPIRKLR

Fig. 20. Sequence of mature VDE from spinach expressed in E. Coli, and marked peptides are those that was identified with MS and confirmed with MS/MS.

3.3.2. Identified disulfide bonds

In fig. 21 and table 1 the peptides with disulfide bonds that were confirmed with MS/MS according to xQuest are shown.

VDALKTCTCLLKECRIELAKCIANPSCAANVACLQTCNNRP
DETECQIKCGDLFANK VVDEFNECAVSRKKCVPQKSDVGE
FPVPDPSVLVKSFNMADFNGKWFISSGLNPTFDAFDCQLHE
FHLEDGKLVGNLSWRIKTPDGGFFTRTAVQKFAQDPSQPGM
LYNHDNAYLHYQDDWYILSSKIENQPDDYVFVYYRGRNDAW
DGYGGAFLYTRSATVPENIVPELNRAAQSVGKDFNKFIRTD
NTCGPEPPLVERLEKTVEEGERTIIKEVEQLEGEIEGDLEK
VGKTEMTLFQRLLEGFQELQKDEEYFLKELNKEERELLEDL
KMEAGEVEKLFGRALPIRKLR

Fig. 21. The sequence for mature VDE in spinach and identified disulfide bonds found in VDE from spinach expressed in E. coli from gel piece marked in fig. 18.

Table. 1. Identified disulfide bonds found in VDE from spinach expressed in E. coli from gel piece marked in fig. 18.

Mass (Da)	Sequence
1566.8	KCVPQK-CGDLFANK
1740.9	CVPQK-TCTCLLKECR
1938.9	CVPQK-VVDEFNECAVSK
2469.2	CVPQK-TDNTCGPEPPLVERLEK

4. DISCUSSION

The thick dominating band (40 kDa) on SDS-PAGE gels of periplasmic proteins from both my own cultivations and the cultivation given with VDE activity has been identified by Mascot and GPS Explorer as ferrochelatase from B. subtilis. The reason why my cultivations and given E. coli pellet with activity contained ferrochelatase from B. subtilis is probably a contamination of a similar plasmid containing the ferrochelatase gene. This ferrochelatase plasmid is present in the same lab and have either been transformed into E. coli containing the VDE plasmid, VDE plasmid transformed into E. coli containing the ferrochelatase plasmid, contamination in the plasmid preparation where both genes have been inserted in the same plasmid or a mixure of E. coli containing the VDE plasmid and E. coli containing the ferrochelatase plasmid.

Both plasmids carry the same ampicilin recistance gene, which means that the contamination plasmid can not be removed by changing antibiotics in the growing medium.

No ferrochelatase activity was detected in the unpurified periplasmic proteins, but it could be a mutated ferrochelatase in the plasmid, with also is used in the same lab. Later experiments showed that the original VDE plasmid preparation was pure and had no contamination. Thus the contamination should be in some later stages

4.1. Obtaining VDE

The ferrochelase contamination of another plasmid will most probably reduce the amount of VDE produced by E. coli. This could be one reason why the amount of VDE produced by E. coli was low compared to other periplasmic proteins.

The periplasmic proteins with VDE activity and ferrochelatase must been grown from a colony from a cell containing two plasmids or several colonies was picked to the graft, one containing the VDE plasmid and another containing ferrochelatase plasmid.

My cultivations did not show any detectable activity at all, but showed the thick ferrochelatase band (40 kDa) also confirmed as ferrochelatase from B. subtilis by MS. The reason to this could be that only colonies containing the ferrochelatase plasmid was picked, even though three different colonies was picked at three times, total of nine colonies without the VDE plasmid.

The size of E. coli pellet after induction of my cultivations was much smaller than the given E. coli pellet with VDE. A reason to this could be that the bacteria grows slower after induction with IPTG compaired to preavously grown E. coli with ferrochelatase or/and VDE plamid/s. It took about the same time for both my cultivations and preavously grown E. coli to reach the same OD_{600} value. So there is no difference before induction with IPTG.

To increse the number of cell walls destroyed in the final step, twice the volyme of the last buffer that destroys the cell wall was added but still no detectable activity was found in my cultivations.

4.2. Purification of VDE

As shown in fig. 8 the biggest improvement in specific activity was when lipid affinity precipitation with MGDG was done with unpurified periplasmic proteins. The downside to this method was that "streaking" appears on the SDS-PAGE gels when running samples that had not been purified with the DEAE column (fig. 18). A combination of DEAE column and lipid affinity precipitation with MGDG was the best way to find VDE.

4.2.1. DEAE

When working in batches with DEAE in eppendorf tubes the release point of VDE was between 100 and 200 mM NaCl without phosphate buffer (fig. 9). With phosphate buffer the release point seems to have widened to between 0 and 200 mM NaCl. Not much differences was detected when incubating with NaCl before incubation with VDE and incubation with VDE before incubation with NaCl (fig. 10), proving that VDE is not denatured in DEAE.

When working with DEAE in a column the release point was observed to be between 50 and 250 mM NaCl.

To concentrate VDE to get a visible band on a SDS-PAGE gel high amount of periplasmic proteins was loaded on a small column then washed with 50 mM NaCl and eluted with 200 mM NaCl. The main problem with this is that the solution with periplasmic proteins is rather thick and to much loading of sample on the column resulted in that the flow stopped and the column had to be repacked to elute the proteins. Diluting the sample 5 times did not avoid the stopping of the system but only delayed it. This thick substance is left in the column after eluating with 200 mM NaCl.

If this eluted fractions with VDE activity was diluted to 100 mM NaCl and reloaded on the column, after being washed with 1.0 M NaCl and equillibrated with 100 mM NaCl, all VDE activity goes straight through. The reason to this could be that the flow rate was doubled at that time to 1.0 ml/min during the loading of the sample and caused VDE to go straight through the column or that there is something in the solution of periplasmic proteins that binds strong to the column and also binds to VDE at low concentrations of NaCl. When this component is removed by reloading eluted VDE on the column withouth this component that binds VDE, VDE will elute at a lower concentration than before. The incubation with NaCl could also contribute to that the release point of VDE was lowered.

When looking at SDS-PAGE gels where periplasmic protein has been concentrated with different metods but not by ionic exchange column one can see that proteins have agreggated and are disolving slowly during the gel run which cause "streaking", which means that there are vertical lines of proteins in the gel contaminating other bands. This phenomena have not been seen in any samples where the periplasmic proteins first have been purified through ionic exchange column. The component causing this streaking and aggregation of proteins must therefor been separated from VDE by the DEAE column.

Using a small column with little amount of DEAE caused the eluted fractions of all proteins to elute in higher concentration. When trying to use a 50 ml DEAE column to avoid clogging of the system instead of 2.4 ml DEAE the proteins were so diluted that no peaks where detected in the UV-detector.

Test showed that the presence of 800 mM NaCl in the unpurified periplasmic proteins did not affect the activity for short incubation.

For analysing the band marked in fig. 12 with MS, two E. coli proteins (GCST_ECO24 and AHPC_ECO57) were in higher concentration than VDE and had to be removed from the mass spectrum to be able to get a significant hit on VDE from spinach.

4.2.2. Ammonium sulfate precipitation

This purification method increased the specific activity 3.7 fold when precipitating between 30 and 50 % saturation on unpurified periplasmic proteins. But when applying this method to fractions from the DEAE column the increase in specific activity was only 1.1 fold. The reason to this could be that about the same proteins were removed in the DEAE column as the proteins removed in two-step precipitation. Alternatively the 200 mM NaCl or 50 mM phosphate buffer was affecting the precipitation. The curves in fig. 16 were obtained from precipitating unpurified periplasmic proteins and may look different if precipitating fractions from the DEAE column instead, containing 200 mM NaCl, 50 mM phosphate buffer and less proteins.

The two-step precipitation concentrated VDE so that the enzyme was identified as a significant hit with Mascot. E. coli proteins (SUCC_ECO24 and GCST_ECO24) that were also found probably needs to be removed from the protein mixure to be able to see mass peaks from peptides containing a disulfide bond.

4.2.3. Lipid affinity precipitation with MGDG

This method increased the specific activity the most, even if most of VDE did not precipitate, the specific activity was increased 7.3 fold when applied to unpurified periplasmic proteins and 5.5 fold when combined with purification with DEAE.

The fractions from the DEAE column had 5.4 times higher activity than the unpurified periplasmic proteins and 5 % of this activity was found in the pellet. 10 % of the activity was found in the supernatant. 85 % of the activity was lost after precipitating DEAE fractions with MGDG. No activity was lost if both the pellet and supernatant's activity was added together when applying this method to unpurified periplasmic proteins.

The reason to the loss of 85 % of VDE could be that the activity on the fractions from the DEAE column was measured two weeks earlier and may have decresed during this time. However no activity decreas was seen for unpurified periplasmic proteins, rather than a slow increse of activity over long time. There could be something in the unpurified periplasmic proteins that protects VDE from denaturing that was removed with the DEAE column or the presence of 200 mM NaCl or 50 mM phosphate buffer maybe increased the denaturing rate in some way.

After using this method on both unpurified periplasmic proteins and fractions from the DEAE column the total activity in the pellet was about the same, 8.2 µg VDE/ml for unpurified periplasmic proteins and 7.0 µg VDE/ml for fractions from the DEAE column. This could mean that this is the amount of VDE that can bind to 3.0 µM MGDG and an increase of this concentration would increase the amount of VDE in the pellet. Or that this is the amount of VDE that can bind to MGDG when 0.11 µM violaxanthin is present and an increase of the violaxanthin concentration will increse the amount of VDE in the pellet.

According to [5] the best concentration of MGDG to percipitate the largest amount of VDE was 2.9 μM MGDG without violaxanthin. When they tested higher concentrations of MGDG less VDE percipitated. When they tested if violaxanthin increased the precipitation they used 14.4 μM MGDG and 0.033 μM violaxanthin and found that it does, but they did not vary the concentrations of violaxanthin.

But if 85 % of VDE in the fractions from the DEAE column was denatured before the precipitation with MGDG it will mean that there was about the same amount of VDE in both the fractions from the DEAE column and the unpurified periplasmic proteins, resulting in about the same amount of VDE in the pellet. Then there are no arguments for that the MGDG or violaxanthin concentration should be saturated by this concentration of VDE.

4.2.4. Ni^{3+}column

Nearly all VDE activity was in the washing fraction and only minimal, barely detectable activity in the first and second eluted fractions. If the His-tag and signal peptide was still left on VDE that could mean that the protein was inactive.

When looking at the SDS-PAGE gel for the eluted fractions from the Ni^{3+} affinity column one band (40 kDa) is present in the last fractions which suggests that this protein was His-tagged. It was former believed that this was unprocessed VDE with the His-tag and signal peptide intact. Identification with MS and MS/MS gave a significant hit of that this was ferrochelatase from B. subtilis.

4.3. Mass spectroscopy

All samples spotted on the target plate was spotted twice, one with standard peptides so that the peaks would be internaly calibrated, and one without standard peptides so that if the intensity of the sample peptides were low they would not be suppressed by a high concentration of standard peptides. Bands from SDS-PAGE gels with VDE always contained a low concentration of VDE probably due to weak intensity of the band. The intensity from peaks for peptide fragments containing a disulfide bond was expected to have lower intensity, due to their larger mass. Therefor the signal to noise ratio was lowered to 10 instead of 50, wich it was when searching for VDE peaks.

The reason to why Mascot identified SUCC_SHIDS instead of SUCC_ECO24 when analyzing the gel piece from two-step precipitation (fig. 17) was that if a protein from different organisms is similar and no differences was found in the mass spectrum the programs reports one of them as highest score and the other organisms is listed further down. In this case SUCC_SHIDS was reported as highest score and SUCC_ECO24 was listed further down, however SUCC_ECO24 was much more probable than SUCC_SHIDS.

4.3.1. Identification of VDE

After purification with DEAE column with large volumes of loaded periplasmic proteins VDE could be identified in marked area in fig. 12 as a significant hit by Mascot after removal of mass peaks from the two dominating E. coli proteins (GCST_ECO24 and AHPC_ECO57).

Other tested purification methods was needed to be combined with purification with DEAE column to be able to see clean bands on the SDS-PAGE gel. Gel runs on samples that were not purified with DEAE column resulted in "streaking", which gave bands contaminated with other proteins.

The following results were from various purification methods combined with DEAE column purification.

The two-step precipitation combined with purification with DEAE column gave an area marked in fig. 17 that Mascot identified containing a mixure of two E. coli proteins (SUCC_ECO24 and GCST_ECO24) and VDE from spinach as a significant hit.

Lipid affinity precipitation with MGDG combined with purification with DEAE column was the only method tested that Mascot reported VDE from spinach as a single significant hit. No other protein was identified as a significant hit by Mascot when analysing the marked area in fig. 18.

4.3.2. Identification of disulfide bonds

The lowering of the signal to noise ratio to 10 when searching for peaks from peptides containing a disulfide bond most probably increase the chance of reporting a "false noise peak" to Peptidemap, the program used to identify possible peptides containing a disulfide bond, which does not look at the intensity of reported masses. That is the reason why all these possible disulfide bonds needed to be confirmed with MS/MS and xQuest. Some of the possible disulfide peaks had too low intensity to get a fragmentation in MS/MS.

The identified disulfide bonds confirmed with MS/MS according to xQuest in fig. 21 and table 1 is suggesting that the same cysteine in peptide (CVPQK) can be connected to four other peptide fragments. If this information is correct this could mean that there is a large amount of VDE with different disulfide bonds and by that means different folding. If there is only one correct folding that gives activity is unknown so which disulfide bond that is the correct can not be decided by these results.

When the lipocalin domain was crystallized the two cysteines was located close to each other, suggesting that these two would form a disulfide bond in the active version of VDE. One of these cysteins (TDNTCGPEPPLVERLEK) was found to connect to a cysteine (CVPQK) in the cysteine rich domain and not to the other cysteine in the lipocalin domain. If this is the disulfide bond formed in the active version of VDE can not be stated by this experiment

Why all the confirmed peptide fragments containing a disulfide bond is linked to the same cysteine could be explained by if this peptide gets very easily airborn in the mass spectrometer and make other peptides more easily airborn by linking them via a disulfide bond. But this peptide was however not identified in the reduced form and confirmed by MS/MS.

The intesity of the peaks does not always correspond to how common the fragments are but instead how easily they get airborn by the laser in the mass spectrometer. If the correct disulfide bond creates a linkage between to large peptides or linking more than two peptides by several disulfide bonds creating a large peptide that hardly gets airborn and therefor not detected. Then mayby a common wrong-folded version of VDE contains some fragments with a disulfide bond that gets more easily airborn will result in that the disulfide bonds in the wrong-folded version of VDE is belived to be the correct disulfide bonds. Using different proteases creating different peptides with common disulfide bonds should be a more secure method to confirm the disulfide bonds.

When the protein is expressed in another organism could result in different folding caused by different conditions and nearby substances. The environment inside the thylakoids of spinach is maybe not reproduced in the periplasm of E. coli where the final folding of VDE is done. Using VDE isolated from spinach may result in higher ratio of correctly folded VDE.

The program used for confirming the disulfide bonds with MS/MS data could also have been wrongly used, because there was no way to measure if a score was significant. Experience is needed to be able to judge if the hit is correct. The reported disulfide bonds could be worng due to lack of experiance.

Possible peaks from peptides containing a disulfide bond that was found in MS but not confirmed with MS/MS could have been a "false noise peak" or had low intensity for proper MS/MS fragmentation or was another peptide with the same mass. That there is a peptide in the sample with the same mass as a possible peptide containing a disulfide bond does not mean that this disulfide bond does not exist. If this other peptide with the same mass as a peptide containing a disulfide bond have much higher intensity than the peptide containing a disulfide bond confirmation with MS/MS is not possible. Either the sample must be purer or these peaks needs to be separated from each other using LC/MS.

5. CONCLUSIONS

- Ferrochelatase contamination found and should be removed to increase the amount of VDE produced by E. coli.
- Lipid affinity precipitation with MGDG on fractions from the DEAE column gave the SDS-PAGE gel where VDE was the only significant hit by Mascot.
- Higher concentration of VDE is neded on the gel to give a stronger band which will improve the intensity of all peptides in the mass spectrum.
- Four disulfide bonds was confirmed with MS/MS according to xQuest.
- Using LC/MS for separating peptides with the same mass to indentify more peptides containing a disulfide bond.

6. ACKNOWLEDGEMENTS

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