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Purification and identification of the violaxanthin deepoxidase as a 43 kDa protein *

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Abstract

Violaxanthin deepoxidase (VDE) has been purified from spinach (*Spinacia oleracea*) leaves. The purification included differential sonication of thylakoid membranes, differential $(NH_4)_2SO_4$ fractionation, gel filtration chromatography and finally either hydrophobic interaction chromatography or anion exchange chromatography. A total purification of more than 5000-fold compared to the original thylakoids enabled the identification of a 43 kDa protein as the VDE, in contrast to earlier reported molecular weight of 54–60 kDa. A detailed comparison was made for the VDE activity and polypeptide pattern for the different fractions throughout the purification and the best correlation was always found for the 43 kDa protein. The highest specific activity obtained was 256 μmol g^{-1} s^{-1} protein, which is at least 10-fold higher than reported earlier. We estimate that there is 1 VDE molecule per 20–100 electron transport chains. The 43 kDa protein was N-terminally sequenced, after protection of cysteine residues with β-mercaptoethanol and iodoacetamid, and a unique sequence of 20 amino acids was obtained. The amino acid composition of the protein revealed a high abundance of charged and polar amino acids and remarkably, 11 cysteine residues. Two other proteins (39.5 kDa and 40 kDa) copurifying with VDE were also N-terminally sequenced. The N-terminal part of the 39.5 kDa protein showed complete sequence identity both with the N-terminal part of cyt b_6 and an internal sequence of polyphenol oxidase.

Abbreviations:
DMSO – dimethylsulfoxid; HIC – hydrophobic interaction chromatography; MGDG – monogalactosyl diacylglycerol; VDE – violaxanthin deepoxidase

Introduction

In nature the intensity of light shows great variation, both temporally and spatially. This variation in the light environment imposes a large demand on the photosynthetic system. At light limiting levels of photosynthesis, light must be captured and utilized with the highest possible efficiency. In the case of excessive light, it is important to avoid over-excitation of the photosynthetic reaction centers, as such over-excitation can result in severe damage to these centers. This problem is further increased in the presence of environmental stresses. A larger proportion of the light becomes excessive when the photosynthetic capacity is lowered. Plants must therefore possess the ability to regulate the flow of excitation energy (Björkman and Adams 1994). One of many photoprotective mechanisms is believed to involve the xanthophyll cycle.

The xanthophyll cycle involves the light-dependent and reversible conversion of violaxanthin to zeaxanthin. Zeaxanthin has been implicated in the protection of the photosynthetic machinery from over-excitation, although the mechanism for this protection is still open for discussion (Björkman and Adams 1994; Gruszecki 1995). The cycle is controlled by two different

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enzymes, violaxanthin deepoxidase (VDE) and zeaxanthin epoxidase. VDE catalyzes the conversion of the diepoxide violaxanthin in two steps to zeaxanthin with antheraxanthin as intermediate. The enzyme is located in the thylakoid lumen (Hager and Holocher 1994) and is indirectly controlled by pH, since the acid form of ascorbate (pK_a 4.1) is the substrate for the reaction (Bratt et al. 1995). Furthermore MGDG was found to be required to obtain an active enzyme (Yamamoto and Higashi 1978).

There are two reports on the isolation of VDE (Hager and Perz 1970; Yamamoto and Higashi 1978). The molecular weight was reported to be 60 kDa based on gel filtration (Yamamoto and Higashi 1978) and 54 kDa (Hager 1975). However, only limited information was given on the results of different steps in the purification and on the final purity of the protein. Particularly, no SDS-gel-electrophoretic analysis was presented, although Hager and Holocher (1994) highlights a band at 58 kDa as VDE, without further evidence, in lanes containing numerous protein bands.

In this work the identity of VDE was established by gel filtration, hydrophobic interaction chromatography (HIC) and DEAE anion exchange chromatography, and the molecular weight was found to be 43 kDa and not 54–60 kDa as reported earlier. VDE was purified more than 5000-fold and showed a unique N-terminal sequence.

Materials and methods

The purification method was a modification and extension of the methods of Hager and Perz (1970) and Yamamoto and Higashi (1978). All purification steps were performed at 4 °C, except for sonication, which was done at 0 °C.

For isolation of thylakoid membranes, 1600 g (in 100 g portions) of spinach (*Spinacia oleracea* L. cv. Carambola) leaves were homogenized for 1 min in 3200 ml 400 mM sorbitol, 50 mM Na-MOPS, pH 7.0 and 10 mM NaCl. The homogenate was filtered through 4 layers of 20 μm nylon cloth and the filtrate was centrifuged at 2500 *g* for 5 min. The chloroplast pellet was resuspended in 800 ml of the same buffer and centrifuged at 5000 *g* for 5 min. The pellet was resuspended in 800 ml distilled water and centrifuged at 37 000 *g* for 10 min. The pellet containing thylakoid membranes was resuspended in 400 ml 100 mM Na-citrate, pH 5.2. Dimethylsulfoxide (DMSO) was added to a final concentration of 5% and the fraction was stored in 100 ml batches at –70 °C or used directly. Compared to freshly prepared material, no loss of VDE activity was caused by the freezing procedure when DMSO was present and the thawing procedure was carried out in a water bath at room temperature.

Freshly thawn thylakoids, 400 ml in 100 mM Na-citrate, pH 5.2, were sonicated in 100 ml batches for 4 min, with a sequence of 5 s sonication and 5 s rest, output 60 (Vibra Cell, Model VC60, with microtip). The sonicated material was centrifuged at 37 000 *g* for 30 min. The supernatant was collected (Son5) for analysis while the pellet was resuspended in 400 ml 180 mM Na-phosphate, pH 7.2, 5 mM $MgCl_2$ and sonicated in 100 ml batches as described above. The sonicated material was centrifuged at 37 000 *g* for 30 min and the supernatant containing VDE was collected (Son7:1), while the pellet was once again resuspended at pH 7.2, sonicated and centrifuged as described above. The resultant supernatant (Sup7:2) was combined with Son7:1 to give Son7. The remaining pellet was resuspended in 50 mM Na-phosphate, pH 7.2, to 400 ml (SonRes7) and saved for analysis.

The combined supernatants (Son7) were subjected to differential $(NH_4)_2SO_4$ fractionation. The $(NH_4)_2SO_4$ was added to 40% of saturation, slowly with gentle stirring until all $(NH_4)_2SO_4$ was dissolved, allowed to rest for 20 min and centrifuged at 37 000 *g* for 30 min. The pellet was resuspended in 60 ml 100 mM Na-phosphate, pH 7.2 (Prec40) and saved for analysis. The supernatant was made 80% saturated with respect to $(NH_4)_2SO_4$, incubated for 3 h and centrifuged at 100 000 *g* for 1 h. The pellet, containing most of the VDE activity, was resuspended in distilled water up to about 5 ml. It was found critical to allow the pellet to dissolve by itself in water for 20 min, in order to retain high activity. The sample was then filtered through a 0.2 μm membrane (Sartorius, Minisart-plus). This fraction was called Prec80.

For gel filtration chromatography, 4.5 ml of the Prec80-fraction was loaded on a Sephacryl S-300HR column (150 × 2.6 cm, Pharmacia) at a flow rate of 0.85 ml min^{-1}, using 100 mM Na-phosphate, pH 7.2. Fractions enriched in VDE activity were either analyzed separately or pooled (GelfPool) for further purification. Calibration of gel filtration was made with bovine serum albumin (BSA), ovalbumin, chymotrypsinogen A and ribonuclease A.

For hydrophobic interaction chromatography, GelfPool was adjusted to 1.25 M $(NH_4)_2SO_4$, 50 mM Na-phosphate, pH 7.2, and subjected to a Poros 20 ET/P column (4.6 × 1000, PerSeptive Biosystems

Inc.), coupled to a Millipore ConSep LC100 system. Pre-equilibration of the column and initial washing after sample application were made at a flow rate of 1 ml min^{-1} with the same medium as above. Elution of bound protein was made with 50 mM Na-phosphate, pH 7.2.

For anion exchange chromatography, a DEAE MemSep 1000HP (Millipore) column was equilibrated with 50 mM Na-phosphate, pH 7.2, at a flow rate of 2.3 ml min^{-1}. The GelfPool-fraction, adjusted to 50 mM Na-phosphate, pH 7.2, was applied to the column, followed by a 3 min wash with 50 mM Na-phosphate, pH 7.2, and eluted with a gradient of 0–600 mM NaCl, 50 mM Na-phosphate, pH 7.2, for 8 min.

The substrate, violaxanthin, was purified from an acetone extract of spinach leaves by thin layer chromatography on Kiselgel HR60 (Merck) using the eluant *n*-hexane/ethylacetate/triethylamine in the proportions of 40:48:12 (by volume). Violaxanthin, the second band from the start position, was extracted with methanol. The purity was verified by HPLC according to Thayer and Björkman (1990).

The violaxanthin deepoxidase activity was determined by dual-wavelength measurements (502–540 nm) in a Shimadzu UV-3000 spectrophotometer according to Yamamoto and Higashi (1978) at 26 °C. The assay mixture (final volume 3 ml) contained 0.33 μM violaxanthin, 9 μM MGDG (Lipid products, South Nutfield, UK), 30 mM Na-ascorbate, 0.1 M citrate-phosphate, pH 5.1.

SDS-PAGE was performed in the buffer system of Laemmli (1970) at 25 °C with a 12–22.5% polyacrylamide gradient without urea. Samples were solubilized at 100 °C for 2 min. Gels were stained with Coomassie brilliant blue R-250. For estimation of apparent molecular weights, the low molecular weight calibration kit from Pharmacia was used.

Protein was determined by the bicinchoninic acid (micro BCA) method of Pierce (No. 23225) with BSA as standard.

For determination of amino acid sequences and composition, proteins from SDS-PAGE were transferred to a polyvinylidene difluoride membrane (PVDF, Millipore) using a JKA-Biotech semidry electroblotter. After Coomassie staining of the PVDF membrane, bands were cut and sequenced on an Applied Biosystems sequenator 477A. In the experiments where protection of cysteine residues were made, the following procedure was adopted (A. Jensen, personal communication). A sample of 35 ml from the gel filtration was desalted on PD10 columns, concentrated until dryness in a speedvac and resuspended to 1200 μl with solubilizing buffer (diluted 1:1 with water). The resuspended sample containing 1.06 M mercaptoethanol was then incubated 3 min at 100 °C. After addition of 1.56 ml 1.0 M iodoacetamide in 1.0 M Tris-HCl, pH adjusted and kept at 7.5, the sample was incubated in darkness for 20 min. The reaction was stopped by addition of 60 μl β-mercaptoethanol and the sample applied to SDS-PAGE as above.

Gel scanning was made on a Personal Densitometer SI (Molecular Dynamics). The pattern was then analyzed using the software ImageQuaNT (Molecular Dynamics).

Results

Washed thylakoids were first sonicated at pH 5.2 to remove loosely attached proteins on the thylakoid membrane as well as soluble stroma and lumen proteins. At this pH the violaxanthin deepoxidase should be bound to the thylakoid membrane (Yamamoto and Higashi 1978; Bratt et al. 1995; Carlsson et al. 1995). Indeed, as shown in Table 1 the supernatant after sonication, Son5, did not contain any VDE activity, while many different proteins were released (Figure 1). Some proteins of the oxygen evolving complex in Photosystem II were present, namely the 33 and 18 kDa (Ljungberg et al. 1986) as well as the 37 kDa ferredoxin-$NADP^+$ oxidoreductase. The 37 kDa protein has previously been identified by N-terminal sequencing by Arvidsson et al. (1993).

The thylakoids were then brought to pH 7.2 and sonicated. This treatment released a large portion of the VDE activity (Table 1, Son7:1). Also here one can see the presence of the 18 kDa protein in roughly equal amount as in Son5 but the 33 kDa protein in lower amounts (Figure 1). The 62 and 54 kDa bands are less likely candidates for VDE, since they were also found in the Son5 fraction which had no activity. Notably, a new weak band was seen at 43 kDa which was absent in the Son5 fraction.

To increase the recovery of VDE activity, the residual thylakoid membranes were sonicated once more. The 43 kDa band was also present in this fraction (Son7:2) but in a lower amount than in the Son7:1 fraction in agreement with the lower activity. The intensity of the 54 kDa band was also decreased compared to that of Son7:1 but was not as low as would be expected from the difference in activity. The other proteins were

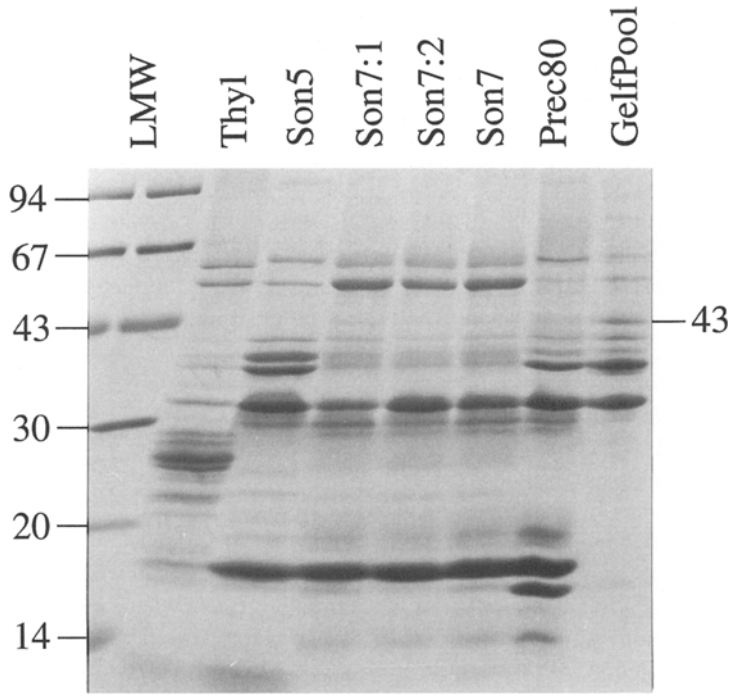

Figure 1. SDS-PAGE of samples from the initial steps of the VDE purification. LMW: low molecular weight standards, values in kDa. Thyl: Thylakoids.

either enriched or showed roughly equal amounts as in Son7:1.

The combined fractions, Son7, contained most of the VDE activity but less than 5% of total protein (Table 1). This lead to a 30- to 60-fold purification of VDE. The uncertainty is related to the activity determination of the thylakoid fraction, that was obscured by ascorbate accessibility to VDE and by the aggregation of thylakoids at low pH. Despite this increase in purity Son7 still contained a large number of different proteins, indicating that VDE is present in low amount in the starting material, compared to other thylakoid-associated proteins.

Precipitation of the Son7 fraction at 40% $(NH_4)_2SO_4$ removed residual membranes, chromophores and one third of total protein but very little (0.5%) of the VDE activity (Prec40, Table 1). At 80% $(NH_4)_2SO_4$ most of the VDE was precipitated together with another third of protein, thus leading to a 2-fold increase in specific activity (Prec80, Table 1). At the same time a 100-fold reduction in volume was obtained. Proteins that were enriched by this precipitation were, the 62 kDa, the 37 kDa ferredoxin $NADP^+$ oxidoreductase, several proteins in the 40–45 kDa range and the 14–20 kDa range. The 59 and 54 kDa proteins were efficiently precipitated already at 40% $(NH_4)SO_4$ (not shown) and were therefore drastically reduced in the Prec80 fraction (Figure 1). As the Prec80 fraction contained most of the VDE activity these results argue against the 59 and 54 kDa proteins as candidates for the VDE.

Upon gel filtration of the Prec80 fraction. around 70% of the loaded activity was recovered and the most active fractions were pooled (GelfPool). The GelPool fraction contained roughly 50% of the VDE activity and only 8% of total protein, compared with the Prec80 fraction applied to the column, resulting in a further 6-fold increase in specific activity in this step. The gel filtration step efficiently removed proteins with a molecular weight below 33 kDa and above 54 kDa. Proteins that showed a strong increase in this step were, the 54 kDa, 43 kDa, 40/39.5 kDa and 36 kDa (Figure 1).

Although the 43 kDa protein showed the best correlation with activity, the GelfPool still contained a large number of proteins. A more detailed evaluation was therefore performed.

A separate gel filtration experiment was run where each individual fraction showing VDE activity was collected and analyzed by SDS-PAGE (Figure 2a). The

Table 1. Purification of violaxanthin deepoxidase from spinach thylakoids. The data presented correspond to 1.6 kg spinach leaves. The values are mean values from 3 different preparations. SonRes7: remaining pellet after sonic treatment at pH 7.2 Supernatant after sonication and centrifugation at pH 5.2 (Son5), first and second supernatant at pH 7.2 (Son7:1 and Son7:2) and the pooled fraction (Son7). Prec40 and Prec80: fractionated $(NH_4)_2SO_4$ precipitation at 40 and 80% saturation, respectively. GelfPool: pooled fraction from a Sephacryl S-300HR gel filtration. HIC and DEAE: representing the most active fractions from a Poros 20 ET/P hydrofobic interaction chromatography (HIC) and from a DEAE MemSep 1000HP anion exchange chromatography, respectively

Fraction	Volume (ml)	Activity (nmol s^{-1})	Protein (mg)	Specific activity (μmol g^{-1} s^{-1})
Thylakoids	400	86	3540	0.024
SonRes7	370	1.8	3030	0.0006
Son5	370	0	149	0
Son7:1	380	143	85	1.7
Son7:2	390	23	33	0.7
Son7	770	166	118	1.4
Prec40	60	0.8	42	0.02
Prec80	5	110	43	2.6
GelfPool	30	52	3.5	14.9
HIC.F4	11	39	0.3	130
DEAE.F5	9	1.9	0.01	190
DEAE.F6	5	2.3	0.009	256
DEAE.F7	3	13	0.09	144
DEAE.F8	3	22	0.36	61

protein bands were quantified by gel scanning. The VDE activity of each fraction and the optical density of each protein band were plotted against fraction number. As shown in Figure 2b, the best correlation was found for the 43 kDa protein. Note particularly that fraction 51 showed the highest specific activity and in addition lacked bands above 43 kDa.

The position of the activity peak corresponded to a molecular weight of 44 kDa when the gel filtration column Sephacryl S300HR was used, which is close to the 43 kDa obtained by SDS-PAGE.

To further purify VDE, HIC was performed with the GelfPool. In order to retain bound activity we found it important to use the weakly hydrophobic column, Poros ET/P with poly(2-hydroxypropylether) as active group, rather than the more hydrophobic Poros PH, Phenyl- and Alkyl-Superose as well as Butyl- and Phenyl-Sepharose CL4B. To further avoid strong interaction with the column, we selected an ionic strength just sufficient for the enzyme to bind to the column. After injection and initial wash, most of the 33–38.5 kDa proteins passed through the column (F1, Figure 3), with only marginal amounts of VDE activity. Then, upon omission of $(NH_4)_2SO_4$ from the medium, the VDE activity could be eluted. This fraction contained most of the 43 kDa protein as well as a 39.5 kDa protein, and showed a 9-fold increase in specific activity (Table 1) compared to the GelfPool fraction. Although higher molecular weight proteins could be detected, they appeared to have roughly the same intensity in fractions 1 and 4 despite a large difference in activity. Thus, these results also point to the 43 kDa protein as VDE, although the 39.5 protein could not be excluded on the basis of this particular experiment.

To further establish the identity of VDE, the GelfPool fraction was subjected to anion exchange chromatography. The following points were found critical in order to retain good activity. First, DEAE columns like DEAE MemSep, used in this experiment, gave high recovery of activity while Q columns like Econo

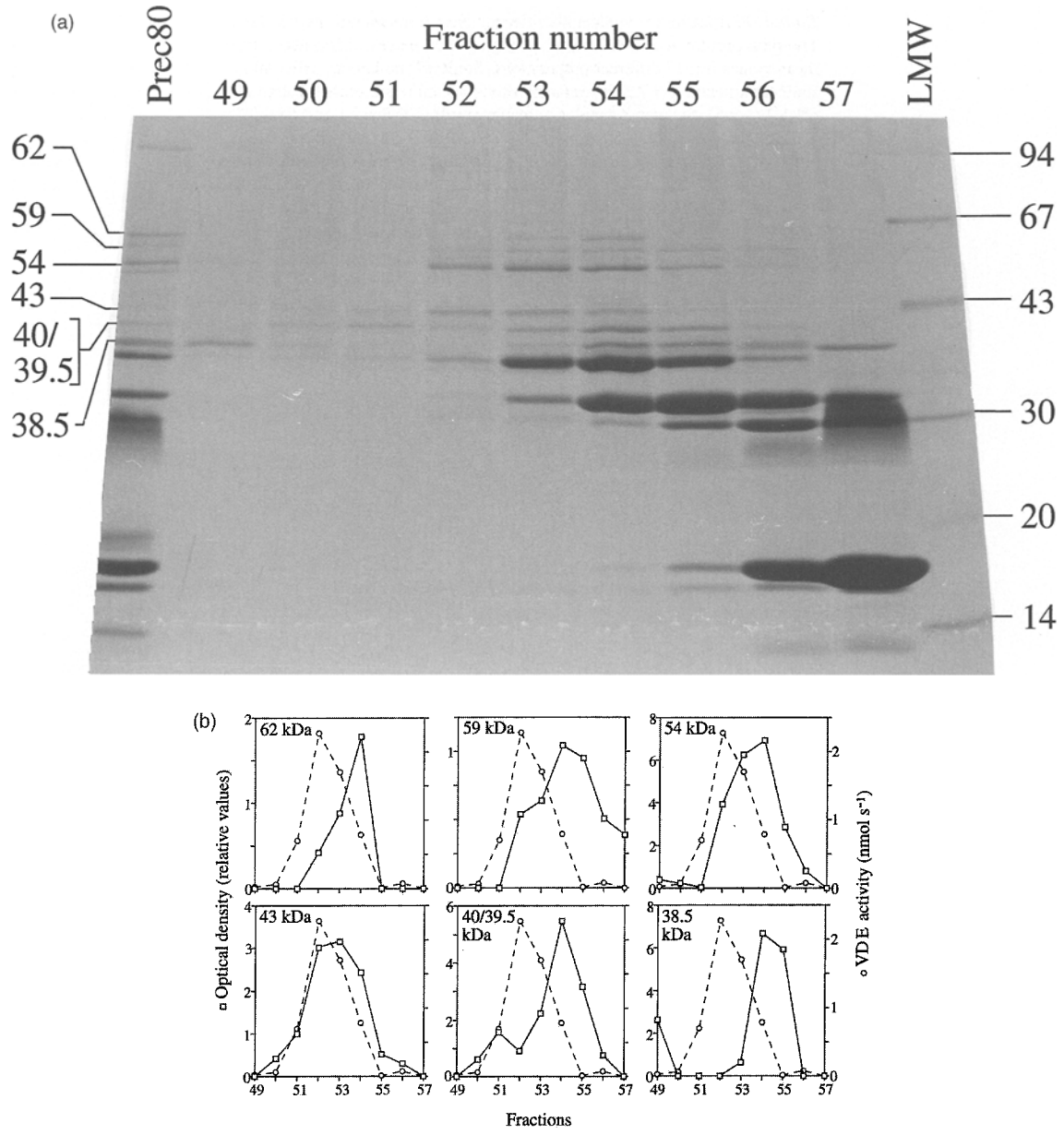

Figure 2. Detailed analysis of the fractions from Sephacryl S300HR gel filtration. (a) SDS-PAGE and (b) activity profile compared to optical density for six of the protein bands with closest resemblance. Densitometry was performed as described in 'Materials and methods'.

Q, Mono Q and Poros Q, gave low recovery. Secondly, the binding conditions had to be such that VDE was just barely bound. Too low ionic strength under the binding step reduced the amount of activity that could be recovered upon elution. Third, the elution required a gradual increase in ionic strength, rather than a step increase. In the latter case no or very little activity was recovered.

As shown in Figure 4a the void fractions (F1–F4) contained the main part of the 33, 36 and 37 kDa proteins. The first fractions obtained upon elution with the salt gradient (F5–F7) were dominated by the 43 kDa protein with two minor components below. In fraction F8 and F9 the 38.5/38, 40/39.5 and 43 kDa proteins dominated. A small amount of the 54 kDa protein was found in the F7 and F8 fractions although it was hardly detectable in the GelfPool fraction used as starting

Table 2. Purification of violaxanthin deepoxidase from spinach thylakoids according to the method of Hager and Perz (1970). Purification was performed using 900 g spinach leaves but the values have been recalculated to correspond to 1.6 kg, in order to facilitate comparison with Table 1. SonRes: remaining pellet after sonic treatment at pH 6.8. Son: resulting supernatant after sonication and centrifugation. DEAE: eluted fraction from a DEAE Sephadex A-25 anion exchange column. Prec50 and Prec70: fractionated $(NH_4)_2SO_4$ precipitation at 50 and 70% saturation respectively. Gelf: pooled fraction from a Sephadex G-100 gel filtration containing three of the most active fractions

Fraction	Volume (ml)	Activity (nmol s^{-1})	Protein (mg)	Specific activity (μmol g^{-1} s^{-1})
Thylakoids	187	140	4490	0.031
SonRes	164	59	3280	0.018
Son	156	77	621	0.12
DEAE	159	47	157	0.3
Prec50	10	4.6	70	0.07
Prec70	6.5	29	25	1.2
Gelf	34	17	2.3	7.4

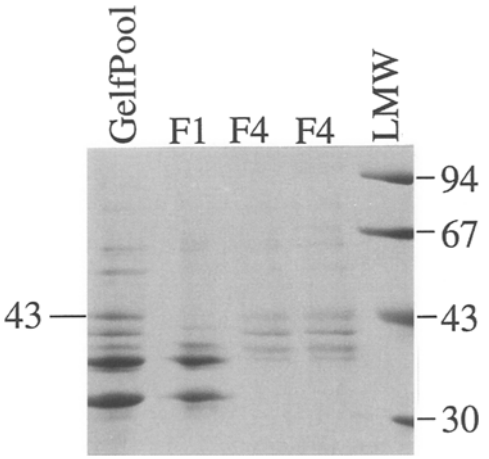

Figure 3. SDS-PAGE of fractions reseived from the hydrophobic interaction chromatography using Poros ET/P. GelfPool represents the fraction applied on the column. F1: the void fraction. F4: fraction eluted containing VDE activity. LMW: low molecular weight standards, values in kDa.

Table 3. Amino acid composition of VDE

Amino acid	mol-%	Number of residues
Ala	7.0	26
Arg	4.6	17
Asx	13.4	51
Cys	3.0	11
Gly	4.4	17
Glx	14.0	53
His	1.2	4
Ile	4.1	15
Leu	10.7	41
Lys	7.5	29
Met	1.6	6
Phe	5.4	20
Pro	5.6	21
Ser	3.0	11
Thr	5.2	20
Trp	ND	ND
Tyr	2.3	9
Val	7.0	27

ND: not determined.

material in this particular experiment. Notably, the proteins in the range of 54–62 varied between preparations, without variations in the VDE activity.

When plotting the VDE activity and band intensity as a function of fraction number a close correlation was only obtained for the 43 kDa protein (Figure 4b). Note particularly the deviations seen for other proteins in F6 and F7. The increase in specific activity in the DEAE step for the fractions F5–F8 was found to be 13-, 17-, 10- and 4-fold, respectively (Table 1).

Before developing the procedure described above we made attempts to isolate VDE according to the early report by Hager and Perz (1970). The results are

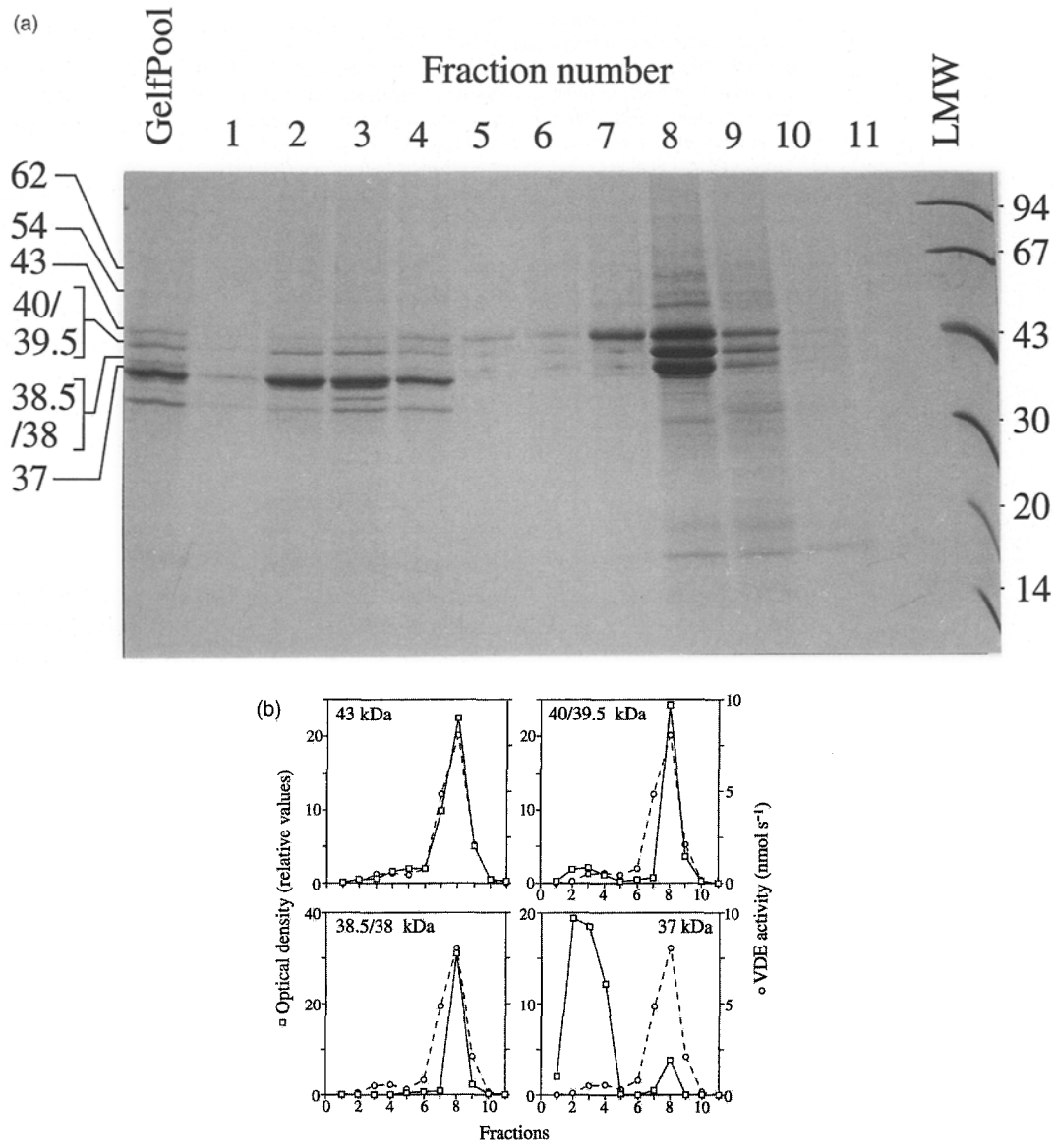

Figure 4. Analysis of results obtained by the DEAE MemSep anion exchange chromatography. (a) SDS-PAGE and (b) activity profile compared to optical density for four of the protein bands with closest resemblance. Densitometry was performed as described in 'Materials and methods'.

summarized in Table 2 and Figure 5. Sonic treatment of thylakoids and passage through a DEAE Sephadex A-25 column resulted in a 2.5-fold increase in purity. Differential $(NH_4)_2SO_4$ (50 and 70%) precipitation was performed, which led to a 4-fold increase in purity. The material was then separated by gel filtration on Sephadex G100 resulting in a 6-fold increase in specific activity. The whole procedure, starting from thylakoids, resulted in a 200-fold increase in purity of VDE, based on the specific activity. An estimate of the molecular weight of VDE was also made from gel filtration, using Sephadex G100, and found to be in the range of 54 kDa, which differs from the 44 kDa obtained on Sephacryl S300HR. We have no explanation for this difference.

Up to this point we obtained essentially the same results as Hager and Perz (1970). However, they did not show a picture of the gel. The SDS-PAGE results from our corresponding purification are presented in Figure 5. The sonication of thylakoids released a large number of proteins. The DEAE-step caused no drastic change in protein pattern. The protein pattern of

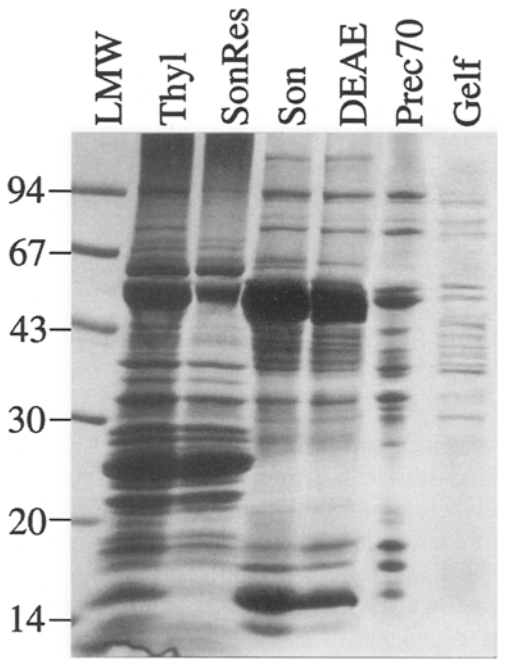

Figure 5. SDS-PAGE of samples obtained from the purification of VDE according to Hager and Perz (1970). LMW: low molecular weight standards, values in kDa. Thyl: Thylakoids. SonRes: remaining pellet after sonic treatment at pH 6.8. Son: resulting supernatant after sonication and centrifugation. DEAE: eluted fraction from a DEAE sephadex A-25 anion exchange column. Prec70: fractionated $(NH_4)_2SO_4$ precipitation at 70% of saturation. Gelf: pooled fraction from a Sephadex G-100 gel filtration containing the three most active fractions.

the most active fraction from $(NH_4)_2SO_4$ precipitation (Prec70) revealed a drastic reduction in a 54 kDa band probably representing Rubisco and a relative increase in several other bands. The presumed Rubisco band was still the most dominat. The final fraction (Gelf) obtained after gel filtration contained more than 10 different protein bands. In our hands this purity was insufficient to conclude which band represented VDE.

The 43 kDa protein isolated by our method was N-terminally sequenced both without and with protection of cysteine residues. The results from the cysteine-protected sample confirmed the results from that without protection and allowed gaps in the sequence to be identified as cysteine (Figure 6). The sequence was found to be unique, and did not show reasonable similarity with any sequence in the data banks. The amino acid composition (Table 3) of the protein revealed a high abundance of charged and polar amino acids, resulting in a polarity index of 48%, which is typical for water-soluble hydrophilic proteins (Capaldi and Vanderkooi 1972). The protein showed a remarkably large number of cysteine residues of which three are seen already in the N-terminal sequence (Figure 6). The significance of this is not clear but it explains the sensitivity of the enzyme towards reducing agents like DTT (Yamamoto and Kamite 1972).

The 39.5 kDa and the 40 kDa proteins were also N-terminally sequenced (Figure 6) and found to be unique and distinct from the 43 kDa protein. In addition, the N-terminal part of the 39.5 kDa protein showed complete sequence identity with the N-terminal part of the Cyt b_6 protein as well as with an internal sequence of polyphenol oxidase (Figure 6). The N-terminal sequence of the 40 kDa protein showed 75% identity with the 39.5 kDa protein, which may indicate that they are related.

Discussion

All our data so far obtained are consistent with the identification of VDE as the 43 kDa protein. The strongest argument for the identification comes from the close correlation between the activity and the optical density of individual protein bands in the DEAE MemSep experiment (Figure 4). However, in all purification steps the amount of the 43 kDa protein correlated with the VDE activity. The highest specific activity obtained here (DEAE MemSep, F6) was 256 μmol $g^{-1}s^{-1}$ protein (Table 1), which correspond to at least a 5000-fold increase compared to the original thylakoids.

When we reproduced the purification method of Hager and Perz (1970), we obtained the same degree of purification as they reported and our purest fraction had a specific activity of 7.4 μmol g^{-1} s^{-1} protein, which is also close to the 10 μmol g^{-1} s^{-1} protein reported by Yamamoto and Higashi (1978). Both Hager and Perz (1970) and Yamamoto and Higashi (1978) reported VDE to be essentially pure but no gel electrophoresis results were presented. The corresponding fraction obtained in our hands had a specific activity of 15 μmol g^{-1} s^{-1} protein and a large number of protein bands. With our purification procedure we obtained an apparent molecular weight for the VDE of 44 kDa on Sephacryl S300HR. Hager and Perz (1970), who first reported on the purification of VDE, used Sephadex G100 in their purification, but they did not report any molecular weight. Later Hager (1975), reported a molecular weight of 54 kDa for the VDE.

Thus, there is a discrepancy between our findings that the identity of the VDE is a 43 kDa protein and previous reports, which have identified the VDE as a protein in the range of 54–60 kDa. Among the proteins

43 kDa:	1:	VDALKTCTCL LKECRIELAG
40 kDa:	1:	APISGDVEKD TEPAXRYAXP
a:	1:	APILPDVEKS TLSDA
39.5 kDa:		APILPDVEKX TLSDA
b:	102:	APILPDVEKC TLSD

Figure 6. Comparison of N-terminal amino acid sequences. Proteins named after their apparent molecular weight on SDS-PAGE from the isolation procedure. a: residue 1–15 of cytochrome b_6-spinach chloroplast (pir S20410, length 15). b: residue 102–115 of spinach polyphenol oxidase precursor (sp P43310, length 639).

of the thylakoid membrane that would be expected to be released by sonication are the CF1 subunit of the ATP-synthase and the stroma protein Rubisco, that is often found associated with thylakoids unless extensively washed. The α- and β-subunits of CF_1 have molecular weights of 62 kDa and 57 kDa, respectively, and the large subunit of Rubisco has a molecular weight of 51–58 kDa. We can detect three bands in this region, namely at 54 kDa, 59 kDa and a 62 kDa. It is reasonable to suspect that some, if not all, of these are CF_1-subunits or the Rubisco subunit. Our argument against these proteins as candidates for VDE is, however, based primarily on the comparison between the intensity of individual protein bands and VDE activity throughout the whole isolation procedure. Furthermore, variations in the amount of the 54, 59 and 62 kDa proteins present in the GelfPool was found between different preparations (i.e., compare the GelfPool fraction in Figures 1 and 4a) without variations in VDE activity.

Recently, Hager and Holocher (1994), reported a series of experiments concerning the localization of VDE within the thylakoid lumen and its mobility in this compartment. They presented an SDS-PAGE of fractions, containing VDE released by freeze thawing. Citing unpublished data the authors highlight a band at 58 kDa, which they claim to be the VDE. However, there were a large number of other proteins released that apparently also followed the activity. Also proteins in the 43 kDa region were released.

We found the two closely spaced bands at 40 kDa more difficult to exclude as candidates for VDE. However, detailed evaluations of band intensities and activity versus fraction number (Figures 2b and 4b) allowed us to exclude these proteins as candidates. In addition, the N-terminal part of the 39.5 kDa protein showed complete sequence identity with the N-terminal part of Cyt b_6 as well as with an internal sequence of polyphenol oxidase. The N-terminal sequence of the 40 kDa protein was similar, although not identical to the 39.5 kDa protein (Table 3), suggesting that they are related.

The amino acid composition of the 43 kDa VDE enzyme indicate a high proportion of the negatively charged amino acids Asp and Glu as well as the presence of 5 His residues. As shown recently (Bratt et al. 1995; Carlsson et al. 1995) the binding of VDE to the thylakoid membrane responds to protons with a cooperativity of 4, with half-binding at pH 6.6. This could reflect protonation of Glu- or Asp-residues with high pKa values or His-residues with low pKa values.

The very large purification factor obtained in this work reflects that the enzyme occurs in low amounts in the thylakoids. When comparing the specific activity found for VDE in the most pure fraction with that of the thylakoids we can estimate that there is 1 VDE molecule per 20–100 electron transport chains.

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References

Arvidsson P-O, Bratt CE, Andréasson L-E and Åkerlund H-E (1993) The 28 kDa apoprotein of CP 26 in PS II binds copper. Photosynth Res 37: 217–225

Björkman O and Demmig-Adams B (1994) Regulation of photosynthethic light energy capture, conversion, and dissipation in leaves of higher plants. In: Schulze A-D and Caldwell MM (eds) Ecophysiology of Photosynthesis, pp 17–47. Springer-Verlag, Berlin

Bratt CE, Arvidsson P-O, Carlsson M and Åkerlund H-E (1995) Regulation of violaxanthin de-epoxidase activity by pH and ascorbate concentration. Photosynth Res 45: 169–175

Capaldi RA and Vanderkooi G (1972) The low polarity of many membrane proteins (solube proteins/polar amino acids / hydrophobicity / polarity index). Proc Natl Acad Sci USA 69: 930–932

Carlsson M, Bratt CE, Arvidsson P-O and Åkerlund H-E (1995) Regulation of violaxanthin de-epoxidase activity by pH and ascorbate concentration. In: Mathis P (ed) Photosynthesis: From Light to Biosphere, Vol. IV, pp 55–58. Kluwer Academic Publishers, Dordrecht

Gruszecki WI (1995) Different aspects of protective activity of the xanthophyll cycle under stress conditions. Acta Physiologiae Plantarum 17: 145–152

Hager A (1975) Die reversiblen, lichtabhängigen Xanthophyllumwandlungen im Chloroplasten. Ber Dtsch Bot Ges 88: 27–44

Hager A and Holocher K (1994) Localization of the xanthophyll-cycle enzyme violaxanthin de-epoxidase within the thylakoid lumen and abolition of its mobility by a (light-dependent) pH decrease. Planta 192: 581–589

Hager A and Perz H (1970) Veränderung der Lichtabsorption eines Carotinoids im Enzym (De-epoxidase)-Substrat (Violaxanthin)-Komplex. Planta (Berl.) 93: 314–322.

Laemmli UK (1970) Cleavage of structual proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685

Ljungberg U, Åkerlund H-E and Andersson B (1986) Isolation and characterisation of the 10-kDa and 22-kDa polypeptides of higher plant Photosystem II. Eur J Biochem 158: 477–462

Thayer SS and Björkman O (1990) Leaf xanthophyll content and composition in sun and shade determined by HPLC. Photosynth Res 23: 331–343

Yamamoto HY and Kamite L (1972) The effects of dithiothreitol on violaxanthin de-epoxidation and absorbance changes in the 500-nm region. Biochim Biophys Acta 267: 558–543

Yamamoto HY and Higashi RM (1978) Violaxanthin de-epoxidase; lipid composition and substrate specificity. Arch Biochem Biophys 190: 514–522