Violaxanthin conversion by recombinant diatom and plant de-epoxidases, expressed in Escherichia coli – a comparative analysis

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The purpose of study presented here was to obtain recombinant violaxanthin de-epoxidases (VDEs) from two species. The first one was VDE of Arabidopsis thaliana (L.) Heynh. (WT Columbia strain) (AtVDE) which catalyzes conversion of violaxanthin (Vx) to zeaxanthin (Zx) via antheraxanthin (Ax) in vivo. The second one was VDE of Phaeodactylum tricornutum Bohlin, 1897 (CCAP 1055/1 strain) (PtVDE) which is responsible for de-epoxidation of diadinoxanthin (Ddx) to diatoxanthin (Dtx). As the first step of our experiments, open reading frames coding for the studied enzymes were amplified and subsequently cloned into the pET-15b plasmid. For recombinant protein production, the Escherichia coli Origami b strain was used. The molecular weight of the produced enzymes was approximately estimated to be 45kDa and 50kDa for AtVDE and PtVDE, respectively. Both enzymes, purified under native conditions by immobilized metal affinity chromatography, displayed comparable activity in an assay mixture and converted up to 90% of Vx in 10 min in a two step enzymatic de-epoxidation, irrespective of the enzyme’s origin. No statistically significant differences were observed when kinetics of the reactions catalyzed by these enzymes were compared. A putative role of the selected amino-acid residues of AtVDE and PtVDE was also considered. Significance of recombinant PtVDE (purified here for the first time ever) is also indicated as a useful tool in various comparative investigations of de-epoxidation reactions in main types of xanthophyll cycles existing in nature.

Key words: diadinoxanthin de-epoxidase, diatoms, light stress, violaxanthin de-epoxidase, xanthophyll cycle

INTRODUCTION

Under oxygenic conditions, excess of light absorbed by photosynthetic pigments of photoautotrophs can result in photodamage of the photosynthetic machinery. To prevent destructive effect of light and oxygen, all photosynthetic organisms carrying out oxygenic photosynthesis have developed various photo-protective mechanisms. One of them is the xanthophyll cycle which is widespread in nature and displays several types depending on the group of photoautotrophs. All kinds of this cycle involve xanthophyll epoxides de-epoxidation by de-epoxidases under light stress, which results in an increase in the number of conjugated double bonds in these xanthophylls, thus increasing their photo-protective efficiency. The most common types of xanthophyll cycles, i.e. violaxanthin (Vx) and diadinoxanthin (Ddx) cycles, can be distinguished based on the de-epoxidase substrate.

During Vx cycle under light stress, Vx, with two epoxide groups, is converted into epoxide-free zeaxanthin (Zx) via antheraxanthin (Ax), which is an intermediate with one epoxy group. This reaction is catalyzed by Vx de-epoxidase (VDE) and occurs in all vascular plants, mosses and some groups of algae, mainly in the green algae (Latowski et al., 2011).

In the Ddx cycle under high light conditions, mono-epoxide – Ddx is transformed into epoxide-free diatixanthin (Dtx). This conversion is catalyzed by an enzyme frequently called Ddx de-epoxidase (DDE). This enzyme has been found in several members of Chromista, first and foremost in brown algae, diatoms and dinoflagellates (Latowski et al., 2011).

Until 2007, it was generally accepted that DDE is the only de-epoxidase in organisms having the Ddx cycle. It was even suggested that in diatoms this de-epoxidase could convert both, Ddx in the Ddx cycle and Vx in the Vx cycle, which also exists in diatoms (Jakob et al., 2001). It should be added that all properties of this enzyme have been determined only for a diatom extract but not for a purified enzyme (Jakob et al., 2007; Goss et al., 2005; Latowski et al., 2007).

However, results of these experiments were helpful in comparison of the most important VDEs and DDEs properties:
1. Both, DDE and VDE are nuclear-encoded and water soluble luminal proteins that undergo a conformational change when pH drops due to formation of light-driven proton gradient across the thylakoid membrane; the change in enzyme conformation is accompanied by functional binding of the enzymes to the thylakoid membrane, where the Vx or Ddx substrates are located;

2. Activity of DDE exhibits a different pH-dependence when compared with VDE:
   a) DDE has pH-optimum at pH 5.5, whereas VDE around pH 5.0;
   b) DDE is also active at pH 7.2, whereas VDE only at a pH below 6.5 (Jakob et al., 2001);

3. Both, DDE and VDE require non-lamellar lipids such as monogalactosyldiacylglycerol (MGDG) or phosphatidylethanolamine (PE) for their activity, but DDE for optimal de-epoxidation requires a lipid/pigment ratio of around 5, while VDE needs a significantly higher ratio of 30 (Latowski et al., 2004; Goss et al., 2005);

4. In addition to substrate and MGDG, both DDE and VDE need ascorbate (Asc); it was suggested, that not the basic form of Asc, but rather the acid form of ascorbate (ascorbic acid, AscH) is required. DDE has a 3–4 times higher affinity for AscH than VDE; it was suggested, that DDE and VDE need ascorbate (Asc); it was suggested, that not the basic form of Asc, but rather the acid form of ascorbate (ascorbic acid, AscH) is required. DDE has a 3–4 times higher affinity for AscH than VDE; for both enzymes, $K_m$ for AscH was independent of the different pH values and was found to be 0.290 mM for VDE; in case of Asc, the $K_m$ value for VDE at pH 5 was determined to be 0.7 mM while for VDE $K_m$ was 2.3 mM at the same pH (Eskling et al., 2006).

In 2007 the presence of two de-epoxidase-encoding genes in one diatom species, Thalassiosira pseudonana, was shown (Montsant et al., 2007). One year later it was demonstrated that the genome of another diatom species, Phaeodactylum tricornutum Bohlin, 1897, contains one gene similar to the gene of VDE, labeled as PtVDE, and two VDE-like genes, designated as PtVVDL1 and PtVVDL2.

All known de-epoxidases belong to the lipocalin family (calycin superfamily) (Bugos et al., 1998; Hieber et al., 2000). A characteristic feature of all known lipocalins is the presence of six or eight antiparallel $\beta$-sheets with one, or two or three structurally conserved regions (SCRs) (Grzyb et al., 2006). In VDE only two of them (SCR1 and SCR3) were identified (Charron et al., 2005). It is postulated that the characteristic structure of $\beta$-sheets of all lipocalins is responsible for creation of a hydrogen-bonded $\beta$-barrel sometimes called “calyx” which is necessary for substrate or ligand binding. The depth of the hollow in examined proteins is about 40 Å (Holden et al., 1987). The same was postulated for VDE on the basis of substrate specificity analysis.

A comparison between the domain structures of the VDE and diatom DDE proteins shows that both, VDE and diatom DDE are being compared for first time.

The cysteine domain, where cysteine residues can form up to six disulfide bridges, is conserved at all cysteine positions in the plant and diatom de-epoxidases and postulated to be essential for activity (Simionato et al., 2015). The distance between SCR1 and SCR3 in the lipocalin domain of diatoms is longer than in known VDEs. The C-terminal Glu-rich domain is less conserved between species compared to the N-terminal and lipocalin domains. Generally, the C-terminal domains of P. tricornutum DDEs display a lower percentage of charged amino acids, including Glu (Coesel et al., 2008).

Previously, our group had achieved for the first time ever an effective production of two of the three P. tricornutum de-epoxidases i.e. PtVDE and PtVVDL2 in Escherichia coli. The level of these proteins’ production was dependent on the type of E. coli strain. Origami b (DE3) (Novagen) appeared to work better for PtVDE, whereas BL21 (Novagen) was more proper for PtVVDL2 (Bojko et al., 2013b). Crude homogenates of both, PtVDE and PtVVDL2, had shown an activity in relation to Vx, with dynamic conversion of Vx into Ax and Zx, depending on the gene expression level (Bojko et al., 2013b). However, up to now, no comparative analysis of the activity of the purified diatom de-epoxidases and VDE was performed. In this paper, activities of recombinant PtVDE and Arabidopsis thaliana (L.) Heynh. VDE (AtVDE) are being compared for first time.

**MATERIALS AND METHODS**

Construction of plasmids with PtVDE and AtVDE. PtVDE and AtVDE open reading frame was amplified using genomic DNA from 5 day old culture of P. tricornutum (CCAP.1055/1 strain), isolated with the help of Genomic Mini AX Plant Gravity kit (A&A Biotechnology). Diatoms were grown on f/2 Guillard medium standard (Guillard & Ryther 1962) supplemented with f/2 vitamins and sodium meta-silicate (3%) with artificial 1.6%...
sea salt (Bio-Active, Tropic Marin) under white light at approximately 40 µmol m⁻² s⁻¹ with 10/14 photoperiod at 15°C (Bojko et al., 2013a). Amplification of Pt/VDE open reading frame was performed using Pfu DNA polymerase (Fermentas), forward (5'-CCGATCTCCATT-GCTGGAGGTTCCTC-3') and reverse (5'-GAGCTTTAAATGGTCTTCCACAG-3') primers (Genomed), according to Bojko et al. (2013b). PCR was performed using the following thermal condition: initial denaturation (3 min), repeated 35 times cycle of denaturation 95°C (30s), annealing 56°C (30 s), extension 72°C (2 min) and final extension at 72°C for 15 min (S1000Thermal Cycler, BioRad). The amplified product and pET-15b vector were digested by NdeI and BamHI restriction enzymes followed by ligation using a ligation kit (Fermentas). The obtained plasmid was named pET-15b/PtVDE.

The sequence of wild type At/VDE open reading frame uses rare codons with a high frequency and contains several negatively cis-acting motifs which might hamper expression in prokaryotes. Therefore, chemical synthesis of At/VDE open reading frame, optimized for E. coli protein production, was ordered (GENEART). The codons of A. thaliana were adapted to the codon bias of E. coli genes. During the optimization process, the following cis-acting sequence motifs were avoided: internal TATA-boxes, chi-sites and ribosomal entry sites; AT-rich or GC-rich sequence stretches; repeat sequences and RNA secondary structures. The optimized open reading frame (length: 1081 bp) with NdeI site at the 5’ terminus and BamHI site at the 3’ terminus site was digested and cloned into pET-15b vector as described for Pt/VDE. The constructed plasmid containing At/VDE was named pET-15b/AtVDE.

PAVDE and AVDE production and enzyme purification. PET-15b/PtVDE and/or pET-15b/AtVDE were used for transformation of E. coli Origami b (DE3) strain cells (Novagen) (Swords 2003). Bacteria were grown in shaken 1 liter LB medium at 37°C. Cultures, at an optical density at 600 nm wavelength (OD₆₀₀) of approximately 0.6, were induced with 0.5 mM isopropyl β-D-1 thiogalactopyranoside (IPTG, Sigma) for 18 hour at 22°C (Bojko et al., 2013b). During induction, samples of about 1 ml were collected at 0, 3rd, 6th, 9th and 12th hour, centrifuged and finally kept at –20°C until used. The studied de-epoxidase proteins, expressed as percentage of total protein amount; (iii) subsequently, the determined purity of the samples was used to estimate the concentrations of PAVDE and AVDE. Obtained values were used to optimize the enzyme concentration in the assay mixture in the enzyme activity studies (see sub-chapter De-epoxidases activity in this section).

Western-Blot analysis. Proteins separated by SDS-PAGE were transferred onto polyvinylidine difluoride (PVDF) membrane with pore size: 0.45 µm (-immobilon-P) by the semi dry method. The membrane was blocked by 0.5% solution of non-fat dry milk with PBS+0.5% Tween 20+5% Blotto for 30 min, and incubated overnight at 4°C with monoclonal anti-polyHistidine primary antibody (Sigma), with dilution 1:3000. After washing (3×5 min, PBS+0.5% Tween 20+5% Blotto) the membrane was incubated with Anti-Mouse IgG-Alkaline Phosphatase antibody produced in goat (Sigma) at a dilution of 1:1000 for 2 h at room temperature. After washing (PBS+0.5% Tween 20) the proteins were detected using p-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) (a sensitive substrate for alkaline phosphatase detection reagent, Sigma) (Bojko et al., 2013b).

De-epoxidases activity. Activities of AVDE and PAVDE were monitored under darkness, at 20°C, in a commonly used de-epoxidation assay mixture containing 0.1 M citrate buffer pH 5.1, 9 µM MGDG, 0.33 µM Vx as a substrate and 0.1 µM enzyme. The enzyme concentration was selected from five independent tested points ranged between 20 nM and 200 nM of AVDE or PAVDE, at which the specific enzyme activity was constant. Reaction was initiated with AsCh which was added to the assay mixture to final concentration of 30 mM. Reaction was stopped at the beginning (zero point) and after 1, 3, 5, 10, 20 and 30 min by mixing 750 µl of the assay mixture with 750 µl of pigment extraction medium 1 (CHCl₃/MeOH:H⁺N⁺H⁺; ratio 1:2,004v/v) and centrifuged (16000×g, 10 min, 4°C). Bottom, organic phases, with reaction pigments, were collected. After evaporation, samples were solubilized with extraction medium 2 (90% Methanol/ammonium acetate (90% Methanol/10% 0.2 M Ammonium acetate): 10% ethylacetate) and centrifuged (16000×g, 10 min, 4°C). Bottom, organic phases, with reaction pigments, were collected. After evaporation, samples were solubilized with extraction medium 2 (90% Methanol/ammonium acetate (90% Methanol/10% 0.2 M Ammonium acetate): 10% ethylacetate) and analyzed by high performance liquid chromatography (HPLC) (Yamamoto 1985; Latowski et al., 2002). Volaxanthin was isolated from daffodil Narcissus pseudonarcissus L. petals as described by Havir and coworkers (Havir et al., 1997).

Statistical analysis. The enzyme activity for each tested concentration of the enzyme, as well as purity of the enzymes, were calculated for five independent analyses and mean values are given with standard deviation (S.D.). The average values and standard deviations of results were compared using Student’s t-test.

RESULTS AND DISCUSSION

The studied de-epoxidase proteins, PAVDE and AVDE, came from two species, P. tricornutum and A. thaliana, being the model organisms commonly used in the diatom and plant research, respectively. DNA electrophoresis confirmed successful incorporation of both cloned VDE open reading frames into pET15b plasmid (Fig. 2A, B). In turn, successful production of both analyzed enzymes in E. coli, Origami b (DE3) strain cells was confirmed (Fig. 2C, D). Protein production level was examined at the initial step of experiment, i.e. simultaneously with IPTG addition, and then at 3, 6, 9, and
18 h. The level of protein production was the highest, for both enzymes, at 18 h after IPTG induction (Fig. 2C, D). The obtained de-epoxidases displayed similar production level and molecular weight of approximately 50 kDa and 45 kDa for PtVDE and AtVDE, respectively (Fig. 2C, D), which is in accordance with the literature data, where mass of PtVDE is postulated at 49.220 kDa (Bowler et al., 2008) and plant VDE at 43 kDa (Rocholl et al., 1996). Comparable level of PtVDE and AtVDE production was additionally confirmed by Western-Blot analysis with anti-polyhistidine-tag antibodies (Fig. 2E, F).

SDS-PAGE was also applied to show the efficiency and degree of protein purification (Fig. 2G, H). As the result of recombinant de-epoxidase purification by the IMAC method, three different fractions were collected. First (Fig. 2h) and second (Fig. 2i) fraction contained unbound or weakly bound proteins to the resin. Elution fraction (j) exhibited proteins with high affinity to the resin, including PtVDE and/or AtVDE with the highest concentration and purity level (Fig. 2G, H). The enzymes’ purity was estimated to be 91.35±1.78% and 93.10±1.58% for PtVDE and AtVDE, respectively (Fig. 2G–J).

In our previous studies, production of PtVDE featured the highest level among all of the three tested genes of identified de-epoxidases (Bojko et al., 2013b). Here, it is additionally shown, that PtVDE was produced in cells of applied E. coli strain at a similar level to AtVDE. In nature, transcript level of PtVDE was increasing the fastest, among all identified de-epoxidase genes of 48-hour-dark-adapted P. tricornutum cells, when they were exposed to 175 mmol m⁻² s⁻¹ continuous white light, or 25 mmol m⁻² s⁻¹ continuous blue light (Coesel et al., 2008). It suggests that PtVDE can appear as the first active de-epoxi-

Figure 2. (A, B) Result of a PtVDE and AtVDE open reading frame cloning into pET15b amplified by PCR. Lanes: (a) pET-15b/PtVDE or (b) pET-15b/AtVDE as a DNA template; (C, D) SDS-PAGE analysis of PtVDE and AtVDE production level in E. coli cells. Lanes: (c) 0 h, (d) 3 h, (e) 6 h, (f) 9 h and (g) 18 h after IPTG induction; (E, F) Western-Blot and (G, H) SDS-PAGE analysis of PtVDE and AtVDE samples from different purification steps. Lanes: (h) proteins unbound to resin, (i) proteins weakly bound to resin and (j) imidazole elution fraction with VDEs; (I, J) Results of densitometry examinations of one of electrophoresis lanes of the “j” section

Figure 3. Putative connection of the Vx and Ddx cycles in Phaeodactylum tricornutum; hypothetical conversion step was indicated by a dashed arrow according to Lohr and Wilhelm (Lohr & Wilhelm, 1999; Lohr & Wilhelm, 2001)
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Figure 4. Violaxanthin de-epoxidation (samples collected at 0, 10 and 30 min of reaction) catalyzed by recombinant PVDE and AVDE, analyzed by level of violaxanthin (A), anteraxanthin (B) and zeaxanthin (C) expressed as percent of xanthophyll pigments sum.

Figure 5. The kinetics of Vx de-epoxidation (samples collected at 0, 1, 3, 5, 10 and 30 min) catalyzed by recombinant AVDE and PVDE.

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..dase in diatom cells under high light conditions and converts Ddx into Dtx. Perhaps, when light stress extends and Dtx achieves the level which could shift equilibrium of Ddx de-epoxidation in the substrate direction, PtVDE starts de-epoxidation of Vx, which is synthesized from Zx and is postulated to be a precursor of Ddx (Fig. 3) (Lohr & Wilhelm, 1999; Lohr & Wilhelm, 2001; Coesel, 2008). To verify this possibility, the de-epoxidation of Vx by recombinant PVDE and AVDE was compared in the assay mixture at the same concentration of both enzymes. As Fig. 4 shows, Vx is just as good substrate for AVDE as it is for PVDE. Levels of each of three xanthophyll cycle pigments showed no statistically significant differences. Above 90% of Vx was de-epoxidized to the comparable levels of Ax and Zx at 10 min. since the beginning of the experiment, irrespective of the enzyme type involved (Fig. 4). At 30 min., i.e. at the last detection step, in the presence of both types of enzymes, approximately 3.5% of Vx, 20% of Ax and up to 75% of the final product of the de-epoxidation were detected.

When kinetics of the reactions catalyzed by AVDE and PVDE were compared, no statistically significant differences were observed (Fig. 5). The activity appeared to be 27.02±5.04 nmol Vx min⁻¹ ml⁻¹ and 28.23±4.34 nmol Vx min⁻¹ ml⁻¹ for AVDE and PVDE, respectively.

The obtained results clearly demonstrate existence of substantial similarities in kinetic properties between AVDE and PVDE despite differences in some conserved amino acid residues of these proteins (Table 1). Comparable activity and kinetic properties presented in this paper seem to be supported by the lack of alterations among the six amino acid residues which are suggested to form active site of AVDE and PVDE (Table 1). These charged or polar, strictly conserved residues, co-localize on one side of the barrel and are indispensable for the enzymatic activity (Arnoux et al., 2009). All of them are also located close to Vx in crystals of the VDE lipocalin domain, and probably they are responsible for substrate binding (Fufezan et al., 2012). However, it is worth to notice that the amino acid composition of PVDE2 and PVDE1 contains up to 50% different residues. On the other hand, the most alterations between AVDE and PVDE are observed among nine amino acids identified as potentially important in the first steps of the enzyme activation caused by a decrease of pH. It is worth to notice that up to 3 of 4 residues suggested to be responsible for pH-dependent conformational changes required for enzymatic activity, such as Asp-98,
Asp-117, Asp-206 and His-168, are different in \( \text{PvVD} \) (Table 1). The fourth alteration does not seem to be important, because Ile-135 in \( \text{AVD} \) is commonly replaced by Leu, not only in \( \text{PvVD} \) but also in other VDEs working in the Vx cycle. In turn, three remaining alterations may explain different pH values required by all known \( \text{VDEs} \) working in the Vx cycle in comparison to that observed for DDE when extracts of diatom cells were used (Jakob et al., 2001).

The His-168 protonation in DDEs probably carries out activation of the enzyme already at higher pHs, while activation of typical VDEs, i.e. engaged in the Vx cycle, is shifted towards more acidic pHs due to the presence of the identified aspartates.

Recombinant \( \text{PvVD} \), which is described in this paper, may be considered as a good model to test this suggestion, as well as to verify the proposed molecular mechanism of de-epoxidases’ activation caused by a decrease in pH. Now, it is postulated that protonation of the fourth mentioned amino acid residues induced some loosening of the barrel and an increased water penetration. These water molecules compete with the Tyr hydroxyl group, thus reducing the strength of the hydrogen bond between His-121 and Tyr-214. The barrel is thought to be opened.

In \( \text{silico} \) studies have also shown that disruption of hydrogen bond between His-121 and Tyr-214 is reversible when the pH increases (Fufezan et al., 2012).

Among two remaining groups of amino acid of de-epoxidases, i.e. a putative Asc binding group, as well as amino acids postulated to be located closely of Vx, all known Vx cycle VDEs and \( \text{PvVD} \) differ only in one position (Table 1). In \( \text{silico} \) docking studies resulted in two possible models obtained from Asc docking in the VDE structure at pH 5. In both cases, binding occurred at the same part of the protein, but Asc orientation and interactions with polypeptide chain were different. In these two models Thr-112, Asp-114, Gln-119, Tyr-198 were postulated to interact with Asc (Fufezan et al., 2012). The four amino acid residues mentioned above are conserved for all known VDEs of the Vx cycle, but not for any diatom VDEs (Table 1). Among amino acid residues, considered to be located closely to Vx, Asn-134 of VDEs involved in the Vx cycle is replaced by Lys in \( \text{PvVD} \).

These single alterations, in light of the presented results, seem not to affect the kinetic properties of the tested enzymes under conditions which were applied in our experiments. On the other hand, active recombinant \( \text{PvVD} \) gives a whole range of possibilities for detailed analysis of the individual amino acids’ significance in various aspect of \( \text{PvVD} \) activity, including structure, affinities to substrates and cofactors or kinetics of the catalyzed reaction.

In our opinion, our data could be also treated as a helpful tool in understanding the role of diatom de-epoxidases in evolutionary success of diatoms in the earth ecosystems.

## Conflicts of Interest

The authors declare no conflict of interest.

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