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Regular paper

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* anteraxanthin (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 *At*VDE and *Pt*VDE, 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

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Abbreviations: Asc, ascorbate; AscH, ascorbic acid; *AtVDE*, violaxanthin de-epoxidase gene of *Arabidopsis thaliana* (L.) Heynh.; *At*VDE, violaxanthin de-epoxidase of *Arabidopsis thaliana* (L.) Heynh.; Ax, antheraxanthin; BamH I, restriction endonuclease isolated from *Bacillus amyloli*; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; DDE, diadinoxanthin de-epoxidase; Ddx, diadinoxanthin; Dtx, diatoxanthin; HPLC, high-performance liquid chromatography; IMAC, immobilized metal affinity chromatography; IPTG, isopropyl β-D-1 thiogalactopyranoside; MGDG, monogalactosyldiacylglycerol; NBT, *p*-nitroblue tetrazolium chloride; Nde I, endonuclease isolated from *Neisseria denitrificans*; OD_{600}, optical density at 600 nm wavelength; PCR, Polymerase Chain Reaction; PVDF, polyvinylidene difluoride; PE, phosphatidylethanolamine; *PtVDE*, violaxanthin de-epoxidase gene of *Phaeodactylum tricornutum* Bohlin, 1897; *Pt*VDE, violaxanthin de-epoxidase of *Phaeodactylum tricornutum* Bohlin, 1897; RCF, relative centrifugal force; RNA, ribonucleic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; VDE, violaxanthin de-epoxidase, Vx, violaxanthin; Zx, zeaxanthin

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 diatoxanthin (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.*, 2001; 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 lumenal 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; for both enzymes, K_m for AscH was independent of the different pH values and was found to be 0.075 mM for DDE and 0.290 mM for VDE; in case of Asc, the K_m value for DDE 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*., 1997; Grouneva *et al*., 2006).

In 2007 the presence of two de-epoxidase-encoding genes in one of 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 *PtVDL1* and *PtVDL2*.

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 ß-sheets with one, 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 β-sheets of all lipocalins is responsible for creation of a hydrogen-bonded ß-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 all indentified DDEs (*Tp*VDE, *Tp*VDL, *PtVDE*, *Pt*VDL1, *Pt*VDL2), consist of:

1) a cysteine-rich N-terminal domain,
2) a lipocalin domain,
3) a C-terminal glutamic acid-rich domain (Fig. 1).

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 plants and diatoms than 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 *PtVDL2* 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 and BL21 (Novagen) was more proper for *PtVDL2* (Bojko *et al*., 2013b). Crude homogenates of both, *Pt*VDE and *Pt*VDL2, 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 *Pt*VDE and *Arabidopsis thaliana* (L.) Heynh. VDE (*At*VDE) are being compared for first time.

MATERIALS AND METHODS

Construction of plasmids with *PtVDE* and *AtVDE*. *PtVDE* 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 Guilliard medium standard (Guillard & Ryther 1962) supplemented with f/2 vitamins and sodium metasilicate (3%) with artificial 1.6%

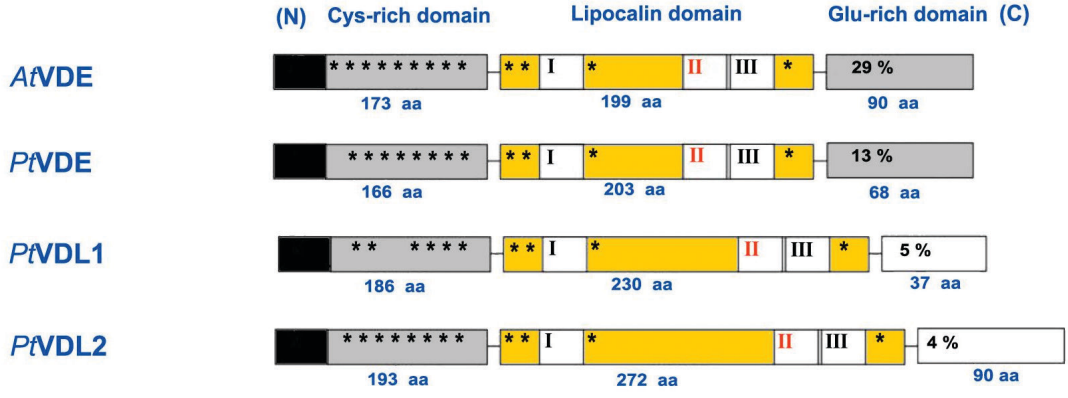

Figure 1. Schematic representation of de-epoxidases.
Black asterisks indicate the positions of conserved Cys residues. The central lipocalin domain contains the lipocalin binding fold. Conserved and divergent lipocalin motifs (roman numbers) are given in black and red, respectively. The C-terminal Glu-rich domain indicates the percentage of Glu residues in this domain (Coesel *et al*., 2008)

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 *PtVDE* open reading frame was performed using Pfu DNA polymerase (Fermentas), forward (5'-CCGGATCCTTATTGCTGGGAGGTTTCTC-3) and reverse (5'-GAGCATATGAAGTTTCTCGGTGTTACCAG-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/*Pt*VDE.

The sequence of wild type *AtVDE* 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 *AtVDE* 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 *PtVDE*. The constructed plasmid containing *AtVDE* was named pET-15b/*At*VDE.

***Pt*VDE and *At*VDE production and enzyme purification**. PET-15b/*Pt*VDE and/or pET-15b/*At*VDE 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 (OD_{600}) 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 18th hour, centrifuged and finally kept at –20°C until electrophoresis was performed in order to confirm the enzyme expression. After induction (18h) the cells were harvested by centrifugation (5 000 × *g*, 10 min, 4°C). The pellet was suspended in buffer A (50 mM Na_2HPO_4/NaH_2PO_4, 300 mM NaCl, pH 7.2) and sonicated 15 min on ice (40% output for 5 sec pulse and 10 sec rest). Subsequently, bacterial lysate was centrifuged (20 000 × *g*, 10 min, 4°C) and incubated on a platform shaker with TALON Metal Affinity Resin (Clontech), equilibrated with buffer A. After incubation the mixture was transferred to a gravity-flow column and the unbound proteins were removed by 5 bed volumes of wash buffer.

Protein bound to the resin was eluted with an elution buffer (150 mM imidazole, 50 mM Na_2HPO_4/NaH_2PO_4, 300 mM NaCl, pH 7.2). After purification, eluted fraction, enriched in *Pt*VDE or *At*VDE was dialyzed in buffer A. Concentrations of studied VDEs produced in *E. coli* cells were estimated as follows: (i) total protein concentration in the samples was assayed by the Lowry method (1951); (ii) known amounts of the samples with determined protein concentration were separated by SDS-PAGE (12% gel) with Coomassie Blue Brilliant staining and next analyzed with a GelAnalyzer software (Laemmli, 1970) to calculate the purity of the isolated enzymes, expressed as percentage of total protein amount; (iii) subsequently, the determined purity of the samples was used to estimate the concentrations of *Pt*VDE and *At*VDE. 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 polyvinylidene 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 *At*VDE and *Pt*VDE 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 *At*VDE or *Pt*VDE, 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 ($CHCl_3$:MeOH:NH_3; ratio 1:2:0,004v/v/v) and centrifuged (16 000 × *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 daffodils *Narcissus jonquilla* 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, *Pt*VDE and *At*VDE, 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

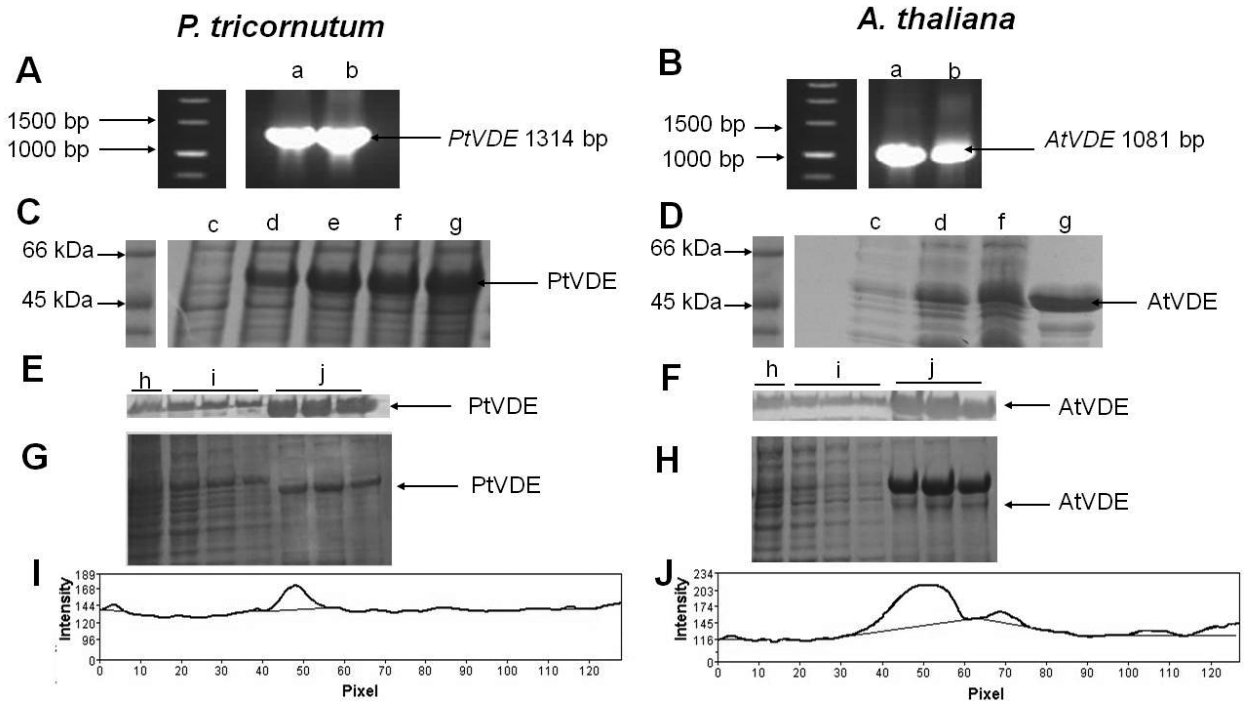

Figure 2. (A, B) Result of a *PtVDE* and *AtVDE* open reading frame cloning into pET15b amplified by PCR. Lanes: (a) pET-15b/*Pt*VDE or (b) pET-15b/*At*VDE as a DNA template; (C, D) SDS-PAGE analysis of *Pt*VDE and *At*VDE production level in *E. coli* cells. Lanes at: (c) 0 h, (d) 3 h, (e) 6 h, (f) 9 h and (g) 18 h after IPTG induction; (E, F) Western-Blot and (G, H) SDS-PAGE analysis of *Pt*VDE and *At*VDE 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

18 h. The level of protein production was the highest, for the 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 *Pt*VDE and *At*VDE, 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 (Rocholm *et al.*, 1996). Comparable level of *Pt*VDE and *At*VDE 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 *Pt*VDE and/or *At*VDE 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 *At*VDE, respectively (Fig. 2G–J).

In our previous studies, production of *Pt*VDE 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 *Pt*VDE was produced in cells of applied *E. coli* strain at a similar level to *At*VDE. In nature, transcript level of *Pt*VDE 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-

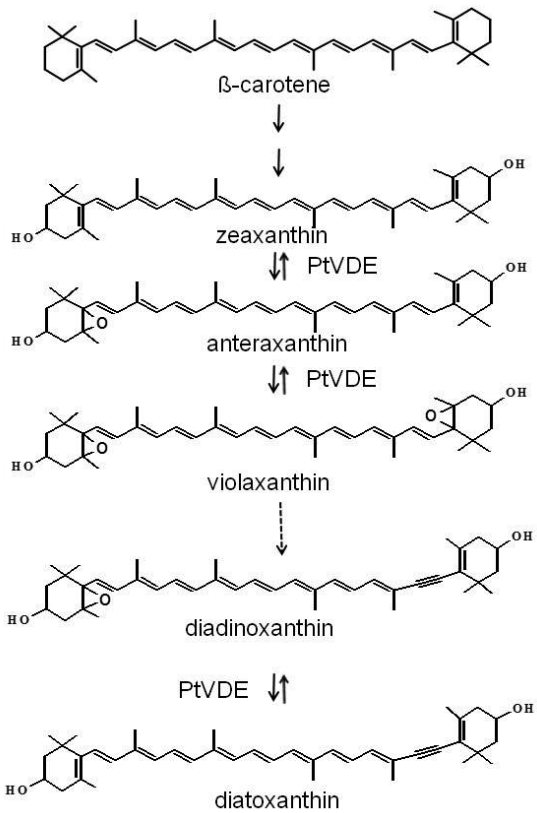

Figure 3. Putative connection of the Vx and Ddx cycles in *Phaeodactylum tricornutum*; hypothetical conversion step was indicated by a dashed arrow according to Lohr and Wilhelm (Lohr & Wilhelm, 1999; Lohr & Wilhelm, 2001)

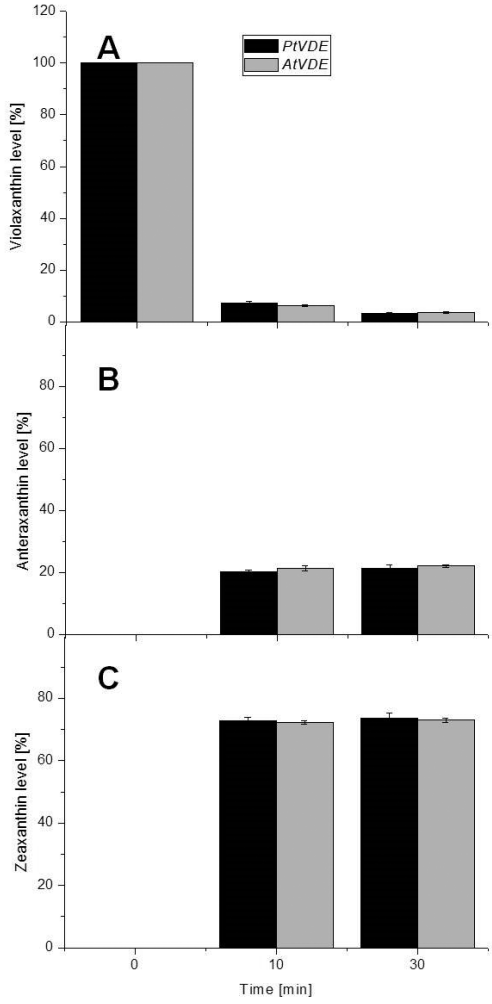

Figure 4. Violaxanthin de-epoxidation (samples collected at 0, 10 and 30 min of reaction) catalyzed by recombinant *Pt*VDE and *At*VDE, analyzed by level of violaxanthin (A), anteraxantin (B) and zeaxanthin (C) expressed as percent of xanthophyll pigments sum

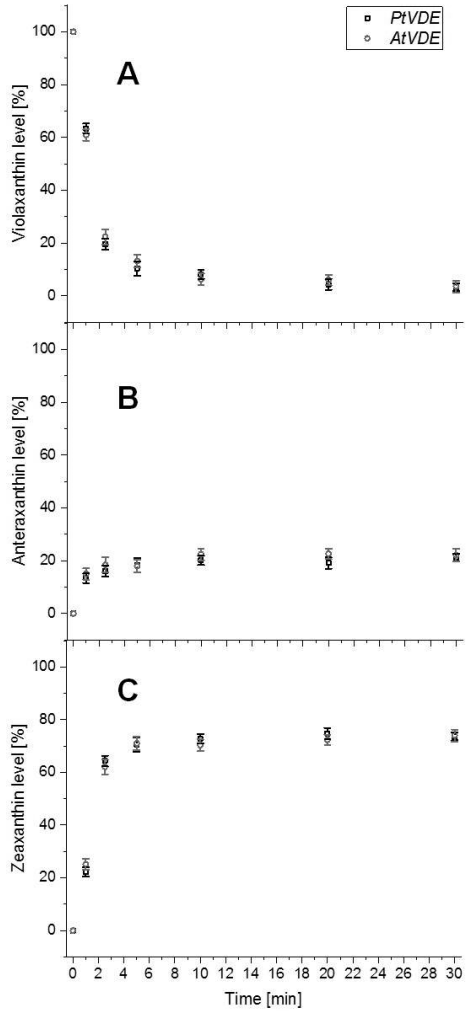

Figure 5. The kinetics of Vx de-epoxidation (samples collected at 0, 1, 3, 5, 10 and 30 min) catalyzed by recombinant *At*VDE and *Pt*VDE

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, *Pt*VDE 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 *Pt*VDE and *At*VDE was compared in the assay mixture at the same concentration of both enzymes. As Fig. 4 shows, Vx is just as good substrate for *At*VDE as it is for *Pt*VDE. 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, aproximatelly 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 *At*VDE and *Pt*VDE 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 *At*VDE and *Pt*VDE, respectively.

The obtained results clearly demonstrate existence of substantial similarities in kinetic properties between *At*VDE and *Pt*VDE 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 *At*VDE and *Pt*VDE (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 *Pt*VDE2 and *Pt*VDE1 contains up to 50% different residues. On the other hand, the most alterations between *At*VDE and *Pt*VDE 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,

Table 1. Comparative analysis of four groups of amino acid residues of *At*VDE and *Pt*VDE important for de-epoxidase activity. Alterations are grey-lighted, *Ile-135 in *At*VDE is commonly replaced by Leu not only in *Pt*VDE but also in other VDEs working in the Vx cycle

Amino acid residues:							
Which are suggested to form active site of de-epoxidases		Potentially important in the enzyme activation by a decrease in pH		Putatively binding ascorbate		Postulated to be located closely to violaxanthin	
*At*VDE	*Pt*VDE	*At*VDE	*Pt*VDE	*At*VDE	*Pt*VDE	*At*VDE	*Pt*VDE
Gln_{153}	Gln	Asp_{98}	Phe	Thr_{112}	Leu	Phe_{123}	Phe
Tyr_{175}	Tyr	Asp_{114}	Asp	Asp_{114}	Asp	Asn_{134}	Lys
Asp_{177}	Asp	Asp_{117}	Pro	Gln_{119}	Gln	Phe_{155}	Phe
Trp_{179}	Trp	His_{121}	His	Tyr_{198}	Tyr	Asn_{167}	Asn
Tyr_{198}	Tyr	Ile_{135}*	Leu	Thr_{245}	Thr	Asp_{178}	Asp
Tyr_{214}	Tyr	Arg_{138}	Arg			Gln_{153}	Gln
		His_{168}	His			Tyr_{175}	Tyr
		Asp_{206}	Glu			Asp_{177}	Asp
		Tyr_{214}	Tyr			Trp_{179}	Trp
						Tyr_{198}	Tyr
						Tyr_{214}	Tyr
						His_{121}	His
						Ile_{135}*	Leu

Asp-117, Asp-206 and His-168, are different in *Pt*VDE (Table 1). The fourth alteration does not seem to be important, because Ile-135 in *At*VDE is commonly replaced by its isomer, Leu, not only in *Pt*VDE but also in other VDEs working in the Vx cycle. In turn, three remaining alterations may explain different range in pH values required by all known VDE 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 *Pt*VDE, which is described in this paper, may be consider 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 four 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-121and Tyr-214. The barrel is thought to be opened. *In silico* studies have also shown that disruption of hydrogen bond between His-121and 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 *Pt*VDE differ only in one position (Table 1). *In 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 *Pt*VDE.

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 *Pt*VDE gives a whole range of possibilities for detailed analysis of the individual amino acids' significance in various aspect of *Pt*VDE 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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