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Characterization of the monolignol oxidoreductase *At*BBE-like protein 15 L182V for biocatalytic applications

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ABSTRACT

Monolignol oxidoreductases from the berberine bridge enzyme-like (BBE-like) protein family (pfam 08031) catalyze the oxidation of monolignols to the corresponding aldehydes. In this report, we explore the potential of a monolignol oxidoreductase from *Arabidopsis thaliana* (*At*BBE-like protein 15) as biocatalyst for oxidative reactions. For this study we employed a variant with enhanced reactivity towards oxygen, which was obtained by a single amino acid exchange (L182V). The pH and temperature optima of the purified *At*BBE-like protein 15 L182V were determined as well as the tolerance toward organic co-solvents; furthermore the substrate scope was characterized. The enzyme has a temperature optimum of 50 °C and retains more than 50% activity between pH 5 and pH 10 within 5 min. The enzyme shows increased activity in the presence of various co-solvents (10–50% v/v), including acetonitrile, 2-propanol, 1,4-dioxane, and dimethyl sulfoxide. Primary benzylic and primary or secondary allylic alcohols were accepted as substrates. The enantioselectivity *E* in the oxidation of secondary alcohols was good to excellent (*E*>34 to >200).

1. Introduction

Flavoproteins are a diverse protein class employing either flavin mononucleotide (FMN) or the flavin adenine dinucleotide (FAD) for catalysis [1,2]. Among them, the BBE-likes (pfam 08031) can be distinguished due to their bicovalent cofactor tethering [3]. The namesake of this protein family is the berberine bridge enzyme (BBE) from California poppy (*Eschscholzia californica*), which catalyzes the oxidative ring closure from (*S*)-reticulin to (*S*)-scoulerine during isoquinoline alkaloid biosynthesis [4,5].

The majority of flavoproteins, including the BBE-likes, catalyze redox reactions, and they are involved in a plethora of biological processes [6]. The catalytically active moiety of the flavin cofactor is the isoalloxazine ring, whose properties are modulated by the protein environment in which it is embedded. The isoalloxazine ring is capable of one or two electron exchange reactions: In addition to the fully reduced (hydroquinone) or oxidized (quinone) state, also stable radical species (semiquinone) can be formed. A comprehensive overview of the function and mechanism of different flavoproteins has been provided by Fagan et al. [6].

The catalytic cycle of a flavoprotein can be divided into two half reactions: In the resting state the flavin cofactor is oxidized. In the reductive half reaction, the flavin is reduced by a given substrate. In the oxidative half reaction, the flavin reacts with an appropriate electron acceptor, thus it becomes reoxidized and is subsequently able to enter another catalytic cycle. The nature of the final electron acceptor is a crucial attribute of flavoproteins. Enzymes that promote the reaction of the flavin cofactor with oxygen are considered to function as oxidases, while enzymes that inhibit the reaction of the reduced flavin with oxygen are defined as dehydrogenases. For BBE-likes it has been shown that a single gatekeeper residue controls the oxygen reactivity of the enzyme [7] (compare Fig. 1 panel A). The rationale behind the engineering of the *At*BBE-like 15 L182V variant is described in [7], creation of the *At*BBE-like variant is published in [8]. We have recently identified two BBE-likes from *Arabidopsis thaliana* as monolignol oxidoreductases (*At*BBE-like protein 13 and *At*BBE-like protein 15) [8]. These enzymes were heterologously expressed in *Komagataella phaffii* (formerly *Pichia*

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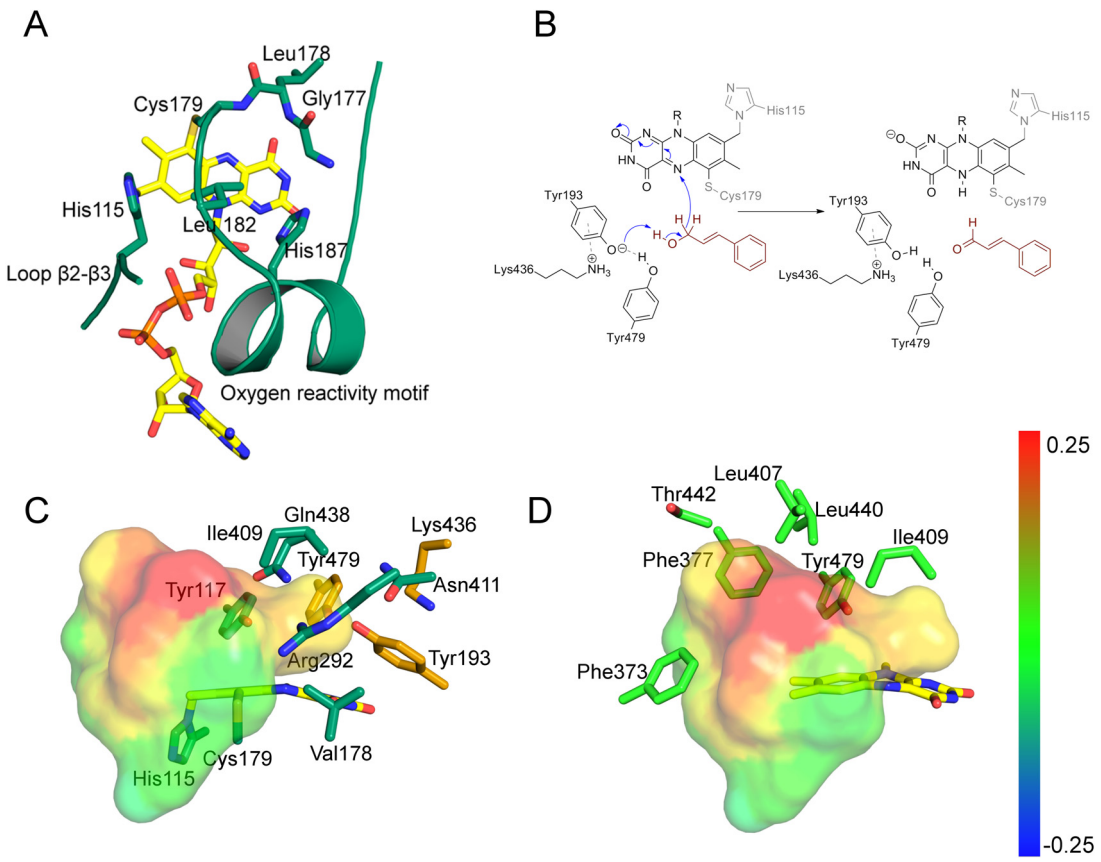

Fig. 1. A: Oxygen reactivity motif of *At*BBE-like protein 15; gate keeper residue Leu182 was changed to valine to turn the enzyme from a dehydrogenase to an oxidase; B: Proposed reaction mechanism; C: Active site and substrate binding pocket colored by hydrophobicity (red: hydrophobic, blue: hydrophilic). Green residues determine the shape of the active site, orange residues also act as a catalytic base. D: Substrate binding pocket; the green residues are responsible for the hydrophobic properties of the substrate binding pocket.

pastoris), and a biochemical and structural characterization has been conducted in our laboratory. Both enzymes were found to oxidize monolignols (*p*-coumaryl **1a**, coniferyl **1b** and sinapyl alcohol **1c**) to the corresponding aldehydes while reacting only sluggishly with molecular oxygen and were hence considered as monolignol dehydrogenases. The physiological electron acceptor for *At*BBE-like proteins 15 and 13 is not known yet; therefore, the sluggish oxidative rate hampers the potential application of *At*BBE-likes as biocatalysts as the turnover rate is determined by the slowest reaction rate. This means that it is virtually impossible to achieve significant turnover rates with a dehydrogenase in the absence of an appropriate electron acceptor. Moreover, while *At*BBE-like 15 was found to be stable and expressed with good yields, expression of *At*BBE-like 13 was very cumbersome and the enzyme was found to be unstable. To overcome these limitations, the oxygen gatekeeper residue in *At*BBE-like protein 15 was changed from leucine to valine to turn the enzyme into an oxidase. The resulting enzyme variant *At*BBE-like protein 15 L182V reacts more than 400 times faster with oxygen compared to the wild type enzyme [8]. This enables the usage of this enzyme for steady state kinetics and the putative application of the enzyme as biocatalyst in oxidative reactions. The structure of the oxygen reactivity motif harboring the variation L182V is shown in Fig. 1A, the proposed reaction mechanism of *At*BBE-like protein 15 is represented in panel B. A representation of an imprint of the active site and the substrate binding site is shown in panel C and D. Figure one is created using the crystal structure of the wild type enzyme (pdb entry: 4UD8).

The structure of the enzyme can be divided in a FAD-binding domain and a substrate binding domain [8]. The oxygen reactivity motif is located in the FAD-binding domain and creates an oxygen pocket that is analogous to the oxyanion hole of serine proteases. In this pocket oxygen is complexed by two backbone amides (compare Fig. 1 panel A: Leu178 and Cys179). From this position the oxygen reacts with the reduced flavin to form a hydroperoxy adduct at the C4a position that will subsequently eliminate hydrogen peroxide to yield oxidized flavin. The accessibility of this pocket is sterically controlled by a single gate keeper residue corresponding to Leu182 in *At*BBE-like 15 (compare Fig. 1, panel A). In order to promote the reaction of the reduced flavin with oxygen oxidases feature a valine in this position, which will allow oxygen to bind in the pocket. In contrast, dehydrogenases sterically block access to the pocket with a larger amino acid side chain and thus prevent the reoxidation of the reduced flavin cofactor [7,9]. The isoalloxazine ring and the active site are located at the interface of both domains. The active site forming residues are shown in Fig. 1C. Tyr 193 is proposed to act as catalytic base. It is stabilized in the deprotonated form by Tyr479 and is positioned by Lys436 via a cation-π interaction (compare Fig. 1B). Tyr193 deprotonates the allylic alcohol to facilitate the hydride transfer to the flavin. The enzyme possesses a wide surface accessible cavity harboring the substrate binding site and the active site [8]. This funnel-like cavity was visualized and analyzed using the CASoX tool (Fig. 1C and D) [10,11]. Residues with a major contribution to the binding site are shown in green in Fig. 1D, also the flavin cofactor shown in yellow contributes to the binding site. In the vicinity of the binding site is the active site. In Fig. 1C in dark green the residues that contribute to the active site are shown. In dark green Tyr117, Gln438, Ile409, Arg292, Val178 and Cys179 are contracting the cavity and thereby form the entrance to the active site. The catalytic base motif (orange residues Fig. 1 panel C), formed by Tyr193, Lys436 and Tyr479, is located opposite to

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this entrance [8]. Asn411 also contributes to the properties of the active site. On the one hand it is involved in the complexation of a water molecule together with Gln438, on the other hand it is a putative binding partner to anchor the monolignols prior to oxidation. His115 and Cys179 are responsible for the bicovalent attachment of the FAD, which confers a very tight steric control of the position of the cofactor to the enzyme (Fig. 1C) [3].

The application of flavin-containing enzymes as oxidative catalysts is highly desirable as they combine the advantage of using molecular oxygen as the most environmentally benign oxidant with the substrate-, regio-, and enantioselectivity of an enzyme [12]. Therefore we initiated a feasibility study to determine the potential of *At*BBE-like protein 15 L182V as a biocatalyst. In particular, the substrate scope, the pH and temperature optima, solvent tolerance, and enantioselectivity were in the focus of our study.

2. Experimental

All chemicals were from Sigma Aldrich or Acros Organics, respectively and were used as received. All solvents were from Roth. NMR spectra were recorded with a Bruker NMR unit at 300 (^{1}H) and 75 (^{13}C) MHz, shifts are given in ppm and coupling constants (J) are given in Hz. The *At*BBE-like L182 V was expressed and purified as described previously [8]; one Unit (U) is defined as the amount of enzyme that forms one μmol substrate per minute. The molecular mass of the catalyst is 60 kDa. Catalase from *Micrococcus lysodeikticus* (CAS 9001-05-2) was from Sigma Aldrich.

All GC–MS measurements were carried out with an Agilent 7890A GC system, equipped with an Agilent 5975C mass-selective detector (electron impact, 70 eV), Helium was used as carrier gas at a flow rate of 0.55 mL/min. HPLC analysis was performed with a Shimadzu HPLC system using the chiral stationary phase as indicated. Optical rotation values were measured with a Perkin Elmer Polarimeter 341.

Analytical thin layer chromatography (TLC) was carried out on Merck TLC silica gel 60 F254 aluminium sheets and spots were visualized by UV light (λ = 254 and/or 366 nm) and/or by staining with potassium permanganate (0.3 g $KMnO_4$, 20 g K_2CO_3, 5 mL 5 % aqueous NaOH in 300 mL H_2O) which were ultimately heated for development of the stains. For preparative product purification a 50 to 100 fold excess of silica gel was used with respect to the amount of dry crude product, depending on the separation problem. The dimensions of the column were selected in such a way that the required amount of silica gel formed a pad between 10 cm and 25 cm. The column was equilibrated first with the solvent or solvent mixture, and the crude product diluted with the eluent was applied onto the top of the silica pad.

2.1. Influence of pH

2.1.1. Activity of At*BBE-like 15 L182V at different pH values*

The pH optimum was determined with an optical oxygen meter FireSting O2 (Pyro Science GmbH, Aachen, Germany) equipped with a retractable needle-type oxygen sensor (Pyro Science GmbH, Aachen, Germany). The reaction was performed in a measuring cell with an integrated magnetic stirrer in triplicate at 25 °C and 500 rpm. 575 μL 50 mM MES buffer pH 7 containing 1.05 μM *At*BBE-like 15 L182 V was stirred until a stable oxygen level was reached. The reaction was started by the addition of 25 μL sinapyl alcohol (**1c**) solution to reach a final substrate concentration of 0.2 mM. The reaction was recorded for at least 5 min. After the initial consumption of 30 μM of oxygen data was not taken in account any more. The consumption of oxygen reflects the conversion of the substrate during the reaction.

2.1.2. Melting point of At*BBE-like protein 15 at different pH values*

Thermofluor experiments were performed using a Biorad® CFX Connect Real time PCR system (BioRad, Hercules, CA, USA). The experiments were performed using Sypro® Orange as fluorescent dye in 50 mM MES buffer pH 7.0. The total volume in each well was 25 μL with a protein concentration of 0.4 mg/mL. The starting temperature of 20 °C was kept for 5 min and then the temperature was increased at a rate of 0.5 °C/min to 95 °C. Melting temperatures were determined using the program Biorad CFX Manager 3.0.

2.2. Influence of temperature

2.2.1. Temperature optimum

The temperature optimum of *At*BBE-like 15 L182V was determined spectrophotometrically by measuring the formation of coniferyl aldehyde (**2b**) from coniferyl alcohol (**1b**). The reaction was performed in 1.5 mL plastic reaction tubes in a thermomixer (Eppendorf, Hamburg, Germany) in a temperature range of 20 °C to 70 °C. 500 μL 50 mM MES buffer pH 7 containing 0.2 μM *At*BBE-like 15 L182V was preheated for 10 min at 500 rpm. The reaction was started by the addition of 100 μL coniferyl alcohol (**1b**) solution to reach a final concentration of 3.33 mM. The reaction was followed over 10 min, whereby every 60 s a sample was taken. To this end, 20 μL of the reaction solution was mixed with 10 μL 1 M TRIS-buffer pH 11. 2 μL of this solution was immediately used to determine the product formation with a NanoDrop 2000 spectrometer (Thermo Fisher Scientific, Waltham, USA). The coniferyl aldehyde (**2b**) concentration was measured at 402 nm, the extinction coefficient of 37120 M^{-1} cm^{-1} was determined with an authentic standard under the given conditions.

2.2.2. Long-term stability of At*BBE-like 15 L182 V at different temperatures*

The enzyme was diluted in 50 mM TRIS/HCl buffer pH 8.0 to a final concentration of 0.125 μM. 950 μL was incubated in plastic reaction tubes in a thermomixer (Eppendorf, Hamburg, Germany) at 20 °C, 30 °C, 40 °C, 50 °C, 60 °C, and 70 °C. After 24 h the solutions were cooled to 20 °C and residual enzyme activity was determined employing a Specord 200 Plus spectrophotometer (Analytik Jena, Jena, Germany). The enzyme solution was transferred to a Hellma SUPRASIL® cuvette (Hellma GmbH & Co. KG, Müllheim, Germany) and the reaction was started by the addition of coniferyl alcohol (**1b**) to a final concentration of 0.5 mM. Product formation was followed for 5 min at 20 °C, the extinction coefficient of coniferyl aldehyde (**2b**) under reaction conditions of 21000 cm^{-1} M^{-1} was determined by an authentic standards. The experiments were performed with and without the addition of glucose to a final concentration of 5 mM.

2.3. Influence of co-solvents

2.3.1. Activity of At*BBE-like 15 L182V in the presence of 10% co-solvents*

The measurements were conducted as described in Section 2.2.1 in the presence of 10% (v/v) of the respective co-solvents. Methanol, ethanol, 2-propanol, 1-butanol, acetone, acetonitrile, tetrahydrofuran (THF), *N,N*-dimethylformamide (DMF), dimethyl sulfoxide (DMSO) and 1,4-dioxane were employed. The activity determined in pure buffer was set to 100%. The presence of co-solvents was not found to influence the extinction coefficient of the coniferyl aldehyde.

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Table 1
GS-MS analytics: Retention times employing a non-chiral stationary phase.

Substance	Method	Retention time starting material [min]	Retention time product [min]	Retention time aldehyde (authentic standard) [min]
Cinnamyl alcohol (**1d**)	M2	13.4	12.9	12.9
Benzyl alcohol (**1g**)	M1	3.4	7.55	8.0
Piperonyl alcohol (**1h**)	M2	14.4	13.8	13.9
trans-2-Hexen-1-ol (**1j**)	M2	6.3	6.0	6.0
cis-2-Hexen-1-ol (**1k**)	M2	6.3	6.0	
Crotyl alcohol (**1i**)	M3	3.2	3.0	3.0
3-Phenyl-2-propyn-1-ol (**1l**)	M1	6.2	5.1	5.1
(*Z*)-2-Fluoro-3-phenyl-prop-2-en-1-ol (**1e**)	M1	6.2	5.7	
1-Phenyletanol (**1p**)	M1	3.6	3.6	
3-Phenyl-1-propanol (**1q**)	M1	12.3	12.3	11.3
Geranylamine (**3a**)	M2	12.3	12.3	12.8

2.3.2. Activity in the presence of 10–50% (v/v) of organic co-solvents

The measurements were conducted as described in Section 2.3.1 in the presence of 10–50% (v/v) co-solvent. Only these solvents were investigated which were found to increase the activity of the enzyme in the experiments described in Section 2.2.1 (ethanol, 2-propanol, 1-butanol, acetonitrile, tetrahydrofuran, dimethyl sulfoxide, and 1,4-dioxane).

2.3.3. Long-term stability in the presence of 30% (v/v) of organic co-solvents

The measurements were conducted as described in section 2.3.1 in the presence of 30% co-solvents. The initial enzyme activity and the residual activity were determined after 24 h incubation at 25 °C and 500 rpm in a thermomixer (Eppendorf, Hamburg, Germany).

2.4. Substrate screening

Reactions were carried out in 50 mM MES buffer pH 7.0 in the presence of 20% (v/v) DMSO. A total volume of 2 mL reaction mixture was incubated in 11 mL glass test tube with screw cap (Pyrex, Darmstadt, Germany) at 30 °C in an orbital shaker at 110 rpm. The reaction was started by the addition of 10 μL enzyme to achieve a final enzyme concentration of 1.2 μM. After 24 h the reaction mixture was extracted with ethyl acetate (2 × 1 mL), the combined organic phase was dried with Na_2SO_4 and subjected to GC analysis to identify putative products.

2.4.1. GC–MS

GC–MS analyses were carried out on an Agilent 7890A GC system equipped with an Agilent J&W HP–5 ms capillary column (30 m × 0.25 mm × 0.25 μm; stationary phase: bonded & cross-linked 5%-phenyl-methylpolysiloxane) (Agilent Technologies, Santa Clara, USA) and coupled to an Agilent 5975C mass-selective detector (electron impact ionisation, 70 eV; quadrupole mass selection) using helium as carrier gas.

2.4.1.1. Method M1. Inlet temperature: 250 °C; split ratio: 90:1; injection volume: 1 μL; column flow rate: 0.7 mL/min; oven program: 100 °C for 0.5 min, 10 °C/min to 300 °C; MS transfer line temperature: 300 °C, MS source temperature: 230 °C, MS quadrupole temperature: 150 °C; MS scan range: m/z = 33–400.

2.4.1.2. Method M2. Inlet temperature: 250 °C; split ratio: 90:1; injection volume: 1 μL; column flow rate: 0.7 mL/min; oven program: 40 °C for 2 min, 10 °C/min to 180 °C, 180 °C for 1 min; MS transfer line temperature: 300 °C, MS source temperature: 230 °C, MS quadrupole temperature: 150 °C; MS scan range: m/z = 33–400.

2.4.1.3. Method M3. Inlet temperature: 250 °C; split ratio: 90:1; injection volume: 1 μL; column flow rate: 0.7 mL/min; oven program: 40 °C for 5 min, 5 °C/min to 100 °C, 25 °C/min to 200 °C, 200 °C for 1 min; MS transfer line temperature: 300 °C, MS source temperature: 230 °C, MS quadrupole temperature: 150 °C; MS scan range: m/z = 33–400.

The products were identified by their retention times determined with authentic standards, which are summarized in Table 1. If no standard was available the products were identified by the MS-spectrum.

2.4.2. Substrate-specific activity of At*BBE-like 15 L182V*

Substrate solutions (2 mM) were provided in MES buffer pH 7.00 in a measuring cell with a magnetic stirrer at 1000 rpm and 25 °C. The reaction was started by the addition of 25 μL enzyme solution. The substrates and the final enzyme concentrations are summarized in Table 2. The course of the reaction was followed by the oxygen consumption determined with an optical oxygen meter FireSting O2 (Pyro Science GmbH, Aachen, Germany) equipped a retractable needle type oxygen probe (OXR50-UHS, PyroScience, Germany). The probe was calibrated with the oxygen saturated buffer 50 mM MES pH 7,00 and a saturated Na_2SO_3-solution.

2.5. Stereo specificity

Kinetic resolutions were carried out with substrates **1l, 1 m 1n** and **1o** as described in Section 2.4. The products were identified by coinjection with authentic samples on GC–FID.

2.5.1. GC–FID (chiral stationary phase)

GC–FID analyses for the optical purity of 3-penten-2-ol (**1n**), 3-octen-2-ol (**1m**), and cyclohex-2-en-1-ol (**1o**) were carried out on an Agilent 7890A GC system equipped with a Varian CP-Chirasil Dex-CB capillary column (25 m × 0.32 mm × 0.25 μm; stationary phase: β-cyclodextrin bonded to dimethylpolysiloxane) (Agilent Technologies, Santa Clara, USA) using hydrogen as carrier gas.

Method **M5** (used for **1n**): Inlet temperature: 220 °C; split ratio: 90:1; injection volume: 1 μL; column flow rate: 1 mL/min; oven program: 60 °C for 1 min, 20 °C/min to 70 °C, 70 °C for 5 min, 20 °C/min to 180 °C, 180 °C for 1 min; detector temperature: 250 °C.

Method **M6** (used for **1o** and **1m**): Inlet temperature: 220 °C; split ratio: 90:1; injection volume: 1 μL; column flow rate: 1 mL/min; oven program: 60 °C for 1 min, 20 °C/min to 95 °C, 95 °C for 9 min, 20 °C/min to 180 °C, 180 °C for 1 min; detector temperature: 250 °C.

4-Phenyl-3-buten-2-ol (**1l**) was converted into the corresponding acetate derivative prior to chiral-phase GC analysis by reaction with acetic anhydride (20 μL/mL sample) and

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Table 2
Reaction mixtures.

Substrate	Enzyme concentration [μM]
Sinapyl alcohol (**1c**)	0.125
Coniferyl alcohol (**1b**)	0.125
p-Coumaryl alcohol (**1a**)	0.125
Cinnamyl alcohol (**1d**)	0.625
Piperonyl alcohol (**1h**)	2.5
4-Phenylbut-3-en-2-ol (**1l**)	2.5
Crotyl alcohol (**1i**)	2.5

Table 3
GC-FID analytics: Retention times employing a chiral stationary phase.

Substance	Method	Retention time alc. 1 [min]	Retention time alc. 2 [min]	Retention time ketone [min]
4-Phenylbut-3-en-2-ol (acetyl derivative) (**1l**)	M7	21.7	22.0	19.3
3-Octen-2-ol (**1m**)	M6	10.2	10.5	9.0
3-Penten-2-ol (**1n**)	M5	5.4	5.6	4.1
Cyclohex-2-en-1-ol (**1o**)	M6	9.8	10.2	7.6

4-dimethylaminopyridine (DMAP; 1 mg/mL sample) at room temperature for 3 h.

Chiral GC–FID analyses of the resulting 4-phenyl-3-buten-2-yl acetate (**1l** acetate) were carried out on an Agilent 7890A GC system equipped with a Restek Rt®-βDEXse capillary column (25 m × 0.32 mm × 0.25 μm; stationary phase: 2,3-di-*O*-ethyl-6-*O*-*tert*-butyldimethylsilyl-β-cyclodextrin added into 14%-cyanopropylphenyl/86%-dimethylpolysiloxane) using hydrogen as carrier gas.

Method **M7** (used for 1l): Inlet temperature: 220 °C; split ratio: 50:1; injection volume: 1 μL; column flow rate: 2 mL/min; oven program: 60 °C for 1 min, 5 °C/min to 180 °C; detector temperature: 250 °C.

The retention times of the observed in these measurements are summarized in Table 3.

2.5.2. HPLC–MS (chiral stationary phase)

HPLC analysis was performed with a Shimadzu HPLC system using equipped with a CHIRALCEL OD-H column (Chiral Technologies Inc., West Chester, United States). Heptane/2-PrOH = 90/10 was employed as mobile phase at flow rate of 0.7 mL/min and 25 °C. For the respective retention times were observed; t_{ret}[(*R*)-enantiomer] = 11.9 min, t_{ret}[(*S*)-enantiomer] = 17.6 min, and t_{ret}(ketone) = 10.5 min.

2.5.3. Scale up kinetic resolution

The substrate **1l** (100 mg, 0.68 mmol) was solubilized in DMSO (14 mL), mixed with MES buffer (50 mM, pH = 7.0, 56 mL) and incubated for 3 h at 30 °C in a shaker at 120 rpm. The oxidase *At*BBE-like 15 (350 μL, 140 μM) and the catalase from *Micrococcus lysodeikticus* (1050 μL, 170000 U/mL) were added and the reaction was incubated for 20 h at 30 °C and 120 rpm. The reaction mixture was extracted with diethyl ether (4 × 40 mL), the combined organic phase was dried over with Na_2SO_4, filtered and concentrated in vacuo. The crude product was purified by flash chromatography (hexane/diethyl ether 5:1). The fractions were collected and concentrated in vacuo to give compounds **1l** (white solid, 35 mg, 0.24 mmol, 35%) and **2l** (yellow oil, 41 mg, 0.28 mmol, 41%) with following physical properties:

(*R*)-**1l**: $[\alpha]_D^{20}$ = +14.7 (c = 1.00, MeOH); lit. :[1] $[\alpha]_D^{20}$ = −17.8 [c = 0.32, MeOH, (*S*)-enantiomer]; ^{1}H NMR (300 MHz, $CDCl_3$): 7.40-7.21 (m, 5H), 6.57 (d, *J* = 15.9, 1H), 6.26 (dd, J_1 = 15.9, J_2 = 6.4, 1H), 4.49 (dquint, J_1 = 6.4, J_2 = 1.0, 1H), 1.70 (bs, 1H), 1.37 (d, *J* = 6.4, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): 136.8, 133.7, 129.5, 128.7, 127.8, 126.6, 69.1, 23.5; MS (EI): *m*/*z*: 148 (54), 130 (47), 115 (66), 105 (100), 91 (60), 77 (45);

HPLC analysis on chiral stationary phase {Daicel Chiralcel OD-H, *n*-heptane/2-propanol 90/10, 0.7 mL/min, 25 °C, t_{ret}(enantiomer 1) = 11.5 min, t_{ret}(enantiomer 2) = 16.8 min}: t_{ret}(major isomer) = 11.5 min, ee = 94%; the physical data is in consistency with literature [12].

2l: ^{1}H NMR (300 MHz, $CDCl_3$): 7.56-7.49 (m, 3H), 7.42-7.39 (m, 3H), 6.72 (d, *J* = 16.3, 1H), 2.39 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): 198.4, 143.4, 134.4, 130.5, 129.0, 128.3, 127.2, 27.6; MS (EI): *m*/*z*: 146 (60), 145 (67), 131 (71), 103 (100), 77 (50); the physical data is in consistency with a commercial sample.

2.6. Synthesis of (Z)-3-fluoro-3-phenyl-prop-2-en-1-ol (1e)

2.6.1. Synthesis of ethyl (Z)-3-fluoro-3-phenylacrylate

The synthesis of ethyl (*Z*)-3-fluoro-3-phenylacrylate was performed according to the work of Li et al. [14]. In a 5 mL round bottom flask AgF (140 mg, 1.10 mmol, 1.9 eq.) was added to a solution of 2 mL acetonitrile and 0.1 mL H_2O. Ethyl 3-phenylpropiolate (94.5 μL, 0.57 mmol, 1.0 eq.) was then added to the brownish suspension and the reaction was heated to 90 °C for 22 h. The reaction mixture was then cooled to room temperature and the solvents were removed under reduced pressure. The crude product was taken up in 10 mL H_2O and washed with Et_2O (3 × 10 mL). The combined organic layers were dried over Na_2SO_4, filtered and concentrated under reduced pressure to give the crude product which was purified via silica column chromatography with cyclohexane/ethyl acetate 50:1 to 20:1 as eluent. The yield was 133 mg (0.685 mmol, 60%) colorless oil (R_f = 0.24, cyclohexane/ethyl acetate 18:1).

^{1}H NMR (300 MHz, $CDCl_3$): δ 7.65 (d, $^3J_{H,H}$ = 6.7 Hz, 2H, H4 + H6), 7.52–7.37 (m, 3H, H1-3), 5.90 (d, $^3J_{H,F}$ = 33.3 Hz, 1H, H8), 4.26 (q, $^3J_{H,H}$ = 7.1 Hz, 2H, H10), 1.33 (t, $^3J_{H,H}$ = 7.1 Hz, 3H, H11).

^{13}C NMR (76 MHz, $CDCl_3$): δ 166.40 (C_q, d, $J_{C,F}$ = 277.6 Hz, C7), 164.18 (C_q, d, $J_{C,F}$ = 2.2 Hz, C9), 131.60 (CH, C1 + C3), 130.81 (C_q, d, $J_{C,F}$ = 26.2 Hz, C5), 128.98 (CH, d, $J_{C,F}$ = 1.9 Hz, C4 + C6), 125.78 (CH, d, $J_{C,F}$ = 7.9 Hz, C2), 97.35 (CH, d, $J_{C,F}$ = 6.9 Hz, C8), 60.55 (CH_2, C10), 14.41 (CH_3, C11).

2.6.2. Reduction of ethyl (Z)-3-fluoro-3-phenylacrylate to (Z)-3-fluoro-3-phenylprop-2-en-1-ol (**1e**)

In a 10 mL Schlenk-tube ethyl (*Z*)-3-fluoro-3-phenylacrylate (100 mg, 0.515 mmol, 1.0 eq.) was dissolved in 3 mL CH_2Cl_2 and cooled to −78 °C via an acetone/dry ice bath. 1.2 mL (1.192 mmol, 2.3 eq.) DIBAL-H (1.0 M in toluene) were slowly added to the colorless solution at −78 °C. The reaction mixture was then warmed to 0 °C over a period of 2 h. Subsequently, the reaction was transferred into an 80 mL Schlenk-tube, diluted with CH_2Cl_2 (15 mL) and quenched by the addition of H_2O (1 mL). Rochelle-salt (sat.) (20 mL) was added and the two phases were stirred vigorously for 19 h. The aqueous phase was then washed with CH_2Cl_2 (3 × 20 mL) and the combined organic layers were washed with 1 M HCl (1 × 50 mL) and brine (1 × 50 mL), dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was purified via column chromatography with cyclohexane/ethyl acetate 5:1 as eluent. The yield was 71 mg (0.466 mmol, 91%) colorless oil (R_f = 0.18, cyclohexane/ethyl acetate 5:1).

^{1}H NMR (300 MHz, $CDCl_3$): δ 7.58–7.30 (m, 5H, H1-6), 5.66 (dt, $^3J_{H,H,F}$ = 36.6, 7.1 Hz, 1H, H8), 4.45 (dd, $^3J_{H,H,F}$ = 7.1, 1.9 Hz, 1H, H9), 1.82 (s, 1H, OH).

^{13}C NMR (76 MHz, $CDCl_3$): δ 158.22 (C_q, d, $J_{C,F}$ = 251.2 Hz, C7), 131.87 (C_q, d, $J_{C,F}$ = 28.6 Hz, C5), 129.44 (CH, C1 + C3), 128.66 (CH, d,

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$J_{C,F}$ = 2.0 Hz, C4 + C6), 124.55 (CH, d, $J_{C,F}$ = 7.2 Hz, C2), 104.92 (CH, d, $J_{C,F}$ = 15.3 Hz, C8), 56.23 (CH_2, d, $J_{C,F}$ = 7.7 Hz, C9).

3. Results and discussion

*At*BBE-like protein 15 L182V was found to oxidize allylic and benzylic alcohols to the corresponding aldehydes. In Scheme 1 the reaction equation and the structure of substances that were used for enzymatic assays are shown.

3.1. pH dependency of the activity of AtBBE-like protein 15 L182V

The pH profile of the enzyme was investigated in a range of 4 to pH 10 (Fig. 2a). Thermofluor experiments were conducted to determine the melting point on the enzyme in a pH-range from 5 to 10 (Fig. 2b).

The enzyme is active over a broad pH range, with highest activity at pH 7, and between pH 5 and pH 10 the enzyme retains more than 50% activity (5 min). The highest melting point of the enzyme was found to be in HEPES buffer pH 7.0 (60 °C).

3.2. Temperature dependent performance of AtBBE-like 15 L182V

The initial enzyme activity was measured in a temperature range of 20 to 70 °C (Fig. 3A). The residual activity of the enzyme was determined after 24 h incubation at temperatures ranging from 20 °C to 70 °C in the presence and absence of glucose (Fig. 3B).

The highest activity was found at 50 °C. The findings are in good agreement with the melting point of the wild type enzyme of 60 °C.

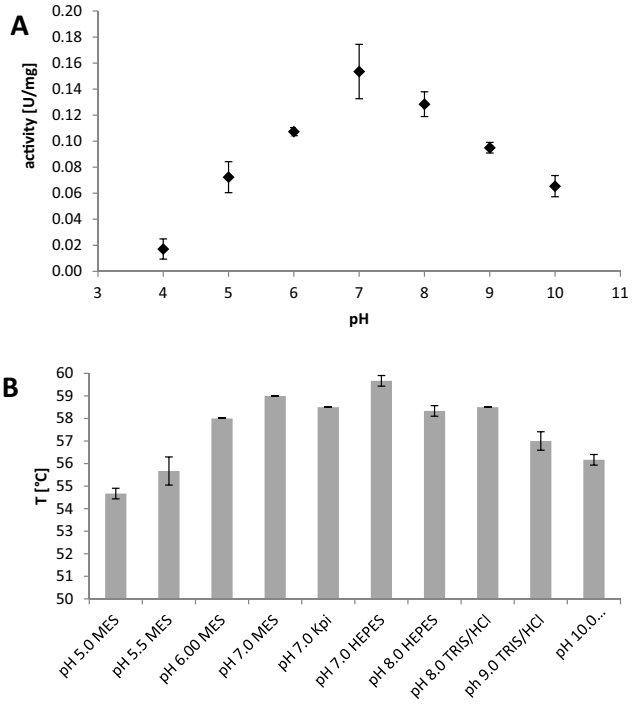

Fig. 2. A: pH optimum of *At*BBE-like 15 L182V. Plotted is the observed consumption of μmol oxygen per mg enzyme per minute. B: Melting point of *At*BBE-like protein 15 at different pH values.

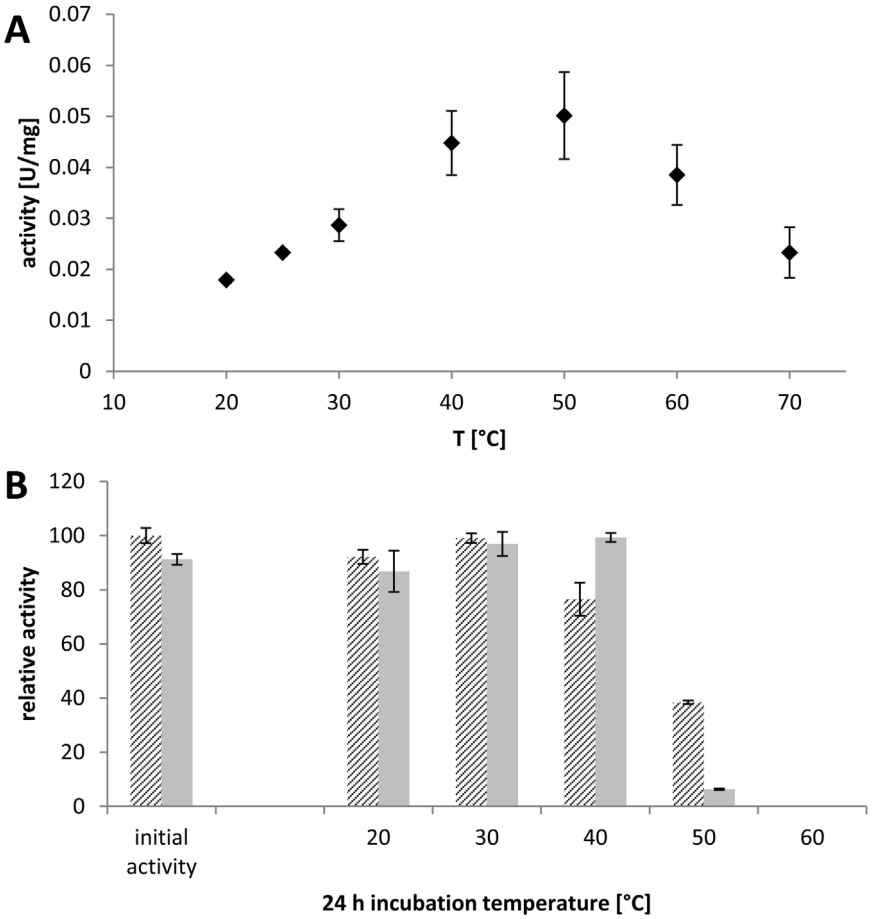

Fig. 3. A: Temperature optimum of *At*BBE-like 15 L182V. B: Residual enzyme activity after incubation at different temperatures for 24 h in the presence (grey bars) and absence (striped bars) of 5 mM glucose. The initial activity determined prior incubation without glucose was set to 100%.

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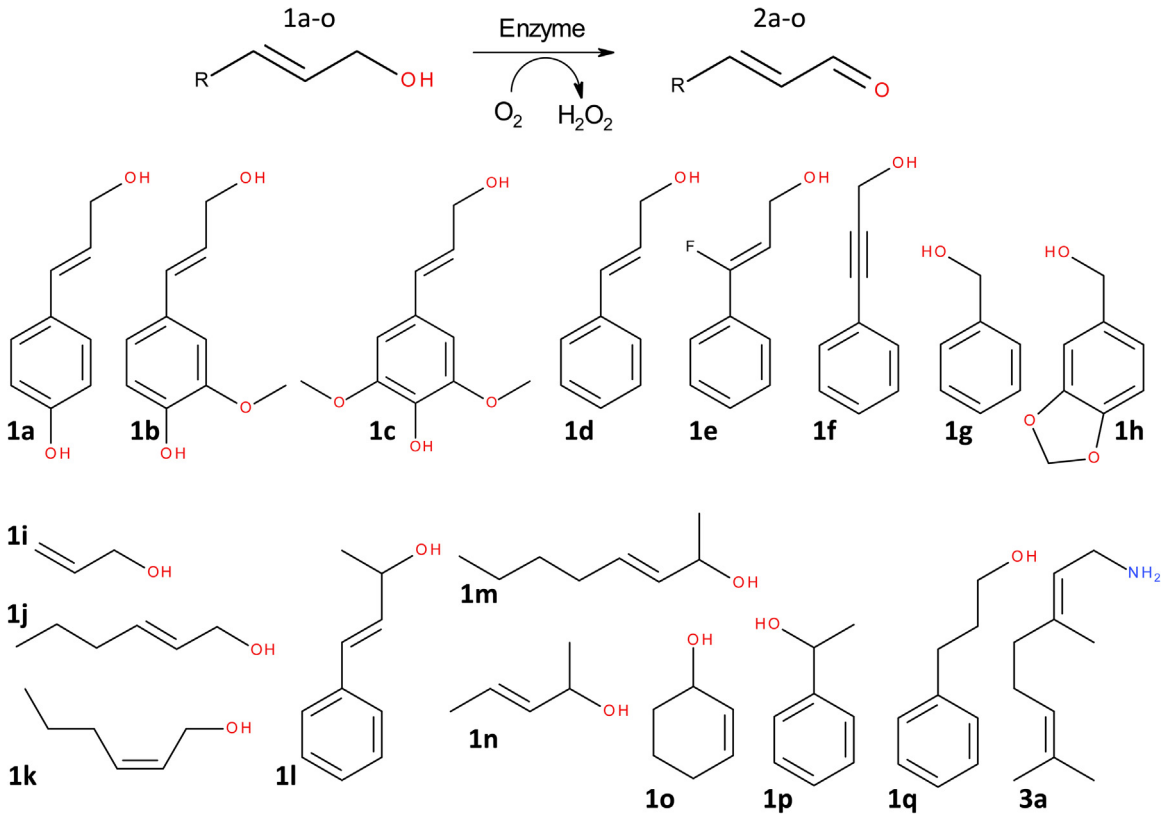

Scheme 1. Reaction equation and substrates used for enzymatic assays.

No loss of activity after 24 h can be observed at 30 °C. At 40 and 50 °C a residual activity of 77% and 38% are found, respectively. Glucose was shown to increase the melting temperature of the enzyme by 15 °C; in the long term experiment, a stabilizing effect of glucose at 40 °C can be observed, while at 50 °C the residual enzyme activity is decreased by glucose [8].

3.3. Activity in the presence of 10% (v/v) of organic co-solvents

While an aqueous system is the natural environment of enzymes the solubility of nonpolar substrates is limited under purely aqueous conditions. This limitation can be overcome by the addition of co-solvents. A high tolerance of the enzyme towards organic solvents is also required if a thermomorphic solvent system is supposed to be applied for enzyme recycling [15]. As oxygen is the final electron acceptor of *At*BBE-like protein 15 L182V, also the oxygen solubility is an important factor for the overall performance of the catalyst. The addition of co-solvent is not only beneficial for substrate solubility but can also enhance the oxygen solubility [16]. To determine the effect of organic solvents on the enzyme stability, measurements in the presence of 10% (v/v) of various organic solvents were conducted. The course of the reaction was followed spectrophotometrically. The enzyme activity in buffer was set to 100%. In Fig. 4 the activities found in the presence of organic solvents are shown.

An increased activity of the enzyme was found in acetonitrile, 2-propanol, 1,4-dioxane, tetrahydrofuran, 1-butanol, dimethyl sulfoxide and ethanol. The highest degree of activation was found for ethanol with 188% of the activity observed in buffer. A decreased activity was determined in the presence of methanol, dimethylformamide and acetone, with the lowest activity found with of methanol (29%).

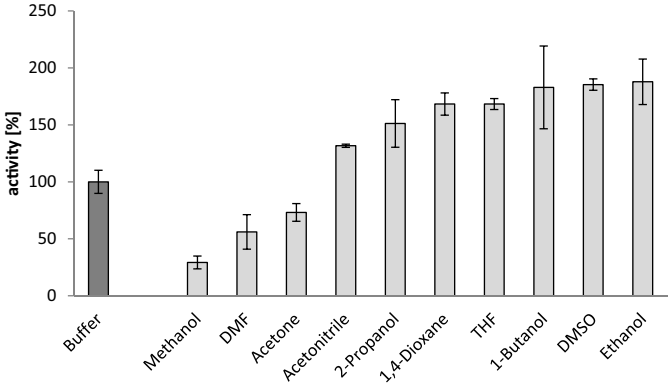

Fig. 4. Activity of *At*BBE-like protein 15 L182V with 10% (v/v) of organic co-solvents.

3.4. Activity in the presence of 10–50% (v/v) of organic co-solvents

The high solvent tolerance of the enzyme encouraged us to further increase the co-solvent concentration to probe the enzyme stability. Experiments were conducted with all solvents that were found to increase the enzyme activity. The enzyme activity was determined with acetonitrile, 2-propanol, 1,4-dioxane, tetrahydrofuran, 1-butanol, dimethyl sulfoxide, and ethanol at concentrations of 10%, 20%, 30%, 40% and 50% (v/v). The measured activities are shown in Fig. 5.

An increasing solvent concentration led to an increased enzyme activity in all cases except for tetrahydrofuran, which was not tolerated at higher concentrations. The highest acitivity was found in 40% ethanol with 346% of the activity measured in buffer. The enzyme was shown not only to tolerate high solvent concentrations, also the activity can be enhanced. Still, high solvent concentrations can lead to the denaturation and thereby deacti-

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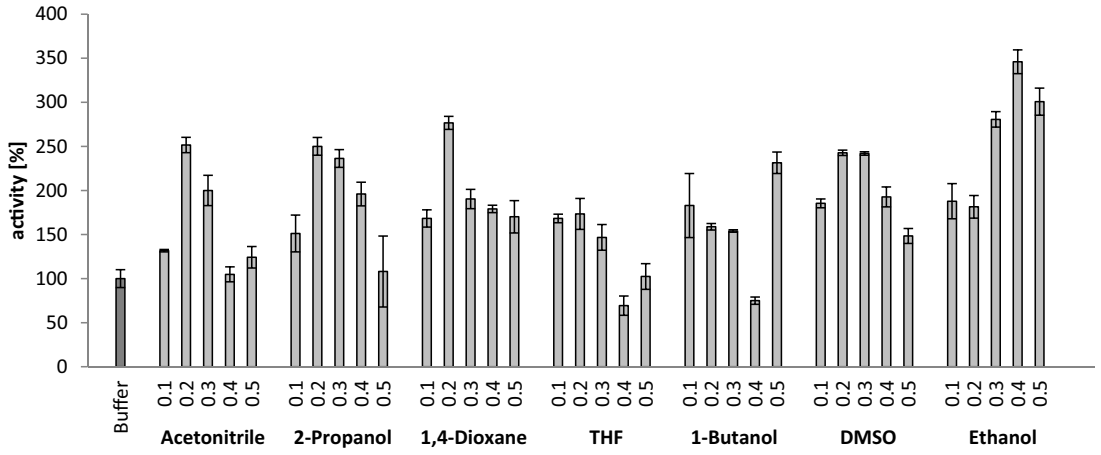

Fig. 5. Effect on the enzyme activity of different solvents at concentrations between 10% and 50% (v/v).

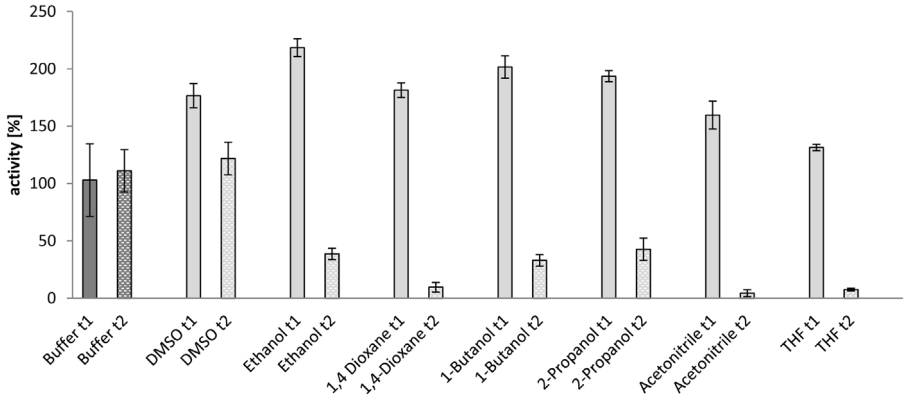

Fig. 6. Long-term activity of *At*BBE-like protein 15 L182V with 30% (v/v) of organic co-solvents with t1 = 0 h, t2 = 24 h.

vation of the enzyme. A good long-term stability of the enzyme under the given reaction conditions is essential to achieve a high space/time yield.

3.5. Long-term stability in the presence of 30% (v/v) of organic co-solvents

The presence of various organic solvents was found to enhance the activity of the enzyme. To test the long-term stability of the enzyme, it was incubated with 30% (v/v) of the respective solvents for 24 h (t2) before the reaction was started. The activity in buffer was set to 100%, the starting and residual activities are depicted in Fig 6.

While no deactivation of the enzyme in buffer was detectable, the presence of organic solvents led to enhanced activity but also a decreased life-time of the enzyme. The highest activity after 24 h incubation was determined in 30% DMSO. The enzyme activity in DMSO was 177% initially and dropped to 122% within 24 h. Also for other solvents a lower but still acceptable retention of the initial activity was determined. The initial activity dropped in ethanol from 218% to 38%, in 2-propanol from 194% to 47% and in 1-butanol from 201% to 33%. Especially the long-term stability in 2-propanol creates interesting options with regard to coupled reactions with NADH dependent alcohol dehydrogenases [17]. 2-Propanol is frequently used to regenerate NADH from NAD^+. In a one-pot process, *At*BBE-like 15 L182V could be employed for kinetic resolutions of secondary allylic alcohols, while an alcohol dehydrogenase with NADH regeneration by 2-propanol could be employed to reduce the allylic ketone formed *in situ* to reach a theoretical yield of 100% of the desired enantiopure alcohol.

Table 4
Substrate conversion determined by GC.

Substance	Conversion [%]
Cinnamyl alcohol (**1d**)	97
(*Z*)-3-Fluoro-3-phenyl-prop-2-en-1-ol (**1e**)	>99
3-Phenyl-2-propyn-1-ol (**1f**)	19
Benzyl alcohol (**1g**)	>99
Piperonyl alcohol (**1h**)	49
Crotyl alcohol (**1i**)	15
trans-2-Hexen-1-ol (**1j**)	47
cis-2-Hexen-1-ol (**1k**)	28
1-Phenyl-ethanol (**1p**)	n.d.
3-Phenyl-propanol (**1q**)	n.d.
Geranylamine (**3a**)	n.d.

3.6. Substrate screening

In order to elucidate the substrate scope of *At*BBE-like 15 L182V, the enzyme was screened for activity with various alcohols. Experiments were conducted in HEPES-buffer pH 7.0 containing 20% DMSO with a substrate concentration of 10 mM.

The substances **1a**-**o** were converted to the corresponding aldehydes, as confirmed by GC–MS analysis. The formation of carboxylic acids, which has been reported as a follow-up reaction in the oxidation of primary alcohols by other flavin-dependent oxidases, has never been observed in our experiments. The conversions observed by GC–MS are summarized in Table 4.

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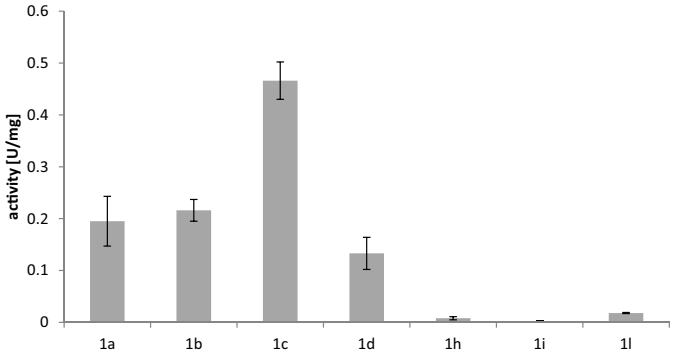

Fig. 7. Specific activity of *At*BBE-like protein 15 L182V with p-coumaryl alcohol (**1a**), coniferyl alcohol (**1b**), sinapyl alcohol (**1c**), cinnamyl alcohol (1d), piperonly alcohol (**1h**), crotyl alcohohl (**1i**) and 4-Phenylbut-3-en-2-ol (**1l**).

Table 5
Kinetic resolutions with *At*BBE-like protein 15 L182V.

Substance	Ketone [%]	Sum alcohols [%]	*e.e.* [%]	*E*
4-Phenylbut-3-en-2-ol (acetyl derivative) (**1l**)	58	42	>99 (*R*)	>34
3-Octen-2-ol (**1m**)	55	45	>99 (*R*)	>47
3-Penten-2-ol (**1n**)	12	88	20 n.d.	>200
Cyclohex-2-en-1-ol (**1o**)	35	65	53 (*R*)	>200

All allylic and primary benzylic alcohols that were tested were accepted as substrate. No conversion was detected (n.d.) for aliphatic and secondary benzylic alcohols (**1p-q**) as well as genanylamine **3a**.

3.6.1. *Substrate specific activity of* At*BBE-like 15 L182V*

To evaluate the activity toward the different substance categories that were found to be converted, the specific activity of the enzyme was assayed employing cinnamyl alcohol derivatives (**1a-d**, **1l**), a benzylic alcohol (piperinyl alcohol **1 h**), and an allyl alcohol (crotyl alcohol **1i**). The determined activities are summarized in Fig. 7.

The activities displayed in Fig. 7 are in good agreement with the conversions summarized in Table 4. The highest activity is found employing cinnamyl alcohol derivatives, ranging from 0.47 U/mg for sinapyl alcohol (**1c**) to 0.13 U/mg for cinnamyl alcohol. The activity towards a benzylic alcohol (piperonyl alcohol **1 h**, 0.008 U/mg) and towards crotyl alchohol (**1i**, 0.002 U/mg) is one and two orders of magnitude lower, respectively.

3.7. *Kinetic resolutions*

As secondary allylic alcohols were found to be converted by the enzyme, this substance class was chosen for testing the enantioselectivity of the enzyme. Kinetic resolutions were conducted starting from racemic alcohols. Enantiomeric excess (*e.e.*) of the remaining substrate as well as conversion were determined by GC analysis on chiral phases, and the enantioselectivity (*E*) was calculated according to Rakels et al. [18]. The results are summarized in Table 5.

The absolute configuration of the remaining alcohols **1l**, **1 m** and **1o** was determined according to [13], [19] and [20], respectively. The results indicate that the enzyme shows a high enantioselectivity in the oxidation of secondary allylic alcohols. While the (*S*)-enantiomer is converted, the (*R*)-enantiomer remains untouched. This is in good agreement with a productive docking mode we have published previously, in that the pro-S proton of the allylic alcohol was found to be oriented towards the N5 position of the flavin, while the pro-R proton was not [8].

4. Conclusions

In the present study we have probed the applicability of a monolignol oxidoreductase for biocatalytic transformations. We have employed the *At*BBE-like 15 L182V variant that was engineered previously toward higher oxygen reactivity. We have elucidated the pH- and temperature optima of the enzyme, the substrate scope and tolerance towards organic co-solvents. *At*BBE-like protein 15 L182V retained more than 50% of activity in a broad pH range between 5 and 10 for 5 min. The highest activity was reached at 50 °C, no deactivation of the enzyme was found after incubation at 30 °C for 24 h. The enzyme exhibits an enhanced activity in the presence of organic co-solvents. The most suitable co-solvent was found to be DMSO: in 30% DMSO the enzyme Exhibits 177% of the activity determined for an aqueous system; after 24 h at 20 °C 122% of enzyme activity is remained. Various allylic and benzylic alcohols were accepted as substrates and converted to the corresponding aldehydes. The enzyme preferably converts *S*-alcohols and was successfully applied in a kinetic resolution in preparative scale. Aliphatic alcohols were not converted by the enzyme. Therefore *At*BBE-like protein 15 L182V could be useful as a chemo- and enantioselective enzyme for future biocatalytic applications.

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