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Colpa, Dana I.; Fraaije, Marco W.

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High overexpression of dye decolorizing peroxidase *TfuDyP* leads to the incorporation of heme precursor protoporphyrin IX

Dana I. Colpa, Marco W. Fraaije*

Molecular Enzymology Group, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

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ABSTRACT

The heterologous overexpression level of the bacterial dye decolorizing peroxidase *TfuDyP* in *Escherichia coli* was increased sixty fold to approximately 200 mg of purified enzyme per liter culture broth by fusing the enzyme to the small ubiquitin-related modifier protein (SUMO). The highly overexpressed SUMO-*TfuDyP* was, however, almost inactive. Analysis of the enzyme by UV-vis absorption spectroscopy and high-resolution mass spectrometry showed that a large fraction of the highly overexpressed enzyme contained the iron deficient heme precursor protoporphyrin IX (PPIX) instead of heme. Here we show that the activity of the enzyme was dependent on the expression level of the protein.

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1. Introduction

Dye decolorizing peroxidases (DyPs) comprise a family of peroxidases which were discovered only two decades ago [1]. The physiological role of these enzymes is unknown, but they display a strikingly broad substrate scope including anthraquinone and azo-dyes [1–4], lignin(-model) compounds [5], β -carotene [6] and aromatic sulfides [7]. Their enigmatic physiological role and broad substrate scope make DyPs interesting targets for biochemical and biotechnological studies. One member of this family is already applied in the food industry to degrade β -carotene in whey-containing beverages [6]. Another application might be the bioremediation of waste water polluted with synthetic dyes. Many DyPs are active on various synthetic dyes, including *TfuDyP* from *Thermobifida fusca*, *BsDyP* from *Bacillus subtilis*, *PpDyP* from *Pseudomonas putida* MET94 and *AnaPX* from *Anabaena* sp. strain PCC 7120 [2–4]. Furthermore, DyPs might become valuable biocatalytic tools in the enzymatic degradation of lignin and lignocellulose containing biomass. It has been shown that the DyP from *Irpex lacteus* promotes the enzyme-mediated degradation of wheat straw and DyPB from *Rhodococcus jostii* RHA1 has been found to be active on lignin and lignin model compounds [5,8].

DyP-type peroxidases have a ferredoxin-like fold and rely on a non-covalently, but tightly, bound heme *b* cofactor for their activity [9]. Heme *b* is the most common heme cofactor, and is found in a wide variety of proteins, e.g. cytochromes P450, hemoglobin, and most peroxidases [10,11]. Heme cofactors are produced through a highly conserved heme synthesis pathway in which heme *b* is synthesized from δ -aminolevulinic acid (δ -ALA) [10]. In the last step ferrous iron is inserted in protoporphyrin IX (PPIX) to form heme *b*.

Our group characterized a thermostable member of the DyP-type peroxidase family, *TfuDyP*. *TfuDyP* from *Thermobifida fusca* is a dimeric, Tat-dependently exported DyP which degrades several types of dyes, oxidizes small aromatic compounds and performs enantioselective sulfoxidations [2,7]. We have recently shown that the enzyme can be exploited as biocatalyst for the production of valuable flavor compound divanillin, using vanillin as substrate. The crystal structure of *TfuDyP* was solved recently [12].

A high expression level in a heterologous host, like *Escherichia coli*, would make DyPs more attractive for industrial applications. In this work we boosted the heterologous overexpression of *TfuDyP* in *E. coli* through a fusion to SUMO (small ubiquitin-related modifier protein). The expression level of *TfuDyP* was efficiently raised from 3 mg *TfuDyP* [7] to approximately 200 mg SUMO-*TfuDyP* from 1 L culture. However, a large fraction of the highly overexpressed enzyme contained the iron deficient heme precursor protoporphyrin IX instead of heme.

Abbreviations: DyP, dye decolorizing peroxidase; δ -ALA, δ -aminolevulinic acid; PPIX, protoporphyrin IX; SUMO, small ubiquitin-related modifier protein; MBP, maltose binding protein.

* Corresponding author.

E-mail address: m.w.fraaije@rug.nl (M.W. Fraaije).

2. Materials and methods

2.1. Reagents and chemicals

Salts, media components, reagents and oligonucleotide primers were obtained from Merck, Fisher chemicals, Sigma-Aldrich and BD (USA). Reactive Blue 19 was from Acros Organics. The In-Fusion HD cloning kit was obtained from Clontech, Phusion DNA polymerase was from Thermo Scientific and the *Pfu*Ultra Hotstart PCR master mix was from Agilent Technologies. Restriction enzymes *Nde*I and *Hind*III were from New England Biolabs and T4 DNA ligase was from Promega.

2.2. Cloning

To improve the expression level of *TfuDyP* (or any other protein) a new pBAD-based expression vector was designed, called pBAD SUMO. Fusing a protein to the C-terminus of SUMO (small ubiquitin-related modifier protein) often improves the expression level and solubility of a protein [13]. The *SUMO* gene (including an N-terminal 6xHis-tag) was amplified from pET SUMO STMO [14] and inserted in front of the multiple cloning site of an empty pBAD NK plasmid. The pBAD NK plasmid is a pBAD/*Myc*-His A-derived expression vector (Invitrogen) in which the *Nde*I site was removed and the *Nco*I site replaced by *Nde*I [15]. The *SUMO* gene was inserted in the first restriction site (*Nde*I) of the multiple cloning site of pBAD NK using In-Fusion cloning (In-fusion HD cloning kit, Clontech). The *Nde*I restriction site was retained on both sides of the gene. To finalize the pBAD SUMO vector, the *Nde*I restriction site in front of *SUMO* was exchanged to an *Nco*I site, while a stop codon (TAA) was created after the multiple cloning site using QuikChange mutagenesis.

In a second step, the *TfuDyP* gene was cloned into the obtained pBAD SUMO vector using restriction enzymes *Nde*I and *Hind*III. The first 35 residues predicted to be the Tat-signal sequence of *TfuDyP* were excluded. The lengths of the signal sequences were predicted using the program TatP [16].

Plasmid pBAD MBP *TfuDyP* was obtained by exchanging SUMO of pBAD SUMO *TfuDyP* with MBP from pBAD MBP AldO using restriction free cloning [17,18]. Plasmid pBAD His-*TfuDyP* was obtained by deleting SUMO, and pBAD His-SviDyP was obtained by replacing the *TfuDyP* gene in plasmid pBAD His-*TfuDyP* with the gene of SviDyP [19] using restriction free cloning [18]. The native gene of SviDyP from *Saccharomonospora viridis* DSM 43017 was synthesized by GenScript. The first 37 residues predicted to be the Tat-signal sequence were excluded.

Plasmid pBAD SUMO *TfuDyP* was used to transform *E. coli* strains TOP10, MC1061, DH5 α , SHuffle, BL21 (DE3), C41 (DE3) and C43 (DE3). pBAD *TfuDyP* *Myc*-His A, pBAD MBP *TfuDyP*, pBAD His-SviDyP and pBAD His-*TfuDyP* were used to transform *E. coli* strain TOP10.

2.3. Enzyme expression and purification

All *E. coli* strains were grown on 5 mL Luria Bertani (LB) medium at 37 °C, 200 rpm, overnight to saturation. The cultures were diluted 1:100 in Terrific Broth (TB) medium and grown at 37 °C, 135 rpm till OD₆₀₀ ~0.6. The cultures were subsequently induced with 0.02% L-arabinose and grown at 30 °C, 130 rpm overnight. *TfuDyP* was expressed in *E. coli* strain TOP10 using a culture volume of 400 mL. SUMO-*TfuDyP* was expressed in *E. coli* DH5 α , SHuffle, BL21 (DE3), C41 (DE3) and C43 (DE3) on a 100-mL scale. All other cultures were grown on 50-mL scale. If required, media were supplemented with 500 μ M δ -aminolevulinic acid (δ -ALA), 10 μ M hemin, 100 μ M ferrous sulfate or 100 μ M ferric citrate. For comparison, cultures of *E. coli* TOP10 expressing *TfuDyP* and SUMO-*TfuDyP* were also induced with 0.2% (100-mL culture, expression at 30 °C) and 0.002% (50-mL culture, expression at 17 °C) L-arabinose, respectively. All

cultures were supplemented with 50 μ g/mL ampicillin and grown in duplicate.

Cells were harvested by centrifugation at 6000 $\times$ g (Beckman Coulter, Avanti JE centrifuge, JLA 14 rotor) for 15 min at 4 °C or at 3000 $\times$ g (Eppendorf centrifuge 5810R) for 30 min at 4 °C. The pellets were washed in Buffer A (50 mM potassium phosphate, 0.5 M NaCl, 5% glycerol, pH 8) after which the cells were disrupted by sonication (5 min total on time with cycles of 10 s on and 10 s off at 70% amplitude). The cell-free extract was obtained by centrifugation at 16,000 $\times$ g for 15 min at 4 °C.

The enzymes were purified from the cell-free extract using a 1-mL His-Trap HP column (GE Healthcare). After loading the cell-free extract onto a pre-equilibrated column, the column was washed with buffer A (see above) followed by Buffer A supplemented with 20 mM imidazole. The enzymes were eluted by a gradient from 20 mM to 300 mM imidazole in Buffer A. After the purification, the buffer was exchanged to buffer B (50 mM potassium phosphate, 150 mM NaCl, 10% glycerol, pH 7.5) using an Econo-Pac 10DG desalting column (BioRad). The enzymes were concentrated using Amicon centrifugal filters with a 30 kDa cut-off (Merck Millipore), flash frozen with liquid nitrogen and stored at -20 °C.

2.4. UV-vis spectral analysis

All purified enzymes were analyzed by UV-vis absorption spectroscopy (V-660 spectrophotometer, Jasco). Absorbance spectra were recorded (250–800 nm) at ambient temperature. Enzyme concentrations were determined using a predicted molecular extinction coefficient at 280 nm (ProtParam tool, Expasy [20]), $\epsilon_{280\text{nm}} = 45950 \text{ M}^{-1} \text{ cm}^{-1}$ for *TfuDyP* and His-*TfuDyP*, $\epsilon_{280\text{nm}} = 47440 \text{ M}^{-1} \text{ cm}^{-1}$ for SUMO-*TfuDyP*, $\epsilon_{280\text{nm}} = 112300 \text{ M}^{-1} \text{ cm}^{-1}$ for MBP-*TfuDyP* and $\epsilon_{280\text{nm}} = 48470 \text{ M}^{-1} \text{ cm}^{-1}$ for His-SviDyP.

2.5. Activity assay

Activity towards Reactive Blue 19 was measured as described before, with minor changes [7]. The activity was analyzed spectrophotometrically (Jasco V-660) in a 50 mM citrate buffer pH 3.5, containing 50 μ M Reactive Blue 19, 100 μ M H₂O₂ and 100 nM enzyme at ambient temperature. Oxidation of Reactive Blue 19 was followed at 595 nm ($\epsilon = 10 \text{ mM}^{-1} \text{ cm}^{-1}$).

2.6. Cofactor extraction and mass spectrometry

The cofactors of purified (SUMO)-*TfuDyP* were extracted using an adaptation of the method of Adamczack et al. [21]. Before extraction, the buffer was exchanged to double-distilled H₂O using an Econo-Pac 10DG desalting column (BioRad). Enzyme concentrations of 25–150 μ M were used to yield a (calculated) heme concentration of 5–40 μ g/mL in the final extract, assuming that the enzyme was fully loaded with heme and all cofactors were extracted. To release the cofactor from the enzyme, hydrochloric acid was added to a concentration of 0.25 M. Two volumes of ethyl acetate were added and samples were vortexed for one minute. To improve phase separation, the samples were centrifuged for 2 min at 16,000 $\times$ g. The red ethyl acetate layer was collected, and the mass of the cofactor was determined by high-resolution mass spectrometry (HR-MS) on an LTQ Orbitrap XL (Thermo Fisher Scientific), with electrospray in positive mode.

Fig. 1. UV-vis absorption spectra of *TfuDyP* (top) and SUMO *TfuDyP* (bottom). The insets show the range between 450 nm and 700 nm enlarged.

3. Results and discussion

3.1. *TfuDyP* versus SUMO-*TfuDyP*

In earlier work, *TfuDyP* was expressed in *E. coli* MC1061 with a yield of 3 mg purified enzyme per liter LB medium [7]. This construct includes the Tat-signal sequence and a C-terminal myc-His tag. The protein yield per liter culture broth could be increased to approximately 25 mg purified enzyme from a culture of *E. coli* TOP10 grown on TB medium.

To increase the expression level of *TfuDyP* further, *TfuDyP* was fused to SUMO. Fusing an enzyme to SUMO often increases the expression level and solubility of an enzyme [13]. By fusing it to SUMO the overexpression level of *TfuDyP* in TB medium was increased to approximately 200 mg enzyme per liter culture broth. Clear differences were, however, observed between *TfuDyP* and SUMO-*TfuDyP*. Purified *TfuDyP* was brown-red while a more purple color was observed for highly overexpressed SUMO-*TfuDyP*. This difference could also be visualized by UV-vis absorption spectroscopy (Fig. 1). The two spectra are on the one hand very similar, as both spectra show a protein peak at 280 nm and a Soret band at around 406–416 nm. Clear differences were on the other hand observed in the range of 450–700 nm; in this range the so-called Q-bands are observed. *TfuDyP* showed a broad peak between 450 and 650 nm, while for SUMO-*TfuDyP* five peaks were observed at 509, 546, 568, 621 and 663 nm.

Fig. 2. Comparison between the protein expression yield per liter culture broth medium and the observed initial activity towards Reactive Blue 19 for variants of *TfuDyP*. The reported expression yields and activities were determined upon purification of the enzyme.

In previous work, we showed that *TfuDyP* could be reduced using sodium dithionite, shifting the Soret band to 431 nm [7]. When sodium dithionite was added to SUMO-*TfuDyP* only minor spectral changes were observed, suggesting that a major part of the cofactors could not be reduced (data not shown). This observation is in agreement with the activity measurements. *TfuDyP* had an observed reaction rate towards Reactive Blue 19 of 5.8 s^{-1} , while for SUMO-*TfuDyP* a relatively low rate of 1.3 s^{-1} was observed.

3.2. Influence of the expression conditions on *TfuDyP*

In an attempt to increase the specific activity of recombinant *TfuDyP* the effects of different expression conditions and protein fusion partners were studied. Hemin and δ -aminolevulinic acid (δ -ALA) are often added to the growth medium when overexpressing heme-containing enzymes [4,22]. Ferrous sulfate and ferric citrate are added to the culture media when overexpressing iron-containing proteins [23]. Addition of δ -ALA, ferrous sulfate or ferric citrate to the culture media had only a minor effect on the specific activity of the purified SUMO-fused enzyme. The protein yields were increased by 35–45% while the activities were increased by only 15–20%. The observed specific activities were still approximately 3.7-fold lower compared to *TfuDyP*. A bigger effect was observed for the addition of hemin to the growth medium, the protein yield was increased by 50% and the activity by a factor of 1.8 to an observed rate of catalysis of 2.4 s^{-1} .

It was hypothesized that SUMO might have an effect on the proper folding, dimerization and/or the accessibility of the active site of *TfuDyP*. To determine whether the fusion of SUMO to *TfuDyP* had an influence on the activity, SUMO was cleaved off using SUMO protease. However, the activity did not increase. To analyze the effect of SUMO further, SUMO was exchanged by another often-used fusion protein, maltose binding protein (MBP). The overexpression of MBP-*TfuDyP* by *E. coli* TOP10 gave comparable results; a highly overexpressed but almost inactive protein was obtained, see Fig. 2. To analyze the effect of any fusion protein (SUMO or MBP) on *TfuDyP*, SUMO was deleted from the plasmid pBAD His-SUMO *TfuDyP* yielding pBAD His-*TfuDyP* (not to be confused with the original plasmid pBAD *TfuDyP* Myc-His A, which includes the Tat-sequence). Expression of His-*TfuDyP* by *E. coli* TOP10 yielded approximately 210 mg purified enzyme per liter culture broth. The highly overexpressed enzyme had again a relatively low specific activity towards Reactive Blue 19 of 1.5 s^{-1} , see Fig. 2. From these results it could be concluded that the lower specific

Fig. 3. Comparison of the expression level and the observed initial reaction rate of SUMO-*TfuDyP* towards Reactive Blue 19, when being overexpressed by seven different *E. coli* strains. *TfuDyP* from *E. coli* TOP10 is added for comparison. The reported expression yields and activities were determined upon purification of the enzyme.

activity of SUMO/MBP-*TfuDyP* was not caused by the protein fusion partner.

3.3. Expression of SUMO-*TfuDyP* by different *E. coli* strains

In an attempt to increase the activity of the highly overexpressed enzyme, several expression strains were screened. Different *E. coli* strains can be used for the heterologous overexpression of enzymes. Here we compared the expression level and the activity of SUMO-*TfuDyP* when being overexpressed by seven different *E. coli* strains, see Fig. 3. Four *E. coli* K-strains (TOP10, MC1061, DH5 α and SHuffle) and three *E. coli* B-strains (BL21 (DE3), C41 (DE3) and C43 (DE3)) were used. Each strain was grown in duplicate in 50–100 mL TB medium, and induced with the same amount of L-arabinose (0.02%). Interestingly, a correlation between the expression level and the enzyme activity was observed. *E. coli* TOP10 and MC1061 produced 190 and 140 mg of purified enzyme per liter culture broth respectively. The enzyme purified from these cultures had a relatively low activity for Reactive Blue 19 of respectively $1.3 s^{-1}$ and $0.8 s^{-1}$. The three *E. coli* B-strains, on the other hand, produced only approximately 20 mg of enzyme per liter culture broth displaying activities between 5.0 – $6.7 s^{-1}$. *E. coli* SHuffle and DH5 α yielded low levels of expression and intermediate specific activities.

3.4. The activity of *TfuDyP* is influenced by the expression level

To prove that the differences between active *TfuDyP* and highly overexpressed but poorly active SUMO-*TfuDyP* were caused by the expression level of the protein, different overexpression conditions were studied. The expression level of *TfuDyP* was boosted through the induction of the *E. coli* TOP10 cultures with 0.2% instead of 0.02% L-arabinose. The expression level increased from 26 to 39 mg per liter culture broth, while the specific activity decreased by 12%. To probe the opposite effect, the relatively high expression level of SUMO-*TfuDyP* was reduced by inducing the respective *E. coli* TOP10 cultures with 0.002% L-arabinose instead of 0.02%.

These cultures were grown at 17 °C for 3 days, instead of at 37 °C overnight. The expression level was indeed efficiently decreased from 194 mg/L to 70 mg/L culture broth, while the observed rate of catalysis improved by 35%. These results show that the activity of the enzyme was dependent on the expression level of the protein.

3.5. Analysis of the cofactor by mass spectrometry

To analyze the differences between active *TfuDyP* and poorly active SUMO-*TfuDyP* further, the heme cofactors were extracted from the enzymes. The red ethyl acetate samples were analyzed by HR-MS. The isolated cofactor of *TfuDyP* had a mass of 616.1758 for the $[M+H]^+$ ion, which is in accordance with the calculated mass of heme (616.1768) (Fig. 4A). On the other hand, revealed the cofactor isolated from the highly overexpressed but almost inactive SUMO-*TfuDyP* three peaks. Intriguingly, the main peak, with a mass of 563.2648 for the $[M+H]^+$ ion, corresponds to the mass of the heme precursor protoporphyrin IX (PPIX, heme deficient in iron) (Fig. 4B). The two smaller peaks correspond to heme (616.1758) and oxidized PPIX (595.2540). PPIX is known to be sensitive to photo-oxidation in the presence of oxygen, which may explain the presence of oxidized PPIX [24]. These results are in agreement with the observed specific activities of the two proteins: fully active *TfuDyP* contained heme, while highly overexpressed and poorly active SUMO-*TfuDyP* was mainly loaded with heme precursor PPIX which cannot support catalysis.

3.6. Highly overexpressed *SviDyP* binds PPIX *in vivo*

To explore whether the *in vivo* binding of PPIX to the recombinantly overexpressed DyP-type peroxidase is specific for *TfuDyP* or a recurrent complicating factor for producing DyPs, we also expressed a DyP from another bacterium. For this we choose the recently described DyP-type peroxidase *SviDyP* [19] from *Saccharomonospora viridis* DSM 43017 which exhibits a different substrate scope and pH profile when compared to *TfuDyP*. Like *TfuDyP*, *SviDyP* also belongs to the subclass of A-type DyPs but only shares 40% sequence identity and displays different enzymatic properties including higher pH and temperature optima [19]. An overexpression level of approximately 100 mg of His-tagged *SviDyP* per liter culture broth was obtained. The UV–vis absorption spectrum of *SviDyP* revealed that it also contains a significant amount of PPIX as it displays the identical Q-bands when compared with the recombinantly produced SUMO-*TfuDyP* (Fig. 5). This shows that the typical PPIX spectral fingerprint in the Q-bands region can be used to verify incorporation of heme or PPIX in DyPs or other hemoproteins.

4. Conclusions

In this study we showed that the specific activity of DyP-type peroxidase *TfuDyP* was dependent on the overexpression level of the enzyme. UV–vis absorption spectroscopy and high-resolution mass spectrometry showed that the inactive enzyme contained, instead of a heme cofactor, the heme precursor PPIX. A heme enzyme lacking the redox active iron is unable to catalyze oxidations. In previous studies, it was shown that apo forms of DyP-type peroxidases Mt-DyP and YfeX could bind PPIX in *in vitro* titration experiments [25,26]. The UV–vis absorption spectra of Mt-DyP and YfeX reconstituted with PPIX are comparable to the spectrum of SUMO-*TfuDyP* loaded with PPIX (Fig. 1). Moreover, hemoproteins nitric oxide synthase from *Geobacillus stearothermophilus* and dye decolorizing peroxidase EfeB from *E. coli* O157, had been described to bind PPIX *in vivo* when being overexpressed in *E. coli* [22,27]. These data indicate that several DyPs have a high affinity towards PPIX, and that the expression host is not always able to provide enough heme to outcompete PPIX binding. It may also (partly)

Fig. 4. A MS analysis of the extracted cofactor of *TfuDyP*. The cofactor had an expected mass of 616.1758 for the $[M+H]^+$ ion. B MS analysis of the extracted cofactor of SUMO-*TfuDyP*. The enzyme contained three different compounds. The main peak corresponds to the $[M+H]^+$ ion of PPIX (563.26 m/z), and the smaller two peaks to heme (616.18 m/z) and oxidized PPIX (592.25 m/z).

explain the observed amounts of PPIX in the studies on EfeB and YfeX [27] leading to the erroneously conclusion that these DyPs are heme dechelates [28]. In this context it is worth noting that *TfuDyP* is not active on hemin [3].

The low affinity of these enzymes towards heme may also offer interesting possibilities. For example, it could potentially be exploited to exchange the heme or PPIX for an artificial porphyrin. Key et al. demonstrated that apo heme enzymes reconstituted with abiological porphyrins show unique catalysis [29].

Laboratory *E. coli* strains are often unable to import or synthesize enough heme for highly overexpressed heme enzymes [22,30,31]. In this work we tried to circumvent this problem by lowering the expression level of the enzyme or through the expression of *TfuDyP*

in alternative *E. coli* strains. To improve heme incorporation one can also opt for engineering the host. For example, overexpression of the heme receptor ChuA, for heme uptake, or heme ferrochelatase, for iron incorporation in heme, have been shown to increase the available amount of heme in the cell [22,30].

This study shows that it is important to carefully tune the conditions for recombinant expression of DyPs in order to obtain enzyme that is fully loaded with the heme cofactor. Members of this newly discovered family of DyP-type peroxidases are currently of great interest concerning their biochemical properties and biotechnological potential [3,5,6,8,9]. Our findings may assist in the development of effective recombinant production methods for DyPs.

Fig. 5. UV-vis absorption spectrum of SviDyP. The inset show the range between 450 nm and 700 nm enlarged.

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