Purification of a Recombinant Bacterial DyP-Type Peroxidase with a Hydrophobic Anion Exchange Resin

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Enzyme Purification

Bulletin 7182

Abstract

DyP (dye-decolorizing peroxidase)-type peroxidases are a family of heme-containing enzymes. They are capable of catalyzing efficient oxidation of a wide array of industrially relevant substrates, including dyes with anthraquinone structure, β-carotene, and aromatic sulfides. Owing to their remarkable expression and robustness, they have many potential uses in a variety of biotechnology applications. Here we show polish purification of bacterially expressed DyPs with a hydrophobic anion exchange mixed-mode resin, Nuvia aPrime 4A, to generate a >95% pure, functionally viable sample. The two-step workflow used in this purification is readily scalable for process production.

Introduction

Peroxidases represent a large family of oxidoreductases that typically catalyze the oxidation of substrate molecules. They are involved in a variety of biochemical processes, including biosynthesis of cell wall material and immunological host-defense responses (Fraaije and van Bloois 2012). The recently discovered DyP-type peroxidases (DyPs; EC 1.11.1.19) represent a superfamily of heme-containing enzymes. They share no similarity in primary sequence or structure to other plant, bacterial, or fungal peroxidases (Colpa et al. 2014).

DyPs possess a broad substrate specificity and low pH optimum. Using hydrogen peroxide as an electron acceptor, they are capable of catalyzing efficient oxidations of a wide array of industrially relevant substrates, including dyes with anthraquinone structure, β-carotene, and aromatic sulfides. Several bacterial DyPs were found to be robust enzymes and thus potent biocatalysts (Loncar et al. 2016, van Bloois et al. 2010). Because they can degrade a variety of synthetic dyes, DyPs can potentially be used in the bioremediation of dye-contaminated waste water. Two fungal DyP-type peroxidases were shown to degrade β-carotene (Scheibner et al. 2008), which is of significant interest in the food industry for enabling the enzymatic whitening of whey-containing foods and beverages. DyP peroxidases also show promise as novel antimicrobial (pro) drug targets (Kong et al. 2010). The large number of potential industrial uses points to the importance of purifying this enzyme at large scale.

In this study, we show polish purification of bacterially expressed DyPs with a hydrophobic anion exchange mixed-mode resin, Nuvia aPrime 4A, to generate a >95% pure, functionally viable sample. The two-step workflow used in its purification is readily scalable for process production. This purification strategy meets the purity requirements for nontherapeutic targets. For therapeutic enzymes and proteins that generally require greater stringency in their purification processes, Nuvia aPrime 4A could be incorporated into a workflow with additional purification steps.

Materials and Methods

General

Pfu polymerase, restriction enzymes, and prestained protein ladders were from Thermo Fisher Scientific. Ni Sepharose HP was from GE Lifesciences. Nuvia aPrime 4A Resin (catalog #12007397) was from Bio-Rad Laboratories. All other chemicals were supplied by Millipore-Sigma and were of analytical grade.

Molecular Cloning and Expression

Histidine-tagged recombinant PfDyPB1 and B2 were expressed in the TOP10 strain of *Escherichia coli* with the pBad*Nde*I/His vector (Invitrogen) according to an established protocol (Loncar et al. unpublished data). Cells expressing PfDyPB1 and PfDyPB2 were grown in LB medium at 37°C to saturation overnight. The following day, the cultures were diluted 1:100 into fresh media and grown until OD 600 = 1.25. Then 0.02% L-arabinose and 0.75 mM hemin were added to induce the expression of PfDyPB and as a supplement to

overcome difficult heme production, respectively. Expression was carried out at 30°C, 180 rpm for 24 hr. Cells were harvested by centrifugation at 6,000 rpm and washed once with 50 mM K_2HPO_4/KH_2PO_4 buffer, pH 8.0, with 0.5 M NaCl. Pelleted cells were resuspended in the same buffer and disrupted by sonication. Cell-free extract was obtained after centrifugation at 11,000 rpm at 4°C for 1 hr.

Purification

For capture purification of histidine-tagged PfDyPB, the cell-free extract was applied to a Ni Sepharose HP Column equilibrated with 50 mM K_2HPO_4/KH_2PO_4 buffer at pH 8.0 and 0.5 M NaCl. Nonspecifically bound proteins were washed away with two column volumes (CV) of 10 mM imidazole in starting buffer followed by elution with 250 mM imidazole in the same buffer. The eluate was further purified on Nuvia aPrime 4A Mixed-Mode Resin. Binding to the resin was achieved with 50 mM K_2HPO_4/KH_2PO_4 buffer at pH 7.8 (buffer A). Stepwise elution was carried out using buffer A with 0.1, 0.2, 0.3, 0.5, and 1.0 M NaCl. Pure fractions were pooled and concentrated using an Amicon stirred cell equipped with a 10 kD cut-off membrane.

Analytical Assays

Protein fractions were analyzed by 10% SDS-PAGE using the SE260 Mighty Small II Deluxe System (Hoefer Inc.) and quantified using ImageJ Software (imagej.net). The UV/Vis absorption spectra of purified DyPB were recorded between 200–800 nm using a Shimadzu UV-1800 Spectrophotometer in 1 cm quartz cuvettes at room temperature.

Enzyme Assays and Steady-State Kinetics

Kinetic parameters of peroxidase activity (k_{cat} and K_m) were measured using different concentrations of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS), o-dianisidine, pyrogallol, Reactive blue 4, and Reactive black 5 with 1 mM H_2O_2 as co-substrate by adding 10 µl of suitably diluted enzyme (final concentration 5.3 nM). For H_2O_2 and t-butyl-peroxide, a fixed concentration of ABTS was used and the concentration of peroxide was varied. Control reactions were included without using enzyme, H_2O_2, or both. Blanks were recorded in parallel with the measurements and subtracted accordingly. The kinetic parameters were calculated by fitting the data with a Michaelis-Menten equation using nonlinear analysis. Inhibition of the peroxidase was tested using 3-amino-1,2,4-triazole, imidazole, dithiothreitol (DTT), cysteine, and sodium azide. The enzyme was assayed for peroxidase activity as previously described after preincubation with the inhibitor for 3 min prior to addition of ABTS.

Influence of pH on Enzyme Activity

The optimal pH of PfDyP was determined using 0.5 mM ABTS and 1 mM H_2O_2 and a set of 0.1 M buffers (Na acetate pH 3–5, MES pH 6, Tris HCl 7–8, glycine/NaOH pH 9–10). Upon determination of the optimal pH, further measurements were made in the appropriate buffer.

Results and Discussion

The predicted molecular weight of the overexpressed *Pseudomonas fluorescens* dye-decolorizing peroxidase (PfDyP) was ~35 kD. The enzyme bound poorly to Ni Sepharose and eluted with less than 10 mM imidazole (chromatogram not shown). This IMAC eluate was less than 10% pure by SDS-PAGE analysis and required further purification for use in any application.

Nuvia aPrime 4A enables purification of biomolecules without feedstream conditioning and can operate across a wide range of salt concentrations and pH. It is designed with a positively charged hydrophobic ligand (Figure 1), which helps in biomolecule binding using both hydrophobic and anion exchange interactions, allowing removal of multiple impurities such as viruses, host cell proteins (HCPs), aggregates, and nucleic acids. Therefore, Nuvia aPrime 4A was used for polish purification of the PfDyPs (Table 1). Binding to Nuvia aPrime 4A was achieved with buffer A. Active peroxidase eluted with 1 M NaCl and showed ~95% purity on SDS-PAGE analysis (Figure 2). The purification procedure yielded ~35 mg of purified histidine-tagged PfDyPs from 1 L of culture broth.

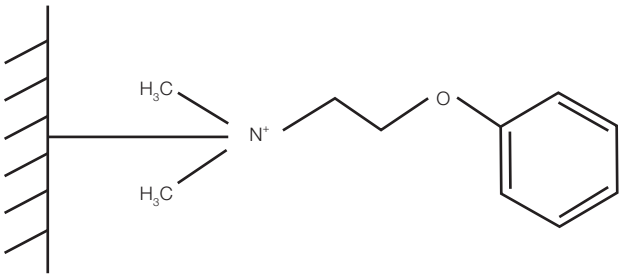

Fig. 1. Mixed-mode ligand for Nuvia aPrime 4A Resin.

Table 1. Nuvia aPrime 4A polish chromatography conditions.

Column	Nuvia aPrime 4A, 2 ml
Equilibration	10 CV of buffer A
Sample	4 ml cell culture supernatant adjusted to pH 7.8 with 6 ml equilibration buffer
Flow rate	120 cm/hr
Wash	20 CV buffer A
Elution	Stepwise elution using 2 CV each of 10, 20, 30, 50, and 100% buffer B
Buffer A	50 mM K_2HPO_4/KH_2PO_4, pH 7.8
Buffer B	1 M NaCl, 50 mM K_2HPO_4/KH_2PO_4,, pH 7.8

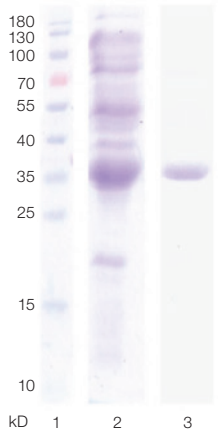

Fig. 2. SDS-PAGE analysis of the enzyme eluted from the Nuvia aPrime 4A Column. Lane 1, molecular markers; lane 2, cell-free extract; lane 3, PfDyP fraction after Nuvia aPrime 4A Mixed-Mode Resin chromatography.

Previous studies have shown that DyPs are members of the heme peroxidase family of proteins and they incorporate heme molecules in their structure (Sugano et al. 2007). We analyzed for the presence of heme to ensure that it was still present in the enzyme and that the enzyme was active. The UV absorption spectrum of the purified enzyme between 200 and 800 nm is shown in Figure 3. The Soret band peak seen at 405 nm indicates that heme moieties are indeed present in the purified PfDyP. Purified PfDyPs exhibited an absorbance maximum at 406–412 nm. The Reinheitzahl value (Rz, the ratio of A_{405}/A_{280}) for the purified enzyme was 1.16.

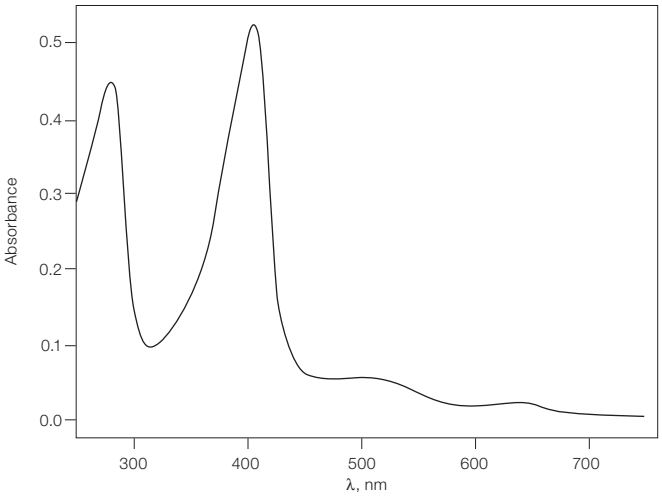

Fig. 3. UV/Vis spectra of PfDyP B2. Soret band (405 nm) is indicative of heme incorporation. An Rz value of 1.16 shows that enzyme is loaded with heme.

The purified enzyme was seen to be most active at pH 4.0 (Figure 4) with moderate activity between pH 3.0 and 6.0, in agreement with other studies (Brown et al. 2012, Santos et al. 2014).

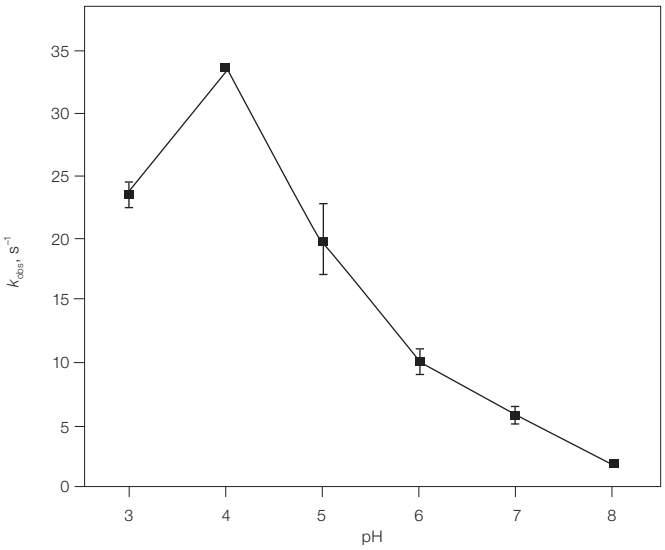

Fig. 4. Optimal pH of the purified PfDyP with the substrate ABTS.

The relative activity of the purified PfDyP enzyme was tested using 0.5 mM ABTS and 0.1 mM H_2O_2 in the presence of various cations (Ca^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Fe^{2+}, and Hg^{2+}) and reducing agents (aminotriazole, EDTA, imidazole, DTT, Cys, and sodium azide). Results show that relative activity was not diminished by the addition of the Ca, Mg, Zn, Mn, and Co ions but was virtually eliminated in the presence of the Fe and Hg ions (Figure 5). Partial reduction in the activity of the purified enzyme was seen with aminotriazole, EDTA, and imidazole, whereas almost complete reduction in activity was seen with DTT, cysteine, and sodium azide (Figure 6).

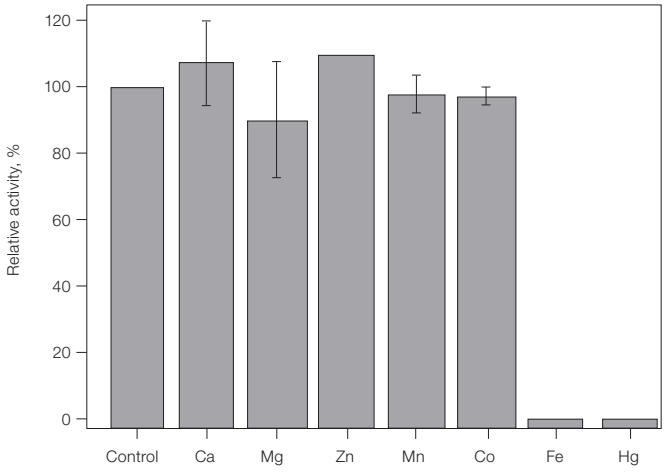

Fig. 5. Relative activity of the purified PfDyP in the presence of different cations.

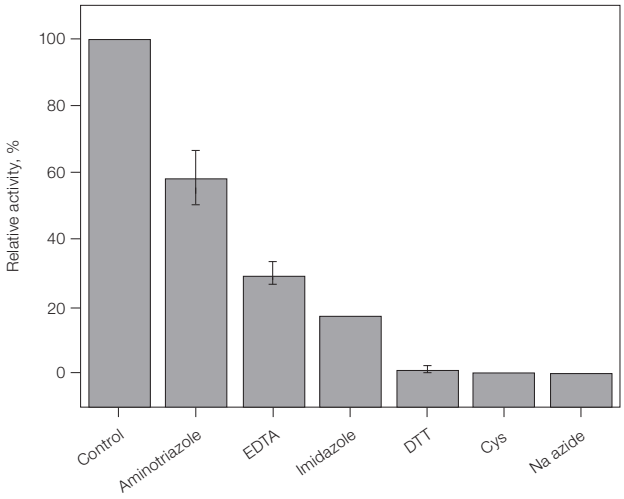

Fig. 6. Relative activity of the purified PfDyP in the presence of different reducing agents.

The substrate specificity of purified PfDyP was tested using various well-known peroxidase substrates. As shown in Table 2, the purified DyP enzyme showed activity with most of the tested substrates, including ABTS and aromatic, azo, and anthraquinone dyes. No activity was seen using guaiacol, 2,6-dimethoxyphenol, manganese, veratryl alcohol, carbobenzoxy-ethanolamine, syringaldehyde, or acetosyringone (data not shown).

Table 2. Substrate scope exploration of Nuvia aPrime 4A–purified PfDyP.

Substrate	λ, nm	ε, $mM^{-1}cm^{-1}$	K_m, mM	k_{cat}, s^{-1}	k_{cat}/K_m, $M^{-1}s^{-1}$
ABTS	414	36.6	0.22	102 ± 6.2	464.12
o-dianisidine	460	11.3	0.003	14.66 ± 1.71	4,360.49
Pyrogallol	430	2.47	1.445	1.98 ± 0.03	1.36
Reactive blue 4	610	4.2	0.010	1.54 ± 0.13	146.61
Reactive black 5	597	37	0.006	0.04 ± 0.006	6.06
H_2O_2*	240	0.0394	0.516	23 ± 0.84	44.57
t-BuOOH*	N/A	N/A	415	97 ± 28	0.23

*Measured indirectly by measuring oxidation of ABTS at 414 nm.
λ, assay wavelength.

Prior studies have also shown that DyP-type peroxidases can degrade lignin (de Gonzalo et al. 2016). Enzymatic lignin depolymerization is highly attractive, as it offers access to a wide array of aromatic precursors that can be further functionalized for production of high added value chemicals. Accordingly, we tested the purified PfDyP to see if it can oxidize kraft lignin. As shown in Figure 7, we detected DyP activity with lignin, which was concentration dependent.

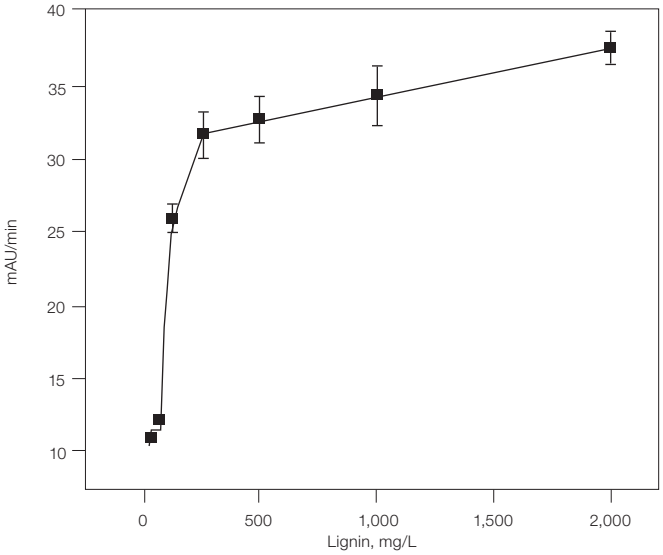

Fig. 7. Steady state activity of *P. fluorescens* DyPB with kraft lignin.

Conclusions

Nuvia aPrime 4A Mixed-Mode Resin can be used to purify crude samples of PfDyP to >95% purity while maintaining the stability and functionality of the active enzyme. This permits the enzyme to be used for biotechnical industry applications. The purification process is scalable and can be used for manufacturing bulk quantities of this enzyme. Although we did not test it in this particular instance, Nuvia aPrime 4A may permit single-step purification directly from the crude feedstock, since it is compatible with high salt concentration and broad pH ranges without the need for feed dilution/reconstitution. This would help in limiting the number of steps required to generate a highly pure enzyme sample.

While this purification strategy meets the purity requirements of targets for nontherapeutic purposes, targets for therapeutic purposes require greater stringency in their purification processes. For such purposes, Nuvia aPrime 4A can be incorporated into a workflow with additional purification steps.

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Bulletin 7182 Ver A US/EG *19-0261 0419 Sig 0119*