

Degradation pathway of an anthraquinone dye catalyzed by a unique peroxidase DyP from *Thanatephorus cucumeris* Dec 1

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Abstract The reactants produced by action of a purified unique dye-decolorizing peroxidase, DyP, on a commercial anthraquinone dye, Reactive Blue 5, were investigated using electrospray ionization mass spectrometry (ESI-MS), thin-layer chromatography (TLC), and ¹H- and ¹³C- nuclear magnetic resonance (NMR). The results of ESI-MS analysis showed that phthalic acid, a Product 2 (molecular weight 472.5), and a Product 3 (molecular weight 301.5), were produced. Product 2 and Product 3 were generated by usual peroxidase reaction, whereas phthalic acid was generated by hydrolase- or oxygenase-catalyzed reaction. One potential associated product, *o*-amino-benzene sulfonic acid, was found to be converted to 2,2'-disulfonyl azobenzene by ESI-MS and NMR analyses. From these results, we propose, for the first time, the degradation pathway of an anthraquinone dye by the enzyme DyP.

Keywords Anthraquinone dye ·
Dye-decolorizing peroxidase · DyP ·
Thanatephorus cucumeris · Phthalic acid

Introduction

Synthetic dyes are widely used in many industries worldwide. Around 10⁶ tons of dyes are produced annually, of which 1–1.5 × 10⁵ tons are released to the environment in wastewater (Stolz 2001). As many dyes are very stable, the discarded dyes remain long-term in the environment and thus accumulate (Anliker 1979; McMullan et al. 2001). Some dyes are converted to harmful compounds such as benzidine (Chung and Stevens 1993). Concerns have been raised that such artificial compounds may be xenobiotics and it is widely considered that they should not be released into the environment. Therefore, microbial degradation of synthetic dyes has been explored. In particular, biodegradation of azo dyes, which make up more than half of the dyes synthesised each year, has been extensively explored and biodegradative mechanisms have been researched in detail (Banat et al. 1996; Fang et al. 2004; Fu and Viraraghavan 2001; Kim et al. 1995; McMullan et al. 2001; Stolz 2001; Young and Yu 1997).

Such dyes are degraded mainly by peroxidases such as lignin peroxidase (LiP), manganese peroxidase (MnP), and versatile peroxidase (Chivukula et al. 1995; Goszczynski et al. 1994; Heinfling et al. 1998; Ollikka et al. 1993; Pasti and Crawford 1991; Vyas and Molitoris 1995). These peroxidases withdraw hydrogen from hydroxyl groups of dyes and generate radical compounds. The radical reaction proceeds and the dyes are either degraded or

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polymerized to other molecules. In fact, several degradation pathways for azo dyes have been proposed (Chivukula et al. 1995; Goszczynski et al. 1994). In the peroxidase reaction, cleavage of azo bonds may occur in dyes with hydroxyl groups (Goszczynski et al. 1994). In contrast, there are but a few reports on biodegradation of anthraquinone dyes, which, along with azo dyes, are widely used in many industries. Little information on the degradation mechanisms of such dyes has been published (Itoh et al. 1996, 1998). To date, degradation of the anthraquinone structure by known peroxidase reactions might be considered to be difficult, because the anthraquinone frame contains no hydrogens that can be withdrawn by peroxidase. In fact, no model of the in vitro degradation of anthraquinone dyes by peroxidase has been published, even though the pseudoperoxidase activity of ferrylmyoglobin promotes oxidative degradation of a hydroxyl anthraquinone compound (Cartoni et al. 2004) and horseradish peroxidase indirectly degrades anthracycline by acting on hydroquinone (Reszka et al. 2005).

Notably, *Thanatephorus cucumeris* Dec 1 (formerly named *Geotrichum candidum* Dec 1), a basidiomycete, degrades anthraquinone dyes as well as azo dyes (Kim et al. 1995). Recently, we found that the complete in vitro decolorization of an anthraquinone dye was achieved by the concerted action of two peroxidases, namely DyP and TcVP1 (Sugano et al. 2006). In particular, DyP is a unique enzyme, for the following reasons. The enzyme contains a heme group and catalyzes peroxidase reactions, but shows no primary structural homology to other known general peroxidases (Sugano et al. 1999, 2000). Recently, DyP has been shown to be a member of a novel heme peroxidase family; this advance became possible when the complete tertiary structure of the enzyme was obtained (Sugano et al. 2007). Other heme proteins similar to DyP have been identified (Scheibner et al. 2008; Sturm et al. 2006; Zubieta et al. 2007a, b). Interestingly, DyP has anthraquinone dye decolorization activity (Kim and Shoda 1999), but does not completely decolorize such dyes. The final reaction products when DyP acts on a deep blue-colored anthraquinone dye, Reactive Blue 5 (RB5), are red-brown. These products were completely decolorized by TcVP1 (Sugano et al. 2006).

To date, we have not identified the red-brown products or presented the degradation pathway of

RB5 treated with DyP. Here, we report the identification of one of the red-brown products obtained by DyP action and propose a reasonable RB5 degradation pathway. This is the first report to confirm an in vitro enzymatic degradation pathway of a hydroxyl-free anthraquinone dye.

Materials and methods

Chemicals

The structure of RB5 is shown in Fig. 1. Aminobenzene sulfonic acid (ABS) and phthalic acid were from Kanto Chemical Co. (Tokyo, Japan). All other chemicals used were of analytical grade and were commercially available unless specified otherwise.

Enzyme

Purified DyP, prepared by the method described in our previous reports (Sugano et al. 1999, 2000), was used for the present study. Enzyme assays were performed as reported previously (Sugano et al. 2000). One unit (U) of enzyme activity was defined as the amount of the enzyme that decolorized (decrease in absorption at 600 nm was measured) 1 μmol of RB5 at 30°C in 1 min.

Preparation of reaction products of RB5 treated with DyP

RB5 (final concentration 0.15 mM) and 2 U of DyP were dissolved in 25 mM citrate buffer (pH 3.2) and H_2O_2 (final concentration 0.2 mM) was added to start

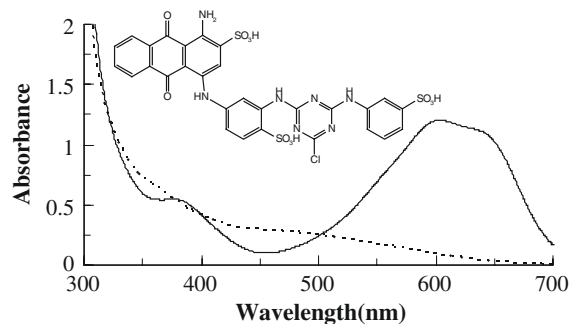


Fig. 1 Spectra of RB5 treated with or without DyP *solid line*, RB5 *broken line*, RB5 treated with DyP. The *inset* structural formula is RB5

the enzyme reaction, at 30°C. After 10 min, the reaction solution was cooled and stored at –80°C. A 0.15 mM authentic phthalic acid sample was prepared in the same buffer and stored at –80°C.

Electrospray ionization mass spectrometry (ESI-MS), together with high performance liquid chromatography (HPLC), for analysis of reaction products, was achieved using the following system. A Waters 2690/996 HPLC equipped with a photo diode array detector and operating at 210–400 nm was used to deliver samples to the MS detector (Waters ZMD Alliance LC/MS system, Nippon Waters Co., Tokyo, Japan). For sample separation, a Symmetry C18 column (2.1 ϕ \times 150 mm) was used. A linear gradient from 0% methanol in 5 mM ammonium acetate, to 80% (v/v) methanol in the same salt solution, over 20 min, at a flow rate of 0.2 ml/min, was applied. Both positive (ES+) and negative (ES–) ion modes were explored.

Preparation of reaction products when ABS was acted upon by DyP

To assist in characterization of the reaction products obtained when DyP degraded RB5, ABS was used as a substrate of the enzymatic reaction. ABS (final concentration 6 mM) was dissolved in 25 mM citrate buffer (pH 3.2) and DyP (final concentration 0.6 μ M) was admixed; the spectrum from 600 to 200 nm was explored. H₂O₂ (final concentration 6 mM) was added to the mixture and incubation proceeded at 30°C for 24 h. To remove DyP, the reaction products were treated by ultrafiltration (cutoff 10 kDa) and the filtrate was recovered. The filtrate was freeze-dried, and the contents later analyzed by thin-layer chromatography (TLC). Otherwise, the filtrate was used directly, without freeze-drying, for HPLC and ESI-MS analyses.

NMR analysis of ABS treated with DyP

Fifty milligram of freeze-dried sample was dissolved in 400 μ l of water and TLC was performed on a silica gel 60 F₂₅₄ glass plate (200 \times 200 mm, Merck Ltd Japan) developed with methanol–ethyl acetate [3:1 (by volume)]. Colored bands were identified visually. Colored reaction products were collected, with silica gel from the glass plate, using a spatula, and suspended in methanol–ethyl acetate [3:1 (by

volume)]. The suspension was centrifuged to remove the silica gel and then filtered through a membrane filter (pore size 0.45 μ m). The solvent was removed by rotary evaporation and the residue analyzed by ¹H and ¹³C nuclear magnetic resonance (NMR) spectroscopy at 300 MHz (and 293 K) with a JNM-AL300 FT-NMR, and at 500 MHz (and 293 K) with a JNM-GX500 FT-NMR (JEOL, Tokyo Japan), respectively. The liquids *d*-DMSO and CD₃OD were used as solvents for ¹³C-NMR and ¹H-NMR, respectively. The integration numbers for ¹H-NMR and ¹³C-NMR analyses were 64 and 1,688, respectively.

ESI-MS analysis of ABS treated with DyP

The reaction mixture after ABS was treated with DyP was applied to HPLC and fractionated. The separation column was an Asahipak NH2-P50 (4.6 ϕ \times 250 mm) and the elution solvent was 50 mM cyclohexyl aminopropane sulfonic acid (CAPS) adjusted to pH 11 with 2 M NaOH. The red–brown-colored elution volume was collected and applied to the ESI-MS (JEOL, Accu TOF CS JMS-T40

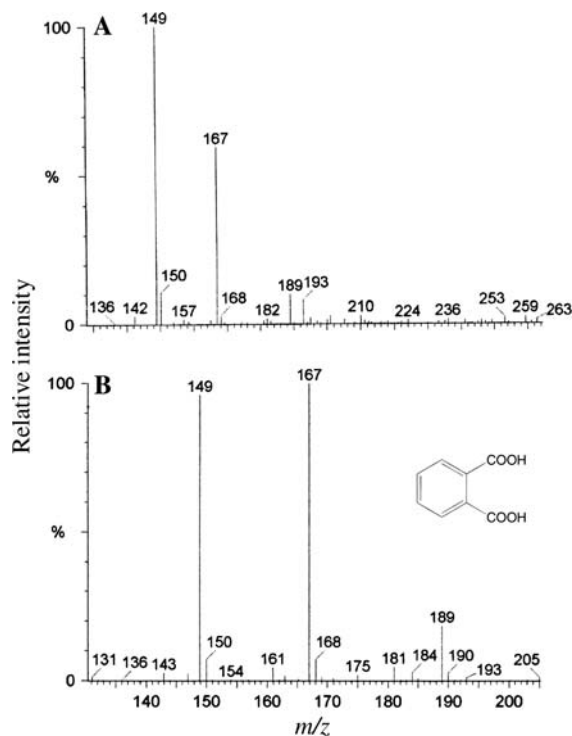


Fig. 2 **a** Mass spectra of a reaction product 1 (MW 166) of RB5 treated with DyP; **b** mass spectra of authentic phthalic acid, of which structural formula is shown as *inset*

instrument) at a constant rate of 0.2 ml/min using H₂O:MeOH [1:1 (by volume)]. The analysis was performed in the positive ion (ES⁺) mode and the scan interval was 1 s. The nebulizing and drying gas flow rates were 600 and 50 l/h of nitrogen, respectively. The nebulizing chamber and ion source temperatures were 250 and 80°C, respectively. The electrospray, orifice, and ring lens voltages were 2.0 kV, 85, and 15 V, respectively.

Results and discussion

Reaction products of RB5 treated with DyP

Changes in the visible spectrum of RB5 treated with DyP are shown in Fig. 1. The absorbance at 600 nm decreased and absorbances at 400–500 nm increased as the color of the reaction solution became red–brown. A molecular ion corresponding to RB5 [i.e.,

m/z of 772(ES[−]) or 774(ES⁺)] was not observed on MS analysis after enzyme reaction. Instead, three distinct molecular ion signals appeared, indicating reaction products. The first product (Product 1) had a molecular ion at m/z of 167(ES⁺) as shown in Fig. 2a. Furthermore, the molecular ion at m/z of 149(ES⁺) suggested that the compound was easily dehydrolyzed. As these mass spectrometric characteristics corresponded to those of authentic phthalic acid, as shown in Fig. 2b, and as the retention times of authentic phthalic acid and the reaction product on HPLC were identical (data not shown), we identified this product as phthalic acid. The second product (Product 2) showed molecular ions at m/z of 471(ES[−]) and 473(ES⁺), as shown in Fig. 3a. The molecular mass of Product 2 corresponded to that of RB5 without the anthraquinone frame [shown as an inset (in Fig. 3a)]. Therefore, phthalic acid was generated by an oxidative ring-opening of the anthraquinone frame. As far as this reaction is

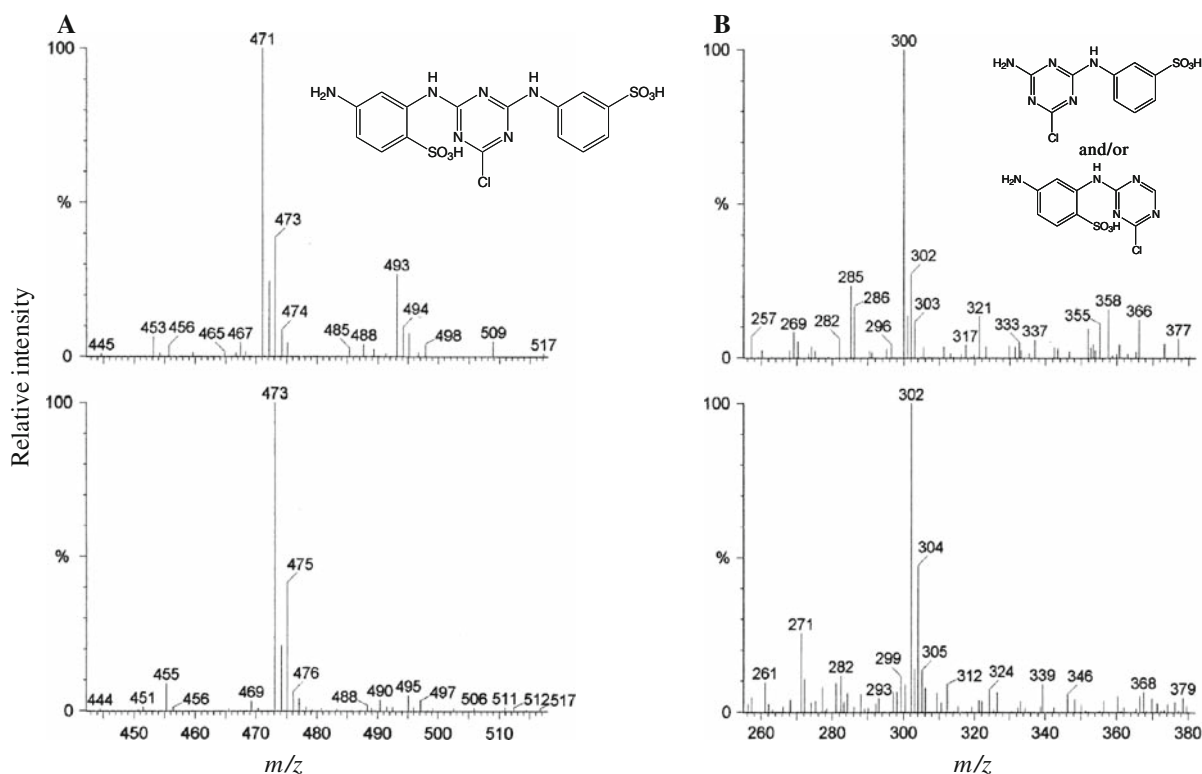


Fig. 3 **a** Mass spectra of Product 2 (MW 472) of RB5 treated with DyP. Above, negative ion mode (ES[−]); Below positive ion mode (ES⁺). Deduced structural formula of Product 2 is shown as *inset*. **b** Mass spectra of Product 3 (MW 301) of RB5

treated with DyP. Above negative ion mode (ES[−]); Below positive ion mode (ES⁺). Deduced structural formula of Product 3 is shown as *inset*

concerned, DyP appears to be a hydrolase or oxygenase rather than a peroxidase, although H_2O_2 was indispensable for the reaction. Jones (2001) reported that water released from H_2O_2 by the heme peroxidase reaction plays a significant role in influencing the reactivity of peroxidases, supporting the idea that DyP might have a unique reaction mechanism in addition to the peroxidase-type activity. The third product (Product 3) showed molecular ions at m/z values of 300(ES⁻) and 302(ES⁺), as shown in Fig. 3b. The molecular mass of Product 3 corresponded to that of Product 2 without ABS [shown as an inset (in Fig. 3b)]. When the enzyme reaction time was prolonged, the molecular ion peak corresponding to Product 2 disappeared, whereas that of Product 3 remained. Therefore, Product 2 was further converted to Product 3, and *m*- or *p*-ABS, by DyP.

In this pathway, *o*-ABS, and *m*- or *p*-ABS, must also be produced. However, we could not detect these chemicals. We speculate that ABS was immediately converted to other products by DyP action. In fact, the color of the reaction products of each aminobenzene sulfonic acid treated by DyP was red–brown, although ABS is colorless. Moreover, this color appeared similar to that of RB5 treated with DyP, as shown in Fig. 4.

Reaction products of ABS treated with DyP

When ABS was used as the substrate for DyP action, the reaction mixture changed to red–brown from

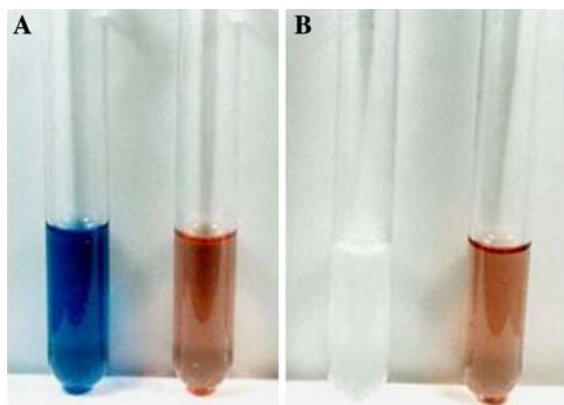


Fig. 4 Decolorization of RB5 (a) and staining of ABS (b) treated with DyP. Each *left* and *right* tubes show before and after DyP reaction, respectively

colorless, as shown in Fig. 4b. The red–brown compound showed an R_f of 0.6 on TLC. In addition, on TLC analysis, an undeveloped product, brown in color, showed a high molecular weight (>80,000) by gel filtration chromatography. This means that polymerization was also accelerated. It has been reported that 2,5-diaminobenzene sulfonic acid was polymerized by horseradish peroxidase (Alva et al. 1997). When the reaction mixture was applied to HPLC, the

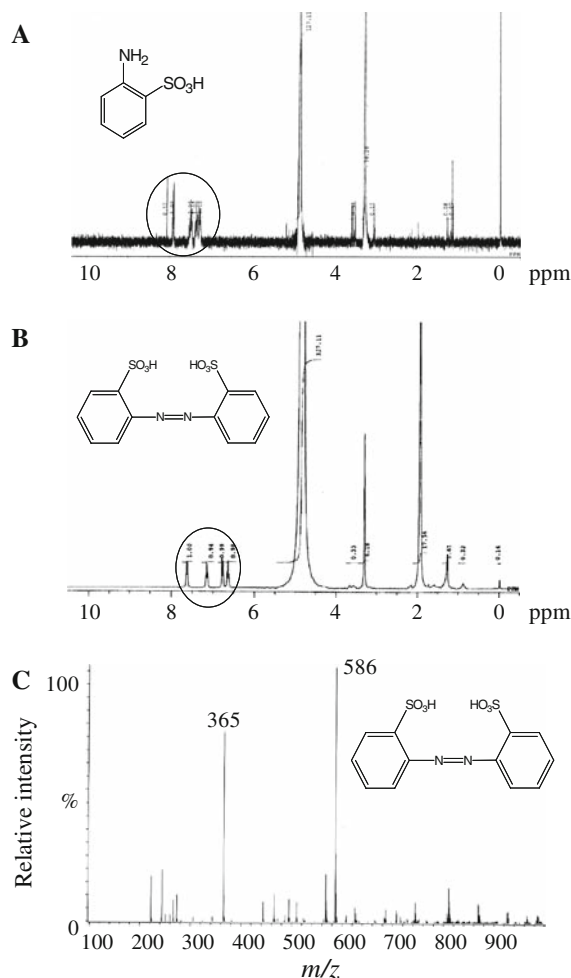


Fig. 5 ^1H -NMR spectra of **a** ABS [(300 MHz, CD_3OD) δ 7.90 (1H, d), 7.48 (1H, d), 7.36 (1H, t), 7.27 (1H, t)] and **b** Product 4 [(300 MHz, CD_3OD) δ 7.61 (1H, d), 7.15 (1H, t), 6.79 (1H, d), 6.63 (1H, t)]. Proton signals indicated by *circles* correspond to those signals from phenyl. Structural formulas of *o*-ABS and 2,2'-disulfonyl azobenzene are shown as *inset* of **a** and **b**, respectively. **c** Mass spectrum of Product 4 at positive ion mode. The ion peaks at 365 and 586 correspond to m/z ($M + \text{Na}$)⁺ and ($M + \text{Na} + \text{CAPS}$)⁺, respectively. Structural formula of identified reaction product is shown as *inset*

red–brown compound eluted at 10.6 min. This compound was named the “ABS derivative (Product 4)”. The $^1\text{H-NMR}$ spectra of *o*-ABS and Product 4 are shown in Fig. 5a and b, respectively. Product 4 was found to have no carbon apart from the benzene ring, from $^{13}\text{C-NMR}$ data [(500 MHz DMSO) δ 148.8, 148.0, 127.9, 113.8, 113.2, 111.5]. In the $^1\text{H-NMR}$ spectra (Fig. 5a, b), the proton signals of Product 4 were similar to those of *o*-ABS, indicating that the product contained an ortho-substituted benzene ring similar to that found in *o*-ABS. Furthermore, the $^1\text{H-NMR}$ signals of the four distinct proton types in the

compound were upfield of those of the protons of *o*-ABS, as shown in Fig. 5, suggesting that the electron-donating group of *o*-ABS ($-\text{NH}_2$) was changed to an electron-withdrawing group. As Product 4 was a colored compound, this group was deduced to be the azo group ($-\text{N}=\text{N}-$). This process is explained by the reaction mechanism of a typical peroxidase (such as horseradish peroxidase) and a spontaneous chemical reaction. The typical peroxidase reactions are two-electron redox pathways consisting of three sequential reactions (Dunford 1999):

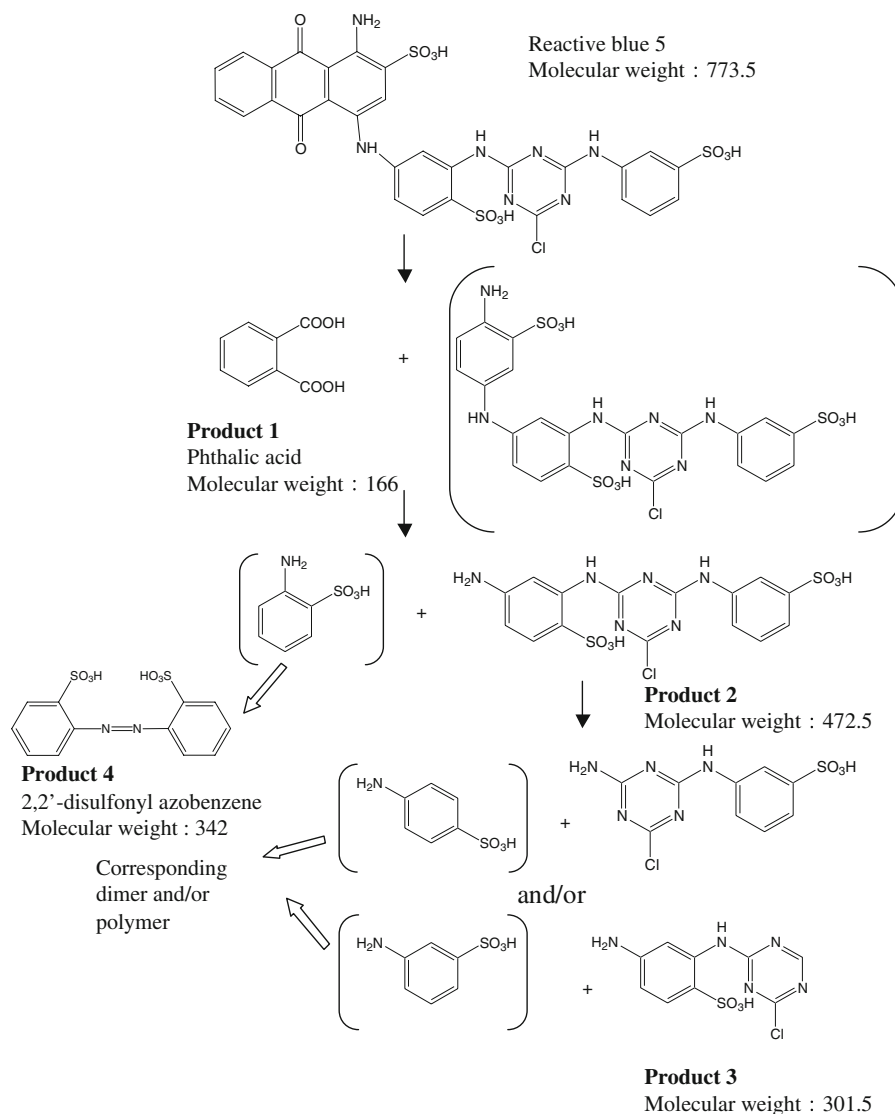


Fig. 6 Deduced degradation pathway of RB5 treated with DyP. Compounds shown in *parenthesis* were not detected by ESI-MS

Resting peroxidase + H₂O₂ → Compound I + H₂O

Compound I + AH₂ → Compound II + AH•

Compound II + AH₂ → Resting peroxidase + AH•

2AH• → A₂H₂ or AH₂

+ A (This is not an enzyme-catalyzed reaction)

If AH₂ corresponds to *o*-ABS, A₂H₂ corresponds to 1,2-bis(2-sulfonylphenyl)hydrazine. As 1,2-bis(2-sulfonylphenyl)hydrazine is also a substrate for a general peroxidase reaction, because hydrogen is available for withdrawal, this material would be further converted to 2,2'-disulfonyl azobenzene by DyP. From the ESI-MS spectrum (Fig. 5c), a molecular ion at *m/z* 365 appeared, indicating that Product 4 is identical to the sodium additive of 2,2'-disulfonyl azobenzene (M + Na)⁺. No molecular ion corresponding to *m/z* 343 (M + H)⁺ was observed because a large amount of Na⁺ was contained in the assay sample; the pH was adjusted to pH 11 with NaOH. Another molecular ion appeared at *m/z* 586, indicating a sodium-plus-cyclohexyl aminopropane sulfonic acid (CAPS) additive to 2,2'-disulfonyl azobenzene (M + Na + CAPS)⁺. This strongly suggests that DyP catalyzes the typical peroxidase reaction in addition to a DyP-specific reaction on anthraquinones. This characteristic might be shared with other DyP-type peroxidases (Sturm et al. 2006; Zubieta et al. 2007a). Based on these results, we can now propose a reasonable degradation pathway of RB5 by DyP, as shown in Fig. 6. It is notable that we have already reported that the red–brown compounds are completely decolorized by a versatile peroxidase, TcVP1, although the end-products of RB5 treated with DyP were red–brown in color (Sugano et al. 2006).

Conclusion

In conclusion, this is the first report to propose a reasonable anthraquinone degradation pathway achieved by a unique peroxidase, DyP. Importantly, phthalic acid and red–brown compounds were generated in the degradation process. One of the red–brown compounds was formed by a general peroxidase reaction, whereas phthalic acid is

suggested to be generated not by a peroxidase effect but via a hydrolase- or oxygenase-catalyzed reaction. This study suggests that DyP is a novel bifunctional enzyme.

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