



## Article

# Enzymatic Degradation of Multiple Major Mycotoxins by Dye-Decolorizing Peroxidase from *Bacillus subtilis*

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**Abstract:** The co-occurrence of multiple mycotoxins, including aflatoxin B<sub>1</sub> (AFB<sub>1</sub>), zearalenone (ZEN) and deoxynivalenol (DON), widely exists in cereal-based animal feed and food. At present, most reported mycotoxins degrading enzymes target only a certain type of mycotoxins. Therefore, it is of great significance for mining enzymes involved in the simultaneous degradation of different types of mycotoxins. In this study, a dye-decolorizing peroxidase-encoding gene *BsDyP* from *Bacillus subtilis* SCK6 was cloned and expressed in *Escherichia coli* BL21/pG-Tf2. The purified recombinant *BsDyP* was capable of oxidizing various substrates, including lignin phenolic model compounds 2,6-dimethylphenol and guaiacol, the substrate 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), anthraquinone dye reactive blue 19 and azo dye reactive black 5, as well as Mn<sup>2+</sup>. In addition, *BsDyP* could efficiently degrade different types of mycotoxins, including AFB<sub>1</sub>, ZEN and DON, in presence of Mn<sup>2+</sup>. More important, the toxicities of their corresponding enzymatic degradation products AFB<sub>1</sub>-diol, 15-OH-ZEN and C<sub>15</sub>H<sub>18</sub>O<sub>8</sub> were significantly lower than AFB<sub>1</sub>, ZEN and DON. In summary, these results proved that *BsDyP* was a promising candidate for the simultaneous degradation of multiple mycotoxins in animal feed and food.

**Keywords:** dye-decolorizing peroxidase; mycotoxin; aflatoxin B<sub>1</sub>; zearalenone; deoxynivalenol; biotransformation

**Key Contribution:** *BsDyP* could degrade multiple major mycotoxins including AFB<sub>1</sub>, ZEN and DON through the catalytic oxidation of Mn<sup>2+</sup>. The predicted biological toxicity of each corresponding degradation product was significantly removed.



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## 1. Introduction

Mycotoxins are a diverse group of toxic secondary metabolites produced by certain filamentous fungi, and they display carcinogenicity, teratogenicity, immune toxicity, neurotoxicity, hepatotoxicity, nephrotoxicity, reproductive and developmental toxicity towards humans and animals [1]. Until now, more than 400 mycotoxins have been identified and reported; however, only a few mycotoxins dominate in contaminated food and animal feed. These are aflatoxins B<sub>1</sub> (AFB<sub>1</sub>), zearalenone (ZEN), deoxynivalenol (DON), ochratoxin A, fumonisin B<sub>1</sub>, citrinin and patulin [2,3]. Meanwhile, the co-occurrence of abovementioned major mycotoxins is frequently observed in cereal-based animal feed and food [4,5], which might lead to synergistic and additive toxicological effects in humans and animals due to different mechanisms of action of mycotoxins at the cellular level [6].

The control of mycotoxins in food and feed depends on two main approaches: the prevention of mycotoxin production and detoxification of mycotoxins [7,8]. Most agronomic, physical and chemical strategies at pre-harvest as well as at post-harvest have been shown to be rather ineffective to control mycotoxin contamination [8]. With the increasing

demand for effective approaches to reducing mycotoxin contamination, researchers have been paying more attention to biological strategies. The detoxification of mycotoxins through biotransformation by microorganisms or their enzymes has gradually become one of the most promising strategies because of its advantages in efficiency, specificity and environmental friendliness [7,9]. As for biotransformation of multiple mycotoxins, several microorganisms such as *Bacillus subtilis*, *Rhodococcus percolatus* and *Pseudomonas putida* have been reported to be capable of degrading AFB<sub>1</sub> and ZEN [7,10–12]. In contrast, only a few enzymes have been identified, purified and characterized for multiple mycotoxin biotransformation.

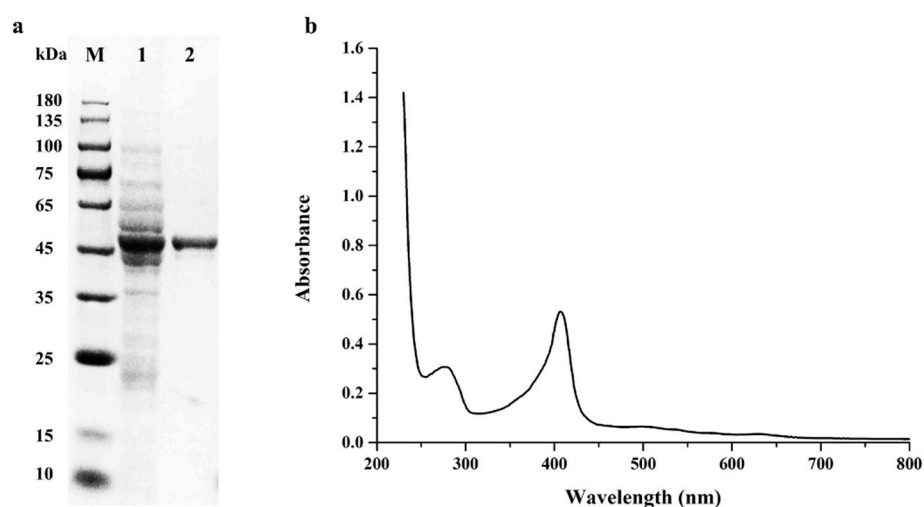
Considering that part of mycotoxins is structurally similar to a lignin monomer or its derivatives, ligninolytic enzymes might be able to degrade different types of mycotoxins. In our previous study, we found that fungal manganese peroxidases could degrade four major mycotoxins, namely AFB<sub>1</sub>, ZEN, DON and fumonisin B<sub>1</sub>, in the presence of a dicarboxylic acid malonate [13]. Moreover, bacterial and fungal laccases could simultaneously degrade AFB<sub>1</sub> and ZEN, using the laccase/mediator systems [14]. In this study, another ligninolytic enzyme dye-decolorizing peroxidase (DyP) from *B. subtilis* SCK6 was expressed in *Escherichia coli*, using the cold shock-inducible expression system coupled with co-expression of chaperones. Then the substrate specificity of purified recombinant BsDyP was characterized. Moreover, the degrading capacity of three major mycotoxins AFB<sub>1</sub>, ZEN and DON by BsDyP was evaluated, and corresponding enzymatic degradation products were identified by mass spectrometry analysis.

## 2. Results and Discussion

### 2.1. Gene Cloning, Expression and Purification of BsDyP from *B. subtilis* SCK6

It has been reported that *B. subtilis* could be able to efficiently degrade different types of mycotoxins, including AFB<sub>1</sub> and ZEN [15,16]. However, very few mycotoxins-degrading enzymes are identified and characterized except for CotA laccase [14]. In the present study, genomic analysis of *B. subtilis* SCK6 revealed that ligninolytic enzymes were composed of DyP and laccase based on the PeroxiBase and CAZy databases annotation. One DyP gene, 1251 bp, was identified in the genome of *B. subtilis* SCK6, which encoded 416 amino acids with an estimated molecular weight of 45.7 kDa and an isoelectric point value of 8.71. The cloned BsDyP from *B. subtilis* SCK6 shared 93% nucleic acid sequence identity and 96% amino acid identity with BsDyP from *B. subtilis* KCTC2023 [17].

*E. coli*, one of the most popular hosts for recombinant protein overexpression, was used as the expressing host to heterologously express BsDyP. Given that the ligninolytic enzyme DyP expressed in *E. coli* accumulated in inclusion bodies which required another refolding process [18–20], the cold shock-inducible expression system coupled with co-expression of chaperones was applied to obtain the active DyP. With the help of molecular chaperones groES, groEL and tig, BsDyP was successfully expressed in *E. coli* BL21 as a soluble form. A significant activity of DyP was detected in the sonicated cell supernatant, indicating the proper formation of disulfide bonds and heme incorporation into recombinant BsDyP. After purification by immobilized metal affinity chromatography, BsDyP showed a single band on SDS-PAGE gel, corresponding to the calculated molecular masses (Figure 1a).



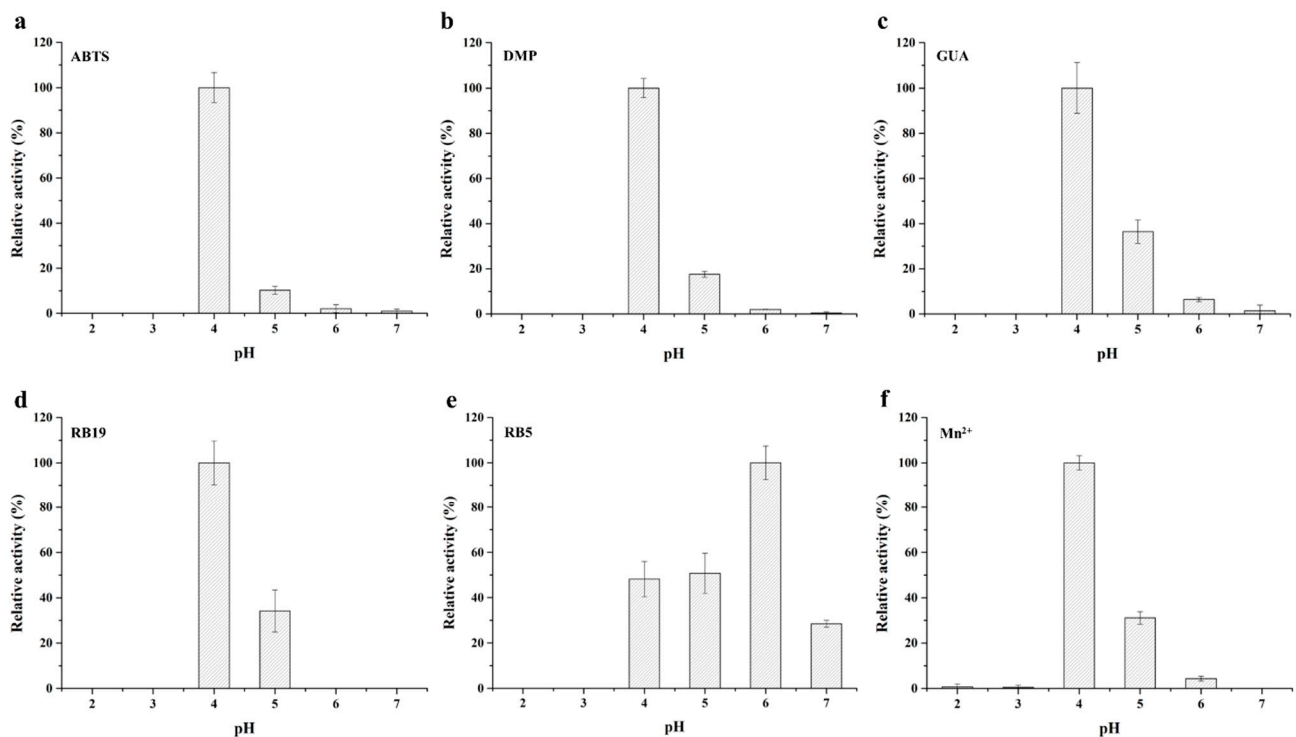
**Figure 1.** The analysis of purified recombinant *BsDyP* by SDS-PAGE (a) and UV-visible spectroscopy (b). Lanes: M, the protein molecular mass marker; 1, the whole-cell lysis protein; 2, the purified recombinant *BsDyP*.

## 2.2. Biochemical Characterization of the Purified Recombinant *BsDyP*

Like other ligninolytic peroxidases, including manganese peroxidase, versatile peroxidase and lignin peroxidase, *DyP* also showed the typical features of heme-containing enzymes with a Soret peak at 404–408 nm [21]. As shown in Figure 1b, *BsDyP* had an obvious absorbance peak at 407 nm, indicating that the heme group was indeed incorporated into the purified recombinant *BsDyP*. The R<sub>z</sub> ( $A_{407}/A_{280}$ ) ratio of the purified *BsDyP* was 1.7.

It had been reported that *DyP* could act on a broad range of substrates, including lignin-derived phenolic and non-phenolic compounds, synthetic high redox potential anthraquinone and azo dyes [19,22]. In order to explore its substrate specificity, *BsDyP* was tested on a series of lignin-derived compounds, synthetic dyes and  $Mn^{2+}$ . The purified recombinant *BsDyP* could oxidize the model substrate ABTS, phenolic compounds DMP and GUA, anthraquinone dye RB19, azo dye RB5 and  $Mn^{2+}$  except for the non-phenolic compound VA (Figure 2). Notably, *BsDyP* had the ability to oxidize  $Mn^{2+}$  to  $Mn^{3+}$ , which belongs to the catalytic properties of manganese peroxidase and versatile peroxidase [23,24]. The results suggested that *BsDyP* might also contain the manganese binding sites.

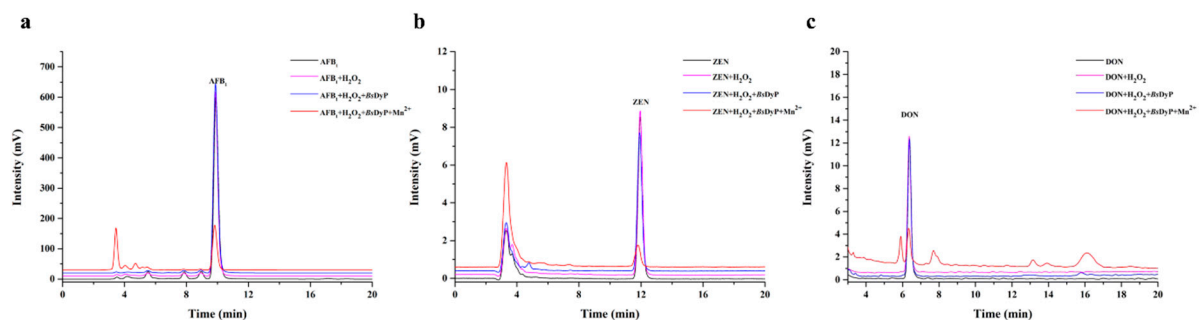
In addition, the optimum pHs of the purified recombinant *BsDyP* oxidizing various substrates, including ABTS, DMP, GUA, RB19 and  $Mn^{2+}$ , were all 4.0, except that a higher optimum pH of *BsDyP* against RB5 was 6.0. Meanwhile, *BsDyP* was completely inactive when the pH was below 3.0, which was different from fungal *DyP* with a wider acidic pH range [22,25]. The specific activity of *BsDyP* for ABTS, DMP, GUA, RB19 and  $Mn^{2+}$  at optimum pH were 7.25, 1.79, 0.03, 0.67 and 0.46 U/mg, respectively. Like other dye-decolorizing peroxidases, *DyPs* from *Pseudomonas putida*, *Thermobifida fusca* and *Thermomonospora curvata* showed higher specific activities towards ABTS and RB19, exhibiting two orders of magnitude higher than that of GUA [17,26,27]. However, the reason for difference in specific activities towards different substrates had not been well elucidated yet.



**Figure 2.** Optimum pH of the purified recombinant *BsDyP* oxidizing different substrates: ABTS (a), DMP (b), GUA (c), RB19 (d), RB5 (e) and Mn<sup>2+</sup> (f). Each assay was performed with three independent biological replicates.

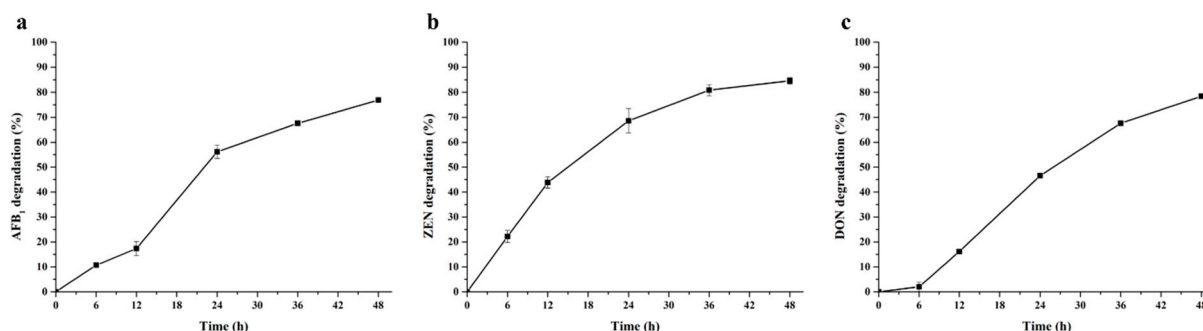
### 2.3. Degradation of Different Types of Mycotoxins by *BsDyP*

Although DyP was reported to be involved in lignin degradation, industrial dyes decolorization [21,28], the great application value of DyP in xenobiotic bioremediation gradually attracted more and more attentions in recent years [29,30]. In this study, the different types of mycotoxins including AFB<sub>1</sub>, ZEN and DON were used to evaluate the mycotoxin degrading capability of *BsDyP* from *B. subtilis* SCK6. As shown in Figure 3, *BsDyP* could efficiently degrade multiple mycotoxins in presence of Mn<sup>2+</sup>. AFB<sub>1</sub>, ZEN and DON were degraded up to 76.93%, 84.65% and 78.42% by the purified *BsDyP* within 48 h, respectively. Unlike AFB<sub>1</sub> and DON, ZEN also could be slightly degraded 11.78% by *BsDyP* in absence of Mn<sup>2+</sup>, indicating that there might be two different mechanisms of ZEN degradation by *BsDyP*. On one hand, it was similar to the detoxification mechanism of manganese peroxidases that oxidized Mn<sup>3+</sup> and malonate acid mediated degradation of mycotoxins through the formation of radicals [13]. On the other hand, ZEN might be directly degraded by binding to the catalytic pocket of *BsDyP*.



**Figure 3.** Degradation of different mycotoxins including AFB<sub>1</sub> (a), ZEN (b) and DON (c) by 1.25 U/mL *BsDyP* in 50 mM malonate buffer (pH 4.0) at 30 °C for 48 h.

The time courses of AFB<sub>1</sub>, ZEN and DON degradation by *BsDyP* in presence of Mn<sup>2+</sup> were shown in Figure 4. The degrading percentages of AFB<sub>1</sub> and ZEN were 10.68% and 11.78% at 6 h, and then gradually ascended to 17.37% and 43.84% at 12 h. More than 50% AFB<sub>1</sub> and ZEN degradation occurred after a 24 h incubation. In contrast, the initial degradation of DON was slower, with degradation percentages of 1.99% at 6 h, but DON content was significantly decreased at 12 h, and then 46.56% DON was degraded over 24 h of incubation.



**Figure 4.** Time-course analysis of AFB<sub>1</sub> (a), ZEN (b) and DON (c) by 1.25 U/mL *BsDyP* in 50 mM malonate buffer (pH 4.0) supplemented with 1 mM MnSO<sub>4</sub> and 0.1 mM H<sub>2</sub>O<sub>2</sub> at 30 °C. Each assay was performed with three independent biological replicates.

#### 2.4. Identification of AFB<sub>1</sub>, ZEN and DON Degradation Products

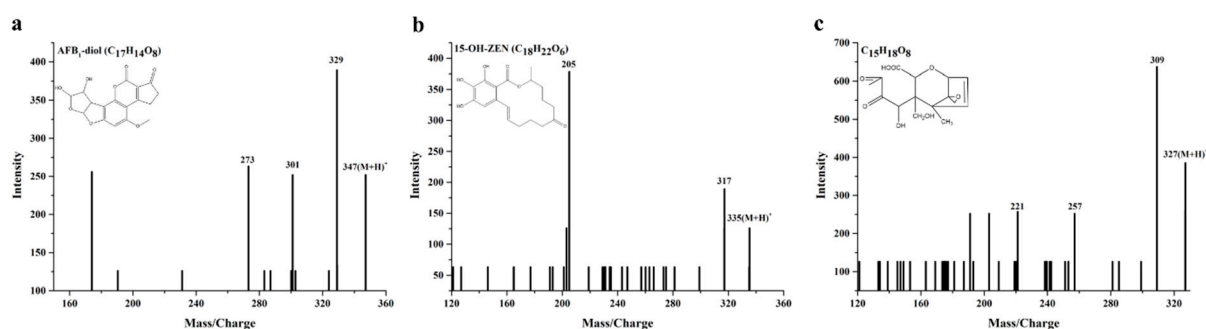
In order to further prove the effective detoxification of multiple mycotoxins by *BsDyP*, it is necessary to identify the corresponding degradation products of mycotoxins and analyze their biological toxicities. Based on the clear relationship between chemical structure and biological activities of AFB<sub>1</sub>, ZEN and DON [31,32], the toxicology of degradation products could judge from chemical structure changes of different mycotoxins.

The main degradation product of AFB<sub>1</sub> was identified by LC-MS/MS. The parent ion appeared at  $m/z$  347.07 [M + H]<sup>+</sup> and produced daughter ions of 329.06 [M – 18 + H]<sup>+</sup>, 301.07 [M – 46 + H]<sup>+</sup> and 273.08 [M – 74 + H]<sup>+</sup> (Figure 5a). These daughter ions were in accordance with the MS/MS fragments of AFB<sub>1</sub>-diol [33], suggesting that the C<sub>8-9</sub> double bond of terminal furan rings in AFB<sub>1</sub> was oxidized by *BsDyP* and two hydroxyl groups were added to the molecular generate AFB<sub>1</sub>-diol. Remarkably, AFB<sub>1</sub>-diol was less toxic than AFB<sub>1</sub> due to the removal of double bond in the terminal furan ring, which was considered as the basis for toxic and carcinogenic activity [32,34]. Similarly, the manganese peroxidase from the white rot fungus *Phanerochaete sordida* YK-624 could convert AFB<sub>1</sub> to AFB<sub>1</sub>-diol and effectively remove the mutagenic activity of AFB<sub>1</sub> [35]. Manganese peroxidases from the white rot fungus *Irpex lacteus* CD2 were able to oxidize AFB<sub>1</sub> to AFB<sub>1</sub>-8,9-epoxide as the intermediate product [13]. Thus, the epoxidation and hydrolysis of AFB<sub>1</sub> to produce AFB<sub>1</sub>-diol might be the common characteristic of AFB<sub>1</sub> degradation by Mn<sup>3+</sup> derived radicals.

The mass spectrum for the corresponding degradation product of ZEN is shown in Figure 5b. Daughter ions were obtained by fragmenting the parent ion with  $m/z$  335.30 [M + H]<sup>+</sup>, including 317.15 [M – 18 + H]<sup>+</sup> and 205.05 [M – 130 + H]<sup>+</sup>. These daughter ions were in agreement with the MS/MS fragments of 15-OH-ZEN [33], indicating that ZEN was hydroxylated at the C<sub>15</sub> position of aromatic moiety by *BsDyP* and 15-OH-ZEN was the major degradation product. The result revealed that there were significant differences between *DyP* and the well-known ZEN degrading enzyme lactonohydrolase ZHD101. Unlike the lactonohydrolase ZHD101, *BsDyP* hydroxylated the aromatic ring rather than cleaved the ester bond of the macrolactone ring in ZEN [36]. Meanwhile, it had been reported that the hydroxylation of aromatic moiety in ZEN exhibited a significantly decreased estrogenicity and the estrogenicity of 15-OH-ZEN was remarkably decreased by



98% compared with ZEN [37]. These indicated that it might be a novel strategy feasible for biodegradation of ZEN by *BsDyP* from *B. subtilis* SCK6.



**Figure 5.** HPLC–MS/MS analysis of AFB1 (a), ZEN (b) and DON (c) degradation products by 1.25 U/mL *BsDyP* in 50 mM malonate buffer (pH 4.0) supplemented with 1 mM  $MnSO_4$  and 0.1 mM  $H_2O_2$  at 30 °C for 48 h.

Although new product peaks of DON degradation by *BsDyP* appeared in HPLC analysis, only one of the main degradation products was identified by LC–MS/MS, exhibiting a parent ion peak at  $m/z$  327.11  $[M + H]^+$ . The corresponding daughter ions were  $m/z$  309.09  $[M - 18 + H]^+$ , 257.10  $[M - 70 + H]^+$  and 221.09  $[M - 106 + H]^+$ , which were consistent with the MS/MS fragments of  $C_{15}H_{18}O_8$  [31]. Although the enzymatic degradation mechanism of DON by *BsDyP* was unclear, the possible structure of the degradation product  $C_{15}H_{18}O_8$  was shown in Figure 5c. According to structure-activity relationship studies, the toxicity of  $C_{15}H_{18}O_8$  might be significantly reduced due to the breakage of  $C_{9-10}$  double bond and the loss of  $C_3$ -hydroxyl group [38]. To our knowledge, this is the first report about biotransformation of multiple mycotoxins, including AFB<sub>1</sub>, ZEN and DON by *DyP*. Moreover, the biological toxicities of each corresponding enzymatic degradation product, namely AFB<sub>1</sub>-diol, 15-OH-ZEN and  $C_{15}H_{18}O_8$ , were significantly lower than AFB<sub>1</sub>, ZEN and DON. Though the other degradation products and their biological toxicity remain to be unveiled, these results might be of great value for practical application of *BsDyP* in the biodegradation of multiple mycotoxins in animal feed and food.

### 3. Conclusions

In this study, a dye-decolorizing peroxidase gene was cloned from *Bacillus subtilis* SCK6, and then successfully expressed in *E. coli* BL21 as a soluble form, using the cold shock-inducible expression system coupled with co-expression of chaperones. The recombinant *BsDyP* could oxidize various lignin-derived phenolic compounds and high redox potential synthetic dyes, as well as  $Mn^{2+}$ . Moreover, *BsDyP* could efficiently degrade different types of mycotoxins, including AFB<sub>1</sub>, ZEN and DON, in the presence of  $Mn^{2+}$ . The major degradation products of multiple mycotoxins were AFB<sub>1</sub>-diol, 15-OH-ZEN and  $C_{15}H_{18}O_8$ , respectively. The predicted biological toxicity of each corresponding degradation product was significantly removed. Moreover, further studies are needed to confirm the detoxification effects for the pretreatment of contaminated food and animal feed. Nonetheless, the *BsDyP* exhibited a great application potential for the enzymatic degradation of different types of mycotoxins in animal feed and food.

### 4. Material and Methods

#### 4.1. Strain and Substrates

*B. subtilis* SCK6 was isolated and preserved in Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing, China. Substrates 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,6-dimethylphenol (DMP), guaiacol (GUA), veratryl alcohol (VA), reactive blue 19 (RB19), reactive black 5 (RB5) and mycotoxins, including AFB<sub>1</sub>, ZEN and DON, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hemin was purchased from TCI (Tokyo, Japan).

#### 4.2. Cloning and Expression of BsDyP

Based on the 5' and 3'-end sequences of the *BsDyP* structural gene, the *BsDyP* was amplified with gene-specific primers (*BsDyP*-NdeI-F: 5' ATCATCATATCGAAGGTAGGCATATGAGC GATGAACAGAAAAAGC 3'; *BsDyP*-XbaI-R: 5' TTTTAAGCAGAGATTACCTATCTAGATGAT TCCAGCAAACGCTG 3'). The PCR product was assembled into the linearized vector pCold I and then transformed into commercial *E. coli* BL21/pG-Tf2 chaperone competent cells (Takara, Japan), which harbored a plasmid pG-Tf2 encoding three molecular chaperones: groES, groEL and tig. The cells harboring pCold I-*BsDyP* were pre-cultured in LB medium supplemented with 100 µg/mL ampicillin and 20 µg/mL chloramphenicol at 37 °C overnight and used as the inocula of 300 mL LB medium containing 5 ng/mL tetracyclin, 100 µg/mL ampicillin and 20 µg/mL chloramphenicol. Tetracyclin was used as an inducer for the expression of molecular chaperones. The cultures were grown at 37 °C for 4 h, followed by the addition of 0.5 mM isopropyl-β-D-thiogalactoside (IPTG) and 20 µM hemin for 12 h induction at 16 °C.

#### 4.3. Purification and Characterization of BsDyP

After induction, the cells were harvested by centrifugation and resuspended in 30 mL binding buffer (20 mM sodium phosphate, 500 mM NaCl, pH 7.4). The cytosol containing the recombinant *BsDyP* was released by sonication at 130 W for 30 min. *BsDyP* was then purified using immobilized metal affinity chromatography with the washing buffer (20 mM sodium phosphate, 500 mM NaCl, 40 mM imidazole, pH 7.4) and elution buffer (20 mM sodium phosphate, 500 mM NaCl, 200 mM imidazole, pH 7.4). The purified recombinant *BsDyP* was verified by SDS-PAGE, using 10% polyacrylamide gel. The protein content was determined by the Bradford method, using bovine serum albumin as the standard.

The DyP activity was measured by monitoring the oxidation of ABTS ( $\epsilon_{420} = 36,000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ ) at 420 nm in the kinetic model by recording absorbance at 30 s intervals for 5 min, in a buffer containing 50 mM malonate, 1 mM ABTS and 0.1 mM H<sub>2</sub>O<sub>2</sub> (pH 5.0 and 25 °C). One unit of enzyme activity was defined as the amount of enzyme that oxidizes 1 µmol of ABTS per min at 25 °C. The purified recombinant *BsDyP* was first subjected to UV-visible spectroscopic analysis in the range of 230 to 800 nm in the 20 mM pH 5.0 malonate buffer. Then the substrate specificity of *BsDyP* was studied for the oxidation of different substrates, including ABTS, DMP ( $\epsilon_{470} = 12,100 \text{ M}^{-1} \cdot \text{cm}^{-1}$ ), GUA ( $\epsilon_{465} = 49,600 \text{ M}^{-1} \cdot \text{cm}^{-1}$ ), VA ( $\epsilon_{310} = 9300 \text{ M}^{-1} \cdot \text{cm}^{-1}$ ), RB19 ( $\epsilon_{595} = 10,000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ ), RB5 and Mn<sup>2+</sup> ( $\epsilon_{270} = 11,590 \text{ M}^{-1} \cdot \text{cm}^{-1}$ ), in 50 mM sodium malonate buffers with pH values ranging from 2.0 to 7.0 at 25 °C. The maximum activity of *BsDyP* was considered to be 100%.

#### 4.4. Degradation of Different Types of Mycotoxins

*BsDyP* (1.25 U/mL) was incubated with different types of mycotoxins (AFB<sub>1</sub> and ZEN 1 µg/mL; DON 10 µg/mL) in 50 mM pH 4.0 malonate buffer supplemented with 0.1 mM H<sub>2</sub>O<sub>2</sub> in the presence or absence of 1 mM MnSO<sub>4</sub>. The reaction was carried out at 30 °C for 48 h. Periodically, samples were taken out and equal volume methanol were added to stop the reaction.

HPLC analysis of AFB<sub>1</sub>, ZEN and DON degradation were performed by using a SHIMADZU 20A series instrument (Kyoto, Japan) equipped with a UV/Vis detector and RF-20A fluorescence detector with a Waters XBridge C18 column (5 µm, 4.6 mm × 150 mm). The elution condition for AFB<sub>1</sub> was set as follows: 45% methanol at a flow rate of 0.8 mL/min; AFB<sub>1</sub> was monitored at 360 nm excitation wavelength and 440 nm emission wavelength. The elution condition for ZEN was set as follows: 45% acetonitrile (ACN) at a flow rate of 0.8 mL/min; ZEN was monitored at 274 nm excitation wavelength and 440 nm emission wavelength. The elution condition for DON was set as follows: 10% ACN at a flow rate of 0.8 mL/min; DON were monitored at 218 nm.

AFB<sub>1</sub>, ZEN and DON degradation products were further analyzed by using LC-MS/MS, which was carried out by coupling a SHIMADZU Nexera UHPLC system (Kyoto,

Japan) to an AB-SCIEX 5600+ Triple TOF mass spectrometer. For LC analysis, the elution program was as follows: 0–70% ACN, 7 min; 70–100% ACN, 5 min; and 100% ACN, 1 min. For MS analysis, the parameters were set as ion source gases GS1 and GS2 and curtain gas, at 55, 55 and 25 psi, respectively; temperature was 500 °C; and ion spray voltage floating was at 5500 V.

**Author Contributions:** X.Q.: Conceptualization, Methodology, Investigation, Data Curation, Writing—Original Draft, Visualization. X.S.: Conceptualization, Investigation, Methodology. T.T.: Formal analysis, Investigation, Funding acquisition. J.Z.: Investigation, Data Curation, Visualization. X.W.: Validation, Formal analysis. Y.W. (Yaru Wang): Resources, Data Curation. Y.W. (Yuan Wang): Data Curation, Visualization. Y.B.: Resources. B.Y.: Supervision, Funding acquisition. H.L.: Conceptualization, Project administration, Writing—Review & Editing, Funding acquisition. H.H.: Resources, Investigation, Project administration. All authors have read and agreed to the published version of the manuscript

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