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Research Paper

Detoxification of aflatoxin B_1 by manganese peroxidase from the white-rot fungus *Phanerochaete sordida* YK-624

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19 mutagenic activity, detoxification

20

21 Running title: detoxification of AFB_1 by MnP

22

Abstract

Aflatoxin B_1 (AFB_1) is a potent mycotoxin with mutagenic, carcinogenic, teratogenic, hepatotoxic and immunosuppressive properties. In order to develop a bioremediation system for AFB_1-contaminated foods by white-rot fungi or ligninolytic enzymes, AFB_1 was treated with manganese peroxidase (MnP) from the white-rot fungus *Phanerochaete sordida* YK-624. AFB_1 was eliminated by MnP. Maximum elimination (86.0%) of AFB_1 was observed after 48 h in a reaction mixture containing 5 nkat of MnP. Addition of Tween 80 enhanced AFB_1 elimination. Elimination of AFB_1 by MnP greatly reduced its mutagenic activity in an umu test, and the treatment of AFB_1 by 20 nkat MnP reduced the mutagenic activity by 69.2%. ^{1}H-NMR and HR-ESI-MS analysis suggested that AFB_1 is first oxidized to AFB_1-8,9-epoxide by MnP and then hydrolyzed to AFB_1-8,9-dihydrodiol. This is the first report that MnP can effectively remove the mutagenic activity of AFB_1 by converting it to AFB_1-8,9-dihydrodiol.

1. Introduction

The human diet can contain a wide variety of natural carcinogens due to the contamination of raw materials or the production of metabolites during food processing or cooking (Osowski et al., 2010). Aflatoxins, a group of potent mycotoxins with mutagenic, carcinogenic, teratogenic, hepatotoxic, and immunosuppressive properties, are of particular importance because of their adverse effects on animal and human health (Lewis et al., 2005). Aflatoxins are produced as secondary metabolites of fungal strains (*Aspergillus flavus* Link:Fries*, A. parasiticus* Speare, and *A. nomius* Kurtzman et al.) that grow on a variety of food and feed commodities (Peltonen et al., 2001; Jiang et al., 2005). Aflatoxin B_1 (AFB_1), which is the most toxic aflatoxin, is of particular interest because it is a frequent contaminant of many food products and one of the most potent naturally occurring mutagens and carcinogens known (Teniola et al., 2005).

White-rot fungi have the apparently unique ability to degrade lignin to the level of CO_2 (Kirk & Farrell, 1987). Lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase are the major extracellular ligninolytic enzymes of white-rot fungi involved in lignin biodegradation (Kirk & Farrell, 1987). There is a great interest in lignin-degrading white-rot fungi and their ligninolytic enzymes because of their

potential for degrading recalcitrant environmental pollutants, such as polychlorinated dibenzodioxin (Kamei et al., 2005), lindene (Bumpus et al., 1985), chlorophenols (Joshi & Gold, 1993), and polycyclic aromatic carbons (Bezalel et al., 1996; Collins et al., 1996). Recently, ligninolytic enzymes such as MnP and laccase were shown to be effective in degrading of methoxychlor (Hirai et al., 2004) and Irgarol 1051 (Ogawa et al., 2004) and in removing the estrogenic activities of bisphenol A, nonylphenol (Tsutsumi et al., 2001), 4-*tert*-octylphenol (Tamagawa et al., 2007), butylparabens (Mizuno et al., 2009), genistein (Tamagawa et al., 2005), and steroidal hormones (Suzuki et al., 2003; Tamagawa et al., 2006). More recently, the degradation of AFB_1 by fungal laccases has been reported (Alberts et al., 2009). However, a degradation product was not detected and the mechanism of degradation remains unclear.

In the present study, we demonstrate the detoxification of AFB_1 by MnP from the white-rot fungus *Phanerochaete sordida* YK-624 which produces LiPs (Sugiura et al., 2003; Hirai et al., 2005) and MnP (Hirai et al., 1994; Kondo et al., 1994) as ligninolytic enzymes. We also detected the metabolites and, on their basis, developed a possible mechanism for their production.

2. Materials and methods

76 *2.1 Fungus*

77 *P. sordida* YK-624 (ATCC 90872) from rotten wood (Hirai et al., 1994) was used
78 in this study. The fungus was maintained on potato dextrose agar slants at 4°C.

79

80 *2.2 Chemicals*

81 AFB_1 was purchased from Wako Pure Chemical Industries, Japan. The umu test
82 with umulac AT (Protein Purify Ltd. Japan) was used to assay mutagenic activity. All
83 other chemicals were extra-pure grade and were used without further purification.

84

85 *2.3 MnP preparation and determination of MnP activity*

86 MnP from *P. sordida* YK-624 was prepared and purified by the modified method
87 described by Kondo et al. (Kondo et al., 1994). The MnP solution did not contain LiP
88 activity, and has been purified to homogeneity in SDS-PAGE. The purified MnP on IEF
89 showed one isoform (data not shown). MnP activity was measured by monitoring the
90 oxidation of 2,6-dimethoxyphenol to coerulignone (ε_{470} = 49.6 $mM^{-1}cm^{-1}$) (Pèriè and
91 Gold, 1991). The reaction mixture (1 ml) contained 2,6-dimethoxyphenol (1 mM),
92 $MnSO_4$ (1 mM), and H_2O_2 (0.2 mM) in 50 mM malonate, pH 4.5. One katal (kat) was
93 defined as the amount of enzyme producing 1 mol of product per second.

94

95 *2.4 MnP treatment of AFB_1*

96 MnP reactions were performed in 1 ml of reaction mixture containing 5 nkat MnP,
97 10 μl of 1 mM AFB_1 in 10% dimethylsulfoxide, 1 mM $MnSO_4$, 0.1% Tween 80, 4 nkat
98 glucose oxidase, and 2.5 mM glucose in 50 mM malonate, pH 4.5. Reactions were
99 performed in triplicate for 24 h at 30°C and mixing at 150 rpm. In some experiments,
100 the amount of MnP (1-20 nkat) and the reaction time (1-48 h) were changed, and Tween
101 80 was omitted. The amount of AFB_1 was determined by high-performance liquid
102 chromatography (HPLC) under the following conditions: column, Wakosil-II 5C18HG
103 (4.6 mm x 150 mm, Wako Pure Chemical Industries, Japan); mobile phase, 40%
104 aqueous methanol; flow rate, 0.5 ml/min; detection wavelength, 365 nm.

105

106 *2.5 Mutagenic activity of AFB_1*

107 The umu test with umulac AT was used to assay the mutagenic activity of AFB_1
108 (Oda et al., 1995). The test was performed with *Salmonella typhimurium* TA1535 and
109 S9 liver homogenate. The TA1535 strain was constructed by subcloning the bacterial
110 *O*-acethyltransferase gene into a plasmid vector pACYC184 and introducing the
111 plasmid into the original strain *S. typhimurium* TA1535/pSK1002 strain harboring an

112 *umuC*'–'*lacZ* fusion gene. Assays were carried out in triplicate using 10 μl of test
113 sample, 10 μl of S9mix (a metabolic activation system based on S9 liver homogenate),
114 and 100 μl of bacterial culture. After incubation for 2 h at 37°C, 100 μl of X-Gal
115 solution was added to each well, and after 1 h at 37°C, the reaction was stopped by the
116 addition of SDS/dimethylsulfoxide solution. The absorbance of the mixture was read at
117 600 nm. The relative mutagenic activity (%) was defined as the percentage of
118 β-galactosidase activity of the AFB_1-containing reaction mixture (with 5, 10, or 20 nkat
119 MnP) divided by the activity of the AFB_1-containing reaction mixture without MnP.
120
121 *2.6 Metabolism experiments*
122 AFB_1 (final concentration 160 μM) was incubated at 30°C for 48 h in a 100-ml
123 reaction mixture containing 750 nkat MnP, 1 mM $MnSO_4$, 0.1% Tween 80, 600 nkat
124 glucose oxidase, and 2.5 mM glucose in 50 mM malonate buffer, pH 4.5. The reaction
125 mixture was extracted twice with 100 ml ethyl acetate. The extract was dried over
126 anhydrous sodium sulfate and then evaporated to dryness. The concentrate was
127 separated by HPLC to isolate the AFB_1 metabolite. The purified metabolite was then
128 analyzed by HR-ESI-MS (JMS-T100LC, JEOL, Japan) and ^{1}H-NMR (Jeol lambda-500,
129 500 MHz, JEOL, Japan). Chemical shifts are expressed in δ relative to the external

130 standard, sodium 3-(trimethylsilyl) propionate.

131

132 **3. Results**

133

134 *3.1 Elimination of AFB_1 by MnP from P. sordida YK-624*

135 We previously showed that ligninolytic enzymes from white-rot fungi can
136 degrade a wide range of aromatic compounds (Tsutsumi et al., 2001; Suzuki et al.,
137 2003; Hirai et al., 2004; Tamagawa et al., 2005; Tamagawa et al., 2006; Tamagawa et
138 al., 2007; Mizuno et al., 2009). In the current study, we examined whether MnP from *P.*
139 *sordida* YK-624 can oxidize AFB_1, which is a difuranocoumarin derivate.

140 After a 24-h reaction using 5 nkat MnP, the level of AFB_1 was reduced by 73.3%
141 (Fig. 1). Further examination of the dose-dependence showed that maximum
142 elimination was obtained at 5 nkat of enzyme. Tween 80, an unsaturated fatty acid that
143 allows MnP to oxidize nonphenolic compounds (Bao et al., 1994), enhanced the
144 elimination of AFB_1 (Fig. 1). Analysis of the time course of AFB_1 elimination by MnP
145 in the presence of Tween 80 (Fig. 2) reveals that AFB_1 was drastically decreased after a
146 4-h treatment, and that 86.0% of AFB_1 was eliminated after a 48-h treatment.

147

148 *3.2 Removal of mutagenic activity of AFB_1*

149 Because removal of toxicity is essential for the biodegradation of environmental
150 pollutants, we examined the mutagenic activity of the metabolites of AFB_1 generated by
151 MnP. Mutagenic activity was measured with the umu test following treatment of AFB_1
152 by a metabolic activation system (S9mix) because, in animals, the toxicity of AFB_1 is
153 activated by cytochrome P450 in the liver (Eaton & Gallagher, 1994). AFB_1 (100 μM)
154 had approximately 7-fold higher mutagenic activity than 2-aminoanthracene (100 μM),
155 a well-known mutagen (Fig. 3). Treatment of AFB_1 by 5 and 20 nkat MnP reduced the
156 mutagenic activity by 49.4% and 69.2%, respectively (Fig. 4).

157

158 *3.3 Identification of an AFB_1 metabolite generated by MnP*

159 HPLC detected a metabolite generated by MnP from AFB_1 with a retention time of
160 10.5 min, whereas AFB_1 has a retention time of 32.8 min (Fig. 5). The metabolite was
161 fractionated and purified by HPLC and then analyzed by ^{1}H-NMR and HR-ESI-MS.
162 The ^{1}H-NMR spectrum in the presence of CD_3OD gave strong C8 and C9 proton
163 signals (δ_H 4.54 and 3.44, respectively) in the upper field compared to AFB_1 (AFB_1 H8
164 [δ_H 6.78], AFB_1 H9 [δ_H 6.44]). HR-ESI-MS, which gave a *m*/*z* of 345.06229 $[M-H]^-$
165 (calcd. for $C_{17}H_{13}O_8$, 345.06104) indicated a molecular formula of $C_{17}H_{14}O_8$, suggesting

166 a molecular mass of 346. The metabolite had a mass 34 greater than the molecular ion
167 of AFB_1. These results indicate that AFB_1 was converted to AFB_1-8,9-dihydrodiol by
168 MnP.
169
170 **4. Discussion**
171
172 The extracellular ligninolytic enzymes produced by white-rot fungi are
173 nonspecific and nonstereoselective enzymes that can degrade not only lignin but also a
174 range of recalcitrant pollutants, making them of great interest for the removal of
175 environmental contamination (Asgher et al., 2008). In the present study, we showed that
176 AFB_1, which is a non-phenolic, difuranocoumarin derivate, can be oxidized by MnP
177 from *P. sordida* YK-624.
178 MnP removed approximately 70% of AFB_1 after 24 h and was capable of
179 removing AFB_1 even in the absence of Tween 80. Although the complete elimination of
180 AFB_1 was not observed in the present study, it is thought that AFB_1 is completely
181 eliminated by the multi-treatment with MnP. Mn(III), which is produced by MnP, could
182 not oxidize AFB_1 directly (data not shown). In the presence of Tween 80, lipid-derived
183 peroxy radicals are produced (Bao et al., 1994) that may directly oxidize AFB_1. On the

184 other hand, formate and superoxide anion radicals, which are generated in the MnP
185 reaction mixture in the absence of Tween 80 (Khindaria et al., 1994), may mediate the
186 oxidation of AFB_1 by MnP alone.

187 AFB_1 -8,9-dihydrodiol was generated as a metabolite generated from AFB_1 by
188 MnP. This metabolite has also been detected in some animals treated with AFB_1 (Wu et
189 al., 2009). AFB_1 -8,9-dihydrodiol is produced in some animals by the hydrolysis of
190 AFB_1-8,9-epoxide, which is formed when the 8,9-vinyl bond is oxidized by the
191 microsomal cytochrome P450 system (Kuilman et al., 2000). Our current results suggest
192 that similar reactions, namely the epoxidation of AFB_1 followed by hydrolysis of
193 AFB_1-8,9-epoxide, occur when AFB_1 is oxidized by MnP. As detailed in Fig. 6, we
194 propose that the 8,9-vinyl bond of AFB_1 can be oxidized by the peroxy radicals of
195 Tween 80, formate radical, superoxide anion radical, or MnP directly (Tuynman et al.,
196 2000) and that the epoxide thus generated is spontaneously hydrolyzed to AFB_1
197 -8,9-dihydrodiol (Guengerich et al., 1996).

198 Removal of toxicity is the most important goal for the biodegradation of
199 environmental pollutions. We showed here that MnP not only removes but detoxifies
200 AFB_1. The metabolite generated from AFB_1 by MnP, AFB_1 -8,9-dihydrodiol, is less
201 toxic than AFB_1 because AFB_1-8,9-dihydrodiol can rearrange and form a reactive

202 dialdehyde that can react with primary amine groups in proteins by Schiff base reactions
203 (Sabbioni et al., 1987). This prevents the formation of DNA adducts, which can cause
204 mutations. Although AFB_1 eliminations by MnP (5-20 nkat) were almost the same, the
205 decrease in mutagenic activity was higher with 20 nkat MnP (69.2%) than with 5 nkat
206 MnP (49.4%), as shown in Fig. 4. It is thought that the amount of AFB_1-8,9-epoxide in
207 the reaction mixture containing 5 nkat MnP was higher than that in the reaction mixture
208 containing 20 nkat MnP.

209 In summary, we show for the first time that MnP can remove the mutagenic
210 activity of AFB_1 by converting it to AFB_1-8,9-dihydrodiol. This system should therefore
211 be useful in the bioremediation of AFB_1-contaminated foods.

212

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214

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319

Figure Legends

Fig. 1. Elimination of AFB_1 in the presence of different activities of MnP. Close circles, with Tween 80; open circle, without Tween 80. MnP reactions were performed in 1 ml of reaction mixture containing 1-20 nkat MnP, 10 μl of 1 mM AFB_1 in 10% dimethylsulfoxide, 1 mM $MnSO_4$, 0.1% Tween 80, 4 nkat glucose oxidase, and 2.5 mM glucose in 50 mM malonate, pH 4.5. Reactions were performed for 24 h at 30°C and mixing at 150 rpm. Values are means ± SD of triplicate samples.

Fig. 2. Time course for AFB_1 elimination by MnP. Reactions contained 5 nkat MnP, 10 μl of 1 mM AFB_1 in 10% dimethylsulfoxide, 1 mM $MnSO_4$, 0.1% Tween 80, 4 nkat glucose oxidase, and 2.5 mM glucose in 50 mM malonate, pH 4.5. Reactions were performed for 24 h at 30°C and mixing at 150 rpm. Values are means ± SD of triplicate samples.

Fig. 3. Mutagenic activity of AFB_1 in the umu test. Close circles, AFB_1; open circle, 2-aminoanthracene. Experimental details were described in Materials and methods. Values are means ± SD of triplicate samples.

338

339 **Fig. 4.** MnP decreases the mutagenic activity of AFB_1. MnP reactions were performed

340 in 1 ml of reaction mixture containing 5-20 nkat MnP, 10 μl of 1 mM AFB_1 in 10%

341 dimethylsulfoxide, 1 mM $MnSO_4$, 0.1% Tween 80, 4 nkat glucose oxidase, and 2.5 mM

342 glucose in 50 mM malonate, pH 4.5. Reactions were performed for 24 h at 30°C and

343 mixing at 150 rpm. Values are means ± SD of triplicate samples.

344

345 **Fig. 5.** Detection of the AFB_1 metabolite by HPLC (a) and ESI-MS spectra of AFB_1

346 metabolite (b) and AFB_1 (c). These compounds were detected by HPLC under the

347 following conditions: column, Wakosil-II 5C18HG; mobile phase, 40% aqueous

348 methanol; flow rate, 0.5 ml/min; detection wavelength, 365 nm.

349

350 **Fig. 6.** Proposed mechanism of AFB_1 oxidation by MnP.

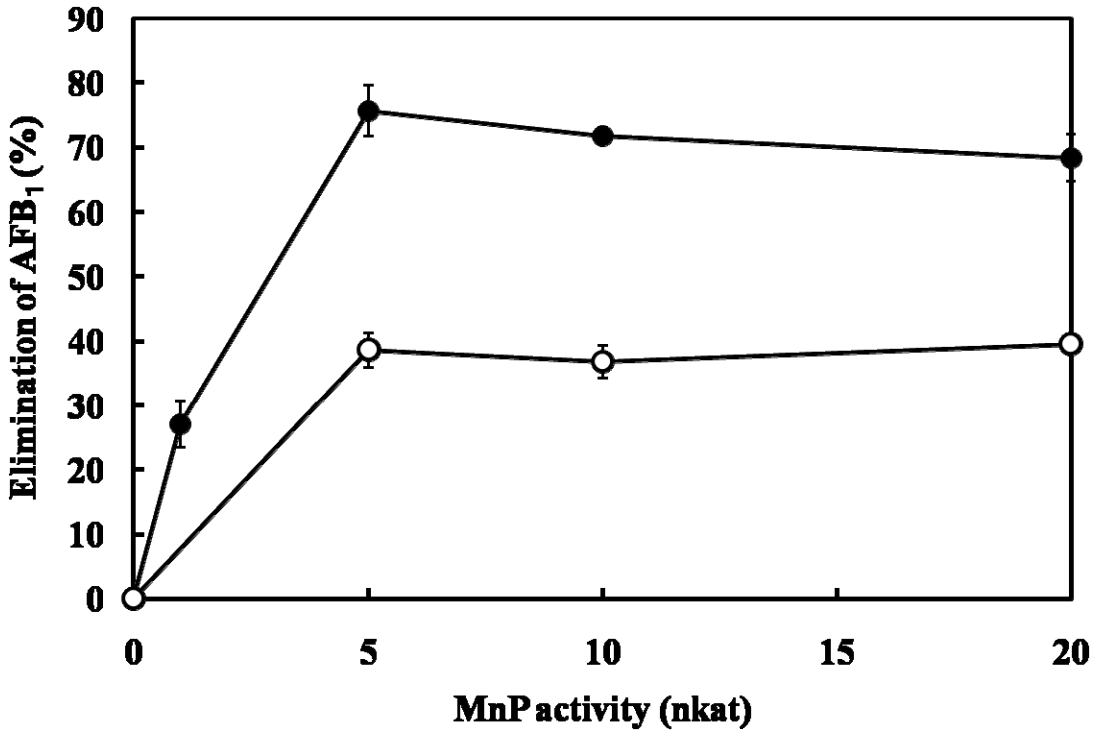
90
80
70
60
50
40
30
20
10
0
Elimination of AFB1 (%)
0
5
10
15
20
MnP activity (nkat)

Fig. 1

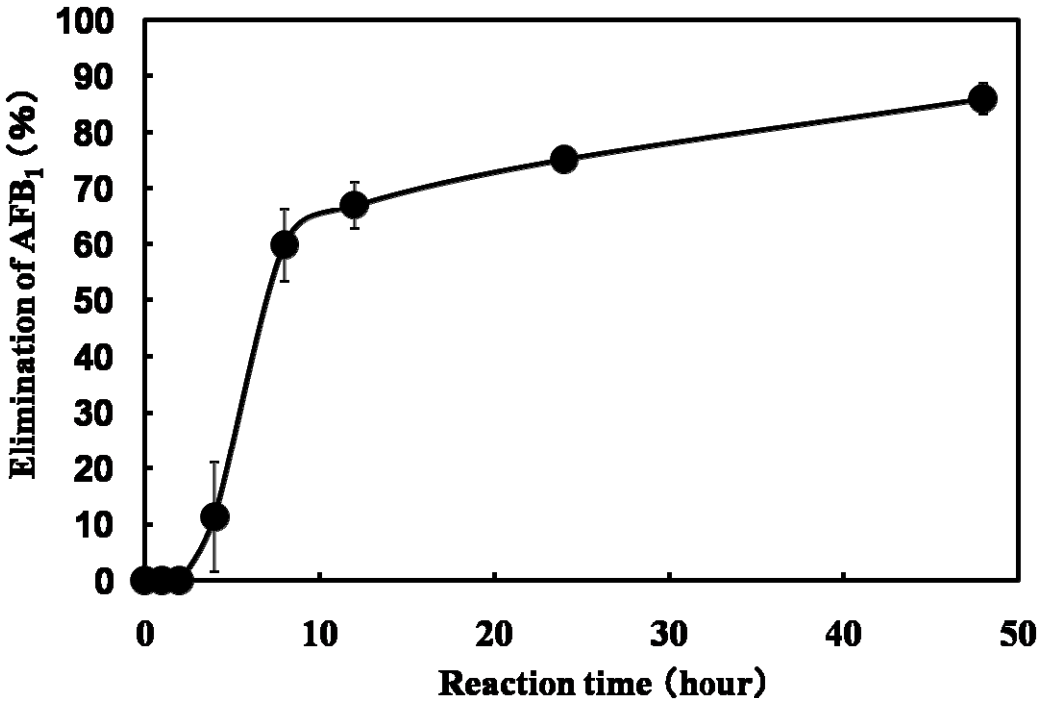
100
90
80
70
60
50
40
30
20
10
0
Elimination of AFB1 (%)
0
10
20
30
40
50
Reaction time (hour)

Fig. 2

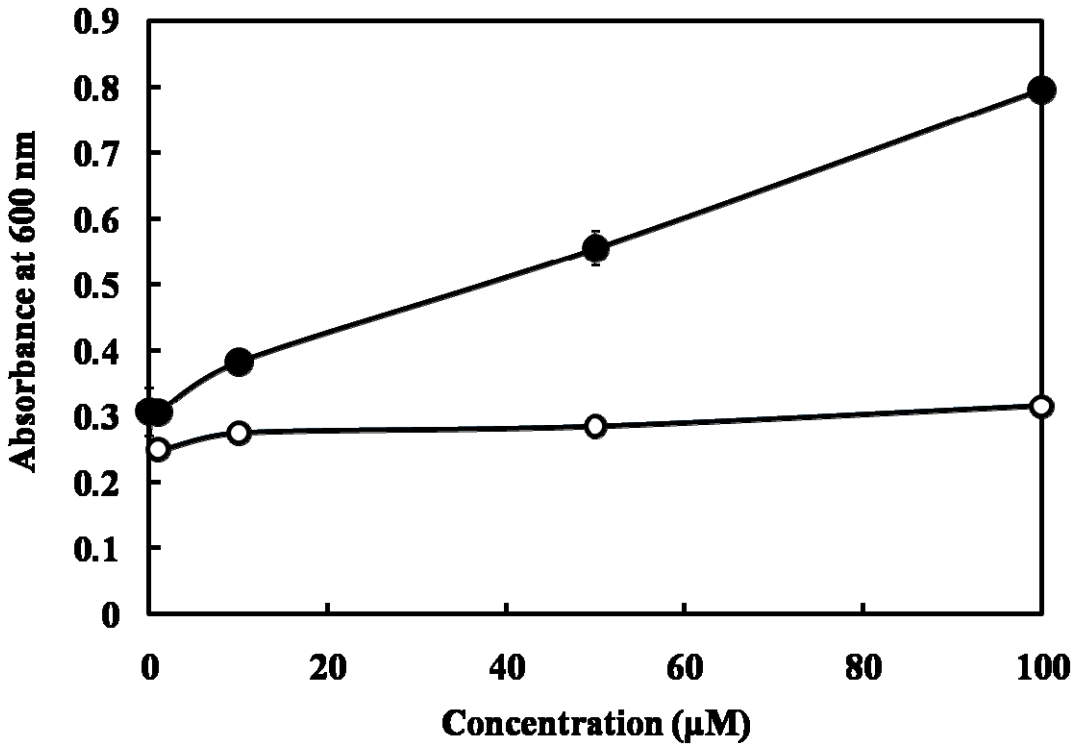
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Absorbance at 600 nm
0
20
40
60
80
100
Concentration (μM)

Fig. 3

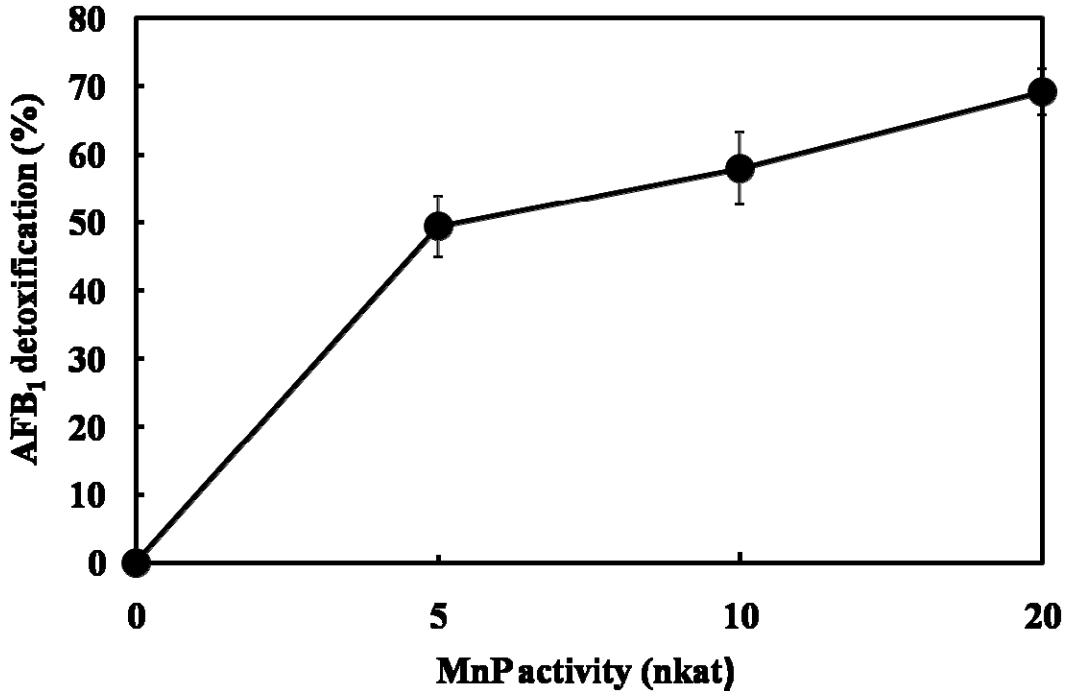
80
70
60
50
40
30
20
10
0
AFB1 detoxification (%)
0
5
10
20
MnP activity (nkat)

Fig. 4

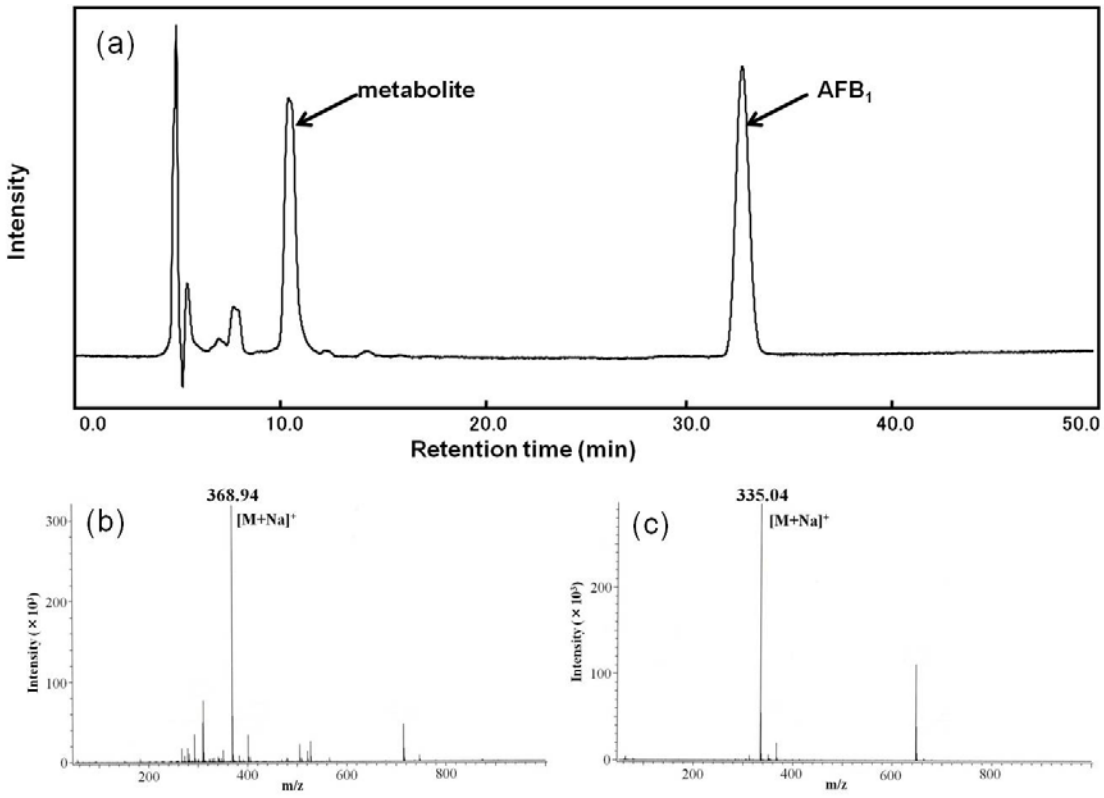
(a)
metabolite
AFB1
Intensity
0.0
10.0
20.0
30.0
40.0
50.0
Retention time (min)
(b)
368.94
[M+Na]+
Intensity (× 10⁴)
m/z
(c)
335.04
[M+Na]+
Intensity (× 10³)
m/z

Fig. 5

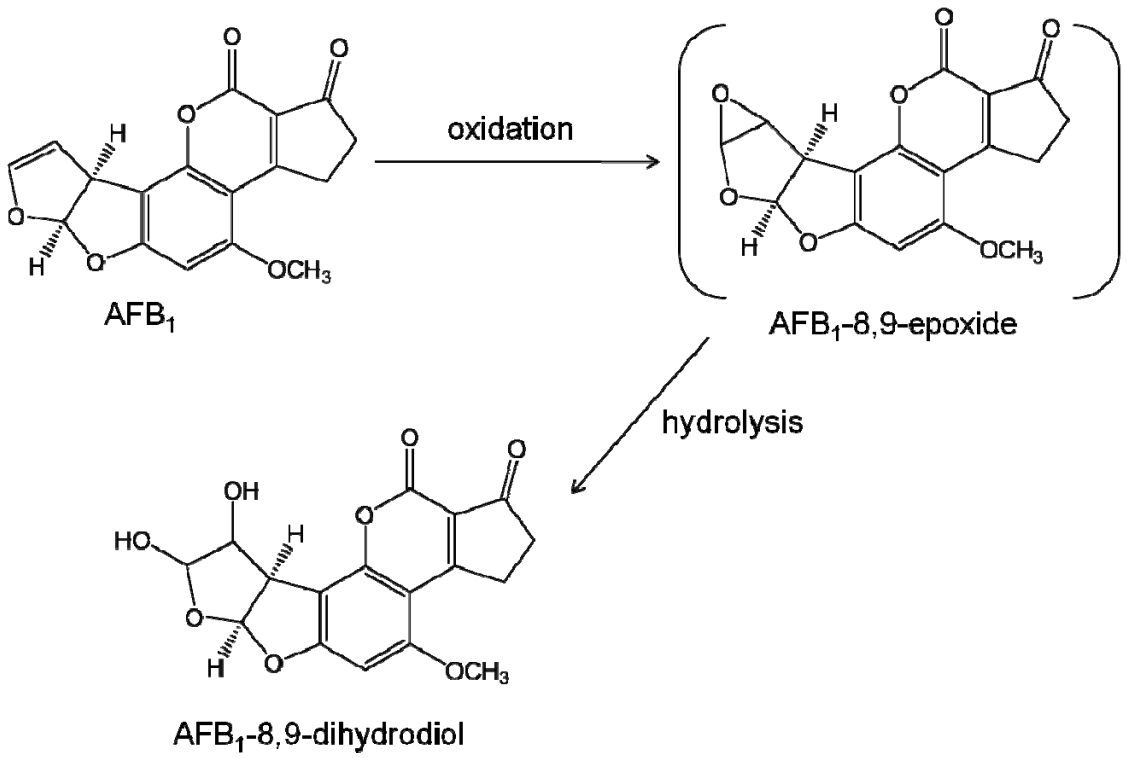
oxidation
AFB1
AFB1-8,9-epoxide
hydrolysis
AFB1-8,9-dihydrodiol

Fig. 6