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Review

Hydrolysis mechanism of carbendazim hydrolase from the strain *Microbacterium* sp. djl-6F

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ABSTRACT

The carbendazim (MBC) hydrolyzing enzyme gene was cloned and heterologously expressed in *Escherichia coli* BL21 (DE3) from a newly isolated MBC-degrading bacterium strain *Microbacterium* sp. strain djl-6F. High performance liquid chromatography-mass spectrometry (HPLC-MS) analysis revealed that purified MheI-6F protein catalyzes direct hydrolysis of MBC into 2-aminobenzimidazole (2-AB) with a high turnover rate and moderate affinity (K_m of 6.69 μmol/L and k_{cat} of 160.88/min) without the need for any cofactors. The optimal catalytic condition of MheI-6F was identified as 45°C, pH 7.0. The enzymatic activity of MheI-6F was found to be diminished by metal ions, and strongly inhibited by sodium dodecyl sulfate (SDS). Through generating amino acid mutations in MheI-6F, Cys16 and Cys222 were identified as the catalytic groups that are essential for the hydrolysis of MBC. This is the first report on the biodegradation of MBC at the enzymatice level.

Introduction

Carbendazim (methyl-1H-benzimidazol-2-ylcarbamate, or MBC) is one of the most active compounds of benzimidazole fungicides which have both protective and curative activities against a wide range of fungal diseases in crops (Boudina et al., 2003; Chen and Zhou, 2009; Wang et al., 2012). MBC is also the active substance and the main degradation product of the other benzimidazole fungicides such as benomyl or thiophanate-methyl (Sandahl et al., 2000; Cuppen et al., 2000; Mazellier et al., 2003). MBC is naturally stable in soil, with a half-life of up to 1 year (Jones et al., 2004); its persistence in soil and water can lead to the contamination of crops (Arora et al., 2008; Carbo et al., 2007). Studies have shown that MBC has toxic effects on mammalian liver and endocrine and reproductive organs (Selmanoglu et al., 2001; Farag et al., 2011). Thus, growing concerns about the nontarget toxicity of MBC have led to the requirement for identifying new methods to eliminate environmental MBC contamination safely and efficiently.

Microbial metabolism has been shown to be the most effective method for degrading MBC in natural soil (Xu et al., 2006). Several research groups have isolated strains of MBC-degrading bacteria from MBC-contaminated soil (Holtman and Kobayashi, 1997; Zhang et al., 2005, 2013;

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Pandey et al., 2010). However, compared with microbial bioremediation, enzymatic bioremediation has stronger substrate affinity and higher substrate specificity. Moreover, enzymes are usually biodegradable in the natural soil (Ahuja et al., 2004). The MBC-hydrolyzing enzyme (MheI) was purified for the first time by molecular-size cut off membranes from the *Nocardioides* sp. strain SG-4G in 2010 (Pandey et al., 2010). In our present work, we cloned and expressed the *mheI* gene from a newly isolated MBC-degrading bacterium strain *Microbacterium* sp. djl-6F. We further investigated the hydrolysis properties of MheI-6F, and characterized the potential of utilizing strain djl-6F in the construction of an MBC-degrading engineered bacteria strain. Through hydrolysate analysis and generating MheI-6F with cysteine mutations, the mechanism of the catalytic process was further addressed in our study.

1. Materials and methods

1.1. Isolation and identification of MBC-degrading strain

Strain djl-6F was isolated from soil samples collected from a vegetable green house in Yangling District (Shaanxi Province, China), which has a 5-year history of repeated MBC applications. The isolate djl-6F is a Gram-negative, aerobic and bulbiform bacterium. The colony morphology of the isolate djl-6F on plain agar plate was orange, smooth, and eminent. Based on the colony morphology, 16S rDNA sequencing, and physical and biochemical characteristics (data not shown), djl-6F was identified as a member of *Microbacterium* sp. Mineral salts medium, supplemented with 40 μmol/L MBC (for 8 ppm solubility in water), was used as the sole carbon source for the culture of *Microbacterium* sp. strain djl-6F.

1.2. *mheI* gene cloning

The coding sequence of the MBC-hydrolyzing esterase derived from the strain djl-6F was cloned by polymerase chain reaction (PCR). The genomic DNA of strain djl-6F was used as the template for PCR. The primers were designed according to the coding sequence of the MBC-hydrolyzing esterase (*mheI*) from *Nocardioides* sp. strain SG-4G (GenBank accession GQ454794) (Pandey et al., 2010). The sequences of the primers were: Mhe1–1 (5′-CGCCATGGGCATGGCCAACTTCGTCCTCGTG-3′) and Mhe1–2 (5′-GCCTCGAGGCCCAGCGCCGCCAGCTTCT-3′). The PCR was performed as the following program: 96°C for 1 min, 35 cycles of 96°C for 15 sec, 64°C for 20 sec, 72°C for 40 sec. The PCR product was examined by gel electrophoresis. The band with the expected size was cut and purified using a gel extraction kit (DP209, TIANGEN Inc., China). Purified DNA was sequenced by Beijing Aoke Biotechnology Co., Ltd., China. The sequencing result was aligned with a known *mheI* sequence using BLAST (www.ncbi.nlm.nih.gov/Blast). The codon of *mheI*-6F was optimized for better heterologous expression, and synthesized by Beijing Aoke Biotechnology Co., Ltd., China. Cloned *mheI*-6F DNA was digested by restriction enzymes *NcoI* and *XhoI* and inserted into the *NcoI-XhoI* site of pET28a (Novagen Co., Germany) by ligation reaction. Plasmid pET28a-*mheI*-6F was transformed into *E. coli* TOP10 and expressed in *E. coli* BL21 (DE3).

1.3. Prokaryotic expression of recombinant MheI-6F

E. coli BL21 (DE3) that was transformed with pET28a-*mheI*-6F plasmid was cultured in 5 mL LB medium supplemented with 50 μg/mL kanamycin, at 37°C overnight. Cultured *E. coli* BL21 (DE3) was then transferred into fresh LB medium at the ratio of 1:100 and cultured for another 2 hr at 37°C, followed by another 8 hr at 25°C with isopropyl-β-D-thiogalactopyranoside (IPTG) at the final concentration of 0.5 mmol/L. Cultured cells were centrifuged at 4°C, 10,000 r/min for 10 min. Selecta sonopuls (SJIA-950W, Sjialab Equipment Co., China) was used to lyse the cells. The cell lysis was centrifuged at 4°C, 7000 r/min for 10 min. Protein in the supernatant phase was purified using cobalt-affinity chromatography. The 2.0 μg purified protein and was then analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Mini Protean 3 Cell, BIO-RAD Co., USA). The molecular mass of the enzyme subunit was estimated by running the protein standards on the same gel. An amicon ultra centrifugal filter unit with an ultracel-10 membrane was used for concentrating the target protein. Protein concentration of the purified enzyme was measured by BCA protein quantification kit (Heart Biological Technology Inc., China).

1.4. Enzyme assays

Enzymatic activity of MheI-6F was examined using MBC as the substrate. Samples were pretreated according to a protocol adapted from Xu et al. (2006) and improved for determining the catalytic activity of MheI-6F in our study. Five milliliters of hydrolyzing system that includes 0.2 mol/L disodium hydrogen phosphate—0.1 mol/L citric acid buffer solution (pH 7.0) and 40 μmol/L MBC was pipetted into three 10 mL flasks. MheI-6F was added to each flask at the final concentration of 0.10 μg/mL. The mixtures were then incubated at 37°C for 1 hr, and warmed in a hot-water bath for 5 min to terminate the reaction. Five milliliters of ethyl acetate was added to each flask to terminate enzyme activity after 1 hr incubation. After 2 min of extraction by ethyl acetate, the organic phase was collected and dried over anhydrous sodium sulfate. Hydrolysis of MBC by MheI-6F was measured by examining the absorbance of MBC at 287 nm by ultraviolet spectrophotometry (U-3310, Hitachi Limited, Japan).

1.5. Determination of MheI-6F activity and stability

To determine the effect of pH on MheI-6F activity, citric acid-disodium hydrogen phosphate buffer solution (pH 4.0–8.0) and glycine-NaOH buffer (pH 9.0–10.0) with 40 μmol/L MBC were used for the assay. Before measuring the enzyme activity, 0.50 μg MheI-6F was incubated in 5 mL buffer at 37°C for 1 hr. To determine the optimal temperature for MheI-6F activity, 40 μmol/L MBC and 0.50 μg MheI-6F were incubated at 4°C, 10°C, 20°C, 30°C, 35°C, 40°C, 45°C, 50°C, 60°C and 70°C for 1 hr with a constant pH of 7.0 in 5 mL citric acid-disodium hydrogen phosphate buffer solution before measuring the enzyme activity. The pH stability of MheI-6F was examined by pre-incubating 0.50 μg MheI-6F in the above citric acid-disodium hydrogen

phosphate buffer (pH 4.0–8.0) and glycine-NaOH buffer (pH 9.0–10.0) for 2 hr at 37°C. Temperature stability was measured by pre-incubating 0.50 μg MheI-6F in pH 7.0 citric acid-disodium hydrogen phosphate buffer solution for 2 hr at different temperatures. The enzyme stability was measured by the remaining MBC hydrolase activity after different treatments.

The effect of various chemicals and metal ions on enzyme activity was investigated by individually adding 1 mmol/L of the following test compounds: K^+, Mg^{2+}, Zn^{2+}, Fe^{3+}, Li^+, Mn^{2+}, Ca^{2+}, Tween-20, sodium dodecyl sulfate (SDS), chelating agents ethylen–ediamine tetraacetic acid (EDTA), β-mercaptoethanol; glycerol and natriumazid. The mixtures were incubated in citric acid-disodium hydrogen phosphate buffer (pH 7.0) at 37°C for 1 hr. The activity of 0.50 μg MheI-6F was then measured using 40 μmol/L MBC as the substrate.

1.6. MBC-hydrolyzing product and kinetic parameters analyses

The metabolites produced during carbendazim biodegradation were analyzed using HPLC-MS after incubation for 72 hr and 90 min with strain djl-6F and hydrolase MheI-6F, respectively. The cultures were centrifuged (5000 r/min for 10 min), and extracted with the same volume of ethylacetate. The organic phase was collected in flat-bottom flasks through anhydrous sodium sulfate and concentrated almost to dryness with a slight nitrogen stream. Methanol was added to dissolve carbendazim and the possible metabolites, and the volume was made up to 5 mL, then analyzed by HPLC-MS. The HPLC was equipped with an ion trap mass spectrometer (LTQ-XL, Thermo, CA, USA) and fitted with an electrospray interface (ESI) source and the Xcalibur software for data acquisition and analysis. Chromatographic separations of MBC and its degradation were carried out using a Waters XTerra C_{18} reverse-phase column (150 × 4.6 mm i.d., 5 μm, Nacalai Tesque, Inc., Japan) with the 0.5-mL/min linear mobile phase gradient. The elution consisted of a linear gradient program from 10% to 100% methanol (A) in aqueous solution containing 0.1% formic acid and 10 mmol/L ammonium formate (B) over 25 min. The gradient conditions were as follows: 10%–80% A (0–5 min); 80%–100% A (5–8 min); 100% A (8–18 min); 100%–10% A (18–20 min); 10% A (20–25 min). The column was set at room temperature (ca. 25°C) and the injection volume was 5 μL. The column elution was directed to a mass spectrometer, operating in full-scan MS acquiring mass spectra in positive mode. Optimal source voltages were established for the probe and ion source components to produce the maximum intensity of $[M + H]^+$ ions of MBC while infusing the standard into the source. The electrospray source was operated at a needle voltage of 4.5 kV with pneumatically assisted nebulization of the liquid flow (nitrogen sheath and auxiliary gas: 35 and 5 L/min) and a heated capillary temperature of 320°C.

The steady-state kinetic parameters of MheI-6F were determined by recording the hydrolysis consumption rate for the concentration of MBC varying between 5 and 50 mmol/L. Michaelis constant (K_m), maximum reaction velocity (V_{max}), and turnover number (k_{cat}) were determined according to the Michaelis–Menten kinetic equation. All experiments were carried out at least in triplicate, and the results are presented as mean ± standard deviation.

1.7. Sulfhydryl blocking

Fifty microliters of 0.2 mol/L sodium iodoacetate was pipetted into a 1.5 mL microcentrifuge tube as the sulfur blocking agent, and 50 μL of 0.10 mg/mL MheI-6F was added to the tube. After 2 hr incubation at 37°C, residual enzyme activity was measured as described above to determine the catalysis of sulfhydryl.

1.8. Cysteine mutation

The oligonucleotide primers, each complementary to the opposite strand of the vector pET28a-*mheI*-6F, were used to generate cysteine mutations, which resulted in replacement of cysteine at the positions 16, 62, 140, 165, and 222 into threonine, respectively. The sequences of the primers are listed in Table 1 for details. Mutations were generated according to the protocol of QuikChange™ site-directed mutagenesis kit (Stratagene Inc., USA). The PCR was performed with 8 cycles of program 1: 96°C for 30 sec, 60°C for 30 sec, 72°C for 5 min with primer 1–1 or 1–2 as the single primer. After mixing 2 PCR products rapidly, the reaction continued with 18 cycles of program 2: 96°C for 30 sec, 60°C for 30 sec, and 72°C for 5 min. The final PCR product was digested with *Dpn* I endonuclease and transformed into *E. coli* TOP10 cells for sequencing. The colonies with the expected sequence were selected for prokaryotic expression in *E. coli* BL21 (DE3) as wild-type MheI-6F.

2. Results and discussion

2.1. Cloning, expression and purification of recombinant MheI-6F

The primers for cloning the *mheI* coding sequence were designed according to the known sequence of the *mheI* gene from *Nocardioides* sp. SG-4G (Pandey et al., 2010). The coding sequence of *mheI*-6F was acquired by using the genomic DNA from strain djl-6F as the template for the PCR reaction. DNA gel electrophoresis of the PCR product showed that there was only 1 band at the position of about 750 bp (Fig. 1). The sequence BLAST result showed that the *mheI*-6F coding sequence had 99% similarity to the sequence of the MBC-hydrolyzing esterase (*mheI*) from *Nocardioides* sp. strain SG-4G (GenBank: GQ454794) and 100% similarity to the sequence of the methyl-1H-benzimidazol-2-ylcarbamate- hydrolyzing esterase from *Rhodococcus erythropolis* strain djl-11 (GenBank: HQ874282).

The cloned *mheI*-6F coding was GC-rich and had a rare codon that caused inefficient gene expression in *E. coli*. For a better heterologous expression, we performed optimization. The optimized *mheI*-6F coding sequence was inserted into the expression vector pET-28a (+) with a C-terminal 6× His tag. The expression of *mheI*-6F was induced by adding 0.5 mmol/L IPTG to the culture of *E. coli* BL21 (DE3) transfected with expression vector pET28a-*mheI*-6F. A clear IPTG-inducible band was observed in SDS-PAGE. The molecular mass of MheI-6F was approximately 25 kDa. These results confirmed the successful expression of the MBC-hydrolyzing enzyme

gene from *Microbacterium* sp. strain djl-6F in *E. coli*. The recombinant protein was detected in both the culture supernatant and the sediment of BL21-MheI-6F (Figs. S1 and S2). We further purified MheI-6F by cobalt-affinity chromatography, which enabled us to carry out detailed functional analysis of MheI-6F (Fig. 2).

2.2. A convenient method for measuring MheI-6F activity

The calibration curve for MBC detection presented good linearity in the concentration range of 5.00 to 50.0 μmol/L with the absorbance of MBC as a vertical coordinate (*y*) and the concentration of MBC as a horizontal coordinate (*x*), whose equation is $y = 0.0080x + 0.0017$ with an R^2 value of 0.9992 when detected at the maximum absorption wavelength (λ_{287}) of MBC by ultraviolet spectrophotometry. The recovery experiment showed values between 70% and 95% for spiked samples, thus indicating that this new method can be applied to examine MheI-6F activity conveniently and efficiently.

2.3. Characterization of MheI-6F

MBC was used as the substrate in the enzymatic stability and activity experiment. First to find out how stable the MheI-6F was under different temperatures, we performed a thermostability assay. MheI-6F was identified as stable when the temperature ranged from 10°C to 45°C, 80% of residual activity still remained after 2 hr incubation. The stability of MheI-6F decreased sharply when the temperature rose above 50°C. MheI-6F lost its enzymatic activity when incubated at 60–70°C for 2 hr. To identify the optimal temperature for MheI-6F activity, the reactions took place with a constant pH value (pH 7.0) but under different temperatures ranging from 4 to 70°C. The activity of MheI-6F gradually increased as the temperature increased from 4°C, and the maximum activity was found at 45°C. MheI-6F activity decreased significantly when the temperature increased above 50°C, and only 40.17% of the activity remained at 70°C, indicating that MheI-6F had lost most of its activity at 70°C (Fig. S3).

The effect of pH on MheI-6F stability and activity was determined with the temperature of the reactions maintained at 40°C, which is the temperature we identified for maximum enzyme thermostability and activity. The pH value of the reactions varied from 3.5 to 10.5. As the pH value increased, both the stability and activity of MheI-6F gradually rose and peaked at pH 7.0. MheI-6F was found relatively more stable under lower pH conditions (4.0–7.0) than it was under higher pH conditions (8.0–10.0). More than 80.0% of the total activity was retained after 1 hr reaction at pH values ranging from 5.0 to 8.0. Only 50% of the total activity remained when the reaction took place at pH 10.0 (Fig. S4).

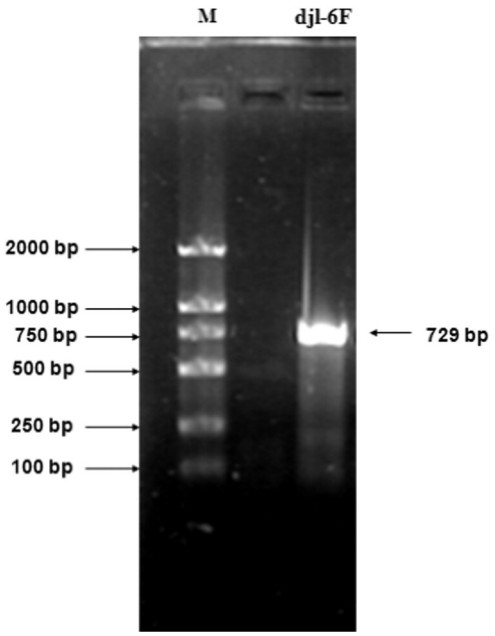

Fig. 1 – Gel electrophoresis of the PCR product of the MheI-6F coding sequence in strain djl-6F. M: 100 bp DNA ladder; djl-6F: PCR product of the MheI-6F coding sequence from djl-6F.

The effects of chemical reagents and metal ions on MheI-6F activity were investigated by adding individual compounds into the reaction mixture at the final concentration of 1 mmol/L. The activity of MheI-6F was shown as a percentage of the activity measured in the absence of the compound. Glycerol, sodium azide, and EDTA did not show significant effects on MheI-6F activity. β-mercaptoethanol and Tween-20 showed slight inhibitory effects on enzyme activity. Surfactant SDS disrupted MheI-6F activity significantly, with only 46% of the activity remaining compared with the control, which had no chemicals present in the reaction. The enzymatic activity of

Table 1 – Primers used to cysteine mutation by quantitative PCR.

Primer	Sequence
C16T1–1	5′-GGCATGGTGGCTGGACCTACCGTGATACC-3′
C16T1–2	5′-GGTATCACGGTAGGTCCAG CCACCATGCC-3′
C62T1–1	5′-CATATTCGCGATGTTCTGGGCACCATCGAAGCCGAAGAACTGG-3′
C62T1–2	5′-CCAGTTCTTCGGCTTCGATGGTGCCCAGAACATCGCGAATATG-3′
C140T1–1	5′-CACTGGA TAAACACACCGGCCTGATGCAACC-3′
C140T1–2	5′-GGTTGCATCAGGCCGGTGTGT TTATCCAGTG-3′
C165T1–1	5′-GTTAATCGTCGCACTGTCCCGCAGG-3′
C165T1–2	5′-CCTGCGGGACAGTGCGACGATTA AC-3′
C222T1–1	5′-CAAGTCGTGAAATTTCCGACTGGTCACGATGTTATGG-3′
C222T1–2	5′-CCATAAC ATCGTGACCAGTCGGAAATTTCACGACTTG-3′

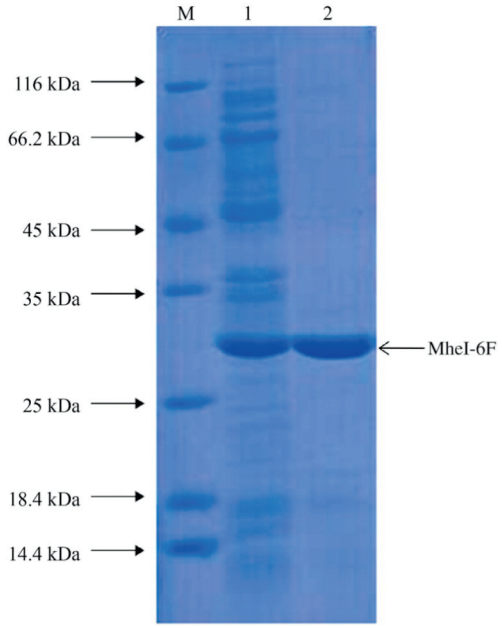

Fig. 2 – SDS-PAGE analysis of purified recombinant MheI-6F. M: protein marker; 1: supernatant of *E. coli* BL21 (DE3) cell lysates; 2: purified recombinant MheI-6F. SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis.

MheI-6F was generally lower when metal ion was present in the reaction. Among the metal ions tested in our study, the addition of Fe^{3+} showed the strongest effects on inhibiting enzyme activity, with only 77% activity remaining compared with the control (Figs. S5 and S6).

2.4. Degradation rate and hydrolytic products of MheI-6F

To further characterize the detailed process of MBC degradation by MheI-6F, we performed HPLC-MS on the hydrolysis reaction at different time points. In the standard MS, the fragment ion peaks at *m/z* 134 $[M + H]^+$ and *m/z* 135 $[M + H]^+$ were analyzed and elucidated as the same as those of authentic 2-AB and 2-hydroxybenzimidazole (2-HB), respectively, for 72 hr incubation of the strain djl-6F, while for 90 min incubation of the hydrolase MheI-6F, only degradation of 2-AB ($[M + H]^+$ of *m/z* = 134) was detected (Fig. 3). The average fortified recovery of carbendazim is 93.67% for this assay.

The HPLC-MS analysis showed that 40 μmol/L carbendazim was degraded completely by strain djl-6F with 5% inoculation (OD_{600} = 1.0) after 72 hr incubation. The same amount of carbendazim was degraded by purified MheI-6F (final concentration 0.10 μg/mL) only after 90 min of incubation at 37°C. The K_m value of MheI-6F was measured as (6.69 ± 2.1) μmol/L. The k_{cat} value of MheI-6F was (160.88 ± 3.3) min^{-1} (Figs. S7, S8, and S9).

2.5. Cysteine mutation

In the sulfhydryl blocking experiment, MheI-6F drastically lost 100% of its activity after being incubated with sodium iodoacetate (0.1 mol/L) at 37°C for 2 hr. This result indicated that sulfhydryl is the active group that contributes to the hydrolyzing activity of MheI-6F on MBC. Based on the amino acid sequence of the enzyme MheI-6F, cysteine, which is the only amino acid with sulfhydryl, is suggested to be the active amino acid in MheI-6F. To test this, we generated mutated MheI-6F (MheI-6F-$^{C\text{-}T}$) by replacing the cysteine at positions 16, 62, 140, 165, and 222 with threonine, respectively.

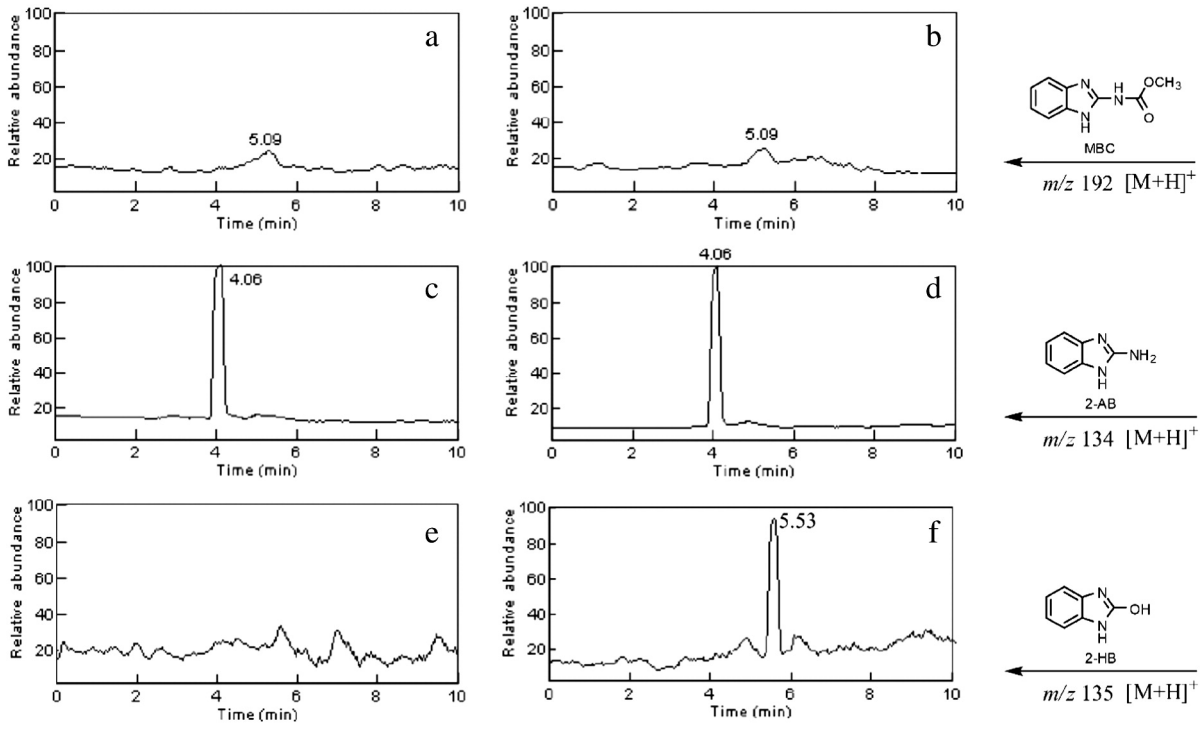

Fig. 3 – Total ion chromatograms of the metabolites of MheI-6F (90 min, a, c, e) and strain djl-6F (72 hr, b, d, f).

Table 2 – Enzyme activities of wild-type and mutant MheI-6F.

Mutant	Relative activity (%)
Control (native MheI-6F)	100.00 ± 1.5
Mutant MheI-6F (C16T)	45.06 ± 5.1
Mutant MheI-6F (C62T)	97.83 ± 2.8
Mutant MheI-6F (C140T)	104.49 ± 4.5
Mutant MheI-6F (C165T)	91.21 ± 5.4
Mutant MheI-6F (C222T)	64.51 ± 3.1
Double mutation MheI-6F (C16T and C222T)	−0.50 ± 3.9

The carbendazim-hydrolyzing data of MheI-6F-$^{C-T}$ showed that the mutations at the sites of 62, 140, and 156 did not show significant effect on enzyme activity. MheI-6F-^{C62T}, MheI-6F-^{C140T}, and MheI-6F-^{C165T} retained more than 90% of their activity compared with wild-type MheI-6F, while mutations at the sites of 16 and 222 significantly compromised MheI-6F activity. MheI-6F-^{C16T} had only 45.06% activity, and MheI-6F-^{C222T} had 64.51% activity compared with wild-type MheI-6F. We generated double-mutated MheI-6F-$^{C16T\&C222T}$ by simultaneously replacing the cysteines with threonine at these 2 sites. MheI-6F-$^{C16T\&C222T}$ was found to have no hydrolyzing activity over MBC (Table 2).

The stability and enzymatic activity of an enzyme determine whether the enzyme has the potential to be used in environmental bioremediation (Sutherland et al., 2004). The dramatic difference in the time consumed for complete degradation of MBC provided further evidence that hydrolase MheI-6F has a significant advantage over strain djl-6F in MBC degradation. The characteristics of MheI-6F, moderate affinity (K_m of 6.69 μmol/L), and high turnover values (k_{cat} of 160.88 min^{-1}) demonstrate the feasibility and efficiency of MheI-6F for remedying MBC contamination in soil and water and removing MBC residue on fruits and vegetables.

The result of sulfhydryl blocking suggested that sulfhydryl (–SH) is the polar group that plays an important role in the hydrolytic process of MheI-6F on MBC. Since sulfhydryl forms disulfide bridges in alkaline and hot solutions, these characteristics also explain why the enzyme activity of MheI-6F became unstable when the pH was greater than 8 or the temperature was higher than 60°C. The difference of the residual enzyme activity between MheI-6F^{C16T} and MheI-6F ^{C222T} suggested that cysteines at the positions of 16 and 222 serve as the main catalytic center of MheI-6F and these 2 cysteines might contribute to the hydrolyzing process of MBC separately.

The degradation pathways of MBC by strain *Pseudomonas* sp. CBW (Fang et al., 2010), strain *Rhodococcus jialingiae* djl-6-2 (Wang et al., 2010), and strain *Rhodococcus qingshengii* djl-6 (Xu et al., 2007) were discussed according to metabolite detection. In our study, the metabolites of strain *Microbacterium* sp. djl-6F on MBC can be summarized as follows: MheI-6F works as an esterase, and the sulfhydryl at the positions 16 and 222 of MheI-6F quickly attack the carbamate group at position 2 of the benzimidazole nucleus of MBC. This process appears to cleave the amide bond in MBC and lowers the toxicity of MBC dramatically in a short amount of time through hydrolyzing MBC to 2-AB (Fig. 4); 2-AB was then transformed to 2-HB. These 2 hydrolysis processes were accomplished by 2 different hydrolases, respectively; MheI-6F was responsible for only the first step. In addition, the degradation of BZ (benzimidazole) was not detected in the degrading pathway of MBC by strain *Microbacterium* sp. djl-6F pure culture, which is different from the degradation process found in strain *Rhodococcus jialingiae* djl-6-2 (Wang et al., 2010) and strain *Rhodococcus qingshengii* djl-6 (Xu et al., 2007). Our preliminary deduction is that there may be some other hydrolase in *Rhodococcus qingshengii* djl-6 and *Rhodococcus jialingiae* djl-6-2 that is different from MheI-6F. This is the first report on the biodegradation of MBC at the enzyme level, and further study to elucidate the entire degradation pathway is underway.

3. Conclusions

Carbendazim hydrolase MheI-6F from a newly isolated MBC-degrading bacteria *Microbacterium* sp. strain djl-6F was found by cloning the *mheI* coding sequence into an expression vector *E. coli* BL21 (DE3). The MheI-6F protein was purified by cobalt-affinity chromatography, and enzymological characterization of Mhe-6F was conducted using a convenient spectrophotometry method. HPLC-MS analysis revealed that

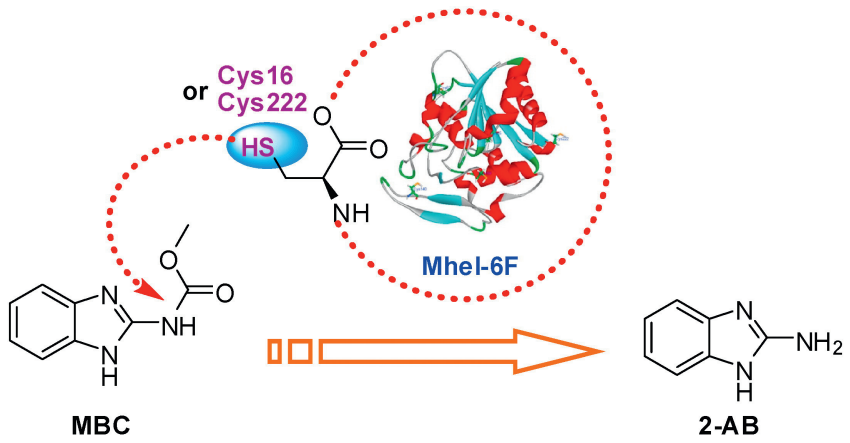

Fig. 4 – Mechanism of the hydrolysis process of MBC by MheI-6F. MBC: carbendazim.

MheI-6F catalyzes the direct hydrolysis of MBC into 2-AB with a high turnover rate and moderate affinity (K_m of 6.69 μmol/L and k_{cat} of 160.88/min) without any cofactors. Through generating sulfhydryl blocking and a cysteine mutation experiment, cysteine16 and cysteine222 were identified as the main catalytic groups that contribute to the hydrolyzing activity of MBC. The mechanism of the detoxifying process of MheI-6F on MBC was reported at the enzyme level for the first time.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jes.2016.05.027.

REFERENCES

Ahuja, S.K., Ferreira, G.M., Moreira, A.R., 2004. Utilization of enzymes for environmental applications. Crit. Rev. Biotechnol. 24 (3), 125–154.

Arora, S., Mukherjee, I., Trivedi, T.P., 2008. Determination of pesticide residue in soil, water and grain from IPM and non-IPM field trials of rice. Bull. Environ. Contam. Toxicol. 81 (4), 373–376.

Boudina, A., Emmelin, C., Baaliouamerb, A., Grenier-Loustalotc, M.F., Chovelon, J.M., 2003. Photochemical behaviour of carbendazim in aqueous solution. Chemosphere 50 (5), 649–655.

Carbo, L., Martins, E.L., Dores, E.F., Spadotto, C.A., Weber, O.L., De-Lamonica-Freire, E.M., 2007. Acetamiprid, carbendazim, diuron and thiamethoxam sorption in two Brazilian tropical soil. J. Environ. Sci. Health, Part B. 42 (5), 499–507.

Chen, Y., Zhou, M.G., 2009. Characterization of *Fusarium graminearum* isolates resistant to both carbendazim and a new fungicide JS399-19. Phytopathology 99 (4), 441–446.

Cuppen, J.G.M., Van den Brink, P.J., Camps, E., Uil, K.F., Brock, T.C.M., 2000. Impact of the fungicide carbendazim in fresh-water microcosms. I. Water quality, breakdown of particulate organic matter and responses of 11 macroinvertebrates. Aquat. Toxicol. 48 (2), 233–250.

Fang, H., Wang, Y.Q., Gao, C.M., Yan, H., Dong, B., Yu, Y.L., 2010. Isolation and characterization of *Pseudomonas* sp. CBW capable of degrading carbendazim. Biodegradation 21 (6), 939–946.

Farag, A., Ebrahim, H., ElMazoudy, R., Kadous, E., 2011. Developmental toxicity of fungicide carbendazim in female mice. Birth Defects Res. B 92 (2), 122–130.

Holtman, M.A., Kobayashi, D.Y., 1997. Identification of *Rhodococcus erythropolis* isolates capable of degrading the fungicide carbendazim. Appl. Microbiol. Biotechnol. 47 (5), 578–582.

Jones, S.E., Williams, D.J., Holliman, P.J., Taylor, N., Baumann, J., Forster, B., et al., 2004. Ring-testing and field-validation of a terrestrial model ecosystem (TME)-an instrument for testing potentially harmful substances: fate of the model chemical carbendazim. Ecotoxicology 13 (1), 61–74.

Mazellier, P., Leroy, E., Laat, J.T., Legube, B., 2003. Degradation of carbendazim by UV/H_2O_2 investigated by kinetic modeling. Environ. Chem. Lett. 1 (1), 68–72.

Pandey, G., Dorrian, S.J., Russell, R.J., Brearley, C., Kotsonis, S., Oakeshott, J.G., 2010. Cloning and biochemical characterization of a novel carbendazim (methyl-1H- benzimidazol-2-ylcarbamate)-hydrolyzing esterase from the newly isolated *Nocardioides* sp. strain SG-4G and its potential for use in enzymatic bioremediation. Appl. Environ. Microbiol. 76 (9), 2940–2945.

Sandahl, M., Mathiasson, L., Jonsson, J.A., 2000. Determination of thiophanate-methyl and its metabolites at thrace level in spiked natural water using the supported liquid membrane extraction and the microporous membrane liquid–liquid extraction techniques combined on-line with high performance liquid chromatography. J. Chromatogr. A. 893 (1), 123–131.

Selmanoglu, G., Barlas, N., Songur, S., Kockaya, E.A., 2001. Carbendazim-induced haematological, biochemical and histopathological changes to the liver and kidney of male rats. Hum. Exp. Toxicol. 20 (12), 625–630.

Sutherland, T.D., Horne, I., Weir, K.M., Coppin, C.W., Williams, M.R., Selleck, M., et al., 2004. Enzymatic bioremediation: from enzyme discovery to applications. Clin. Exp. Pharmacol. Physiol. 31 (11), 817–821.

Wang, Z.C., Wang, Y.Y., Gong, F.F., Zhang, J., Hong, Q., Li, S.P., 2010. Biodegradation of carbendazim by a novel actinobaeterium *Rhodococcus jialingiae* djl-6-2. Chemosphere 81 (5), 639–644.

Wang, X.G., Song, M., Wang, Y.Q., Gao, C.M., Zhang, Q., Chu, X.Q., et al., 2012. Response of soil bacterial community to repeated applications of carbendazim. Ecotoxicol. Environ. Saf. 75, 33–39.

Xu, J.L., Gu, X.Y., Shen, B., Wang, Z.C., Wang, K., Li, S.P., 2006. Isolation and characterization of a carbendazim-degrading *Rhodococcus* sp. djl-6. Curr. Microbiol. 53 (1), 72–76.

Xu, J.L., He, J., Wang, Z.C., Wang, K., Li, W.J., Tang, S.K., et al., 2007. *Rhodococcus qingshengii* sp. nov. djl-6, a carbendazim-degrading bacterium. Int. J. Syst. Evol. Microbiol. 57 (12), 2754–2757.

Zhang, G.S., Jia, X.M., Cheng, T.F., Ma, X.H., Zhao, Y.H., 2005. Isolation and characterization of a new carbendazim-degrading *Ralstonia* sp. strain. World J. Microbiol. Biotechnol. 21 (3), 265–269.

Zhang, X.J., Huang, Y.J., Harvey, P.R., Li, H., Ren, Y., Li, J.S., et al., 2013. Isolation and characterization of carbendazim-degrading *Rhodococcus erythropolis* djl-11. PLoS One 8 (10), 1–6.