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SulE, a Sulfonylurea Herbicide De-Esterification Esterase from *Hansschlegelia zhihuaiae* S113

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De-esterification is an important degradation or detoxification mechanism of sulfonylurea herbicide in microbes and plants. However, the biochemical and molecular mechanisms of sulfonylurea herbicide de-esterification are still unknown. In this study, a novel esterase gene, *sulE*, responsible for sulfonylurea herbicide de-esterification, was cloned from *Hansschlegelia zhihuaiae* S113. The gene contained an open reading frame of 1,194 bp, and a putative signal peptide at the N terminal was identified with a predicted cleavage site between Ala37 and Glu38, resulting in a 361-residue mature protein. SulE minus the signal peptide was synthesized in *Escherichia coli* BL21 and purified to homogeneity. SulE catalyzed the de-esterification of a variety of sulfonylurea herbicides that gave rise to the corresponding herbicidally inactive parent acid and exhibited the highest catalytic efficiency toward thifensulfuron-methyl. SulE was a dimer without the requirement of a cofactor. The activity of the enzyme was completely inhibited by Ag^{+}, Cd^{2+}, Zn^{2+}, methamidophos, and sodium dodecyl sulfate. A *sulE*-disrupted mutant strain, Δ*sulE*, was constructed by insertion mutation. Δ*sulE* lost the de-esterification ability and was more sensitive to the herbicides than the wild type of strain S113, suggesting that *sulE* played a vital role in the sulfonylurea herbicide resistance of the strain. The transfer of *sulE* into *Saccharomyces cerevisiae* BY4741 conferred on it the ability to de-esterify sulfonylurea herbicides and increased its resistance to the herbicides. This study has provided an excellent candidate for the mechanistic study of sulfonylurea herbicide metabolism and detoxification through de-esterification, construction of sulfonylurea herbicide-resistant transgenic crops, and bioremediation of sulfonylurea herbicide-contaminated environments.

Sulfonylurea herbicides are an important class of herbicides used worldwide for controlling weeds in all major agronomic crops. The herbicides inhibit acetohydroxy acid synthase (AHAS), a key enzyme in the biosynthesis pathway of branched-chain amino acids valine, leucine, and isoleucine in bacteria, fungi, and plants (3, 4, 8, 13). The use of sulfonylurea herbicides has developed rapidly because of their high efficacies at low dosages and multicrop selectivities. The sulfonylurea products are now the second most common kind of herbicides after the glyphosates, and more than 30 products have been commercialized.

Most sulfonylurea herbicides are weak acids, vulnerable to acid hydrolysis under acidic conditions. However, in neutral to alkaline soils, some of the herbicides, such as metsulfuron-methyl, chlorsulfuron, and ethametsulfuron-methyl, are degraded at a very slow rate and persist from several months to more than 1 year (15, 20, 24, 30). The residues of herbicides in the soil seriously damage subsequent rotation of sulfonylurea-sensitive crops, like legumes and oilseeds, which can result in serious agricultural loss. Thus, great concern and interest have been raised regarding the environmental behavior and degradation mechanism of sulfonylurea herbicide residues in soil.

Laboratory and field studies have demonstrated that in neutral to alkaline soils, biodegradation plays a major role in the dissipation of sulfonylurea herbicides (5, 9, 10, 26). To date, several microorganisms capable of degrading sulfonylurea herbicides have been described (5, 17, 21, 22, 27, 32, 45, 46). The reported biodegradation pathways of sulfonylurea herbicides include oxidation, de-esterification, and cleavage of the sulfonylurea bridge (27, 28, 32, 45, 46). Two sulfonylurea oxidation cytochrome P450 monooxygenases, P450SU1 and P450SU2, were identified in *Streptomyces griseolus* ATCC 11796 (31, 32); their coding genes, *suaC* and *subC*, have been cloned and characterized (34). De-esterification is another important microbial metabolic mechanism of sulfonylurea herbicides (9, 22, 27, 45). However, all the evidence for de-esterification of sulfonylurea herbicide has come from metabolite identification; the molecular basis of the pathway has not been described.

S113, a bacterial strain, capable of degrading a variety of sulfonylurea herbicides, was isolated from sulfonylurea herbicide-contaminated soil and identified as *Hansschlegelia zhihuaiae* (21, 42). In this study, we have demonstrated that the strain degrades sulfonylurea herbicides via de-esterification to generate corresponding parent acids which are herbicidally inactive. A novel esterase gene involved in the de-esterification of sulfonylurea herbicide, *sulE*, was cloned, and the protein encoded by the enzyme, SulE, was characterized. Furthermore, our results have shown that *sulE* enhances the resistance of strain S113 and *Saccharomyces cerevisiae* to thifensulfuron-methyl and metsulfuron-methyl.

MATERIALS AND METHODS

Chemicals and media. The sulfonylurea herbicides (bensulfuron-methyl, chlorsulfuron, chlorimuron-ethyl, cinosulfuron, ethametsulfuron-methyl, metsulfuron-methyl, and thifensulfuron-methyl) were purchased from Sigma-Aldrich Chemical Co. (Shanghai, China). Stock solutions of each of the sulfonylurea herbicides (40 mM) were prepared in methanol and sterilized by membrane filtration with a pore size of 0.22 μm. Luria-Bertani

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TABLE 1 Strains and plasmids used in this study

Strains and plasmids	Relevant characteristics	Reference or origin
Strains		
E. coli DH10B	F$^-$ *endA1 recA1 galU galK deoR nupG rpsL* Δ*lacX74* ϕ80*lacZ*ΔM15 *araD139* Δ(*ara leu*)*7697 mcrA* Δ(*mrr-hsdRMS-mcrBC*) λ$^-$	Invitrogen
E. coli DH10B (*ilvG*$^+$)	DH10B derivative, *ilvG*$^+$	This study
E. coli BL21(DE3)	F$^-$ *ompT hsdS*B(r_B^- m_B^-) *dcm gal* λ(DE3)	Invitrogen
H. zhihuaiae S113	Capable of degrading sulfonylurea herbicides (DSM 18984, CCTCC AB 206143, KCTC 12880), Ap^r	21
H. zhihuaiae Δ*sulE*	*sulE*-disrupted mutant of strain S113	This study
S. cerevisiae BY4741	*MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	23
S. cerevisiae BYSulE	BY4741 harboring pRS 427-P_{ADH1}-sulE	This study
Plasmids		
pUC118	Clone vector, Ap^r	TaKaRa
pET-29a(+)	Expression vector, Km^r	Novagen
pET-sulE	pET-29a(+) carrying the *sulE* gene	This study
pJQ200SK	Suicide vector used for gene disruption, Gm^r	37
pJQ-dsulE	pJQ200SK carrying *sulE* deletion fragment	This study
pGADT7	AD vector with P_{ADH1}	Clontech
pRS425	*S. cerevisiae-E. coli* shuttle vector, Ap^r Leu$^+$	29
pRS 427-P_{ADH1}-*sulE*	pRS427 carrying P_{ADH1}-sulE	This study

(LB), R2A, yeast extract-peptone-dextrose (YPD), and yeast extract-nitrogen base (YNB) media were purchased from Difco Laboratories (Detroit, MI). The synthetic complete (SC) medium consisted of the following (in g liter^{-1}): 6.7 YNB, 20 glucose, 0.1 urea, 0.2 methionine, and 0.2 histidine. The mineral salts medium (MSM) consisted of the following (in g liter^{-1}): 1.0 NH_4NO_3, 1.0 NaCl, 1.5 K_2HPO_4, 0.5 KH_2PO_4, 0.2 $MgSO_4 \cdot 7H_2O$; pH 7.0. MSGM was MSM supplemented with 5 g liter^{-1} glucose. MSMM was MSM supplemented with 1 ml liter^{-1} methanol. For solid medium, 15.0 g of agar was added per liter. The media were sterilized by autoclaving at 121°C for 30 min. All of the enzymes used in DNA manipulations were obtained from TaKaRa Biotechnology (Dalian, China).

Bacterial strains, plasmids, and culture conditions. The strains and plasmids used in this study are listed in Table 1. All of the *Escherichia coli* strains were grown aerobically at 37°C in LB medium supplemented with appropriate antibiotics. Strain S113 was grown aerobically at 30°C in R2A medium or MSMM. *Saccharomyces cerevisiae* BY4741 was grown aerobically at 30°C in YPD or SC medium.

Degradation experiment and metabolite identification. Cells of strain S113, cultured in R2A medium for 48 h, were harvested by centrifugation, washed twice with MSM, and resuspended in MSM (the cell density was adjusted to an approximate optical density at 600 nm [OD_{600}] of 2.0). An aliquot of the cells (10%, vol/vol) was inoculated in 50 ml of MSM supplemented with 200 μM thifensulfuron-methyl (or a 50 μM concentration of the other sulfonylurea herbicides, respectively) in a 150-ml Erlenmeyer flask. The cultures were incubated at 30°C and 150 rpm on a rotary shaker. At 8-h intervals the cultures were analyzed by high-performance liquid chromatography (HPLC) or HPLC-mass spectrometry (HPLC-MS), as described by Lu et al. (27). Each treatment was performed in three replicates; the control experiments without inoculation were carried out under the same conditions.

Construction of the *E. coli* DH10B (*ilvG*$^+$) strain. *E. coli* DH10B has two AHAS isozymes, AHAS I (encoded by *ilvB*) and AHAS II (encoded by *ilvG*). AHAS II is highly sensitive to sulfonylurea herbicides but is in an inactive form due to a frameshift mutation (1, 13). The *ilvG* gene was activated using Red-mediated recombination, according to the methods of Datsenko et al. (12) and Ellis et al. (14). Briefly, a single-strand oligonucleotide (5′-ATGCTCTGTTACCAGCATTACAGCAGCCGTTAAAT ATCAATGACTGGCAGCAACACTGCGCGCAGCTGCGTGAT-3′) was designed to eliminate the frameshift mutation by introducing 2 nucleotides (underlined). This single-strand oligonucleotide was electrotransferred into *E. coli* DH10B, which has acquired plasmid pKD46 carrying the arabinose-inducible λred gene. Mutant *E. coli* DH10B (*ilvG*$^+$) cells were selected by plating transformants on MSGM plates supplemented with 200 mg liter^{-1} valine. The pKD46 plasmid was cured from *E. coli* DH10B (*ilvG*$^+$) by nonselective growth at 37°C. The *ilvG* gene sequences of *E. coli* DH10B (*ilvG*$^+$) were verified through PCR.

Cloning of the sulfonylurea herbicide de-esterification gene and sequence analysis. General DNA manipulation was carried out as described by Sambrook and Russell (38). The genomic DNA of strain S113 was digested with Sau3AI. The fractions containing approximately 4- to 6-kb DNA fragments were pooled, ligated into the BamHI site of the cloning plasmid pUC118, and transformed into *E. coli* DH10B (*ilvG*$^+$). The library was spread on MSGM plates containing 100 mg liter^{-1} ampicillin, 200 mg liter^{-1} valine, 200 mg liter^{-1} leucine, and 50 μM thifensulfuron-methyl and incubated at 37°C for 36 h. The visible colonies were picked, purified, and tested further for the ability to degrade thifensulfuron-methyl using HPLC analysis.

The inserted fragment in the positive transformant was sequenced using an automated sequencer (model 3730; Applied Biosystems). The nucleotide and deduced amino acid sequence analyses were performed using OMIGA 2.0 software (Oxford Molecular Ltd.). The Blastn and Blastp tools (www.ncbi.nlm.nih.gov/Blast) were used for the nucleotide sequence and deduced amino acid identity searches, respectively. The signal peptide was predicted using the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP) (2).

Gene expression and purification of the recombinant enzyme. The *sulE* coding region, minus the signal peptide, was amplified by PCR with one primer pair of sulEF1 (5′-CTGATTGCATATGGAAACTGACAACG TGGAGCT-3′; the NdeI site is underlined, corresponding to sites 112 to 131 of the gene) and sulER1 (5′-TACAAGCTTGCTTTCGTTCTGATCT AAGC-3′; HindIII site is underlined, corresponding to sites 1175 to 1194 of the gene). The PCR products were digested with NdeI and HindIII and inserted into pET-29a(+); the recombinant plasmid was transformed into *E. coli* BL21(DE3). The induction and purification of the recombinant SulE were performed according to the methods described by Wang et al. (41). The protein concentrations were determined by the Bradford method using bovine serum albumin as the standard (7).

Enzyme assay. The enzymatic activities toward various sulfonylurea herbicides were assayed in 1 ml of 50 mM potassium phosphate buffer (pH 7.5) at 40°C for 10 min. The reactions were initiated by the addition of purified SulE (40 μg ml^{-1}) to the final concentration of 0.02 μg ml^{-1} for thifensulfuron-methyl, and 0.4 μg ml^{-1} for other sulfonylurea herbi-

cides. Reactions were stopped by adding 1 ml methanol and cooling in liquid nitrogen. Thawed samples were centrifuged at 14,000 rpm for 10 min; the supernatants were filtered through a 0.2-μm-pore-size filter and placed in crimp seal HPLC vials. HPLC analysis was used to calculate initial rates of substrate disappearance. Hydrolytic activity for *p*-nitrophenyl esters was assayed according to the method described by Wu et al. (44). One unit of enzyme activity was defined as the amount of enzyme that converted 1 μmol of each sulfonylurea herbicide to its parent acid form. Control samples without enzyme were analyzed in parallel; no spontaneous activity was detected. For kinetic studies, the sulfonylurea herbicides were appropriately diluted into seven different concentrations, from 5 to 50 μM, around the dissociation constant (*Km*) value. Kinetic values were obtained from the Hanes-Woolf equation.

Biochemical characterization. The molecular mass of the denatured protein was determined by SDS-PAGE (25). The molecular mass of the native protein was determined by gel filtration (44). The pH range of the enzyme was determined by incubating the enzyme, with 50 μM thifensulfuron-methyl as the substrate, for 30 min at a pH range of 3 to 11. For pH stability determination, the enzyme was incubated in buffer at a pH range of 3 to 11 at 40°C for 1 h. The optimal reaction temperature was determined under standard conditions ranging from 15 to 50°C. For the thermal stability determination, the enzyme was preincubated in a water bath at different temperatures for 1 h; the remaining activity was assayed. The effects of potential inhibitors on the enzyme were determined through addition of various chemical agents to the reaction mixture and incubation at 40°C for 30 min. The activity of the enzyme was assayed as described above and expressed as a percentage of the activity displayed in the absence of the added compound.

Construction of a *sulE*-disrupted mutant of strain S113. A 547-bp fragment (corresponding to sites 308 to 854 of *sulE*) was used for homologous recombination; the directing sequence was amplified from the genomic DNA of strain S113 with the primer pair of sulEF2 (5′-CGCGGATCCCTCGTGGGCTGCCTCTAGTCTTT-3′; the BamHI site is underlined, corresponding to sites 308 to 330 of *sulE*) and sulER2 (5′-AAAACTGCAGAGAGGCGGTATCTCAGGCACTTT-3′; PstI underlined, corresponding to sites 832 to 854 of *sulE*) and inserted into the BamHI-PstI site of the suicide plasmid pJQ200SK to yield pJQdsulE, which was then electrotransformed into strain S113. The mutant created by the single recombination event was selected based on its resistance to ampicillin and gentamicin on R2A plates. One *sulE*-disrupted mutant was screened and designated Δ*sulE*. The single recombination event in Δ*sulE* was checked by PCR and its ability to degrade sulfonylurea herbicides.

Expression of *sulE* in *S. cerevisiae*. The *sulE* gene, minus the signal peptide, was amplified from the genomic DNA of strain S113 with the primer pair sulEF3 (5′-CGCGGATCCTCAGCTTTCGTTCTGATCTAAGC-3′; BamHI site underlined) and sulER3 (5′-CAATCAACTCCAAGCTTTGCAAAGATGGAAACTGACAACGTGGAGCTTG-3′). The P_{ADH} promoter sequence was amplified from vector pGADT7 by the primer pair PF1 (5′-ATCACTGCAGGCCTGCAGGTCGAGATCCGGGATCG-3′; PstI site underlined) and PR1 (5′-CAAGCTCCACGTTGTCAGTTTCCATCTTTGCAAAGCTTGGAGTTGATTG-3′). The two PCR fragments were spliced by overlap extension (19), digested with PstI and BamHI, and cloned into the corresponding site of the *S. cerevisiae-E. coli* shuttle vector pRS 427 to generate pRS 427-P_{ADH1}-sulE, which was then transformed into *S. cerevisiae* BY4741. The positive transformant (designated BYSulE) was selected from the SC plate.

Herbicide resistance assay. Strain S113 and its *sulE*-disrupted mutant Δ*sulE* were inoculated at a ratio of 10% (vol/vol) into 50 ml of MSMM supplemented with or without 200 μM thifensulfuron-methyl (or 50 μM metsulfuron-methyl and chlorsulfuron), respectively. The cultures were incubated at 30°C and 150 rpm on a rotary shaker. The degradation of the herbicides was determined as described above; the bacterial growth was monitored based on the OD_{600} at 8-h intervals. To determine the sulfonylurea resistance of the recombinant yeast strain BYSulE, an overnight culture of BYSulE was inoculated in SC medium in the presence or absence of 100 μM thifensulfuron-methyl (or 20 μM metsulfuron-methyl). *S. cerevisiae* BY4741, with empty vector, was used as the control. The cells were grown at 30°C and 150 rpm on a rotary shaker. The degradation of the herbicides was determined, and the growth of *S. cerevisiae* was monitored based on the OD_{600} at 2-h intervals.

Nucleotide sequence accession number. The nucleotide sequence of the *sulE* gene has been deposited in the GenBank database under the accession number JN617866.

RESULTS

Metabolite identification. Strain S113 completely degraded 200 μM thifensulfuron-methyl in MSM within 40 h. One metabolite, with a retention time of 11.25 min, appeared in the HPLC chromatogram (see Fig. S1 in the supplemental material); the quantity of this metabolite reached the maximum level at 40 h. An additional 48 h of incubation failed to decrease the metabolite's level, indicating that this metabolite could not be degraded further by strain S113. The metabolite was identified by HPLC-tandem MS (HPLC-MS/MS) as the herbicidally inactive thifensulfuron acid by reference to authentic standards (see Fig. S1). Furthermore, other sulfonylurea herbicides with an ester structure in the aryl ring (e.g., metsulfuron-methyl, bensulfuron-methyl, ethametsulfuron-methyl, and chlorimuron-ethyl) were also be de-esterified to their corresponding parent acid forms by strain S113, while sulfonylurea herbicides, without such an ester structure (e.g., chlorsulfuron and cinosulfuron) were not degraded (data not shown). The cell extract of strain S113, grown on R2A in the presence or absence of sulfonylurea herbicide, showed the same sulfonylurea herbicide de-esterification activities, indicating that the esterase responsible for sulfonylurea herbicide de-esterification is constitutively expressed in the strain.

Construction of the screening model for the sulfonylurea herbicide de-esterification esterase gene. *E. coli* DH10B shows moderate resistance to sulfonylurea herbicide because it contains an active isozyme, AHAS I, which is highly sensitive to valine but resistant to sulfonylurea herbicide. Another isozyme, AHAS II, which is highly sensitive to sulfonylurea herbicide, is in an inactive form caused by a frameshift mutation that leads to a premature stop codon (1, 13). Using Red-mediated recombination, the frameshift mutation of the inactivated AHAS II was eliminated to generate *E. coli* DH10B (*ilvG*$^+$), which was highly sensitive to sulfonylurea herbicide. When grown on an MSGM plate containing 200 mg liter^{-1} valine and 200 mg liter^{-1} leucine, 50 μM thifensulfuron-methyl was sufficient to completely inhibit the growth of *E. coli* DH10B (*ilvG*$^+$) (see Fig. S2 in the supplemental material). When a thifensulfuron-methyl de-esterification gene was introduced and functionally expressed, the transformant grew well due to its ability to convert thifensulfuron-methyl to thifensulfuron acid.

Cloning and sequence analysis of the sulfonylurea herbicide de-esterification esterase gene. Using *E. coli* DH10B (*ilvG*$^+$) as the recipient strain for library construction, one transformant that was able to develop a visible colony on the plate was obtained (see Fig. S2). A degradation experiment showed that this transformant was able to degrade about 85% of the initially added 50 μM thifensulfuron-methyl within 24 h of incubation and, simultaneously, produce an equal amount of thifensulfuron acid (data not shown). The results suggested that the insert fragment of the transformant contains the sulfonylurea herbicide de-esterification esterase gene. The sequencing result showed that the inserted fragment in the transformant was 5,143 bp long; nine

TABLE 2 Substrate specificities and apparent kinetic constants of SulE[a]

Substrate	Sp act (μmol/min/mg)	k_{cat} (s^{-1})	K_m (mM)	Catalytic efficiency k_{cat}/K_m (mM^{-1} s^{-1})
Thifensulfuron-methyl	27.4 ± 1.30	19.0 ± 0.90	0.0195 ± 0.0009	974.4
Metsulfuron-methyl	1.98 ± 0.07	1.37 ± 0.05	0.0172 ± 0.0005	79.7
Ethametsulfuron-methyl	1.76 ± 0.04	1.22 ± 0.03	0.0167 ± 0.0009	73.1
Bensulfuron-methyl	1.72 ± 0.09	1.19 ± 0.06	0.0207 ± 0.0011	57.5
Chlorimuron-ethyl	1.36 ± 0.06	0.94 ± 0.04	0.0181 ± 0.0007	51.9
Chlorsulfuron	0	0	NM	NM
Cinosulfuron	0	0	NM	NM
p-Nitrophenyl acetate	35.4 ± 1.6	24.5 ± 1.1	0.36 ± 0.02	68.2
p-Nitrophenyl butyrate	5.05 ± 0.20	3.5 ± 0.14	0.32 ± 0.03	10.9
p-Nitrophenyl caproate	0	0	NM	NM

[a] Values are means ± standard deviations from three experiments. NM, not measurable.

complete open reading frames (ORFs) were identified by computer analysis. According to the results of the Blastp program, one ORF shared similarities with some putative or hypothetical esterases (highest identity, 37%). The ORF was subcloned to the linearized vector pMD18-T and transformed into *E. coli* DH10B. The resting cells of the subclone showed the ability to degrade thifensulfuron-methyl. Therefore, we concluded that this ORF was the target gene encoding the sulfonylurea herbicide de-esterification esterase and designated *sulE*.

Sequence analysis indicated that *sulE* consists of 1,194 bp, with a GC content of 51% and encodes a protein of 398 amino acids. A putative signal peptide at the N terminal was identified by using the SignalP 3.0 server, with the most likely cleavage site situated between amino acids Ala37 and Glu38, and resulted in a 361-residue mature protein. The results of a Blastp search in the NCBI protein databases revealed that SulE showed low sequence similarities (highest identity, 37%) with many hypothetical or putative alpha/beta-hydrolase fold proteins whose secondary structures are composed of alternating α-helices and β-strands along the backbone. Among the characterized proteins, SulE shared only 29% similarity with esterase 731 (GenBank accession number 1QLW), which catalyzes the hydrolysis of halogenated cyclic compounds from an *Alcaligenes* sp. strain (6), and less than 20% similarity with other characterized proteins.

Gene expression and purification of the recombinant SulE. The *sulE* gene, minus the signal peptide, was cloned into pET-29a(+) to generate pET-sulE and expressed in *E. coli* BL21(DE3) as a C-terminally His-tagged recombinant protein. After induction, most of the sulfonylurea herbicide de-esterification activity and the recombinant protein were detected in the supernatant of the cell lysate. The recombinant SulE was purified to homogeneity using Ni-nitrilotriacetic acid affinity chromatography (see Fig. S3 in the supplemental material). The molecular mass of the denatured enzyme was determined to be approximately 40 kDa, which matched the calculated mass of the tagged protein (41,579 Da). Gel filtration indicated a molecular mass of 84 kDa. Comparison of this value with the calculated molecular mass suggested that the enzyme was a homologous dimer.

Substrate specificity of SulE. The substrate specificities of the enzyme were tested with various sulfonylurea herbicides as the substrates (Table 2). All the sulfonylurea herbicides with a methyl or ethyl ester were substrates of the enzyme, and the hydrolysis rates descended as follows: thifensulfuron-methyl > metsulfuron-methyl > ethametsulfuron-methyl > bensulfuron-methyl > chlorimuron-ethyl. Sulfonylurea herbicides without an ester structure, such as chlorsulfuron and cinosulfuron, were not substrates of the enzyme. The HPLC-MS/MS results demonstrated that sulfonylurea herbicides with a methyl or ethyl ester were de-esterified to corresponding parent acids by the enzyme (see Fig. S4 to S8 in the supplemental material). SulE was able to hydrolyze *p*-nitrophenyl acetate and *p*-nitrophenyl butyrate, but not *p*-nitrophenyl caproate (Table 2), indicating that the hydrolysis activities of the enzyme decreased with the increase of aliphatic chain length of *p*-nitrophenyl esters.

Characteristics of SulE. The optimal pH of SulE was observed to be 7.5 to 8.0. The enzyme was stable at the pH range 6.0 to 9.0, retaining more than 85% of the original activity after preincubation in the buffer at that pH range for 1 h. The activity of SulE was maximal at 40°C. The enzyme was fairly stable up to 45°C, retained approximately 85% of its activity at 45°C for 1 h, retained 20 to 40% of its residual activity at 55°C, and was completely inactivated at 65°C. The enzymatic activity was inhibited by more than 90% with the metal ions Ag^+, Cd^{2+}, and Zn^{2+} (1.0 mM), the organic phosphorus pesticide methamidophos (2.0 mM), and the surfactant SDS (1.0 mM), while the metal ion Ni^{2+} (1.0 mM) caused 40 to 50% inhibition of the SulE activity. The chelators EDTA and the surfactant Tween 80 failed to inhibit the enzyme (data not shown).

***sulE* increased the resistance of strain S113 to thifensulfuron-methyl and metsulfuron-methyl, but not chlorsulfuron.** To study the possible physiological function of *sulE* in strain S113, a *sulE*-disrupted mutant, Δ*sulE*, was constructed by insertion of a suicide vector pJQ200SK into the *sulE* gene. The single recombination event was confirmed by PCR and sequencing analysis (data not shown). The degrading activities and resistance to thifensulfuron-methyl, metsulfuron-methyl, and chlorsulfuron of wild-type strain S113 (wt) and its mutant Δ*sulE* were investigated. As shown in Fig. 1A and B, wt degraded almost 100% of the 200 μM thifensulfuron-methyl within 48 h and 84.4% of the 50 μM metsulfuron-methyl within 72 h, while Δ*sulE* lost the degradation ability, which indicated that *sulE* was the only gene responsible for sulfonylurea herbicide de-esterification in strain S113. The Δ*sulE* mutant and wt showed no growth differences in MSMM. However, wt showed significantly better growth than Δ*sulE* in the presence of thifensulfuron-methyl or metsulfuron-methyl (Fig. 1A and B), indicating that wt showed higher resistance to thifensulfuron-methyl and metsulfuron-methyl than Δ*sulE*. Moreover, Fig. 1C shows that wt, like Δ*sulE*, could not degrade chlorsulfuron; its growth was seriously inhibited.

***sulE* increased the sulfonylurea resistance of *S. cerevisiae*.** To

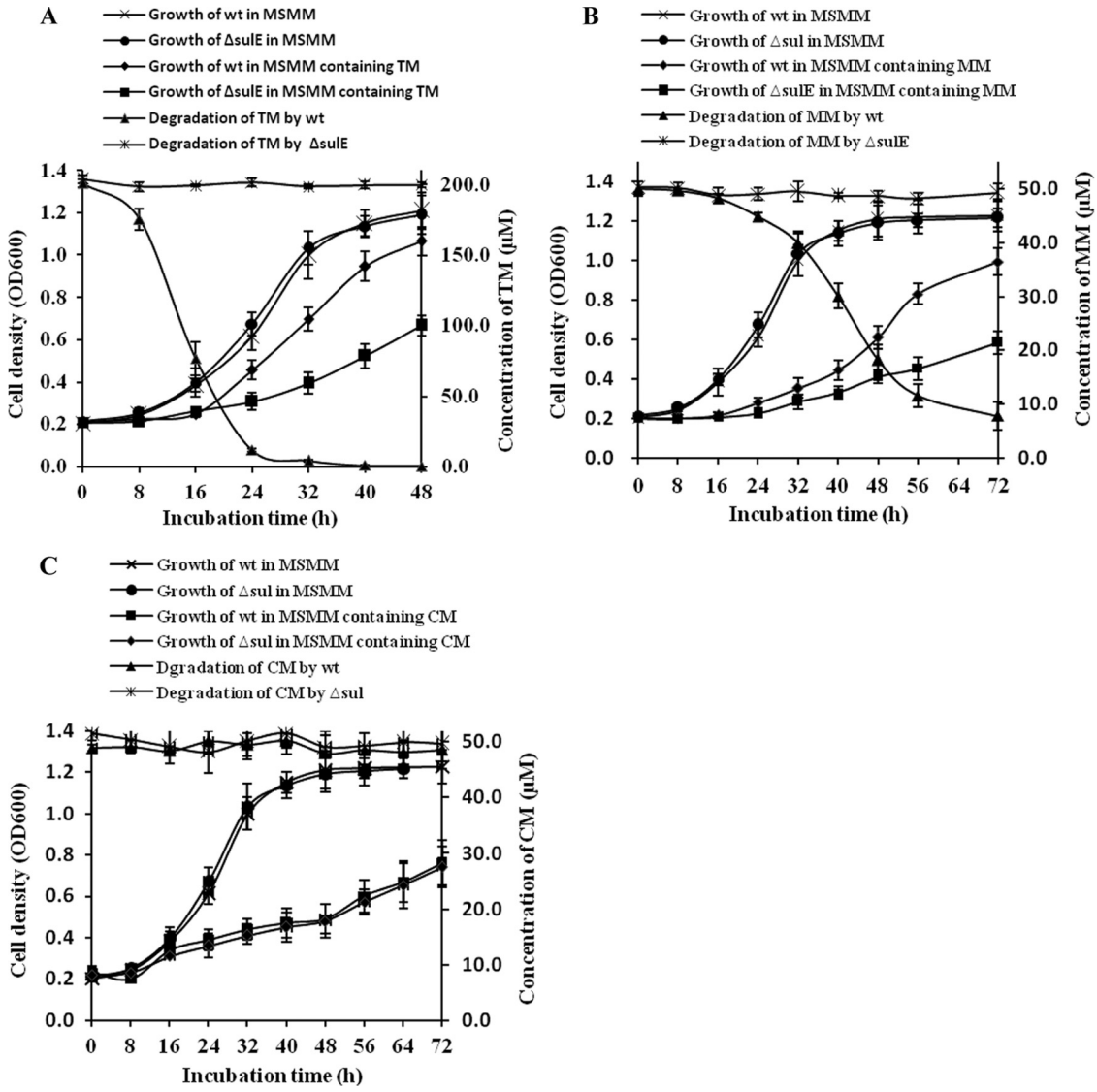

FIG 1 Growth of the wt and Δ*sulE* mutant of strain S113 and degradation of sulfonylurea herbicides in MSMM supplemented with different sulfonylurea herbicides. The cells of wt and Δ*sulE* strain S113 were inoculated in MSMM that contained 200 μM thifensulfuron-methyl (A), 50 μM metsulfuron-methyl (B), or 50 μM chlorsulfuron (C). The cells were grown at 30°C with aeration. TM, thifensulfuron-methyl; MM, metsulfuron-methyl; CM, chlorsulfuron.

evaluate if the *sulE* gene could be functionally expressed in a eukaryote, the *sulE* gene was ligated into the *S. cerevisiae-E. coli* shuttle vector pRS 427 and transformed into yeast strain *S. cerevisiae* BY4741 to generate recombinant strain BYSulE. As shown in Fig. 2, BYSulE almost completely degraded 100 μM thifensulfuron-methyl and 88.1% of 20 μM metsulfuron-methyl within 48 h of incubation, and it displayed significantly better growth than BY4741, which could not degrade the two herbicides, suggesting that *sulE* increased the sulfonylurea resistance of *S. cerevisiae*.

DISCUSSION

Resistance to sulfonylurea herbicides can be conferred by site modification of AHAS and metabolic detoxification. The former is a common mechanism for sulfonylurea resistance; in most cases, the resistance is due to a single point mutation (13). Rapid metabolic detoxification of the herbicides by cytochrome P450s is also a reported resistance mechanism (43). The results of the present study have demonstrated that strain S113 can de-esterify sulfonylurea herbicides to herbicidally inactive parent acids, increasing its resistance against herbicides. Δ*sulE* (the *sulE*-disrupted mutant of strain S113) has lost its de-esterification ability, and so it is significantly more sensitive to the herbicides than the wild-type strain; this confirmed the role of de-esterification in sulfonylurea resistance of strain S113. These results suggested another mechanism of metabolic detoxification of sulfonylurea herbicides in bacteria in which the herbicides are de-esterified to herbicidally inactive acid forms by an esterase. It is interesting that the esterase for the de-esterification of sulfonylurea herbicides is constitutively expressed in strain S113. A possible reason is that this esterase may have other unknown physiological functions in strain S113; sulfonylurea herbicides, which are artificial chemicals, are not the natural substrates of the enzyme. Its ability to degrade sulfonylurea herbicide is incidental or due to mutation.

Esterases have played important roles in the hydrolysis of a

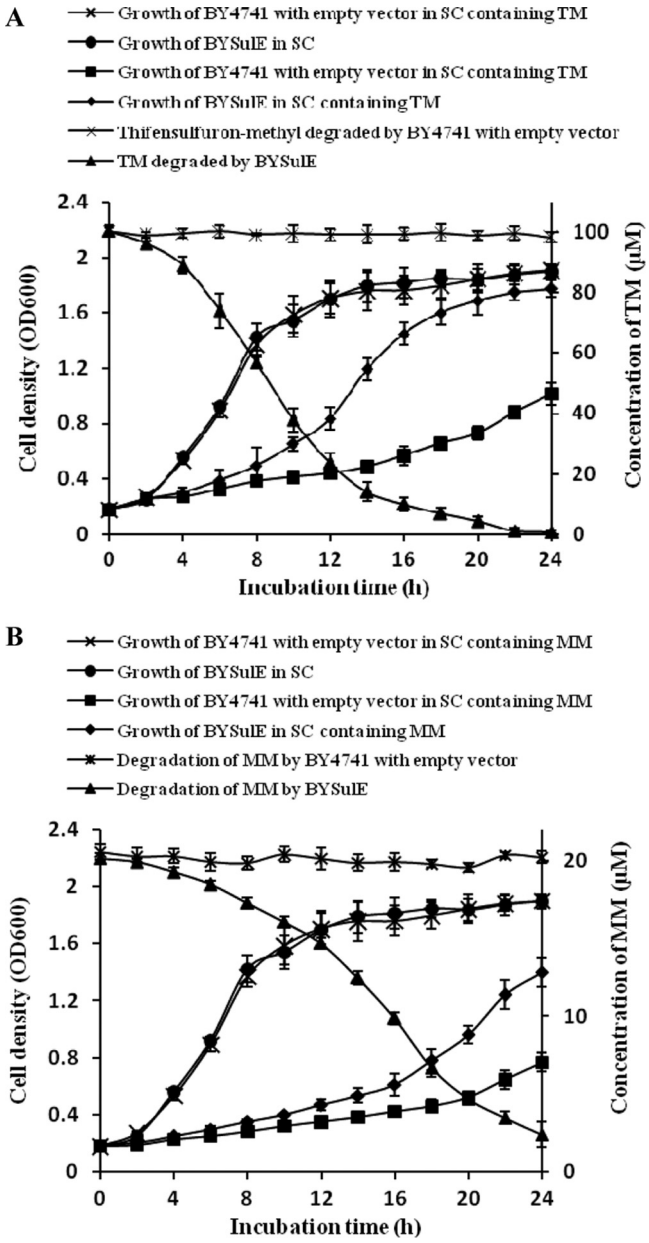

FIG 2 Growth of *S. cerevisiae* BY4741 with empty vector and BYSulE and degradation of sulfonylurea herbicides in MSMM supplemented with different sulfonylurea herbicides. The cells of BY4741 with empty vector and BYSulE were inoculated in the SC medium containing 100 μM thifensulfuron-methyl (A) or 20 μM metsulfuron-methyl (B). The cells were grown at 30°C with aeration. TM, thifensulfuron-methyl; MM, metsulfuron-methyl.

wide range of ester-containing xenobiotics. The genes for esterases, involved in the hydrolysis of many pesticides and herbicides, such as carbaryl, carbofuran, carbendazim, phenmedipham, parathion, phenylurea, and pyrethroids, have been cloned and characterized (11, 18, 35, 36, 39, 40, 41). To the best of our knowledge, SulE is the first reported esterase that can hydrolyze sulfonylurea herbicides. The results from a Blastp search of the NCBI protein databases revealed that SulE has very low sequence similarities with characterized esterases (less than 30% identity). The sequence alignment of SulE with alpha/beta-hydrolase fold proteins indicated that the enzyme contains the typical catalytic triad of alpha/beta-hydrolase fold proteins, consisting of Ser245-His369-Glu268. However, the conserved pentapeptide sequence (Gly-X_1-Ser-X_2-Gly) around the catalytic serine residue of most alpha/beta-hydrolase fold proteins has not been found in SulE. The results suggest that SulE differs from previously reported esterases by the absence of sequence relatedness and substrate difference.

SulE can de-esterify a wide range of sulfonylurea herbicides with a methyl or ethyl ester. It is interesting that SulE has a similar Michael's constant K_m for each of the sulfonylurea herbicides with an ester; however, the catalytic constant k_{cat} for thifensulfuron-methyl is at least 10-fold higher than that for the other four sulfonylurea herbicides with an ester that is hydrolyzed at a similar catalytic efficiency. The results indicate that the substitution of thiophene with an aryl ring significantly reduces the catalytic efficiency; the structure of heterocyclic (pyrimidine or triazine) rings seems to have no significant influence on the de-esterification efficiency.

Genes which confer resistance to herbicides have great potential application in construction of transgenetic herbicide-resistant crops. For example, the glyphosate resistance gene (EPSPS) has been successfully used to construct glyphosate-resistant crops; they have been commercialized and widely planted (16). As an esterase, SulE shares the following advantages: (i) broad-spectrum substrate specificity; (ii) a low molecular weight with only one component; (iii) no cofactor or energy requirement. SulE can degrade or detoxify a variety of sulfonylurea herbicides, such as thifensulfuron-methyl, metsulfuron-methyl, bensulfuron-methyl, ethametsulfuron-methyl, and chlorimuron-ethyl through de-esterification. The mature SulE is a homologous dimer with a subunit that consists of 361 amino acids, and it does not need any cofactor and energy for its activity, while the reported cytochrome P450 system is a multicomponent system and requires the presence of ferredoxin reductase, ferredoxin, and the cofactor NADH (31, 32, 34). Thus, in comparison to the cytochrome P450 system, SulE is more applicable in degrading or detoxifying sulfonylurea herbicides. *S. cerevisiae* BYSulE, which has acquired the *sulE* gene, can degrade thifensulfuron-methyl and metsulfuron-methyl; it has shown enhanced resistance against the two herbicides, and this indicates that it is possible to functionally express *sulE* in a eukaryote. The overall results suggest that *sulE* is an excellent candidate for genetic engineering of sulfonylurea herbicide-resistant crops and bioremediation of sulfonylurea herbicide-contaminated environments.

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