BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Purification of an amide hydrolase DamH from *Delftia* sp. T3-6 and its gene cloning, expression, and biochemical characterization

Fei Wang · Ying Hou · Jie Zhou · Zhoukun Li · Yan Huang · Zhongli Cui

Abstract A highly active amide hydrolase (DamH) was purified from *Delftia* sp. T3-6 using ammonium sulfate precipitation, diethylaminoethyl anion exchange, hydrophobic interaction chromatography, and Sephadex G-200 gel filtration. The molecular mass of the purified enzyme was estimated to be 32 kDa by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. The sequence of the N-terminal 15 amino acid residues was determined to be Gly-Thr-Ser-Pro-Gln-Ser-Asp-Phe-Leu-Arg-Ala-Leu-Phe-Gln-Ser. Based on the N-terminal sequence and results of peptide mass fingerprints, the gene (*damH*) was cloned by PCR amplification and expressed in *Escherichia coli* BL21(DE3). DamH was a bifunctional hydrolase showing activity to amide and ester bonds. The specific activities of recombinant DamH were 5,036 U/mg for 2′-methyl-6′-ethyl-2- chloroacetanilide (CMEPA) (amide hydrolase function) and 612 U/mg for 4-nitrophenyl acetate (esterase function). The optimum substrate of DamH was CMEPA, with K_m and k_{cat} values of 0.197 mM and 2,804.32 s^{-1}, respectively. DamH could also hydrolyze esters such as 4-nitrophenyl acetate, glycerol tributyrate, and caprolactone. The optimal pH and temperature for recombinant DamH were 6.5 and 35 °C, respectively; the enzyme was activated by Mn^{2+} and inhibited by Cu^{2+}, Zn^{2+}, Ni^{2+}, and Fe^{2+}. DamH was inhibited strongly by phenylmethylsulfonyl and SDS and weakly by ethylenediaminetetraacetic acid and dimethyl sulfoxide.

Keywords *Delftia* sp. T3-6 · Amide hydrolase · Purified · Cloning · Characterization

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Introduction

Chloroacetanilide herbicides are one of the most widely used herbicide families. Members of this family share a molecular core consisting of 2-chloroacetanilide and differ only in the type and arrangement of substitutions (Fig. 1) (Saha et al. 2012). Acetochlor (2-chloro-*N*-(ethoxymethyl)-*N*-(2-ethyl-6-methylphenyl)-acetamide) is an important chloroacetanilide herbicide. Chloroacetanilide herbicides are selective pre-emergent herbicides that are used to control broadleaf weeds and annual grasses in corn fields (Xu et al. 2006). Acetochlor has been used worldwide for more than 40 years; the estimated annual consumption is 35,000,000 lbs in the USA and >100,000,000 lbs in China (Grube et al. 2011; Hua 2011), mostly for corn cultivation. Previous studies have revealed that acetochlor negatively impacts both the environment and agricultural ecosystems. Acetochlor exerts inhibitory effects on soil microorganisms, which could affect the growth and reproduction of earthworms (Zhou et al. 2006; Yasmin and D'Souza 2010). It also exhibits certain toxic effects in humans and animals (Crump et al. 2002; Liu et al. 2006; Xiao et al. 2006; Zafeiridou et al. 2006). As such, acetochlor has been identified as a class B-2 carcinogen by the US Environmental Protection Agency (Coleman et al. 2000; Xiao et al. 2006).

Microorganisms play major roles in the degradation of acetochlor (Liu et al. 2002). Acetochlor biodegradation pathways include dechlorination, hydroxylation, *N*-dealkylation, *C*-dealkylation, and dehydrogenation (Xu et al. 2006), resulting in breakdown products, including 2-ethyl-6-

Fig. 1 Structure of chloroacetanilide herbicides. R_1-ethyl, R_2-methyl—alachlor. R_2-butyl—butachlor. R_1-methyl, R_2-ethyl—acetochlor. R_2-iso-propyl—propisochlor

methylaniline (MEA), chloroacetyl-indoline, and 2′-methyl-6′-ethyl-2-chloroacetanilide (CMEPA) (Jablonkai 2000; Dagnac et al. 2002; Ye et al. 2002).

The hydrolysis of an amide bond in the structure is an important reaction during the microbial degradation of acetanilide herbicides. In recent years, several arylamidases that play roles in the degradation of acetanilide herbicides have been cloned and characterized (Zhang et al. 2011, 2012; Shen et al. 2012). These enzymes belong to the amidase family and commonly contain a GGSS motif and Ser-Ser-Lys catalytic triads (Mayaux et al. 1990; Shin et al. 2002).

We previously isolated an efficient CMEPA-hydrolyzing strain T3-6 from acetochlor-polluted soil. This strain was identified as *Delftia* sp. T3-6 and isolated from a microbial consortium that could mineralize acetochlor completely. One member of this consortium could transform acetochlor into CMEPA, whereas another could grow using MEA as its sole carbon source and supply carbon sources for other consortium members. In this study, an amide hydrolase that hydrolyzed CMEPA into MEA was purified from *Delftia* sp. T3-6, and its encoding gene was cloned and expressed in a heterologous manner in *Escherichia coli*.

Materials and methods

Chemicals

CMEPA (96.5 % purity) and 2′,6′-diethyl-2-chloroacetanilide (CDEPA; 96.7 % purity) were purchased from Qingdao Vochem Co. Ltd. (Shandong, China). Acetochlor, butachlor, metamifop, propanil, and MEA (96.7 % purity) were purchased from Shanghai Jingchun Co. Ltd. All other chemicals used in this study were purchased from Sigma (St. Louis, MO, USA) and were analytical grade or higher purity.

Bacterial cultivation and crude extract preparation

Delftia sp. T3-6 (CCTCC No. M 2012526), which exerted strong hydrolase activity on CMEPA, was isolated from soil samples. The strain was precultured in Luria–Bertani medium (LB; containing 10.0 g/L tryptone, 5.0 g/L yeast extract, and

10.0 g/L NaCl, pH 7.0), harvested by centrifugation at 6,000× g at 4 °C for 5 min, washed, and resuspended in 20 mM sodium phosphate buffer (PBS; pH 7.0). The cells were disrupted by sonication for 20 min (Sonicator 201 M, Kubota, Japan); cell suspensions were then centrifuged at 12,000 rpm for 20 min at 4 °C. The supernatants obtained from this step were referred to as crude extracts. Protein concentrations were determined using the Bradford (1976) method.

Purification of CMEPA hydrolase DamH

All purification steps were performed at temperatures ≤4 °C to avoid any possible enzyme denaturation. The cell extracts were fractionated using ammonium sulfate, and the precipitate (60–80 % saturation) was harvested by centrifugation at 12,000 rpm for 25 min. Precipitates were dissolved in ~50 mL of 20 mM PBS (pH 8.0) and dialyzed. The dialyzed enzyme fraction was then applied to a DEAE-Toyopearl 650 M (Toyopearl, Japan) column. The active fractions were pooled for hydrophobic interaction chromatography. The fractions containing CMEPA hydrolase activity were collected and concentrated. The enzyme was then subjected to gel filtration on a Sephadex G-200 column (2.6×150 cm, GE Healthcare, USA). Fractions containing CMEPA hydrolase activity were collected (Mayaux et al. 1990).

Zymogram analysis of CMEPA hydrolase

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to the method of Laemmli (1970). After electrophoresis, the gel was cut into two parts. One part was stained with 0.1 % Coomassie brilliant blue R-250 and used to analyze the size of DamH; the other was washed twice with 2.5 % (v/v) Triton X-100 for 30 min to remove the SDS. After washing, the gel was incubated with 0.1 M Tris-HCl buffer (pH 8.0) for 1 h at 4 °C to renature DamH and then stored in 20 mM Tris-HCl buffer (pH 8.0) at 4 °C for zymogram analysis. Gel sections with renatured DamH were incubated on 20 mM Tris-HCl agar plates containing 200 mg/L CMEPA. After incubation at 37 °C for 30 min, the gel was stained with 0.04 M potassium hexacyanoferrate(III) and 0.02 M 4-aminoantipyrene solution (El-Dib et al. 1975; Norwitz and Keliher 1980); the location where DamH existed became visible as a purple band. The stained gels were then compared with zymogram, and the molecular weight of the CMEPA hydrolase was determined (Bano et al. 2011).

Standard assay for the enzyme activities with cell extracts

DamH activity was determined by measuring the release of MEA formed during CMEPA hydrolysis. A reaction mixture

containing 2 mM CMEPA and 10 μL enzyme diluted in 3 mL 20 mM Tris-HCl (pH 7.0) was incubated at 30 °C for 20 min. After incubation, 30 μL 0.2 M potassium hexacyanoferrate(III) and 30 μL 0.1 M 4-aminoantipyrene were added to the reaction mixture. MEA reacted with 4-aminoantipyrene to form a purple compound with a maximum absorption at 545 nm (Kotresha and Vidyasagar 2008). One unit of CMEPA hydrolase activity was defined as the amount of enzyme needed to release 1 μmol of MEA in 1 min.

Protein assay

Proteins were quantified using the Bradford (1976) method using bovine serum albumin as the standard; protein concentrations during purification studies were then calculated from the standard curve. The eluted fractions from the chromatographic separations were monitored at 280 nm.

Protein sequencing and mass spectroscopy analysis

N-terminal amino acid sequencing and peptide mass fingerprint analysis of the DamH sequence were performed using the Edman degradation method on an ABI Precise 494 protein sequencer (Shanghai GeneCore Bio Technologies Co. Ltd.). The stained gel band corresponding to the position of the purple band in the renatured gel was excised and used for peptide mass fingerprint analysis (Bo-Yuan Biological Technology Co. Ltd.). The resulting peptide fragments were analyzed by searches in Mascot website databases (http://www.matrixscience.com) to determine the sequences of peptide fragments.

Gene cloning, expression, and purification of the recombinant DamH

Genomic DNA was extracted from *Delftia* sp. T3-6 cells and purified as described previously to serve as the PCR template (Sambrook and Russell 2001). Oligonucleotide primers were designed to amplified the *damH* gene (Table 1). Primer DamPF (forward) and DamPR (reverse) was applied to amplify the internal 214 bp of *damH*. The 5′-nucleotide sequence (1–876 bp of *damH*) was amplified using primers DamNF and DamPR. The 3′-nucleotide sequence was amplified by thermal asymmetric interlaced (TAIL)-PCR using the specific primes F-SP2 and F-SP1, arbitrary primers ARB1, ARB2, and ARB3 (Liu and Whittier 1995; Terauchi and Kahl 2000). The complete gene was PCR-amplified using the following primers: Dam-F (forward), containing an *Nde*I site (underlined) at the initiation site of the *damH* gene; and Dam-R (reverse), containing a *Sal*I site (underlined) after the stop codon and a 6× His tag before the stop codon.

The PCR products were digested using *Nde*I and *Sal*I and inserted into the *Nde*I-*Sal*I sites of pET29a(+) to obtain the plasmid pET29a-*damH*. The plasmid was then transformed into *E. coli* BL21(DE3). The transformants were grown in LB medium containing 50 μg/mL kanamycin at 37 °C until they reached mid-log phase; they were then induced with 0.2 mM IPTG at 18 °C for 24 h.

Crude enzyme extracts of *E. coli* BL21(DE3) were prepared using ultrasonic disruption, as described previously. The recombinant DamH was purified with Ni^{2+}-NTA resin (Qiagen, Valencia, CA, USA) (Janknecht et al. 1991). The fractions containing recombinant DamH were collected and dialyzed against 20 mM Tris-HCl (pH 7.0) overnight at 4 °C to remove imidazole.

Biochemical properties of the purified recombinant DamH

Substrate specificity

The substrate specificity of DamH was determined using propanil, acetochlor, butachlor, metamifop, formamide, acetamide, propanamide, acrylamide, benzamide, benzeneacetamide, nicotinamide, pyrazinamide, anthranilamide, CMEPA, CDEPA, formanilide, acetanilide, 4-nitroacetanilide, 4-acetamido phenol, glyceryl triacetate, glycerol tributyrate, glycerol tricaproate, 4-nitrophenyl acetate, 4-nitrophenyl butyrate, 4-nitrophenyl caprylate, and ε-caprolactone. Substrate solutions were prepared in 20 mM Tris-HCl (pH 7.0) at a concentration of 1 mM. Amidase activity was determined by the release of ammonia using the phenol-hypochlorite ammonia detection method (Weatherburn 1967), esterase activity was assessed using the release of fatty acids (Ihara et al. 1991; Wei et al. 2003; Kaiser et al. 2006; Rashamuse et al. 2009), and arylamidase activity was determined under standard assay conditions, as described previously.

DamH activity on different concentrations (0.025–2 mg/mL) of the test substrates was determined, as described above. The kinetic rate constants K_m and V_{max} were calculated using a Lineweaver–Burk plot (Dowd and Riggs 1965) at a concentration range of 0.025–4 mg/mL.

Effects of pH and temperature on activity and stability

The optimal reaction pH was assessed in several buffers of varying pH at 30 °C. The following buffers were used: 20 mM citrate buffer, pH 3.0–6.0; 20 mM PBS, pH 6–8.0; 20 mM Tris-HCl buffer, pH 7.5–8.8; and 0.05 M glycine NaOH buffer, pH 8.8–11.0. The optimal reaction temperatures were determined using the optimal pH at temperatures from 4 to 50 °C.

To measure pH stability, the enzyme was incubated at 4 °C for 24 h in different buffers, and the residual activity was determined using the enzyme assay conditions described above. The thermal stability of DamH was assessed by

Table 1 Oligonucleotide primers used in PCR

Primer	Sequence (5′ to 3′)	Design basis	Position in *damH* gene
DamPF	GCTGGCCAATCCGCTCAA	Peptide fragments 1	663–680 in *damH* gene
DamPR	GCGGCGCAGTTCGTCATC	Peptide fragments 2	859–876 in *damH* gene
DamNF	ATGGGCACSTCSCCSCAG	N-amino acid sequences	1–18 in *damH* gene
ARB1	GGCCACGCGTCGACTAGTACNNNNNNNNNNNGATAT	Arbitrary primer for Tail PCR	None
ARB2	GGCCACGCGTCGACTAGTACNNNNNNNNNNNACGCC	Arbitrary primer for Tail PCR	None
ARB3	GGCCACGCGTCGACTAGTAC	Arbitrary primer for Tail PCR	None
F-SP1	GCCGAAACCCTGCTGGACAA	Specific primer for Tail PCR	727–746 in *damH* gene
F-SP2	ACGGCATGCAGCACGTCTTC	Specific primer for Tail PCR	809–828 in *damH* gene
Dam-F	<u>CATATG</u>GGCA CGTCGCCGCAGTCTGAT	Forward primer for *damH*	1–24 in *damH* gene
Dam-R	<u>GTCGAC</u>TCAGTGGTGGTGGTGGTGGTGGGCGCCCTTGAACCACTGGG	Reverse primer for *damH*	884–903 in *damH* gene

incubating the enzyme preparations at different temperatures. Aliquots were removed at specific time intervals, and the activity remaining was measured using the enzyme assay conditions described above. Non-heated enzyme was used as the control (100 %).

Effect of metal ions and chemical agents on enzyme activity

The effects of potential inhibitors or activators on the enzymatic activity of DamH were determined by adding 1 mM of various metal salts (Cu^{2+}, K^{+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Fe^{3+}, Zn^{2+}, Ca^{2+}, Mg^{2+}, Cr^{3+}, Ba^{2+}, and Mn^{2+}), chemical agents (Triton X-100, Tween-80, SDS, dimethyl formamide (DMF), and PMSF), and organic reagents (methanol, ethanol, acetone, acetonitrile, isopropanol, and DMSO) to the reaction mixture that had been pre-incubated for 10 min at 30 °C. Enzyme activity without any additive was used as the control and defined as 100 %.

Nucleotide sequence accession number

The sequence for the amide hydrolase gene *damH* has been deposited in the GenBank database under the accession number KF638270.

Results

Purification of DamH from *Delftia* sp. T3-6

The CMEPA hydrolase DamH is a constitutive enzyme in *Delftia* sp. T3-6. When cultivated in LB media, T3-6 showed high DamH activity (up to 233 U/mg) without the addition of any inductive compounds. A summary of the purification process for DamH is shown in Table 2. After the four-step purification process, DamH was purified by 17.7-fold, with a 0.3 % recovery. SDS–PAGE was then used to analyze the purified DamH to verify its purity and determine its molecular weight. As shown in Fig. 2, the enzyme with CMEPA hydrolysis activity formed a single band on SDS–PAGE with a molecular weight of ~32 kDa.

Cloning and expression of the *damH* gene

The N-terminal 15-residue amino acid sequence of DamH was determined to be NH$_2$-GTSPQSDFLRALFQS. Peptide fingerprint analysis also identified two additional peptide fragments of DamH as NH$_2$-APEADDELRR and NH$_2$-TNPLANPLKASYQGFPR. These amino acid sequences were used to design primers to amplify the *damH* gene from the genome of strain T3-6. The primers DamPF and DamPR

Table 2 Purification of CMEPA hydrolase DamH from *Delftia* sp. T3-6

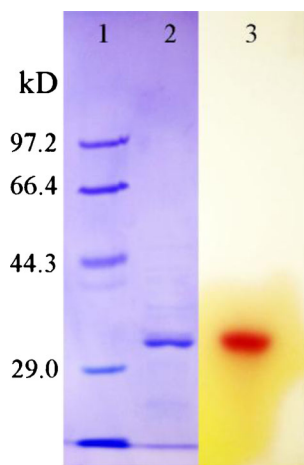| Purification step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Purification fold	Yield (%)
Crude extract	286.2	66,763.2	233.3	1	100
Ammonium sulfate precipitation	60.2	52,442.0	870.7	3.7	78.6
DEAE-Sepharose chromatography	20.7	33,739.1	1,627.1	7.0	50.5
Hydrophobic interaction chromatography	1.3	2,872.7	2,221.6	9.5	4.3
Sephadex G-200 Gel filtration	0.1	190.7	4,126.4	17.7	0.3

Fig. 2 Zymogram analysis and molecular weight measurement of the DamH by SDS–PAGE. *Lane 1* protein molecular weight markers; *lane 2* purified CMEPA; *lane 3* potassium hexacyanoferrate(III) and 4-aminoantipyrene solution staining on CMEPA plate

Fig. 4 Analysis of the expression and purification of recombinant DamH. *Lane 1* protein molecular weight marker; *lane 2* extract of a 300-mM imidazole-eluted sample; *lane 3* extract of a 200-mM imidazole eluted sample; *lane 4* extract of a 100-mM imidazole-eluted sample; *lane 5* extract of a 50-mM imidazole-eluted sample; *lane 6* extract of a 0-mM imidazole eluted sample; *lane 7* total protein of uninduced BL21(DE3) harboring recombinant damH in pET-29a(+)

were used to amplify the internal 214 bp of *damH*, the 3′ terminal nucleotide sequence of *damH* was amplified by TAIL-PCR, and the 5′ terminal nucleotide sequence of *damH* was amplified by DamNF and DamPR. Based on the 5′ and 3′ information obtained from these reactions, oligonucleotides for the complete nucleotide sequence were designed, which was then PCR-amplified using genomic DNA as the template. The cloned *damH* gene was 903 bp in length, encoding a 300-amino acid protein with a calculated molecular mass of 32 kDa. The corresponding protein was searched against the GenBank database using the BLASTP program (http://blast.ncbi.nlm.nih.gov). The searches revealed that the most related proteins were an arylamidase (98 % identity) and acetyl-hydrolase from *Acinetobacter* sp. SE19 (67 % identity) (Fig. 3). DamH contained the sequence (G-X-S-X-G)
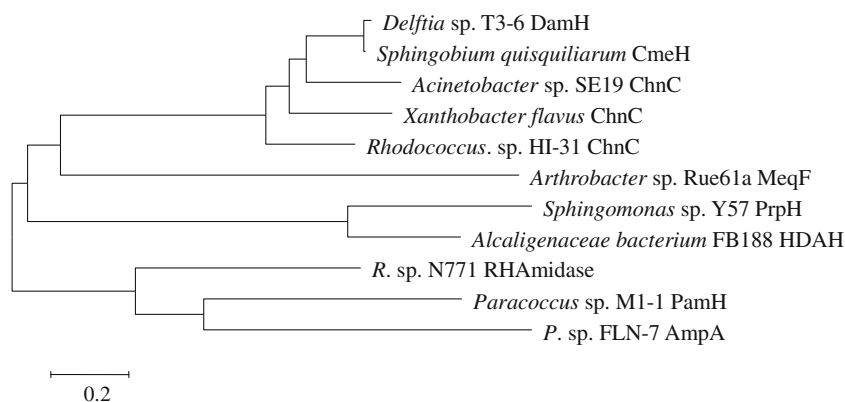
(Bornscheuer 2002), which is highly conserved and characteristic of the esterase family. A catalytic triad that is also highly conserved in this family, Ser-Glu-His (Ser149, Glu244, and His274), was also identified.

The overexpression of *damH* in *E. coli* BL21 was successful. The expressed and purified recombinant DamH was analyzed using SDS–PAGE (Fig. 4).

Substrate specificity

The substrate specificity of the purified enzyme was examined using various amide and ester compounds. Data revealed that DamH catalyzed anilide compounds such as CMEPA, CDEPA, formanilide, acetanilide, 4-nitroacetanilide, and 4-acetamido phenol but could not hydrolyze propanil, acetochlor, butachlor, metamifop, formamide, acetamide,

Fig. 3 Phylogenetic tree of DamH and related esterase/arylamidase constructed by the neighbor-joining method. DamH was aligned with the following proteins, with their preferred structure indicated: *Sphingobium quisquiliarum* arylamidase/esterase, *Acinetobacter* sp. SE19 acetyl-hydrolase (Cheng et al. 2000), *Xanthobacter flavus* 6-hexanolactone hydrolase (Van Beilen et al. 2003), *Rhodococcus* sp. HI-31 caprolactone hydrolas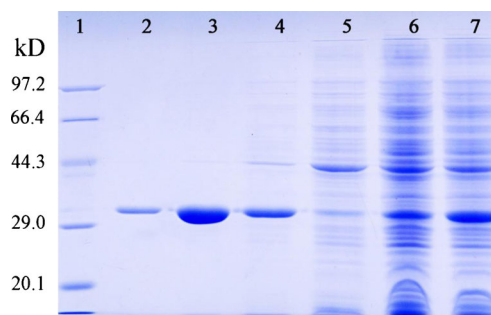e (Mirza et al. 2009), *Arthrobacter* sp. Rue61a *N*-acetylanthranilate amidase (Kolkenbrock et al. 2006), *Sphingomonas* sp. Y57 propanil hydrolase (Zhang et al. 2011), *Alcaligenaceae bacterium* FB188 histone deacetylase (Nielsen et al. 2005), *Rhodococcus* sp. N771 amidase (Ohtaki et al. 2010), *Paracoccus* sp. M1-1 aryl acylamidase (Shen et al. 2012), *Paracoccus* sp. FLN-7 aryl-amidase (Zhang et al. 2012)

Table 3 Substrate specificity and apparent kinetic constants of DamH

Substrate	Sp act (U mg^{-1})	K_m (mM)	k_{cat} (s^{-1})	Catalytic efficiency (k_{cat}/K_m [μM^{-1} s^{-1}])
CMEPA	5,036.28	0.197	2,804.32	14.24
CDEPA	4,973.45	0.278	1,733.83	6.24
Formanilide	3,112.36	0.276	1,659.89	6.01
Acetanilide	3,895.60	0.436	2,048.50	4.70
4-Nitroacetanilide	2,556.89	0.232	1,395.21	6.01
4-Acetamido phenol	2,616.07	0.194	1,363.65	7.03
Glyceryl triacetate	185.60	1.65	98.98	0.06
Glyceryl tributyrate	198.24	1.31	105.73	0.08
Glycerol tricaproate	77.95	3.29	41.57	0.01
4-Nitrophenyl acetate	611.94	4.77	326.36	0.07
4-Nitrophenyl butyrate	394.87	5.26	210.59	0.04
4-Nitrophenyl caprylate	260.35	7.12	138.85	0.02
ε-Caprolactone	141.24	1.66	75.33	0.05

propanamide, acrylamide, benzamide, benzeneacetamide, nicotinamide, pyrazinamide, or 2-aminobenzamide. Although it shares homology and a similar catalytic triad like typical carboxylesterases, its amidase activity was much higher than its esterase activity. The substrate specificity and K_m and k_{cat} values of DamH were determined using various amide substrates (Table 3). The specific activities of DamH were 5,036.28 U/mg (CMEPA) and 611.94 U/mg (4-nitrophenyl acetate). The most suitable substrate was CMEPA, with K_m and k_{cat} values of 0.197 mM and 2,804.32 s^{-1}, respectively.

Effects of pH and temperature on enzyme activity and stability of recombinant DamH

The effects of temperature and pH on the CMEPA hydrolase activity of DamH were assayed using purified enzyme. Recombinant DamH exerted high levels of activity at
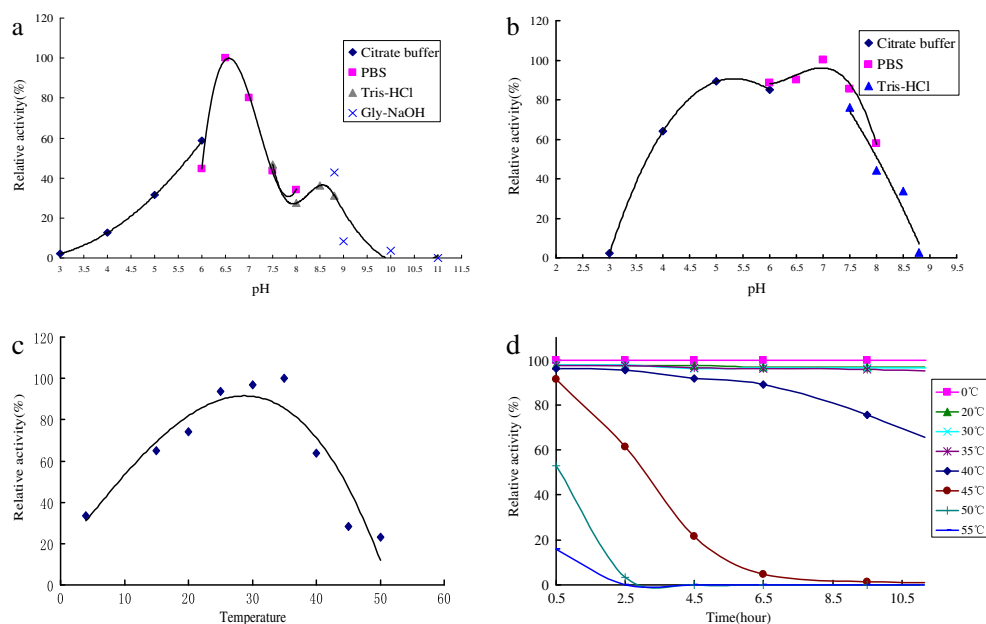

Fig. 5 Effects of pH and temperature on enzyme activity and stability of the recombinant DamH. **a** Determination of the optimal pH. Assays were carried at 35 °C for 15 min in buffers of varying pH. **b** pH stability. Activity was measured under optimal conditions (20 mM PBS, pH 6.5, 35 °C, 15 min) after incubation of the purified enzyme with buffers of varying pH at 4 °C for 24 h. **c** Determination of the optimal temperature. Activity was measured in 20 mM PBS, pH 6.5, at 5–50 °C for 15 min. **d** Thermal stability. Activity was measured under optimal conditions after incubation of the enzyme at indicated temperatures for 11 h

Table 4 Effect of metal ions on enzyme activity of DamH

Metal ions	Concentration (mM)	Relative activity (%)
No addition	0	100±0.01
Cu^{2+} ($CuCl_2$)	1	26.3±2.0
Fe^{2+} ($FeCl_2$)	1	35.5±1.8
Ni^{2+} ($NiCl_2$)	1	46.8±3.6
Zn^{2+} ($ZnCl_2$)	1	47.7±1.2
Mg^{2+} ($MgCl_2$)	1	78.6±8.5
Ba^{2+} ($BaCl_2$)	1	85.9±0.5
K^+ (KCl)	1	87.1±1.5
Co^{2+} ($CoCl_2$)	1	94.9±4.0
Fe^{3+} ($FeCl_3$)	1	99.0±2.1
Ca^{2+} ($CaCl_2$)	1	100.2±3.5
Cr^{3+} ($CrCl_3$)	1	103.4±2.8
Mn^{2+} ($MnCl_2$)	1	146.7±12.2

pH 6.5–7.0, with an optimum pH of 6.5 (Fig. 5a). Little activity was detected at a pH below 3.0 or above 8.0, but DamH retained >50 % activity after storage at pH 4.0–8.0 for 24 h (Fig. 5b). The enzyme was active at 5–50 °C, with an optimum temperature of 35 °C (Fig. 5c). The thermal stability of the purified enzyme was assessed by incubating DamH for 11 h in the absence of substrate at 0–55 °C (Fig. 5d). DamH was stable and retained >60 % residual activity for 11 h at temperatures <40 °C but was unstable at temperatures >45 °C. This suggests that DamH is a mesophilic enzyme.

Effect of metal ions and chemical agents on the enzymatic activity of DamH

Metal ions play an important role in the activity of enzymes. As shown in Table 4, 1 mM Mn^{2+} stimulated DamH enzyme

Table 5 Effect of chemical agent on enzyme activity of DamH

Chemical agent	Concentration	Relative activity (%)
No addition		100.00±1.53
Methanol	10 %	32.05±0.96
Ethanol	10 %	15.30±0.44
Acetone	10 %	5.99±1.19
Isopropanol	10 %	11.28±0.74
Acetonitrile	10 %	24.75±1.51
Tween 80	20 mg/mL	34.55±2.55
Triton X-100	20 mg/mL	24.46±5.25
SDS	2 mg/mL	0.00±0.00
PMSF	5 %	9.52±0.74
DMSO	5 %	80.36±2.00
DMF	5 %	46.50±5.65
EDTA	10 mM	96.96±1.27

activity strongly, whereas Mg^{2+}, Ba^{2+}, K^+, and Co^{2+} all reduced activity slightly, and Cu^{2+}, Fe^{2+}, Ni^{2+}, and Zn^{2+} inhibited enzyme activity strongly. Ca^{2+} and Cr^{3+} ions had little effect on the enzyme activity at a concentration of 1 mM. The effect of various chemical agents on enzyme activity was also assessed (Table 5). Ethylenediaminetetraacetic acid (EDTA) did not inhibit the enzyme activity at a concentration of 10 mM. In contrast, detergents and most organic solvents inhibited the activity of DamH. Taken together, these data suggest that DamH is a metal ion-independent hydrolase, and EDTA did not chelate a possible metal ion required for the activity of the enzyme.

Discussion

Acetochlor is an important herbicide widely used for weeds controlling due to its high efficacy and low cost. Long-term application of acetochlor caused serious residue in soil. Microbial degradation is an important removal pathway of such compounds from the environment (Dictor et al. 2008). Many studies have focused on the microbial degradation of acetochlor, and several biodegradation intermediates have been identified (Coleman et al. 2000; Dagnac et al. 2002; Ye et al. 2002; Xu et al. 2006, 2008; Dictor et al. 2008). CMEPA is the key metabolic intermediate during the biodegradation of acetochlor; similar structures are also formed during the degradation of alachlor, butachlor, metolachlor, pretilachlor, and propisochlor. Till now, no strain that could mineralize acetochlor had been isolated (Xu et al. 2006, 2008). *Delftia* sp. T3-6 is a member of an acetochlor-degrading consortium which can completely mineralize acetochlor. Strain T3-6 can transform CMEPA to MEA, but enzyme and gene involved in the transformation process are unknown.

Propanil is an acetanilide herbicide with similar core structure with CMEPA and can be hydrolyzed by amidases (Shen et al. 2012; Zhang et al. 2012) and histone deacetylase-like propanil hydrolase PrpH (Zhang et al. 2011). The amidases AmpA (Zhang et al. 2012) and PamH (Shen et al. 2012) possess fatty acyl amidase and aryl arylamidase activities and can act on various short-chain aliphatic amines and aryl acylamides. Both amidases could not hydrolyze chloroacetamide herbicides such as acetochlor or butachlor, and their activity on CMEPA and CDEPA was not reported. PrpH is a histone deacetylase-like propanil hydrolase PrpH which could hydrolyze propanil only (Zhang et al. 2011). However, DamH showed different substrates specificity compared with PrpH, AmpA, and PamH. DamH could catalyze the hydrolysis of aryl acylamides and various ester substrates. DamH showed no activity on acetochlor or butachlor but could hydrolyze their degradation intermediates CMEPA and CDEPA; we deduced that steric hindrance effects of the ethoxymethyl/butoxymethyl groups of the parental compounds inhibited the approach of the these substrates to the active

sites of enzyme. Meanwhile, DamH could not hydrolyze fatty acid amides and propanil, indicating that its catalytic mechanism is different from PamH and AmpA.

Sequence alignment analysis of hydrolases that act on amides or arylamides revealed that they could be clustered into three groups (Fig. 3). PamH and AmpA formed a group of amidases, whereas PrpH is a histone deacetylase homolog that formed an independent group. PamH and AmpA possess the typical amidase GGSSXG signature sequence and the Lys-Ser-Ser catalytic triad, suggesting that they belong to typical amidase family (Capel et al. 1995; Patricelli et al. 1999; Shen et al. 2012; Zhang et al. 2012). In contrast, DamH possessed the common G-X-S-X-G (amino acid residues 147 to 151) esterase motif (Bornscheuer 2002) and a S149, E244, H274 catalytic triad. Like many "GX class" esterases and lipases, DamH contains an HGX motif ([79]HGG[81]), indicating that it belongs to the "GX-type hydrolases (Kolkenbrock et al. 2006). Although it shares homology and a similar catalytic triad like typical carboxylesterases (Arpigny and Jaeger 1999), the enzyme clearly shows the highest activity in the hydrolysis of the model compounds CMEPA, which is, however, a tertiary amine. Hence, the enzyme is a special esterase with amide hydrolase activity. DamH showed much higher catalytic efficacy compared with other amidases. It worked five times faster in catalytic efficacy and 50 times more specific than AmpA on 4-acetamido phenol.

Of the enzymes with reported amides and ester hydrolytic activity, most were classified predominantly as amidases, with weaker esterase activity (Schmidt et al. 2007; Kourist et al. 2008; Wang et al. 2009; Syrén and Hult 2011). Biochemical characterization revealed that DamH was able to hydrolyze aryl arylamides and various esters. Similar phenomenon was observed in several aryl arylamide hydrolases (Pohlenz et al. 1992; Akutsu-Shigeno et al. 2006; Kolkenbrock et al. 2006). Amq from *Arthrobacter nitroguajacolicus* Rü61a is a bifunctional hydrolase that also acts on aryl-acylamides and esters (Kolkenbrock et al. 2006). Amp is predominantly an esterase with phenylacetate as the optimal substrate, whereas DamH tended to function as an amidase, using CMEPA as the optimal substrate. Pcp from *Arthrobacter oxydans* P52 is also an amide hydrolase classified as carboxyesterase which could hydrolyze *p*-nitrophenylbutyrate (Pohlenz et al. 1992). In recent years, many enzymes have been identified that catalyze more than one chemical transformation, which is known as catalytic promiscuity. This process plays an important role in the evolution of enzymes and in enzyme engineering (Khersonsky et al. 2006). The unique catalytic activity of DamH makes it an interesting research entity for the evolution and mechanisms of catalytic promiscuity.

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