Biotechnol Lett (2012) 34:1107–1113
DOI 10.1007/s10529-012-0880-0

ORIGINAL RESEARCH PAPER

Promiscuous esterase activities of the C–C hydrolases from *Dyella ginsengisoli*

Hao Zhou · Yuanyuan Qu · Chunlei Kong · Yingge Wu · Kang Zhu · Jie Yang · Jiti Zhou

Received: 27 December 2011 / Accepted: 10 February 2012 / Published online: 24 February 2012

Abstract A C–C hydrolase gene ($bphD_{LA\text{-}4}$) from strain *Dyella ginsengisoli* LA-4 was cloned and expressed in *Escherichia coli* BL21 (DE3). $BphD_{LA\text{-}4}$ together with another hydrolase $MfphA_{LA\text{-}4}$, which derived from the same strain, possessed esterase activities. *p*-Nitrophenyl butyrate was the best substrate for both enzymes. $BphD_{LA\text{-}4}$ had high catalytic efficiency to *p*-nitrophenyl benzoate, whereas $MfphA_{LA\text{-}4}$ had no activity. Homology modeling and docking studies demonstrated that the proper hydrogen bond interaction was important for the reactivity of specific substrate.

Keywords C–C bond hydrolase · Esterase · Homology modeling · Molecular docking

Introduction

The α/β-hydrolase superfamily is constituted of enzymes with similar folds but different catalytic functions (Jochens et al. 2011). These enzymes can catalyze various hydrolytic reactions on a broad range of substrates, such as esters (lipase/esterase), amides (amidase), epoxides (epoxide hydrolase) and alkyl halides (dehalogenase). The catalytic promiscuity of the α/β-fold hydrolase has drawn great attention because the enzyme can catalyze distinctly different chemical transformations in a single active site (Bornscheuer and Kazlauskas 2004; Hult and Berglund 2007; Leitgeb and Nidetzky 2010). For example, the esterase from *Bacillus subtilis* not only catalyzes the C–O ester bond but C–N amide bond cleavage (Kourist et al. 2008). Proline aminopeptidase has a normal hydrolysis activity towards the C–N bond in proline amides but can also hydrolyze the P–F bond in diisopropyl fluorophosphates (Bornscheuer and Kazlauskas 2004). The common mechanism of these reactions, nucleophilic attack, provides the possibility of the catalytic promiscuity (Khersonsky and Tawfik 2010).

C–C bond hydrolases (E.C. 3.7.1.8), which are identified in the degradation pathway of aromatic compounds, can catalyze a rare C5–C6 bond cleavage of the 2-hydroxy-6-oxo-hexa-2,4-dienoate (HODA) (Seah et al. 1998). As an exception, they have a different hydrolytic mechanism from most members of the α/β-hydrolase superfamily. The serine of catalytic triad does not act as a nucleophile in the catalytic reaction and obeys the general base mechanism (Speare et al. 2004). However, a C–C hydrolase, MhpC from *Escherichia coli*, catalyses the hydrolysis of a monoethyl adipate ester, while another C–C

H. Zhou · Y. Qu (✉) · C. Kong · Y. Wu · K. Zhu · J. Yang · J. Zhou
State Key Laboratory of Fine Chemicals and Industrial Ecology and Environmental Engineering (Ministry of Education, China), School of Environmental Science and Technology, Dalian University of Technology, Dalian 116024, China
e-mail: qyy@dlut.edu.cn

hydrolase, BphD from *Burkholderia xenovorans* LB400, could hydrolyze *p*-nitrophenyl esters (Scheme 1), which expanded our insights into the catalytic promiscuity of C–C hydrolases (Li and Bugg 2007; Li et al. 2008). Therefore, it is necessary to further explore whether the esterase activities exist extensively in different C–C hydrolases and the possible enzyme–ester substrate interactions.

Previously, the whole biphenyl degradation gene cluster from strain *Dyella ginesengisoli* LA-4 was obtained, and a recombinant C–C bond hydrolase $MfphA_{LA-4}$ was purified and characterized (Li et al. 2010). In this study, the cloning and heterologous expression of another C–C bond hydrolase $BphD_{LA-4}$ in *E. coli* were performed. The esterase activities of purified $MfphA_{LA-4}$ and $BphD_{LA-4}$ were determined using *p*-nitrophenyl esters. Furthermore, the enzyme–substrate interactions were simulated with homology modeling and molecular docking in order to explain the substrate specificities.

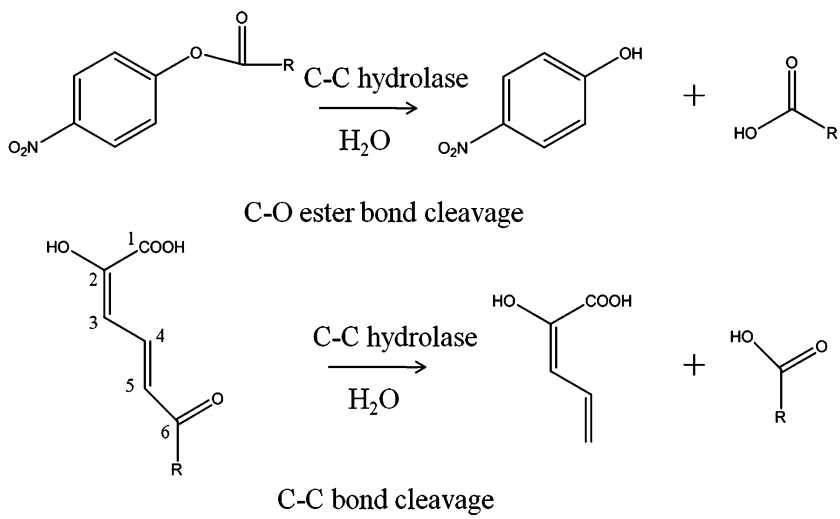
Materials and methods

Chemicals, plasmids and growth conditions

All the *p*-nitrophenyl esters were purchased from Sigma-Aldrich. 2-Hydroxy-6-oxo-6-methyl-hexa-2,4-dienoate (6M-HODA), HODA and 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (HOPDA) were enzymatic synthesized using BphC (see Seah et al. 1998). All other chemicals were of analytical grade. *E. coli* JM109 (TaKaRa) and *E. coli* BL21 (DE3)/pET28-a (Novagen) were used for plasmid construction and gene expression, respectively. *E. coli* were grown in Luria–Bertani medium at 37°C.

Cloning, expression and purification of $BphD_{LA-4}$

The gene encoding $BphD_{LA-4}$ was amplified by standard PCR using the genomic DNA of *D. ginsengisoli* LA-4 as template. PCR primers were: BphD-F (5′-TCGCGGATCCGAATTCATGACGGCACTTACCGAAAG-3′) and BphD-R (5′-GTGCGGCCGCAAGCTTTCATTCATTCATGCTTGAGAAAAT-3′). The purified PCR product was inserted into the pET28-a vector and transformed into *E. coli* BL21 (DE3). The recombinant *E. coli* BL21 (DE3) harboring plasmid (pET28-a-bphD) was grown at 37°C in LB medium with 30 μg kanamycin/ml until the OD_{600} reached 0.4–0.6, and then induced with 1 mM IPTG at 37°C for 3 h. The cells were harvested by centrifugation at 5,000×*g* for 10 min at 4°C, suspended in 200 mM sodium phosphate buffer (PBS, pH 7.0), and finally disrupted by sonication. After centrifugation (58,545×*g*, 20 min, 4°C), the supernatants were used as the crude extracts and purified by a prepacked His-Trap FF column using an ÄKTA Explorer 100 chromatography system. The target peak fraction was pooled and dialyzed overnight to remove imidazole. The molecular weight and purity of the enzyme were estimated by SDS-PAGE. Protein concentration was determined by Bradford method using bovine serum albumin as standard.



Scheme 1 Promiscuous activities of C–C hydrolase. For C–O ester bond cleavage reaction, R = ethyl (PNPA), butyl (PNPB) and phenyl (PNPBen); for C5–C6 bond cleavage reaction, R = hydrogen (HODA), methyl (6 M-HODA) and phenyl (HOPDA)

Enzyme assays of $BphD_{LA-4}$ and $MfphA_{LA-4}$

The esterase activity and C–C hydrolase activity were determined in 200 mM PBS (pH 7.0) containing 50 μM substrate. The reactions were initiated by adding the purified $BphD_{LA-4}$ and $MfphA_{LA-4}$, and the activity was determined spectrophotometrically at 405 nm (*p*-nitrophenol), 388 nm (6 M-HODA), 375 nm (HODA) and 434 nm (HOPDA). The optimal pH was assayed by incubating the enzymes in different buffers for 30 min. The buffer solutions used were PBS (pH 6.0–7.5, 200 mM) and Tris/HCl (pH 8.0–9.0, 200 mM). The thermal stability of $MfphA_{LA-4}$ and $BphD_{LA-4}$ were determined by heating the enzymes for 30 min at different temperatures and stopped by immersion the solutions in an ice bath. To investigate the stability in the presence of denaturing agents, i.e. SDS, diethylpyrocarbonate (DEPC) and PMSF, the enzymes were incubated for 30 min in solutions which contained varying denaturant concentrations. One unit of enzymatic activity was defined as the quantity of enzyme required to consume 1 μM HODAs or produce 1 μM *p*-nitrophenol/min. For kinetic assays, appropriate equations were fitted to the initial velocities determined at different substrate concentrations by using the non-linear regression function of Origin 7.0.

Sequence analysis, homology modeling and docking

The sequence of $BphD_{LA-4}$ was obtained from GenBank (ACH87189.1). Sequence analysis was conducted using BLAST (http://blast.ncbi.nim.nih.gov/Blast.cgi). The 3D model of $BphD_{LA-4}$ and $MfphA_{LA-4}$ was built with SWISS-MODEL using the crystal structure of $BphD_{LB400}$ (PDB ID: 2OG1) and $CumD_{IP01}$ (PDB ID: 1IUP) as template, respectively. The initial models were further optimized by Gromacs 4.5.1. The quality of the 3D model was evaluated by PROCHECK analysis and Verify3D. To perform docking studies, the coordinate files of ligands were prepared in PRODRG server (http://davapc1.bioch.dundee.ac.uk/prodrg/index.html). AutoDock 4.2 was employed to generate ligand–protein complexes and calculate the relative binding free energy. The results were visualized using the PyMOL program.

Results and discussion

Cloning and sequence analysis of $BphD_{LA-4}$

The gene encoding $BphD_{LA-4}$ (858 bp) was successfully obtained by direct PCR method. The deduced

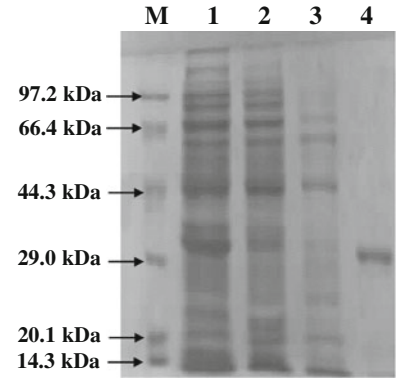


Fig. 1 SDS-PAGE of purified $BphD_{LA-4}$. *E. coli* containing pET28-a-$bphD_{LA-4}$ was lysed and run through Ni–NTA resin. The column was then subsequently washed with increasing concentrations of imidazole (100–325 mM) in stepwise increments. *Lane M* Protein weight marker (*broad*), *lane 1* lysate, *lane 2* 100 mM imidazole, *lane 3*, 200 mM imidazole, *lane 4* 325 mM imidazole

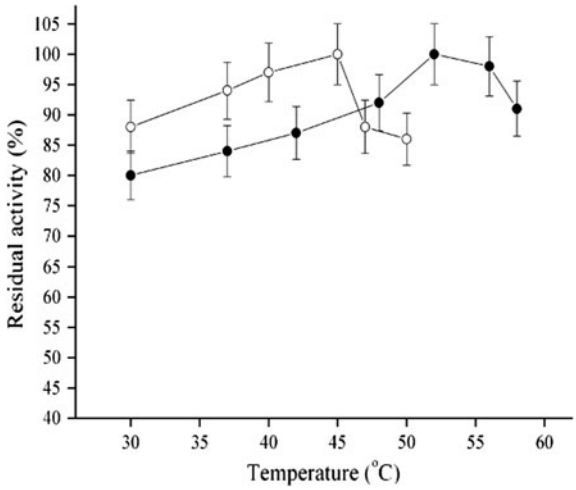


Fig. 2 Thermal stabilities of $BphD_{LA-4}$ and $MfphA_{LA-4}$. The enzyme solutions were pre-incubated in 0.2 M sodium phosphate buffer (pH 7.0) for 30 min and immersed in an ice bath before determined at 405 nm. The residual activity was expressed as the percentage of the activity of enzyme incubated at optimal temperature (8.2 U/mg for $BphD_{LA-4}$ at 52°C, 4.5 U/mg for $MfphA_{LA-4}$ at 44°C), which were taken as 100%

amino acid sequence, composed of 286 residues, exhibited the highest similarity (89%) with C–C bond hydrolase from *Pseudomonas pseudoalcaligenes* KF707. According to the sequence alignment of $BphD_{LA-4}$ and related C–C bond hydrolases, the residues Ser112, Asp237 and His265 formed the catalytic triad, which were conserved in all C–C bond hydrolases.

Purification and biochemical characterization of $BphD_{LA-4}$ and $MfphA_{LA-4}$

The $BphD_{LA-4}$ was successfully expressed in a soluble form at 37°C or lower. The protein was purified to homogeneity by nickel chelate affinity chromatography, and dialysed at 4°C overnight. The purified protein with a molecular weight of 34 ± 1 kDa was a

Table 1 Apparent kinetic parameters of $BphD_{LA-4}$ and $MfphA_{LA-4}$

Enzyme	Substrate[a]	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM)
$BphD_{LA-4}$	PNPA	2.7 ± 0.3	0.6 ± 0.02	0.22
	PNPB	3.7 ± 0.3	12 ± 0.16	3.2
	PNPBen	3.4 ± 0.2	4.9 ± 0.27	1.4
	HOPDA	0.75 ± 0.03	9.6 ± 0.02	12.8
	6M-HODA	32 ± 0.7	2.2 ± 0.06	0.68
	HODA	31 ± 0.6	0.1 ± 0.01	0.03
$MfphA_{LA-4}$	PNPA	15 ± 0.3	8.7 ± 0.16	0.58
	PNPB	2.3 ± 0.1	3.1 ± 0.12	1.3
	PNPBen	ND	ND	ND
	6M-HODA	5.3 ± 0.2	1.1 ± 0.08	0.21
	HOPDA	475 ± 0.4	0.28 ± 0.04	0.0006
	HODA	32 ± 0.3	2.4 ± 0.08	0.076

The kinetic parameters were calculated via non-linear regression using Origin 7.0. The reactions were performed in 0.2 M sodium phosphate buffer (pH 7.0). The molar extinction coefficients of *p*-nitrophenol, HOPDA, HODA and 6M-HODA were: 18,000, 12,800, 40,900 and 11,000 M^{-1} cm^{-1}

ND not detected

[a] Substrate concentrations were: 0.5–100 μM PNPA, 0.5–80 μM PNPB, 0.5–80 μM PNPBen, 0.25–20 μM HOPDA and 1–60 μM 6M-HODA

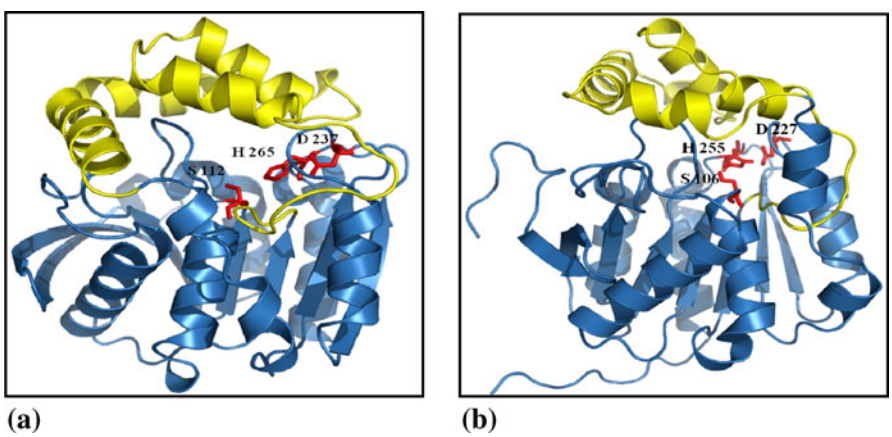


Fig. 3 Homology modeling of $BphD_{LA-4}$ and $MfphA_{LA-4}$. **a** Three-dimensional carton model of $BphD_{LA-4}$. The crystal structure of $BphD_{LB400}$ (PDB ID: 2OG1) was used as the template (88% amino acid sequence identity). **b** Three-dimensional carbon model of $MfphA_{LA-4}$. The crystal structure of $CumD_{IP01}$ (PDB ID: 1IUP) was used as the template (62% amino acid sequence identity). The lid domain, core domain and catalytic triad were labeled in *orange*, *blue* and *red*, respectively

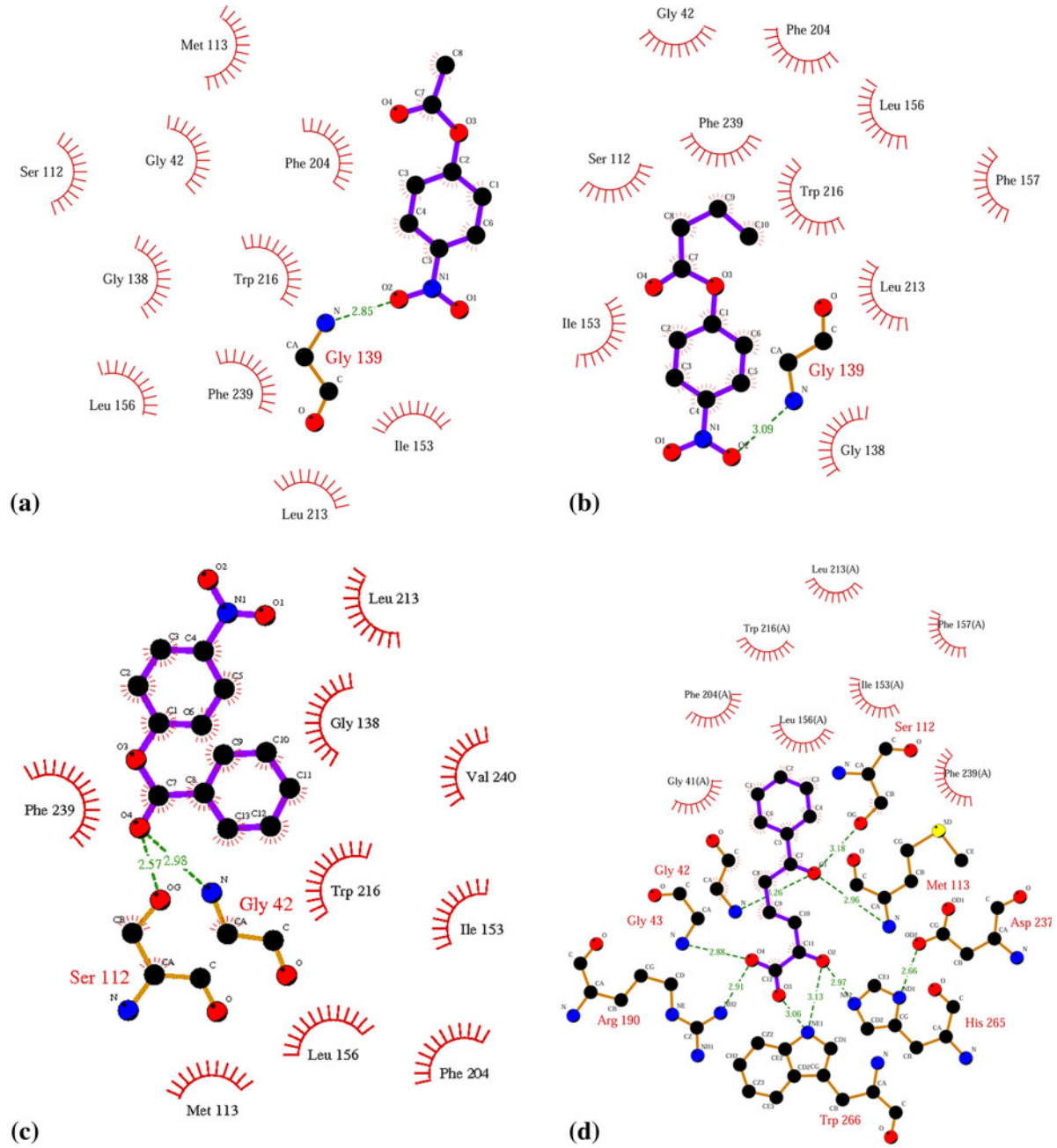


Fig. 4 2D view of enzyme–substrate interactions. The hydrogen bonds were labeled by *dash line*. The non-ligand residues and corresponding atoms in ligand involved in hydrophobic contact were labeled as *spline segment*. **a** BphD-PNPA. **b** BphD-PNPB. **c** BphD-PNPBen. **d** BphD-HOPDA. **e** MfphA-PNPA. **f** MfphA-PNPB. **g** MfphA-PNPBen. **h** MfphA-6M-HODA

little larger than the prediction molecular weight deduced from the sequence (32 kDa), which was due to the existence of *N*-terminal histidine tag (Fig. 1). The purified $BphD_{LA-4}$ could utilize *p*-nitrophenyl acetate (PNPA), *p*-nitrophenyl butyrate (PNPB) and *p*-nitrophenyl benzoate (PNPBen) as substrates. However, the purified $MfphA_{LA-4}$ could only catalyze PNPA and PNPB hydrolysis, but had no detectable activity toward PNPBen. Using PNPB as substrate, the optimal pH for $MfphA_{LA-4}$ and $BphD_{LA-4}$ were

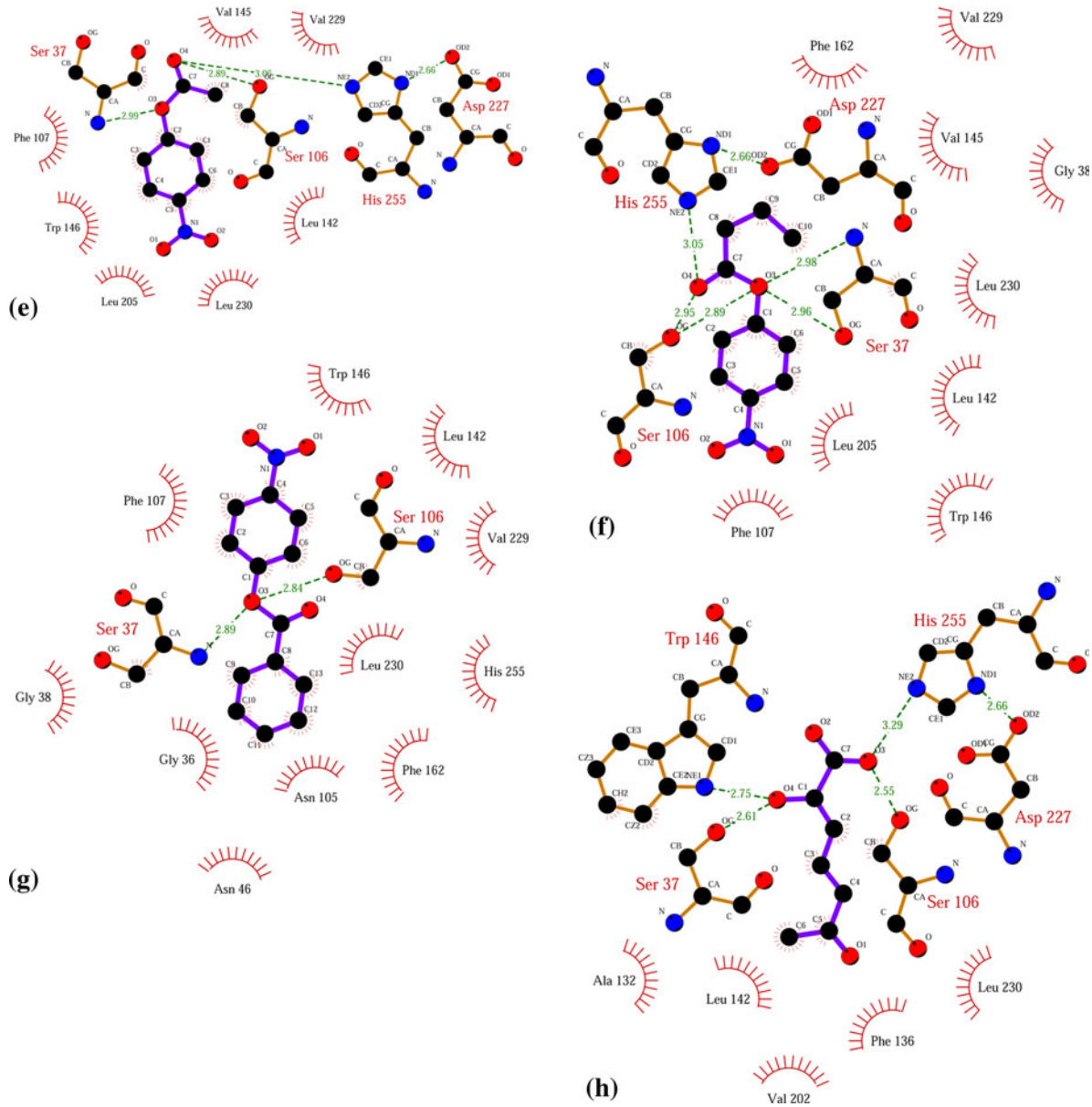


Fig. 4 continued

both 7.0, while the $BphD_{LA-4}$ possessed higher thermostability than $MfphA_{LA-4}$. The optimal temperature for the esterase activity of $BphD_{LA-4}$ and $MfphA_{LA-4}$ were 52 and 44°C, respectively (Fig. 2). SDS, DEPC and PMSF could significantly inhibit the esterase activities of $MfphA_{LA-4}$ and $BphD_{LA-4}$, which indicated that the oligomeric form of native proteins, His265 and Ser112, were indispensable for esterase activity.

Apparent kinetics of $BphD_{LA-4}$ and $MfphA_{LA-4}$

The kinetic parameters of $BphD_{LA-4}$ and $MfphA_{LA-4}$ on esters and HODAs were illustrated in Table 1. For $BphD_{LA-4}$, all the tested *p*-nitrophenyl esters and HODAs were readily hydrolyzed, among which $BphD_{LA-4}$ exhibited the highest catalytic efficiency for PNPB (esterase activity) and HOPDA (C–C hydrolase activity). However, the catalytic efficiency

for ester hydrolysis were 1.7 (PNPA), 25.5 (PNPB) and 11.2 (PNPBen) percent of the HOPDA hydrolysis, respectively. On the other hand, $MfphA_{LA-4}$ could hydrolyze PNPA and PNPB effectively but not PNPBen. Interestingly, the catalytic efficiency of $MfphA_{LA-4}$ for ester hydrolysis were 2.8-fold (PNPA) and 6.4-fold (PNPB) of the 6M-HODA hydrolysis, which demonstrated that the physiological role of $MfphA_{LA-4}$ needed to be further clarified.

Homology modeling and molecular docking

In order to understand the enzyme–substrate interactions, the structure models of $BphD_{LA-4}$ and $MfphA_{LA-4}$ were constructed by homology modeling and modified through energy minimization. The overall structures of two enzymes were canonical α/β-hydrolase fold, and the conserved catalytic triad was located at the cleft between the lid and core domain (Fig. 3). The quality validation indicated that the comparative models were suitable for further docking studies.

Molecule docking studies were used to explain different reactivity of substrates according to one criterion: the hydrogen bond formed between serine catalytic triad and substrate. The serine catalytic triad of C–C bond hydrolase had been inferred to play an important role in stabilizing the intermediate via hydrogen-bonding (Li et al. 2007). Hydrogen bonds involved in hydroxyl side-chain of Ser112 and carbonyl oxygen atom of ligands were observed in $BphD_{LA-4}$-PNPBen (Fig. 4c), $BphD_{LA-4}$-HOPDA (Fig. 4d), $MfphA_{LA-4}$-PNPA (Fig. 4e), $MfphA_{LA-4}$-PNPB (Fig. 4f) and $MfphA_{LA-4}$-6M-HODA (Fig. 4h). For both $BphD_{LA-4}$-PNPA and $BphD_{LA-4}$-PNPB (Fig. 4a, b), the hydrogen-bond interaction was observed between Gly139 and ligand, which had been proved to stabilize interaction between HOPDA and $BphD_{LB400}$ (Horsman et al. 2006). In particular, an improper hydrogen bond was formed between Ser106 of $MfphA_{LA-4}$ and phenolic oxygen atom of PNPBen (Fig. 4g), which should prevent the necessary rotation of oxyanion intermediate, resulting in an undetectable reactivity of PNPBen.

Conclusions

Both hydrolases $BphD_{LA-4}$ and $MfphA_{LA-4}$ hydrolyzed specific *p*-nitrophenyl esters with different specificities. According to the docking studies, the proper hydrogen bond formed between substrates and hydrolases were prerequisite for esterase activities.

Acknowledgments The authors would like to thank the National Natural Science Foundation of China (Nos. 21176040 and 51078054) for their financial supports.

References

Bornscheuer UT, Kazlauskas R (2004) Catalytic promiscuity in biocatalysis: using old enzymes to form new bonds and follow new pathways. Angew Chem Int Ed Engl 43: 6032–6040

Horsman GP, Ke J, Dai S, Seah SY, Bolin JT, Eltis LD (2006) Kinetic and structural insight into the mechanism of BphD, a C–C bond hydrolase from the biphenyl degradation pathway. Biochemistry 45:11071–11186

Hult K, Berglund P (2007) Enzyme promiscuity: mechanism and applications. Trends Biotechnol 25:231–238

Jochens H, Hesseler M, Stiba K, Padhi SK, Kazlauskas RJ, Bornscheuer UT (2011) Protein engineering of α/β-hydrolase fold enzymes. Chembiochem 12:1508–1517

Khersonsky O, Tawfik DS (2010) Enzyme promiscuity: a mechanistic and evolutionary perspective. Annu Rev Biochem 79:471–505

Kourist R, Bartsch S, Fransson L, Hult K, Bornscheuer UT (2008) Understanding promiscuous amidase activity of an esterase from *Bacillus subtilis*. Chembiochem 9:87–89

Leitgeb S, Nidetzky B (2010) Enzyme catalytic promiscuity: the nonheme Fe^{2+} center of beta-diketone-cleaving dioxygenase Dke1 promotes hydrolysis of activated esters. Chembiochem 11:502–505

Li JJ, Bugg TD (2007) Investigation of a general base mechanism for ester hydrolysis in C–C hydrolase enzymes of the alpha/beta-hydrolase superfamily: a novel mechanism for the serine catalytic triad. Org Biomol Chem 5:507–513

Li C, Hassler M, Bugg TD (2008) Catalytic promiscuity in the alpha/beta-hydrolase superfamily: hydroxamic acid formation, C–C bond formation, ester and thioester hydrolysis in the C–C hydrolase family. Chembiochem 9:71–76

Li A, Qu YY, Zhou JT, Ma F, Zhou H, Shi SN (2010) Characterization of a novel meta-fission product hydrolase from *Dyella ginsengisoli* LA-4. Proc Biochem 45:94–100

Seah SY, Terracina G, Bolin JT, Riebel P, Snieckus V, Eltis LD (1998) Purification and preliminary characterization of a serine hydrolase involved in the microbial degradation of polychlorinated biphenyls. J Biol Chem 273:22943–22949

Speare DM, Fleming SM, Beckett MN, Li JJ, Bugg TD (2004) Synthetic 6-aryl-2-hydroxy-6–ketohexa -2,4-dienoic acid substrates for C–C hydrolase BphD: investigation of a general base catalytic mechanism. Org Biomol Chem 2: 2942–2950