Microbiological Research 178 (2015) 27–34

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

Short Communication

Probing role of key residues in the divergent evolution of *Yarrowia lipolytica* lipase 2 and *Aspergillus niger* eruloyl esterase A

Guilong Wang[a], Zimin Liu[a], Li Xu[a], Houjin Zhang[a], Yunjun Yan[a,b,*]

[a] Key Laboratory of Molecular Biophysics of the Ministry of Education, College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan, PR China
[b] Shenzhen R & D Center of Huazhong University of Science and Technology, Shenzhen, PR China

ARTICLE INFO

Article history:
Received 26 January 2015
Received in revised form 9 April 2015
Accepted 16 May 2015
Available online 11 June 2015

Keywords:
Yarrowia lipolytica lipase 2
Aspergillus niger feruloyl esterase A
Site-directed mutagenesis
Interfacial activation
Divergent evolution

ABSTRACT

Yarrowia lipolytica lipase 2 (YLLip2) and *Aspergillus niger* feruloyl esterase A (AnFaeA) are enzymes of similar structures but with different functions. They are both classified into the same homologous family in Lipase Engineering Database (LED). The major difference between the two enzymes is that YLLip2 exhibits interfacial activity while AnFaeA does not. In order to better understand the interfacial activation mechanisms of YLLip2, structure guided site-directed mutagenesis were performed, mutants were constructed, kinetics parameters and lipase properties were detected. Mutant enzymes showed enhanced catalytic efficiency towards *p*-nitrophenyl butyrin (*p*NPB) but their catalytic efficiency decreased towards *p*-nitrophenyl palmitate (*p*NPP), their catalysis behavior was more close to feruloyl esterase. Moreover, the mutant enzymes exhibited enhanced thermostability compared with their wild type. These results indicate that I100 and F129 are probably cut-off point of divergent functions between the two enzymes during evolution.

© 2015 Elsevier GmbH. All rights reserved.

1. Introduction

Lipases (EC 3.1.1.3) are carboxylic esters hydrolases that can hydrolyze long-chain acyl-triglycerides into di- and monoglycerides, glycerol, and free fatty acids at lipid/water interface (Jaeger et al., 1999). Whereas, feruloyl esterases (EC 3.1.1.73) are enzymes responsible for cleaving the ester link between ferulic acid and the polysaccharide main chain of xylans or pectins (Garcia-Conesa et al., 1999). So far, both lipases and feruloyl esterases are widely used in the biotechnological industry. Among all the lipases, the lipase 2 from *Yarrowia lipolytica* (YLLip2) has been extensively studied because of its unique characteristics. YLLip2 arouses high research interests in protein purification and expression, structure determination, lipase immobilization and application, and protein engineering for enhancing enantioselectivity and thermostability (Fickers et al., 2011). Out of the feruloyl esterases, the most thoroughly studied one is the feruloyl esterase A from *Aspergillus niger* (AnFaeA). The relevant researches mainly focus on enzyme production and application (Koseki et al., 2009), structure determination (Hermoso et al., 2004; McAuley et al., 2004), thermostability enhancement (Zhang and Wu, 2011; Zhang et al., 2012) and structure–function relationship (Faulds et al., 2005; Benoit et al., 2006; Koseki et al., 2005). In terms of functions, the two enzymes are very different from each other and AnFaeA does not exhibit lipase activity (Aliwan et al., 1999).

However, the structure of AnFaeA (pdb access number: 1USW) is very similar to that of two other fungal lipases in their open conformation (Hermoso et al., 2004; McAuley et al., 2004). One is *Rhizomucor miehei* lipase (RML, pdb access number: 4TGL), the other is *Thermomyces lanuginosus* lipase (TLL, pdb access number: 1GT6). Interestingly, the structure of YLLip2 in its closed form has been resolved, it is very similar to the structures of RML and TLL in their closed conformation (Bordes et al., 2010). Indeed, according to the classification in Lipase Engineering Database (LED), the four enzymes belong to the same superfamily abH23 (Filamentous fungi lipases) and homologous family abH23.01 (*Rhizomucor miehei* lipase like) (Fischer and Pleiss, 2003). Moreover, structure analysis showed that the four enzymes have same structural organizations. They display a typical α/β hydrolase fold with same catalytic triads (Ser-Asp-His) and oxyanion holes (Thr-Leu), their catalytic mechanism is called nucleic attack (Hermoso et al., 2004; Bordes et al., 2010; Jaeger et al., 1999). The three lipases have a unique property–interfacial activation (Jaeger et al., 1999; Bordes et al., 2010). Specifically, when in aqueous phase, lipases are in closed

* Corresponding author at: Key Laboratory of Molecular Biophysics of the Ministry of Education, College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan, PR China. Tel.: +86 27 87792213; fax: +86 27 87792213.
E-mail addresses: yanyunjun@hust.edu.cn, yanyunjun@tom.com (Y. Yan).

http://dx.doi.org/10.1016/j.micres.2015.05.011
0944-5013/© 2015 Elsevier GmbH. All rights reserved.

conformation, while at oil–water interface, their conformation changes to an open one, which involves the “lid” movement that plays a key role in the substrate access to the binding pocket to approach the catalytic center, enabling the nucleophilic reaction (Jaeger et al., 1999; Verma et al., 2008; Reis et al., 2009). However, AnFaeA does not show any interfacial activity because its active site is exposed to solvent though it has a cap region equivalent to the lid of RML or TLL in their open conformation (Hermoso et al., 2004; McAuley et al., 2004). Based on the above information, we speculate that the structure of YLLip2 in its open conformation is also similar to the structure of AnfaeA though it has not been resolved. We hypothesized that the four enzymes perhaps share the same ancestral gene and during a long evolution process the gene diverged into several new genes coding new proteins with different functions. But which residues caused the divergent function of YLLip2 and AnFaeA is still unknown. In this research, we analyzed the structure of AnFaeA and YLLip2 in its open conformations to find out the conserved and non-conserved amino acid residues in their substrate binding pocket. It is supposed that the structure equivalent amino acid residues (conserved amino acid residues) play similar roles during nucleic catalysis but non-conserved residues probably cause functional differentiation. Subsequently, site-directed mutagenesis was conducted, and the enzymatic properties of the mutants were examined. We hope a better understanding of the structure–function relationship between the two enzymes in their divergent evolution will be achieved in this study.

2. Materials and methods

2.1. Plasmids, strains and reagents

Wild-type lipase Lip2 with N-terminal 6× histidine tag expression vector pYL-H6-mlip2 was constructed in our previous research (Wang et al., 2014). Protein expression was driven by the hp4d promoter and its secretion was mediated by the Xpr2pre signal region. *Escherichia coli* DH5α was used as a host for plasmid amplification while *Yarrowia lipolytica* lipase deficient strain JMY1212 was used for protein expression. Plasmid extraction kit and DNA purification kit were purchased from Omega (USA). Quikchange™ Site-Directed Mutagenesis Kit was bought from Agilent Technologies Inc (California, USA). Restriction endonucleases were procured from Takara (Dalian, China). Casein Acids Hydrolysate and lipase substrates (*p*-nitrophenyl butyrin, *p*NPB, *p*-nitrophenyl caprylate, *p*NPC, and *p*-nitrophenyl palmitate, *p*NPP) were commercially available from Sigma-Aldrich (St. Louis, USA).Yeast Nitrogen Base without Amino Acids, Yeast extract and peptone were obtained from Difco. All organic solvents and other reagents were of analytical grade and were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China) unless otherwise stated.

2.2. Site-directed mutagenesis

Single-point mutations of YLLip2 were carried out by Quikchange™ site-directed mutagenesis kit under the guide of the operation manual provided by the supplier using pYL-H6-mlip2 as a template. The double substitution was conducted using single-point mutation as a template. The primers used for mutagenesis were listed in Table 1. The purified plasmid PCR products were transformed into *E. coli* DH5α competent cells and plated on LB solid plates (0.5% yeast extract, 1% peptone, 1% sodium chloride, 2% agar) containing 40 μg/mL kanamycin. After 24 h incubation at 37 °C, the colonies were picked for further cultivation and the mutagenesis was confirmed by DNA sequencing.

Table 1
Primers used for site-directed mutagenesis.

Primers	Sequence (5′-3′)
I100Y-f	5′-gtcataaccgacatccgatacatgcaggctcctctgac-3′
I100Y-r	5′-gtcagaggagcctgcatgtatcggatgtcggttatgac-3′
F129Y-f	5′-tcttgtccacaatggctacatccagtcctacaaca-3′
F129Y-r	5′-tgttgtaggactggatgtagccattgtggacaaga-3′

The mutant codons were underlined.

2.3. *Yarrowia lipolytica* transformation, protein expression and purification

For *Y. lipolytica* transformation, the mutant plasmids were first digested with NotI. Long fragments harboring target gene expression cassette and selection marker were recovered and then transformed into the *Y. lipolytica* JMY1212 competent cells according to the method described by Xuan et al. (1988). Next, the mixtures were plated on YNBDcasa solid medium (6.7% YNB, 1% D-dextrose, 0.2% casein acid hydrolysates, 1.5% agar). After 48 h cultivation at 28 °C the colonies were transferred to YNBT solid agar (6.7% YNB, 1% tributyrin, 1.5% agar) for activity assay. The colonies with clear halos surrounded were identified as positive clones for further study. For protein expression, the positive clones of wild-type YLLip2 and mutants were inoculated in 5 mL liquid YPD (1% yeast extract, 2% peptone, 2% D-dextrose) medium for overnight cultivation, and then transferred to liquid YT_2D5 (1% yeast extract, 2% peptone, 5% D-dextrose) medium for enzyme production. After 72 h cultivation at 28 °C, the fermentation broth was centrifuged (10,000 × g, 4 °C) and the collected supernatant was filtered through a 0.22 μm filter membrane and then loaded into the column for one-step immobilized metal affinity chromatography. The eluted protein was dialyzed against 50 mM phosphate buffer solution (PBS, pH 8.0) to remove imidazole and glycerol. The purity of lipase was checked by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentrations of the purified YLLip2 and its mutants were determined according to the method described by Bradford using bovine serum albumin (BSA) as the standard (Bradford, 1976).

2.4. Enzyme kinetics determination

The lipase kinetics was determined by the initial reaction rate of substrates hydrolysis under different concentrations. The activities were detected according to the method described by Kordel et al. (1991) with some modifications. The *p*NP-esters used in this study were *p*-nitrophenyl butyrate (*p*NPB, C4), *p*-nitrophenyl caprylate (*p*NPC, C8), and *p*-nitrophenyl palmitate (*p*NPP, C16). They respectively stand for short, middle, and long fatty acid chain esters. The reaction mixture contained 10 μL *p*NP-esters dissolved in absolute ethanol (5% final concentration), and 980 μL 50 mM phosphate buffer solution (pH 8.0). After incubation at 40 °C for 3 min, 10 μL properly diluted enzyme solution were added to the mixture. Two minutes later, the reaction was stopped by putting the mixture on ice-bath. Then, the absorbance was assayed at 348 nm which corresponds to the isobestic point of *p*-nitrophenol, where the ionization degree (due to pH modification during fatty acid released by enzymes) did not affect the molar extinction coefficient ($\varepsilon = 4789\ M^{-1}\ cm^{-1}$) (Rhee et al., 2005). The initial reaction rates were calculated. The Michaelis-Menton constant (K_m) and the maximum velocity (V_{max}) were determined via Lineweaver–Burk plot (Nawani and Kaur, 2000). One unit of lipase activity was defined as the amount of enzyme that liberated 1 μM of *p*-nitro phenol per min under the assay condition.

Download English Version:

https://daneshyari.com/en/article/2092975

Download Persian Version:

https://daneshyari.com/article/2092975

Daneshyari.com