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Expression and Characterization of a Novel Lipase from *Aspergillus fumigatus* with High Specific Activity

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Abstract A novel lipase gene from *Aspergillus fumigatus*, *afl*1-1, was cloned and expressed with a molecular mass of 38 kDa in *Escherichia coli* for the first time. The recombinant lipase had a preference for short carbon chain *p*-nitrophenyl esters, especially toward C2 *p*-nitrophenyl ester and exhibited potent hydrolysis activity that had not been observed. The optimum pH and temperature of this new enzyme were 8.5 and 65 °C, respectively. The recombinant lipase (AFL1-1) is an alkaline enzyme which was stable in the pH range 6.0~8.5 for 16 h (at 4 °C) and at 30~50 °C for 1 h. It is an intracellular enzyme which was purified approximately 8.47-fold with an overall yield of 86.1% by single-step Ni-NTA affinity purification, with a very high specific activity of approximately 1.00×10^3 U mg^{-1} on a standard substrate of *p*-nitrophenyl acetate. The Michaelis–Menten kinetic parameters V_{max} and K_m of the lipase were 1.37 mM mg^{-1} min^{-1} and 14.0 mM, respectively. Ca^{2+} and other metal ions could not activate the lipase. According to the homology analysis and site-directed mutagenesis assay, the catalytic triad of the recombinant lipase was identified as Ser-165, Asp-260, and His-290 residues.

Keywords *Aspergillus fumigatus* · Recombinant lipase · Expression · Characterization · Active site

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Introduction

Lipases (E.C. 3.1.1.3) are a vital group of ubiquitous enzymes that can catalyze the hydrolysis of triacylglycerols to glycerol and fatty acids at the interface of lipid and water [1]. It exists in almost all living organisms and can be produced by microorganisms, plants, and animals [2]. Currently, industrial lipases used were mainly produced by microorganisms because of their wide variety of sources, good stability, diversified enzymatic properties, and various applications compared with those enzymes from animals and plants [2–4].

Fungi were widely recognized as preferred resources for screening different lipases with unique features. Previous studies showed that many useful lipases were isolated from filamentous fungi, such as *Geotrichum*, *Rhizopus*, *Mucor*, *Rhizomucor*, *Aspergillus*, and *Penicillium* [5]. For instance, some well-known lipases from *Aspergillus niger* and *Aspergillus oryzae* have been recognized as GRAS (generally regarded as safe) enzymes by the FDA [5].

Aspergillus fumigatus is a saprotroph that is widespread in nature, typically found in soil and decaying organic matter such as compost heaps, where it plays an essential role in carbon and nitrogen recycling. *A. fumigatus* was once screened on glycerol tributyrate agar plate, olive oil, and pig fat-containing medium with a view to identify an economically viable lipase producer [6]. Here, we first reported a new kind of lipase gene which was cloned from the genomic DNA of *A. fumigatus* (CGMCC 2873). To our surprise, the recombinant enzyme expressed (abbreviated as AFL1-1) showed extremely high hydrolytic activity toward *p*-nitrophenyl esters of short-chain carboxylic acids, such as *p*-nitrophenyl acetate (*p*NPA) (C2). A strategy was successfully used in which the ORF sequence of a putative lipase from NCBI was selected and amplified for the AFL1-1 gene cloning. This gene (AF_lip_EU0001) of lipase AFL1-1 has been submitted to GenBank with an accession number of HQ231780. A catalytic triad of AFL1-1 was also predicated with the strategies of homology analysis and site-directed mutagenesis assay.

Materials and Methods

Strains and Materials

The host strain *Escherichia coli* DH5α, *E. coli* BL21 (DE3), and expression vector pET28a were obtained from Novagen (USA); the strain of *A. fumigatus* Af293 used in this work was a fungus strain purchased from China General Microbiological Cultures Collection, with an accession number of CGMCC 2873. Ex-Taq DNA polymerase, T4 DNA ligase, pMD-18T vector, restriction endonucleases, and DNA marker were purchased from TaKaRa Co. (Dalian, China). All kinds of *p*-nitrophenyl esters were prepared from *p*-nitrophenol and various fatty acids [7]. The site-directed mutagenesis on plasmid was performed using KOD-Plus-Mutagenesis Kit purchased from Toyobo Co. (Shanghai, China). All the other chemicals were also available commercially and of analytical regent grade.

Extraction of *A. fumigatus* Genomic DNA

A. fumigatus existed predominantly as mycelia and hyphae, and grinding with mortar and pestle was suggested to be the most efficient means of disrupting the rigid fungal cell wall of hyphae and conidia [8]. The CTAB method was used for DNA extraction.

The mycelium of the *A. fumigatus* was scraped and grounded to a fine powder in a pre-frozen mortar and pestle in liquid nitrogen. Then, the powder was transferred into a 1.5-ml centrifuge tube with 0.5 ml of extraction buffer (preheated at 65 °C) that contained 2% *w*/*v* CTAB (hexadecyl trimethyl ammonium bromide), 100 mM Tris–HCl pH 8.0, 20 mM EDTA, and 1.4 M NaCl. The mixture was incubated at 65 °C for 1 h and mixed every 10 min. An equal volume of phenol–chloroform alcohol (1:1, *v*/*v*) was added to the crude extract and mixed at room temperature for 30 min. The extract was then centrifuged at 12,000 rpm for 10 min at 4 °C, and the supernatant was carefully decanted. Two-thirds (*v*/*v*) of isopropanol was added and then allowed to stand at −20 °C for 10 min. The mixture was centrifuged at 7,000 rpm for 10 min at 4 °C. The recovered precipitate was washed with 0.5 ml of 75% ethanol twice and then washed with 0.5 ml absolute ethyl alcohol. After removing all traces of ethanol, the precipitate was dissolved in 100 μl of TE (10 mM Tris–HCl, 1 mM EDTA, pH 7.5) containing 50 μg/ml of DNase free RNase A and incubated for 30 min at 37 °C.

Cloning, Sequencing, and Overexpression of the Lipase Gene

According to the putative lipase ORF sequence (Gene ID: 3504452) from NCBI, a pair of primers was designed. Forward primer was 5′-cgcatatggcttctccaattctgc-3′ (*Nde* I cutting sites is underlined); reverse primer was 5′-cgtaagcttctagataccgtcaggc-3′ (*Hind* III cutting sites is underlined). DNA fragments containing the gene of AFL1-1 were polymerase chain reaction (PCR) amplified from chromosomal DNA under the following conditions: 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s; and a final extension at 72 °C for 10 min. The amplification products were directly cloned into pMD18T and sequenced (Majorbio, Shanghai, China). The gene of AFL1-1 was obtained by the digestion of pMD18T-*afl*1-1 with *Nde* I and *Hind* III, gel-purified, and sub-cloned into the corresponding restriction sites of pET28a to obtain an expression plasmid.

E. coli BL21 (DE3) cells were transformed with the expression vector containing the encoding sequence of AFL1-1. The desired clones were selected and inoculated into 100-ml LB medium containing kanamycin (50 μg/ml) and grown at 37 °C for 12 h. Each culture was then diluted 1:100 (*v*/*v*) into fresh medium and grown at 37 °C. At $OD_{600\ nm}$=0.8–1.0, IPTG was added to a final concentration of 0.2 mM for induction of the fusion gene expression. The cells were harvested (15 °C, 15 h after induction) by centrifugation at 3,000 rpm for 20 min.

Purification of the Recombinant Lipase

Ni-NTA agarose affinity chromatography was used for enzyme purification. The cells harvested above were suspended in 10-ml cold binding buffer (50 mM Tris–HCl, pH 8.0) and lysed by sonication at 400 W using a 50% pulsed mode for 15 min. The supernatant was collected by centrifugation at 12,000 rpm for 10 min (4 °C), and the recombinant proteins were purified on Ni-NTA His-Bind resin column (ø1.0×10 cm), which was pre-equilibrated with 50 mM Tris–HCl (pH 8.0). After being washed with the same buffer, the recombinant proteins were eluted with solutions of 20, 200, and 500 mM imidazole dissolved in Tris–HCl (50 mM, pH 8.0). The active protein fractions were collected and applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 13.5%, *w*/*v*) for analysis. The activities of the active protein fractions were measured.

Assay of Lipase Activity and Protein Content

*p*NPA (100 mM) was used as the substrate. The procedure for assay of lipase activity has been described previously [9]. The activity measurement was carried out at 30 °C, pH 7.5 (100 mM KPB).One unit of lipase activity was defined as the amount of enzyme releasing 1.0 μmol of *p*-nitrophenol per minute under the assay conditions [10]. All the assays were performed in triplicate, and the average values were taken. Relative activity was defined as the percentage of the maximum enzyme activity value. Bradford method with bovine serum albumin as the standard was used to determine the protein content [11].

Characterization of the Lipase

The Kinetic Parameters of the Lipase

The Michaelis–Menten kinetic parameters (V_{max} and K_m) of the lipase were calculated using different concentrations (5, 10, 12.5, 25, and 50 mM) of *p*NPA as the substrate at 30 °C, pH 7.5. V_{max} and K_m parameters were calculated by Lineweaver–Burk plots, supposing that the reaction follows a simple Michaelis–Menten kinetics.

Effects of Temperature and pH on Lipase Activity

The effect of temperature on the hydrolytic activity was determined by measuring the hydrolytic activity at different temperatures ranging from 30 °C to 75 °C. The stability of the lipase to temperature was investigated by measuring the residual activity after incubating the purified lipase at 30~80 °C for 1 h.

The optimum pH was determined by measuring the hydrolytic activity in buffers with various pH values. The different pH buffers (50 mM) consisted of citrate phosphate (pH 3.0~8.0), Tris–HCl (pH 8.5), and glycine–NaOH (pH 9.0~10.0). The effect of pH on lipase stability was determined by analyzing the residual activity after incubating the purified lipase in the buffers with different pH values (pH 4.0~10.0) at 4 °C for 16 h.

Effect of Metal Ions and Chemical Additives on Lipase Activity

The effects of EDTA, various metal ions, and surfactants on the hydrolytic activity were determined by measuring the residual activity at 30 °C, pH 7.5 after incubating the purified lipase containing EDTA (2 mM), each of various metal ions (2 mM), and each of surfactants (0.1%, *w*/*v*) in a 50-mM Tris–HCl buffer (pH 8.0) at 30 °C for 0.5 h, respectively. The sample without metal ions and chemical additives under the same experimental conditions was set as the control.

Effect of Organic Solvent on Lipase Activity

The enzyme was incubated in the presence of 10% and 50% (*v*/*v*) various organic solvents at 4 °C for 12 h [12]. The control was the sample without organic solvent under the same experimental conditions. Residual activity was measured under the assay condition (30 °C, pH 7.5) with *p*NPA as the substrate. Ethanol, methanol, isopropanol, acetone, 1-butanol, and DMSO were used for the experiments of the organic solvent effects to the enzyme activity.

Substrate Specificity

The substrate specificity of the lipase was studied using *p*-nitrophenyl fatty acid esters (C2–C16) with various acyl chain lengths under the activity assay conditions as described above.

Prediction of the Active Site of the Lipase

On the basis of homology analysis and sequences alignments in PDB (protein data bank), some amino acids were chosen as presumed active sites. These putative active-site amino acids were mutated to alanine respectively by site-directed mutation, and the activity of these mutant lipases was measured as described above.

Results and Discussion

Cloning of the Lipase Gene and Construction of the Expression Plasmids

Genomic DNA (Fig. 1a) obtained from the *A. fumigatus* with the method described above was used as a PCR template. A DNA fragment of about 972 bp length was obtained with PCR amplification (Fig. 1b) with primers described above. After sequence analysis and assembling by DNAMAN software, the amplified fragment sequence had 99.99% similarity with the putative lipase gene (accession number XM_742008) from NCBI described above which contains no intron.

The expression plasmid was constructed by introducing the coding region of AFL1-1 into pET28a at the *Nde* I and *Hind* III sites and sequenced to verify its correctness and authenticity. The result was consistent with the prediction.

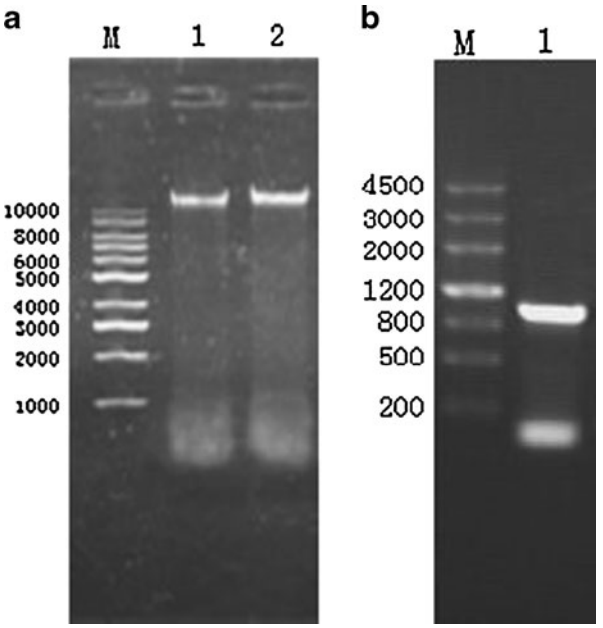

Fig. 1 **a** Agarose gel electrophoresis analysis of DNA extracted from *Aspergillus fumigatus*. *Lanes 1* and *2* DNA extracted from *A. fumigatus*, *lane M* 1 kb DNA ladder marker. **b** PCR amplification of the gene of AFL1-1. *Lane M* DNA ladder marker III, *lane 1* PCR product of AFL1-1 gene

Expression and Purification of the Recombinant Lipase AFL1-1

The recombinant plasmid was transformed into *E. coli* BL21 (DE3) for protein expression. Many physical and chemical factors such as IPTG concentration, incubation time, and temperature may affect the production and correct folding of the protein overexpressed in *E. coli* [9]; therefore, the induction conditions were optimized for enhancing the soluble expression of the recombinant enzyme. In order to collect massive soluble protein, 15 °C was chosen for induction to avoid amount of inclusion body being produced at 37 °C (data not shown). The maximum lipase production could reach 3.17×10^6 U/l under the condition of 0.2 mM IPTG, 15 °C for 15 h. The molecular weight of this fusion protein (38 kDa) on SDS-PAGE (Fig. 2a) was found consistent with the expected molecular weight size. To our knowledge, this is the first report about the expression of lipase gene from *A. fumigatus* in *E. coli*.

Lipase AFL1-1 was purified by Ni-NTA affinity chromatography to near homogeneity, which appeared as a single band at the expected 38-kDa position on SDS-PAGE gel (Fig. 2b), and the purification fold reached about 8.47 by single-step affinity purification. The specific activity of the purified enzyme was 1.00×10^3 U mg^{-1} using *p*NPA (100 mM) as the substrate at 30 °C, pH 7.5, and the recovery of total activity was 86.1% (Table 1). These results reveal the possibility of using bio-engineered bacteria for AFL1-1 production.

Characterization of the Recombinant Lipase AFL1-1

The Kinetic Parameters of the Recombinant Lipase AFL1-1

The Michaelis–Menten kinetic parameters (V_{max} and K_m) of the lipase were calculated using *p*NPA as the substrate with different concentrations at 30 °C, pH 7.5. From Fig. 3, the results calculated were 14.0 mM (K_m) and 1.37 mM mg^{-1} min^{-1} (V_{max}), respectively. It was a 1×10^6 of magnitude higher than the K_m value (70 nM) of HerE [13].

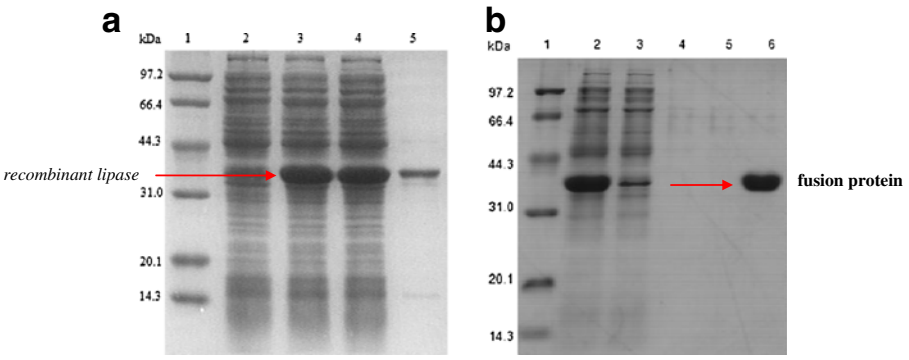

Fig. 2 SDS-PAGE analysis of expression (**a**) and purification (**b**) of AFL1-1 lipase. The gel was stained by Coomassie brilliant blue R-250. The *left arrow* indicates the position of the fusion protein. The *right arrow* indicates the position of the purified fusion protein. **a** SDS-PAGE analysis of the expression of AFL1-1 lipase. *Lane 1* protein molecular weight markers, *lane 2* lysates of uninduced recombinant cell, *lane 3* lysates of induced recombinant cells, *lane 4* supernatant of the cell extract, *lane 5* inclusion body of the cell extract. **b** SDS-PAGE analysis of the purification of AFL1-1 lipase. *Lane 1* protein molecular weight markers, *lane 2* supernatant of cell extract, *lane 3* collection of flow-through, *lane 4* elute with 50 mM Tris–HCl buffer (pH 8.0), *lane 5* elute with 50 mM Tris–HCl buffer containing 20 mM imidazole (pH 8.0), *lane 6* elute with 50 mM Tris–HCl buffer containing 200 mM imidazole (pH 8.0)

Table 1 Purification process of the recombinant AFL1-1

Step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Purification fold	Yield (%)
Supernatant of cell lysate	122	1.44×10^4	118	1.00	100
Ni-NTA agarose	12.4	1.24×10^4	1.00×10^3	8.47	86.1

The lipase AFL1-1 was eluted with an imidazole gradient (20, 200, and 500 mM) at pH 8.0 (50 mM, Tris–HCl)

Effect of pH on Lipase AFL1-1 Activity

The lipase activity was detected in the pH range of 3.0 to 10.0 using *p*NPA as substrate, the optimal pH for AFL1-1 was 8.5, and the activity dropped dramatically at pH below 9.0 (Fig. 4a). As shown in Fig. 4b, at pH 7.0, the activity retained nearly 100% of the maximal activity. Between pH 6.0 and 8.5, the lipase showed good stability; the activity remained above 75% after 16 h incubation in buffers with various pH values at 4 °C. Majority of the lipases of *Aspergillus* origin showed acidic pH optima (5.0–6.0) [5] except lipase from *Aspergillus carneus* [14], which exhibited alkaline pH optimum of 9.0. Therefore, the recombinant AFL1-1 is an alkaline lipase.

Effect of Temperature on Lipase AFL1-1 Activity

As shown in Fig. 5a, the optimal temperature of the pure lipase activity was 65 °C. Above the optimal temperature, the activity decreased sharply with almost 80% loss of its original activity at 75 °C. Between 30 °C and 50 °C, the activity of the lipase was not affected much but still remained 60% after incubated for 1 h (Fig. 5b). The characters of AFL1-1 were consistent with the report that the fungal lipases are generally active and stable at 40~50 °C [15].

Effect of Metal Ions and Chemical Additives on Lipase AFL1-1 Activity

Effects of metal ions, EDTA, PEG, and SDS on the recombinant lipase activity were studied (Table 2). Among the various metal ions tested, Fe^{2+}, Cu^{2+}, and Zn^{2+} decreased lipase activity greatly by 32.8%, 52.5%, and 67.8%, respectively. Even though calcium was known to stabilize lipolytic enzymes [16, 17], it was not observed under the conditions

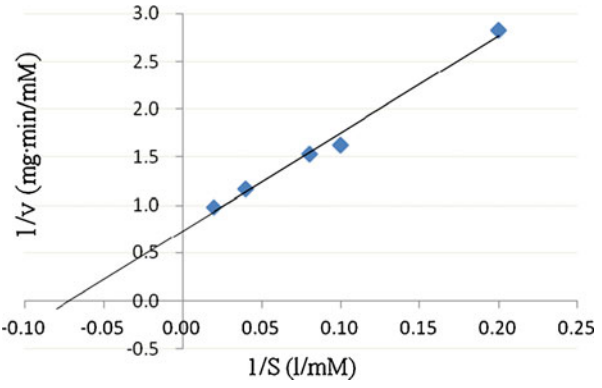

Fig. 3 Lineweaver–Burk plot for recombinant lipase. The recombinant lipase was assayed using varying concentrations of *p*NPA. Lineweaver–Burk plot was used to calculate the kinetic parameters K_m and V_{max}

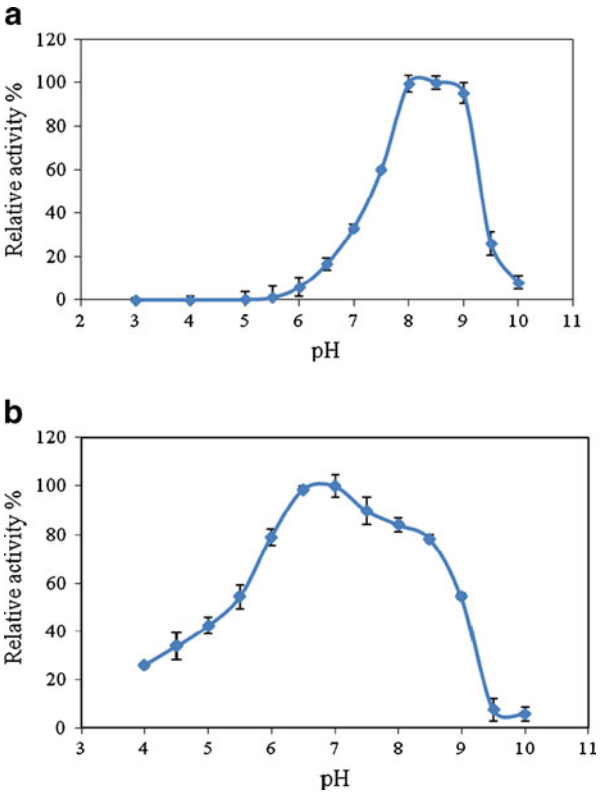

Fig. 4 Effects of pH on activity (**a**) and stability (**b**) of the recombinant lipase. **a** Purified lipase samples were assayed in various buffers from pH 3.0 to pH 10.0 using *p*NPA (100 mM) as substrate at 30 °C. **b** Purified lipase samples were diluted in buffers with different pH values, pH adjusted, and incubated for 16 h at 4 °C, and the residual activity was measured at pH 7.5, 30 °C. Values are means±SD (n=3)

tested, and no metal ions significantly activated the enzyme. Furthermore, the activity of AFL1-1 was almost independent of EDTA concentration within a certain range (Table 2). It was suggested that this recombinant lipase did not belong to metalloenzyme as those lipases from *Serratia marcescens*, *Bacillus licheniformis*, *Mucor* sp., and *Bacillus thermoleovorans* [9, 18–20].

Surfactants have been widely applied to lipase-catalyzed reactions of insoluble substrates to increase the lipid–water interfacial area, which in turn enhance the reaction rate of the kinetic resolution [21]. As shown in Table 2, non-ionic polymer or surfactants such as PEG 20000, PVA, and Triton X-100 at 0.1% (*v*/*v*) increased the activity of the lipase by 18~28%. However, the lipase was sensitive to anionic surfactant SDS, which almost inhibited the whole hydrolytic activity of the lipase. These results will be helpful to optimize the activity by selectively adding proper surfactants to the reaction system.

Effect of Organic Solvents on the Stability of Lipase AFL1-1

Lipases have diverse sensitivities to different solvents, and polar solvents have greater destabilization effects to the enzyme than non-polar solvents generally [22, 23]. Table 3 shows that there was not much influence on the lipase activity in the presence of 10% concentration of different organic solvents except 1-butanol. However, in a higher concentration (e.g., 50%), the enzyme was almost inactivated under the solvents listed in Table 3. These results inferred that AFL1-1 was sensitive to high concentrations of organic solvents.

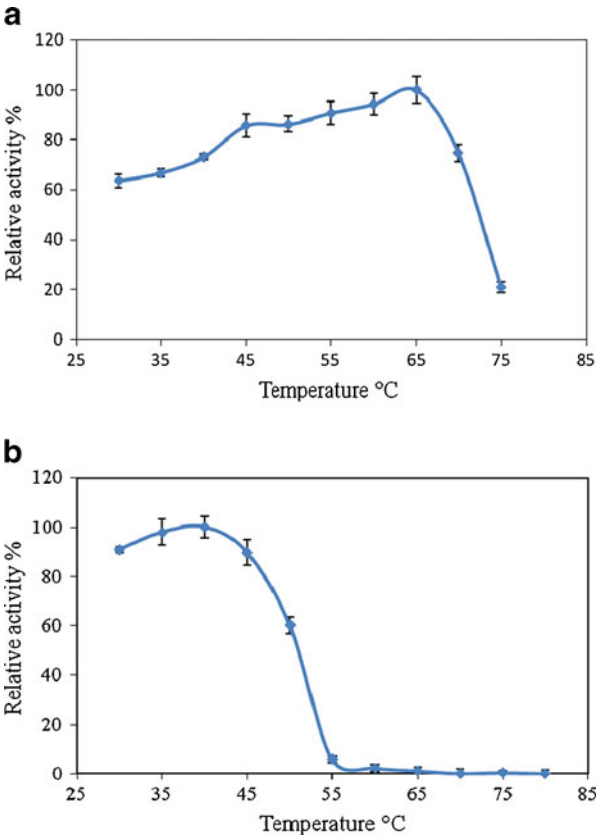

Fig. 5 Effects of temperature on activity (**a**) and stability (**b**) of the recombinant lipase. **a** Temperature–activity profile was determined by measuring the relative activity at different temperatures at pH 7.5. **b** Temperature–stability profile was determined by measuring the residual activity at pH 7.5 and 30 °C after incubating the purified lipase at different temperatures for 1 h. Values are means±SD (n=3)

Table 2 Effects of various metal ions, EDTA, and various surfactants on the activity of the recombinant lipase

Metal ions/surfactants	Concentration (mM)	Relative activity (%)
Control	–	100
Ca^{2+}	2	97.1
Fe^{2+}	2	57.2
Mn^{2+}	2	95.1
Ni^{2+}	2	99.6
Cu^{2+}	2	47.5
Mg^{2+}	2	85.0
Zn^{2+}	2	32.2
Ba^{2+}	2	92.3
Li^{+}	2	76.4
EDTA	2	93.2
Triton X-100	0.1% (*w*/*v*)	128
PVA	0.1% (*w*/*v*)	118
PEG 20000	0.1% (*w*/*v*)	124
SDS	0.1% (*w*/*v*)	0.201

The lipase, metal ions, and chemical additives were incubated in a 50-mM Tris–HCl buffer (pH 8.0) at 30 °C for 0.5 h, respectively

Table 3 Effect of various organic solvents on the activity of AFL1-1

Organic solvent	Concentration (*v*/*v* %)	Residual activity (%)
Control	–	100
Methanol	10	108
	50	0.613
Ethanol	10	100
	50	2.14
Isopropanol	10	83.8
	50	0
Acetone	10	113
	50	0.917
1-Butanol	10	4.28
	50	0.917
DMSO	10	99.1
	50	57.2

The lipase was incubated in the presence of 10% and 50% (*v*/*v*) various organic solvents at 4 °C for 12 h

Substrate Specificity of Lipase AFL1-1

To study the chain length specificity of the lipase, the initial hydrolysis rate of various fatty acids esters, including *p*-NP acetate (C2), *p*-NP propionate (C3), *p*-NP butyrate (C4), *p*-PN decanoate (C10), *p*-NP laurate (C12), *p*-NP myristate (C14), and *p*-NP palmitate (C16), was measured with purified lipase. The activity can be classified into the following order: *p*-NP acetate (C2)>*p*-NP butyrate (C4)>*p*-NP propionate (C3)>*p*-PN decanoate (C10)>*p*-NP myristate (C 14). The enzyme showed almost no activity on long-chained acyl ester, such as *p*-NP palmitate (C16) (Fig. 6). The result showed that this novel enzyme had a considerable activity toward *p*-NP acetate (C2) that had not been found in previous reports. Further experiments also showed that AFL1-1 had potential enantioselectivity for the ability to catalyze methyl 3-(4-methoxyphenyl) glycidate and methyl 3-phenylglycidate (data not shown), and the catalytic mechanism remained to be fully clarified.

Identification of the Active Site of the Recombinant Lipase AFL1-1

For better understanding the catalytic mechanism of recombinant AFL1-1, the catalytic triad of AFL1-1 was predicated with the homology analysis. Comparison of the various

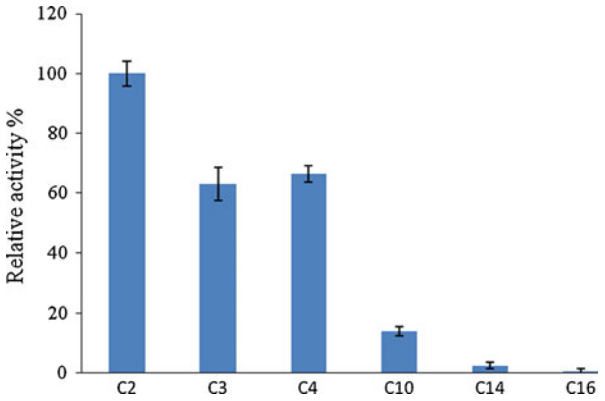

Fig. 6 Substrate specificity of the lipase from *Aspergillus fumigatus* towards *p*-nitrophenyl fatty acid esters with varied acyl chain lengths. Values are means±SD (*n*=3)

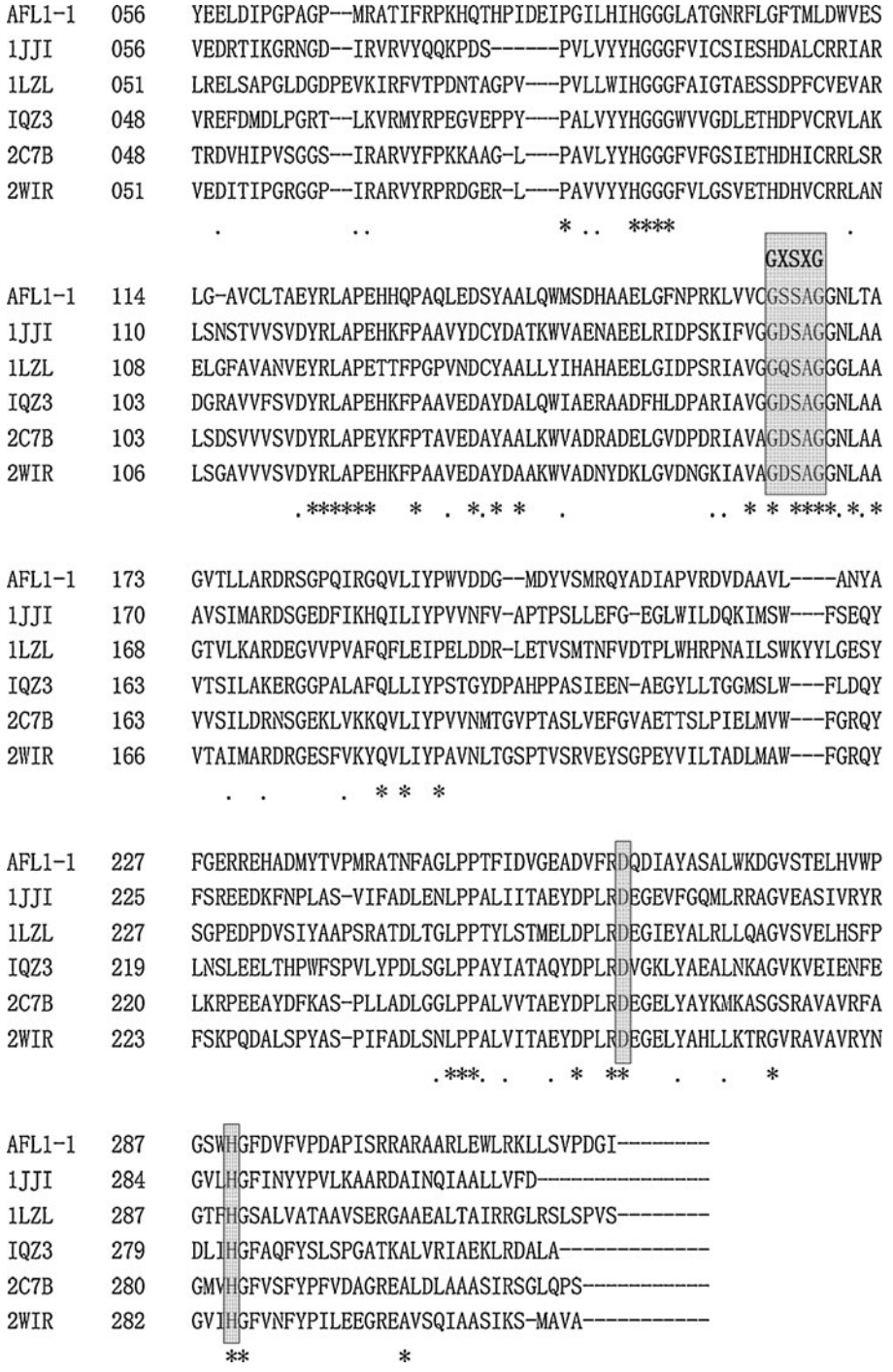

Fig. 7 Manual multiple amino acid sequence alignment of AFL1-1 with other lipases. The conserved GXSXG motif is shown as a *gray box*. The putative active site acids aspartate (*D*) and histidine (*H*) are marked in *gray*

known triacylglycerol lipase crystal structures leads to the identification of a common structural feature shared by both lipases and esterases, known as the α/β hydrolase fold, with a catalytic triad consisting of three residues: serine, histidine, and aspartate/glutamate [24]. The serine as a nucleophile is located in a strand-turn-helix nucleophilic elbow, while the histidine interacts with serine via a hydrogen bond, and the aspartate or glutamate acts as an acid in the reaction [25]. A conserved motif GXSXG (where X is any amino acid) characteristic for the active serine was an important sequence for the hydrolase family [26]. The amino acid sequence of AFL1-1 was analyzed with a superfamily search program (http://supfam.org/SUPERFAMILY/index.html) and suggested that AFL1-1 belonged to the α/β hydrolase family. Ser-165 was found located in the conserved motif GXSXG and probably acted as the nucleophilic contributor in the catalytic triad. Another search with the amino acid sequence from AFL1-1 in PDB (protein data bank) was carried out to further confirm this prediction. Five proteins with known structure showed high identities to AFL1-1: 1LZL 39%, 2WIR 32%, 1QZ3 34%, 2C7B 30%, and 1JJI 28% (shown by PDB code), and were therefore chosen for homology analysis. Among the five proteins, an acetyl esterase called HerE (PDB code: 1LZL) showed the highest identities (39%) with AFL1-1, and the prototypic catalytic triad of HerE was identified as Ser-160, Asp-260, and His-290, as reported [13]. The result of the manual multiple amino acid sequences alignments of AFL1-1 with other lipases revealed the conserved motif GXSXG and the conserved amino acids aspartate (D) and histidine (H) (Fig. 7). To sum up all the points above, Ser-165, Asp-260, and His-290 of AFL1-1 were concluded as the probably conserved positions in the catalytic triad. Subsequent experiments were employed with site-directed mutagenesis to change these putative active-site amino acids to an inert amino acid (alanine), respectively [27]. Another two amino acids (Asp-264 and His-283) were also collected for mutagenesis because they were very close to the putative active site Asp-260 and His-290. The importance of individually mutated amino acids for the catalytic activity was revealed by measuring the activity of the purified mutant lipases.

As shown in Table 4, for the Ser165Ala mutant, the enzymatic activity decreased dramatically from 3.12×10^{5} U mg^{-1} of the wild-type enzyme to only 0.154 Umg^{-1}. Compared to the value of 24.0 for the His283Ala mutant, the purified His290Ala mutant gave a value of 1.05×10^{-2} that was far lower than the wild type. According to the Asp260Ala and Asp264Ala mutants, it can be seen that the enzymatic activity of the Asp264Ala variant dropped not very significantly in comparison with the very low activity (4.58×10^{-2} U mg^{-1}) of the Asp260Ala mutant. It can be deduced that the catalytic triad of AFL1-1 consists of Ser-165, Asp-260, and His-290 residues. These results also demonstrated that Asp-264 and His-283 had important contribution to enzyme activity.

In the light of the high homology identity (39%) between the HerE with the known 3D structure and AFL1-1, HerE was chosen as a template for homology modeling (see Electronic Supplementary Material). Upon viewing the 3D structure predicated, Ser-165 is

Table 4 Results from hydrolytic enzymatic activity of AFL1-1 and mutant proteins

	Enzyme	U mg^{-1} protein
1	AFL1-1, wild-type	3.12×10^{3}
2	Ser165Ala	0.154
3	His290Ala	1.05×10^{-2}
4	His283Ala	24.0
5	Asp260Ala	4.58×10^{-2}
6	Asp264Ala	47.9

One unit (U) of activity was defined as the amount of enzyme releasing 1 μmol *p*-nitrophenol per minute under the assay conditions

located in a strand-turn-helix nucleophilic elbow, and His-290 was located following the final central parallel β-sheet. It is in accordance with the report about the catalytic triad of lipase [25, 28].

Conclusion

The novel recombinant lipase AFL1-1 from *A. fumigatus* has been reported for the first time in this paper. The lipase demonstrates very high activity towards C2 fatty acids and exhibits optimal activity under the condition of 65 °C and pH 8.5. For better understanding the mechanism of this novel lipase, the catalytic triad of AFL1-1 was predicated with the homology analysis and confirmed with site-directed mutagenesis assay. This article indicates that *A. fumigatus* is a new source of lipase. Further study about application of recombinant lipase AFL1-1 is in progress.

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