High-level soluble expression and enzymatic characterization of Burkholderia sp. lipase with steryl ester hydrolysis activity in E. coli

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Abstract

Background *Burkholderia cepacia* lipase is an important industrial biocatalyst for biodiesel production and chiral pharmaceutical synthesis. Heterologous soluble expression of lipase lipA gene from *B. cepacia* in *Escherichia coli* highly depends on co-expression of its cognate foldase gene, lipB. However, the interaction between recombinant lipase LipA and chaperonin LipB is rather complicated and confusing. In this research, various systems of lipA/lipB co-expression combinations are investigated to obtain high-level soluble expression of lipA, respectively. **Results** The best co-expression combination system for lipA and lipB is *E. coli* Origami 2 (DE3)/pETDuet-lipB(MCS1)/lipA(MCS2). The soluble expression level of lipA is 100.4 U/OD600 towards 4-nitrophenyl laurate hydrolysis. The recombinant LipA can be rapidly isolated from cell-free supernatant of recombinant *E. coli* lysate using HisTrap HP affinity chromatography column, and the lipA/LipB complex is obtained. Enzymatic characterization analysis shows that the purified LipA is a mesothermal and alkaline enzyme. LipA displays preference for medium-chain-length acyl groups (C10-C12) and sn-1,3 regioselectivity. Besides triacylglycerol hydrolase activity (EC. 3.1.1.3), LipA also displays steryl ester hydrolase activity (EC. 3.1.1.13). The specific activity of LipA towards 4-nitrophenyl decanoate and cholesterol linoleate are 638.9 U/mg and 1111.5 mU/mg, respectively. **Conclusions** Host strain *E. coli* Origami 2 (DE3), lipB locus at MCS1 on the dual expression cassette plasmid pETDuet, and low-temperature induction contribute to the soluble expression of lipA. Recombinant LipA displays both triacylglycerol hydrolase activity and steryl ester hydrolase activity.

Background

Lipase (triacylglycerol hydrolase, EC 3.1.1.3) hydrolyzes triacylglycerols into glycerol and fatty acids, or the reverse reaction of esterification. Besides hydrolysis reaction, microbial lipase also catalyzes alcoholysis reaction, ammonolysis reaction, or transesterification reaction in non-aqueous system, in which lipase displays strict enantioselectivity, and regioselectivity [1]. As an important industrial biocatalyst, microbial lipase is used widely in many industrial fields, including biodiesel synthesis, biodegradable polymers production, and pharmaceuticals preparation [2,3].

Among the known microbial lipase resources, *Burkholderia cepacia* lipase is known as an enantioselective biocatalyst for synthesis reactions because of its' distinctive structural characterization of the funnel-like active site [4,5,6]. Moreover, *B. cepacia* lipase also exhibits excellent organic solvent tolerance and is a promising biocatalyst for biodiesel production [7,8]. Disulfide bond and calcium ion in the 3D molecular structure contributes to the stability of *B. cepacia* lipase, respectively [9,10]. According to the classification standard for lipolytic enzyme family, *B. cepacia* lipase belongs to I.2 subfamily of true lipases [11,12]. The functional expression and secretion in an active form of I.2 subfamily lipases highly depends on a chaperone protein (also named as lipase-specific foldase) [13,14].

Because most *B. cepacia* strains are human opportunistic pathogens, it is severely regulated to construct recombinant *B. cepacia* strains using homologous expression system for lipase production in industry [8,15–17]. To obtain high-yield functional recombinant lipase from *Burkholderia* sp., cell-free protein

synthesis systems and foldase-assisted refolding of recombinant lipase *in vitro* are used to produce the functional recombinant lipase at the early stage, respectively [18,19]. In recent years, two-plasmid co-expression systems or dual expression cassette plasmid systems are used to co-express lipase gene (*lipA*) with its cognate foldase gene (*lipB*), and a method which enabled to obtain high yields of functional recombinant lipase LipA is obtained [20–23].

In this research, various systems of *lipA/lipB* co-expression combinations are screened and then the best co-expression combination system, *E. coli* Origami 2 (DE3)/pETDuet-*lipB*(MCS1)/*lipA*(MCS2), is selected for high-yield production of functional LipA. The recombinant LipA is purified and enzymatic characterization is determined. Besides triacylglycerol hydrolase activity (EC. 3.1.1.3), recombinant LipA also displays steryl ester hydrolysis activity (EC. 3.1.1.13).

Results

Effects of different co-expression combination systems on the soluble expression of lipA

In the dual expression cassette plasmid system, gene loci of *lipA* and *lipB* on the plasmid were the key factors for the functional soluble expression of *lipA*. The lipase activity from the supernatant of recombinant *E. coli* lysate reached 6.5 ± 0.2 U/OD₆₀₀ when *lipA* and *lipB* were inserted into MCS2 and MCS1 of pACYCDuet, respectively. In contrast, the lipase activity was only 1.8 ± 0.1 U/OD₆₀₀ when *lipA* and *lipB* were inserted into MCS1 and MCS2 of pACYCDuet, respectively (Table 1, *E. coli* BL21 (DE3) as the expression host). A similar experimental result was also observed when pETDuet was selected as the dual expression cassette plasmid. The lipase activity increased from 1.7 ± 0.1 U/OD₆₀₀ to 3.9 ± 0.1 U/OD₆₀₀ by gene locus interchange between *lipA* and *lipB* (Table 1, *E. coli* BL21 (DE3) as the expression host). Aamand et al. (1994) also reported that the presynthesized LipB could activate the recombinant LipA [24].

In the two-plasmid co-expression systems, types of plasmid carrying *lipA* and *lipB* showed the important effect on the functional soluble expression of *lipA*. When *lipA* and *lipB* was inserted into MCS of pET28a and MCS1 of pACYCDuet, respectively, no lipase activity was detected from the supernatant of recombinant *E. coli* lysate (Table 1, *E. coli* BL21 (DE3) as the expression host). In contrast, a low lipase activity, 2.6 ± 0.1 U/OD₆₀₀, was obtained when *lipA* and *lipB* were inserted into MCS1 of pACYCDuet and MCS of pET28a, respectively. Ihara et al. (1995) reported that it was the recombinant LipA, rather than the recombinant LipB, that was prone to form inclusion body under the control of T7 promoter [25]. Low-level expression of *lipA* using low-copy number plasmid (pACYCDuet or pETDuet used in this study) contributed to form functional soluble LipA. Among the investigated two-plasmid co-expression systems, plasmid combinations of pACYCDuet with pETDuet showed excellent effect on the functional soluble expression of *lipA*, in which lipase activity was as high as from 9.9 ± 0.4 U/OD₆₀₀ to 12.6 ± 1.0 U/OD₆₀₀ (Table 1, *E. coli* BL21 (DE3) as the expression host).

In previous report, co-expression of an additional copy of chaperone *lipB* gene resulted in a considerable increase of functional LipA yield in native host strain [14,26]. However, no statistical significance of

functional LipA yield was observed in *E. coli* expression system when an additional copy of *lipB* was introduced into the co-expression combination system of *lipA* and *lipB*. Lipase activity was 6.5 ± 0.2 U/OD₆₀₀ in the dual expression cassette plasmid system of pACYCDuet-B1A2. However, lipase activity still was 6.3 ± 0.1 U/OD₆₀₀ in the two-plasmid co-expression system of pACYCDuet-B1A2 and pETDuet-B1, in which an additional copy of *lipB* was introduced using plasmid pETDuet-B1 (Table 1, *E. coli* BL21 (DE3) as the expression host).

E. coli expression strain was another key element for the functional soluble expression of *lipA* from *Burkholderia* sp.. In the 3D structural model of LipA from *B. cepacia*, a [disulfide bond](#) existed between Cys¹⁹⁰ and Cys²⁷⁰ [10]. Refolding *in vitro* of LipA from *B. cepacia* indicated that the functional LipA was highly depended on the correct formation of a [disulfide bond](#) between Cys¹⁹⁰ and Cys²⁷⁰ [27]. The functional soluble expression yield of *lipA* from *Burkholderia* sp. was improved by fusing LipA with the N-terminal peptide tags of Dsb-family chaperones [28]. Compared with that in *E. coli* BL21 (DE3), soluble expression yield of *lipA* in *E. coli* Origami 2 (DE3) was increased by 1.3-fold to 7.9-fold (Table 1).

Effects of culture temperature on the functional soluble yield of *lipA*

From Table 1, a significantly positive effect of low temperature on functional soluble LipA yield was observed. The soluble LipA yield reached 100.4 ± 0.7 U/OD₆₀₀ when *E. coli* Origami 2 (DE3)/pETDuet-B1A2 was cultured at 20°C. While *E. coli* Origami 2 (DE3)/pETDuet-B1A2 was cultured at 30°C, soluble LipA yield decreased to 30.9 ± 0.3 U/OD₆₀₀ (Table 3, *E. coli* Origami 2 (DE3) as the expression host). Same results were obtained when other co-expression combinational systems were induced at 20°C and 30 °C, respectively. Low temperature culture contributed to not only the correct folding of recombinant protein, but also improving stability, which resulted in productivity enhancement of soluble recombinant protein [29,30].

During exponential phase, growth rates of recombinant *E. coli* BL21(DE3) strains were higher than that of recombinant *E. coli* Origami 2 (DE3) strains at 20°C (Fig. 1A). It were 15 hours for recombinant *E. coli* BL21(DE3) strains that were taken to reach [stationary phases](#) at 20°C. While for recombinant *E. coli* Origami 2 (DE3) strains, 25 hours were needed to reach [stationary phases](#) at 20°C (Fig. 1A). No significant difference of cell density existed between recombinant *E. coli* BL21(DE3) strains and recombinant *E. coli* Origami 2 (DE3) strains during [stationary phases](#) (Fig. 1A). However, significant differences of soluble LipA yield existed between recombinant *E. coli* Origami 2 (DE3) strains and recombinant *E. coli* BL21(DE3) strains during [stationary phases](#) (Fig. 1B). The highest soluble expression level of *lipA* from *E. coli* Origami 2 (DE3)/pETDuet-B1A2 reached 100.4 U/OD₆₀₀ towards 4-nitrophenyl laurate hydrolysis. The functional soluble yields of *lipA* reached 132 mg/L or 73000 U/L.

Purification of recombinant LipA

There were a 34-kDa band and a 32-kDa band from the purified LipA preparation shown on the SDS-PAGE gel (lane 2, Fig. 2). In the dual expression cassette plasmid system of *E. coli* Origami 2 (DE3)/pETDuet-B1A2, *lipA* was inserted into the MCS2, while *lipB* were inserted into the MCS1 of pETDuet. Consequently, S-tag and His-tag should be fused to the C-terminus of LipA and N-terminus of LipB, respectively. It was confirmed using western blotting analysis of anti-His tag antibody that the 32-kDa band originated from LipB (lane 1, Fig. 2). Given the characteristic of HisTrap HP affinity chromatography column and interaction between LipA and LipB, it was LipB that was firstly combined with His-tag of matrix materials and LipA was then combined with LipB. Recombinant LipA showed the same relative molecular weight, 34 kDa (lane 2, Fig. 2), as the native extracellular lipase from *Burkholderia* sp. ZYB002 [31]. In other reports on co-expression of *lipA/lipB* from *B. cepacia* or *P. aeruginosa* in *E. coli*, the purified preparation also simultaneously contained the recombinant LipA and LipB [20,21]. Pauwels and Van Gelder (2008) even developed a rapid affinity-based purification method of a bacterial lipase through steric chaperone interactions [32].

Using one-step purification procedure of HisTrap HP affinity chromatography column, recombinant LipA was purified with a recovery of 68.6% and a fold purification of 2.6. The specific activity of recombinant LipA towards 4-nitrophenyl laurate hydrolysis was 547.0 U/mg. Because recombinant LipA exists in the form of LipA/LipB complex, the actual value of specific activity of LipA is approximate two times as much as the tested value, and is same as that of the native extracellular lipase from *Burkholderia* sp. ZYB002 [31].

Enzymatic characterization of LipA

As shown in Fig. 3A and Fig. 3B, the recombinant LipA was a **mesothermal** and alkaline enzyme. The optimum temperature and pH of LipA was 40°C and 8.0, respectively. The half-time of the recombinant LipA was about 36 min at 40°C (Fig. 3C). The stability of LipA was lower in acidic buffer solution than in alkaline buffer solution (Fig. 3D). However, the residual activity of LipA was only 70% of the initial activity after incubation in 20 mmol/L pH 8.0 Na₂HPO₄-NaH₂PO₄ buffer for 4 h at 4 °C (Fig. 3D). Among the investigated bivalent metal ions and EDTA, only Mg²⁺ was found to enhance LipA activity, whereas, Fe²⁺, Zn²⁺ and Cu²⁺ ions strongly inhibited LipA activity (Fig. 4).

Partial hydrolysis of triolein by the recombinant LipA resulted in three kinds of hydrolysis products, including 1,2-diolein, oleic acid, 1-monoolein (Fig. 5), which showed that LipA cleaved only the 1,3-positioned ester bonds and displayed *sn*-1,3 regioselectivity. Trace amounts of 1-monoolein in the hydrolyzed products might be related to acyl migration of *sn*-2 to *sn*-1, which occurs spontaneously in glycerides [33].

The recombinant LipA showed a preference for medium chain length fatty acid esters (C10-C12) when assayed using 4-nitrophenyl derivatives (Table 2). Recombinant LipA exhibited a simple Michaelis-Menten kinetics for 4-nitrophenyl decanoate hydrolysis. The values of K_m and V_{max} of LipA were 0.57

mmol·L⁻¹ and 22964 μmol·min⁻¹·mg⁻¹, respectively. Among the determined four steryl esters, LipA showed the highest hydrolysis activity towards cholesterol linoleate, and specific activity reached 1111.5 mU/mg (Table 2). Kontkanen et al. (2004) also reported that some commercial lipase preparations displayed steryl esterase activity. However, the steryl esterase activity from lipase preparations was considerably lower than the native lipase activity [34].

Discussion

In native *Burkholderia cepacia* strain, *lipA* and *lipB* usually form an operon suggesting a 1 to 1 ratio for both *lipA* and *lipB* expression [35,36]. However, LipB acted as multi-turnover catalysts to direct lipase folding, and expression yield of *lipB* was lower than *lipA* in native host strains [14,37]. While LipA and LipB formed a stable complex, in which LipB acted as single-turnover catalysts to direct lipase folding in heterologous host, *E. coli* strains [21,38]. Both El Khattabi et al. (2000) and Quyen et al. (1999) reported that an excess of LipB was the prerequisite for correct LipA folding in *E. coli* host [19,38]. Moreover, high-level active LipA could be obtained only when LipB was synthesized first in two-plasmid co-expression systems in *E. coli* host [24]. The similar result was also observed in this research. High level soluble LipA was obtained only when *lipB* and *lipA* was inserted into the MCS1 site and MCS2 site on the dual expression cassette plasmid pETduet, respectively. Compared with that from pETDuet-A1B2, soluble expression yield of LipA from pETDuet-B1A2 was increased by 28.7 fold (Table 1, *E. coli* Origami 2 (DE3) as the expression host).

DsbA-DsbB disulfide bond formation system existed in native *B. cepacia* strains [39]. Disulfide bond in *Burkholderia* sp. lipase played a key role in activating lipolytic activity and stabilizing the 3D structure [9,40]. In heterologous strain, the soluble expression yield of *Burkholderia* sp. lipase was improved by co-expression of cytoplasmic chaperone GroEL/ES, which could facilitate refolding of disulfide-bond [28,41]. Host strain was another substitution strategy for high-level soluble expression of lipase with disulfide-bond in 3D structure. To produce recombinant *Rhizopus oryzae* lipase in *E. coli*, the insoluble and inactive protein pellet was obtained when *E. coli* BL21(DE3) or *E. coli* Rosetta was selected as the expression host strain. While recombinant *Rhizopus oryzae* lipase was expressed as soluble and active forms when *E. coli* Origami (DE3) strain was used as the expression host strain [42]. In this research, soluble expression level of LipA from *E. coli* Origami 2 (DE3)/pETDuet-B1A2 was 100.4 U/OD₆₀₀, while from *E. coli* BL21(DE3)/pETDuet-B1A2, soluble expression level of LipA was only 49.7 U/OD₆₀₀.

When *E. coli* was selected as host strain for co-expression of *lipA* and *lipB* from *Burkholderia* sp., LipA/LipB complex would be formed and it was difficult to isolate LipA from LipA/LipB complex [43]. In previous research work, a low-*Mr* compound was purified from cell-free lysate of *Pseudomonas* sp. and then identified as Glutathione, which could facilitate the dissociation of LipA/LipB complex and liberate free active lipase [44,45]. In this work, *E. coli* Origami 2 (DE3) was selected as the expression host, which would enhance disulfide bond formation. However, recombinant LipA was still aggregated with recombinant LipB. To facilitate the dissociation of LipA/LipB complex, 1% (v/v) Triton X-100, 1mol/L Urea, 1mol/L ammonium sulfate, or 2 mmol/L CaCl₂ was added into the elution buffer solution for

HisTrap HP affinity chromatography column, respectively. However, the recombinant LipA was not still dissociated from LipA/LipB complex (data not shown). Moreover, Kim et al. (2001) reported that lipase-Proline¹¹²Glutamine mutant could correctly fold and display lipase activity in the absence of chaperone LipB. It is necessary to further investigate the molecular mechanism on folding of LipA and dissociation of LipA/LipB complex [46].

Microbial steryl esterase (EC. 3.1.1.13) have tremendous application potential in steroids synthesis [47]. Nevertheless, sources of microbial steryl esterases were extremely limited and most of the reported microbial steryl esterases should, in fact, be classified as microbial lipases (EC. 3.1.1.3)[48]. Steryl esterase from *B. cepacia* ST200 was firstly reported by Takeda et al. (2006) and enzymatic characterization for steryl esterase activity was determined. However, lipase activity of this enzyme was not tested [49]. The amino acid sequence identity was over 93% between steryl esterase from *B. cepacia* ST200 and LipA from *Burkholderia* sp. ZYB002 [49,50]. In this research, both lipase activity and steryl esterase activity were determined from LipA from *Burkholderia* sp. ZYB002 (Table 2). Moreover, microbial lipase could act on some non-triglyceride substrates and displayed promiscuous activity [51,52]. In previous reports, a few microbial lipases could catalyze hydrolysis reaction of steryl esters [34]. Because of the structural difference between triglyceride and steryl ester, hydrolysis efficiency of steryl ester catalyzed by lipase was always low, and it was necessary to improve catalysis activity towards steryl esters using protein engineering technology.

Conclusions

Host strain, gene loci of *lipA* and *lipB* on the dual expression cassette plasmid pETDuet, and low-temperature induction (20°C) impacted positive effect on the soluble expression level of *lipA*. The best expression system was *E. coli* Origami 2 (DE3)/pETDuet-B1A2, in which *lipA* was inserted into MCS2 and *lipB* was inserted into MCS1. Under the above conditions, the highest soluble expression level of *lipA* reached 100.4 U/OD₆₀₀ towards 4-nitrophenyl laurate hydrolysis. The functional soluble yields of LipA reached 132 mg/L or 73000 U/L. Besides triacylglycerol hydrolase activity, recombinant LipA also displayed steryl ester hydrolase activity.

Methods

Strains, plasmids and reagents

Bacterial strains and plasmids used in this study were listed in Table 3. Briefly, *E. coli* DH5α was used as the host strain for plasmid amplification, while *E. coli* BL21 (DE3) or *E. coli* Origami 2 (DE3) were used to express lipase gene (*lipA*) and the cognate foldase gene (*lipB*), respectively.

Restriction endonucleases, high-fidelity DNA polymerases, PCR product purification kit, T₄-DNA ligase, DNA gel extraction kit, DNA marker and protein marker were purchased from TaKaRa Biotechnology Co. Ltd. (Dalian, China). DNA sequencing and oligonucleotide primers synthesis was performed by Sangon

Biotechnology Co. Ltd. (Shanghai, China). Various *p*-nitrophenyl fatty acid esters, cholesterol esters, triolein, oleic acid, 1,3-diolein, 1,2-diolein and 1-monoolein were purchased from Sigma-Aldrich (China). Silica gel GF254 was purchased from Haiyang Chemical Co. Ltd. (Qingdao, China). HisTrap HP affinity chromatography column (1 mL) was purchased from GE Healthcare Life Sciences (China). All other Chemical reagents were of analytical grade unless otherwise stated and purchased from Sinopharm Chemical reagent Co. Ltd. (China).

Construction of recombinant plasmids

To discriminate lipase and its cognate lipase-specific foldase, lipase and foldase from *Burkholderia* sp. ZYB002 were named as LipA and LipB, respectively. The corresponding gene symbols of LipA and LipB were designated as *lipA* and *lipB*, respectively. Recombinant plasmids used in this research were listed in Table 3. Insertion sites of *lipA* and (or) *lipB* were shown on the restriction maps of the recombinant plasmids, respectively (Fig. S1). Construction of recombinant plasmids was as follows:

Recombinant plasmids pETDuet-A1B2, pACYCDuet-A1, and pETDuet-B2 were derived from pEDSF-*lipB*-*lipA*, respectively [50]. Plasmid pEDSF-*lipB*-*lipA* was firstly double digested by the restriction endonucleases, *Bam*H I and *Hind*III, followed by *Bgl*II and *Xho*I digestion. The recovered DNA fragments of *lipA* and (or) *lipB* were ligated into the corresponding endonuclease-digested plasmid, pETDuet, pACYCDuet, and pETDuet, respectively.

Except for the above mentioned recombinant expression plasmids, other recombinant expression plasmids listed in Table 3 were constructed as follows. The DNA fragments of *lipA* and *lipB* were amplified by PCR using plasmid pMD18T-*lipAB* as the template [50]. The oligonucleotide sequences of PCR primers, PCR primer pairs, annealing temperatures, and PCR products were listed in Table 4. The resulting PCR products were digested with the restriction endonucleases (shown in the oligonucleotide sequences of PCR primer in Table 4) and then ligated into the corresponding endonuclease-digested expression vector pACYCDuet, pETDuet, and pET28a, respectively.

All of the resulting recombinant plasmids were transformed into *E. coli* DH5 α , and the reading frames were confirmed by DNA sequencing.

Co-expression of *lipA* with *lipB* in *E. coli*

To obtain high-yield soluble expression of *lipA*, various systems of *lipA*/*lipB* co-expression combinations were screened, including two-plasmid co-expression systems and dual expression cassette plasmid systems. Total eleven co-expression combination systems of *lipA* with *lipB* were investigated (Table 1).

Effect of culture temperature and types of expression host strain on the soluble expression yield of *lipA* were further investigated. 30°C and 20°C were set for the culture temperature, respectively.

Simultaneously, *E. coli* BL21 (DE3) and *E. coli* Origami 2 (DE3) were selected as the expression host strain, respectively.

50 mL of Luria-Bertani medium supplemented with antibiotic in 250-mL conical flask was used to produce the soluble LipA on orbital shaking incubator at 220 rpm. The final concentration of Chloramphenicol, Kanamycin, and Ampicillin was 150 µg/mL, 50 µg/mL, and 60 µg/mL, respectively. When the cell density ($OD_{600\text{ nm}}$) reached 0.6, IPTG was added to the culture medium to the final concentration of 0.5 mmol/L. After 24 h induction culture, the cell density ($OD_{600\text{ nm}}$) of every recombinant strains were determined, and then the cell pellets were collected by centrifugation and resuspended in 50 mL 20 mmol/L $\text{Na}_2\text{HPO}_4\text{-NaH}_2\text{PO}_4$ buffer (pH7.4). *E. coli* cells were lysed using sonication and then the supernatant was collected for lipase activity assay, respectively. The soluble expression level of LipA was evaluated using total lipase activity per OD_{600} of the recombinant cell.

Purification of recombinant LipA

E. coli Origami 2 (DE3)/pETDuet-B1A2 displayed the highest-yield of soluble LipA among the total eleven co-expression combination systems of *lipA* with *lipB*, and was selected for the following large-scale production of functional soluble LipA.

After induction culture, recombinant *E. coli* was collected and then re-suspend in loading buffer composed of 20 mmol/L pH7.4 $\text{Na}_2\text{HPO}_4\text{-NaH}_2\text{PO}_4$ buffer, 20 mmol/L imidazole, and 500 mmol/L NaCl. *E. coli* cells were lysed using sonication and then the supernatant was loaded on a HisTrap HP affinity chromatography column (1 mL, GE Healthcare) pre-equilibrated with loading buffer. After the column was rinsed with 10 mL of loading buffer, recombinant LipA was eluted with 20 mL of 20–500 mmol/L imidazole step gradients in the same buffer with a flow rate of 0.3 mL/min. The purity of the active fractions was monitored by SDS-PAGE on a 10% separating gel [53]. The fractions with pure LipA was pooled and dialyzed against 20 mmol/L $\text{Na}_2\text{HPO}_4\text{-NaH}_2\text{PO}_4$ (pH7.4) buffer overnight at 4°C. The protein concentration was analyzed using the method of Bradford, with bovine serum albumin as the standard [54].

Lipase activity determination of recombinant LipA

Lipase activity was determined using spectrophotometric assay method [55] with slight modifications. The reaction mixture consisted of 0.4 mmol/L of each *p*-nitrophenol ester in 20 mmol/L $\text{Na}_2\text{HPO}_4\text{-NaH}_2\text{PO}_4$ (pH8.0) buffer and 30 µL the appropriately diluted lipase solution. The kinetics was detected for 5 min at 410 nm. Under the above condition used, the molar extinction coefficient (ϵ_{410}) of *p*-nitrophenol was $1.15 \times 10^{-2} \text{ L}/\mu\text{mol.cm}$.

All reactions were carried out at 40 °C and 20 mmol/L Na₂HPO₄-NaH₂PO₄ (pH8.0) buffer. One unit of lipase activity was defined as the amount of lipase that liberated 1 μmol of *p*-nitrophenol from *p*-nitrophenol esters per min. All measurements were carried out three times and the average value was taken.

Cholesterol ester hydrolase activity assay of LipA

Cholesterol ester hydrolase activity of LipA was assayed using spectrophotometric method as described by Stępień and Gonchar (2013) [56], and the instruction manual for *Pseudomonas* sp. cholesterol esterase from TOYOBO (USA) with slight modifications. The reaction mixture was composed of 20 mmol/L Na₂HPO₄-NaH₂PO₄ (pH8.0) buffer, 1.5 mmol/L 4-aminoantipyrine, 22 mmol/L 3,5-dichlorodihydroxy benzenesulfonic acid, 10 U/mL cholesterol oxidase, 5 U/mL horseradish peroxidase and maximum concentration of dissolved cholesterol esters. The final concentration of cholesterol oleate, cholesterol linoleate, cholesterol palmitate and cholesterol stearate in reaction mixture were 0.07 mmol/L, 0.2 mmol/L, 0.12 mmol/L, and 0.1 mmol/L, respectively. The kinetics was detected for 45 min at 516 nm. Under the above condition used, the molar extinction coefficient (ϵ_{516}) of *p*-nitrophenol was 2.08×10^{-2} L/μmol.cm.

All reactions were carried out at 40 °C. One unit of cholesterol ester hydrolase activity was defined as the amount of LipA that liberated 1 μmol of cholesterol from cholesterol ester per min. All measurements were carried out three times and the average value was taken.

Effect of pH on activity and stability

The optimal pH for lipase activity was determined by incubating lipase substrates in a suitable buffer at various pH ranging from 6.5 to 9.0, and the maximum lipase activity was considered 100%. To determine the effect of pH on lipase stability at pH ranging from 6.5 to 10.0, aliquots of the concentrated lipase preparation were diluted five-fold in the corresponding buffer and then incubated for 4 h at 4 °C. The residual lipase activity after incubation was determined and lipase activity at the start was taken as 100%. The corresponding buffers were Na₂HPO₄-NaH₂PO₄ (pH6.5–7.5), Tris-HCl (pH8.0–8.5), and Gly-NaOH (pH9.0–10.0), respectively. The final concentrations of various buffers were 20 mmol/L.

Effect of temperature on lipase activity and stability

The optimal temperature for lipase activity was determined by incubating the standard reaction mixture at different temperatures ranging from 25°C to 55°C, and the maximum lipase activity was considered 100%. To determine the effect of temperature on lipase stability, the lipase preparation was incubated at 40°C and aliquots were continuously taken at 5-min interval to assay the residual activity. The lipase activity at the start was taken as 100%. Half-life of thermal inactivation was calculated using the method as described by Zhao and Arnold [57].

Effect of metal ions and EDTA on lipase activity

The purified LipA was dialyzed against 20 mmol/L $\text{Na}_2\text{HPO}_4\text{-NaH}_2\text{PO}_4$ buffer (pH 7.4) overnight at 4°C, and then the various metal ions and EDTA were added into the dialyzed LipA solution to a final concentration of 1 mmol/L. After incubation at 4°C for 1 h, the lipase activity was determined. The lipase activity of the dialyzed LipA solution at the start was considered 100%.

Positional specificity assay

Positional specificity was determined by analyzing lipolysis products of triolein using thin-layer chromatography (TLC) on silica gel GF254. The experimental procedure was carried out as described by Shu et al. (2016) with slight modifications [50]. In brief, the reaction mixtures consisted of 0.1 mmol/L of triolein, 1955 μL $\text{Na}_2\text{HPO}_4\text{-NaH}_2\text{PO}_4$ (20 mmol/L, pH8.0), and 2 U (25 μL) LipA solution. After incubation at 40°C for 20 min, the reaction products were extracted with *n*-Hexane and then analyzed by TLC.

Determination of Michaelis–Menten constants

Enzyme assays with 30 μL of purified LipA were performed in 20 mmol/L $\text{Na}_2\text{HPO}_4\text{-NaH}_2\text{PO}_4$, pH8.0 at 40°C with increasing concentration of 4-Nitrophenyl decanoate from 0.06 to 2.0 mmol/L. Lineweaver-Burk plots were used to determine the Michaelis-Menten kinetic parameters, V_{max} and K_{m} , assuming that simple Michaelis-Menten kinetics was followed.

Statistical analysis

All experiments were carried out three times independently. Data are presented as the average $\pm$ standard deviation. The data were statistically analyzed using SPSS software and groups were compared using Student's *t*-test with significant differences defined as $P < 0.05$, whereas $P < 0.01$ represented a highly significant difference.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional files.

Additional files

Additional file 1: Figure S1. Restriction maps of the recombinant plasmids containing lipA and (or) lipB in this study.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ZY Shu designed and supervised the work. Feng Li participated in the construction of various recombinant plasmids, and purification of the recombinant lipase. WJ Jia produced the recombinant LipA. H Lin and XD Mu determined the triacylglycerol hydrolysis activity and the steryl ester hydrolysis activity of the recombinant LipA, respectively. YR Liu provided advice for the discussion. This manuscript was drafted by F Li and revised by ZY Shu. JZ Huang gave final approval of the version to be published. All authors have read and approved the final manuscript.

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Tables

Table 1 Effect of different combination co-expression systems and induction temperatures on the soluble expression yield of *lipA*.

Host strain	Recombinant plasmids	Induction temperature			
		30 °C Lipase activity (U/OD ₆₀₀)	SA	20 °C Lipase activity (U/OD ₆₀₀)	SA
<i>E. coli</i> BL21 (DE3)	pEDSF- <i>lipB-lipA</i>	1.8±0.1		2.8±0.1	
	pACYCDuet-B1A2	6.5±0.2	*	53.0±1.1	*
	pACYCDuet-B1A2 and pETDuet-B1	6.3±0.1	*	30.4±0.3	
	pETDuet-A1B2	1.7±0.1		2.9±0.04	
	pETDuet-B1A2	3.9±0.1	*	49.7±0.6	*
	pETDuet-B1A2 and pACYCDuet-B1	3.7±0.3	*	38.7±1.0	
	pET28a- <i>lipB</i> and pACYCDuet- A1	2.6±0.1		17.1±0.2	
	pET28a- <i>lipA</i> and pACYCDuet- B1	ND		ND	
	pACYCDuet-B1A2 and pETDuet-A1B2	12.6±1.0	#	38.2±1.4	#
	pACYCDuet-B1A2 and pETDuet-B1A2	9.9±0.4	#	36.6±1.1	#
	pEDSF- <i>lipB-lipA</i> and pETDuet-B1A2	12.4±0.5	#	36.2±0.9	#
	pEDSF- <i>lipB-lipA</i>	2.4±0.6		4.0±0.04	
	pACYCDuet-B1A2	42.9±1.0	*	73.7±1.3	*
	pACYCDuet-B1A2 and pETDuet-B1	30.3±0.9		40.5±0.6	
<i>E. coli</i> Origami 2 (DE3)	pETDuet-A1B2	2.3±0.3		3.5±0.03	
	pETDuet-B1A2	30.9±0.3	*	100.4±0.7	*
	pETDuet-B1A2 and pACYCDuet-B1	22.5±0.4		70.8±1.0	
	pET28a- <i>lipB</i> and pACYCDuet- A1	8.0±0.1		34.7±0.3	
	pET28a- <i>lipA</i> and pACYCDuet- B1	ND		0.3±0.02	
	pACYCDuet-B1A2 and pETDuet-A1B2	31.6±1.6	#	78.9±1.1	#
	pACYCDuet-B1A2 and pETDuet-B1A2	34.7±1.7	#	77.9±1.1	#
	pEDSF- <i>lipB-lipA</i> and pETDuet-B1A2	34.4±1.4	#	76.1±1.5	#

SA: statistical analysis. #: $P < 0.05$; *: $P < 0.01$.

Table 2 Substrate specificity of the recombinant LipA towards 4-nitrophenyl esters and steryl esters

Substrate for lipase activity	Specific activity (U/mg)	Substrate for steryl esterase activity	Specific activity (mU/mg)
4-nitrophenyl palmitate (C ₁₆)	253.8 ± 6.3	Cholesterol linoleate	1111.5 ± 6.2
4-nitrophenyl myristate (C ₁₄)	229.0 ± 4.8	Cholesterol oleate	80.8 ± 2.7
4-nitrophenyl laurate (C ₁₂)	547.0 ± 6.7	Cholesterol stearate	47.0 ± 3.5
4-nitrophenyl decanoate (C ₁₀)	638.9 ± 10.3	Cholesterol palmitate	288.9 ± 1.8
4-nitrophenyl octanoate (C ₈)	371.4 ± 7.2		
4-nitrophenyl butyrate (C ₄)	129.4 ± 9.6		

Strains and plasmids	Description	Source
Strains		
<i>E. coli</i> DH5α	Host strain for plasmid amplification	TAKARA
<i>E. coli</i> BL21(DE3)	Expression host strain	Novagen
<i>E. coli</i> Origami 2 (DE3)	Expression host strain	Novagen
Plasmids		
pMD18T- <i>lipAB</i>	pMD18T containing the PCR-amplified <i>lipA</i> and <i>lipB</i>	Reference 50
pEDSF- <i>lipB-lipA</i>	pACYCDuet with insertion of <i>lipA</i> at MCS1 and <i>lipB</i> at MCS2	Reference 50
pACYCDuet-B1A2	pACYCDuet with insertion of <i>lipB</i> at MCS1 and <i>lipA</i> at MCS2	This study
pETDuet-A1B2	pETDuet with insertion of <i>lipA</i> at MCS1 and <i>lipB</i> at MCS2	This study
pETDuet-B1A2	pETDuet with insertion of <i>lipB</i> at MCS1 and <i>lipA</i> at MCS2	This study
pACYCDuet-A1	pACYCDuet with insertion of <i>lipA</i> at MCS1	This study
pACYCDuet-B1	pACYCDuet with insertion of <i>lipB</i> at MCS1	This study
pETDuet-B1	pETDuet with insertion of <i>lipB</i> at MCS1	This study
pETDuet-B2	pETDuet with insertion of <i>lipB</i> at MCS2	This study
pET28a- <i>lipA</i>	Expression plasmid containing <i>lipA</i> gene	This study
pET28a- <i>lipB</i>	Expression plasmid containing <i>lipB</i> gene	This study

Table 3 Strains and plasmids used in the current study

Table 4 Oligonucleotide primers used in the current study^a

Primers	Oligonucleotide sequence (5' to 3')	Annealing temperature (°C)	PCR products
lipBEF1	CGCGGATCCGCCGCCGTCGCTCGC	60	The coding region for the truncated LipB
lipBER1	CCCAAGCTTTTACTGCATGCTGCCG		(for pACYCDuet-B1A2 and pETDuet-B1A2 construction)
lipAEF1	GAAGATCTCGCCGATGGCTACG	55	The coding region for the mature LipA
lipAER1	GAGATATCTTACACGCCCCGCCA		(for pACYCDuet-B1A2 and pETDuet-B1A2 construction)
1ipAEF2	TATGGATCCGCCGATGGCTACGCGGCGACGC	58	The coding region for the mature LipA
1ipAER2	CTTAAGCTTTTACACGCCCCGCCAGCTTCAG		(for pET28a- <i>lipA</i> construction)
lipBEF2	CGCGGATCCCCGCCGTCGCTCGCC	59	The coding region for the truncated LipB
lipBER1	CCCAAGCTTTTACTGCATGCTGCCG		(for pET28a- <i>lipB</i> construction)

^aunderlined nucleotides: restriction endonuclease site.

Figures

Figure 1

Growth curves (A) and kinetic curves for soluble LipA (B) of various recombinant E. coli strains carrying co-expression combination system of lipA and lipB cultivated at 20°C. 1: E. coli BL21 (DE3)/pACYCDuet-B1A2; 2: E. coli BL21 (DE3)/pETDuet-B1A2; 3: E. coli BL21 (DE3)/pACYCDuet-B1A2 plus pETDuet-B1A2; 4: E. coli Origami 2 (DE3)/pACYCDuet-B1A2; 5: E. coli Origami 2 (DE3)/pETDuet-B1A2; 6: E. coli Origami 2 (DE3)/pACYCDuet-B1A2 plus pETDuet-B1A2.

Figure 2

SDS-PAGE analysis and western blotting analysis of the purified LipA/LipB complex from cell-free supernatant of E. coli Origami 2 (DE3)/pETDuet-B1A2 lysate using HisTrap™ HP affinity chromatography. Lane 1: western blotting analysis of anti-His tag antibody of the purified LipA/LipB complex; lane 2: SDS-PAGE analysis of the purified LipA/LipB complex; M: protein molecular weight marker.

A

B

C

D

Figure 3

Enzymatic characterization of the purified LipA. (A): Effect of temperature on LipA activity; (B): Effect of pH on LipA activity; (C): Effect of temperature on LipA stability; (D): Effect of pH on LipA stability.

Figure 4

Effect of different bivalent salt ions and EDTA on the recombinant LipA activity. 1: Control; 2: NaCl; 3: KCl; 4: BaCl₂; 5: MgCl₂; 6: FeCl₃; 7: CaCl₂; 8: ZnCl₂; 9: CuCl₂; 10: AlCl₃; 11: MnCl₂; 12: EDTA.

Figure 5

Thin-layer chromatogram of the hydrolysis products of triolein catalyzed by LipA. Lane 1: Triolein; Lane 2: 1,2-diolein; Lane 3: 1,3-diolein; Lane 4: 1-monoolein; Lane 5: oleic acid; Lane 6: hydrolysis products of triolein.

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