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Full Length Research Paper

Purification and characterization of a novel cold-adapted lipase from *Burkholderia anthina NT15*

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A cold-adapted lipase (LIP-BA) of *Burkholderia anthina NT15* was purified by using PEG1000/potassium phosphate based aqueous two-phase systems, chromatography separation on a DEAE-cellulose-32 column and a Sephadex G100 column. Molecular weight of LIP-BA was determined to be approximately 44.5 kDa by SDS-PAGE. The optimum temperature for the activity of Lipase-BA was found to be 30°C. The optimum pH for the activity was 9.5.LIP-BA activity could be inhibited by Ca^{2+}, Fe^{2+}, La^{3+}, Mn^{2+}, K^{+}, Sr^{2+}, Fe^{3+}, Cu^{2+} and Zn^{2+} or increased by Na^{+} and Mg^{2+}. LIP-BA was stable in the solvent of isopentanol, ether and n-butanol. Tween-40 and Tween-60 could enhance the enzyme activity. The K_m, V_{max} and K_{cat} values of LIP-BA were 0.10 mM, 430 U/mg and 210 S^{-1}, respectively.

Key words: Lipase, *Burkholderia anthina*, purification, characterization.

INTRODUCTION

Lipases (triacylglycerol acyl hydrolases, EC 3.1.1.3) are useful biocatalysts which are widely used in food-processing industry, detergent industry, organic synthesis, oleochemical industry, textile industry, pharmaceutical industry, etc. (Hasan et al., 2006; Park et al., 2009; Kim et al., 2009).

Burkholderia strains are the important sources of lipase because of its high stability in organic solvents and exquisite enantioselectivity (Tomic and Ramek, 2006). *Burkholderia cepacia* lipases had been used for enzymatic synthesis of alpha, beta-dipeptides (D'Arrigo et al., 2009), hydrolysis of beta-heteroaryl-beta-amino esters (Tasnadi et al., 2009), preparation of methyl (R)-N-(2,6-dimethylphenyl) alaninate (Park and Lee, 2005) and other enantioselectivitive esterifications.

In our previous work, we had isolated a lipase producing strain (*Burkholderia anthina NT15*). The aim of this follow-up work was to purify and characterize this lipase (named LIP-BA).

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MATERIALS AND METHODS

Microorganism and culture conditions

B. anthina NT15 was isolated from the soil of North China with at about 10°C and was a stock culture of the School of Life Sciences, Nantong University, Nantong, Jiangsu province, China. The medium for lipase production contained (per liter): 30 g glucose, 5 g yeast extract, 10 g peptone, 3 g NaCl, 40 ml olive oil and 5 ml Triton X-100. The initial pH was adjusted to 7.2 with NaOH after autoclaving. *B. anthina NT15* was inoculated into a 250 mL Erlenmeyer flask containing 50 mL of the medium and cultivated at 30°C on a reciprocal shaker at 200 rpm for 72 h.

Purification of lipase and determination of lipase molecular weight

After 72 h of growth, the culture broth was centrifuged to remove the cells. The cell-free fermentation broth was dried by freeze-drying and stored at -20°C for later use. Firstly, lipase was partially purified by PEG1000/potassium phosphate based aqueous two-phase systems (Yuan et al., 2010). The obtained enzyme solvent was dialyzed for 24 h with three changes in 20 mM Tris-HCl buffer (pH 7.2). The dialyzed enzyme solution was applied to a column of DEAE-cellulose-32 (2.6 × 40 cm) that had been equilibrated with Tris-HCl Buffer (pH 7.2) and washed with a negative linear gradient of 0–1.0 M NaCl at a flow rate of 2 ml/min. The active fractions were

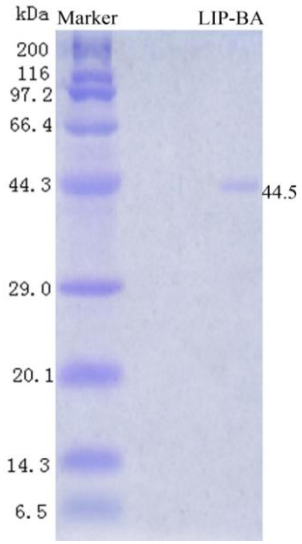

Figure 1. SDS-PAGE analysis of purified LIP-BA. Lane 1, molecular markers; lane 2, purified LIP-BA.

collected and loaded onto the Sephadex G75 column (1.6 × 60 cm) equilibrated with Tris–HCl buffer (pH 7.2). The column was then washed with the same buffer at a flow rate of 0.5 ml/min. The lipase-active fractions were pooled, dialyzed, concentrated by ultra-filtration and stored at -20°C.

SDS-PAGE [10] was performed using 5% polyacrylamide-stacking gel and a 15% polyacrylamide-resolving gel. The standard marker was the mixture of myoglobulin (200 kDa), β-galactosidase (116 kDa), phosphorylase b (97.2 kDa), bovine serum albumin (66.4 kDa), albumin egg (44.3 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20.1 kDa), lysozyme (14.3 kDa), and aprotinin (6.5 kDa). Protein bands were stained with Coomasie brilliant blue R250. The protein concentration was determined according to the Bradford method with BSA as the standard (Bradford, 1976).

Lipase activity assay

Lipase activity was determined by the modified *p*-nitrophenol (*p*-NP) method (Margesin et al., 2002). 3.7 ml 0.1 M Tris-HCl (pH 9.0) and 0.2 ml 10 mM *p*-nitrophenyl butyrate (*p*-NPB) were equilibrated at 30°C. To this solution, 0.1 ml of suitable dilution of lipase was added and incubated at 30°C for 15 min. The amount of liberated *p*-NP was recorded at 405 nm. One unit of lipase activity was defined as the amount of lipase that catalyzed the formation of 1 µMol of *p*-NP per min.

pH effects on lipase activity and stability

The effects of pH on the purified lipase activity were determined by incubating the purified enzyme at various pH values (5.0-10.5) using the standard lipase assay method. The Tris-HCl buffer was used as a buffer system. The relative lipase activity at different pH values was calculated taking that with pH 9.5 as 100%.

To determine the effect of pH on lipase stability, the enzyme was equilibrated at 30°C for 8 h in buffers of pH 5.0-11.0. The residual lipase activity was determined at pH 9.5 under standard assay conditions. The relative lipase activity was calculated by taking the lipase activity without buffer treatment with pH 9.5 as 100%.

Temperature effects on lipase activity and stability

The optimum temperature of lipase activity was measured by assaying its hydrolytic activities toward p-NPB at different temperature ranged from 10 to 70°C under standard assay conditions. The relative lipase activity at different temperature was calculated by taking that at 30°C as 100%. The thermal stability was studied by incubating lipase at various temperatures (10 - 55°C) and measuring the residual activity with time under standard assay conditions. The relative lipase activity was calculated by taking the non-heated lipase activity as 100%.

Substrate specificity

Substrates *p*-nitrophenyl fatty acid esters of varying chain length were used to measure enzyme activity under standard assay conditions. The substrates (Sigma) were *p*-nitrophenyl utyrate (*p*-NPB, C2), *p*-nitrophenyl acetate (*p*-NPA, C4), *p*-nitrophenyl caproate (*p*-NPC, C8), and *p*-nitrophenyl palmitate (*p*-NPP, C16).

Effect of metal ions on lipase activity

The enzyme sample was incubated in various metal ions (K^+, Na^+, Ca^{2+}, La^{3+}, Sr^{2+}, Fe^{2+}, Fe^{3+}, Cu^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+}), respectively for 10 min at 30°C and activity was determined by using standard assay conditions and the obtained activity values were compared to activity without supplement of surfactant.

Effects of surfactant on lipase activity

The enzyme sample was incubated in various surfactants (Sodium deoxycholate, SDS, Tween-20, Tween-40, Tween-60, Tween-80, Trition X-100), respectively for 30 min at 30°C and activity was determined by using standard assay conditions and the obtained activity values were compared to activity without supplement of surfactant.

Effects of organic solvent on lipase activity

Various organic solvents (2-butanone, acetone, ether, ethanol, benzyl alcohol, n-butanol, isopentanol) were added to the purified lipase solutions, respectively. Then it was incubated for 30 min at 30°C. The activities were determined by using standard assay conditions and the obtained activity values were compared to activity without supplement of organic solvent.

Determination of kinetic parameters

The K_m and V_{max} values of lipase were determined for *p*-NPB substrate. Kinetic constants were calculated by using Lineweaver-Burk plot (Lineweaver and Burk, 1934).

RESULTS AND DISCUSSION

Purification of LIP-BA

The overall level of recovery was about 41.3%. The SDS-PAGE electrophoresis (Figure 1) showed a single band for LIP-BA. The molecular mass of denatured LIP-BA

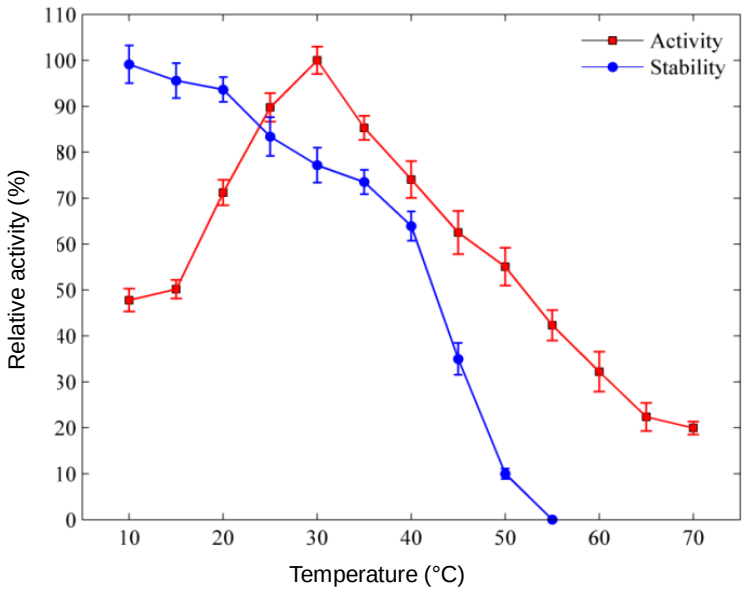

Figure 2. Effects of temperature on enzyme activity.

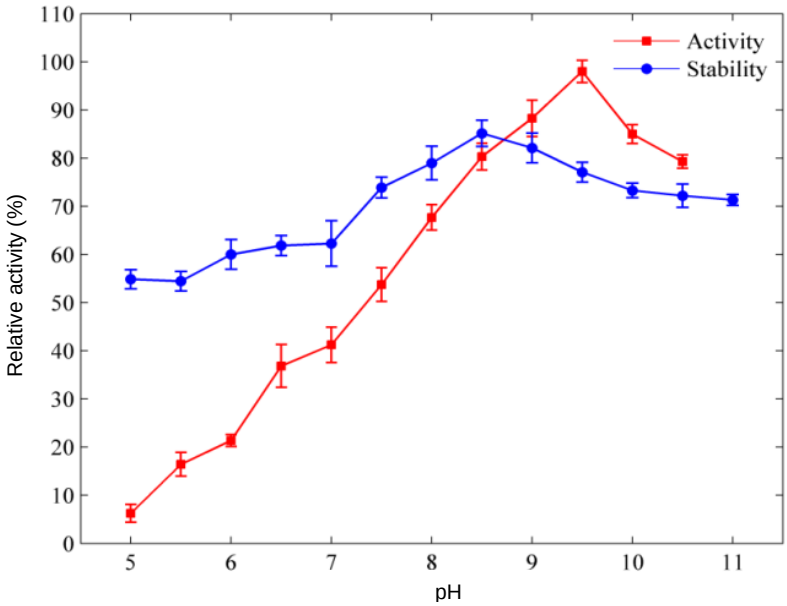

Figure 3. Effects of pH on enzyme activity.

estimated from the relative mobility of standard proteins on SDS-PAGE was about 44.5 kDa.

Effect of temperature

The optimum temperature of Lipase-BA was 30°C. Lipase-B had 45% activity at 10°C. When temperature was higher than 15°C, the activity of Lipase-B would decrease significantly. LIP-BA was stable at the low temperature. It could retain about 100% at 10°C (Figure 2).

Effect of pH

Optimum enzyme activities of the two lipases were observed at pH 9.5. About 50% of optimum activity was detectable at pH 8. The results showed that it was alkaline lipase (Figure 3).

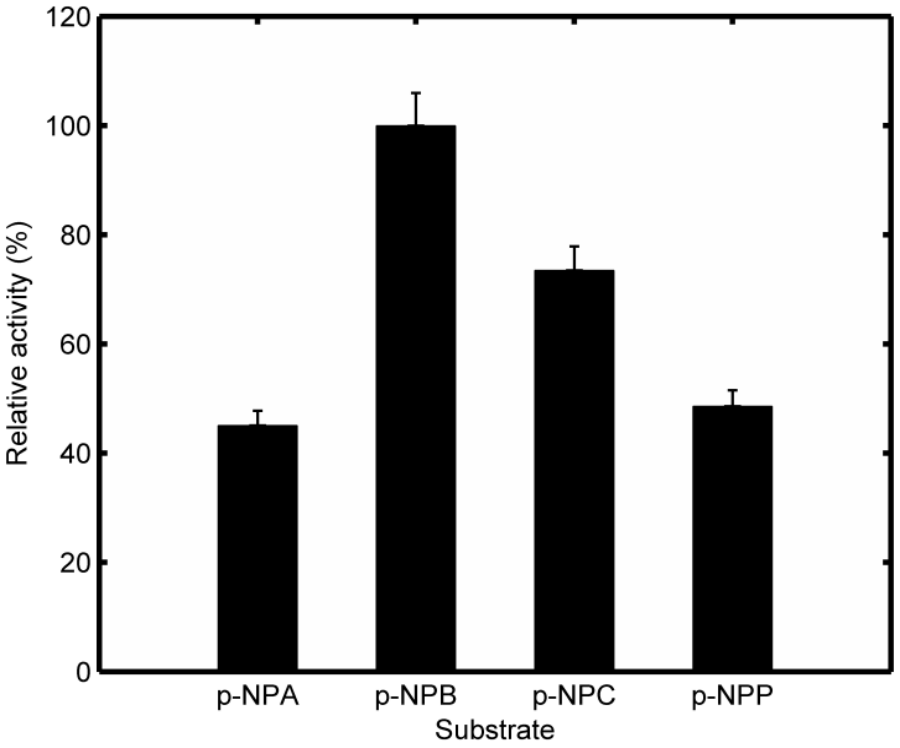

Figure 4. Substrate specificities of LIP-BA.

Table 1. Effect of metal ion and EDTA on the activity of LIP-BC produced by *Burkholderia* sp. SYBC LIP-Y.

Metal ions Type	Concentration (Mm)	1	5	10
		Residual lipase activity (%)		
Na^{+}		117.3	130.8	190.1
Mg^{2+}		121.1	140.5	179.2
Ca^{2+}		102.8	106.1	102.8
Fe^{2+}		107.7	97.1	89.8
La^{3+}		102.8	98.6	97.0
Mn^{2+}		96.5	102.0	90.9
K^{+}		95.9	97.4	89.7
Sr^{2+}		95.6	97.9	87.4
Fe^{3+}		100.4	95.0	83.8
Cu^{2+}		90.9	78.0	62.3
Zn^{2+}		54.7	56.4	40.0
EDTA		93.6	86.5	79.9

Substrate specificity

LIP-BA showed best hydrolysis efficiency for *p*-NPB (C4). The activity on *p*-NPA (C2), *p*-NPC (C8) and *p*-NPP (C16) were higher than 40% (Figure 2). The similar substrate specificity was found in case of two cold-adapted lipases from a newly isolated mesophilic *Geotrichum* sp. They also showed the highest activity on *p*-NPB (Cai et al., 2009) (Figure 4).

Effect of metal ions on LIP-BA activity

The effect of various metal ions on the activity of LIP-BA was listed in Table 1. The results indicated that Na^{+} and Mg^{2+}could significantly increase the activity of LIP-BA.

Table 2. Effect of organic solvent on the activity of LIP-BC produced by *Burkholderia* sp. SYBC LIP-Y.

Organic solvent Type	Concentration (%)	10	30	50
		Residual lipase activity (%)		
2-butanone		54.1	46.0	38.2
acetone		57.1	41.2	35.3
ethanol		90.9	75.6	57.9
benzylalcohol		81.9	71.6	63.8
isopentanol		107.4	105.5	99.2
ether		108.8	106.2	100.8
n-butanol		113.1	107.3	104.0

Table 3. Effect of surfactant on the activity of LIP-BC produced by *Burkholderia* sp. SYBC LIP-Y.

Surfactant Type	Concentration (%)	0.1	0.3	0.5
		Residual lipase activity (%)		
SDS		98.9	99.1	92.8
Tween-20		99.5	99.8	98.3
Tween-40		108.8	108.3	104.6
Tween-60		126.3	119.3	120.2
Tween-80		87.8	80.9	79.6
Triton X-100		103.4	99.4	97.9
Sodium deoxycholate,		99.7	101.5	95.9

LIP-BA activity was found to be inhibited in presence of Ca^{2+}, Fe^{2+}, La^{3+}, Mn^{2+}, K^{+}, Sr^{2+}, Fe^{3+}, Cu^{2+} and Zn^{2+}. Cu^{2+} and Zn^{2+} were the strongest inhibitor. The enzyme activity was reduced by the metal chelator EDTA indicating that LIP-BA was a metalloenzyme. An alkaline thermostable lipase from *Aspergillus carneus* also showed that Na^{+} and Mg^{2+} stimulated lipase activity while Cu^{2+} and Zn^{2+} caused inhibition. In contrast, there was no effect of EDTA on this lipase activity (Saxena et al., 2003). A *Burkholderia* sp. GXU56 lipase activity was strongly inhibited by Zn^{2+}, Cu^{2+}, Co^{2+}, Fe^{2+} and Fe^{3+}, while it was stimulated by Li+ and Ca^{2+} ions (Wei et al., 2008). The *Burkholderia cepacia* ATCC 25416 lipase activity was strongly activated by Mg^{2+}, Ca^{2+}, Cu^{2+}, Zn^{2+} and Co^{2+} (Wang et al., 2009).

Effect of organic solvent on LIP-BA activity

Effects of various organic solvents were shown in Table 2. LIP-BA was stable in the presence of isopentanol, ether and n-butanol. Ethanol, 2-butanone, acetone, and benzyl alcohol could reduce the activity significantly. Obviously, high-polar organic solvents could inhibit LIP-BA activity by reducing the water activity around the protein molecules and promoter the structural denaturation (Tanford, 1968). Other research also found that *B. cepacia* strains showed no tolerance to any of alcohol (Shu et al., 2009).

Effect of surfactant on LIP-BA activity

As listed in Table 3, Tween-40 and Tween-60 could enhance the enzyme activity. SDS, Tween-20, Trition X-100 and sodium deoxycholate slightly caused reduction in activity. However, Tween-80 could reduce the activity about 20% at 0.3% concentration. For a lipase from *B. cepacia* ATCC 25416, all the tested non ionic surfactants could activate the lipase; however, ionic surfactants slightly inhibited the enzyme activity (Wang et al., 2009).

The kinetic constants

The kinetic constants of LIP-BA were determined on the basis of Michaelis-Menten behavior. The K_m, V_{max} and K_{cat} values of LIP-BA were 0.10 mM, 380 U/mg and 210 S^{-1}, respectively. This compares very similar with the reported values of a lipase from *B.* cepacia (ATCC 25609) where K_m and V_{max} were 430 U/mg and 0.11 mM, respectively (Dalal et al., 2008). However, for a *B.*

cepacia ATCC 25609 lipase, the K_m value was 11 mM (Perez et al., 2007). LIP-BA had a high affinity for the substrate.

Conclusion

In this work, we purified and characterized a new lipase. This lipase have unique profile: wide range pH and temperature stabilities with cold-adapted features, solvent and surfactant stability, utilizing wide range of substrate. These characters made it a potential candidate for its use in industry.

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