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Gene cloning, overexpression and characterization of a novel organic solvent tolerant and thermostable lipase from *Galactomyces geotrichum* Y05

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Abstract

Although the lipase of *Geotrichum candidum* has been extensively reported, little attention has been focused on molecular genetic and biochemical characterizations of *Galactomyces geotrichum* lipases. A lipase gene from *G. geotrichum* Y05 was cloned from both genomic DNA and cDNA sources. Nucleotide sequencing revealed that the *ggl* gene has an ORF of 1692 bp without any introns, encoding a protein of 563 amino acid residues, including a potential signal sequence of 19 amino acid residues. The amino acid sequence of this lipase showed 86% identity to lipase of *Trichosporon fermentans* WU-C12. The mature lipase gene was subcloned into pPIC9K vector, and overexpressed in methylotrophic *Pichia pastoris* GS115. Active lipase was accumulated to the level of 100.0 U/ml (0.4 mg/ml) in the shake-flask culture, 10.4-fold higher than the activity of the original strain (9.6 U/ml). This yield dramatically exceeds that previously reported with 23–50 U/ml, 0.06 mg/ml and 0.2 mg/ml. The purified lipase exhibited several properties of significant industrial importance, such as pH and temperature stability, wide organic solvent tolerance and broad hydrolysis on vegetable oils. Such a combination of properties makes it a promising candidate for its application in non-aqueous biocatalysis, such as biodiesel production, selective hydrolysis or esterification for enrichment of PUFAs and oil-contaminated biodegradation, which have been drawn considerable attention currently.

Keywords: *Galactomyces geotrichum* Y05; Lipase; Cloning; Overexpression; Characterization

1. Introduction

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are unique in catalyzing the hydrolysis of triacylglycerols into fatty acids and glycerol at the water–lipid interface and its reverse reaction in non-aqueous solvents [1,2], which occupy a place of prominence among biocatalysts owing to their novel and multifold applications in oleochemistry, resolution of racemic mixtures, synthesis of fine chemicals and pharmaceuticals. Lipases with chemo-specificity, organic solvents tolerance and thermostability are capable of catalyzing a variety of important reactions, thereby presenting a fascinating field for future research and causing tremendous interest among scientists and industrialists [3,4].

The lipase produced by the fungus *Geotrichum candidum* was one of the important lipases investigated, and many reports on its production, isolation, activity and selectivity were published in recent decades. Lipase I and lipase B from *G. candidum* present an unique substrate specificity for long-chain fatty acids containing *cis*-9 double bond, thus they are widely used in industry for the alcoholysis and intersterification reactions [5,6]. Recent applications of the *cis*-9-selective enzyme in the hydrolysis or esterification of conjugated linoleic acid (CLA), a mixture of octadecadienoic acids differing in the position of their conjugated double bonds, has attracted considerable attention because of its anticarcinogenic and antiatherogenic properties [7].

Shimada et al. initiated molecular studies on *G. candidum* lipase by cloning and sequencing two closely related lipase genes lipI and lipII [8]. Then Bertolini et al. further demonstrated the polymorphism of the lipase genes from *G. candidum* strains [9]. Recently, few investigations have been done on the expression of *G. candidum* lipases in *Saccharomyces cerevisiae* and *Picha pastoris* with low yield of 0.06 mg/ml and 50 U/ml, respectively

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[10,11]. Catalytic and biochemical properties of these recombinant lipases make them suitable for potential applications in selective hydrolysis and esterification of CLA [12], resolving racemic mixtures of *cis*- or *trans*-isomer of 2-(4-methoxybenzyl) cyclohexyl acetates [13]. This enzyme also showed moderate activity with esters containing polyunsaturated fatty acids (PUFAs) such as EPA (eicosapentaenoic acid) or DHA (docosahexaenoic acid) [14,15]. Selective hydrolysis and esterification for enrichment of PUFAs fraction present in fish oils have been drawn tremendous attention [7,16].

Galactomyces geotrichum is the teleomorph of *G. candidum*. Both are on the borderline between the typical yeasts and moulds, phylogenetically with ascomycetous yeast-like fungi [17]. The genus *Geotrichum* link has 23 recognized taxa, 14 species with *Dipodascus* and 2 with *Galactomyces* teleomorphs [18]. Although there were few previous studies on *G. geotrichum* lipase, there is still dearth of *G. geotrichum* lipases possessing features such as thermostability and organic solvents tolerance that favor them to be excellent industrial biocatalyst [18,19]. Some lipase genes of *G. candidum* have previously been expressed in *S. cerevisiae* and *P. pastoris*. However, the yields and quality of the recombinant lipases are still unsatisfactory. In this paper, we describe the cloning, overexpression, purification and characterization of a novel lipase from *G. geotrichum* Y05, and provide more comprehensive information of the recombinant lipase, which may have tremendous potential in areas such as biodiesel production, selective hydrolysis or esterification for enrichment of PUFAs and oil-contaminated biodegradation. The cloned *ggl* gene encoding for lipases with distinct differences in substrate specificity, thermostability and organic solvent tolerance, provides unique gene resource to further address the molecular basis of the substrate specificity and identify the regions of the molecule involving in thermostability and organic solvent tolerance.

2. Materials and methods

2.1. Strains, plasmids and culture mediums

A fungus *G. geotrichum* Y05 producing lipase was isolated from oil-contaminated soil samples from Luzhou City, Sichuan Province, China. *Pichia pastoris* GS115 (his4) and pPIC9K (Invitrogen, USA) were used as host and vector for heterologous expression of the lipase. *Escherichia coli* DH5α was used for all plasmid constructions. Plasmid pMD18-T (Takara, Ltd.) was used for gene cloning and sequencing. *E. coli* was grown in Luria–Bertani medium (LB) containing 100 μg/ml ampicillin at 37 °C. *P. pastoris* was grown in shake-flasks at 30 °C, in BMGY medium containing 1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate buffer (pH 6.0), 1.34% (w/v) yeast nitrogen base, 4×10^{-5}% (w/v) biotin, 1% (v/v) glycerol before the induction, or BMMY medium containing the same composition as BMGY medium with the exception that 0.5% (v/v) methanol was added instead of glycerol for the induction. MD medium (1.34% (w/v) yeast nitrogen base, 4×10^{-5}% (w/v) biotin, 2% (w/v) dextrose, 2% (w/v) agar) were used for selection of *P. pastoris* transformants. YEPD medium (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose, 2% (w/v) agar) containing G418 at a final concentration of 0.25, 0.5, 0.75, 1.0, 1.5, 1.75, 2.0, 3.0 and 4.0 mg/ml, were used for screening of multiple copy inserts transformants. YEPD-rhodamine B-olive oil plates containing 2×10^{-4}% (w/v) rhodamine B and 1% (v/v) olive oil were used for assaying active secretion of extracellular lipase.

2.2. Cloning and sequencing of complete ggl gene

Genomic DNA from *G. geotrichum* Y05 was extracted according to the method described by Cheng et al. [20]. Isolation of total RNA was performed using TRIZOL reagent (Invitrogen, USA) according to manufacturer's instructions. PCR and RT-PCR amplification were carried out as described by Sambrook [21]. Based on the complete nucleotide sequences of lipase genes of *Geotrichum* link (Accession No. E02678, Accession No. X78032, Accession No. AB000260, Accession No. DQ313172), two degenerated primers F1 (5′ATGGTWTCCAAAAVCTT KTTYTTRGC3′) and R1 (5′TTAACCGTAGAGATTAACGTCAGWCTC 3′ (W-A, T; V-G, A, C; K-G, T; Y-C, T; R-A, G)) were designed for PCR and RT-PCR amplification. The cloned fragments were then ligated into pMD18-T vector and sequenced by ABI 3730 DNA analyzer.

2.3. Expression of ggl gene in P. pastoris

Two primers F2 (5′CTGAATTCCAAGCTCCTACGGCTGTTCT3′) and R2 (5′CTTGCGGCCGCTTAACCGTAGAGATTAACGT 3′) were designed on the basis of the complete *ggl* gene cloned above to introduce *Eco* RI and *Not* I restriction sites in the 5′ and 3′ ends of the mature *ggl* gene, respectively (underlined sequences correspond to introduced restriction sites *Eco* RI and *Not* I). The amplified mature *ggl* gene was digested with *Eco* RI/*Not* I and ligated into expression vector pPIC9K linearized with the same enzymes, giving rise to recombinant expression plasmid pPIC9K-*ggl*. Both pPIC9K-*ggl* and pPIC9K were linearized with SacI, and then transformed into *P. pastoris* GS115 cells by electroporation according to Invitrogen's recommendations. Transformants were sequentially screened on MD medium, YPED-G418 medium and YEPD-rhodamine B-olive oil medium plates. The best transformant was chosen to grown in BMMG medium until they reached an $OD_{600} = 2–6$, and then transferred to BMMY medium for lipase induction and secretion by adding 0.5% of methanol at 24 h intervals. The recombinant with pPIC9K was used as control for background for the expression analysis.

2.4. Lipase purification

Recombinant *P. pastoris* was grown in BMMY liquid medium as described above. Cells from the cultures were removed by centrifugation (10,000 × *g*, 5 min, 4 °C). Then ammonium sulfate was added to 300 ml of cell-free supernatant to the final concentration of 50% saturation and the suspension was kept at 0 °C for 6 h under gentle stirring. The precipitated

was collected by centrifugation at 10,000 × *g* for 10 min and further dissolved in 50 ml of 20 mM Tris–HCl buffer (pH 7.0). The enzyme solution was dialyzed overnight against the same buffer and the insoluble materials were discarded by centrifugation. The enzyme solution was loaded onto a DEAE-Sepharose Fast Flow (2.0 cm × 20 cm) anion exchange chromatograph column previously equilibrated with 20 mM Tris–HCl buffer (pH 7.0). The enzyme was eluted by a linear gradient from 0 to 1 M NaCl in the same buffer. Fractions showing lipase activity were concentrated and dissolved in 50 ml 50 mM Tris–HCl buffer (pH 8.0), and then loaded onto a Sephadex-G75 (1.6 cm × 60 cm) gel filtration chromatograph column previously equilibrated with 50 mM Tris–HCl buffer (pH 8.0) and eluted with the same buffer. The active fractions were pooled from each purification step and assayed for lipase activity and protein concentration. The specific activity of the purified enzyme was compared with that of crude enzyme and purification fold was calculated. Protein concentration was determined spectrophotometrically according to the method of Bradford using BSA as standard [22]. SDS-PAGE was performed as described by Laemmli using a 10% separating gel and a 5% stacking gel. The gels were stained by Commassie Brilliant Blue R-250 for protein detection [23].

2.5. Effect of temperature and pH on enzyme activity and stability

The optimal temperature for activity was determined using olive oil as substrate at different temperatures (20–65 °C), at pH 8.0. For determination of temperature stability, 1 ml of the enzyme solution were incubated for 24 h in 50 mM Tris–HCl buffer (pH 8.0), at 20–65 °C, and the residual activity was measured. Optimal pH was determined similarly, at 50 °C in 50 mM buffer solutions of pH values ranging from 4 to 11 (sodium citrate–phosphate (pH 4–8); Tris–HCl (pH 7–9); glycine–NaOH (pH 9–10); Na_2HPO_4–NaOH (pH 11)). The effect of pH on enzyme stability was analyzed by measuring residual activity after the pre-incubation of 1 ml of enzyme solution for 24 h at room temperature in 5 ml of the above-mentioned buffer solutions (pH 4–11).

2.6. Effect of chemicals and detergents on lipase

The effect of a variety of detergents and chemicals on enzyme activity was analyzed by incubating an enzyme aliquot (1 ml) for 6 h at room temperature in 50 mM Tris–HCl buffer (pH 8.0), containing 1% (v/v) of the detergents (Triton X-100, Tween-80 and SDS) and 1 mM of $BaCl_2$, $CaCl_2$, $MgCl_2$, NaCl, KCl, LiCl, $HgCl_2$, EDTA and phenylmethyl-sulfonyl fluoride (PMSF), respectively. Activity was measured after pre-incubation.

2.7. Effect of organic solvents on lipase

The effect of organic solvents on enzyme activity was determined by measuring residual activity after pre-incubation of 4 ml of enzyme solution for 6 h at room temperature, in 4 ml of methanol, ethanol, acetone, isopropanol, glycerol, butanol, chloroform, hexane, *n*-heptane, cyclohexane, isooctane, benzene and diethyl ether, respectively.

2.8. Substrate specificity

Lipase substrate specificity was analyzed by using a variety of fat and oil substrates including olive oil, corn oil, soybean oil, castor oil, tung oil, rape seed oil, tea oil, cotton seed oil, castor oil and lard. Specific activities of recombinant enzymes against a series of triacylglycerol substrates (trioctanoin ($C_{8:0}$), tripalmitin ($C_{16:0}$), tristearin ($C_{18:0}$), triolein ($C_{18:1}$, *cis*-9), trivaccinin ($C_{18:1}$, *trans*-9), trilinolein ($C_{18:2}$, *cis*-9,12) and trilinolenin ($C_{18:3}$, *cis*-9, 12, 15)) differing in chain length and saturation were determined similarly.

2.9. Lipase activity assay

Activity determination was carried out titrimetrically as described previously using 50 mM NaOH as titrant [24]. The reaction mixture contained 5 ml of 50 mM Tris–HCl buffer (pH 8.0), 4 ml of olive oil as substrate. Unless stated otherwise, incubations were carried out at 50 °C and pH 8.0 for 10 min. The enzymatic reaction was initiated by addition 1 ml of the enzyme solution to the reaction mixture and stopped by the addition of 15 ml of ethanol. Control was carried out similarly, except that the enzyme solution was added after the addition of ethanol. One unit of lipase activity was defined as the amount of enzyme that caused the release of 1 μmol of free fatty acid per minute under test condition. The method for the determination of other substrate specificity is similar to the above-mentioned, except for differences in substrates.

3. Results and discussion

3.1. Cloning and sequence analysis of the ggl gene

The predicted fragments were both amplified from genomic DNA and cDNA sources by PCR and RT-PCR. Nucleotide sequencing of the fragments revealed that the *ggl* gene has an ORF of 1692 bp without any introns, encoding a protein of 563 amino acid residues, including a potential signal sequence of 19 amino acid residues. The complete nucleotide sequence of *ggl* gene was deposited at GenBank under Accession No. DQ841279.

Multiple sequence alignment showed GGL (Accession No. DQ841279) has 86% identity to lipase I precursor of *Trichosporon fermentans* WU-C12 (Accession No. AB000260), 85% identity to lipase of *G. candidum* strain lip42 (Accession No. DQ313172), and 81% identity to extracellular lipase of *G. geotrichum* CBS 772.71 (Accession No. X78032). Moreover, the *ggl* gene encoded a -Gly^{234}-Glu-Ser-Ala-Gly^{238}- conservative motif, characteristic of the triacylglycerol hydrolases. In all these alignment lipases, the catalytic triad -Ser^{236}-Glu^{373}-His^{482}- were found at perfectly conserved positions. The protein contained two potential glycosylation sites (-Ala^{383}-Asn-Thr^{385}- and -Ala^{456}-Asn-Thr^{458}-) and four Cys residues involving in two disulfide bridges (-Cys^{80}∼Cys^{124}- and

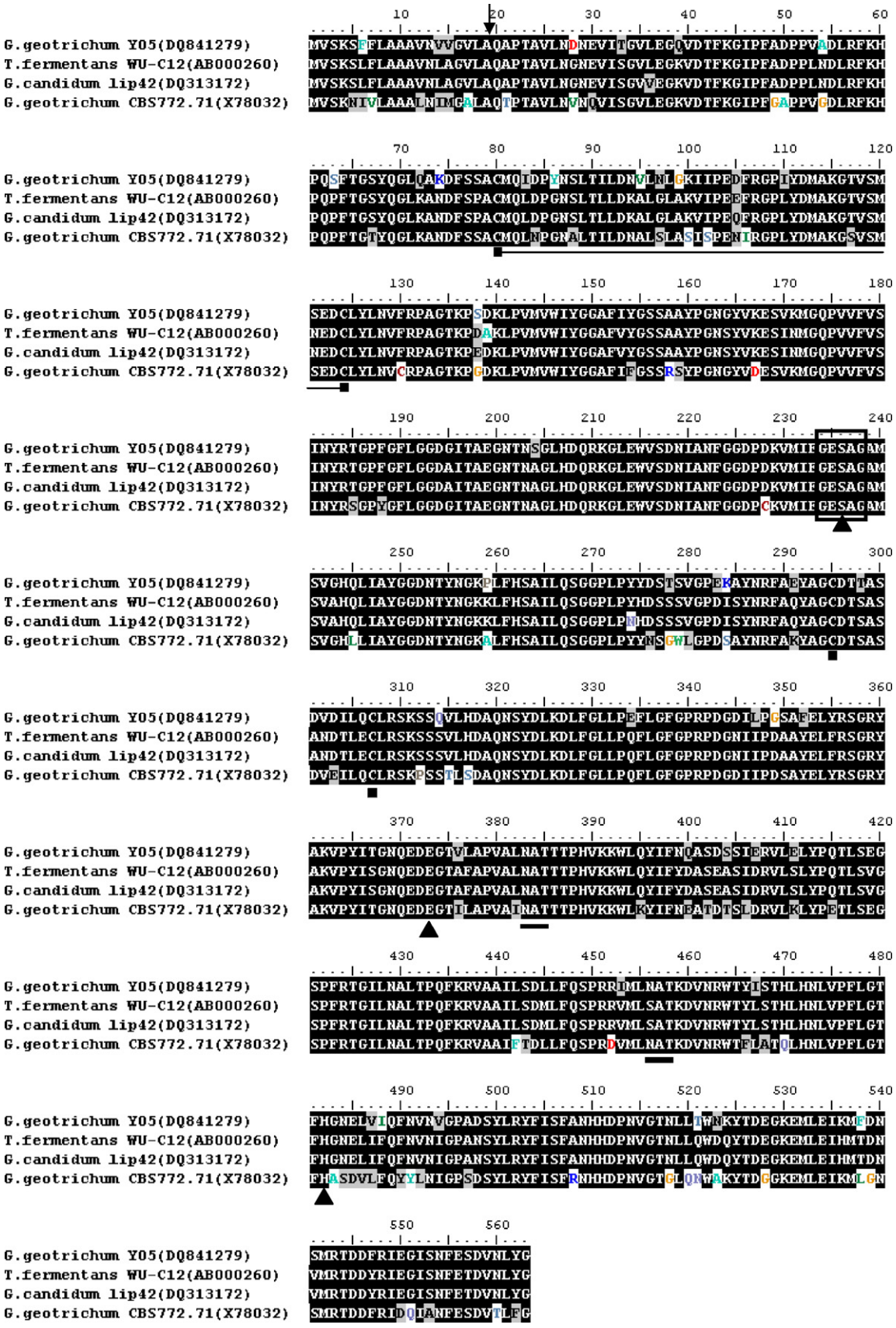

Fig. 1. Multiple sequence alignment of the complete lipase of *G. geotrichum* Y05 (GGL) against other related complete lipases. The catalytic triad -Ser^{236}-Glu^{373}-His^{482}- is marked by triangles. The conservative motif -Gly^{234}-Glu-Ser-Ala-Gly^{238}- is boxed. Line indicates flap domain and bold lines indicate the potential glycosylation sites. Rectangles and arrowhead show the disulfide bonds and signal peptide proteolytic cleavage, respectively.

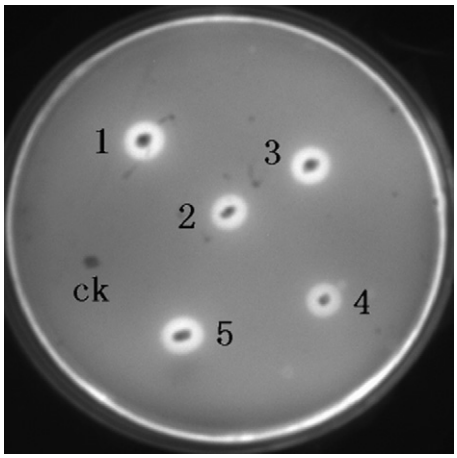

Fig. 2. The fluorescent halos produced by transformants hyper-resistance to G418 on YEPD-rhodamine B-olive oil plate when exposed by UV light. CK: transformant containing plasmid pPIC9K as control; 1–5: transformants containing recombinant vector pPIC9K-*ggl*.

-Cys^{295}∼Cys^{307}-). As previously reported [18,25], the characteristic flap covering the active site of the lipase-encompassed residues Met^{81}-Asp^{123} (Fig. 1).

3.2. Overexpression of the ggl gene in P. pastoris

The mature *ggl* gene in frame with α factor signal sequence was placed under the control of the methanol inducible alcohol oxidase promoter (AOX1). Integration of the recombinant expression vector pPIC9K-*ggl* into *P. pastoris* genome resulted in transformants that could express and secrete recombinant GGL.

Hundreds of transformants were obtained by electroporation on MD plates. However, only five muti-copy transformants were screened on YEPD plate containing the highest G418 concentration 4.0 mg/ml and observed obvious fluorescent halos on YEPD-rhodamine B-olive oil plate when exposed under 350 nm UV irradiation (Fig. 2). The best clone producing the highest lipase yield (216.67 U/mg protein) was chosen for further lipase purification and characterization. In cultures of *P. pastoris* transformant grown in BMMY medium, lipase production was detected in the supernatant after induction for 24 h with 0.5% methanol. The lipase production kept increasing during the next 72 h of induction and attained maximal activity 100.0 U/ml, enhanced 10.4-fold compared with activity of original strain (9.6 U/ml). In the negative control cells transformed with vector pPIC9K without lipase gene, no lipase production could be detected. The lipase quantities (0.4 mg/ml) obtained here increase dramatically in the levels of expression compared with previously reported yield of 23–50 U/ml, 0.06 mg/ml and 0.2 mg/ml, respectively [10,11,25].

3.3. Purification

The cell-free crude preparation from culture broth containing 100.0 U/ml of lipase have been purified to homogeneity with an overall yield of 32% and a purification fold of 3.2 employing a

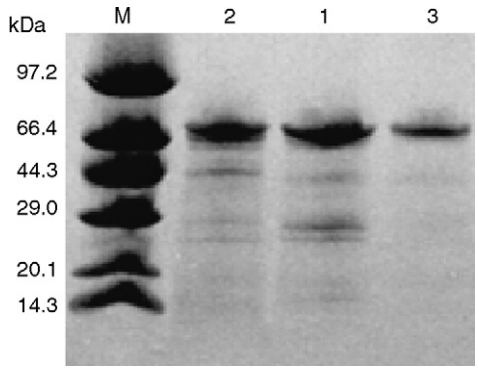

Fig. 3. SDS-PAGE analysis of the column purified GGL. Lane M: protein marker; lane 1: pooled lipase fraction from ammonium sulfate precipitation; lane 2: pooled lipase fraction from the DEAE-Sepharose; lane 3: pooled lipase fraction from the Sephadex-G75.

series of purification steps (Table 1). Since *P. pastoris* secreted few native proteins at low levels to the medium, the GGL was approximately free from contaminating proteins. Further purification is only necessary if a very high purity lipase is desired. For general catalysis or kinetic investigations of recombinant lipase produced by *P. pastoris*, the cultured *P. pastoris* cells may simply be separated from the culture broth by centrifugation to yield a solution that only needs to be desalted and concentrated to give a lipase product with high purity. The lipase was confirmed to be a single band with a relative molecular mass of 64 kDa determined by SDS-PAGE analysis (Fig. 3), corresponding to the molecular size range of 50–66 kDa reported for the native lipase isoforms and recombinant lipases from *G. candidum* depending on the different glycosylation extent [26,27].

3.4. Characterization of the recombinant lipase

3.4.1. Effect of pH and temperature on lipase activity and stability

The purified enzyme exhibited optimum lipolytic activity at pH 8.0 (Fig. 4), different from recombinant lipases of *G. candidum* CMICC 335426 and ATCC 34614 whose optimum pH was at range of pH 7.0–7.5 [10,11,25]. The enzyme retained more than 60% residual activity after incubation at pH 6.0–10.0 for 24 h at room temperature, while at pH 11 there was only

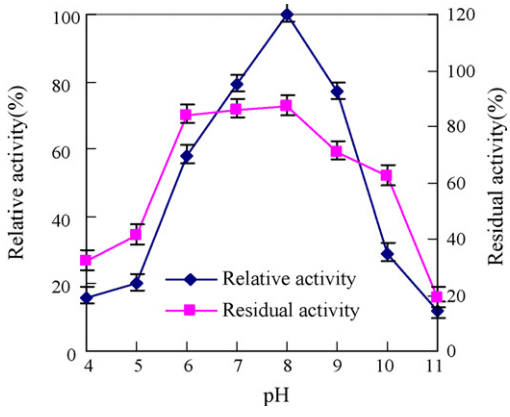

Fig. 4. Effect of pH on lipase activity and stability.

Table 1
Purification of GGL from culture supernatant of *P. pastoris*

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude lipase	30,000	138.46	216.67	100	1
Ammonium sulfate precipitation	18,720	71	263.66	96	1.22
DEAE-Sepharose chromatograph	7,020	11.57	606.74	36	2.8
Sephadex-G75 chromatograph	6,240	9.00	693.33	32	3.2

19% residual activity. The optimum catalytic temperature for GGL was 50 °C, much higher than previous reported for lipases of *G. geotrichum* CBS 772.71 and *G. candidum* (25–40 °C) [6,10,11,19]. Below 60 °C the lipase was stable at least for 12 h at pH 8.0, but above 60 °C rapid inactivation occurred, only 36% residual activity (Fig. 5). However, Catoni et al. reported that lipase B from *G. candidum* CMICC 335426 was rapidly inactivated above 37 °C [11]. Thermostable lipases have been isolated from many sources including *Pseudomonas* sp. [28,29] and *Bacillus* sp. [30–34], but there was no report on lipase from *Geotrichum* sp. with high temperature tolerance. It would be favorable for industrial use if the lipase could work up to 60 °C.

3.4.2. Effect of chemicals and detergents on lipase

The effect of different metal ions and detergents on the activity of the lipase is shown in Table 2. Similar to previous report [35], the lipase activity was strongly inhibited in the presence of Hg^{2+}, Ag^{+}, which may be due to altering enzyme conformation. However, the metal-chelating agent EDTA did not affect the activity, suggesting that the enzyme was not a metalloenzyme. In contrast with lipase from *Rhizopus miehei* [36], surfactants Triton X-100, Tween-80 and SDS reduced GGL activity to 33, 5 and 5%, respectively. To our surprise, this enzyme activity was lightly enhanced by the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (124%), suggesting that the addition of PMSF to the culture medium did not inactivated the secreted lipase but inhibit harmful intracellular protease released as a result of minor *P. pastoris* cell lysis during cultivation. Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+}, Ba^{2+}, Li^{+}, Fe^{2+}, NH_4^{+} and Ni^{2+} had no significant effect on the enzyme activity (81–114%), whereas Zn^{2+} decreased activity by 43%. It has been suggested that the effect of metal ions could be attributed to a change in the solubility and the behavior of the ionized fatty acids at interface.

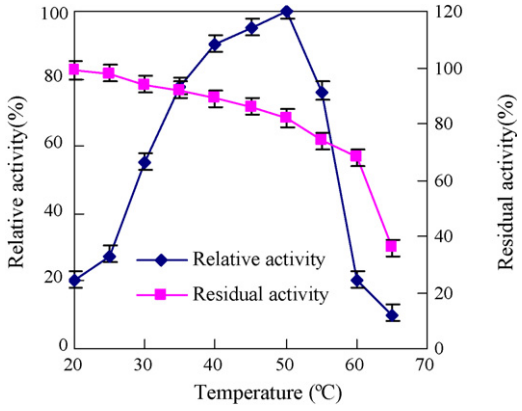

Fig. 5. Effect of temperature on lipase activity and stability.

Table 2
Effect of chemicals and detergents on lipase

Metal ions (1 mM) and detergents (1%)	Relative activity (%)[a]
Control	100
Na^{+}	90
K^{+}	100
Mg^{2+}	95
Ca^{2+}	114
Mn^{2+}	110
Cu^{2+}	105
Ba^{2+}	81
Li^{+}	110
Fe^{2+}	110
NH_4^{+}	100
Ni^{2+}	114
Zn^{2+}	57
Ag^{+}	1
Hg^{2+}	0
Triton X-100	33
Tween-80	5
SDS	5
EDTA	109
PMSF	124

[a] The activities are expressed as the mean of three determinations.

3.4.3. Effect of organic solvents on lipase activity

As described in Table 3, the stability of GGL was investigated in various polar and nonpolar organic solvents. Lipases are diverse in their sensitivity to solvents, but there is general agreement that polar water miscible solvents are more destabilizing than water immiscible solvents [37]. In this study, the GGL showed stability in the presence of both water miscible and immiscible organic solvents, since it retained more than 80%

Table 3
Effect of organic solvents on lipase activity

Organic solvents (50%)	Relative activity (%)[a]
Control	100
Methanol	80
Ethanol	0
Isopropanol	13
Glycerol	107
Butanol	27
Acetone	27
Hexane	87
Cyclohexane	80
n-Heptane	80
Isooctane	98
Chloroform	0
Benzene	83
Diethyl ether	83

[a] The activities are expressed as the mean of three determinations.

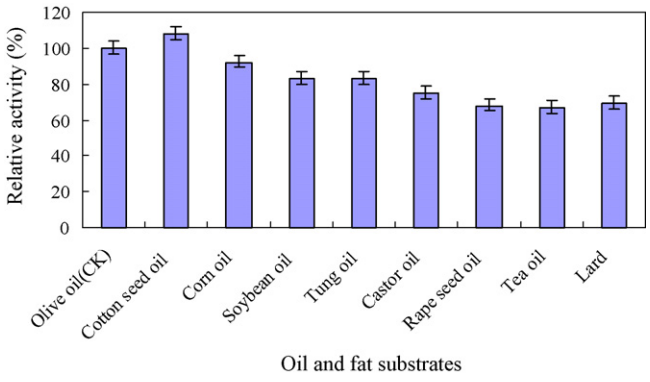

Fig. 6. Substrate specificity of GGL towards oil and fat substrates.

residual activity after exposure by 6 h in 50% methanol, glycerol, hexane, *n*-heptane, cyclohexane, isooctane, benzene and diethyl ether. Glycerol of 50% (v/v) slightly activated the enzyme activity. It has been proposed that a thin layer of water molecules tightly bound to the enzyme acting as a protective sheath along the enzyme's hydrophilic surfaces and allowing retention of the native conformation [37]. On the contrary, ethanol, chloroform, acetone, isopropanol and butanol decreased enzyme activity to 13–27%, probably because they provoked a rapid protein denaturation or disturbing the interface of the reaction mixture. Stability of this lipase in organic solvents suggests it could be used as a biocatalyst in non-aqueous medium, and the tolerance to methanol and glycerol which both existing in system of biodiesel production is a fascinating feature [38].

3.4.4. *Oil substrates specificity*

The substrate preferences of the recombinant GGL were characterized with various oil and triacylglycerol substrates. As shown in Figs. 6 and 7, relative activity on each substrate is expressed as the percentage of that on olive oil. GGL showed relative high activity on various emulsified oils (67–108%), especially on cotton seed oil (108%). An explanation for this can be vegetable oils and lard containing a large number of long, unsaturated fatty acyl chains, such as oleic acid. The above results suggest that this fungal lipase may play a significant part in the biodegradation of oil when oil spills in soil or coastal environments [4]. Moreover, its broad hydrolysis of various oil

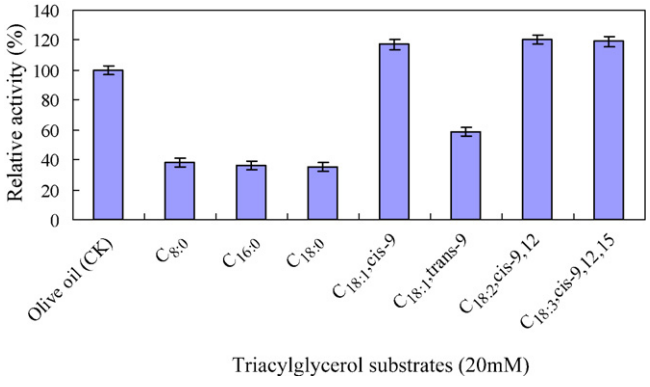

Fig. 7. Substrate specificity of GGL towards triacylglycerol substrates.

sources also indicates that this lipase can be potentially applied in the production of biodiesel [38]. As shown in Fig. 7, a series of triacylglycerols were chosen as substrates to determine the effects of the chain length and unsaturation level on the specific activity. Among the substrates tested, GGL shows a distinct preference for long, unsaturated fatty acyl chains. The specific activities for substrates with *cis*-9 unsaturation ($C_{18:1}$, *cis*-9; $C_{18:2}$, *cis*-9, 12; $C_{18:3}$, *cis*-9, 12, 15) are approximately twice higher than the specific activity for trivaccinin ($C_{18:1}$, *trans*-9) and approximately triple or more higher than the activities for the saturated triacylglycerols tested ($C_{8:0}$, $C_{16:0}$, $C_{18:0}$). This property is very valuable and can be applied in oleochemistry, such as the enzymatic restructuring of oils and fats into products with defined fatty acid composition.

4. Conclusions

G. geotrichum Y05 lipase possessing features such as thermostability and organic solvents tolerance is an excellent industrial biocatalyst. The cloned *ggl* gene provides valuable gene resource for further research on mutated or directional evolution lipases and molecular mechanism of enzyme characteristics. The *ggl* gene can be overexpressed and efficiently secreted in *P. pastoris* in larger amounts compared with original strain and early reports, which lays a good foundation for lipase production in large scale. The characterization results suggest that the recombinant GGL possesses several properties of significant industrial importance, such as pH and temperature stability, tolerance to organic solvents and broad hydrolysis on oils. Such a combination of properties makes it a promising candidate for its application in non-aqueous biocatalysis, such as biodiesel production, selective hydrolysis or esterification for enrichment of PUFAs and oil-contaminated biodegradation, which have been drawn considerable attention currently.

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