

Purification and characterization of a secretory lipolytic enzyme, *MgLIP2*, from *Malassezia globosa*

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Malassezia globosa is a lipid-dependent yeast that is found on the human skin and is associated with various skin disorders, including dandruff and seborrhoeic dermatitis (SD). Despite its important role in skin diseases, the molecular basis for its pathogenicity is poorly understood. The current hypothesis is that dandruff and SD are linked to fatty acid metabolism and secretory lipolytic enzymes, which hydrolyse sebaceous lipids and release irritating free fatty acids. A previous genomic analysis of *M. globosa* identified a family of 13 homologous genes predicted to encode secreted lipases. We have also reported that *M. globosa* had significantly higher extracellular lipase activity compared with other species. To identify the major secretory lipases of this yeast during its growth, we successfully purified and characterized an extracellular lipase *MgLIP2*. Based on MALDI-TOF MS, the peptide mass fingerprint of a tryptically digested protein *MgLIP2* corresponded to ORF MGL_4054 of *M. globosa*. This lipase showed high esterase activity against 4-nitrophenyl palmitate and 1-naphthyl palmitate but not 1-naphthyl acetate. This enzyme had optimal activity at 30 °C and pH 5.0. Furthermore, the activity significantly increased in the presence of Triton X-100 and was partially inhibited by PMSF but was unaffected by univalent and divalent metal ions.

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INTRODUCTION

The lipid-dependent yeasts of the genus *Malassezia* are part of the normal cutaneous microflora and are commonly found on human skin. Certain conditions, such as high humidity, greasy skin and immunodeficiency, can cause these yeasts to become pathogenic, resulting in several skin diseases such as dandruff, pityriasis versicolor, atopic dermatitis and seborrhoeic dermatitis (Kieffer *et al.*, 1990; Guého *et al.*, 1998; Ashbee & Evans, 2002; Cafarchia & Otranto, 2008; Guillot *et al.*, 2008). *Malassezia* yeasts also cause systemic infections in neonates as well as many common skin disorders in animals, such as canine dermatitis and otitis externa (Dankner *et al.*, 1987; Outerbridge, 2006).

With the exception of *Malassezia pachydermatis*, all of the other *Malassezia* species show lipid dependency and require external lipids for growth (Midgley, 2000; Batra *et al.*, 2005). It was suggested that this lipid dependency is due to a defect in the synthesis of myristic acid, a precursor of long-chain fatty acids (Porro *et al.*, 1976). As a result, these yeasts take up external lipids, and produce lipolytic enzymes that digest sebum glycerides as the first step in lipid consumption. Moreover, it was shown that the *Malassezia*-mediated lipid metabolic process is likely

involved in the most common form of hyperproliferative dermatitis and seborrhoeic dermatitis (DeAngelis *et al.*, 2007). Therefore, extracellular lipolytic enzymes are considered to be important *Malassezia* virulence factors.

Lipases (EC 3.1.1.3) are a class of hydrolases that primarily catalyse the hydrolysis of ester bonds of neutral lipids, such as triacylglycerols, to release free fatty acids. Previous studies have shown that some *Malassezia* species have lipase activity. Lipase activity has been detected in the soluble fractions of *Malassezia furfur* cell lysates (Ran *et al.*, 1993). Plotkin *et al.* (1996) detected lipase activity in both the culture supernatant and cell homogenates of *M. furfur* and further characterized three different lipolytic activities in the soluble fraction by chromatography. In other studies, the culture supernatants of *Malassezia pachydermatis* have also been shown to have lipase activity (Mancianti *et al.*, 2001). Recently, *Malassezia* lipase and esterase genes were cloned from *M. furfur* (Brunke & Hube, 2006), *M. pachydermatis* (Shibata *et al.*, 2006) and *M. globosa* (DeAngelis *et al.*, 2007). Of these, only a lipase in *M. pachydermatis* has been naturally isolated and characterized as a secretory protein using the culture supernatant. However, *M. pachydermatis* does not commonly colonize the human skin, so this yeast is not thought to be closely related to human skin diseases (Aspiroz *et al.*, 1999; Gemmer *et al.*, 2002). Whereas the *M. globosa* lipase LIP1 was extracted from cells (DeAngelis *et al.*, 2007), the lipase gene *MfLIP1* was cloned from *M. furfur* mRNA and then

Abbreviations: 1-NA, 1-naphthyl acetate; 1-NP, 1-naphthyl palmitate; 4-NPP, 4-nitrophenyl palmitate.

expressed in *Pichia pastoris* (Brunke & Hube, 2006). *M. globosa* has been noted as the most frequently isolated species on lesions of human skin, followed by *M. restricta* (Sugita *et al.*, 2001; Morishita *et al.*, 2005). A recent genome sequence analysis of *M. globosa* showed that the *M. globosa* genome encodes abundant hydrolase genes (e.g. lipases, phospholipases, proteases and sphingomyelinases) (Xu *et al.*, 2007). This fungus has 14 lipase gene homologues, 13 of which are thought to be secreted. Previously, we compared the lipase activity of secretory proteins that are naturally produced by several *Malassezia* species and found that *M. globosa* had the highest activity, while the other species had only minimal activity (Juntachai *et al.*, 2009). Therefore, in this study, we attempted to isolate the secretory proteins of *M. globosa* and identify a secretory lipase with high lipase activity using a proteomics approach that combines chromatography and MALDI-TOF MS. In addition, we further characterized several properties of this lipase. This work is a fundamental step in understanding the molecular basis of the pathogenicity of fungi in the genus *Malassezia*.

METHODS

Strains and culture conditions. *M. globosa* CBS 7966 was obtained from the Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre, The Netherlands. The fungus was grown on YPD agar medium (1 % yeast extract, 2 % bacto peptone, 2 % glucose, 2 % agar) supplemented with 0.1 % Tween 40, 0.1 % Tween 80 and 1 % olive oil at 30 °C for 7 days. Tween 40 and 80 were purchased from ICN Biomedicals and olive oil was purchased from Nacalai Tesque.

Purification of extracellular lipase. To extract extracellular proteins, yeast cells were cultured and then removed from the agar medium. The top of the agar medium was washed twice with distilled water, then crushed and incubated in 20 ml protein extraction buffer [20 mM Tris/HCl, 1 mM EDTA, 5 mM MgCl₂, 50 mM NaCl, 5 % (v/v) glycerol, pH 7.5] per agar plate, and then stirred overnight at 4 °C. The mixture was filtered using a paper filter to remove the agar. The resulting solution containing extracellular proteins was filtered again through a 0.2 µm membrane filter (Advantec) to remove the remaining particles in the solution and then concentrated with an

Amicon Ultra-15 centrifugal filter (Millipore) with a molecular mass cut-off of 30 kDa.

The sample protein was loaded onto a Toyopearl-Phenyl 650S column (1 × 5 cm), equilibrated in buffer A [22 mM 2-morpholinoethanesulfonic acid (MES; Nacalai Tesque), 8 mM KCl and 1 mM CaCl₂; pH 5.5] containing 2 M ammonium sulfate, at a flow rate of 1 ml min⁻¹. The column was eluted with a linear gradient of 2–0 M ammonium sulfate in buffer A and then with deionized water to elute the residual highly hydrophobic proteins. The flow rate was maintained at 1 ml min⁻¹ in all steps and the eluted fractions were collected at 5 min intervals. The active fractions were concentrated by ultrafiltration with a molecular mass cut-off of 30 kDa (Millipore) to remove any contaminating low-molecular-mass substances, dialysed against deionized water, lyophilized and stored at -20 °C. The amount of eluted proteins was monitored by measuring the absorbance at 280 nm and the protein concentration of each fraction was determined by using the Bradford protein assay, with BSA as the standard.

Enzymic assay. Lipase activity against 4-nitrophenyl palmitate (4-NPP) (Sigma) was measured spectrophotometrically as described previously (Juntachai *et al.*, 2009). The assay mixture was prepared in a final volume of 0.3 ml, containing 0.5 mM 4-NPP, 0.5 % Triton X-100, 100 mM citrate buffer (pH 5.6) and an appropriate amount of the sample protein. The assay mixture was incubated at 30 °C for 1 h. To terminate the reaction and adjust the pH, two vols 1 M Tris/HCl (pH 8.0) were added to the assay mixture before measuring the absorbance at 405 nm. The lipase activity was determined based on the increase in absorption at 405 nm, which was converted into the concentration of 4-nitrophenol (4-NP) generated from 4-NPP.

SDS-PAGE. Analytical protein electrophoresis was performed by using the Laemmli method (Laemmli, 1970) under reducing conditions on 10 % polyacrylamide gels. The protein samples were denatured at 95 °C for 5 min using 6 × sample buffer (125 mM Tris/HCl, 4 % SDS, 20 % glycerol, 0.012 % bromophenol blue and 10 % 2-mercaptoethanol). The protein samples and the molecular mass standard were loaded into the wells and separated by electrophoresis at a constant current of 20 mA. After separation, the gel was silver-stained to visualize the proteins.

MALDI-TOF-MS analysis of the extracellular lipase. After the purified enzyme was separated by SDS-PAGE as described above, the protein was excised from the gel for MS analysis. Prior to the MS analysis, the excised protein band was destained and the protein was

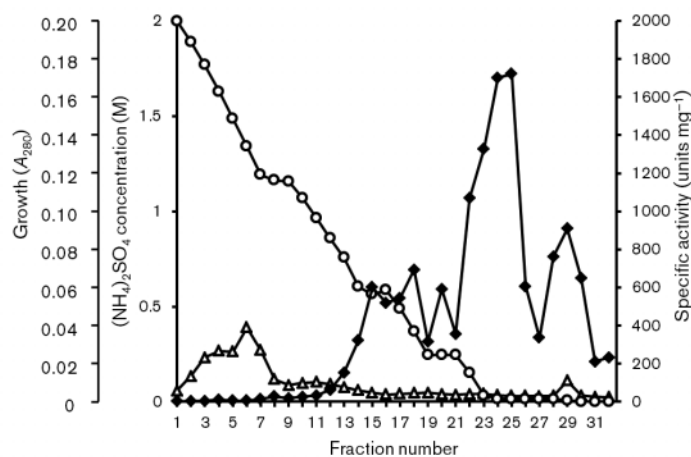


Fig. 1. Chromatography of secreted *M. globosa* proteins. Secreted *M. globosa* proteins were applied to a Toyopearl Phenyl 650S column that was equilibrated in MES buffer with 2 M ammonium sulfate (pH 5.6). Proteins with lipase activity were eluted with a linear gradient of ammonium sulfate (2–0 M) in MES buffer at a flow rate of 1 ml min⁻¹. A_{280} (Δ), (NH₄)₂SO₄ concentration ($\circ$) and specific activity ($\blacklozenge$) are shown.

Table 1. Purification summary for lipases secreted from *M. globosa*

The purification was performed and the enzyme activity was determined by the absorption photometry of 4-NP released from 4-NPP as a lipase substrate. The protein concentrations were determined using the Bradford method. A unit of enzyme activity was defined as the amount of released 4-NP nmol h⁻¹ at 30 °C.

Step	Protein yield (mg)	Total activity (units)	Specific activity (units mg ⁻¹)	Recovery (%)	Purification (fold)
Crude	2.25	118.94	52.86	100	1
Phenyl 650S	0.066	38.54	583.93	32.40	11.0
Ultrafiltration	0.014	15.17	1083.57	12.75	20.5

reduced by incubating the excised gel in 50 µl 10 mM dithiothreitol (DTT) and 25 mM NH₄HCO₃ at 56 °C for 1 h. After protein reduction, the gel was allowed to cool. An alkylation step with 50 µl 55 mM iodoacetamide (Wako Pure Chemical Industries) and 25 mM NH₄HCO₃ was performed in the dark at room temperature for 45 min. The resulting carbamidomethylated protein was in-gel digested overnight at 37 °C with 5 µl 10 µg trypsin gold ml⁻¹ (Promega, Mass Spectrometry grade), which was diluted in 25 mM NH₄HCO₃. The tryptic peptides were extracted from the gel by sonicating in 50 % acetonitrile (Wako Pure Chemical Industries) and 0.1 % TFA at room temperature for 10 min. The digest mixture was concentrated in a vacuum desiccator and analysed by MALDI-TOF-MS. The data were screened against the NCBI database using the MASCOT search program.

Characterization of enzyme activity. All experiments were performed in triplicate. 4-NPP was used as a substrate for lipase activity unless specified otherwise and the lipase activity was measured as described above. The thermal stability of the enzyme was determined by measuring the residual activity at 30 °C after incubation for 1 h at various temperatures. The effect of pH (range 3.0–8.8) on the lipase activity was determined as described above

using 0.1 M citrate buffer, 0.2 M acetate buffer, 0.2 M phosphate buffer and 0.5 M Tris/HCl.

To examine the effects of metal ions and various reagents on lipase activity, Triton X-100, EDTA, PMSF or various metal salt solutions (NaCl, KCl, CaCl₂, MgCl₂, MnCl₂ and ZnCl₂) were added to the reaction mixtures at the desired concentration. Metal salt solutions, EDTA and Triton X-100 were prepared in deionized water. PMSF was prepared in 2-propanol.

For the substrate specificity assays, the diazo coupling reaction was improved and performed using 1-naphthyl acetate (1-NA) and 1-naphthyl palmitate (1-NP) (Sigma) as substrates (Miller & Karn, 1980). The appropriate amount of protein sample was added to the reaction solution to obtain a final concentration of 0.1 % Triton X-100, 0.125 mM 1-NA or 1-NP, and 50 mM citrate buffer (pH 5.6) in a total volume of 1 ml. The reaction was then initiated by incubating the reaction mixture at 30 °C for 1 h, and the reaction was stopped by adding 0.1 ml 0.1 % Fast Blue RR salt in 1 % SDS. After the reaction mixture was incubated for 15 min in the dark at room temperature, the concentration of released 1-naphthol coupled to the Fast Blue RR salt that formed the diazo dye complex was determined by measuring absorbance at 510 nm.

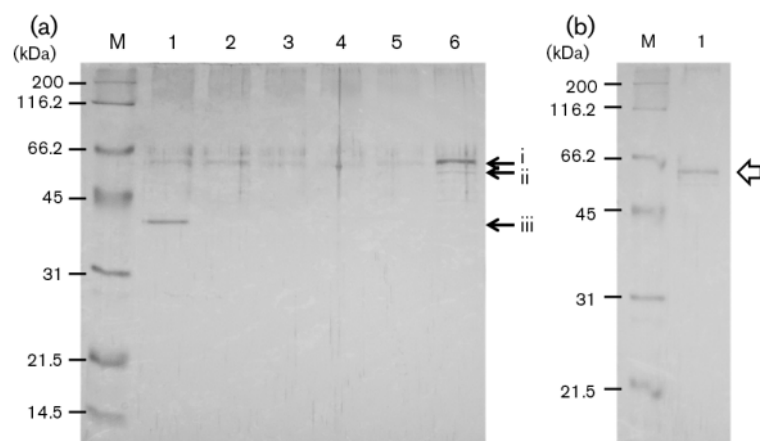


Fig. 2. SDS-PAGE analysis of purified *M. globosa* lipase. Proteins in the active fractions after hydrophobicity interaction chromatography (a) and ultrafiltration (b) were separated by using denaturing SDS-PAGE and visualized by silver staining. (a) Lanes: M, protein molecular mass marker as shown on the left; 1, fraction no. 18; 2–5, fraction nos 22–25; 6, fraction no. 29. All protein bands in each lane were excised and MALDI-TOF MS yielded the following identities: i, hypothetical secretory lipase (MGL_4054); ii, unidentified; and iii, hypothetical aspartyl protease (MGL_4053). (b) Proteins from fraction nos 22–25 were collected and concentrated by ultrafiltration using a 30 kDa molecular mass cut-off, and then analysed by using SDS-PAGE. Lanes: M, protein molecular mass marker as shown on the left; 1, the concentrated active sample. The open arrow indicates a single band corresponding to the hypothetical secretory lipase (MGL_4054) that was analysed by MALDI-TOF MS.

RESULTS

Purification of extracellular lipase from *M. globosa*

Since it is difficult to culture *M. globosa* in liquid medium, the fungus was cultured on solid agar and the fungal secretory proteins were extracted from the agar, concentrated and purified by hydrophobic interaction chromatography on Toyopearl-Phenyl as described in Methods.

Three peaks of lipase activity appeared in fractions 15–18 and 22–25, which were eluted with MES buffer containing approx. 0.2 and 0 M ammonium sulfate, respectively, and in fractions 28–30, which were eluted with deionized water (Fig. 1). In this chromatography step, 32 % lipase activity was recovered (Table 1).

Because fractions 22–25 had the highest lipase activity, they were ultrafiltered using an Amicon Ultra-15 centrifugal filter. After ultrafiltration, there was a relatively large increase in the enzyme-specific activity of the filtered proteins that resulted from successfully removing the contaminating low-molecular-mass substances in the eluate. The final yield, specific activity, purification and recovery of the purified lipase were 0.014 mg, 1083.57 U (mg protein)⁻¹, 20.5-fold and 12.75 %, respectively.

MALDI-TOF-MS analysis of lipase and carbamidomethylated lipase

SDS-PAGE analysis of the fractions that contained lipase activity at the purification stage shared a common band

with a molecular mass of approximately 55 kDa (Fig. 2). Additional protein bands were found in fractions 18 and 29 with molecular masses of approximately 40 and 50 kDa, respectively (Fig. 2a). However, fractions 22–25 (the most significant lipase activity peak) had only a single band of 55 kDa even after the fractions were pooled and concentrated (Fig. 2b).

To determine the primary structure of the purified protein, all protein bands from fractions with enzyme activity were excised, carbamidomethylated and initially in-gel digested with trypsin prior to the MS analysis as described above. The resulting peptides were analysed by MALDI-TOF-MS and the MS profiles of the peptides were subsequently analysed using the MASCOT search program. An analysis of the peptide mass fingerprints showed that the 55 kDa protein bands corresponded to a hypothetical secretory lipase in *M. globosa* (MGL_4054, XM_001728887, 49.74 kDa). Two other protein bands, of 40 kDa in fraction 18 and 50 kDa in fraction 29, closely matched a hypothetical aspartyl protease (MGL_4053, XM_001728886, 43.31 kDa) and an unidentified protein, respectively.

The peptide mass fingerprint of the 55 kDa protein band had a sequence coverage score of 40 % with 21 peptides that matched MGL_4054 (data not shown). The major mass peaks and amino acid sequences are shown in Fig. 3. On the other hand, there were no peptide mass peaks in the purified protein that corresponded to other *M. globosa* lipases. This suggested that we had successfully identified a lipase with high activity among *M. globosa* secretory proteins. This novel identified enzyme was named MgLIP2.

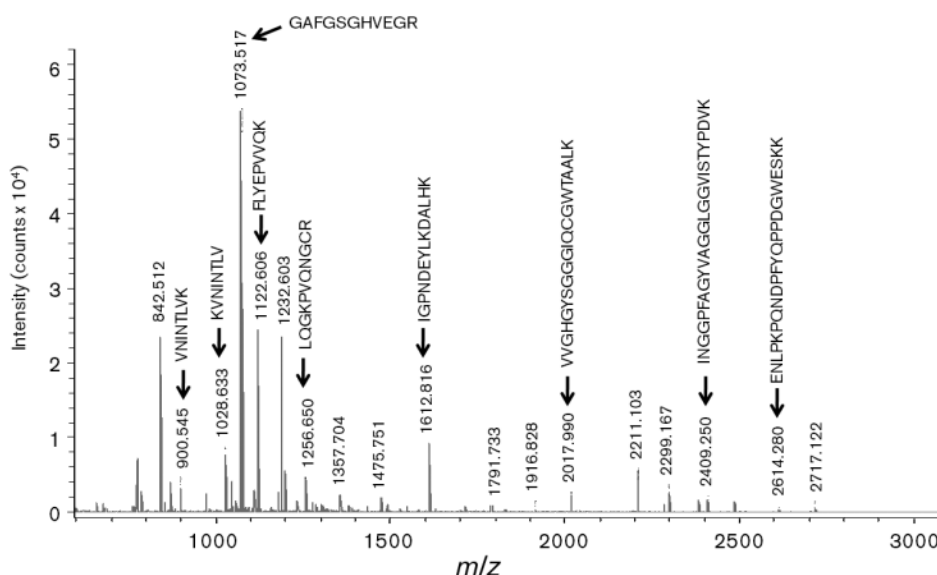


Fig. 3. The MALDI-TOF-MS profile of the purified enzyme. The band corresponding to the purified lipase was in-gel trypsin digested and the mass of the peptides was analysed by MALDI-TOF MS. The prominent mass peaks were screened using a database search for the peptide mass fingerprint with the MASCOT search program. The arrows indicate the major mass peaks and the corresponding amino acid sequences that were identified using the MASCOT search.

Effects of pH and temperature on the lipase activity of MgLIP2

To determine the properties of MgLIP2, the enzyme was concentrated and tested in different buffers with a pH range of 3.0–8.8. In citrate buffer, MgLIP2 showed a significantly higher activity at a pH range of 4.2–5.8, which peaked at pH 5.0. However, in acetate buffer, the activity dropped to approximately 20–30 % compared with that of citrate buffer at the same pH value. Above pH 6.0, the lipase activity of MgLIP2 was significantly reduced and almost no activity could be detected at a pH over 7.0 (Fig. 4a). In addition, the lipase activity of MgLIP2 was stable in deionized water or a 20 % ethanol solution at 4 °C for several weeks.

The effect of temperature on the lipase activity of MgLIP2 was examined in citrate buffer as described above. The activity reached approximately 80 % at 25 °C, stably

increased, and then peaked at approximately 30 °C. The activity dropped rapidly at 40 °C and the residual activity was lower than 20 % at 55 °C (Fig. 4b).

Effects of different metal ions and reagents on the enzyme activity

Because the substrates that are widely used to measure lipase assay, such as triglycerides and 4-NPP, are insoluble in water, surfactants such as the Tween series and Triton X-100 are necessary to facilitate the aqueous solubilization of these substrates. However, the surfactant potency of detergents usually affects protein stability. To investigate the influence of Triton X-100 on MgLIP2, Triton X-100 was added to the assay mixture at different concentrations ranging from 0.1 to 2.5 %. Compared with the mixture without Triton X-100, adding 0.25 % Triton X-100 significantly increased the lipase activity, while a greater increase in the Triton X-100 concentration led to a decrease in the lipase activity. There was maximum activity at a concentration of 0.5 % Triton X-100, which was almost twice that at 2.5 % Triton X-100 (Fig. 5a). Moreover, in respect to the substrate specificity, MgLIP2 hydrolysed not only 4-NPP but also 1-NP. On the other hand, 1-NA, which was used as an esterase substrate, was not recognized by MgLIP2 (data not shown).

Treating with EDTA and PMSF had different effects on the lipase activity. The activity of MgLIP2 was virtually unaffected by EDTA. In contrast, the activity was partially inhibited in the presence of PMSF. Under the conditions with 25 mM PMSF, the lipase activity decreased to 40 % of the activity in the absence of PMSF (Fig. 5b).

To investigate the effects of metal ions on the lipase activity, different univalent and divalent metal ions were added to the reaction mixture. Even when the concentration of metal ions was 10 mM, there was no significant inhibition or enhancement (Fig. 5c).

DISCUSSION

Since members of the genus *Malassezia* are unable to synthesize fatty acids, the fatty acids that are essential for metabolism have to be obtained from the environment. The ability of this yeast to hydrolyse extracellular lipids is closely associated with its ability to survive on the host skin and to induce pathogenicity. Although *M. globosa* is the species most frequently found on patient skin, there have been very few molecular studies on *M. globosa* secretory enzymes. DeAngelis *et al.* (2007) described lipase activities in seven *Malassezia* species and isolated LIP1 from the supernatant of *M. globosa* cells using anion-exchange chromatography. According to their report, the lipase activity of *M. globosa* was insignificant and was far less than that of *M. pachydermatis*. Moreover, the activity of LIP1 was different from that of intact cells, suggesting the presence of another lipase. In contrast, our previous study showed that *M. globosa* produced enzymes with much

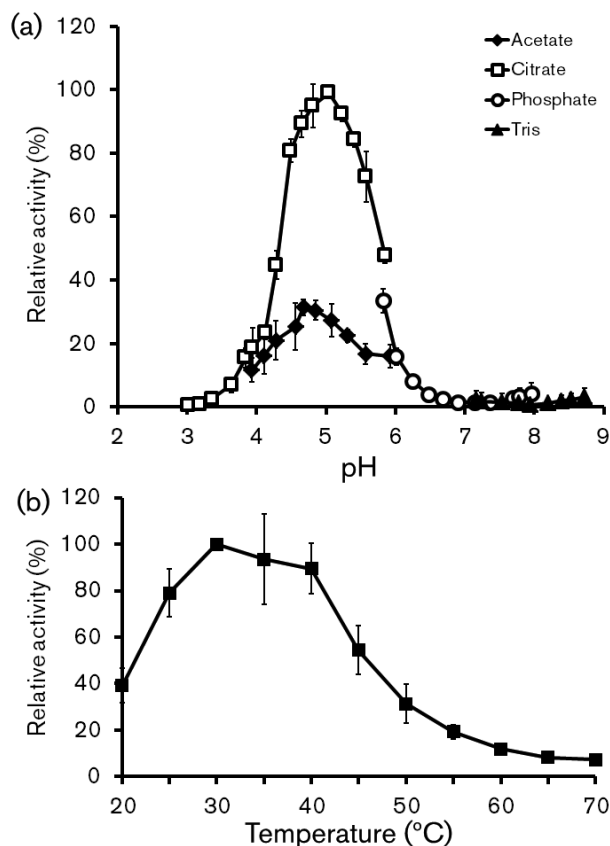


Fig. 4. Effects of pH and temperature on the lipase activity of MgLIP2. (a) Effects of pH on the enzyme activity. The results represent the mean $\pm$ SD of the relative activity compared with the activity at pH 5.0 in citrate buffer. (b) The thermal stability of the enzyme was determined by measuring the enzyme activity after incubation for 1 h at various temperatures. The results represent the mean $\pm$ SD of the relative activity compared to the activity at 30 °C. Experiments were performed independently three times.

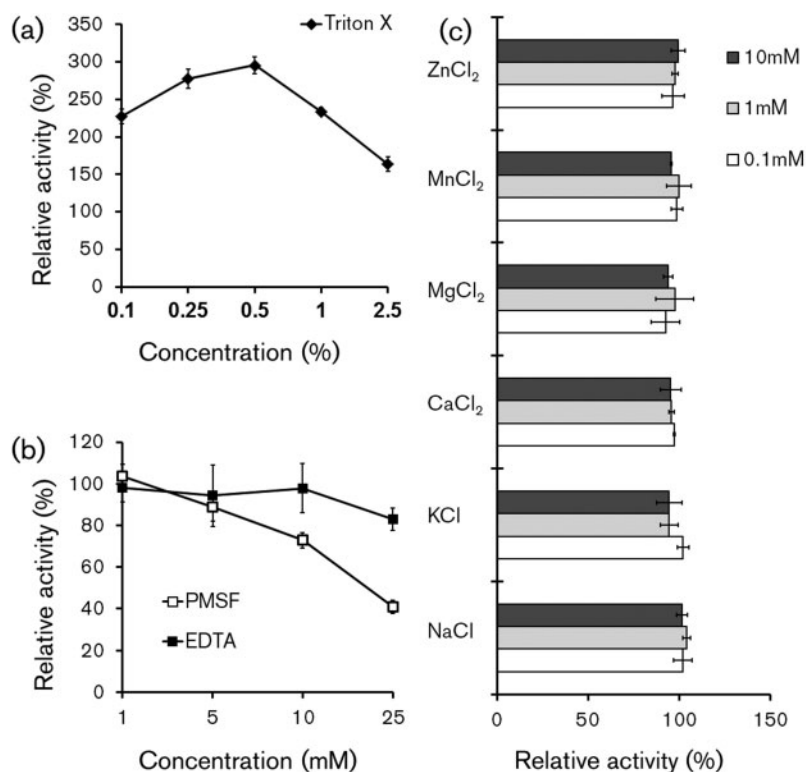


Fig. 5. Effects of reagents and ions on the lipase activity of *MgLIP2*. (a) Effects of Triton X-100 on lipase activity. The results represent the mean $\pm$ SD of the relative activity values without Triton X-100. (b) Effects of EDTA and PMSF on lipase activity. The results represent the mean $\pm$ SD of the relative activity compared to the activity in the absence of these reagents. (c) Effects of univalent and divalent metal ions on lipase activity. The results represent the mean $\pm$ SD of the relative activity with no metal ions. All experiments were performed in triplicate and error bars were calculated from three independent experiments.

higher extracellular lipase activity than those of other *Malassezia* species under the same growth conditions (Juntachai *et al.*, 2009). Here, we performed the first identification and characterization of a major secretory lipase *MgLIP2* from growing *M. globosa* cells.

Our attempt to isolate *M. globosa* secretory lipases from extracellular proteins by anion-exchange chromatography was unsuccessful because most lipases did not bind to the column, suggesting that another chromatography strategy was required. Thus, hydrophobicity interaction chromatography was selected to purify extracellular *M. globosa* lipases since the lipase activity might be unaffected by the high concentration of ammonium sulfate under acidic conditions. Prior to purification, the optimum pH of lipase activity in the crude extracellular proteins was from 3.9 to 5.8, suggesting that there are several lipases among secreted *M. globosa* proteins. However, the MS analysis results showed that *MgLIP2* (MGL_4054, XP_001728887) was the only lipase found in the fractions with high lipase activity. This suggests that *M. globosa* secreted an abundant amount of *MgLIP2* as a major lipase among the secreted proteins, while other lipases were not expressed or showed minimal lipase activities under this culture condition.

The biochemical properties of *MgLIP2* are consistent with the ability of *M. globosa* to colonize host skin. The activity is stable around 30–40 °C, which is consistent with the human body temperature (32–37 °C). In addition, the active pH range of approximately 4.5–5.6 and optimum pH

of 5.0 are consistent with the normal pH of human skin (pH 4.5–6.0). Surprisingly, *MgLIP2* seemed to be sensitive to alkaline conditions and the activity was irreversibly diminished when the pH was over 7.8 (data not shown).

Most yeasts have more than one lipase gene, and 14 putative lipase genes have been identified in the *M. globosa* genome. These genes can be classified into two large families according to the PFAM categories: a typical triglyceride lipase family LIP and a lipase class 3 family which is not closely related to other lipase families. Of these, 13 lipases are predicted to be secreted (Xu *et al.*, 2007). Unlike LIP1 which belongs to a lipase class 3, *MgLIP2* can be classified into the LIP family along with *M. pachydermatis* esterase (*MpEST*) and *M. furfur* lipase (*MfLip1*). There are several notable differences in the properties of *MgLIP2* and other fungal LIPs, including those of *Malassezia* species. The activities of *MpEST* and *MfLip1* are inhibited in the presence of EDTA, PMSF and metal ions such as Ca^{2+} and Zn^{2+} (Brunke & Hube, 2006; Shibata *et al.*, 2006), whereas the activity of *MgLIP2* was not inhibited or enhanced by metal ions and only partially inhibited by PMSF. Interestingly, the lipase activity of *MgLIP2* was significantly decreased in acetate buffer compared to that in citrate buffer at the same pH. Moreover, this lipase could hydrolyse only substrates that were esterified to fatty acids, suggesting that this enzyme specifically recognizes fatty acids.

Although lipolytic activity is essential for *Malassezia* yeasts, the role of lipolytic enzymes in the *M. globosa* life cycle is

unclear. In *Candida albicans*, some lipase genes are differentially expressed *in vitro* and *in vivo* under conditions with different carbon sources, possibly reflecting a broad lipolytic activity, which may contribute to the persistence and virulence of this fungus on human tissue (Hube *et al.*, 2000). Similarly, the production of extracellular and cell-bound lipases of *Yarrowia lipolytica* was reported to depend on the carbon and nitrogen source and the lipase levels seemed to be regulated by cell morphology (Ota *et al.*, 1982; Novotný *et al.*, 1994). Our previous study also found that the extracellular lipase activity of *M. furfur* increased under lipid-limited culture conditions (unpublished). Accordingly, the lipase genes in *M. globosa* may show different expression patterns, depending on the external lipid source or environmental conditions. However, to date, there has been no description of the activities of these secreted lipases and it was unclear which lipase provides the major activity under cell growth conditions.

The role of secretory lipolytic enzymes in *M. globosa* pathogenicity could be further examined by constructing a gene deletion. However, there is currently no established protocol to introduce genes into this fungus. In addition, developing this technique will be challenging because of the fastidious nature of this yeast in culture and the difficulty of introducing DNA fragments through the thick cell wall. Therefore, an ELISA using an MgLIP2-specific antibody should be developed to investigate the role of this enzyme in pathogenicity or to directly detect *M. globosa* on the human skin.

In summary, we have isolated and identified a secretory lipase MgLIP2 that belongs to the LIP family of secreted *M. globosa* proteins using hydrophobicity interaction chromatography and a proteomic analysis. Furthermore, we have characterized the major biochemical properties of this enzyme. Our data show that MgLIP2 significantly contributes to the extracellular lipase activity of *M. globosa* and is a major secreted lipase, suggesting that MgLIP2 plays an important role in lipid metabolism known to correlate with the pathogenicity of this fungus.

REFERENCES

- Ashbee, H. R. & Evans, E. G. (2002). Immunology of diseases associated with *Malassezia* species. *Clin Microbiol Rev* **15**, 21–57.
- Aspiroz, C., Moreno, L. A., Rezusta, A. & Rubio, C. (1999). Differentiation of three biotypes of *Malassezia* species on human normal skin. Correspondence with *M. globosa*, *M. sympodialis* and *M. restricta*. *Mycopathologia* **145**, 69–74.
- Batra, R., Boekhout, T., Guého, E., Cabañes, F. J., Dawson, T. L., Jr & Gupta, A. K. (2005). *Malassezia* Baillon, emerging clinical yeasts. *FEMS Yeast Res* **5**, 1101–1113.
- Brunke, S. & Hube, B. (2006). *MfLIP1*, a gene encoding an extracellular lipase of the lipid-dependent fungus *Malassezia furfur*. *Microbiology* **152**, 547–554.
- Cafarchia, C. & Otranto, D. (2008). The pathogenesis of *Malassezia* yeasts. *Parassitologia* **50**, 65–67.
- Dankner, W. M., Spector, S. A., Fierer, J. & Davis, C. E. (1987). *Malassezia* fungemia in neonates and adults: complication of hyperalimentation. *Rev Infect Dis* **9**, 743–753.
- DeAngelis, Y. M., Saunders, C. W., Johnstone, K. R., Reeder, N. L., Coleman, C. G., Kaczvinsky, J. R., Jr, Gale, C., Walter, R., Mekel, M. & other authors (2007). Isolation and expression of a *Malassezia globosa* lipase gene, *LIP1*. *J Invest Dermatol* **127**, 2138–2146.
- Gemmer, C. M., DeAngelis, Y. M., Theelen, B., Boekhout, T. & Dawson, T. L., Jr (2002). Fast, noninvasive method for molecular detection and differentiation of *Malassezia* yeast species on human skin and application of the method to dandruff microbiology. *J Clin Microbiol* **40**, 3350–3357.
- Guého, E., Boekhout, T., Ashbee, H. R., Guillot, J., Van Belkum, A. & Faergemann, J. (1998). The role of *Malassezia* species in the ecology of human skin and as pathogens. *Med Mycol* **36** (Suppl 1), 220–229.
- Guillot, J., Hadina, S. & Guého, E. (2008). The genus *Malassezia*: old facts and new concepts. *Parassitologia* **50**, 77–79.
- Hube, B., Stehr, F., Bossenz, M., Mazur, A., Kretschmar, M. & Schäfer, W. (2000). Secreted lipases of *Candida albicans*: cloning, characterisation and expression analysis of a new gene family with at least ten members. *Arch Microbiol* **174**, 362–374.
- Juntachai, W., Oura, T., Murayama, S. Y. & Kajiwara, S. (2009). The lipolytic enzymes activities of *Malassezia* species. *Med Mycol* **47**, 477–484.
- Kieffer, M., Bergbrant, I. M., Faergemann, J., Jemec, G. B., Ottevanger, V., Stahl Skov, P. & Svejgaard, E. (1990). Immune reactions to *Pityrosporum ovale* in adult patients with atopic and seborrheic dermatitis. *J Am Acad Dermatol* **22**, 739–742.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Mancianti, F., Rum, A., Nardoni, S. & Corazza, M. (2001). Extracellular enzymatic activity of *Malassezia* spp. isolates. *Mycopathologia* **149**, 131–135.
- Midgley, G. (2000). The lipophilic yeasts: state of the art and prospects. *Med Mycol* **38** (Suppl. 1), 9–16.
- Miller, R. B. & Karn, R. C. (1980). A rapid spectrophotometric method for the determination of esterase activity. *J Biochem Biophys Methods* **3**, 345–354.
- Morishita, N., Sei, Y., Takiuchi, I. & Sugita, T. (2005). [Examination of the causative agent of pityriasis versicolor]. *Nippon Ishinkin Gakkai Zasshi* **46**, 169–170 (in Japanese).
- Novotný, C., Dolezalová, L. & Lieblová, J. (1994). Dimorphic growth and lipase production in lipolytic yeasts. *Folia Microbiol (Praha)* **39**, 71–73.
- Ota, Y., Gomi, K., Kato, S., Sugiura, T. & Minoda, Y. (1982). Purification and some properties of cell bound lipase from *Saccharomycopsis lipolytica*. *Agric Biol Chem* **46**, 2885–2893.
- Outerbridge, C. A. (2006). Mycologic disorders of the skin. *Clin Tech Small Anim Pract* **21**, 128–134.
- Plotkin, L. I., Squiquera, L., Mathov, I., Galimberti, R. & Leoni, J. (1996). Characterization of the lipase activity of *Malassezia furfur*. *J Med Vet Mycol* **34**, 43–48.
- Porro, M. N., Passi, S., Caprilli, F., Nazzaro, P. & Morpurgo, G. (1976). Growth requirements and lipid metabolism of *Pityrosporum orbiculare*. *J Invest Dermatol* **66**, 178–182.
- Ran, Y., Yoshiike, T. & Ogawa, H. (1993). Lipase of *Malassezia furfur*: some properties and their relationship to cell growth. *J Med Vet Mycol* **31**, 77–85.
- Shibata, N., Okanuma, N., Hirai, K., Arikawa, K., Kimura, M. & Okawa, Y. (2006). Isolation, characterization and molecular cloning of a lipolytic enzyme secreted from *Malassezia pachydermatis*. *FEMS Microbiol Lett* **256**, 137–144.

Sugita, T., Suto, H., Unno, T., Tsuboi, R., Ogawa, H., Shinoda, T. & Nishikawa, A. (2001). Molecular analysis of *Malassezia* microflora on the skin of atopic dermatitis patients and healthy subjects. *J Clin Microbiol* **39**, 3486–3490.

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(2007). Dandruff-associated *Malassezia* genomes reveal convergent and divergent virulence traits shared with plant and human fungal pathogens. *Proc Natl Acad Sci U S A* **104**, 18730–18735.

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