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ORIGINAL ARTICLE

Purification and Characterization of Class III Lipase from a White-Rot Fungus *Pleurotus ostreatus*

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Abstract

Pleurotus ostreatus is an edible white-rot fungus with lignocellulosic biomass degrading enzymes that have been studied extensively. However, until now, lipolytic enzymes from *P. ostreatus*, which degrade extractives in lignocellulosic biomass, have not been purified and characterized. In this study, *P. ostreatus* was inoculated into the rapeseed oil containing culture to induce lipase. The lipase in the culture broth was successfully purified to homogeneity by chromatographic methods. The molecular weight of the purified lipase was 27 kDa, and its optimal pH and temperature were 5.0 and 30 °C, respectively. The purified lipase showed high activity with the substrates 4-methylumbelliferyl (4-MU) decanoate (C10:0) and 4-MU oleate (C18:1), and no activity with 4-MU acetate (C2:0) and 4-MU butyrate (C4:0). The amino acid sequences and substrate specificities of the purified lipase suggested that it belonged to class III. Kinetic parameters measurements (Km and Vmax) showed that 4-MU palmitate had a high affinity for the purified lipase, and it was the substrate most efficiently hydrolyzed by the purified lipase.

Keywords *Pleurotus ostreatus* · Lipase · Triacylglycerol hydrolases · White-rot fungi

Introduction

Lipases (triacylglycerol hydrolases, E.C. 3.1.1.3) are a class of hydrolases that catalyze the hydrolysis of long-chain triglycerides (≥ 10 carbon atoms), while carboxylesterases (E.C. 3.1.1.1) hydrolyze shorter chain triglycerides (< 10 carbon atoms) [1–3]. Lipases are produced by various kinds of microorganisms [4], and have been applied in food, detergent, chemical, pulp and paper, and pharmaceutical industries [5, 6]. White-rot fungi produce various kinds of enzymes such as cellulases, hemicellulases, and lignin-degrading enzymes, which degrade lignocellulosic biomass [7, 8]. As lignocellulosic biomass contains extractives such as triglycerides, terpenoids, and steroids, in addition to cellulose, hemicellulose, and lignin [9], white-rot fungi produce various kinds of enzymes that degrade the extractives [10, 11]. *Pleurotus ostreatus*, an edible white-rot fungus, has

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been extensively studied, especially for lignin-degrading enzymes such as manganese peroxidase, versatile peroxidase, and laccase [12, 13]. However, few articles have been published on lipases from *P. ostreatus*. When *P. ostreatus* was grown on pulp and paper industry waste, lipase activity was detected [14]. However, as *p*-nitrophenyl butyrate was used for the enzymatic activity measurement, the carboxylesterase activity from *P. ostreatus* would be measured instead of lipase activity. Another study reported the presence of 53 putative lipases and 34 putative carboxylesterases-coding genes in the *P. ostreatus* genome. The heterologous expression of *P. ostreatus* lipases, PleoLip 241 and PleoLip 369, was conducted using *Pichia pastoris* as the host strain [15]. Although the two lipases from *P. ostreatus* were successfully expressed in *P. pastoris* and their lipase properties were characterized, the lipase properties expressed in *P. pastoris* could be different from those of *P. ostreatus* due to yeast-specific glycosylation [16]. In this study, lipase from *P. ostreatus*, which was grown in the presence of triglycerides (rapeseed oil), was successfully purified to homogeneity and its properties were characterized. To our knowledge, this is the first report on the purification and characterization of a lipase from *P. ostreatus*.

Materials and Methods

Organism and Culture Conditions

The fungal strain used was *Pleurotus ostreatus* (No. 11, Akiyama Mycological Institute, Yamanashi, Japan). The stock cultures of *P. ostreatus* were maintained on potato-dextrose (PD) agar (BD, Sparks, MD, USA) at 4 °C. Ten agar plugs (0.5-cm diameter) were taken from the stock cultures and homogenized with 100 mL of sterilized water by a Waring blender (low speed, 10 s). The homogenized mycelium (3 mL) was inoculated into a 300-mL Erlenmeyer flask containing 100 mL of glucose peptone (GP) medium (2% glucose, 0.5% hipolypeptone, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, pH 5.0) and incubated at 26 °C for 1 week with shaking at 100 rpm. The precultures of the three flasks were filtered with Miracloth (Millipore, Burlington, MA, USA), and the mycelium was homogenized with 100 mL of sterilized water by a Waring blender (low speed, 10 s). The homogenized mycelium (3 mL) was inoculated into a 300-mL Erlenmeyer flask containing 100 mL of induction medium in which 750 µL of rapeseed oil (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was used as a carbon source instead of glucose in the GP medium. Aliquots of the culture were sampled every 24 h and lipase activity was measured.

Lipase Activity Assays

The lipase activity was determined using 4-methylumbelliferyl (4-MU) oleate (Cayman Chemical, Ann Arbor, MI, USA) as the substrate. The substrate (0.1 mM) was dissolved in 2-methoxy ethanol and the sample with lipase activity was reacted in 50 mM sodium-phosphate buffer (pH 5.0) for 30 min at 40 °C. The lipase activity was stopped with 250 mM glycine-sodium hydroxide (pH 10.5). The amount of 4-MU liberated from 4-MU oleate was measured fluorometrically at an emission wavelength of 460 nm and an excitation wavelength of 355 nm using a Mithras LB 940 multimode microplate reader (Berthold Technologies, Bad Wildbad, Germany).

Lipase Purification

When maximum lipase activity was observed (4 d) in the presence of rapeseed oil, the culture was filtered using Miracloth followed by membrane filters (0.4 and 0.2 μm). The clarified supernatant was concentrated by ultrafiltration (Vivaflow 50, MWCO 10,000, Sartorius, Goettingen, Germany). The concentrated supernatant was applied to a HiPrep diethylaminoethyl (DEAE) column (Cytiva, Marlborough, MA, USA) equilibrated with 20 mM sodium-phosphate buffer (pH 7.0). The column was washed with a stepwise salt gradient [NaCl 0 mM (fr.1–10), 100 mM (fr.11–20), 250 mM (fr.21–40), and 1000 mM (fr.41–60)] at a flow rate of 2.0 mL/min. From fr.1 to 20, 10 mL of the eluent was collected in each test tube. From fr.21 to 60, 5 mL of the eluent was collected in each test tube. Fraction 14 with lipase activity was collected and then applied to a Sephacryl S-200 column (Cytiva) previously equilibrated with 20 mM sodium-phosphate buffer (pH 7.0, NaCl 50 mM) and washed at a flow rate of 0.5 mL/min. Fraction 26, which had the highest lipase activity, was collected and desalted using a PD-10 desalting column (Cytiva) and then applied to a HiTrap Q column (Cytiva) equilibrated with 20 mM sodium-phosphate buffer (pH 7.0). The column was washed with a stepwise salt gradient (25, 50, 100, 250, and 1000 mM NaCl) at a flow rate of 1.0 mL/min. The fraction with lipase activity (fr.15) was collected, concentrated, and desalted using Amicon Ultra-0.5 centrifugal filter unit (10 kDa, Millipore). The purified lipase was stored at 4 °C.

Electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was conducted using a Mini-PROTEAN Tetra cell system (Bio-Rad, Hercules, CA, USA) with a 10% precast polyacrylamide gel. The proteins were visualized by staining with Coomassie blue.

Effects of pH and Temperature on Lipase Activity and Stability

The optimal pH of the lipase activity was determined using 4-MU oleate from pH 2.0 to 9.0 using different pH buffer solutions [50 mM glycine–hydrochloric acid buffer (pH 2.0–4.0), 50 mM sodium-acetate buffer (pH 4.0–6.0), 50 mM sodium-phosphate buffer (pH 6.0–8.0), 50 mM Tris–HCl (pH 8.0, 9.0)] at 40 °C. The optimal temperature for the lipase activity was measured from 20 to 70 °C in 50 mM sodium-acetate buffer (pH 5.0). The pH stability of the lipase was assessed by incubating the lipase from pH 2.0 to 9.0 for 8 h at 40 °C, and the remaining lipase activity was measured using 4-MU oleate for 30 min at 40 °C. The thermostability of the lipase was determined by incubating the lipase from 20 to 70 °C for 30 min in 50 mM sodium-acetate buffer (pH 6.0), and the remaining lipase activity was measured using 4-MU oleate for 30 min at 40 °C. All measurements were conducted in triplicate.

Substrate Specificity

To determine the substrate specificity of the purified lipase, 4-MU esters containing acyl chains of different lengths were used. Specifically, 4-MU acetate (C2:0, FUJIFILM Wako Chemical Corporation), 4-MU butyrate (C4:0, Sigma-Aldrich, St. Louis, MO, USA),

4-MU decanoate (C10:0, Toronto Research Chemicals, Toronto, ON, Canada), 4-MU palmitate (C16:0, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and 4-MU oleate (C18:1) were used as the substrates. The purified lipase was reacted in 50 mM sodium-phosphate buffer (pH 5.0) for 30 min at 40 °C in the presence of the substrate (0.1 mM), and the lipase activity was stopped with 250 mM glycine-sodium hydroxide (pH 10.5). The amount of 4-MU liberated from 4-MU containing substrates was measured fluorometrically at an emission wavelength of 460 nm and an excitation wavelength of 355 nm using a Mithras LB 940 instrument. All measurements were conducted in triplicate.

Kinetic Parameters Determination

A Lineweaver–Bulk plot was used to determine the Km and Vmax values using 4-MU decanoate, 4-MU palmitate, and 4-MU oleate as the substrate from 0.01 to 10 mM. The reaction was conducted at pH 5.0 (50 mM sodium-acetate buffer) for 30 min at 40 °C. Lipase activity was stopped with 250 mM glycine-sodium hydroxide (pH 10.5). The amount of 4-MU liberated from 4-MU containing substrates was measured fluorometrically at an emission wavelength of 460 nm and an excitation wavelength of 355 nm.

Protein Concentration

Protein concentrations were determined by the quick start Bradford protein assay (Bio-Rad) with bovine gamma globulin (Bio-Rad) as a standard.

Amino Acid Sequencing

After SDS-PAGE, the gel slippage was reduced by 100 mM dithiothreitol (DTT) and alkylated by 100 mM iodoacetamide. After washing, the gels were incubated with trypsin overnight at 30 °C. The recovered peptides were desalted using a ZipTip C18 (Millipore). Samples were analyzed by nano liquid chromatography with tandem mass spectrometry (LC/MS/MS) systems (DiNa HPLC system KYA TECH Corporation/QSTAR XL Applied Biosystems). Mass data acquisitions were piloted by Mascot software.

Results and Discussion

Lipase Production and Purification

P. ostreatus was inoculated in rapeseed oil-containing culture to induce lipase activity, and the time course of lipase activity in the culture broth was measured (Fig. 1). Maximum lipase activity was observed on day 4 (38.4 U/mL), which was much higher than that previously reported (30 U/L) in *P. ostreatus* [15]. The lipase activity obtained in this study was almost the same as that of other fungal species [*Rhizopus rhizopodiformis* (43.0 U/mL) and *Penicillium chrysogenum* (40 U/mL)] [5]. The difference in lipase activity between this and the previous study could be attributed to the use of different strains [*P. ostreatus* No.11 (Akiyama Mycological Institute), *P. ostreatus* (ATCC MYA-2306)], different substrates (4-MU oleate, *p*-nitrophenyl decanoate), and different carbon sources (rapeseed oil, olive oil) for inducing lipase. The culture was collected on day 4 and filtered to remove

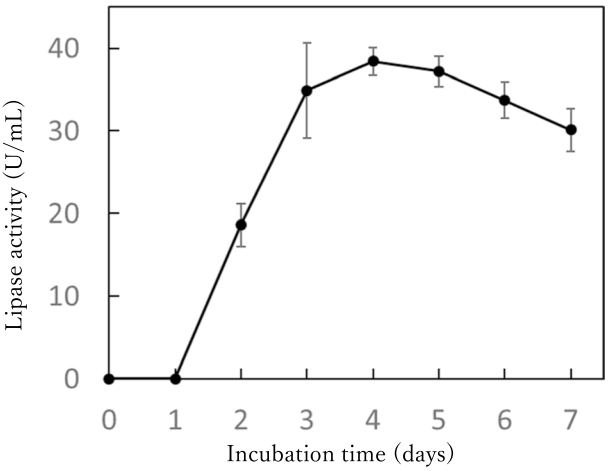

Fig. 1 Time course of lipase activity by *P. ostreatus*. Extracellular lipase activity of *P. ostreatus* incubated in the presence of rapeseed oil was measured using 4-methylumbelliferyl oleate as the substrate

the mycelium, because the lipase was an extracellular enzyme. The culture broth was ultrafiltered to concentrate the lipase, but lipase activity decreased during ultrafiltration (Table 1). The reason for the decrease in lipase activity would be the adsorption of lipase onto the ultrafiltration membrane, as previously reported [17]. Alternative methods to concentrate lipase, such as ammonium sulfate precipitation are required to increase lipase yields in future studies. The concentrated lipase was purified by three chromatography columns: HiPrep DEAE column (anionic exchange), Sephacryl S-200 column (gel filtration chromatography), and HiTrap Q column (anionic exchange) (Table 1). When the concentrated culture broth was fractionated using a HiPrep DEAE column, four different lipase activity peaks were observed, suggesting that at least four lipases were produced by *P. ostreatus* in the presence of rapeseed oil (Fig. 2). Although the four lipases showed similar activities (Fig. 2), the lipase in the second lipase peak (fr.14) was further separated because it was more stable with NaCl, which was inevitable for the separation columns. After separation using a Sephacryl S-200 column, fr. 26, which had the highest lipase activity (Fig. 3), was further separated using a HiTrap Q column, and fr. 15, with lipase activity, was analyzed by SDS-PAGE, which showed that the lipase was purified to homogeneity with a molecular weight of 27 kDa (Fig. 4). In this study, we successfully purified one of the

Table 1 Purification of *P. ostreatus* lipase

Purification step	Total protein[a] (mg)	Total activity[b] (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Culture filtration	199	412,549	2073	100	1.0
Ultrafiltration	0.87	4,560	5241	1.1	2.5
HiPrep DEAE	4.6×10^{-2}	258	5609	6.3×10^{-2}	2.7
Sephacryl S-200	2.2×10^{-2}	126	5727	3.1×10^{-2}	2.7
HiTrap Q	5.0×10^{-3}	56	11,200	1.4×10^{-2}	5.4

[a]Protein was measured by Bradford method

[b]Activity was measured with 4-methylumbelliferyl oleate as the substrate

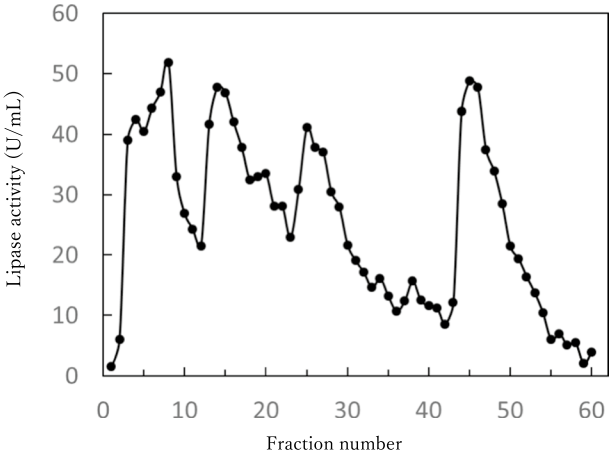

Fig. 2 Separation of lipases from *P. ostreatus* by a HiPrep DEAE. The concentrated supernatant from *P. ostreatus* was applied to a HiPrep DEAE column equilibrated with 20 mM sodium-phosphate buffer (pH 7.0). The column was washed with a stepwise salt gradient [NaCl 0 mM (fr.1–10), 100 mM (fr.11–20), 250 mM (fr.21–40), and 1000 mM (fr.41–60)] at a flow rate of 2.0 mL/min. From fr.1 to 20, 10 mL of the eluent was collected in each tube. From fr.21 to 60, 5 mL of eluent was collected in each tube. The lipase activity was measured using 4-methylumbelliferyl oleate as the substrate

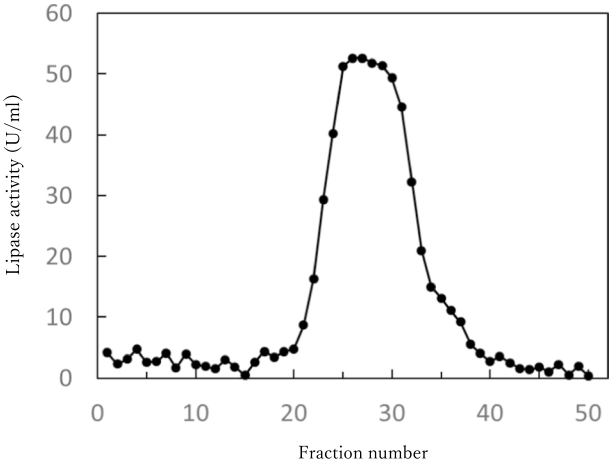

Fig. 3 Separation of lipase (fr. 14 from HiPrep DEAE) by a Sephacryl S-200. The fr. 14 from a HiPrep DEAE was applied to a Sephacryl S-200 column which was separated with 20 mM sodium-phosphate buffer (pH 7.0, NaCl 50 mM) at a flow rate of 0.5 mL/min. The eluent (5 mL) was collected in each tube. The lipase activity was measured using 4-methylumbelliferyl oleate as the substrate

lipases produced by *P. ostreatus*. Further studies are required to characterize other lipases from *P. ostreatus* to elucidate their lipid hydrolysis mechanisms.

Amino Acid Sequences

The amino acid sequences of the purified lipase, which were identified by nano LC–MS/MS, were as follows: SVIVAHQGTDTSK, SIQVHNGFSEAQAR, AAPAVLAAVK, TAMSQFSATR, TVTFGMPR, and GDIVPIVPGR. These amino acid sequences are identical to those of *P. ostreatus* PC15 lipase [class 3(PF 01,764); Protein ID 1044280] [18–20]. When the genome sequence database of *P. ostreatus* PC15 (v 2.0) was analyzed, 17 class III lipases were

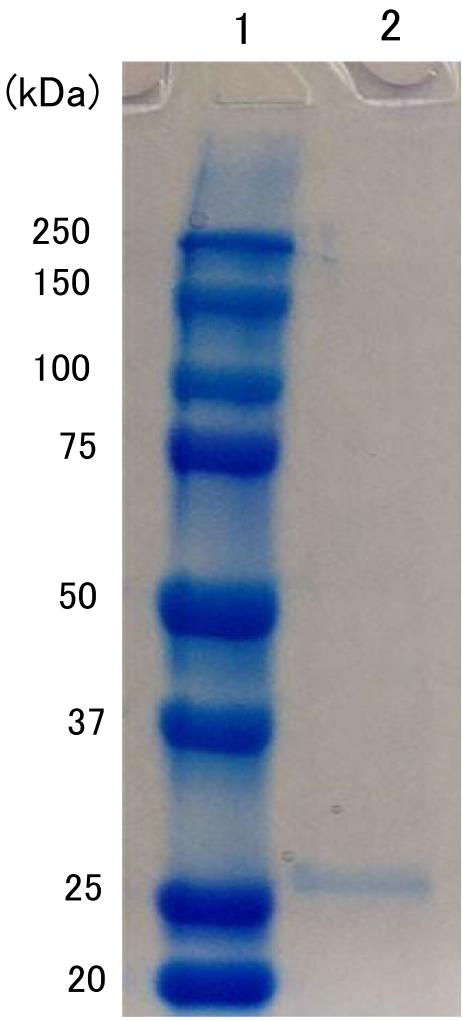

Fig. 4 SDS–PAGE of purified lipase. Lane 1, molecular weight markers; lane 2, purified lipase

detected [18–20]. The molecular weight of the class III lipase from *P. ostreatus* PC15 (Protein ID 1,044,280) without the putative signal peptides (19 amino acids) was estimated to be 30 kDa, while that of the purified lipase in this study was 27 kDa (Fig. 4). One of the reasons that the different molecular weights were observed could be that the amino acid sequences of these two lipases were partially different, because different strains, *P. ostreatus* No.11 (Akiyama Mycological Institute), were used in this study. In addition, the amino acid sequences of the purified lipase were different from those of the previously reported *P. ostreatus* lipases, which were identical to *P. ostreatus* PC15 lipases (Protein ID 1,091,241 and 1,060,369) [15]. Class III lipases were reported to be the largest gene family in *P. ostreatus* lipases and the most highly expressed in two strains (*P. ostreatus* PC9 and PC15) and all incubation conditions (static and shaking cultures), which accounted for between 56.86 and 95.25% of the lipase family expression [20]. These results suggest that the class III lipase purified in this study plays an important role in the hydrolyze of triacylglycerol by *P. ostreatus*.

Effects of pH and Temperature on Lipase Activity and Stability

The effects of pH and temperature on lipase activity were measured. The optimal pH and temperature of the lipase were 5.0 and 30 °C, respectively (Fig. 5a, b). The optimal pH of the *P. ostreatus* lipases (PleoLip 241 and PleoLip 369), which were heterologously expressed using *P. pastoris*, was 7.0 [15], whereas the optimum pH of the purified lipase in this study was weak acids (Fig. 5a).

The pH and thermostability of the purified lipase were measured. The purified lipase showed maximum stability at pH 6.0 and was relatively stable from pH 3.0 to 7.0 (Fig. 5c). The lipase showed maximum thermostability at 20 °C (Fig. 5d). Increasing the temperature decreased the lipase activity, and the activity completely disappeared when the lipase was maintained above 60 °C. A previous study reported that the pH stability of *P. ostreatus* lipases (PleoLip 241 and PleoLip 369) were pH 6–9, and their thermostability was 30–50 °C [15]. The purified lipase in this study was more stable at acidic pH than previously reported lipases.

Specificities for Substrates

The substrate specificity of the purified lipase was measured using fluorescent substrates. Higher lipase activity was observed when 4-MU decanoate (C10:0), 4-MU oleate (C18:1), and 4-MU palmitate (C16:0) were used as substrates, whereas hydrolytic activity was not

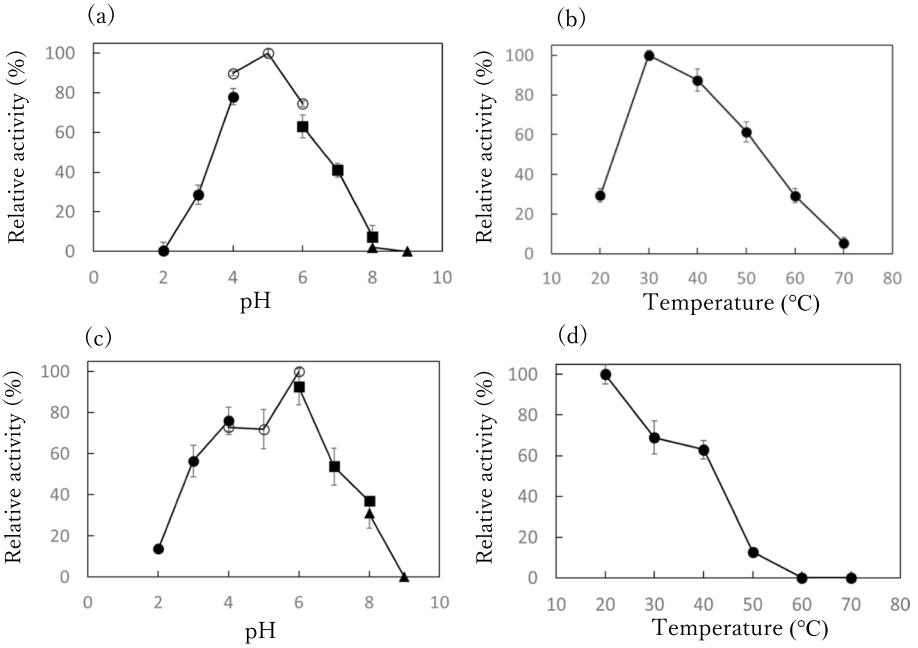

Fig. 5 Effects of pH and temperature on lipase activity and stability. **a** pH profiles of lipase; **b** temperature profiles of lipase; **c** pH stability of lipase; **d** thermostability of lipase. pH profiles and pH stability were determined with 4-MU oleate in glycine-hydrochloric acid buffer (●, pH 2–4), sodium acetate buffer (○, pH 4–6), sodium phosphate buffer (■, pH 6–8), Tris–HCl buffer (▲, pH 8, 9). Temperature profiles and thermostability were determined with 4-MU oleate in sodium acetate buffer (pH 5.0)

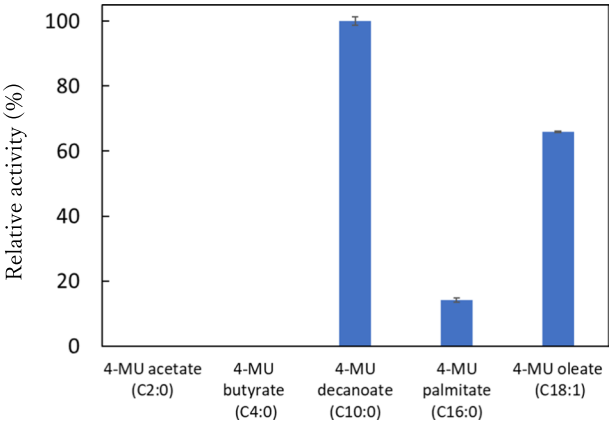

Fig. 6 Substrate specificity of the purified lipase from *P. ostreatus*

Table 2 Substrate specificity of the purified lipase from *P. ostreatus*

	Km (μmol/L)	Vmax (μmol/min)
4-MU decanoate (C10:0)	789	0.14
4-MU palmitate (C16:0)	38	0.01
4-MU oleate (C18:1)	350	0.03

observed when 4-MU acetate (C2:0) and butyrate (C4:0) were used (Fig. 6). The purified lipase preferentially hydrolyzed longer chain substrates (≥ 10 carbon atoms) compared with short-chain substrates (< 10 carbon atoms). Thus, the purified lipase had a typical lipase activity rather than carboxylesterase activity [3]. It was reported that lignocellulosic biomass contains lipolytic compounds such as palmitic acid (C16:0), oleic acid (C18:1), and linoleic acid (C18:2) [9, 21]. The results of this study indicate that *P. ostreatus* produces lipases that hydrolyze and utilize lipolytic compounds in lignocellulosic biomass. In addition, the results showed that the lipases from *P. ostreatus* have a potential to be used for pulp and paper industry to remove lipolytic compounds, as the lipases derived from other microorganisms have been used for this purpose [5, 6]. The Km and Vmax values of purified lipase were measured using 4-MU decanoate, oleate, and palmitate (Table 2). As Km was increased 4-MU palmitate, oleate, and decanoate in this order, it is suggested that 4-MU palmitate had the highest affinity with the purified lipase under the experimental conditions. The Vmax was increased by 4-MU palmitate, oleate, and decanoate in this order. These results suggest that 4-MU palmitate was the substrate most efficiently hydrolyzed by the purified lipase. The amino acid sequences and substrate specificities suggest that the purified lipase from *P. ostreatus* in this study belongs to class III.

Conclusions

Lipases that hydrolyze triacylglycerol are useful enzymes applicable to various industries; thus, lipases from various kinds of microorganisms have been purified and characterized [5]. Although white-rot fungi have the potential to produce various kinds of

lipases, because they degrade extractives in lignocellulosic biomass, few studies have been reported to date [13, 15]. In this study, lipase from *P. ostreatus* was purified from the culture broth with rapeseed oil as a carbon source, and the properties of the purified lipase were observed. The molecular weight of the lipase was 27 kDa, and its optimal pH and temperature were 5.0 and 30 °C, respectively. The purified *P. ostreatus* lipase showed higher activity with 4-MU decanoate and 4-MU oleate as substrates, while it did not show activity with 4-MU acetate and 4-MU butyrate. The amino acid sequences and substrate specificity suggested that the purified lipase belonged to class III.

Further studies should include the purification and characterization of the remaining three lipases from *P. ostreatus* that were observed in this study. When the key enzymes for lipid hydrolysis were elucidated, a molecular biological method will be used to improve the productivity of lipases and the properties. Comprehensive studies on *P. ostreatus* lipases will clarify the availability of lipases for industrial use, such as the reduction of lipolytic compounds in pulp and paper production processes.

Author Contribution Conceptualization, S.N.; methodology, S.N., H.M, N.M.; validation, S.N., H.M, N.M.; investigation, S.N., H.M, N.M.; resources, S.N.; data curation, S.N., H.M, N.M.; writing-original draft preparation, S.N., H.M, N.M.; writing-review and editing, S.N.; visualization, S.N., H.M, N.M.; supervision, S.N.; project administration, S.N.; funding acquisition, S.N.

Data Availability The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Conflict of Interest The authors declare no competing interests.

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