Biochemical Characterization of Ketapang Lipase: Its Preference to Short-Chain Fatty Acids Despite the Long-Chain Fatty Acids Dominant Content

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ABSTRACT. Lipases are versatile enzymes with high specificity toward lipid substrate. They have many industrial applications, such as in food, pharmacy, and green fuel. So far, most explored lipases are from microbial and animal sources, whereas those from plants are less studied. The present study aims to characterize ketapang (*Terminalia catappa* Linn) lipase. The lipase was isolated from germinating ketapang seeds. The activity was determined by hydrolysis of virgin coconut oil (VCO). Biochemical characterization of ketapang lipase includes the optimum temperature, pH, kinetics, metal ions addition, and analysis of substrate specificity. It was shown that ketapang lipase has an optimum temperature of 45 °C, pH 7.5. Ca^{2+} increases the lipase activity, whereas Na^+, K^+, Mg^{2+}, Zn^{2+}, Fe^{2+}, and Cu^{2+} inhibit ketapang lipase to various extents. A comparison of SDS-PAGE and native-PAGE analysis showed that ketapang lipase consists of several protein subunits. A further test by in-gel assay revealed that the 54 kDa, 35 kDa, two bands at ~16 kDa, and 12 kDa proteins showed lipolytic activity against α-naphthyl palmitate substrate. When tested on various chromogenic fatty acid substrates, ketapang lipase showed the highest specificity against short-chain fatty acids (C4 and C8), despite the fact that ketapang oil seed composes mainly of long fatty acid (C18). Since lipases that have high lipolytic activity toward short fatty acids are considered esterases, the esterase activity of ketapang lipase is yet to be determined.

Keywords: in-gel lipase assay, ketapang lipase, lipolytic activity, substrate specificity

INTRODUCTION

The ketapang plant (*Terminalia catappa* Linn) is native to Southeast Asia (Anand et al., 2015) and widely grows in Asia, Africa, Australia, and tropical regions (Nampoothiri et al., 2022). Phytochemical studies regarding the content of its parts, such as roots, stems, and leaves, have been widely carried out. Likewise, its use as an antimedicine such as antimicrobial, antifungal, antidiabetic, antioxidant, and anti-cancer activities, has been extensively reported (Anand et al., 2015; Terças et al., 2017).

The tree has horizontal branches and an upright, symmetrical crown that reaches a height of 35 meters. Its typical branch arrangement is tier-based. The leaves are big, ovoid, lustrous, dark green, and leathery, measuring 15–25 cm long and 10–14 cm wide. The fruit is a drupe that is 5–7 cm long, 3–5.5 cm wide, and contains a single seed. It is first green, then turns yellow, and ultimately turns red when ripe.

Ripe ketapang seed is rich in lipids which are estimated to be 54 to 60% (Santos et al., 2022), and when fully ripe, the fruit's seed is edible and has since been considered a potential dietary lipid source (Janporn et al., 2015; Weerawatanakorn et al., 2015). Moreover, oil from various seeds has been investigated for the production of biodiesel (Cavalcante et al., 2021), including those from ketapang seed (Suhendra et al., 2017). They are also an interesting substrate for pharmaceuticals and surfactants (Gunawan et al., 2018). As a rule of thumb, seeds rich in lipids are potential sources of lipase, especially in the germinating phase of the seed. The seeds require lipolytic activity to mobilize triacyl glycerol storage for energy and nutrition during growth (Dhouibi et al., 2021; Guzmán-Ortiz et al., 2019).

Lipase (EC 3.1.1.3) is a versatile enzyme that hydrolysis triacyl glycerol into glycerol and fatty acids.

Lipases work on the interface for the hydrophobic and aqueous environment. Due to their ability to catalyze a specific reaction, lipases are intensively studied and applied in an array of applications such as in the bioenergy, food and flavor, pharmaceuticals, cosmetics, and detergents industries (Melani et al., 2020; Sarmah et al., 2018). Studies on lipases so far have been dominated by microbial and animal sources, and fewer studies have been dedicated to plant-based lipase (Sankar & Ponnuraj, 2020; Seth et al., 2014). As a plant whose seed is rich in lipase, very limited studies have been reported for ketapang lipase. In the present study, we characterized the biochemical properties of ketapang lipase.

EXPERIMENTAL SECTION
Materials
On the Indonesian island of Lombok, a local orchard provided the ketapang seeds from mature ketapang fruit. Buffer components for crude extract preparation included KNO_3, NaOH, Na_2HPO_4, and NaH_2PO_4 (Merck, Darmstadt, Germany). The lipase substrates were virgin coconut oil (VCO, from a local vendor "Lombok VCO") and chromogenic substrates 4-nitrophenyl butyrate, 4-nitrophenyl octanoate, 4-nitrophenyl laurate, 4-nitrophenyl myristate, 4-nitrophenyl palmitate, and 4-nitrophenyl stearate (supplied by Sigma-Aldrich, Missouri USA), as well as 4-nitrophenyl decanoate (Santa Cruz Biotechnology, California USA). The buffer required for specificity assay using chromogenic substrate consisted of n-propanol, Sodium deoxycholate, Arabic gum, and Tris-Cl (provided by Sigma-Aldrich, Missouri, USA). n-hexane, acetone, and 96% ethanol (Merck, Darmstadt, Germany) were also solvents. The end-point indicator for titration employed was phenolphthalein (Sigma-Aldrich, Missouri, USA). $CuSO_4$ solution, Na-K-Tartrate, 7.5% NaOH solution, and standard casein solution (all from Merck, Darmstadt, Germany) made up the components for the protein content analysis. Acrylamide, bis-acrylamide, sodium dodecyl sulfate, 10% ammonium persulfate, Dithiothreitol (DTT), glycine (all from Bio Basic, Toronto Canada), and N,N-dimethylformamide, N,N,N,N-Tetramethyl ethylenediamine (TEMED), glycerol, β-mercaptoethanol, bromophenol blue, Coomassie brilliant blue, Tritone X-100, and fast blue B salt (Sigma-Aldrich, Missouri USA), α-naphthyl palmitate, protein marker (Thermo Scientific, Massachusetts USA), were the ingredients used in the electrophoresis and in-gel lipase assay. Metal ions tested their lipase inhibition activity were supplied by Merck (Darmstadt, Germany). Graphs were prepared by using Prism 7.0 tool (GraphPad Software Inc., California, USA).

Sample Preparation and Lipase Activity
Ketapang imbibition is done by storing ketapang seeds in moist media. Ketapang germination was observed after about 20 days of imbibition. Ketapang fruit kernels in this phase were collected and crushed, then suspended in a buffer solution with a seed-to-buffer ratio of 1 : 3 (w/v). Samples were centrifuged at 8000 rpm to separate the cream, skim, and debris phases. The skim phase, herein referred to as a crude extract, was stored at -20 °C for subsequent use.

Protein determination followed the method described elsewhere (Hayes, 2020) with modification. The biuret reagent was created by combining 0.375 g of solid $CuSO_4$ with 1.51 g of solid K-Na-Tartrate. This mixture was then dissolved in 100 mL of distilled water, and 100 mL of a 7.5% (w/v) NaOH solution was then added. The solution was added to a 250 mL volumetric flask and diluted to the proper concentration with distilled water.

A modified version of the technique developed by Khor and colleagues was used to measure the activity of lipase (Khor et al., 1986). A water bath shaker was used to incubate 5 g of VCO (Virgin Coconut Oil), 2.5 mL n-hexane, 5 mL of 100 mM phosphate buffer pH 7.5, and 1 mL of lipase enzyme for 45 minutes at 37 °C. A few drops of the phenolphthalein indicator and 25 mL of acetone-ethanol (1:1, v/v) were added to the mixture after incubation. The mixture was then titrated with 0.01 M NaOH. The only difference between the blank treatment and the sample treatment was the absence of enzymes.

$$Lipase\ activity = \frac{(V_{sample}-V_{blank})x\ [NaOH]x\ 1000)}{V_{enzym}\ x\ t} \quad (1)$$

Optimum Reaction Condition and Kinetics of Ketapang Lipase
The above method was used to investigate the optimum hydrolysis reaction condition of ketapang lipase. This included the temperature and pH of ketapang lipase and was determined by varying the incubation temperature and buffer pH. Accordingly, the investigation of ketapang lipase kinetics was determined by applying the reaction at various concentrations of VCO substrate from 0 to 3.6 mg/mL.

Effect of Ion Metals on Ketapang Lipase Activity
The effect of ion metals on ketapang lipase activity was performed the same way as in the determination of ketapang lipase activity in the presence of various metal ions at concentrations of 10 mM or in the absence of them as a control. The metal ions being tested were alkaline metal ions Na^+ and K^+, alkaline earth metal ions Ca^{2+} and Mg^{2+}, as well as transition metal ions Zn^{2+}, Fe^{2+}, and Cu^{2+}.

Identification of Active Lipase Protein
Denatured crude ketapang lipase extract was subjected to SDS-PAGE that followed the Laemmli method (Laemmli, 1970) with modification. Briefly, 20 μg of crude extract was loaded into each well. Staining was undertaken by using Coomassie brilliant blue dye. For native-PAGE, a similar procedure was done, except the sample was not denatured by heating or the addition of β-mercaptoethanol or DTT, and there was no SDS used in the preparation of the gel and running buffer.

An in-gel assay with α-naphthol palmitate was used to investigate the active lipase proteins. Briefly, the sample of ketapang lipase was initially separated by using SDS-PAGE (without prior heating of samples). After the SDS-PAGE separation process was completed, the proteins or enzymes in the gel were renatured to remove SDS by immersing the electrophoretic gel with a washing solution (0.05 M phosphate buffer solution pH 7 and 2.5% (v/v) Triton X-100) for 3 x 30 minutes. The gel was incubated at 37 °C with a developer solution (α-Naphtyl Palmitate, *N,N*-Dimethylformamide, Fast Blue B Salt, 0.1 M phosphate buffer pH 7) in a dark room for 30 minutes. The gel was washed again with aquadest for 3 x 30 minutes using a shaker. Bands of yellow color appear from active lipase proteins.

Substrate Specificity of Ketapang Lipase

The substrate specificity of ketapang lipase was investigated by using chromogenic substrates as previously described (Savalas et al., 2021). Prior to the assay, a time course curve was generated by applying ketapang lipase to standard substrate 4-nitrophenyl palmitate at various incubation times, and the intensity of the released yellow color of 4-nitrophenol was measured at 400 nm (Susilo et al., 2022). To test the specificity of ketapang lipase, an incubation time of 6 minutes was chosen for various chain lengths of substrates. The substrate preference of ketapang lipase was further investigated by kinetics study by using C8 fatty acid (4-nitrophenyl octanoate) and C18 fatty acid (4-nitrophenyl stearate) at concentration from 0.2 μM to 50 μM. The Vmax and K_M data were generated by using Prism 7.0 tool.

RESULTS AND DISCUSSION
Ketapang Lipase Activity

Tropical regions are blessed with high diversity in both microorganisms and higher plants. Seeding plants are potential sources of lipase, especially for food, biodiesel, and pharmaceutical production (Seth et al., 2014). However, despite their potential application, plant lipases still need to be explored (Sankar & Ponnuraj, 2020; Seth et al., 2014). In this study, we describe the characteristics of ketapang lipase. Ketapang seed is rich in oil, especially when it is ripe (**Figure 1**). Depending on the isolation process and the variant of ketapang, the reported oil content of ketapang seed may differ. However, most reports revealed that ketapang seed is estimated to contain 54 to 60% of oil (Gunawan et al., 2018; Janporn et al., 2015; Ng et al., 2015; Santos et al., 2022). During kernel ripening and germination, lipase from the seed shows increasing activity to enable the mobilization of lipid storage for seed growth. Germination was achieved by imbibition, i.e., by storing ripe or mature ketapang seeds in humid for c.a. 20 days.

The activity of ketapang activity was determined by hydrolysis of triacylglycerol contained in VCO using the crude extract of ketapang lipase. The released free fatty acids were determined by titration with sodium hydroxide. It is revealed in this study that lipase from germinating ketapang has an activity and specific activity of 1.90 and 0.42 U/mg, respectively. To our knowledge, there has yet to be a report about the activity or specific activity of ketapang lipase. The activity of ketapang lipase, with similar preparation, is higher than the activity of coconut lipase we have previously reported. Coconut lipase has an activity and specific activity of 0.56 and 0.30 U/mg, respectively (Savalas et al., 2021). Under different preparation conditions, the crude extract of sunflower seeds has a specific activity of 0.55 U/mg (Al-Haidari et al., 2020).

Optimum Temperature and pH

The optimum temperature assay showed that ketapang lipase has an optimum temperature at 45 °C and is still showing significant activity at slightly lower and higher temperatures. A sharp increase in the activity is shown from 37 to 40 °C (**Figure 2A**). With a few exceptions, most seed lipases have optimum temperature from ambient to mesophilic. Among the few examples are almond seed (*Amygdalus communis* L.), oat seed (*Avena fatua*), and rice seed (*Oryza sativa*) lipases that have an optimum temperature of 65, 75, and 80 °C, respectively (Barros et al., 2010).
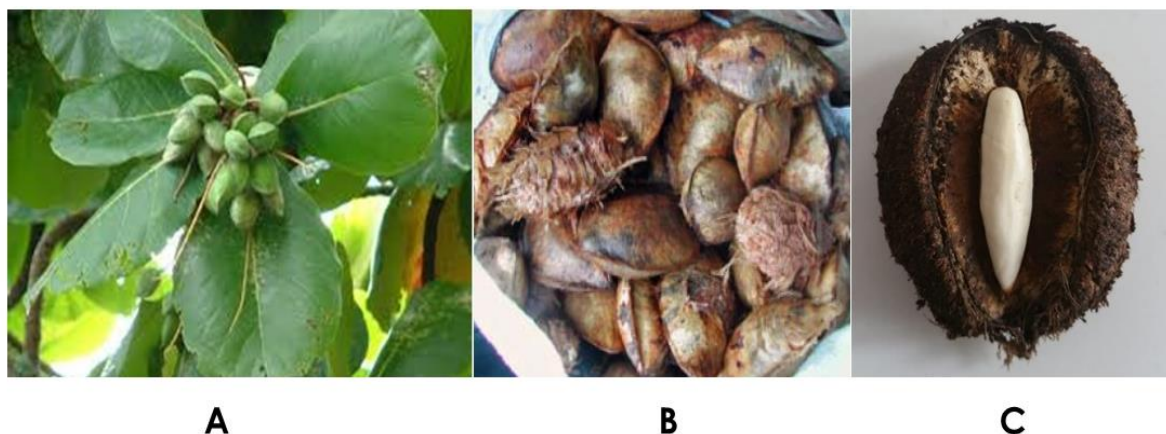

Figure 1. Ketapang fresh fruits (**A**), mature and dried fruits (**B**), and germinated seed (**C**)
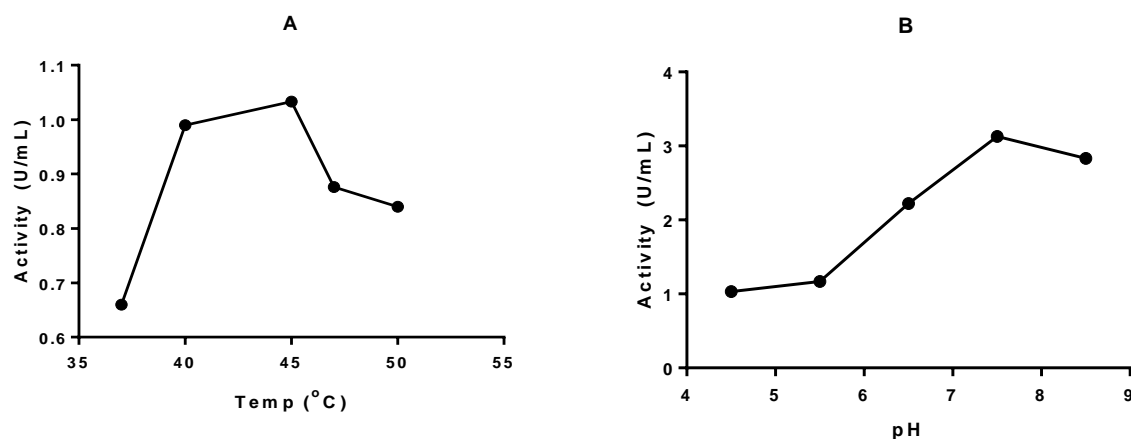
A

B

Figure 1. Optimum temperature and pH of ketapang lipase
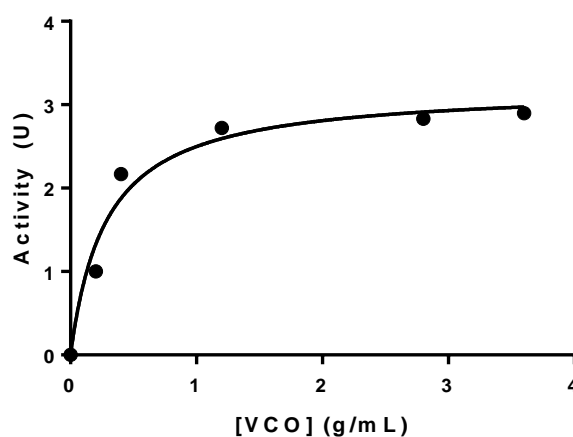

Figure 2. Michaelis-Menten curve of ketapang lipase. A plot of ketapang lipase's initial activity against substrate concentration shows a K_M value of 0.082 g/mL for VCO substrate.

Figure 2B shows that the optimum pH of ketapang lipase is 7.5, and it tends to retain 91% of its optimum activity at pH 8.5. This profile needs more study to evaluate the pH stability in a more basic environment since, for many applications, lipase activities in a basic environment are demanded. In general, many seed lipases were reported to have optimum pH at neutral conditions, although acid and basic lipases are also occurring, for example, Castor bean seed (*Phaseolus vulgaris*), Oat seed (*Avena fatua*), and Rice seed (*Oryza sativa*) have an optimum pH of 4.5, 9.0 and 11, subsequently (Barros et al., 2010).

In order to reveal the kinetics of ketapang lipase, the activity of ketapang lipase was tested in the increase of substrate concentration. In this study, VCO was employed as a substrate. Like coconut oil, triacyl glycerol from VCO is rich in lauric acid moieties (Prapun et al., 2016), and **Figure 7** shows that lauric acid (C12) belongs to a good substrate of ketapang lipase. Ketapang lipase shows that it follows Michaelis-Menten kinetics (**Figure 3**). Analysis of the graph in **Figure 3** revealed a K_M value of 0.082 gram/mL VCO. The K_M value resembles the popular commercial lipase

from *Candida rugosa*, which has a K_M value of 0.083 g/mL against palm oil substrate (Rashid et al., 2014). The low K_M value shows that the affinity of ketapang lipase to the VCO substrate is very high.

Effect of Metal Ions on Ketapang Lipase Activity

The presence (or absence of) metal ions is critical to lipase activity (Goswami et al., 2013). Here we showed that ketapang lipase is slightly activated by Ca^{2+} ion. Calcium ion has been reported to activate many lipases, regardless of their sources. Alkaline metal ions (Na^+ and K^+) and related alkaline earth metal ions Mg^{2+}, as well as transition metal ions Zn^{2+}, Fe^{3+}, and Cu^{2+}, reduce ketapang lipase activity (**Figure 4**). The fact that calcium ion increase ketapang lipase activity suggests the presence of a calcium-binding motif, which upon calcium binding, can increase lipase activity. Such observation was discussed in much literature (Hertadi & Widhyastuti, 2015; Mohammadi et al., 2016). In *Pseudomonas aeruginosa* lipase, calcium ions help the correct positioning of a histidine residue in the catalytic triad (El Khattabi et al., 2003). Since the occurrence of catalytic triad is commonly found in lipases, the

presence of calcium ions is considered to have a similar role. Alternatively, calcium might also speed up the removal of the liberated free fatty acids from the interface of oil-water molecules (Rmili et al., 2019). As a consequence, the removal of free fatty acids facilitates further cleavage of triacyl glycerol and increases lipolysis. However, the exact role of calcium ions in the activity of ketapang lipase is yet to be investigated. Other metal ions tend to inhibit ketapang lipase (**Figure 4**). Unlike calcium ions that are prone to activate seed lipases, other metal ions can either activate or inhibit seed lipase. An example is shown by Cu^{2+}, which inhibits Rapeseed (*Brassica napus* L.) and Almond seed (*Amygdalus communis* L.) but activates Laurel seed (*Laurus nobilis* L.) (Barros et al., 2010).

Identification of Lipase-Active Subunits

SDS-PAGE of ketapang lipase shows it consists of several bands. However, on native-PAGE, only fewer bands with high molecular weight are observed

(**Figure 5**). This may lead to concluding that several bands observed on SDS-PAGE may form more complex proteins of several subunits. A similar observation was also recently reported for lipase from coconut (Savalas et al., 2021).

To assess whether various bands observed on SDS-PAGE have lipase activity, an in-gel assay has been performed. A lipase-active subunit hydrolyzes the α-naphthyl palmitate substrate into a yellow color of α-naphthol (Zienkiewicz et al., 2015). The middle lane in **Figure 5** shows that several yellow bands appear as a result of the lipolytic activity of respective proteins, i.e., a protein of ~ 54 kDa, a protein of ~ 35 kDa, two protein bands of ~ 16, and two bands of ~ 12 kDa. This finding underlies that those subunits are active lipases. The presence of six active proteins or subunit proteins showed that ketapang lipase consists of several active subunits. A similar observation is found in the case of coconut lipase (Savalas et al., 2021).
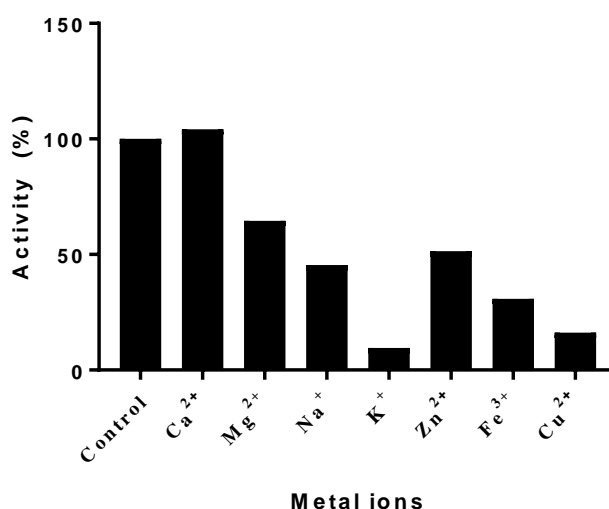

Figure 4. Effect of metal ions on ketapang lipase activity. Most metal ions tested reduce ketapang lipase activity, except Calcium ions.
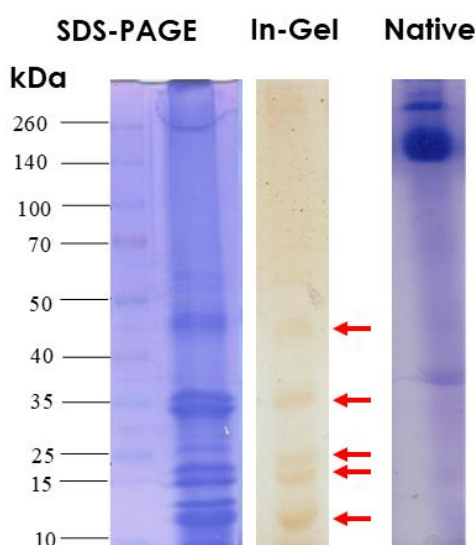

Figure 5. SDS-PAGE, in-gel lipase assay, and native-PAGE of ketapang lipase.

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Left lanes: SDS-PAGE of crude extract of ketapang lipase with protein markers. Middle lane: In-gel lipase assay to reveal the active lipase from the crude extract. Right lane: Native-PAGE of crude extract. Major bands that appear on SDS-PAGE are 54 kDa, two bands of ~ 35 kDa, two bands of~ 16 kDa, and two bands of ~ 12 kDa. Those bands correspond to the hydrolysis of α-naphthyl palmitate substrate in the in-gel assay. On native-PAGE, only a single clear band appears at ~ 180 kDa, which may represent a complex protein consisting of several lipase active subunits.

Substrate Specificity of Ketapang Lipase

Prior to the determination of the substrate specificity of ketapang lipase, a time course curve was generated. By using 4-nitrophenyl palmitate as substrate, ketapang lipase shows good linearity between 4 to 10 minutes (**Figure 6**). For further analysis of ketapang lipase substrate specificity with various chromogenic fatty acids, an incubation time of 6 minutes was chosen.

Ketapang seed has been reported to be rich in oil. The composition of ketapang seed oil has been reported by several working groups. All reports showed that saturated, mono, and di-unsaturated C18 fatty acids represent c.a. 60% of ketapang seed oil, whereas c.a. 30% are palmitic acids. On the opposite, the short fatty acids (C8) are only found in trace (less than 1%), and C4 is hardly detected (Janporn et al., 2015; Ng et al., 2015; Santos et al., 2022). Other studies suggested that seed lipase preferred the major fatty acid substrate in the respected seed (Lin et al., 1986). The data has led us to expect the preference of ketapang lipase to the long fatty acid C18 or C16. Surprisingly, our result shows that ketapang lipase prefers the short and middle-chain fatty acids (C4 and C8) instead of the long fatty acids C18 or C16 (**Figure 7**). The overwhelming long fatty acids content of ketapang seeds does not correlate with the specificity of ketapang lipase, which prefers short (C4 and C8) fatty acids.

The preference of ketapang lipase against short fatty acid instead of long fatty acid is confirmed by kinetics analysis of ketapang lipase against 4-nitrophenyl octanoate and 4-nitrophenyl stearate substrates. The K_M value against 4-nitrophenyl octanoate is lower than that against 4-nitrophenyl stearate, i.e., 3.14 and 10.87 μM of the respective substrate, subsequently (**Figure 8**). The data show that the affinity of ketapang lipase to 4-nitrophenyl octanoate is higher than that of 4-nitrophenyl stearate.
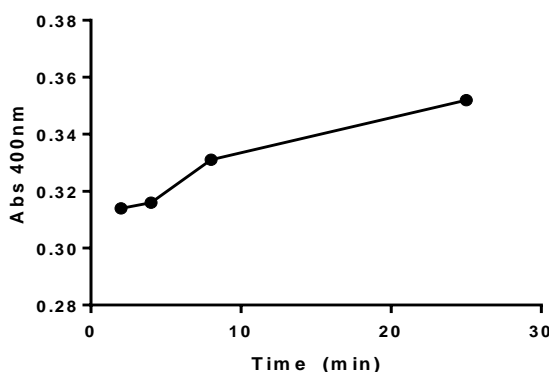

Figure 6. Time course of ketapang lipase activity. Ketapang lipase shows linear activity over time (first-order reaction) from 4 to 10 minutes by using a 4-nitrophenyl palmitate substrate.
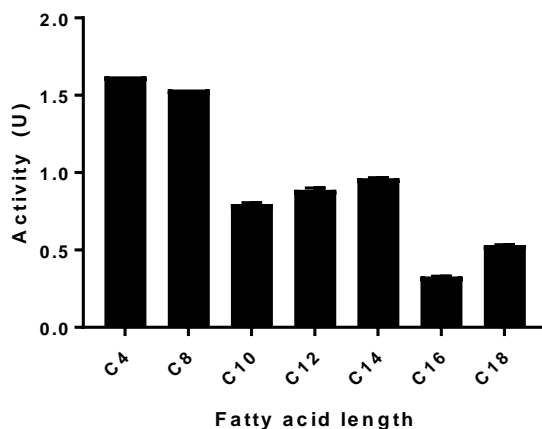

Figure 7. Substrate specificity of ketapang lipase. C4: 4-nitrophenyl butyrate; C8: 4-nitrophenyl octanoate; C10: 4-nitrophenyl decanoate; C12: 4-nitrophenyl laurate; C14: 4-nitrophenyl myristate; C16: 4-nitrophenyl palmitate; C18: 4-nitrophenyl stearate.
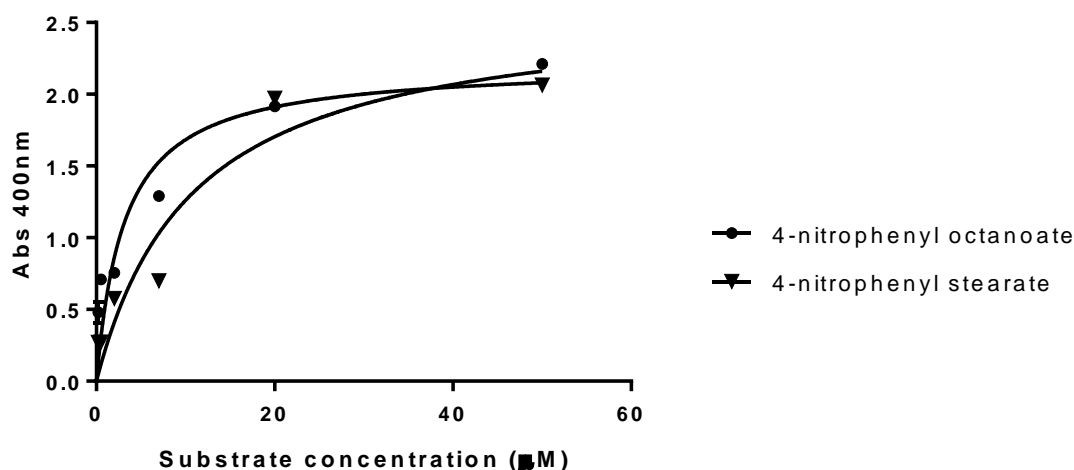
Figure 8. Kinetics of ketapang lipase against 4-nitrophenyl octanoate and 4 -nitrophenyl stearate substrates. The Vmax values for 4-nitrophenyl octanoate and 4 -nitrophenyl stearate are 2.21 and 2.63, respectively, whereas the K_M is 3.14 μM and 10.87 μM, subsequently.

This finding could be an important starting point to explore ketapang lipase more thoroughly, especially its potential as esterase in addition to its lipase activity. A recent review revealed that lipase that prefers short fatty acid might have more esterase activity than lipolytic activity (Casas-Godoy et al., 2018; Lee & Park, 2019; Vaquero et al., 2016). Hence, it is of scientific interest to investigate the esterase activity of ketapang lipase.

CONCLUSIONS

The present study shows the characteristics of ketapang lipase. It has an optimum lipolytic temperature and pH of 45 °C and 7.5, respectively. The enzyme follows Michaelis-Menten Kinetics with a K_M value of 0.082 g/mL VCO substrate. Ketapang lipase is activated by Ca^{2+} ions, while K^+, Na^+, Mg^{2+}, Zn^{2+}, Fe^{2+}, and Cu^{2+} ions inhibit the enzyme. SDS-PAGE and native-PAGE results suggest that ketapang lipase is a complex protein consisting of various proteins. The in-gel assay further suggests that lipase activity is shown by several protein subunits. Despite having long fatty acid (C18) as the major fatty acid component of ketapang seed, ketapang lipase has the highest preference for short fatty acids (butyric and octanoate fatty acid). Further study deems necessary to explore the potential activity of ketapang lipase as an esterase.

ACKNOWLEDGMENTS

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