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Exploration of Novel Lipase from Plant Seeds and Plant Latexes

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Abstract

As the demand for fatty acids increases, the enzymatic process of triglyceride hydrolysis emerges as a promising technology. Compared to microbial lipase, utilization of plant lipase is more practical due to its ease of preparation and cost-efficiency. This work aimed to verify the degree of lipolysis of several novel lipase sources from plants. Novel lipase sources investigated were seeds of kapok (*Ceiba pentandra*), java almond (*Sterculia foetida*), pongam (*Milletia pinnata*), sea mango (*Cerbera manghas*), tamanu (*Calophyllum inophyllum*), latex of sea mango, aveloz (*Euphorbia tirucalli*), and jackfruit (*Artocarpus heterophyllus*). Several acknowledged plant lipase sources were also compared, i.e. seeds of castor bean (*Ricinus communis*), physic nut (*Jatropha curcas*), rice bran (*Oryza sativa*), latex of frangipani (*Plumeria rubra*) and papaya (*Carica papaya*). Plant lipase was utilized in the hydrolysis of olive oil at room temperature. Results for seed and latex lipase were compared and technical issues were reported. Several plant lipases are remarkably active and potential to compete with microorganism lipases in industrial applications.

Keywords: fatty acid, lipolysis, lipase, plant latex, plant seed

INTRODUCTION

Oleochemicals industry market was valued at $25.6 billion in 2020 and is expected to grow 7.5% between 2021-2030 (Sushant, Amit, & Eswara, 2022). The two principal intermediate products of the oleochemical industry are fatty acids and their methyl esters. The fatty acids market accounted for $29.47 billion in 2022 and is projected to reach $48.42 billion by 2032 (Presedence Research, 2023). Fatty acids are used for the production of chemical and pharmaceutical products, and recently, it is also processed to produce hydrocarbons with decarboxylation or pyrolysis (Elizabeth et al., 2021; Liu & Li, 2020; Puspawiningtiyas et al., 2021; Santner et al., 2023). Unlike biodiesel from FAME or oxygenated fuel (such as bioethanol), this plant-sourced hydrocarbon can be directly mixed with conventional fuel due to their identical structure (Bruder, Moldenhauer, Lemke, Ledesma-Amaro, & Kabisch, 2019; Karatzos, Van Dyk, McMillan, & Saddler, 2017; Silva et al., 2022). It represents a new potential area of fatty acid utilization and could result in a large increase in industrial fatty acid demands in the future.

In the last decades, a low-temperature route for the production of fatty acid has been developed through the utilization of enzymes. Triglyceride hydrolysis with lipase (also known as lipolysis) is preferable to the conventional uncatalyzed thermal hydrolysis of triglyceride, which thermodynamically requires high temperature and high pressure (Istyami, Soerawidjaja, & Prakoso, 2018). The challenge of lipase utilization in fatty acid production is the limited lipase source. Commercial lipases are commonly produced from microbial sources, which requires complex separation and high production costs.

Compared to microbial lipase, plant lipase is an attractive option due to its ease of preparation and the cost-efficiency of the source (Avelar et al., 2013). It is more abundantly available and easier to handle, without necessitating complex separation. Plant-seed lipase investigations have been reported, mostly in the form of oil-free powder or acetone powder (Avelar et al., 2013; Barros, Fleuri, & Macedo, 2010). The latter preparation method involves delipidation of oilseed with acetone, a solvent that could dissolve oil as well as water but would not solubilize enzyme. The use of

acetone was also claimed to improve the stability of seed lipase (Cavalcanti-Oliveira et al., 2007). Other report claims similar activity between original and oil-free powder, and original activity shows higher stability during storage time (Tavares, Petry, Sackser, Borba, & da Silva, 2018). The lipolytic activity of plant lipase differs when this method is applied to various types of plant seeds using different solvents (A. Istyami, Soerawidjaja, Prakoso, & Kresnowati, 2016).

In the last two decades, investigations have been reported on the activity of lipase from various seeds, such as castor bean, corn, rapeseed, elm, mustard, palm, and pinus seed (Barros et al., 2010). Plant latex lipases that have been reported are those *of Carica papaya, Asclepias curassavica, A. syriaca, Euphorbia characias* (Giordani, Moulin, & Verger, 1991), *Plumeria rubra* (A. N. Istyami, Purwadi, Kresnowati, Prakoso, & Soerawidjaja, 2019), etc. Besides plant seeds and plant latexes, rice bran also shows significant lipolytic activity during its storage time, which was reported to be minimized under temperature -20 ^{0}C and with the presence of hydrochloric acid (Singh & Sogi, 2016).

The main challenge of plant lipase utilization is its low activity, resulting in long reaction times and voluminous solvent requirements. For example, 98.7% conversion in castor oil lipolysis with oat seed powder requires a 39:1 solvent/oil ratio and more than 300 hours of reaction time (Patent No. US5932458A, 1999). A remarkable progress was shown by achieving 88.2% degree of lipolysis using dormant castor bean oil-free powder (1:5 powder/oil ratio) within 2 hours (Avelar et al., 2013). This result confirmed previous common knowledge that castor bean lipase has been known as the most feasible plant lipase for lipolysis so far. Nevertheless, other sources of comparable or even more effective lipases are still desirable. Exploration of other plant seeds and plant latexes is necessary to find a plant lipase source with better economic feasibility.

The present research aims to investigate and compare the degree of lipolysis of several plant-based lipases. Novel sources investigated were seeds of kapok (*Ceiba pentandra*), java almond (*Sterculia foetida*), pongam, (*Milletia pinnata*), sea mango (*Cerbera manghas*), tamanu (*Calophyllum inophyllum*), and latexes of sea mango (*Cerbera manghas*), aveloz (*Euphorbia tirucalli*), and jackfruit (*Artocarpus heterophyllus*). Other lipase sources were also investigated, i.e. seeds of castor bean (*Ricinus communis*), physic nut (*Jatropha curcas*), rice bran (*Oryza sativa*), and latexes of frangipani (*Plumeria rubra*), and papaya (*Carica papaya*). All seeds are utilized in a dormant state. Species were chosen for their abundance, especially in tropical areas. Plant seed and plant latex lipase were utilized in 1%-mass-of-substrate-oil to identify the feasibility of low-dosage enzyme lipolysis. The degree of lipolysis was evaluated for plant lipases in the form of oil-free (delipidated) powder as well as original (non-delipidated) powder.

METHODOLOGY

Materials and Instrumentals

Rice bran was purchased from a local rice mill in Cimahi, Indonesia. Sea mango seeds and latex and the latexes of aveloz, jackfruit, papaya, and frangipani were collected from local trees in Bandung, Indonesia. Pongam seeds were collected from local trees in Kupang, Indonesia. The seeds of physic nut, kapok, tamanu, and java almond were collected from a local plantation in Majalengka, Indonesia. Castor beans were purchased from Semarang, Indonesia. Acetone was technical grade. All other chemical reagents were analytical grade. The tool used in this research was a Thermo Scientific SP88850105 heating plate.

Preparation of Plant Lipase

Plant seeds and rice bran were utilized in the form of original (non-delipidated) powder and oil-free (delipidated) powder. Original plant powders were prepared by grinding the plant seeds with Philips Food Processor. The oil-free powder was prepared by grinding the plant seeds immersed in cold acetone followed by filtration as described by Avelar, et al. (2013). Plant latexes were prepared in the form of naturally dried powder, centrifugated particulate as well as the corresponding supernatant, and immobilized latex. Centrifugation was established with Hermle Z 366 K centrifuge. Natural drying of latex was done at room temperature (26 ^{o}C) for 3 days. Centrifugated particulate and the supernatant were prepared by centrifuging distilled water diluted fresh plant latex (9:1 v/v) at 6000 rpm for 10 minutes followed by decantation. Immobilization was done by dry impregnation of plant latex onto an oil-free powder of java almond seeds.

Enzymatic Hydrolysis of Triglyceride

Triglyceride hydrolysis was conducted by mixing 25 g of olive oil, 1%-mass of lipolytic catalyst, 8% of water, and adjusted amount (0%, 0.4%, 8%, 10%,

20%, or 40%) of buffer with desirable pH (7.0, 8.0, 9.0, 10.0, or 11.0). Lipolysis proceeded in heating plate Thermo Scientific SP88850105 for some desirable time (4 or 24 hours) at adjusted temperatures (26 ºC, 40 ºC, 60 ºC, or 80 ºC). The acid value of lipolysis products was analyzed by titration with 0.1 N alcoholic KOH. The degree of lipolysis was calculated using Eq. (1) (Rooney & Weatherley, 2001):

$$Lipolysis\ (\%) = \frac{v_{KOH} \times 10^{-3} \times M_{KOH} \times MM}{Wt \times f} \times 100 \quad (1)$$

where V_{KOH} is the volume of potassium hydroxide solution (KOH) required (mL), M_{KOH} is the molarity of KOH solution (0.1 M), MM is the average molecular mass of olive oil fatty acids, and Wt is sample weight (g) and f is oil fraction in the reactant.

RESULTS AND DISCUSSION

Lipolysis with Plant Seeds and Rice Bran

To evaluate the activity of various seed lipases, degrees of lipolysis after 4 and 24 hours of hydrolysis were determined. In Table 1, significant differences between the degrees of lipolysis achieved at the 4th and 24th hours with castor seed lipases suggest that the reaction was still proceeding toward equilibrium during this period. The rate of conversion is much slower in the absence of agitation (results not shown), suggesting that the reaction is mass transfer controlled. Lipase in the form of oil-free powder tends to produce a higher degree of lipolysis than that of the original powder, confirming the positive effect of oil and water removal on lipolytic activity. The highest lipolytic degree achieved by castor seed confirmed that castor seed is by far the most feasible plant source of lipase. Compared to castor seed lipase, physic nut lipase shows low activity although the source trees are closely related by taxonomy. Next to the lipase oil-free powder of castor seed, that of sea mango shows some potential, although its lipolytic activity is significantly lower.

To evaluate the effect of buffer, lipolysis was also conducted with increased buffer content (8%). The results, shown in Table 2, show the comparison between two buffer concentrations: 0.4% (low concentration) and 8% (high concentration) to show the significance. At 0.4% concentration and lower, the effect of the buffer is practically absent. Meanwhile, an 8% concentration of buffer is considered practical, especially in terms of downstream processing. Table 2 indicates that the increase in buffer addition from 0.4% to 8% increased the degree of lipolysis achieved by oil-free powder up to thirteen-fold, possibly due to better enzyme conformation stability.

Among the novel seeds evaluated in Table 2, the kapok seed produced the highest lipolytic activity. The level of lipase activity does not seem to depend on the plant family. The effect of pH and temperature on kapok seed lipase activity is provided in Figure 1. It shows that kapok seed lipase was, surprisingly, still active at relatively high temperatures (i.e. 80 ºC). This property is useful in applications where solventless lipolysis of saturated triglycerides (with melting point up to 80 ºC) is needed, such as in the lipolysis of palm stearin for making pharmaceutical-grade products. The shortcoming of oil processing at high temperatures is the darkening of product color, as shown in Figure 2 (product of 40 ^{0}C and 80 ^{0}C lipolysis). The product color of lipolysis at 26 ^{0}C and 60 ºC (not shown) was similar to the 40 ^{0}C. Thus, for saturated oil fats which are solid at room temperature, lipolysis with kapok seed lipase at 40 ºC is recommended, because the activity is similar to that of 60 ºC and 80 ºC, with minimum discoloration effect. Buffer with a pH of 8 is preferable in all temperatures evaluated, indicating that kapok seed is a source of alkaline lipase, similar to physic nut, coconut, and laurel seed (Barros, Fleuri, & Macedo, 2010).

Figure 3 shows that the presence of buffer significantly increases kapok seed lipase activity, particularly when the added buffer is in the range of 20 – 40 %. It confirms the effect of pH stability on the performance of lipase. Most plant lipases are active in neutral to basic pH (7-9) (Amaturrahim, Yusak, & Sebayang, 2020; Barros et al., 2010; Kumar et al., 2020). Several groups in the enzyme are ionizable, thus the change in pH will affect the enzyme conformation, which contributes to its catalytic performance (Schuler & Kargi, 2001). The pH stability of the reaction medium facilitates lipase to convert more triglyceride substrate although more fatty acids are produced. Although buffer concentration increases the lipolytic activity of lipases, it should be kept on practical value to avoid costly downstream processing due to highly basic wastewater. The lipase activity in Figure 3 seems to be very active only for 15 minutes, and no proceeding hydrolysis afterward. A short reaction period is a strong advantage in industry, especially with mild operating conditions, and it is unnecessary to add other features to improve the kinetics.

Some of seed lipases show better selectivity for the dominating fatty acid in its seed oil (Hellyer, Chandler, & Bosley, 1999). This was confirmed in Figure 4, in which kapok seed lipase shows higher selectivity towards kapok seed oil than olive oil. Considering that kapok seed oil contains higher linoleic and linolenic acid content than olive oil (Anwar, Rashid, Shahid, & Nadeem, 2014), its lipase seems to have higher selectivity towards polyunsaturated than monounsaturated triglycerides. Further potential investigation of seed lipases is immobilization, which was also done on rice bran (Dali, Firdaus, & Rusman, 2017; Firdaus, Dali, & Rusman,2017).

Table 1. Degree of lipolysis with 1% lipase from plant seed lipase powder, 0.4% buffer, and 8% water

Seed Lipase Source	Family	Degree of lipolysis			
		4 hours, original powder	24 hours, original powder	4 hours, oil-free powder	24 hours, oil-free powder
Kapok	Malvaceae	0.35%	0.63%	0.35%	0.63%
Wild almond	Malvaceae	0.49%	0.63%	0.35%	0.49%
Sea mango	Apocynaceae	0.35%	0.49%	0.92%	2.19%
Pongam	Fabaceae	0.57%	0.56%	0.48%	0.67%
Tamanu	Calophyllaceae	0.42%	0.28%	0.21%	0.28%
Physic nut	Euphorbiaceae	0.28%	0.49%	0.28%	0.42%
Rice bran	Poaceae	0.21%	0.77%	0.42%	0.91%
Castor	Euphorbiaceae	0.35%	3.50%	0.70%	9.69%

Table 2. Degree of lipolysis with 1% lipase from plant seed lipase powder, 8%, buffer, and 8% water

Seed Lipase Source	Family	Degree of lipolysis	
		4 hours, oil-free powder, 8% buffer	4 hours, oil-free powder, 8% buffer
Kapok	Malvaceae	0.35%	5.50%
Wild almond	Malvaceae	0.35%	3.48%
Sea mango	Apocynaceae	0.92%	1.78%
Pongam	Fabaceae	0.48%	2.95%
Tamanu	Calophyllaceae	0.21%	2.88%
Rice bran	Poaceae	0.28%	5.60%
Physic nut	Euphorbiaceae	0.42%	3.37%
Castor	Euphorbiaceae	0.70%	9.00%

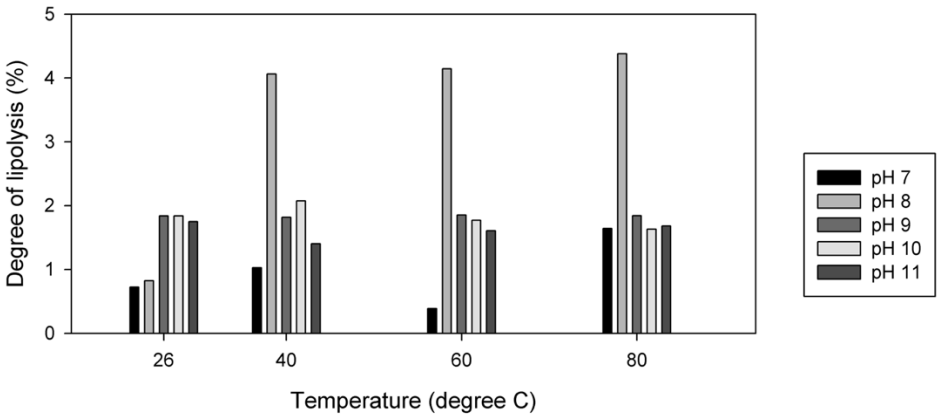

Figure. 1. Kapok seed lipase performance in olive oil lipolysis at various levels pH and temperature (1% kapok seed powder, 8% water, 5% buffer, 6 hours of reaction)

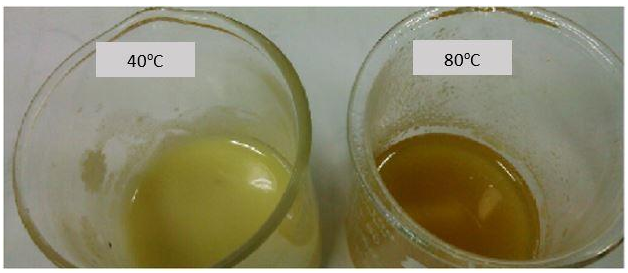

Figure. 2. Product of olive oil lipolysis with kapok seed powder lipase in temperature of 40^0C (left) and 80^0C (right)

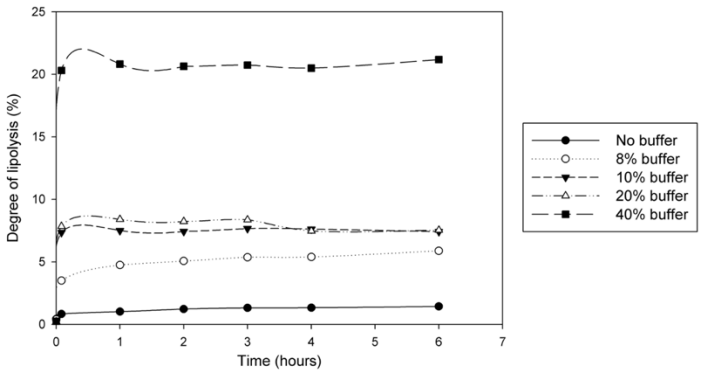

Figure 3. Kapok seed lipase performance in olive oil lipolysis in various level of buffer pH 8 (1% kapok seed powder, 8% water)

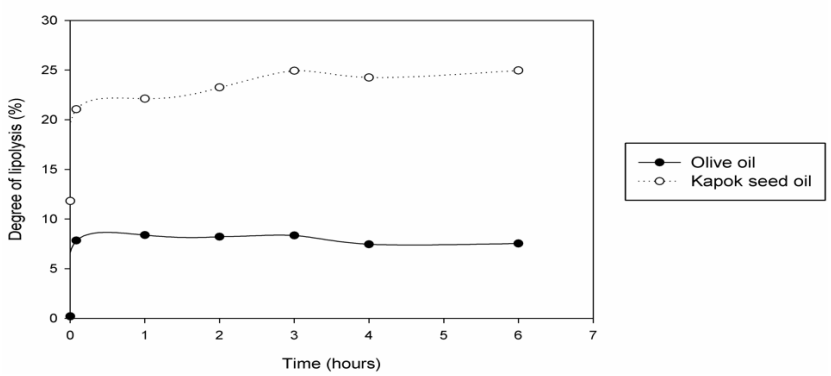

Figure 4. Kapok seed lipase performance in olive oil and kapok seed oil lipolysis (1% kapok seed powder, 20% buffer pH 8, and 8% water)

Lipolysis with Plant Latex

The lipolytic activities of some naturally-dried plant latexes are shown in Table 3. Comparing the results with those of Table 1, it was shown that plant latex lipases produce a higher degree of lipolysis than plant seed lipases. Sea mango latex and frangipani latex in particular show the highest activity. They are both from a family of Apocynaceae, similar to *Asclepias currasavica* which has been reported to exhibit high lipolytic activity (Giordani et al., 1991). This suggests that latex from Apocynaceae has superior lipolytic activity compared to other plant lipases.

Both frangipani and sea mango latex were further evaluated with various pretreatment before lipolysis tests. The results, presented in

T, show that the supernatant fraction of centrifugated latex has very low lipolytic activities, thus indicating that almost all lipolytic activity resides in the particulate fraction, similar to that found by Giordani et al. (1991) with *Carica papaya* latex. Papaya latex is more acknowledged for its papain enzyme (Malle, Tellusa, & Lasamahu, 2015).

Frangipani latex and sea mango latex were also pretreated with immobilization. Delipidated powder of java almond seeds was used as support. As a cellulosic-based support that naturally contains lipase, the delipidated powder of java almond gives moderate performance of immobilization. Table 4 shows that immobilized latexes have lower activities than free latexes. The effect of immobilization methods and kind of support on the degree of lipolysis of frangipani latex is under investigation.

The high activities of lipases from frangipani and sea mango latexes, combined with the potential availabilities of the latexes, indicate a good possibility of commercial exploitation. Frangipani trees are drought and salt-tolerant. They are frequently seen as landscape trees. They are also cultivated for their fragrance and medicinal uses (Dey & Mukherjee, 2015). Sea mango (*Cerbera manghas*) is a coastal tree and is often associated with mangrove forests with various chemical constituents and anticancer properties (Chan, 2016). This species has not been utilized as crops, but with its abundance in tropical areas and high oil content, it is one of the potential vegetable oil trees to be utilized further (Ong, Silitonga, Mahlia, Masjuki, & Chong, 2014).

Our experience showed that frangipani latex is technically easier to utilize commercially than sea mango latex. Sea mango latex thickens immediately after harvesting. It indicates the possibility of high content of agglutinating proteins such as lectin, which is a typical protein that works as a plant defense protein and causes latex agglutination (Santana et al., 2014). Agglutination slowed the drying process and caused difficulties in centrifugation. The pretreatment method is necessary to develop the utilization of sea mango latex without compromising its lipolytic activity.

Table 3. Degree of olive oil hydrolysis with lipases of dried plant latexes (1%), 0.04% buffer, and 8% water

Latex Lipase Source	Family	Degree of lipolysis (%)
Sea mango	Apocynaceae	34.93%
Jackfruit	Moraceae	0.73%
Aveloz	Euphorbiaceae	0.31%
Frangipani	Apocynaceae	25.00%
Papaya	Caricaceae	9.16%

Table 4. Degree of triglyceride hydrolysis with 1% lipase from plant latex lipase, 0.04% buffer, and 8% water, with variation of pretreatment.

Latex Lipase Source	Pretreatment	Degree of lipolysis	
		4 hours	24 hours
Frangipani	Natural drying	25.00%	54.17%
	Particulate fraction	46.48%	68.22%
	Soluble fraction	1.07%	1.86%
	Immobilized	13.18%	20.77%
Sea mango	Natural drying	34.93%	55.62%
	Particulate fraction	21.63%	41.78%
	Soluble fraction	0.97%	1.79%
	Immobilized	13.38%	29.41%

CONCLUSION

Exploration of plant seeds and plant latexes is necessary to find lipase sources with high economic feasibility. In this research, we report several novel and acknowledged plant lipase activity. Among the novel lipase investigated, kapok seed lipase oil-free powder shows the highest activity, up to 20% degree of lipolysis on olive oil, and even showed higher activity towards kapok seed oil. It implies the potential for lipolysis of polyunsaturated triglycerides.

It is also active at high temperatures (80 ^{o}C), although discoloration appeared on the product, and a temperature of 40 ^{o}C was considered practical for its optimum activity. The optimum pH was 8.0, with significant activity among other pH of 7.0-11.0. Compared to seed lipases, most plant latexes show higher lipolytic activity. The latexes of frangipani (*Plumeria rubra*) and sea mango (*Cerbera manghas*) show remarkable potential for commercial utilization. Shortly, optimum operating conditions should be investigated to discover further potential of latex lipases in industrial applications.

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