BUKTI KOREPONDENSI

DAFTAR ISIAN PENELITIAN

JURNAL ILMIAH

JURNAL INTERNASIONAL BEREPUTASI DAN BERFAKTOR DAMPAK

NO	JENIS ISIAN	ISIAN
1	**Judul Artikel**	Biochemical properties of coconut (Cocos nucifera L) lipase
2	**Penulis**	**1. Lalu Rudyat Telly Savalas**, 2. Sirodjudin Sirodjudin, 3. Erin R. Gunawan, 4. Ro'yal Aini, 5. Dedy Suhendra, 6. Nurul H. Basri, 7. Jannatin 'Ardhuha, and 8. Baiq Nila S. Ningsih
3	**Nama Jurnal**	The Philippines Journal of Science
4	**Tahun Terbit**	2021
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13	**Link Index**	https://www.scimagojr.com/journalsearch.php?q=19700175735&tip=sid&exact=no
14	**Apakah ini syarat khusus**	Tidak, tetapi dapat melengkapi syarat khusus publikasi bereputasi internasional dengan SJR > 0,1

KRONOLOGI KOREPONDENSI

No	Tanggal	Aktivitas	Keterangan
1	4 Maret 2021	Email submission	Email ke Editor Philippines Journal of Science (PJS)
2	8 Maret 2021	Submission acknowledgment	Email dari Asisten Editor
3	8 Maret 2021	Submission ID didapatkan	Email dari Asisten Editor
4	13 April 2021	Permintaan revisi	Email dari Asisten Editor
5	13 April 2021	Pengantar dan Komentar reviewer	Attachment email
6	21 April 2021	Jawaban terhadap reviewer	Itemized response dan perbaikan manuskrip oleh author
7	22 April 2021	Acknowledgement hasil revisi	Email dari Asisten Editor
8	23 April 2021	Konfirmasi review ronde ke-2	Email dari Asisten Editor, naskah dikirim ke reviewer
9	11 Mei 2021	Acceptance	Email dari Editor-in-chief
10	12 Mei 2021	Response atas acceptance	Email ke Editor-in-Chief
11	12 Mei 2021	Perbaikan dari author	Email ke Managing Editor
12	12 Mei 2021	Acknowledgement revisi dari author	Email dari Managing Editor
13	21 Juni 2021	Copyedit/first draft	Email dari Managing Editor
14	21 Juni 2021	Permintaan revisi terhadap first draft	Emai ke Managing Editor
15	5 Juli 2021	Copyedit/second draft	Email dari Managing Editor
16	6 Juli 2021	Persetujuan author terhadap second draft	Email ke Managing Editor
17	6 Juli 2021	Galley/naskah final	Email dari Managing Editor
18	6 Juli 2021	Author approval	Persetujuan author atas naskah final
19	6 Juli 2021	Copyright transfer agreement	Oleh author dengan saksi
20	6 Juli 2021	Co-author agreement	Tanda tangan elektronik semua co-author

Dokumen di bawah ini disusun menurut kronologi seperti pada tabel di atas.

K1 Manuscript submission 4 Maret 2021

Lalu Rudyat Telly Savalas <telly@unram.ac.id>

PJS submission

1 message

Lalu Rudyat Telly Savalas <telly@unram.ac.id> 4 March 2021 at 08:32
To: philjournsci@gmail.com

The Editor-in-Chief
Philippine Journal of Science
Science and Technology Information Institute (STII)
DOST Compound, Bicutan, Taguig City, 1631 PHILIPPINES

Dear Editor-in-Chief,
please find our submission as attached files to be considered for PJS publication.

Should you have further concern regarding our submission, please contact us. Thank you.

Sincerely yours,
on behalf of authors
L RT Savalas (corresponding author)

==========
Dr.rer.nat. Lalu Rudyat Telly Savalas
Dept of Chemistry, Faculty of Teacher Training and Education
University of Mataram
Jl. Majapahit No. 62 Mataram
Nusa Tenggara Barat 83125
Indonesia
Phone +62 370 623873
Fax +62 370 634918
E-mail: telly@unram.ac.id
==========

5 attachments

1. Lalu RT SAVALAS et al Cover letter PJS submission 4 March 2021.pdf
119K

4. Lalu RT SAVALAS et al PJS submission Authorship_Statement 4 March 2021.pdf
275K

3. Lalu RT SAVALAS et al PJS submission List of possible reviewers.pdf
289K

2b. Lalu RT SAVALAS et al PJS manuscript submission 4 March 2021.pdf
648K

2a. Lalu RT SAVALAS et al PJS manuscript submission 4 March 2021.doc
1595K

K2 Submission acknowledgment 8 Maret 2021

Lalu Rudyat Telly Savalas <telly@unram.ac.id>

Re: PJS submission

1 message

Philippine Journal of Science <philjournsci@gmail.com> 8 March 2021 at 15:22
To: Lalu Rudyat Telly Savalas <telly@unram.ac.id>

Dear Dr. Savalas:

This is to confirm the receipt of your complete submission of requirements. I will send another email for the issuance of your reference number.

Thank you for considering the Philippine Journal of Science as a venue for reporting your research findings.

Sincerely,
David Matthew C. Gopilan
Editorial Assistant

For Caesar A. Saloma
Editor-in-Chief

On Thu, Mar 4, 2021 at 8:32 AM Lalu Rudyat Telly Savalas <telly@unram.ac.id> wrote:

> The Editor-in-Chief
> Philippine Journal of Science
> Science and Technology Information Institute (STII)
> DOST Compound, Bicutan, Taguig City, 1631 PHILIPPINES
>
> Dear Editor-in-Chief,
> please find our submission as attached files to be considered for PJS publication.
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> Sincerely yours,
> on behalf of authors
> L RT Savalas (corresponding author)
>
> ==========
> Dr.rer.nat. Lalu Rudyat Telly Savalas
> Dept of Chemistry, Faculty of Teacher Training and Education
> University of Mataram
> Jl. Majapahit No. 62 Mataram
> Nusa Tenggara Barat 83125
> Indonesia
> Phone +62 370 623873
> Fax +62 370 634918
> E-mail: telly@unram.ac.id
> ==========

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Science and Technology Information Institute
Department of Science and Technology
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Scopus: https://www.scopus.com/sourceid/19700175735

K3 Submission ID didapatkan 8 Maret 2021

Lalu Rudyat Telly Savalas <telly@unram.ac.id>

PJS Reference Number Ms 21-049 | Biochemical properties of coconut lipase

1 message

Philippine Journal of Science <philjournsci@gmail.com> 8 March 2021 at 15:24
To: telly@unram.ac.id, Caesar Saloma <caesar.saloma@gmail.com>

Dear Dr. Savalas:

In reference to your manuscript entitled, **"Biochemical Properties of Coconut (*Cocos nucifera* L.) Lipase"** which was submitted for possible publication in the Philippine Journal of Science, your reference number is **Ms 21-049**.

Your paper will be forwarded to the Editor-in-Chief and reviewers for evaluation.

Thank you.

Sincerely,
David Matthew C. Gopilan
Editorial Assistant

Forwarded Conversation

Subject: PJS submission

From: **Lalu Rudyat Telly Savalas** <telly@unram.ac.id>
Date: Thu, Mar 4, 2021 at 8:32 AM
To: <philjournsci@gmail.com>

The Editor-in-Chief
Philippine Journal of Science
Science and Technology Information Institute (STII)
DOST Compound, Bicutan, Taguig City, 1631 PHILIPPINES

Dear Editor-in-Chief,
please find our submission as attached files to be considered for PJS publication.

Should you have further concern regarding our submission, please contact us. Thank you.

Sincerely yours,
on behalf of authors
L RT Savalas (corresponding author)

==========
Dr.rer.nat. Lalu Rudyat Telly Savalas
Dept of Chemistry, Faculty of Teacher Training and Education
University of Mataram
Jl. Majapahit No. 62 Mataram
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Indonesia
Phone +62 370 623873
Fax +62 370 634918
E-mail: telly@unram.ac.id
==========

From: **Philippine Journal of Science** <philjournsci@gmail.com>
Date: Mon, Mar 8, 2021 at 3:22 PM

To: Lalu Rudyat Telly Savalas <telly@unram.ac.id>

Dear Dr. Savalas:

This is to confirm the receipt of your complete submission of requirements. I will send another email for the issuance of your reference number.

Thank you for considering the Philippine Journal of Science as a venue for reporting your research findings.

Sincerely,
David Matthew C. Gopilan
Editorial Assistant

For Caesar A. Saloma
Editor-in-Chief

--

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Science and Technology Information Institute
Department of Science and Technology
DOST Complex, Gen. Santos Ave., Bicutan 1631
Taguig City, Metro Manila, Philippines
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Website: http://philjournalsci.dost.gov.ph
Scopus: https://www.scopus.com/sourceid/19700175735

K4 Email permintaan revisi 13 April 2021

Lalu Rudyat Telly Savalas <telly@unram.ac.id>

Comments on PJS Paper Ms 21-049

1 message

Philippine Journal of Science <philjournsci@gmail.com> 13 April 2021 at 11:59
To: Lalu Rudyat Telly Savalas <telly@unram.ac.id>, Caesar Saloma <caesar.saloma@gmail.com>

LALU RUDYAT T. SAVALAS
Department of Chemistry Education
Faculty of Teacher Training and Education
University of Mataram
Mataram, Indonesia

Dear Dr. Savalas:

This refers to your paper entitled, **"Biochemical Properties of Coconut (*Cocos nucifera* L.) Lipase" [Ms 21-049],** which was submitted for possible publication in the Philippine Journal of Science.

On behalf of Dr. Caesar Saloma, I am sending you the letter of the Editor-in-Chief and the comments of the reviewers regarding its need for revision. Attached also is a copy of your manuscript with comments written on it.

Please submit an itemized list of your answers to the said comments together with the revised version of your paper. You may also provide rebuttal should you not agree with the comments. Kindly notify us upon receiving this letter.

Thank you very much. I look forward to receiving your revised paper.

Sincerely,
David Matthew C. Gopilan
Editorial Assistant

--

Philippine Journal of Science
Science and Technology Information Institute
Department of Science and Technology
DOST Complex, Gen. Santos Ave., Bicutan 1631
Taguig City, Metro Manila, Philippines
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Email: philjournsci@gmail.com
Website: http://philjournalsci.dost.gov.ph
Scopus: https://www.scopus.com/sourceid/19700175735

2 attachments

Ms 21-049 Review Notes.pdf
147K

Ms 21-049 Reviewer 1 Comments on Manuscript.doc
1613K

K5 Pengantar dan komentar reviewer 13 April 2021

Republic of the Philippines
DEPARTMENT OF SCIENCE AND TECHNOLOGY
SCIENCE AND TECHNOLOGY INFORMATION INSTITUTE

ISO 9001:2015

13 April 2021

DR. LALU RUDYAT T. SAVALAS
Department of Chemistry Education
Faculty of Teacher Training and Education
University of Mataram
Mataram, Indonesia

Dear Dr. Savalas:

Thank you for considering the **Philippine Journal of Science** as a venue for publication of your research paper.

After a thorough evaluation of specialists in your field, it is recommended that your paper entitled, **"Biochemical Properties of Coconut (*Cocos nucifera* L.) Lipase" [Ms 21-049],** can be considered for publication only after the following revisions/comments are answered and complied with.

Attached is a copy of the reviewers' comments and recommendations on your paper. Please submit a copy of your revised paper and a checklist of your point-for-point answers to reviewers' comments not later than one (1) month upon receipt of this letter. Otherwise, we will consider the paper as new submission. You may send it through email philjournsci@gmail.com.

Thank you. We hope to receive your revised manuscript soon.

Sincerely yours,

CAESAR A. SALOMA
Editor-in-Chief, PJS
Professor, National Institute of Physics
University of the Philippines Diliman
Quezon City, Philippines

Encl: a/s

COMMENTS ON THE PAPER

Biochemical Properties of Coconut (*Cocos nucifera* L.) Lipase

GENERAL

The manuscript presents a biochemical profile of coconut lipase and its subunits which merit further characterization.

The evaluation of the manuscript has gained favorable recommendations from the two reviewers. The first reviewer commented that the control conditions must be described. Both reviewers commented that the manuscript needs a careful language editing, while more recent studies should be consulted for a more robust interpretation of findings.

In this regard, the manuscript needs a minor revision to enhance its scientific merit and overall presentation. The specific comments of the two reviewers are discussed below in detail.

SPECIFIC

Reviewer 1

1. Overall recommendation is to accept for publication subject to the detailed comments which are given in the attached file.
2. A few edits on the language need to be done, in particular, with respect to the active form such as: “In this study, we isolate lipase from germinating coconut seed. We further performed biochemical characterization of coconut lipase, especially for its specificity and its subunits. By using various chromogenic ester of fatty acids, we showed that lauric acid is the most preferred substrate for coconut lipase esterase reaction.”
3. All of the Control conditions need to be clearly described.
4. I suggest that the authors add the following references with the appropriate discussion:
    a. Nguyen et al., Hydrolysis Activity of Virgin Coconut Oil Using Lipase from Different Sources, Scientifica Volume 2018, Article ID 9120942, 6 pages
    b. Chua et al., Hydrolysis of Virgin Coconut Oil Using Immobilized Lipase in a Batch Reactor, Enzyme Research, Volume 2012, Article ID 542589, 5 pages
    c. Subashri et al., Extraction and partial purification of lipase from coconut seeds, International Journal of Research in Pharmaceutical Sciences, 2018; 9 (2): 442-445.

Please see attached file with detailed comments and suggestions.

Reviewer 2

Overall, this study gave a comprehensive understanding in characterization of lipase from coconut oil. The methodologies were well described which helped viewers easily access to this field of study. The revealed results were relatively adequate to demonstrate the characteristics of obtained lipase from coconut oil. However, the writing style should be changed in the whole content. Passive tense and past tense should be used to describe or discuss results from previous reports and author's results. More explanation of the results should be included to strongly support author's finding.

Pay attention to typos.

line	Comments and Recommendations
187-196	This paragraph should be in the context of introduction rather than discussion part
125	Enzyme activity should be presented in U/mL or U/mg enzyme
198-208	Is this paragraph better to be in methodology section?
215	Could authors describe the result in Figure 2 in details?
224	Why did calcium ions activate enzyme activity while the other ions showed an inhibitory effect?
28, 30, 107, 134, many others	Passive tense should be used
224, 231, many others	Previous study should be discussed in past tense. Misspellings

OTHERS

Please include an itemized list of your answers to the above comments in the revised version of your paper.

Biochemical Properties of Coconut (*Cocos nucifera* L.) Lipase

Lalu Rudyat T. Savalas[1*], Sirodjudin Sirodjudin[2], Erin R. Gunawan[2], Ro'yal Aini[2], Dedy Suhendra[2], Nurul H. Basri[2], Jannatin 'Ardhuha[3], Baiq Nila S. Ningsih[1,4]

[1] Department of Chemistry Education, Faculty of Teacher Training and Education, University of Mataram, Mataram 83125, Indonesia. *Corresponding author e-mail: telly@unram.ac.id; phone +62 370 623873, fax +62 370 634918

[2] Department of Chemistry, Faculty of Mathematics and Natural Sciences, University of Mataram; Mataram 83125, Indonesia.

[3] Department of Physics Education, Faculty of Teacher Training and Education, University of Mataram, Mataram 83125, Indonesia.

[4] Division of Physical Science, Faculty of Science, Prince of Songkla University, Hat Yai, Songkla 90110, Thailand.

Running head: biochemical properties of coconut lipase

Keywords: coconut lipase, substrate specificity, native electrophoresis, lipase subunits, in-gel assay

*Corresponding Author: telly@unram.ac.id; Phone +62 (0370) 623873

ABSTRACT

Ubiquitous in nature, Lipases represent an example of enzymes with high versatility. Nevertheless, they offer specificity for various applications. Plant seeds are potential sources of lipase, and they are attracting more attention for specific purposes. In this study, we isolate lipase from germinating coconut seed. We further performed biochemical characterization of coconut lipase, especially for its specificity and its subunits. By using various chromogenic ester of fatty acids, we showed that lauric acid is the most preferred substrate for coconut lipase esterase reaction. Calcium ions enhance its activity, whereas other metal ions such as magnesium, nickel, sodium, and potassium reduce it. Electrophoresis under native conditions showed that coconut lipase is a single protein. Since electrophoresis under denaturing conditions revealed four subunits, coconut lipase is likely a complex enzyme. It is further shown that all subunits are active, as evident in an in-gel hydrolysis assay. However, they also hint that they do not have an equal catalytic rate against the 16 carbon length palmitate derivative. This finding thus opens up a notion that those subunits have different substrates specificity yet to be determined.

Commented [A1]: Suggest to delete this sentence.

Commented [A2]: The age of coconut in months should be specified.

INTRODUCTION

Fatty acids are widely used in modern life and hence are of the critical industrial concern. The utilization of fatty acids spans from essential ingredients in many industries, such as in coating (Rajput *et al.* 2014), surfactants (Semblante *et al*. 2009),

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lubricants (Ruths *et al.* 2008), essential fatty acids, nutraceuticals productions (Sande *et al.* 2018), personal cares (Tavares *et al.* 2018), and bioremediation (Melani *et al.* 2019). Several methods achieve fatty acid production from fats, such as the mechanical separation process, alkaline chemical hydrolysis, and enzymatic process. Mechanical separation requires high pressure and temperature. It causes the process costly, although beneficial in terms of yield. An additional drawback with this method is that the separation of undesirable by-products needs extra production effort. These may reduce the revenues of fatty acid production via this route (Sande *et al.* 2018). The Colgate-Emery process operating at 250 °C, and pressure as high as 4.82 MPa is an example of fatty acid production by mechanical separation. The process is accompanied by oxidation and dehydration products despite its high conversion rate (Tavares *et al.* 2018). Likewise, alkaline hydrolysis also offers a practical method. However, efforts are required to separate unwanted products (Sande *et al.* 2018). In contrast, enzymatic hydrolysis operates at mild conditions (low temperature and pressure). It offers ease in the recovery process (Jain and Mishra, 2015) and product loss due to minimized overheating (Barros *et al.* 2010).

Commented [A3]: This introductory section (lines 42 to 60) is too long. This paragraph should be compressed and combined with the next paragraph.

Lipases or glycerol ester hydrolases (EC 3.1.1.3) are enzymes that can perform hydrolysis, esterification, and transesterification reactions under mild conditions. Which reaction takes place largely depends on the reaction environment (Tavares *et al.* 2018). Lipases act on different ester compounds, with acylglycerols become their prominent substrates. Significant sources of lipases are microbes and animals. Many enzymes

Commented [A4]: replace with "principal"

Commented [A5]: Suggest to delete this sentence. All oil seed plants have significant amounts of lipases.

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serving as immobilized catalysts in industries are derived from those origins (Santana *et al.* 2011). Plant-based lipases are increasingly become the researcher's interest due to low production cost and high specificity (Tavares *et al.* 2018; Villeneuve, 2003). They also have an easy pharmacological acceptance due to their eukaryotic source (Seth *et al.* 2014). Essential sources of plant-based lipases are plant seeds, especially the seeds in their germinating phases. Examples are lipases from *Carica papaya* (Campillo-Alvarado and Tovar Miranda, 2013), *Pentaclethra macrophylla* (Enujiugha *et al.* 2004), Linseed (Sammour, 2005), and coconut (Ejedegba *et al.* 2013). However, significant lipase activity from non-germinating seeds also exists, such as in castor beans (Eastmond, 2004; Tavares *et al.* 2018).

Coconut trees grow almost in every region in the tropics. The physical appearance of coconut fruits is very distinct and easy to handle. As a consequence, their utilization as lipase sources is foreseeable. To date, coconut lipase's biochemical characterization has not been sufficiently reported, thus limited its applications. In this context, the present study investigates the biochemical characterization of coconut lipase. The work includes the study of coconut lipase substrate specificity and the property of its subunits. A thorough understanding of the biochemical properties of coconut lipase will lead to its plausible application.

Commented [A6]: This is an inaccurate reference for this statement.

Commented [A7]: Mainly along coastal areas of the tropics.

Commented [A8]: Awkward.

MATERIALS AND METHODS

*Corresponding Author: telly@unram.ac.id; Phone +62 (0370) 623873

Material

Golden coconut (local: gading coconut) was obtained from a local garden in Lombok Island of Indonesia. Reagents for buffer and electrophoresis of p.a. grades were obtained from major chemical suppliers. We bought the virgin coconut oil (VCO) from a local vendor. The artificial lipase substrates were *p*-nitrophenyl butyrate, *p*-nitrophenyl octanoate, *p*-nitrophenyl-decanoate, *p*-nitrophenyl dodecanoate, *p*-nitrophenyl myristate, and *p*-nitrophenyl palmitate, and *p*-nitrophenol (Merck/Sigma-Aldrich). Reagents for in-gel lipase assays were Fast Blue B salt (Sigma-Aldrich) and alpha-naphthyl palmitate (Santa Cruz Biotechnology, USA). Assay buffer contained Arabic gum (Sigma-Aldrich) and sodium deoxycholate (Sigma-Aldrich). Protein determination used a bicinchoninic acid (BCA) kit from Thermo scientific. We used Prism 7 (GraphPad) for graphical preparation and Image-J for dye density calculation.

Commented [A9]: Spell out.

Commented [A10]: "Virgin coconut oil (VCO) was purchased from a local vendor."

Methods

Coconut germination, crude extract preparation, and protein determination

Ripe and dried coconut (*Cocos nucifera* L.) was allowed to germinating in humid, and when this stage was reached (c.a. a month), coconut flesh was collected. The flesh was shredded and resuspended in 5 mM phosphate buffer, pH 7.0. We filtered the suspension by using a filter cloth. The resulted in coconut milk was centrifuged at 3,000 rpm for 20 mins at 4 °C. The floating cream was removed from a 50 mL conical centrifuge tube. The skim fraction was decanted and further subjected to freeze-drying to reduce water content. The resulted in 15 mL concentrated coconut lipase was stored at -20 °C for further analysis. Protein concentration was determined using the BCA kit

Commented [A11]: Confusing:
1. "Ripe" needs to be defined. The of age of the coconut should be given in terms of months.
2.What do they mean by "dried coconut"? This should be described more quantitatively.

*Corresponding Author: telly@unram.ac.id; Phone +62 (0370) 623873

according to the manufacturer's instruction. The developed color was measured at 562 nm by a spectrophotometer (MultiSkan GO, Thermo scientific).

Enzyme assay

Coconut lipase activity was assayed for its hydrolytic activity against virgin coconut oil as a substrate (Khor *et al*. 1986). The reaction mixture consisted of 5 grams VCO, 2.5 mL n-hexane, 5 mL 100 mM phosphate buffer pH 7.5, and 1 mL of the enzyme. The mixture was incubated in a 35 °C water bath shaker for 45 mins, and after this period, 25 mL of acetone/methanol (1:1) was added. The liberated free fatty acids were determined by titration. We used 0.01 M sodium hydroxide for the titration following the addition of a few drops of phenolphthalein. Sodium hydroxide was previously standardized against sodium oxalate. Lipase activity was calculated as follows:

$$\text{Lipase activity (U)} = \frac{(Vsample - Vblank)\,x\,[NaOH]\,x\,1000}{Venzyme\,x\,t}\ \text{U}$$

Where V_{sample} = titrant volume for sample
V_{blank} = titrant volume for blank
V_{enzyme} = coconut lipase volume
[NaOH] = sodium hydroxide concentration

Coconut lipase activity in the presence of metal ions

Coconut lipase activity was assayed against VCO, as previously described, in the presence of several metal ions. We used 10 mM magnesium, calcium, sodium, potassium, iron, copper, and zinc ions.

*Corresponding Author: telly@unram.ac.id; Phone +62 (0370) 623873

Substrate specificity of coconut lipase

We first determined the assay condition that allows kinetics analysis of coconut lipase. We chose hydrolysis assay of *p*-nitrophenyl palmitate by serial dilution coconut lipase. The intensity of the liberated *p*-nitrophenol was measured at 405 nm. Hydrolysis profiles of *p*-nitrophenyl palmitate were recorded every 5 minutes with lipase dilution range from 1: 3,000 to 1: 100,000.

For different pNP-fatty acids, an 8-minute reaction with 1:100,000 dilution of lipase stock was further employed. For each reaction, the pNP-fatty acid substrates were prepared as follows: 2 mL of 8 mM pNP-fatty acid in n-propanol was added to 18 mL of an emulsifier solution. The emulsifier contained 20 mg of Arabic gum and 41.4 mg sodium deoxycholate in 50 mM Tris-Cl buffer pH 8.0. The substrate solution was kept in the dark before use. The final concentration of pNP-fatty acid in the substrate solution was 0.8 mM. Hydrolysis assay was performed by pre-incubating of 2.7 mL substrate solution at 37 °C for 5 minutes before the addition of 0.3 mL diluted lipase. The yellow color formation was recorded after 8 minutes at 405 nm. We tested coconut lipase specificity against *p*-nitrophenyl butyrate, *p*-nitrophenyl octanoate, *p*-nitrophenyl decanoate, *p*-nitrophenyl dodecanoate, *p*-nitrophenyl myristate, and *p*-nitrophenyl palmitate. One unit activity (U) is defined as micromole(s) of *p*-nitrophenol released upon hydrolysis by 1 mL enzyme at 37 °C under assay conditions (Kanwar *et al*. 2005).

SDS-PAGE and Native PAGE

SDS-PAGE was undertaken according to the method initially developed by Laemmli (1970) on 12.5% separating gel and 5% focusing gel. Briefly, a sample containing 50 µg

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of coconut lipase was precipitated by the addition of an equal volume of cold absolute-ethanol. The resulted precipitate was resuspended in a 5X sample buffer and boiled for 2 minutes prior to electrophoresis. Electrophoresis was accomplished by applying 150 Volt electric current in a mini gel (MiniVe SDS-PAGE apparatus, GEHealthcare) for c.a. 2 hours. The resulted gel was stained with Coomassie Brilliant Blue (CBB). For native-PAGE, coconut lipase was subjected to electrophoresis under non-denaturing conditions, i.e., by omitting SDS from the gel and running buffer. The sample buffer was also void of 2-mercaptoethanol or dithiothreitol. Ammonium sulfate fractionation was undertaken according to Sana and coworkers (Sana *et al*. 2004). Briefly, ammonium sulfate threshold of 0-30%, 30-45%, 45-60%, 60-75%, and 75-90% saturation was added to the protein sample. The excess of salt was removed by dialysis from each fraction. The resulted fractions were subjected to both SDS and native PAGE.

In-gel hydrolysis assay

The activity of lipase subunits was assayed after lipase was separated in 12.5% gel SDS-PAGE. In this case, the sample was not boiled to keep the protein intact. After separation, SDS content was washed by soaking the gel in 50 mM phosphate buffer pH 7.0 and 2.5% (v/v) Triton X-100. It was done with gentle shaking for 30 minutes. The washing step was repeated twice. The gel loaded with lipase was incubated in a developing solution for 30 minutes in a dark container to allow hydrolysis to proceed. The developing solution contained alpha-naphthyl palmitate and Fast Blue B salt. Unbound dye was removed by three-time washing in aquadest, 10 minutes each. The hydrolysis of alpha-naphthyl palmitate corresponds to the lipase subunit activity. An

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active subunit releases a yellow color of alpha-naphthol (Zienkiewicz *et al.* 2014) that appears on the gel. We also prepared an identical gel stained by Coomassie Brilliant Blue for comparison.

RESULTS AND DISCUSSION

Plant base lipases have recently attracted more researcher's attention due to their unique properties. As a fruit with a lot of lipid content, coconut is a potential source of lipase. The focus of coconut exploration in lipase research was limited to the use of coconut as a medium for lipase-producing fungi (Benjamin and Pandey, 1997). It is also used in the immobilization study of other lipases (Brigida *et al.* 2007). Additionally, coconut provides a substrate for lipase reaction (Ibrahim *et al.* 2008). In this study, we isolate coconut lipase from germinating coconut and investigate its properties. Since many biochemical properties of coconut lipase remain unclear, coconut lipase's biochemical characterization is necessary, and the results will facilitate further utilization of coconut lipase.

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Germination of coconut fruit is attained by storing coconut fruit in humid. The coconut shoot's appearance showed germination after c.a. a month of storage (Figure 1). The germination process is accompanied by the development of haustorium inside coconut fruit (Figure 1). As the coconut flesh is the primary food storage, coconut lipase is isolated only from the part. Nevertheless, literature reported that all parts of germinating coconut have lipase activity, with the shoot being the most active part (Su'i and Suprihana, 2013). The coconut of average size results in c.a. 200 grams of meat. The

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fraction containing coconut lipase is obtained by centrifugation of coconut flesh suspension. The cream fraction is removed, and the skimmed fraction containing lipase is used for enzyme assay (Figure 1). Protein determination using the bicinchoninic acid method (BCA kit) resulted in a typical 3 mg/mL protein concentration.

Virgin coconut oil (VCO) was used as the substrate for coconut lipase hydrolysis activity instead of using popular olive oil since it offers a more comprehensive composition of fatty acids ester from various chain lengths. The isolated coconut lipase has an activity of 0.56 U/mL corresponding to a specific activity of 0.30 U/mg protein. These results resemble those reported by Su'i and Suprihana (Su'i and Suprihana, 2013). Figure 2 shows that coconut lipase has a very high esterase activity. A sample dilution by a factor of 50 thousand times would lead to an immediate saturation curve. The high lipase activity from various germinating seeds has been reported (Barros *et al.* 2010) with Castor bean (Eastmond, 2004), and Egusi melon seed (Barros *et al.* 2010) are only a few exceptions as their ungerminated seeds also show significant lipase activity.

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Many lipases have their activity altered in the presence of specific metal ions. Here we tested the effect of several metal ions on the esterase activity of coconut lipase. Figure 3 shows that calcium ions act as coconut lipase activators. A literature survey also suggests that calcium ions activate many plant lipases, such as those from white melon kern (Eze and Ezema, 2012). On the other hand, Fe^{3+}, Cu^{2+}, Zn^{2+}, and Mg^{2+}, as well as alkali ions K^{+} and Na^{+}, decrease the esterase activity of coconut lipase. The effect of

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metal ions on various plant base lipase activity is depicted in Table 1. To our knowledge, only a few lipases are inhibited by magnesium ions, such as rice bran (Bhardwaj *et al.* 2001), almond seed lipase (Yesiloglu and Baskurt, 2013), and Africa bean seed (Enujiugha *et al.* 2004) lipases (Table 1). Coconut lipase adds a new member~~t~~ to the relatively short list of plant seed lipases inhibited by magnesium ions.

The substrate specificity of coconut lipase was analyzed using various *p*-nitrophenyl fatty acid esters of different chain lengths. Figure 4 shows that *p*-nitrophenyl laurate (C12) gives the highest hydrolysis product, and the longer fatty acids (C14 *p*-nitrophenyl myristate and C16 *p*-nitrophenyl palmitate) come the next. The shorter fatty acids (C4 *p*-nitrophenyl butyrate, C8 *p*-nitrophenyl octanoate, and C10 *p*-nitrophenyl decanoate) give lower hydrolysis products in the same incubation time. Lauric acid (C12) is the predominant fatty acid of coconut that belongs to the medium-chain fatty acid (Manohar *et al*. 2019; Dayrit, 2014). The aforementioned result indicates that coconut lipase, in order of preference, hydrolyzes medium, long, and short-chain fatty acid esters.

SDS-PAGE and native-PAGE of coconut lipase are shown in Figure 5. SDS-PAGE electroforegram reveals at least four protein bands of approximately 54 kDa, 32 kDa, 21 kDa, and 15 kDa. However, native-PAGE reveals only a single band of c.a. 130 kDa. After thresholds of ammonium sulfate precipitation, separation of coconut lipase also shows a single complex band in native PAGE for all fractions (Figure 6). Together, these suggest that coconut lipase is a heteromeric enzyme. In humans, hormone-

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sensitive lipase, an enzyme involves in the mobilization of lipid storage in adipose tissue, has long been shown to be more active in its ~160 kDa dimer. It is 40 times more active than the ~85 kDa monomer form (Shen *et al.* 2000). A reverse situation is recently reported for the human Lipoprotein lipase, whose 55 kDa monomer has similar activity to its 110 kDa homodimer (Beigneux *et al.* 2019). The fact that coconut lipase consists of several subunits and that it is not universal that all subunits of given lipase are functional highlights the need to dissect whether all coconut lipase subunits are active. To address the above question, we performed an in-gel cleavage assay.

In-gel hydrolysis assay allows us to analyze whether a specific protein can hydrolyze fatty acyl ester after the separation of proteins by electrophoresis. An active protein within the gel cleaves the substrate alpha naphthyl palmitate to release a yellow coloring of naphthol (Figure 7a), following SDS removal from the gel (Zienkiewicz *et al.* 2015). Figure 7b shows that all coconut lipase subunits can hydrolyze alpha naphthyl palmitate, which indicates that all coconut lipases are active. Two subunits with equal intensity on coomassie staining produce a different naphthol intensity, demonstrated by the 54 kDa dan 21 kDa subunits (Figure 7b). It suggests that the two subunits have a different affinity to alpha naphthyl palmitate, with the latter has a lower affinity. However, this does not nullify the possibility that the 21 kDa subunit has a higher esterase activity for shorter fatty acids. It is worth testing whether its cleavage to medium-chain and short-chain fatty acids give the same pattern. There are faint protein bands at c.a. 40 kDa and 45 kDa in addition to the four distinct subunits. We speculate that the two

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proteins are glycosylated forms of the 32 kDa subunit. Our finding that coconut lipase consists of several active subunits may explain confronting reports on plant seed lipase activities, such as those from rice *Oryza sativa* lipase (Table 1).

The data presented in this study shows that all coconut lipase subunits can cleave fatty acid esters, regardless of their hydrolysis rate. However, it is worth noting that the stereoselectivity of coconut lipase remains unclear. To address this issue, the hydrolysis of triacylglycerol substrates will provide the required data. From the present experiment, we expect that coconut lipase has the highest affinity to trilaurin. Moreover, to give details of individual subunits' activity, it is deemed necessary to separate the subunits and test their specificity. Such a study may reveal the contribution of subunits to the coconut lipase as a whole. Furthermore, if cloning and heterologous expression are desired, this can be directed to the study of individual subunits, especially at the current circumstance when the coconut genome is emerging on the horizon (Xiao *et al.* 2017). Accordingly, biochemical characterization of various subunits (optimum temperature and pH reaction, substrate specificity, metal ions effect, and detergent effect) would provide more detailed information.

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"However, it is worth noting that the regioselectivity and stereoselectivity of coconut lipases remain unclear."

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Commented [A19]: Suggested rewording:
"From the present experiment, we conclude that coconut lipase gives the highest activity with lauryl esters."

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CONCLUSION

By using a simple procedure, we have been able to isolate coconut lipase. A direct comparison of SDS-PAGE and native PAGE readily hint that coconut lipase is a

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complex enzyme. This enzyme consists of four subunits of 54 kDa, 32 kDa, 21 kDa, and 15 kDa. In its complex form, lauric acids are the most preferred substrate for coconut lipase. The enzyme is activated by Ca^{2+} ion, whereas Fe^{3+}, Cu^{2+}, Zn^{2+}, Mg^{2+}, K^{+}, and Na^{+} decrease coconut lipase activity. Each subunit can cleave the fatty acid ester bond; hence this enzyme might be regarded as a cluster of smaller active proteins. Since all coconut lipase subunits are active as esterases, specificity determination of subunits and further biochemical characterization of the subunits are yet to be investigated. We argue that a similar approach can be applied for the initial study of other plant or seed-based lipases.

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"In its complex form, the coconut lipase shows highest preference for lauryl esters."

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ACKNOWLEDGMENTS

This research was partially funded by the Ministry of Education and Culture Republic of Indonesia through the Insinas research grant. Additional support was from the Research and Community Service Institute of the University of Mataram. The authors thank Siti Rosidah for technical assistance.

STATEMENT ON CONFLICT OF INTEREST

All authors declare to have no conflict of interest.

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419 **Table 1.** Properties of some plant-based lipases

No	Lipase Source	MW (kDa)	Activator	Inhibitor	Reference
1[a]	Rice bran (*Oryza sativa*)	~ 10	Potassium acetate, sodium acetate, NaCl	CHAPS, digitonin, SDS, NP-40, Triton X-100, Mg^{2+}, Mn^{2+}, Cu^{2+}, Cd^{2+}	(Barros *et al.* 2010)
	Rice Bran Lipase II	33	n.a.	n.a.	(Aizono *et al.* 1976)
	Rice Bran	40	n.a.	n.a.	(Kim, 2004)
2[b]	Castor bean (*Ricinus communis* L.)	60	Ca^{2+}	p-Chloromercuribenzoic, $HgCl_2$	(Eastmond, 2004)
	Castor bean	n.a.	Slightly activated by Na^{+}, K^{+}, and dithiothreitol	Mg^{2+}, Ca^{2+}	(Muto and Beevers, 1974)
	Castor bean	60	Mn^{2+}, Na^{+}, K^{+}, Al^{3+} and Li^{+}	Zn^{2+}, Co^{2+}, Pb^{2+} and Cu^{+}	(Su *et al.* 2010)
3	Linseed (*Linum usitatissimum)*	42	Mg^{2+}, K^{+}	Triton x-100, Tween 80	(Sammour, 2005)
4	Almond seed *(Amygdalus communis* L.)	n.a.	Ca^{2+}, Fe^{2+}, Mn^{2+}, Co^{2+}, Ba^{2+}	Mg^{2+}, Cu^{2+}, Ni^{2+}	(Yesiloglu and Baskurt, 2013)
5	Africa Bean seed (*Pentachlethra macrophylla* Benth)	n.a.	Ca^{2+}	NaCl, $MgCl_2$, EDTA	(Enujiugha *et al.* 2004)
6	Sunflower seed (*Helianthus annuus* L)	40-50	Ca^{2+}, Mg^{2+}	Hg^{2+}, EDTA	(Sadeghipour and Bhatla, 2003)
7	Canola lipase (*Brassica napus*)	n.a.	Ca^{2+}, Bi^{3+}	Fe^{2+}, Fe^{3+}, Zn^{2+}, Hg^{2+} and Cu^{2+}	(Sana *et al.* 2004)

420 MW: Molecular Weight; n.a.: not available; FA: Fatty Acid; TAG: Triacylglycerol
421 [a]) different reports of lipases from rice bran.
422 [b]) different reports for lipase from these seeds suggest that they have at least two
423 lipases, i.e., the acid and alkaline lipase
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426 **Figure 1.** Preparation of coconut lipase from the germinated coconut fruit

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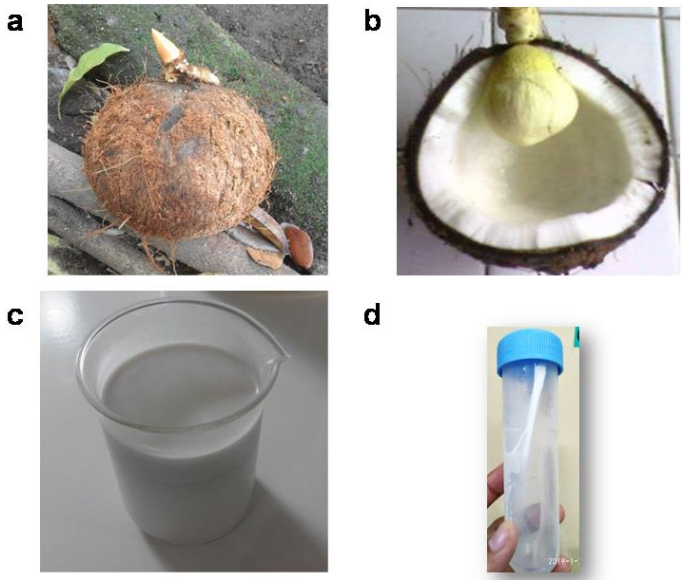

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442 **Figure 1.** Preparation of coconut lipase from the germinated coconut fruit

443 a. Coconut shoot appears after a month of germination. b. Inside the hard shell,
444 haustorium is developing. Mucilage or coconut flesh was removed and further used as
445 the source of coconut lipase. c. Coconut milk prepared by suspending shredded
446 coconut flesh in 5 mM phosphate buffer, pH 7.0. d. Following centrifugation, the cream
447 fraction was removed. The clear fraction of coconut milk was decanted and stored for
448 electrophoresis and enzyme assays.

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Figure 2. Coconut lipase activity

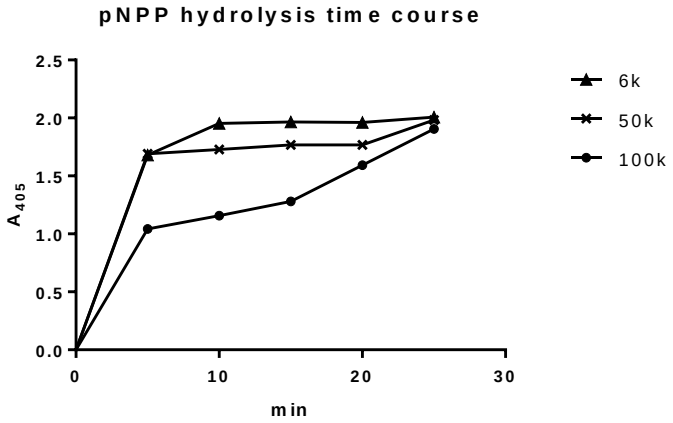

Figure 2. Coconut lipase activity

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The activity of coconut lipase of different dilutions was assayed at different time points. The yellow color showed lipase activity as a result of *p*-nitrophenyl palmitate hydrolysis. After ten minutes of reaction, the saturated curve is observed by 6,000 times diluted lipase. For the more diluted lipase, saturation is delayed. In the substrate specificity assay, 8 minutes of incubation times were chosen, with the sample diluted by 100,000 factors.

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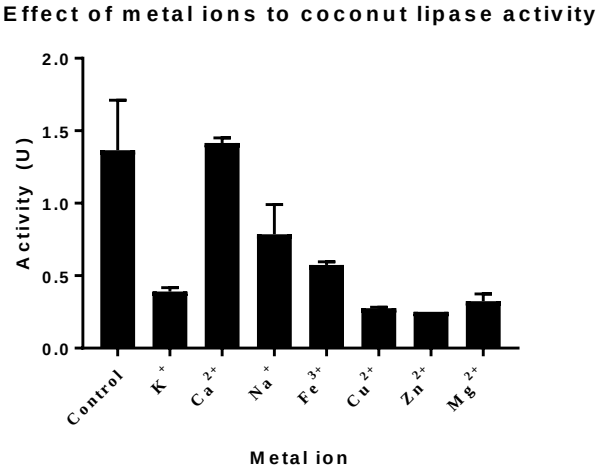
462 **Figure 3.** The activity of coconut lipase with the presence of metal ions.

Commented [A24]: The Control condition needs to be described in detail.



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465 **Figure 3.** The activity of coconut lipase with the presence of metal ions.

466 The effect of metal ions on coconut lipase activity was assayed by the inclusion of 10

467 mM of respective metal ions in the assay mixture. The released free fatty acids were

468 titrated by using sodium hydroxide. Measurements were made triplicate.

Commented [A25]: "made in triplicate."

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Figure 4. Substrate specificity of coconut lipase

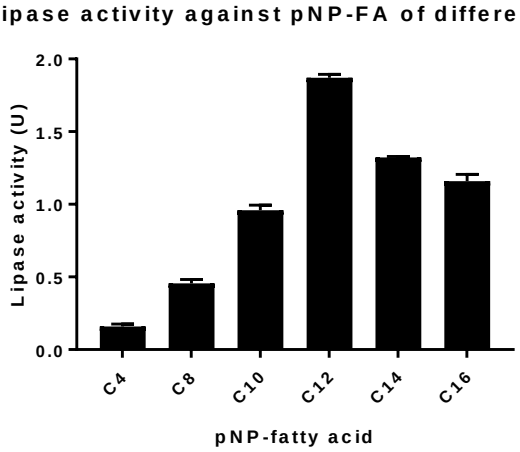

Figure 4. Substrate specificity of coconut lipase

Diluted coconut lipase was allowed to hydrolyze various esters of fatty acids (*p*-nitrophenyl butyrate, *p*-nitrophenyl octanoate, *p*-nitrophenyl decanoate, *p*-nitrophenyl dodecanoate, *p*-nitrophenyl myristate, and *p*-nitrophenyl palmitate) for 8 minutes of reaction. The released *p*-nitrophenol was measured at 405 nm, and the obtained values were converted to lipase activity.

Commented [A26]: Indicate how many replicates.

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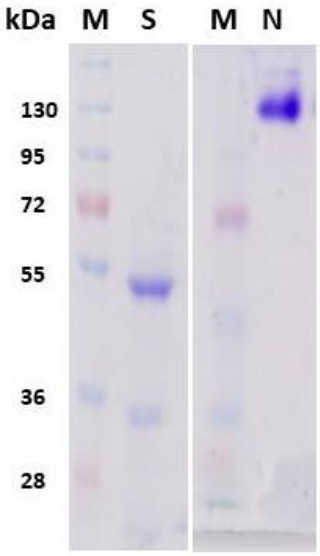
479 **Figure 5.** Coconut lipase separation in SDS-PAGE and Native-PAGE



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481 **Figure 5**. Coconut lipase separation in SDS-PAGE and Native-PAGE

482 Coconut lipase separated on a 10% gel of SDS-PAGE (S) shows different protein
483 bands, i.e., 54 kDa, 32 kDa, and 21 kDa. The bands are from a single complex protein
484 of c.a. 130 kDa, as shown in native-PAGE (N). Note: the smallest band of c.a. 15 kDa is
485 not shown here but obvious on a 12.5% gel (Figure 6 and 7b).

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Figure 6. Ammonium sulfate fraction

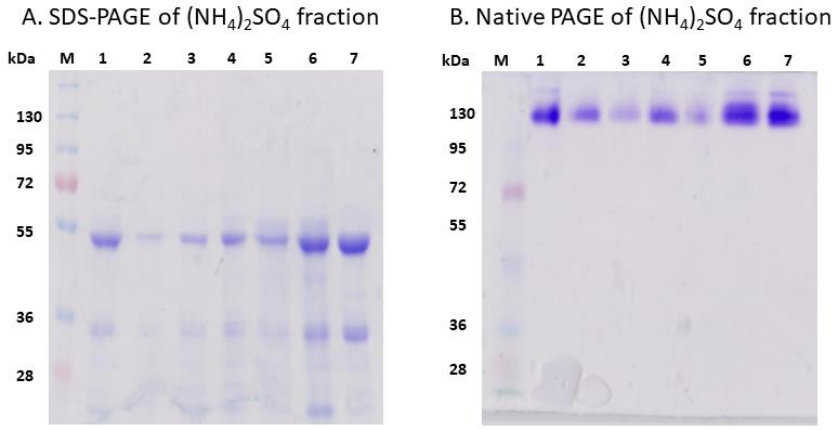

Figure 6. Ammonium sulfate fractions of coconut lipase separated by SDS-PAGE (A) and native PAGE (B). M: protein marker; 1: crude extract; 2: fraction 0-15%; 3: fraction 15-30%; 4: fraction 30-45%; 5: fraction 45-60%; 6: fraction 60-75%; and 7: fraction 75-90%.

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Figure 7a. Hydrolysis of alpha-naphthyl palmitate by lipase.

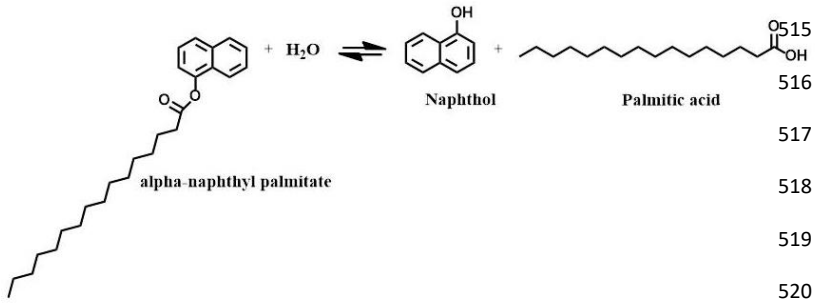

Figure 7a. Hydrolysis of alpha-naphthyl palmitate by lipase.

An active lipase or esterase cleaves alpha-naphthyl palmitate to release naphthol. The yellow color of naphthol is measured spectrophotometrically at 405 nm.

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Figure 7b. In-gel activity assay of coconut lipase.

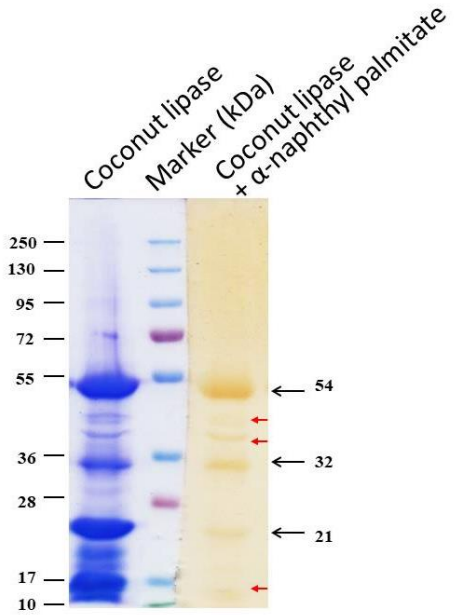

Figure 7b. In-gel activity assay of coconut lipase.

Coconut lipase is separated in a 12.5% polyacrylamide gel under denatured conditions, except for the boiling step. The gel was cut for coomassie staining (left) and an in-gel assay(right). At least four distinct bands are noticed upon coomassie staining, including the smallest subunit of c.a. 15 kDa. Additional faint bands are seen above the 32 kDa. The corresponding hydrolysis products by lipase subunits appear as yellow bands. It represents the results of alpha naphthyl palmitate hydrolysis by respective lipase subunits. The pixel density ratio of naphthol to coomassie for 54 kDa and 21 kDa subunits is 77.5% and 43.2%, respectively. Note: several distinct bands above the 32 kDa band (red arrows) are shown to be active in hydrolyzing the alpha naphthyl

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552 substrate. It gives a hint that the 32 kDa is glycosylated. We also speculate that a
553 smeary pattern observed in the native gel (Figure 5) indicates the glycosylation of native
554 protein.

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K6 Jawaban terhadap komenter reviewer 21 April 2021

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Ms 21-049 Response to reviewer's notes and revised manuscript

1 message

Lalu Rudyat Telly Savalas <telly@unram.ac.id> 21 April 2021 at 23:05
To: Philippine Journal of Science <philjournsci@gmail.com>

CAESAR A. SALOMA
Editor-in-Chief, PJS
Professor, National Institute of Physics
University of the Philippines Diliman
Quezon City, Philippines

Dear Prof. Saloma,
in response to the reviewer notes to our submitted manuscript entitled: “Biochemical Properties of Coconut (Cocos nucifera L.) Lipase” [Ms 21-049], here we attach itemized answers to the reviewer's notes, as well as the revised version of our manuscript.

We hope that our manuscript can be proceeded for publication in the coming issue of the Philippine Journal of Science.

Kind regards,

On behalf of all authors,

Dr. Lalu Rudyat T. Savalas
corresponding author

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2 attachments

Ms 21-049 Response to Reviewer's notes.docx
39K

Ms 21-049 Manuscript PJS REVISION.doc
933K

Mataram, Indonesia, 21 April 2021

CAESAR A. SALOMA
Editor-in-Chief, PJS
Professor, National Institute of Physics
University of the Philippines Diliman
Quezon City, Philippines

Dear Prof. Saloma,

Thank you for your positive response to our submitted manuscript **("Biochemical Properties of Coconut (*Cocos nucifera* L.) Lipase" [Ms 21-049]).** On behalf of all author, I also thank for forwarding the reviewer's comments and recommendations for our manuscript.

Enclosed is our itemized response to the comments and recommendations of both reviewers. The revised version of our manuscript is attached in a separate file. We have addressed all reviewer concerns and, wherever applicable, added explanation to previous text that was pooply delivered.

We hope that our manuscript will finally be accepted for publication in the coming issue of the Philippine Journal of Science. Should there are future concerns, we would be available for further correction.

Thank you in advance and we look forward to hearing from you.

On behalf of authors,

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Faculty of Teacher Training and Education
University of Mataram
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RESPONSE to REVIEWER 1

No	Reviewer notes	Authors response
1	Overall recommendation is to accept for publication subject to the detailed comments which are given in the attached file	All author appreciate the reviewer views and recommendations to our manuscript. We have done our best to accomodate reviewer notes, and wherever necessary elaborated few texts that need further explanation.
2	A few edits on the language need to be done, in particular, with respect to the active form such as: "In this study, we isolate lipase from germinating coconut seed. We further performed biochemical characterization of coconut lipase, especially for its specificity and its subunits. By using various chromogenic ester of fatty acids, we showed that lauric acid is the most preferred substrate for coconut lipase esterase reaction."	We have changed the text into passive form: "In this study, coconut lipase was isolated from germinating coconut seed. Biochemical characterization of coconut lipase was undertaken to reveal its substrate specificity and its subunits properties. By using various chromogenic ester of fatty acids, it was demonstrated that lauric acid is the most preferred substrate for coconut lipase esterase reaction". *(Line 27-31)* Other parts of the text have also been modified, as suggested by both Reviewer 1and Reviewer 2.
3	All of the Control conditions need to be clearly described.	Control condition has been described, especially for Comment A24 of the reviewed manuscript
4	I suggest that the authors add the following references with the appropriate discussion: a. Nguyen et al., Hydrolysis Activity of Virgin Coconut Oil Using Lipase from Different Sources, Scientifica Volume 2018, Article ID 9120942, 6 pages b. Chua et al., Hydrolysis of Virgin Coconut Oil Using Immobilized Lipase in a Batch Reactor, Enzyme Research, Volume 2012, Article ID 542589, 5 pages c. Subashri et al., Extraction and partial purification of lipase from coconut seeds, International Journal of Research in Pharmaceutical Sciences, 2018; 9 (2): 442-445.	*Ref a and b.* Authors thank for the suggestion. Ref Nguyen *et al*. and Chua *et al*. have been integrated in the discussion section in the context that VCO had been investigated in many lipase optimation studies (Line 201-203). We additionally referred to the two articles to give insight that, whereas VCO is valuable in the study of complete hydrolysis study, various pNP-acyl esters are advantagous in a kinetics study to determine substrate preference of lipase (Line 241-243) *Ref c.* Subashri *et al*. report has very limited data and only identified a coconut lipase with molecular weight between 29 and 43 kDa (which corresponds to the 32 kDa subunit in our manuscript). However, a piece of important information (although very limited) is found in the Material and Method section of Subhashri report that showed they used a long chain fatty acid ester as substrate (as they analysed oleic acid hydrolysis product). This may support our suggestion that individual subunits have different preferrence to difference fatty acid esters.

		We have integrated Subashri report in the discussion section (Line 274-278).

ITEMIZED RESPONSE TO REVIEWER 1 COMMENTS AND RECOMMENDATIONS

Nr	Reviewer Comment	New Line nr	Author response
A1	Suggest to delete this sentence.	26	The sentence has been deleted
A2	The age of coconut in months should be specified.	28	The germinating coconut preparation is decribed in more detail in Methodology section. We opt not to specify the age of the coconut here in the abstract to avoid distraction with long explanation. The coconut fruits are of 11-12 months and experienced one month germinating process prior to extraction of its lipase (described in the Methodology section).
A3	This introductory section (lines 42 to 60) is too long. This paragraph should be compressed and combined with the next paragraph.	41-53	This part has been compressed
A4	replace with “principal”	58	The word “prominent” has been replaced by “principal”
A5	Suggest to delete this sentence. All oil seed plants have significant amounts of lipases.	59	The sentence: “Significant sources of lipases are microbes and animals” has been deleted.
A6	This is an inaccurate reference for this statement.	59	Since the previous sentence has been deleted, the next sentence (“Many enzymes serving as immobilized catalysts in industries are derived from those origins (Santana *et al.* 2011)” has also been deleted as the later refers to the previous sentence.
A7	Mainly along coastal areas of the tropics.	70-71	The new sentence: “Coconut trees grow almost in every region in the tropics, mainly along coastal areas of the tropics.”
A8	The word “plausible” is awkward.	81	The wordy word has been deleted
A9	Spell out (“p.a.”)	86	Replaced by “pro analysis”
A10	“Virgin coconut oil (VCO) was purchased from a local vendor.”	87-88	The active sentence has been replaced by a passive sentense as suggested.
A11	The sentence: “Ripe and dried coconut....” Confusing: 1. “Ripe” needs to be defined. The of age of the coconut	100 – 106	The reviewer concerns have been described in a clearer way. In response to Reviewer 2 note, correction for A11 and A13 comment have been moved to the methodology section. The new sentences are: “The coconut fruits were pickup from coconut tree

A13	should be given in terms of months. 2. What do they mean by “dried coconut”? This should be described more quantitatively. The % humidity and temperature should be specified.		after they turned dry as indicated by the brown color of their shell. The condition is typically reached by the fruits at the age of 11 to 12 months. To observe the germination, the outer shell of the fruit was partially removed (Figure 1a). Germination of coconut fruit was attained by storing coconut fruit in the direct sunlight protected open air condition in our region with an average humidity of above 80% and temperature between 23 °C to 28 °C. The humid environment was kept by watering the fruit everyday. The coconut shoot's appearance showed germination after c.a. a month of storage (Figure 1a). “
A12	“Plant base lipases ..... (Ibrahim *et al.* 2008). Transfer to Introduction section.	72-76	This part has been transferred into introduction section
A13			See A11 above
A14	“The high lipase activity from various germinating seeds has been reported (Barros *et al.* 2010) with Castor bean (Eastmond, 2004), and Egusi melon seed (Barros *et al.* 2010) are only a few exceptions as their ungerminated seeds also show significant lipase activity.“ Reword this sentence so that the same citation is not repeated.	215	We have changed the cited article for Egusi melon to Bege et al (instead of Barros et al. 2010). Article from Bege et al is the original repot for Egusi melon. “The high lipase activity from various germinating seeds has been reported (Barros *et al.* 2010) with Castor bean (Eastmond, 2004), and Egusi melon seed (Bege *et al*. 2015) are only a few exceptions as their ungerminated seeds also show significant lipase activity.”
A15	Replace with “contradictory”?	284	The word “confronting” has been replaced by “contradictory”
A16	regardless of their hydrolysis rate “at different rates of hydrolysis.”	287	The sentence: “...regardless of their hydrolysis rate” has been replaced by” “at different rates of hydrolysis.”
A17	The sentence: “However, it is worth noting that the stereoselectivity of coconut lipase remains unclear.” Suggest to reword as: “However, it is worth noting that the regioselectivity and stereoselectivity of coconut lipases remain unclear.”	287-288	The sentence has been modified as suggested

A18 A19	"To address this issue, the hydrolysis of triacylglycerol substrates will provide the required data" This statement should be elaborated. From the present experiment, we expect that coconut lipase has the highest affinity to trilaurin Suggested rewording: "From the present experiment, we conclude that coconut lipase gives the highest activity with lauryl esters."	286-292	A18 and A19 Thank you for the suggestion. Here we would like to make it clearer that A19 refers to previous sentence ("To address this issue, the hydrolysis of **triacylglycerol** substrates will provide the required data"). Since it was clear that coconut lipase prefers lauryl esters, as tested by using substrate *p*-nitrophenyl laurate, we expect the lipase has the highest activity against trilaurin, i.e. 1,2,3-glyceryl trilaurate (a triacylglycerol with all acyl groups are lauryl) The conclusive sentence suggested in A19 is described elsewhere The data presented in this study show that all coconut lipase subunits can cleave fatty acid esters at different rate of hydrolysis. However, it is worth noting that the regioselectivity and stereoselectivity of coconut lipase remain unclear. To address this issue, the hydrolysis of triacylglycerol substrates of various acyl group lengths will provide the required data. From the present experiment, we expect that coconut lipase has the highest affinity to trilaurin. Hydrolysis study with 1-monolaurin, 1,3-dilaurin and 2,3-dilaurin substrates will reveal regioselectivity of coconut lipase.
A20	"Moreover, to give details of individual subunits' activity" Change "give" into "obtain"	293	The word "give" has been replaced by "obtain"
A21	The sentence: "In its complex form, lauric acids are the most preferred substrate for coconut lipase." Should be replaced by: "In its complex form, the coconut lipase shows highest preference for lauryl esters."	307-308	The sentence has been modified as suggested
A22	"We argue that a similar...." Reviewer suggests to replace "argue" with "propose"	313	The word "argue" has been replace by "propose"
A23	Coconut lipase activity Figure 2 caption needs to be replaced by: "Coconut lipase activity at different dilutions".	465	The caption has been replaced as suggested
A24	Figure 3 The Control condition needs to be described in detail.	479-480	The control condition has been describe by inserting the sentence: "... . The released free fatty acids were titrated by using sodium hydroxide. Control was provided by...."

A25	"...were made triplicate."	480	measuring lipase activity against VCO substrate in the absense of metal ions. All measurements were made in triplicate." The statement: " ....were made triplicate." hase been corrected into: "...were made in triplicate."
A26	Figure 4 Indicate how many replicates.	490	The last part of Figure 4 caption becomes: "The released *p*-nitrophenol was measured at 405 nm, and the obtained values were converted to lipase activity. All measurements were made triplicate."

ITEMIZED RESPONSE TO REVIEWER 2 NOTES

Line	Comments and Recommendations	New line nr	Authors response
187-196	This paragraph should be in the context of introduction rather than discussion part	72-76	We have transferred this part to introduction section
125	Enzyme activity should be presented in U/mL or U/mg enzyme	131	We have checked the equation and the enzyme activity should be presented in U/mL. Accordingly, we have changed the y axis of Figure 3 and Figure 4. The changes do not affect the values of lipase activity since the equation itself lead to the activity per mL enzyme.
198-208	Is this paragraph better to be in methodology section?	100- 112	We have transferred this part to methodology section under heading coconut germination, crude extract preparation and protein determination. To avoid repetition, the text has been integrated with previous text under the same heading of methodology.
215	Could authors describe the result in Figure 2 in details?	208-213	Figure 2 is described as follows: "Figure 2 shows that coconut lipase has a very high esterase activity against artificial substrate *p*-nitrophenyl palmitate. Sample dilution by factors of 6 and 50 thousand times would lead to immediate saturation curves after five minutes of incubation. Sample dilution by factor of 100 thousand times showed a delayed saturation curve, namely after 20 minutes of reaction. This dilution factor was used for specificity assay below since it meets the requirement of a first order kinetics in its initial reaction."
224	Why did calcium ions activate enzyme activity while the other ions showed an inhibitory effect?	222-228	We have added the explanation as follows: "Calcium ion is a well known activator for different sources of lipases, presumably be stabilizing the three dimensional stucture of lipase during catalysis (Rosenstein and Gotz, 2000). On the other hand, Fe^{3+}, Cu^{2+}, Zn^{2+}, and Mg^{2+}, as well as alkali ions K^{+} and Na^{+}, decreased the esterase activity of coconut lipase (Table 1). It

			suggests that those ions induce different conformational levels of the lipase that unfavor esterase activity (Hertadi and Widhyastuti, 2015), although a deep structural study is necessary to understand the effect of various metal ions."
28, 30, 107, 134, many others	Passive tense should be used	28-29	The new sentence: "Biochemical characterization of coconut lipase was undertaken to reveal its substrate specificity and its subunits properties"
		29-31	The new sentence: "By using various chromogenic ester of fatty acids, it was demonstrated that lauric acid is the most preferred substrate for coconut lipase esterase reaction."
		113-114	The new sentence: "The suspension was filtered by using a filter cloth."
		128-129	The new sentence: "Sodium hydroxide of 0.01 M was used for the titration following the addition of a few drops of phenolphthalein."
		140-142	The new sentence: "Magnesium, calcium, sodium, potassium, iron, copper, and zinc ions were added to each lipase reaction mixture with a final concentration of 10 mM."
		145-147	The new sentence: "In order to allow kinetics analysis of coconut lipase, a suitable assay condition was first determined. It was performed by hydrolysing the artificial substrate *p*-nitrophenyl palmitate by serial dilution coconut lipase."
		158-160	The new sentence: "The coconut lipase specificity was tested against *p*-nitrophenyl butyrate, *p*-nitrophenyl octanoate, *p*-nitrophenyl decanoate, *p*-nitrophenyl dodecanoate, *p*-nitrophenyl myristate, and *p*-nitrophenyl palmitate."
		191-192	The new sentence:" An identical gel stained by Coomassie Brilliant Blue was prepared for comparison."
224, 231, many others	Previous study should be discussed in past tense. Misspellings	194-195	The sentence has been changed to: "In this study, lipase was isolated from germinating coconut and its biochemical properties were investigated".
		222-222	The sentence has been changed to: "A

			literature survey also suggested that calcium ions activate many plant lipases.....”
		224-225	The sentence has been changed to: “.....alkali ions K^+ and Na^+, decreased the esterase activity of coconut lipase.”
		232	Typo has been corrected

Biochemical Properties of Coconut (*Cocos nucifera* L.) Lipase

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Running head: biochemical properties of coconut lipase

Keywords: coconut lipase, substrate specificity, native electrophoresis, lipase subunits, in-gel assay

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ABSTRACT

Ubiquitous in nature, Lipases represent an example of enzymes with high versatility. Plant seeds are potential sources of lipase, and they are attracting more attention for specific purposes. In this study, coconut lipase was isolated from germinating coconut seed. Biochemical characterization of coconut lipase was undertaken to reveal its substrate specificity and its subunits properties. By using various chromogenic ester of fatty acids, it was demonstrated that lauric acid is the most preferred substrate for coconut lipase esterase reaction. Calcium ions enhance its activity, whereas other metal ions such as magnesium, nickel, sodium, and potassium reduce it. Electrophoresis under native conditions showed that coconut lipase is a single protein. Since electrophoresis under denaturing conditions revealed four subunits, coconut lipase is likely a complex enzyme. It is further shown that all subunits are active, as evident in an in-gel hydrolysis assay. However, they also hint that they do not have an equal catalytic rate against the 16 carbon length palmitate derivative. This finding thus opens up a notion that those subunits have different substrates specificity yet to be determined.

INTRODUCTION

Fatty acids are widely used in modern life and hence are of the critical industrial concern. The utilization of fatty acids spans from essential ingredients in many industries, such as in coating (Rajput *et al.* 2014), surfactants (Semblante *et al*. 2009), lubricants (Ruths *et al.* 2008), essential fatty acids, nutraceuticals productions (Sande *et*

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al. 2018), personal cares (Tavares *et al.* 2018), and bioremediation (Melani *et al.* 2019). Several methods achieve fatty acid production from fats, such as the mechanical separation process, alkaline chemical hydrolysis, and enzymatic process. Mechanical separation requires high pressure and temperature that causes the process costly. Likewise, alkaline hydrolysis also offers a practical method. However, efforts are required to separate unwanted products (Sande *et al.* 2018). In contrast, enzymatic hydrolysis operates at mild conditions (low temperature and pressure). It offers ease in the recovery process (Jain and Mishra, 2015) and product loss due to minimized overheating (Barros *et al.* 2010).

Lipases or glycerol ester hydrolases (EC 3.1.1.3) are enzymes that can perform hydrolysis, esterification, and transesterification reactions under mild conditions. Which reaction takes place largely depends on the reaction environment (Tavares *et al.* 2018). Lipases act on different ester compounds, with acylglycerols become their principal substrates. All oil seed plants have significant amounts of lipases. Plant-based lipases are increasingly become the researcher's interest due to low production cost and high specificity (Tavares *et al.* 2018; Villeneuve, 2003). They also have an easy pharmacological acceptance due to their eukaryotic source (Seth *et al.* 2014). Essential sources of plant-based lipases are plant seeds, especially the seeds in their germinating phases. Examples are lipases from *Carica papaya* (Campillo-Alvarado and Tovar Miranda, 2013), *Pentaclethra macrophylla* (Enujiugha *et al.* 2004), Linseed (Sammour, 2005), and coconut (Ejedegba *et al.* 2013). However, significant lipase activity from non-

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germinating seeds also exists, such as in castor beans (Eastmond, 2004; Tavares *et al.* 2018).

Coconut trees grow almost in every region in the tropics, mainly along coastal areas of the tropics. The physical appearance of coconut fruits is very distinct and easy to handle. As a consequence, their utilization as lipase sources is foreseeable. The focus of coconut exploration in lipase research was limited to the use of coconut as a medium for lipase-producing fungi (Benjamin and Pandey, 1997), immobilization study of other lipases (Brigida *et al.* 2007), and to the potential of coconut as a substrate for lipase reaction (Ibrahim *et al.* 2008). In contrast to its potential, biochemical characterization of coconut lipase has not been sufficiently reported, thus limited its applications. In this context, the present study investigates the biochemical characterization of coconut lipase. The work includes the study of coconut lipase substrate specificity and the property of its subunits. A thorough understanding of the biochemical properties of coconut lipase will lead to its application.

MATERIALS AND METHODS

Material

Golden coconut (local: gading coconut) was obtained from a local garden in Lombok Island of Indonesia. Reagents for buffer and electrophoresis of pro analysis grades were obtained from major chemical suppliers. Virgin coconut oil (VCO) was purchased from a local vendor. The artificial lipase substrates were *p*-nitrophenyl butyrate, *p*-nitrophenyl octanoate, *p*-nitrophenyl-decanoate, *p*-nitrophenyl dodecanoate, *p*-

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nitrophenyl myristate, and *p*-nitrophenyl palmitate, and *p*-nitrophenol (Merck/Sigma-Aldrich). Reagents for in-gel lipase assays were Fast Blue B salt (Sigma-Aldrich) and alpha-naphthyl palmitate (Santa Cruz Biotechnology, USA). Assay buffer contained Arabic gum (Sigma-Aldrich) and sodium deoxycholate (Sigma-Aldrich). Protein determination used a bicinchoninic acid (BCA) kit from Thermo scientific. The Prism 7 tool (GraphPad) and Image-J were used graphical preparation and dye density calculation, respectively.

Methods

Coconut germination, crude extract preparation, and protein determination

The coconut fruits were pickup from coconut the tree after they turned dry, as indicated by the brown color of their shell. The condition was typically reached by the fruits at the age of 11 to 12 months. To observe the germination, the outer shell of the fruit was partially removed (Figure 1a). Germination of coconut fruit was attained by storing coconut fruit in the direct sunlight protected open-air condition in our region with an average humidity of above 80% and temperature between 23 °C to 28 °C. The humid environment was kept by watering the fruit every day. The coconut shoot's appearance showed germination after c.a. a month of storage (Figure 1a). The germination process was accompanied by the development of haustorium inside coconut fruit (Figure 1b). As the coconut flesh is the primary food storage, coconut lipase was isolated only from the part. Nevertheless, literature reported that all parts of germinating coconut have lipase activity, with the shoot being the most active part (Su'i and Suprihana, 2013). The coconut of average size resulted in c.a. 200 grams of meat.

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The flesh was shredded and resuspended in 5 mM phosphate buffer, pH 7.0. The suspension was filtered by using a filter cloth. The resulted in coconut milk was centrifuged at 3,000 rpm for 20 mins at 4 °C. The floating cream was removed from a 50 mL conical centrifuge tube. The skim fraction was decanted and further subjected to freeze-drying to reduce water content. The resulted in 15 mL concentrated coconut lipase was stored at -20 °C for further analysis. Protein concentration was determined using the BCA kit according to the manufacturer's instruction. The developed color was measured at 562 nm by a spectrophotometer (MultiSkan GO, Thermo scientific).

Enzyme assay

Coconut lipase activity was assayed for its hydrolytic activity against virgin coconut oil as a substrate (Khor *et al*. 1986). The reaction mixture consisted of 5 grams VCO, 2.5 mL n-hexane, 5 mL 100 mM phosphate buffer pH 7.5, and 1 mL of the enzyme. The mixture was incubated in a 35 °C water bath shaker for 45 mins, and after this period, 25 mL of acetone/methanol (1:1) was added. The liberated free fatty acids were determined by titration. Sodium hydroxide of 0.01 M was used for the titration following the addition of a few drops of phenolphthalein. Sodium hydroxide was previously standardized against sodium oxalate. Lipase activity was calculated as follows:

$$\text{Lipase activity (U/mL)} = \frac{(Vsample - Vblank)\,x\,[NaOH]\,x\,1000}{Venzyme\,x\,t}\ \text{(U/mL)}$$

Where V_{sample} = titrant volume for sample
V_{blank} = titrant volume for blank
V_{enzyme} = coconut lipase volume

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[NaOH] = sodium hydroxide concentration

Coconut lipase activity in the presence of metal ions

Coconut lipase activity was assayed against VCO, as previously described, in the presence of several metal ions. Magnesium, calcium, sodium, potassium, iron, copper, and zinc ions were added to each lipase reaction mixture to a final concentration of 10 mM.

Substrate specificity of coconut lipase

In order to allow kinetics analysis of coconut lipase, a suitable assay condition was first determined. It was performed by hydrolysing the artificial substrate *p*-nitrophenyl palmitate by serial dilution of coconut lipase. The intensity of the liberated *p*-nitrophenol was measured at 405 nm. Hydrolysis profiles of *p*-nitrophenyl palmitate were recorded every 5 minutes with lipase dilution range from 1: 3,000 to 1: 100,000.

For different *p*NP-fatty acids, an 8-minute reaction with 1:100,000 dilution of lipase stock was further employed. For each reaction, the *p*NP-fatty acid substrates were prepared as follows: 2 mL of 8 mM *p*NP-fatty acid in n-propanol was added to 18 mL of an emulsifier solution. The emulsifier contained 20 mg of Arabic gum and 41.4 mg sodium deoxycholate in 50 mM Tris-Cl buffer pH 8.0. The substrate solution was kept in the dark before use. The final concentration of *p*NP-fatty acid in the substrate solution was 0.8 mM. Hydrolysis assay was performed by pre-incubating of 2.7 mL substrate solution at 37 °C for 5 minutes before the addition of 0.3 mL diluted lipase. The yellow color formation was recorded after 8 minutes at 405 nm. The coconut lipase specificity was

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159 tested against *p*-nitrophenyl butyrate, *p*-nitrophenyl octanoate, *p*-nitrophenyl decanoate,
160 *p*-nitrophenyl dodecanoate, *p*-nitrophenyl myristate, and *p*-nitrophenyl palmitate. One
161 unit activity (U) is defined as micromole(s) of *p*-nitrophenol released upon hydrolysis by
162 1 mL enzyme at 37 °C under assay conditions (Kanwar *et al*. 2005).
163
164 **SDS-PAGE and Native PAGE**
165 SDS-PAGE was undertaken according to the method initially developed by Laemmli
166 (1970) on 12.5% separating gel and 5% focusing gel. Briefly, a sample containing 50 µg
167 of coconut lipase was precipitated by the addition of an equal volume of cold absolute-
168 ethanol. The resulted precipitate was resuspended in a 5X sample buffer and boiled for
169 2 minutes prior to electrophoresis. Electrophoresis was accomplished by applying 150
170 Volt electric current in a mini gel (MiniVe SDS-PAGE apparatus, GEHealthcare) for c.a.
171 2 hours. The resulted gel was stained with Coomassie Brilliant Blue (CBB). For native-
172 PAGE, coconut lipase was subjected to electrophoresis under non-denaturing
173 conditions, i.e., by omitting SDS from the gel and running buffer. The sample buffer was
174 also void of 2-mercaptoethanol or dithiothreitol. Ammonium sulfate fractionation was
175 undertaken according to Sana and coworkers (Sana *et al*. 2004). Briefly, ammonium
176 sulfate threshold of 0-30%, 30-45%, 45-60%, 60-75%, and 75-90% saturation was
177 added to the protein sample. The excess of salt was removed by dialysis from each
178 fraction. The resulted fractions were subjected to both SDS and native PAGE.
179
180 **In-gel hydrolysis assay**

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The activity of lipase subunits was assayed after lipase was separated in 12.5% gel SDS-PAGE. In this case, the sample was not boiled to keep the protein intact. After separation, SDS content was washed by soaking the gel in 50 mM phosphate buffer pH 7.0 and 2.5% (v/v) Triton X-100. It was done with gentle shaking for 30 minutes. The washing step was repeated twice. The gel loaded with lipase was incubated in a developing solution for 30 minutes in a dark container to allow hydrolysis to proceed. The developing solution contained alpha-naphthyl palmitate and Fast Blue B salt. Unbound dye was removed by three-time washing in aquadest, 10 minutes each. The hydrolysis of alpha-naphthyl palmitate corresponded to the lipase subunit activity. The active subunit released a yellow color of alpha-naphthol (Zienkiewicz *et al.* 2014) that appeared on the gel. An identical gel stained by Coomassie Brilliant Blue was prepared for comparison.

RESULTS AND DISCUSSION

In this study, lipase was isolated from germinating coconut and its biochemical properties were investigated. Since many biochemical properties of coconut lipase remain unclear, coconut lipase's biochemical characterization is necessary, and the results will facilitate further utilization of coconut lipase.

Virgin coconut oil (VCO) was used as the substrate for coconut lipase hydrolysis activity instead of using popular olive oil since it offers a more comprehensive composition of fatty acids ester from various chain lengths. VCO has also been investigated in the optimation of many lipases, such as immobilized *Mucor mihei* lipase (Chua *et al*. 2012),

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Candida rugosa and porcine pancreas lipases (Nguyen *et al*. 2018). The isolated coconut lipase has an activity of 0.56 U/mL corresponding to a specific activity of 0.30 U/mg protein. These results resemble those reported by Su'i and Suprihana (Su'i and Suprihana, 2013).

Figure 2 shows that coconut lipase has a very high esterase activity against artificial substrate *p*-nitrophenyl palmitate. Sample dilution by factors of 6 and 50 thousand times would lead to immediate saturation curves after five minutes of incubation. Sample dilution by a factor of 100 thousand times showed a delayed saturation curve, namely after 20 minutes of reaction. This dilution factor was used for the specificity assay below since it met the requirement of first-order kinetics in its initial reaction. The high lipase activity from various germinating seeds has been reported (Barros *et al.* 2010) with Castor bean (Eastmond, 2004), and Egusi melon seed (Bege *et al*. 2015) are only a few exceptions as their ungerminated seeds also show significant lipase activity.

Many lipases have their activity altered in the presence of specific metal ions. Here, the effect of several metal ions on the esterase activity of coconut lipase was tested. Figure 3 shows that calcium ions act as coconut lipase activators. A literature survey also suggested that calcium ions activate many plant lipases, such as those from white melon kern (Eze and Ezema, 2012). Calcium ion is a well-known activator for different sources of lipases, presumably by stabilizing the three-dimensional structure of lipase during catalysis (Rosenstein and Gotz, 2000). On the other hand, Fe^{3+}, Cu^{2+}, Zn^{2+}, and

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225 Mg^{2+}, as well as alkali ions K^+ and Na^+, decreased the esterase activity of coconut
226 lipase (Table 1). It suggests that those ions induced different conformational levels of
227 the lipase that unfavoured esterase activity (Hertadi and Widhyastuti, 2015), although a
228 deep structural study is necessary to understand the effect of various metal ions. To our
229 knowledge, only a few lipases are inhibited by magnesium ions, such as rice bran
230 (Bhardwaj *et al.* 2001), almond seed lipase (Yesiloglu and Baskurt, 2013), and Africa
231 bean seed (Enujiugha *et al.* 2004) lipases (Table 1). Coconut lipase adds a new
232 member to the relatively short list of plant seed lipases inhibited by magnesium ions.
233
234 The substrate specificity of coconut lipase was analyzed using various *p*-nitrophenyl
235 fatty acid esters of different chain lengths. Figure 4 shows that *p*-nitrophenyl laurate
236 (C12) gives the highest hydrolysis product in a given time at the initial period of reaction,
237 and the longer fatty acids (C14 *p*-nitrophenyl myristate and C16 *p*-nitrophenyl palmitate)
238 come the next. The shorter fatty acids (C4 *p*-nitrophenyl butyrate, C8 *p*-nitrophenyl
239 octanoate, and C10 *p*-nitrophenyl decanoate) give lower hydrolysis products in the
240 same incubation time. Lauric acid (C12) is the predominant fatty acid of coconut that
241 belongs to the medium-chain fatty acid (Manohar *et al*. 2019; Dayrit, 2014). The
242 complete hydrolysis of VCO by other lipases reported by Chua *et al* (2012) and Nguyen
243 *et al* (2018) confirmed that lauric acid is the predominant fatty acid of coconut. Instead
244 of using complete hydrolysis, the kinetics study reported here took advantage of the use
245 of various *p*NP-fatty acid substrates to allow the investigation at the initial period of
246 reaction, from which the fatty acid preference of coconut lipase can easily be

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247 determined. The aforementioned result indicates that coconut lipase, in order of
248 preference, hydrolyzes medium, long, and short-chain fatty acid esters.
249
250 SDS-PAGE and native-PAGE of coconut lipase are shown in Figure 5. SDS-PAGE
251 electroforegram reveals at least four protein bands of approximately 54 kDa, 32 kDa, 21
252 kDa, and 15 kDa. However, native-PAGE reveals only a single band of c.a. 130 kDa.
253 After thresholds of ammonium sulfate precipitation, separation of coconut lipase also
254 shows a single complex band in native PAGE for all fractions (Figure 6). Together,
255 these suggest that coconut lipase is a heteromeric enzyme. In humans, hormone-
256 sensitive lipase, an enzyme involves in the mobilization of lipid storage in adipose
257 tissue, has long been shown to be more active in its ~160 kDa dimer. It is 40 times more
258 active than the ~85 kDa monomer form (Shen *et al.* 2000). A reverse situation is
259 recently reported for the human Lipoprotein lipase, whose 55 kDa monomer has similar
260 activity to its 110 kDa homodimer (Beigneux *et al.* 2019). The fact that coconut lipase
261 consists of several subunits and that it is not universal that all subunits of given lipase
262 are functional highlights the need to dissect whether all coconut lipase subunits are
263 active. To address the above question, an in-gel cleavage assay was performed.
264
265 In-gel hydrolysis assay allows us to analyze whether a specific protein can hydrolyze
266 fatty acyl ester after the separation of proteins by electrophoresis. An active protein
267 within the gel cleaves the substrate alpha naphthyl palmitate to release a yellow
268 coloring of naphthol (Figure 7a), following SDS removal from the gel (Zienkiewicz *et al.*

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2015). Figure 7b shows that all coconut lipase subunits can hydrolyze alpha naphthyl palmitate, which indicates that all coconut lipases are active. Two subunits with equal intensity on coomassie staining produce a different naphthol intensity, demonstrated by the 54 kDa dan 21 kDa subunits (Figure 7b). It suggests that the two subunits have a different affinity to alpha naphthyl palmitate, with the latter has a lower affinity. However, this does not nullify the possibility that the 21 kDa subunit has a higher esterase activity for shorter or longer fatty acids. Subashri and coworkers have identified coconut lipase with a molecular weight between 29 and 43 kDa (Subashri *et al*. 2018), which is comparable to the 32 kDa subunit in the present study. Since Subahsri *et al*. used ester of oleic acid as substrate, one can speculate that the 32 kDa subunit of coconut lipase has a preference for the C18 fatty acid ester. Hence, it is worth testing whether the cleavage of medium-chain and short-chain fatty acids by coconut lipase give the same pattern. There are faint protein bands at c.a. 40 kDa and 45 kDa in addition to the four distinct subunits. We speculate that the two proteins are glycosylated forms of the 32 kDa subunit. Our finding that coconut lipase consists of several active subunits may explain contradictory reports on plant seed lipase activities, such as those from rice *Oryza sativa* lipase (Table 1).

The data presented in this study show that all coconut lipase subunits can cleave fatty acid esters at a different rate of hydrolysis. However, it is worth noting that the regioselectivity and stereoselectivity of coconut lipase remain unclear. To address this issue, the hydrolysis of triacylglycerol substrates of various acyl group lengths will

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provide the required data. From the present experiment, we expect that coconut lipase has the highest affinity to trilaurin. Hydrolysis study with 1-monolaurin, 1,3-dilaurin and 2,3-dilaurin substrates will reveal regioselectivity of coconut lipase. Moreover, to obtain details of individual subunits' activity, it is deemed necessary to separate the subunits and test their specificity. Such a study may reveal the contribution of subunits to the coconut lipase as a whole. Furthermore, if cloning and heterologous expression are desired, this can be directed to the study of individual subunits, especially at the current circumstance when the coconut genome is emerging on the horizon (Xiao *et al.* 2017). Accordingly, biochemical characterization of various subunits (optimum temperature and pH reaction, substrate specificity, metal ions effect, and detergent effect) would provide more detailed information.

CONCLUSION

By using a simple procedure, we have been able to isolate coconut lipase. A direct comparison of SDS-PAGE and native PAGE readily hint that coconut lipase is a complex enzyme. This enzyme consists of four subunits of 54 kDa, 32 kDa, 21 kDa, and 15 kDa. In its complex form, the coconut lipase shows highest preference for lauryl esters. The enzyme is activated by Ca^{2+} ion, whereas Fe^{3+}, Cu^{2+}, Zn^{2+}, Mg^{2+}, K^{+}, and Na^{+} decrease coconut lipase activity. Each subunit can cleave the fatty acid ester bond; hence this enzyme might be regarded as a cluster of smaller active proteins. Since all coconut lipase subunits are active as esterases, specificity determination of subunits and further biochemical characterization of the subunits are yet to be investigated. We

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also propose that a similar approach can be applied for the initial study of other plant or seed-based lipases.

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STATEMENT ON CONFLICT OF INTEREST

All authors declare to have no conflict of interest.

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446 **Table 1.** Properties of some plant-based lipases

No	Lipase Source	MW (kDa)	Activator	Inhibitor	Reference
1[a]	Rice bran (*Oryza sativa*)	~ 10	Potassium acetate, sodium acetate, NaCl	CHAPS, digitonin, SDS, NP-40, Triton X-100, Mg^{2+}, Mn^{2+}, Cu^{2+}, Cd^{2+}	(Barros *et al.* 2010)
	Rice Bran Lipase II	33	n.a.	n.a.	(Aizono *et al.* 1976)
	Rice Bran	40	n.a.	n.a.	(Kim, 2004)
2[b]	Castor bean (*Ricinus communis* L.)	60	Ca^{2+}	p-Chloromercuribenzoic, $HgCl_2$	(Eastmond, 2004)
	Castor bean	n.a.	Slightly activated by Na^{+}, K^{+}, and dithiothreitol	Mg^{2+}, Ca^{2+}	(Muto and Beevers, 1974)
	Castor bean	60	Mn^{2+}, Na^{+}, K^{+}, Al^{3+} and Li^{+}	Zn^{2+}, Co^{2+}, Pb^{2+} and Cu^{+}	(Su *et al.* 2010)
3	Linseed (*Linum usitatissimum)*	42	Mg^{2+}, K^{+}	Triton x-100, Tween 80	(Sammour, 2005)
4	Almond seed *(Amygdalus communis* L.)	n.a.	Ca^{2+}, Fe^{2+}, Mn^{2+}, Co^{2+}, Ba^{2+}	Mg^{2+}, Cu^{2+}, Ni^{2+}	(Yesiloglu and Baskurt, 2013)
5	Africa Bean seed (*Pentachlethra macrophylla* Benth)	n.a.	Ca^{2+}	NaCl, $MgCl_2$, EDTA	(Enujiugha *et al.* 2004)
6	Sunflower seed (*Helianthus annuus* L)	40-50	Ca^{2+}, Mg^{2+}	Hg^{2+}, EDTA	(Sadeghipour and Bhatla, 2003)
7	Canola lipase (*Brassica napus*)	n.a.	Ca^{2+}, Bi^{3+}	Fe^{2+}, Fe^{3+}, Zn^{2+}, Hg^{2+} and Cu^{2+}	(Sana *et al.* 2004)

447 MW: Molecular Weight; n.a.: not available; FA: Fatty Acid; TAG: Triacylglycerol
448 [a]) different reports of lipases from rice bran.
449 [b]) different reports for lipase from these seeds suggest that they have at least two
450 lipases, i.e., the acid and alkaline lipase
451

452

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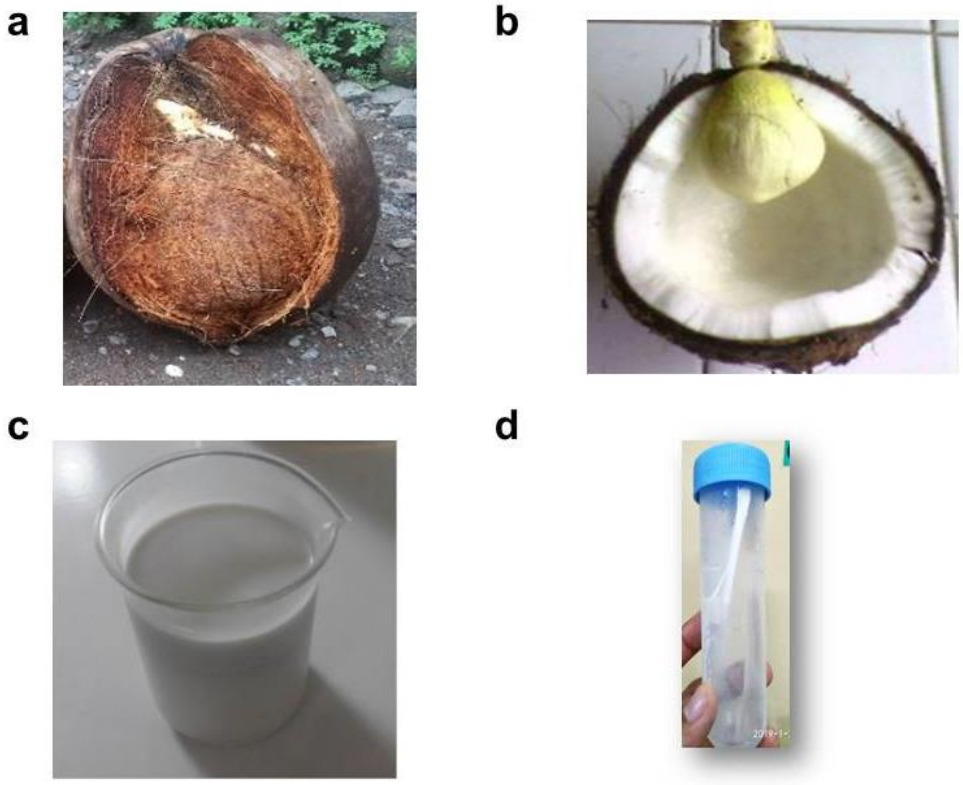
453 **Figure 1.** Preparation of coconut lipase from the germinated coconut fruit



454

455 **Figure 1.** Preparation of coconut lipase from the germinated coconut fruit

456 a. Coconut shoot appears after a month of germination. b. Inside the hard shell,
457 haustorium is developing. Mucilage or coconut flesh was removed and further used as
458 the source of coconut lipase. c. Coconut milk prepared by suspending shredded
459 coconut flesh in 5 mM phosphate buffer, pH 7.0. d. Following centrifugation, the cream
460 fraction was removed. The clear fraction of coconut milk was decanted and stored for
461 electrophoresis and enzyme assays.

462

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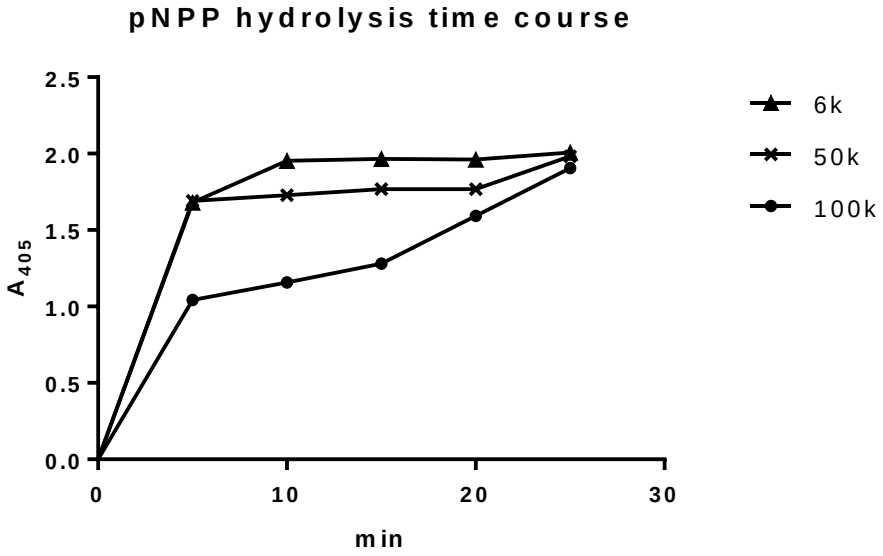
463 **Figure 2.** Coconut lipase activity



464

465 **Figure 2**. Coconut lipase activity at different dilutions.

466 The activity of coconut lipase of different dilutions was assayed at different time points.
467 The yellow color showed lipase activity as a result of *p*-nitrophenyl palmitate hydrolysis.
468 After ten minutes of reaction, the saturated curve is observed by 6,000 times diluted
469 lipase. For the more diluted lipase, saturation is delayed. In the substrate specificity
470 assay, 8 minutes of incubation times were chosen, with the sample diluted by 100,000
471 factors.

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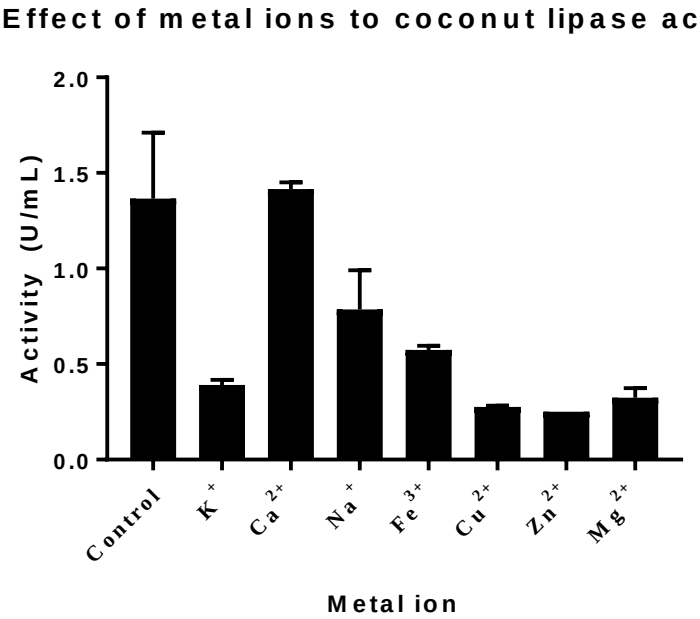
474 **Figure 3.** The activity of coconut lipase with the presence of metal ions.



475

476 **Figure 3.** The activity of coconut lipase with the presence of metal ions.

477 The effect of metal ions on coconut lipase activity was assayed by the inclusion of 10
478 mM of respective metal ions in the assay mixture. The released free fatty acids were
479 titrated by using sodium hydroxide. Control was provided by measuring lipase activity
480 against VCO substrate in the absense of metal ions. All measurements were made in
481 triplicate.

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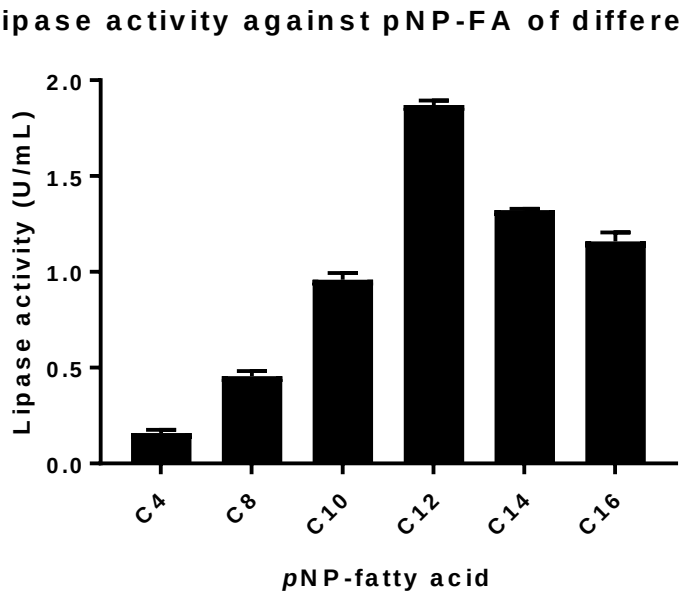
483 **Figure 4.** Substrate specificity of coconut lipase



484

485 **Figure 4.** Substrate specificity of coconut lipase

486 Diluted coconut lipase was allowed to hydrolyze various esters of fatty acids (*p*-
487 nitrophenyl butyrate, *p*-nitrophenyl octanoate, *p*-nitrophenyl decanoate, *p*-nitrophenyl
488 dodecanoate, *p*-nitrophenyl myristate, and *p*-nitrophenyl palmitate) for 8 minutes of
489 reaction. The released *p*-nitrophenol was measured at 405 nm, and the obtained values
490 were converted to lipase activity. All measurements were made triplicate.

491

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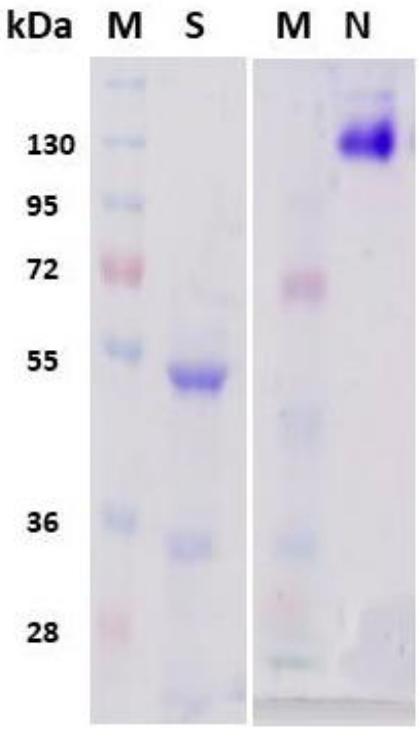
492 **Figure 5.** Coconut lipase separation in SDS-PAGE and Native-PAGE



493

494 **Figure 5**. Coconut lipase separation in SDS-PAGE and Native-PAGE

495 Coconut lipase separated on a 10% gel of SDS-PAGE (S) shows different protein
496 bands, i.e., 54 kDa, 32 kDa, and 21 kDa. The bands are from a single complex protein
497 of c.a. 130 kDa, as shown in native-PAGE (N). Note: the smallest band of c.a. 15 kDa is
498 not shown here but obvious on a 12.5% gel (Figure 6 and 7b).

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Figure 6. Ammonium sulfate fraction

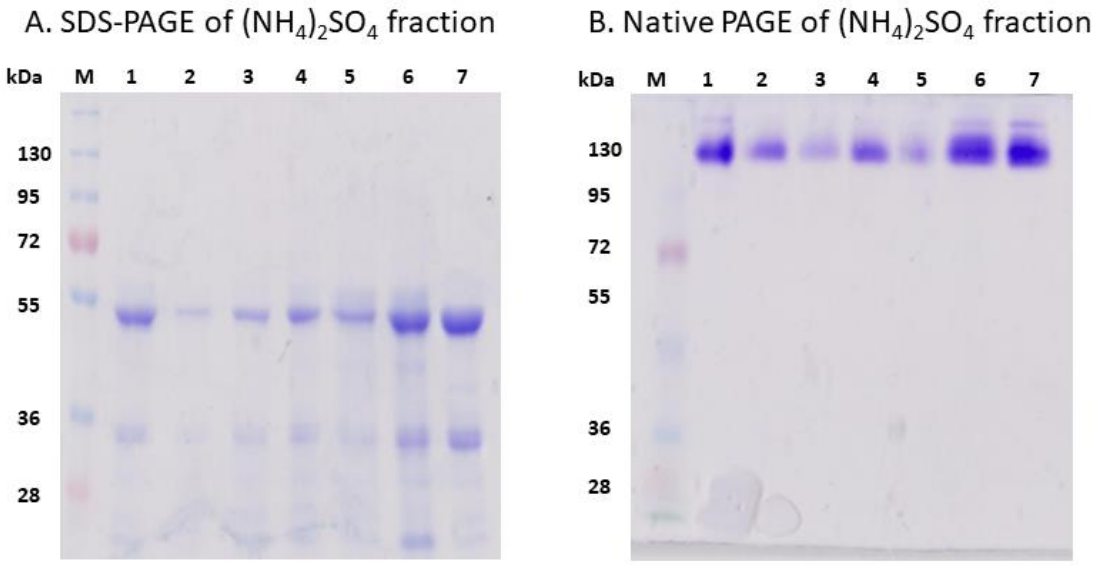

Figure 6. Ammonium sulfate fractions of coconut lipase separated by SDS-PAGE (A) and native PAGE (B). M: protein marker; 1: crude extract; 2: fraction 0-15%; 3: fraction 15-30%; 4: fraction 30-45%; 5: fraction 45-60%; 6: fraction 60-75%; and 7: fraction 75-90%.

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Figure 7a. Hydrolysis of alpha-naphthyl palmitate by lipase.

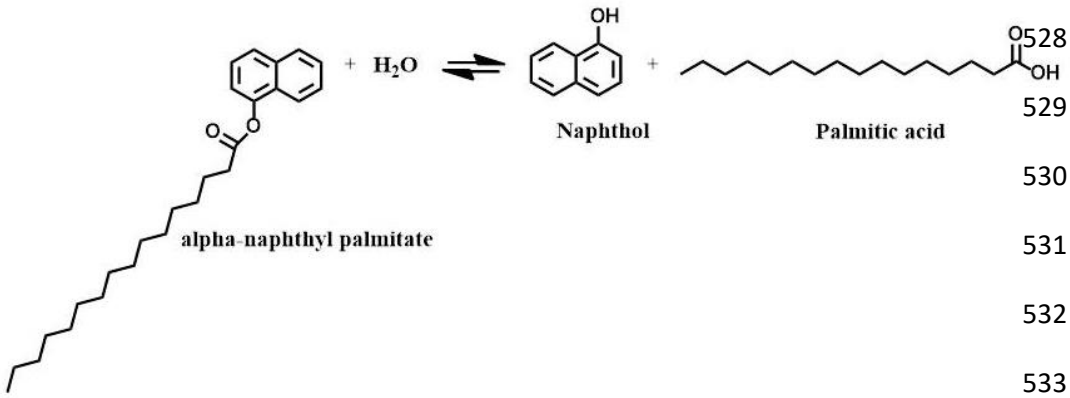

Figure 7a. Hydrolysis of alpha-naphthyl palmitate by lipase.

An active lipase or esterase cleaves alpha-naphthyl palmitate to release naphthol. The yellow color of naphthol is measured spectrophotometrically at 405 nm.

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Figure 7b. In-gel activity assay of coconut lipase.

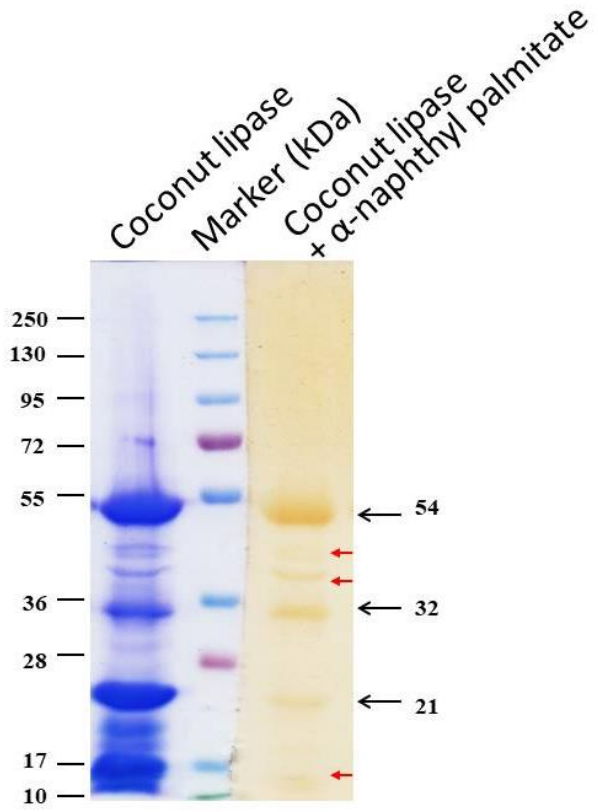

Figure 7b. In-gel activity assay of coconut lipase.

Coconut lipase is separated in a 12.5% polyacrylamide gel under denatured conditions, except for the boiling step. The gel was cut for coomassie staining (left) and an in-gel assay(right). At least four distinct bands are noticed upon coomassie staining, including the smallest subunit of c.a. 15 kDa. Additional faint bands are seen above the 32 kDa. The corresponding hydrolysis products by lipase subunits appear as yellow bands. It represents the results of alpha naphthyl palmitate hydrolysis by respective lipase subunits. The pixel density ratio of naphthol to coomassie staining for the 54 kDa and the 21 kDa subunits is 77.5% and 43.2%, respectively. Note: several distinct bands above the 32 kDa band (red arrows) are shown to be active in hydrolyzing the alpha

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565 naphthyl substrate. It gives a hint that the 32 kDa is glycosylated. We also speculate
566 that a smeary pattern observed in the native gel (Figure 5) indicates the glycosylation of
567 native protein.

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K7 Acknowledgment hasil revisi 22 April 2021

Lalu Rudyat Telly Savalas <telly@unram.ac.id>

Re: Ms 21-049 Response to reviewer's notes and revised manuscript

1 message

Philippine Journal of Science <philjournsci@gmail.com> 22 April 2021 at 10:36
To: Lalu Rudyat Telly Savalas <telly@unram.ac.id>

Dear Dr. Savalas:

This is to confirm receipt of your revised **Ms 21-049** paper and your answers to the reviewers' comments. These will be forwarded to the reviewers for another round of evaluation.

Thank you.

Sincerely,
David Matthew C. Gopilan
Editorial Assistant

For Caesar A. Saloma
Editor-in-Chief

On Wed, Apr 21, 2021 at 11:05 PM Lalu Rudyat Telly Savalas <telly@unram.ac.id> wrote:

> CAESAR A. SALOMA
> Editor-in-Chief, PJS
> Professor, National Institute of Physics
> University of the Philippines Diliman
> Quezon City, Philippines
>
> Dear Prof. Saloma,
> in response to the reviewer notes to our submitted manuscript entitled: “Biochemical Properties of Coconut (Cocos nucifera L.) Lipase” [Ms 21-049], here we attach itemized answers to the reviewer's notes, as well as the revised version of our manuscript.
>
> We hope that our manuscript can be proceeded for publication in the coming issue of the Philippine Journal of Science.
>
> Kind regards,
>
> On behalf of all authors,
>
> Dr. Lalu Rudyat T. Savalas
> corresponding author
>
> Department of Chemistry Education
> Faculty of Teacher Training and Education
> University of Mataram
> Mataram, Indonesia
> E-mail telly@unram.ac.id

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Philippine Journal of Science
Science and Technology Information Institute
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DOST Complex, Gen. Santos Ave., Bicutan 1631
Taguig City, Metro Manila, Philippines
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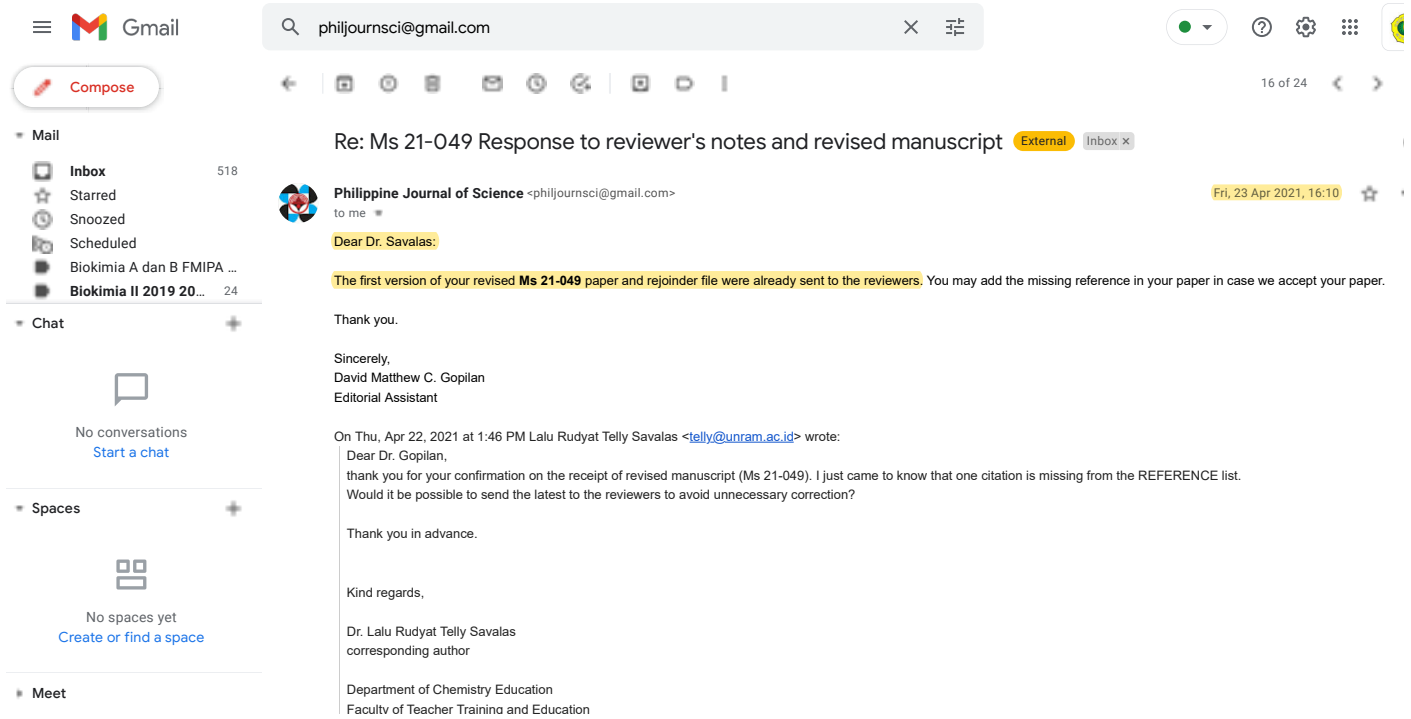
K8 Konfirmasi hasil review 23 April 2021



K9 Decision: Accepted 11 Mei 2021

Lalu Rudyat Telly Savalas <telly@unram.ac.id>

From Caesar Saloma/11 May 2021/ Acceptance/MS 21-049R

1 message

Caesar Saloma <caesar.saloma@gmail.com> 11 May 2021 at 21:01
To: telly@unram.ac.id
Cc: Philippine Journal of Science <philjournsci@gmail.com>

11 May 2021

DR LALU RUDYAT SAVALAS
Department of Chemistry Education
Faculty of Teacher Training and Education
University of Mataram
Mataram, Indonesia
Email: telly@unram.ac.id

Subject: MS 21-049R
Title: Biochemical Properties of Coconut (Cocos nucifera L.) Lipase
Authors: LR Savalas, S Sirodjudin, E Gunawan, R Aini,
D Suhendra, NH Basri, J Ardhuha and BN Ningsih

Dear Dr Savalas:

We are pleased to inform you that your revised manuscript has been accepted for publication as a Regular Article in the next available issue of the Philippine Journal of Science

Kindly submit a final version that strictly complies with the format of a Regular Article as explained in the PJS Author's Guide found in: http://philjournalsci.dost.gov.ph/index.php/author-s-guide.

Please send it to the PJS Managing Editor, Mr Allyster Endozo at: philjournsci@gmail.com. It will be used to produce the galley proofs of your article.

We look forward to hearing from you soon so as not to delay the publication of your work.

Kindly direct to the PJS Managing Editor any future inquiry regarding the publication status of your article.

Thank you.

Sincerely yours,
Caesar Saloma (Signed)
Editor-in-Chief
The Philippine Journal of Science

FINAL COMMENTS OF REVIEWERS
REVIEWER 1
1st evaluation - acceptable; minor revision

REVIEWER 2
1st evaluation - Reconsider only after the comments/recommendations are clarified and/or complied with.
2nd evaluation – Acceptable

I accept the corrections of the revised Ms 21-049 paper.

Article History:
Receipt of submission: 8 Mar 2021
Comments sent to author: 13 Apr 2021
Receipt of revision: 22 Apr 2021

END

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Caesar Saloma, PhD NAST FSPP SMOSA
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Quezon City 1101, Philippines
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http://en.wikipedia.org/wiki/Caesar_Saloma

K10 Respons atas acceptance 12 Mei 2021

Lalu Rudyat Telly Savalas <telly@unram.ac.id>

Re: From Caesar Saloma/11 May 2021/ Acceptance/MS 21-049R

1 message

Lalu Rudyat Telly Savalas <telly@unram.ac.id> 12 May 2021 at 10:44
To: Caesar Saloma <caesar.saloma@gmail.com>

PROF. DR. CAESAR SALOMA
Editor-in-Chief
The Philippines Journal of Science,

Dear Prof. Dr. Saloma,
thank you very much for the positive outcome of our manuscript (Ms 21-049) evaluation.
On behalf of all authors I forward the appreciation to efforts that have been dedicated by both editorial and reviewing teams.
As requested, I will send the final version of our manuscript.

Kind regards,

Lalu Rudyat Telly Savalas

==========
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Indonesia
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Fax +62 370 634918
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==========

On Tue, 11 May 2021 at 20:01, Caesar Saloma <caesar.saloma@gmail.com> wrote:

> 11 May 2021
>
> DR LALU RUDYAT SAVALAS
> Department of Chemistry Education
> Faculty of Teacher Training and Education
> University of Mataram
> Mataram, Indonesia
> Email: telly@unram.ac.id
>
> Subject: MS 21-049R
> Title: Biochemical Properties of Coconut (Cocos nucifera L.) Lipase
> Authors: LR Savalas, S Sirodjudin, E Gunawan, R Aini,
> D Suhendra, NH Basri, J Ardhuha and BN Ningsih
>
> Dear Dr Savalas:
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> We are pleased to inform you that your revised manuscript has been accepted for publication as a Regular Article in the next available issue of the Philippine Journal of Science
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> Kindly submit a final version that strictly complies with the format of a Regular Article as explained in the PJS Author's Guide found in: http://philjournalsci.dost.gov.ph/index.php/author-s-guide.
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> Please send it to the PJS Managing Editor, Mr Allyster Endozo at: philjournsci@gmail.com. It will be used to produce the galley proofs of your article.
>
> We look forward to hearing from you soon so as not to delay the publication of your work.
>
> Kindly direct to the PJS Managing Editor any future inquiry regarding the publication status of your article.

Thank you.

Sincerely yours,
Caesar Saloma (Signed)
Editor-in-Chief
The Philippine Journal of Science

FINAL COMMENTS OF REVIEWERS
REVIEWER 1
1st evaluation - acceptable; minor revision

REVIEWER 2
1st evaluation - Reconsider only after the comments/recommendations are clarified and/or complied with.
2nd evaluation – Acceptable

I accept the corrections of the revised Ms 21-049 paper.

Article History:
Receipt of submission: 8 Mar 2021
Comments sent to author: 13 Apr 2021
Receipt of revision: 22 Apr 2021

END

--
Caesar Saloma, PhD NAST FSPP SMOSA
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K11 Perbaikan dari author 12 Mei 2021

Lalu Rudyat Telly Savalas <telly@unram.ac.id>

(no subject)

1 message

Lalu Rudyat Telly Savalas <telly@unram.ac.id> 12 May 2021 at 10:53
To: Philippine Journal of Science <philjournsci@gmail.com>

MR. ALLYSTER ENDOZO
Managing Editor
The Philippines Journal of Science

Dear Mr. Endozo,
Following the positive decision to our manuscript Ms 21-049 entitled:
Biochemical Properties of Coconut (Cocos nucifera L.) Lipase, herewith I send the final version of the manuscript that referred to the journal instruction.
Should there be further concern regarding the manuscript, please do not hesitate to inform us.

Thank you very much and I look forward to further hints.

Best regards,

Lalu RT Savalas
University of Mataram
Indonesia

==========
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Ms 21-049 PJS Author's final.doc
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K12 Acknowledgment revisi dari author

Lalu Rudyat Telly Savalas <telly@unram.ac.id>

Re:

1 message

Philippine Journal of Science <philjournsci@gmail.com> 12 May 2021 at 13:29
To: Lalu Rudyat Telly Savalas <telly@unram.ac.id>

Dear Dr. Savalas,

Greetings!

This refers to your paper entitled, "Biochemical Properties of Coconut (*Cocos nucifera* L.) Lipase" accepted for publication in the Philippine Journal of Science. We are now preparing a draft of your article based on the attached manuscript, which will be presented to you once it is ready.

Thank you for your assistance and valuable support.

Sincerely,
Mr. ALLYSTER A. ENDOZO
Managing Editor

On Wed, May 12, 2021 at 10:54 AM Lalu Rudyat Telly Savalas <telly@unram.ac.id> wrote:

> MR. ALLYSTER ENDOZO
> Managing Editor
> The Philippines Journal of Science
>
> Dear Mr. Endozo,
> Following the positive decision to our manuscript Ms 21-049 entitled:
> Biochemical Properties of Coconut (Cocos nucifera L.) Lipase, herewith I send the final version of the manuscript that referred to the journal instruction.
> Should there be further concern regarding the manuscript, please do not hesitate to inform us.
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> Thank you very much and I look forward to further hints.
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> Best regards,
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> Lalu RT Savalas
> University of Mataram
> Indonesia
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> ==========
> Dr. Lalu Rudyat Telly Savalas
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K13 Copyedit/first draft 21 Juni 2021

Lalu Rudyat Telly Savalas <telly@unram.ac.id>

1st Draft of PJS Article Ms 21-049

1 message

Philippine Journal of Science <philjournsci@gmail.com> 21 June 2021 at 08:50
To: Lalu Rudyat Telly Savalas <telly@unram.ac.id>

Dear Dr. Savalas,

Greetings!

Attached below is the first draft of your article entitled, "Biochemical Properties of Coconut (*Cocos nucifera* L.) Lipase" [Ms 21-049] accepted for publication in the Philippine Journal of Science. Kindly review this copy and should you have no further corrections, provide us your approval for printing.

We hope to hear from you on the matter within 48 hours after the receipt of this letter. We shall consider this version of your paper as the galley proof for final editorial processing in the absence of a response within the cut-off time.

Thank you very much.

Sincerely,
Mr. ALLYSTER A. ENDOZO
Managing Editor

--

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Philippine Journal of Science
150 (5): 887-896, October 2021
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Biochemical Properties of Coconut (*Cocos nucifera* L.) Lipase

Lalu Rudyat T. Savalas[1]*, **Sirodjudin Sirodjudin**[2], **Erin R. Gunawan**[2], **Ro'yal Aini**[2], **Dedy Suhendra**[2], **Nurul H. Basri**[2], **Jannatin 'Ardhuha**[3], and **Baiq Nila S. Ningsih**[1,4]

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Ubiquitous in nature, lipases represent an example of enzymes with high versatility. Plant seeds are potential sources of lipase, and they are attracting more attention for specific purposes. In this study, coconut lipase was isolated from germinating coconut seed. Biochemical characterization of coconut lipase was undertaken to reveal its substrate specificity and its subunits properties. By using various chromogenic ester of fatty acids, it was demonstrated that lauric acid is the most preferred substrate for coconut lipase esterase reaction. Calcium ions enhance its activity, whereas other metal ions such as magnesium, nickel, sodium, and potassium reduce it. Electrophoresis under native conditions showed that coconut lipase is a single protein. Since electrophoresis under denaturing conditions revealed four subunits, coconut lipase is likely a complex enzyme. It was further revealed that all subunits are active, as evident in an in-gel hydrolysis assay. However, they also hint that they do not have an equal catalytic rate against the 16-carbon-length palmitate derivative. This finding, thus, opens up a notion that those subunits have different substrates specificity yet to be determined.

Keywords: coconut lipase, in-gel assay, lipase subunits, native electrophoresis, substrate specificity

INTRODUCTION

Fatty acids are widely used in modern life and, hence, are of critical industrial concerns. The utilization of fatty acids spans from essential ingredients in many industries, such as in coating (Rajput *et al.* 2014), surfactants (Semblante *et al*. 2009), lubricants (Ruths *et al.* 2008), essential fatty acids, nutraceuticals production (Sande *et al.* 2018), personal care products (Tavares *et al.* 2018), and bioremediation (Melani *et al.* 2019). Several methods achieve fatty acid production from fats, such as the mechanical separation process, alkaline chemical hydrolysis, and enzymatic process. Mechanical separation requires high pressure and temperature that causes the process costly. Likewise, alkaline hydrolysis also offers a practical method. However, efforts are needed to separate unwanted products (Sande *et al.* 2018). In contrast,

*Corresponding Author: telly@unram.ac.id

enzymatic hydrolysis operates at mild conditions (low temperature and pressure). It offers ease in the recovery process (Jain and Mishra 2015) and product loss due to minimized overheating (Barros *et al.* 2010).

Lipases or glycerol ester hydrolases (EC 3.1.1.3) are enzymes that can perform hydrolysis, esterification, and transesterification reactions under mild conditions. Which reaction takes place largely depends on the reaction environment (Tavares *et al.* 2018). Lipases act on different ester compounds, with acylglycerols become their principal substrates. All oilseed plants have significant amounts of lipases. Plant-based lipases are increasingly become the researcher's interest due to low production cost and high specificity (Tavares *et al.* 2018; Villeneuve 2003). They also have an easy pharmacological acceptance due to their eukaryotic source (Seth *et al.* 2014). Essential sources of plant-based lipases are plant seeds, especially the seeds in their germinating phases. Examples are lipases from *Carica papaya* (Campillo-Alvarado and Tovar Miranda 2013), *Pentaclethra macrophylla* (Enujiugha *et al.* 2004), linseed (Sammour 2005), and coconut (Ejedegba *et al.* 2013). However, significant lipase activity from non-germinating seeds also exists, such as in castor beans (Eastmond 2004; Tavares *et al.* 2018).

Coconut trees grow almost in every region in the tropics, mainly along coastal areas of the tropics. The physical appearance of coconut fruits is very distinct and easy to handle. As a consequence, their utilization as lipase sources is foreseeable. The focus of coconut exploration in lipase research was limited to the use of coconut as a medium for lipase-producing fungi (Benjamin and Pandey 1997), immobilization study of other lipases (Brigida *et al.* 2007), and to the potential of coconut as a substrate for lipase reaction (Ibrahim *et al.* 2008). In contrast to its potential, biochemical characterization of coconut lipase has not been sufficiently reported, thus limiting its applications. In this context, the present study investigates the biochemical characterization of coconut lipase. The work includes the analysis of coconut lipase substrate specificity and the property of its subunits. A thorough understanding of the biochemical properties of coconut lipase will lead to its application.

MATERIALS AND METHODS

Materials

Golden coconut (local: *gading* coconut) was obtained from a local garden in Lombok Island of Indonesia. Reagents for buffer and electrophoresis of pro hy grades were obtained from major chemical suppliers. Virgin coconut oil (VCO) was purchased from a local vendor. The artificial lipase substrates were *p*-nitrophenyl butyrate, *p*-nitrophenyl octanoate, *p*-nitrophenyl-decanoate, *p*-nitrophenyl dodecanoate, *p*-nitrophenyl myristate, and *p*-nitrophenyl palmitate, and *p*-nitrophenol (Merck/Sigma-Aldrich). Reagents for in-gel lipase assays were Fast Blue B salt (Sigma-Aldrich) and alpha-naphthyl palmitate (Santa Cruz Biotechnology, USA). Assay buffer contained Arabic gum (Sigma-Aldrich) and sodium deoxycholate (Sigma-Aldrich). Protein determination used a bicinchoninic acid (BCA) kit from Thermo scientific. The Prism 7 tool (GraphPad) and Image-J were used graphical preparation and dye density calculation, respectively.

Methods

Coconut germination, crude extract preparation, and protein determination. The coconut fruits were pickup from coconut the tree after they turned dry, as indicated by the brown color of their shell. The condition was typically reached by the fruits at the age of 11–12 mo. To observe the germination, the outer shell of the fruit was partially removed (Figure 1a). Germination of coconut fruit was attained by storing coconut fruit in the direct sunlight protected open-air condition in our region with an average humidity of above 80% and temperature between 23–28 °C. The humid environment was kept by watering the fruit every day. The coconut shoot's appearance showed germination after c.a. a month of storage (Figure 1a). The germination process was accompanied by the development of haustorium inside coconut fruit (Figure 1b). As the coconut flesh is the primary food storage, coconut lipase was isolated only from the part. Nevertheless, literature reported that all parts of germinating coconut have lipase activity, with the shoot being the most active part (Su'i and Suprihana 2013). The coconut of average size resulted in c.a. 200 grams of meat.

The flesh was shredded and resuspended in 5 mM phosphate buffer, pH 7.0. The suspension was filtered by using a filter cloth. The resulted in coconut milk was centrifuged at 3,000 rpm for 20 min at 4 °C. The floating cream was removed from a 50-mL conical centrifuge tube. The skim fraction was decanted and further subjected to freeze-drying to reduce water content. The resulting 15-mL concentrated coconut lipase was stored at –20 °C for further analysis. Protein concentration was determined using the BCA kit according to the manufacturer's instruction. The developed color was measured at 562 nm by a spectrophotometer (MultiSkan GO, Thermo Scientific).

Enzyme assay. Coconut lipase activity was assayed for its hydrolytic activity against VCO as a substrate (Khor *et al*. 1986). The reaction mixture consisted of 5 g VCO, 2.5 mL n-hexane, 5 mL 100 mM phosphate buffer pH 7.5, and 1 mL of the enzyme. The mixture was incubated

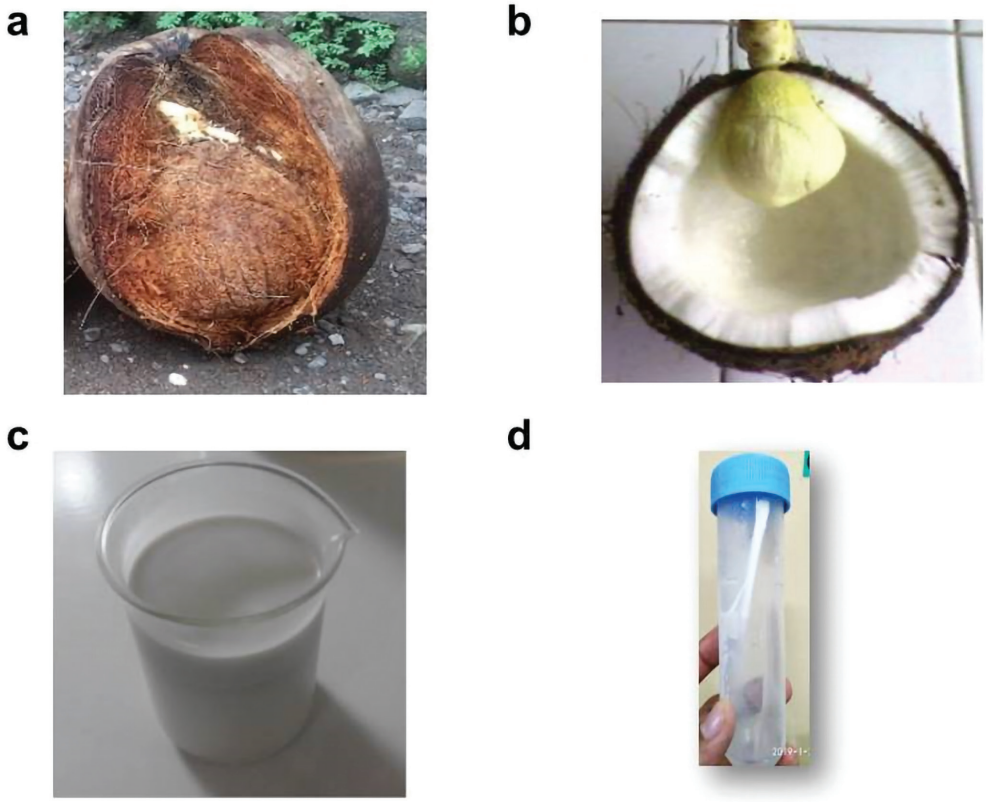

Figure 1. Preparation of coconut lipase from the germinated coconut fruit. a) Coconut shoot appears after a month of germination. b) inside the hard shell, haustorium is developing. Mucilage or coconut flesh was removed and further used as the source of coconut lipase. c) Coconut milk prepared by suspending shredded coconut flesh in 5 mM phosphate buffer, pH 7.0. d) Following centrifugation, the cream fraction was removed. The clear fraction of coconut milk was decanted and stored for electrophoresis and enzyme assays.

in a 35 °C water bath shaker for 45 min and, after this period, 25 mL of acetone/methanol (1:1) was added. The liberated free fatty acids were determined by titration. Sodium hydroxide of 0.01 M was used for the titration following the addition of a few drops of phenolphthalein. Sodium hydroxide was previously standardized against sodium oxalate. Lipase activity was calculated as follows:

$$\text{Lipase activity (U/mL)} = \frac{(V_{sample} - V_{blank})\, x\, [\text{NaOH}]\, x\, 1000}{V_{enzyme}\ x\ t}\ \text{(U/mL)}$$

where:

V_{sample} = titrant volume for sample

V_{blank} = titrant volume for blank

V_{enzyme} = coconut lipase volume

[NaOH] = sodium hydroxide concentration

Coconut lipase activity in the presence of metal ions. Coconut lipase activity was assayed against VCO, as previously described, in the presence of several metal ions. Magnesium, calcium, sodium, potassium, iron, copper, and zinc ions were added to each lipase reaction mixture to a final concentration of 10 mM.

Substrate specificity of coconut lipase. In order to allow kinetics analysis of coconut lipase, a suitable assay condition was first determined. It was performed by hydrolyzing the artificial substrate *p*-nitrophenyl palmitate by serial dilution of coconut lipase. The intensity of the liberated *p*-nitrophenol was measured at 405 nm. Hydrolysis profiles of *p*-nitrophenyl palmitate were recorded every 5 min with lipase dilution range from 1:3,000 to 1:100,000.

For different *p*NP-fatty acids, an 8-min reaction with 1:100,000 dilution of lipase stock was further employed. For each reaction, the *p*NP-fatty acid substrates were prepared as follows: 2 mL of 8 mM *p*NP-fatty acid in n-propanol was added to 18 mL of an emulsifier solution. The emulsifier contained 20 mg of Arabic gum and 41.4 mg sodium deoxycholate in 50 mM Tris-Cl buffer pH 8.0. The substrate solution was kept in the dark before

use. The final concentration of *p*NP-fatty acid in the substrate solution was 0.8 mM. Hydrolysis assay was performed by pre-incubating of 2.7 mL substrate solution at 37 °C for 5 min before the addition of 0.3-mL diluted lipase. The yellow color formation was recorded after 8 min at 405 nm. The coconut lipase specificity was tested against *p*-nitrophenyl butyrate, *p*-nitrophenyl octanoate, *p*-nitrophenyl decanoate, *p*-nitrophenyl dodecanoate, *p*-nitrophenyl myristate, and *p*-nitrophenyl palmitate. One unit activity (U) is defined as micromole(s) of *p*-nitrophenol released upon hydrolysis by 1 mL enzyme at 37 °C under assay conditions (Kanwar *et al*. 2005).

SDS-PAGE and native PAGE. SDS-PAGE was undertaken according to the method initially developed by Laemmli (1970) on 12.5% separating gel and 5% focusing gel. Briefly, a sample containing 50 µg of coconut lipase was precipitated by the addition of an equal volume of cold absolute-ethanol. The resulted precipitate was resuspended in a 5X sample buffer and boiled for 2 min prior to electrophoresis. Electrophoresis was accomplished by applying 150 V of electric current in a mini gel (MiniVe SDS-PAGE apparatus, GEHealthcare) for c.a. 2 h. The resulted gel was stained with Coomassie Brilliant Blue (CBB). For native-PAGE, coconut lipase was subjected to electrophoresis under non-denaturing conditions, *i.e.* by omitting SDS from the gel and running buffer. The sample buffer was also void of 2-mercaptoethanol or dithiothreitol. Ammonium sulfate fractionation was undertaken according to Sana and coworkers (2004). Briefly, ammonium sulfate threshold of 0–30, 30–45, 45–60, 60–75, and 75–90% saturation was added to the protein sample. The excess of salt was removed by dialysis from each fraction. The resulted fractions were subjected to both SDS and native PAGE.

In-gel hydrolysis assay. The activity of lipase subunits was assayed after lipase was separated in 12.5% gel SDS-PAGE. In this case, the sample was not boiled to keep the protein intact. After separation, SDS content was washed by soaking the gel in 50 mM phosphate buffer pH 7.0 and 2.5% (v/v) Triton X-100. It was done with gentle shaking for 30 min. The washing step was repeated twice. The gel loaded with lipase was incubated in a developing solution for 30 min in a dark container to allow hydrolysis to proceed. The developing solution contained alpha-naphthyl palmitate and Fast Blue B salt. Unbound dye was removed by three-time washing in aquadest, 10 min each. The hydrolysis of alpha-naphthyl palmitate corresponded to the lipase subunit activity. The active subunit released a yellow color of alpha-naphthol (Zienkiewicz *et al.* 2014) that appeared on the gel. An identical gel stained by CBB was prepared for comparison.

RESULTS AND DISCUSSION

In this study, lipase was isolated from germinating coconut and its biochemical properties were investigated. Since many biochemical properties of coconut lipase remain unclear, coconut lipase's biochemical characterization is necessary, and the results will facilitate further utilization of coconut lipase.

VCO was used as the substrate for coconut lipase hydrolysis activity instead of using popular olive oil since it offers a more comprehensive composition of fatty acids ester from various chain lengths. VCO has also been investigated in the optimation of many lipases, such as immobilized *Mucor mihei* lipase (Chua *et al*. 2012), *Candida rugosa*, and porcine pancreas lipases (Nguyen *et al*. 2018). The isolated coconut lipase has an activity of 0.56 U/mL corresponding to a specific activity of 0.30 U/mg protein. These results resemble those reported by Su'i and Suprihana (2013).

Figure 2 shows that coconut lipase has a very high esterase activity against artificial substrate *p*-nitrophenyl palmitate. Sample dilution by factors of 6 and 50 thousand times would lead to immediate saturation curves after 5 min of incubation. Sample dilution by a factor of 100 thousand times showed a delayed saturation curve, namely after 20 min of reaction. This dilution factor was used for the specificity assay below since it met the requirement of first-order kinetics in its initial reaction. The high lipase activity from various germinating seeds has been reported (Barros *et al.* 2010) with castor bean (Eastmond 2004), and egusi melon seed (Bege *et al*. 2015) are only a few exceptions as their ungerminated seeds also show significant lipase activity.

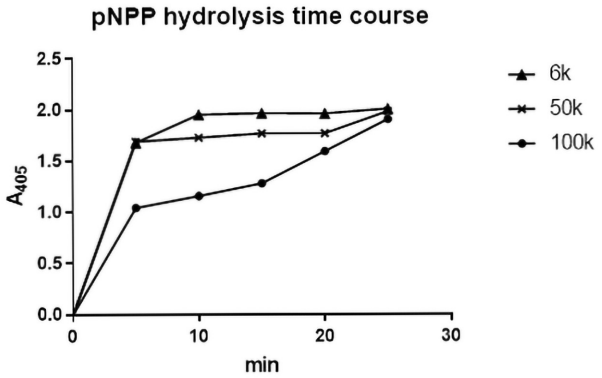

Figure 2. Coconut lipase activity at different dilutions. The activity of coconut lipase of different dilutions was assayed at different time points. The yellow color showed lipase activity as a result of *p*-nitrophenyl palmitate hydrolysis. After ten minutes of reaction, the saturated curve is observed by 6,000 times diluted lipase. For the more diluted lipase, saturation is delayed. In the substrate specificity assay, 8 min of incubation times were chosen, with the sample diluted by 100,000 factors.

Many lipases have their activity altered in the presence of specific metal ions. Here, the effect of several metal ions on the esterase activity of coconut lipase was tested. Figure 3 shows that calcium ions act as coconut lipase activators. A literature survey also suggested that calcium ions activate many plant lipases, such as those from white melon kern (Eze and Ezema 2012). Calcium ion is a well-known activator for different sources of lipases, presumably by stabilizing the three-dimensional structure of lipase during catalysis (Rosenstein and Gotz 2000). On the other hand, Fe^{3+}, Cu^{2+}, Zn^{2+}, and Mg^{2+} – as well as alkali ions K^+ and Na^+ – decreased the esterase activity of coconut lipase (Table 1). It suggests that those ions induced different conformational levels of the lipase that unfavored esterase activity (Hertadi and Widhyastuti 2015), although a deep structural study is necessary to understand the effect of various metal ions. To our knowledge, only a few lipases are inhibited by magnesium ions, such as rice bran (Bhardwaj *et al.* 2001), almond seed (Yesiloglu and Baskurt 2013), and Africa bean seed (Enujiugha *et al.* 2004) lipases (Table 1). Coconut lipase adds a new member to the relatively short list of plant seed lipases inhibited by magnesium ions.

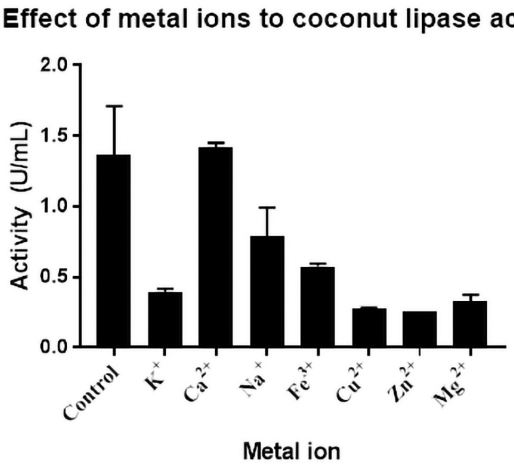

Figure 3. The activity of coconut lipase with the presence of metal ions. The effect of metal ions on coconut lipase activity was assayed by the inclusion of 10 mM of respective metal ions in the assay mixture. The released free fatty acids were titrated by using sodium hydroxide. Control was provided by measuring lipase activity against VCO substrate in the absence of metal ions. All measurements were made in triplicate.

The substrate specificity of coconut lipase was analyzed using various *p*-nitrophenyl fatty acid esters of different chain lengths. Figure 4 shows that *p*-nitrophenyl laurate (C12) gives the highest hydrolysis product in a given time at the initial period of reaction, and the longer fatty acids (C14 *p*-nitrophenyl myristate and C16 *p*-nitrophenyl palmitate) come next. The shorter fatty acids (C4 *p*-nitrophenyl butyrate, C8 *p*-nitrophenyl octanoate, and C10 *p*-nitrophenyl decanoate) give lower hydrolysis products in the same incubation time. Lauric acid (C12) is the predominant fatty acid of coconut that belongs to

Table 1. Properties of some plant-based lipases.

No.	Lipase source	MW (kDa)	Activator	Inhibitor	Reference
1[a]	Rice bran (*Oryza sativa*)	~ 10	Potassium acetate, sodium acetate, NaCl	CHAPS, digitonin, SDS, NP-40, Triton X-100, Mg^{2+}, Mn^{2+}, Cu^{2+}, Cd^{2+}	Barros *et al.* (2010)
	Rice bran lipase II	33	n/a.	n/a	Aizono *et al.* (1976)
	Rice bran	40	n/a	n/a	Kim (2004)
2[b]	Castor bean (*Ricinus communis* L.)	60	Ca^{2+}	p-chloromercuribenzoic, $HgCl_2$	Eastmond (2004)
	Castor bean	n/a	Slightly activated by Na^+, K^+, and dithiothreitol	Mg^{2+}, Ca^{2+}	Muto and Beevers (1974)
	Castor bean	60	Mn^{2+}, Na^+, K^+, Al^{3+} and Li^+	Zn^{2+}, Co^{2+}, Pb^{2+}, Cu^+	Su *et al.* (2010)
3	Linseed (*Linum usitatissimum*)	42	Mg^{2+}, K^+	Triton x-100, Tween 80	Sammour (2005)
4	Almond seed (*Amygdalus communis* L.)	n/a	Ca^{2+}, Fe^{2+}, Mn^{2+}, Co^{2+}, Ba^{2+}	Mg^{2+}, Cu^{2+}, Ni^{2+}	Yesiloglu and Baskurt (2013)
5	Africa bean seed (*Pentachlethra macrophylla* Benth)	n/a	Ca^{2+}	NaCl, $MgCl_2$, EDTA	Enujiugha *et al.* (2004)
6	Sunflower seed (*Helianthus annuus* L.)	40-50	Ca^{2+}, Mg^{2+}	Hg^{2+}, EDTA	Sadeghipour and Bhatla (2003)
7	Canola lipase (*Brassica napus*)	n/a	Ca^{2+}, Bi^{3+}	Fe^{2+}, Fe^{3+}, Zn^{2+}, Hg^{2+}, Cu^{2+}	Sana *et al.* (2004)

MW – molecular weight; n/a – not available; FA – fatty acid; TAG – triacylglycerol
[a]Different reports of lipases from rice bran
[b]Different reports for lipase from these seeds suggest that they have at least two lipases, *i.e.* the acid and alkaline lipase

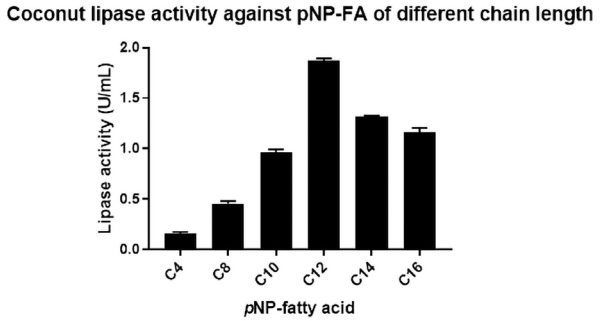

Figure 4. Substrate specificity of coconut lipase. Diluted coconut lipase was allowed to hydrolyze various esters of fatty acids (*p*-nitrophenyl butyrate, *p*-nitrophenyl octanoate, *p*-nitrophenyl decanoate, *p*-nitrophenyl dodecanoate, *p*-nitrophenyl myristate, and *p*-nitrophenyl palmitate) for 8 min of reaction. The released *p*-nitrophenol was measured at 405 nm, and the obtained values were converted to lipase activity. All measurements were made triplicate.

the medium-chain fatty acid (Manohar *et al*. 2019; Dayrit 2014). The complete hydrolysis of VCO by other lipases reported by Chua *et al.* (2012) and Nguyen *et al.* (2018) confirmed that lauric acid is the predominant fatty acid of coconut. Instead of using complete hydrolysis, the kinetics study reported here took advantage of the use of various *p*NP-fatty acid substrates to allow the investigation at the initial period of reaction, from which the fatty acid preference of coconut lipase can easily be determined. The aforementioned result indicates that coconut lipase – in order of preference – hydrolyzes medium, long, and short-chain fatty acid esters.

SDS-PAGE and native-PAGE of coconut lipase are shown in Figure 5. SDS-PAGE electroforegram reveals at least four protein bands of approximately 54 kDa, 32 kDa, 21 kDa, and 15 kDa. However, native-PAGE reveals only a single band of c.a. 130 kDa. After thresholds of ammonium sulfate precipitation, separation of coconut lipase also shows a single complex band in native PAGE for all fractions (Figure 6). Together, these suggest that coconut lipase is a heteromeric enzyme. In humans, hormone-sensitive lipase – an enzyme involves in the mobilization of lipid storage in adipose tissue – has long been shown to be more active in its ~ 160 kDa dimer. It is 40 times more active than the ~ 85 kDa monomer form (Shen *et al.* 2000). A reverse situation is recently reported for the human lipoprotein lipase, whose 55 kDa monomer has similar activity to its 110 kDa homodimer (Beigneux *et al.* 2019). The fact that coconut lipase consists of several subunits and that it is not universal that all subunits of given lipase are functional highlights the need to dissect whether all coconut lipase subunits are active. To address the above question, an in-gel cleavage assay was performed.

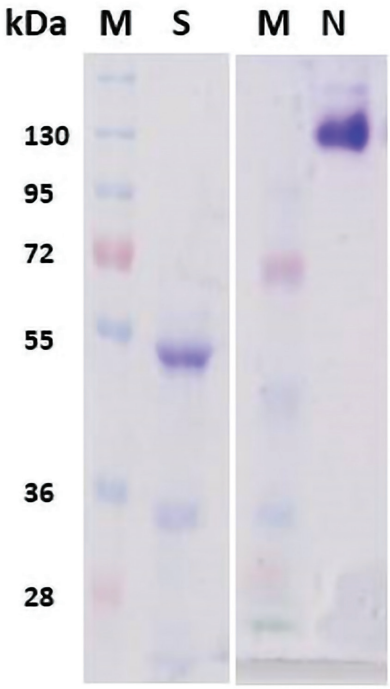

Figure 5. Coconut lipase separation in SDS-PAGE and native-PAGE. Coconut lipase separated on a 10% gel of SDS-PAGE (S) shows different protein bands, *i.e.* 54 kDa, 32 kDa, and 21 kDa. The bands are from a single complex protein of c.a. 130 kDa, as shown in native-PAGE (N). Note: the smallest band of c.a. 15 kDa is not shown here but is obvious on a 12.5% gel (Figures 6 and 7b).

In-gel hydrolysis assay allows us to analyze whether a specific protein can hydrolyze fatty acyl ester after the separation of proteins by electrophoresis. An active protein within the gel cleaves the substrate alpha naphthyl palmitate to release a yellow coloring of naphthol (Figure 7a), following SDS removal from the gel (Zienkiewicz *et al.* 2015). Figure 7b shows that all coconut lipase subunits can hydrolyze alpha naphthyl palmitate, which indicates that all coconut lipases are active. Two subunits with equal intensity on CBB staining produce different naphthol intensity, demonstrated by the 54 kDa dan 21 kDa subunits (Figure 7b). It suggests that the two subunits have a different affinity to alpha naphthyl palmitate, with the latter has a lower affinity. However, this does not nullify the possibility that the 21 kDa subunit has a higher esterase activity for shorter or longer fatty acids. Subashri and coworkers (2018) have identified coconut lipase with a molecular weight between 29–43 kDa , which is comparable to the 32 kDa subunit in the present study. Since Subahsri *et al*. used ester of oleic acid as substrate, one can speculate that the 32 kDa subunit of coconut lipase has a preference for the C18 fatty acid ester. Hence, it is worth testing whether the cleavage of medium-chain and short-chain fatty acids by coconut lipase gives the same

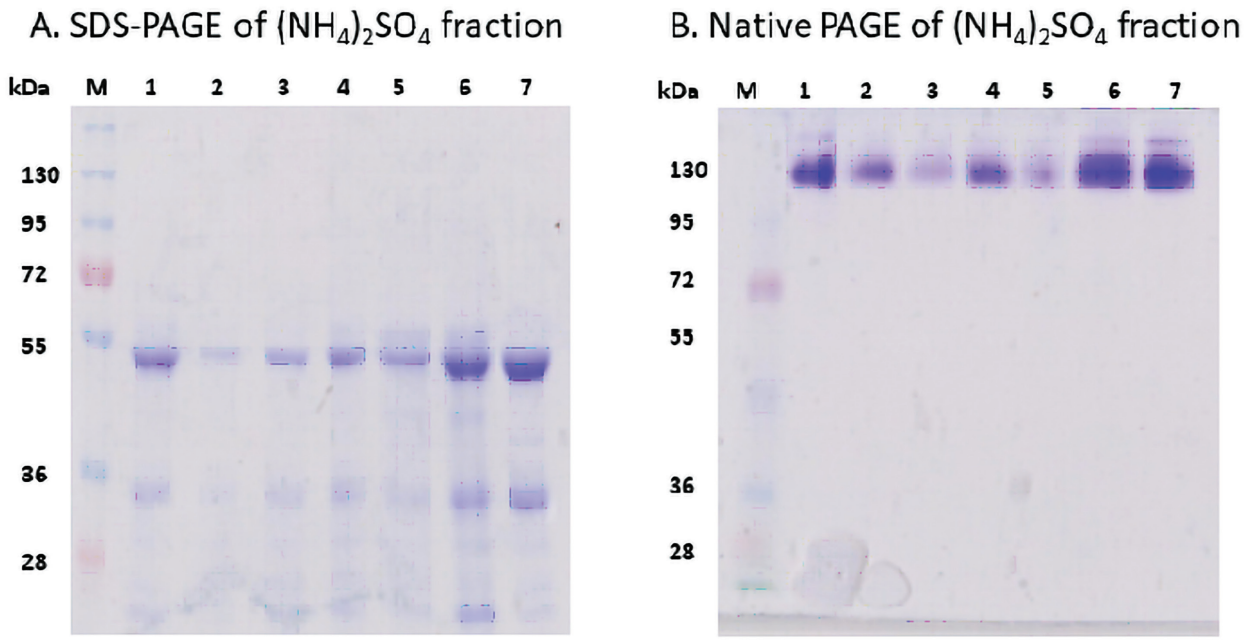

Figure 6. Ammonium sulfate fractions of coconut lipase separated by SDS-PAGE (A) and native PAGE (B). M – protein marker; 1 – crude extract; 2 – fraction 0–15%; 3 – fraction 15–30%; 4 – fraction 30 –45%; 5 – fraction 45–60%; 6 – fraction 60-75%; 7: fraction 75–90%.

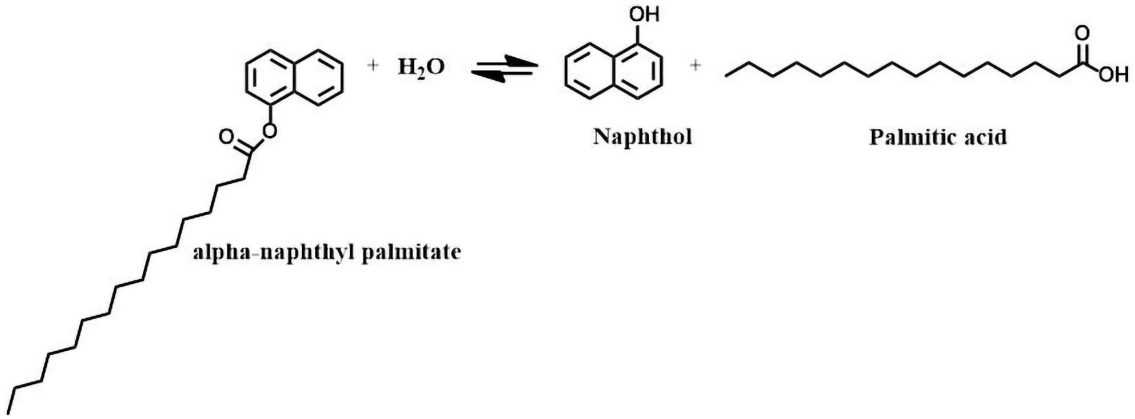

Figure 7a. Hydrolysis of alpha-naphthyl palmitate by lipase. An active lipase or esterase cleaves alpha-naphthyl palmitate to release naphthol. The yellow color of naphthol is measured spectrophotometrically at 405 nm.

pattern. There are faint protein bands at c.a. 40 kDa and 45 kDa in addition to the four distinct subunits. We speculate that the two proteins are glycosylated forms of the 32 kDa subunit. Our finding that coconut lipase consists of several active subunits may explain contradictory reports on plant seed lipase activities, such as those from rice *Oryza sativa* lipase (Table 1).

The data presented in this study show that all coconut lipase subunits can cleave fatty acid esters at a different rate of hydrolysis. However, it is worth noting that the regioselectivity and stereoselectivity of coconut lipase remain unclear. To address this issue, the hydrolysis of triacylglycerol substrates of various acyl group lengths will provide the required data. From the present experiment, we expect that coconut lipase has the highest affinity to trilaurin. Hydrolysis study with 1-monolaurin, 1,3-dilaurin, and 2,3-dilaurin substrates will reveal regioselectivity of coconut lipase. Moreover, to obtain details of individual subunits' activity, it is deemed necessary to separate the subunits and test their specificity. Such a study may reveal the contribution of subunits to the coconut lipase as a whole. Furthermore, if cloning and heterologous expression are desired, this can be directed to

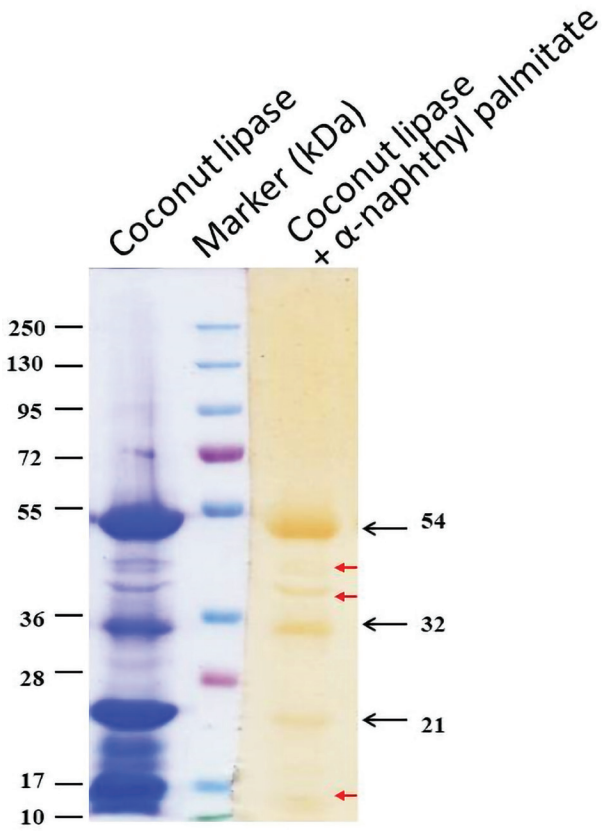

Figure 7b. In-gel activity assay of coconut lipase. Coconut lipase is separated in a 12.5% polyacrylamide gel under denatured conditions, except for the boiling step. The gel was cut for CBB staining (left) and an in-gel assay(right). At least four distinct bands are noticed upon CBB staining, including the smallest subunit of c.a. 15 kDa. Additional faint bands are seen above the 32 kDa. The corresponding hydrolysis products by lipase subunits appear as yellow bands. It represents the results of alpha naphthyl palmitate hydrolysis by respective lipase subunits. The pixel density ratio of naphthol to CBB staining for the 54 kDa and the 21 kDa subunits is 77.5% and 43.2%, respectively. Note: several distinct bands above the 32 kDa band (red arrows) are shown to be active in hydrolyzing the alpha naphthyl substrate. It gives a hint that the 32 kDa is glycosylated. We also speculate that a smeary pattern observed in the native gel (Figure 5) indicates the glycosylation of native protein.

the study of individual subunits, especially at the current circumstance when the coconut genome is emerging on the horizon (Xiao *et al.* 2017). Accordingly, biochemical characterization of various subunits (optimum temperature and pH reaction, substrate specificity, metal ions effect, and detergent effect) would provide more detailed information.

CONCLUSION

By using a simple procedure, we have been able to isolate coconut lipase. A direct comparison of SDS-PAGE and native PAGE readily hint that coconut lipase is a complex enzyme. This enzyme consists of four subunits of 54, 32, 21, and 15 kDa. In its complex form, coconut lipase shows the highest preference for lauryl esters. The enzyme is activated by Ca^{2+} ion, whereas Fe^{3+}, Cu^{2+}, Zn^{2+}, Mg^{2+}, K^{+}, and Na^{+} decrease coconut lipase activity. Each subunit can cleave the fatty acid ester bond; hence, this enzyme might be regarded as a cluster of smaller active proteins. Since all coconut lipase subunits are active as esterases, specificity determination of subunits and further biochemical characterization of the subunits are yet to be investigated. We also propose that a similar approach can be applied for the initial study of other plant or seed-based lipases.

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STATEMENT ON CONFLICT OF INTEREST

All authors declare to have no conflict of interest.

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Lalu Rudyat Telly Savalas <telly@unram.ac.id>

Re: 1st Draft of PJS Article Ms 21-049

1 message

Lalu Rudyat Telly Savalas <telly@unram.ac.id> 21 June 2021 at 11:45
To: Philippine Journal of Science <philjournsci@gmail.com>

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Managing Editor

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with best regards,

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K15 Copyedit/second draft 5 Juli 2021

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Re: 1st Draft of PJS Article Ms 21-049

1 message

Philippine Journal of Science <philjournsci@gmail.com> 5 July 2021 at 10:28
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Biochemical Properties of Coconut (*Cocos nucifera* L.) Lipase

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Ubiquitous in nature, lipases represent an example of enzymes with high versatility. Plant seeds are potential sources of lipase, and they are attracting more attention for specific purposes. In this study, coconut lipase was isolated from germinating coconut seed. Biochemical characterization of coconut lipase was undertaken to reveal its substrate specificity and its subunits properties. By using various chromogenic ester of fatty acids, it was demonstrated that lauric acid is the most preferred substrate for coconut lipase esterase reaction. Calcium ions enhance its activity, whereas other metal ions such as magnesium, nickel, sodium, and potassium reduce it. Electrophoresis under native conditions showed that coconut lipase is a single protein. Since electrophoresis under denaturing conditions revealed four subunits, coconut lipase is likely a complex enzyme. It was further revealed that all subunits are active, as evident in an in-gel hydrolysis assay. However, they also hint that they do not have an equal catalytic rate against the 16-carbon-length palmitate derivative. This finding, thus, opens up a notion that those subunits have different substrates specificity yet to be determined.

Keywords: coconut lipase, in-gel assay, lipase subunits, native electrophoresis, substrate specificity

INTRODUCTION

Fatty acids are widely used in modern life and, hence, are of critical industrial concerns. The utilization of fatty acids spans from essential ingredients in many industries, such as in coating (Rajput *et al.* 2014), surfactants (Semblante *et al*. 2009), lubricants (Ruths *et al.* 2008), essential fatty acids, nutraceuticals production (Sande *et al.* 2018), personal care products (Tavares *et al.* 2018), and bioremediation (Melani *et al.* 2019). Several methods achieve fatty acid production from fats, such as the mechanical separation process, alkaline chemical hydrolysis, and enzymatic process. Mechanical separation requires high pressure and temperature that causes the process costly. Likewise, alkaline hydrolysis also offers a practical method. However, efforts are needed to separate unwanted products (Sande *et al.* 2018). In contrast,

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enzymatic hydrolysis operates at mild conditions (low temperature and pressure). It offers ease in the recovery process (Jain and Mishra 2015) and product loss due to minimized overheating (Barros *et al.* 2010).

Lipases or glycerol ester hydrolases (EC 3.1.1.3) are enzymes that can perform hydrolysis, esterification, and transesterification reactions under mild conditions. Which reaction takes place largely depends on the reaction environment (Tavares *et al.* 2018). Lipases act on different ester compounds, with acylglycerols become their principal substrates. All oilseed plants have significant amounts of lipases. Plant-based lipases are increasingly become the researcher's interest due to low production cost and high specificity (Tavares *et al.* 2018; Villeneuve 2003). They also have an easy pharmacological acceptance due to their eukaryotic source (Seth *et al.* 2014). Essential sources of plant-based lipases are plant seeds, especially the seeds in their germinating phases. Examples are lipases from *Carica papaya* (Campillo-Alvarado and Tovar Miranda 2013), *Pentaclethra macrophylla* (Enujiugha *et al.* 2004), linseed (Sammour 2005), and coconut (Ejedegba *et al.* 2013). However, significant lipase activity from non-germinating seeds also exists, such as in castor beans (Eastmond 2004; Tavares *et al.* 2018).

Coconut trees grow almost in every region in the tropics, mainly along coastal areas of the tropics. The physical appearance of coconut fruits is very distinct and easy to handle. As a consequence, their utilization as lipase sources is foreseeable. The focus of coconut exploration in lipase research was limited to the use of coconut as a medium for lipase-producing fungi (Benjamin and Pandey 1997), immobilization study of other lipases (Brigida *et al.* 2007), and to the potential of coconut as a substrate for lipase reaction (Ibrahim *et al.* 2008). In contrast to its potential, biochemical characterization of coconut lipase has not been sufficiently reported, thus limiting its applications. In this context, the present study investigates the biochemical characterization of coconut lipase. The work includes the analysis of coconut lipase substrate specificity and the property of its subunits. A thorough understanding of the biochemical properties of coconut lipase will lead to its application.

MATERIALS AND METHODS

Materials

Golden coconut (local: *gading* coconut) was obtained from a local garden in Lombok Island of Indonesia. Reagents for buffer and electrophoresis of pro hy grades were obtained from major chemical suppliers. Virgin coconut oil (VCO) was purchased from a local vendor. The artificial lipase substrates were *p*-nitrophenyl butyrate, *p*-nitrophenyl octanoate, *p*-nitrophenyl-decanoate, *p*-nitrophenyl dodecanoate, *p*-nitrophenyl myristate, and *p*-nitrophenyl palmitate, and *p*-nitrophenol (Merck/Sigma-Aldrich). Reagents for in-gel lipase assays were Fast Blue B salt (Sigma-Aldrich) and alpha-naphthyl palmitate (Santa Cruz Biotechnology, USA). Assay buffer contained Arabic gum (Sigma-Aldrich) and sodium deoxycholate (Sigma-Aldrich). Protein determination used a bicinchoninic acid (BCA) kit from Thermo scientific. The Prism 7 tool (GraphPad) and Image-J were used graphical preparation and dye density calculation, respectively.

Methods

Coconut germination, crude extract preparation, and protein determination. The coconut fruits were pickup from coconut the tree after they turned dry, as indicated by the brown color of their shell. The condition was typically reached by the fruits at the age of 11–12 mo. To observe the germination, the outer shell of the fruit was partially removed (Figure 1a). Germination of coconut fruit was attained by storing coconut fruit in the direct sunlight protected open-air condition in our region with an average humidity of above 80% and temperature between 23–28 °C. The humid environment was kept by watering the fruit every day. The coconut shoot's appearance showed germination after c.a. a month of storage (Figure 1a). The germination process was accompanied by the development of haustorium inside coconut fruit (Figure 1b). As the coconut flesh is the primary food storage, coconut lipase was isolated only from the part. Nevertheless, literature reported that all parts of germinating coconut have lipase activity, with the shoot being the most active part (Su'i and Suprihana 2013). The coconut of average size resulted in c.a. 200 grams of meat.

The flesh was shredded and resuspended in 5 mM phosphate buffer, pH 7.0. The suspension was filtered by using a filter cloth. The resulted in coconut milk was centrifuged at 3,000 rpm for 20 min at 4 °C. The floating cream was removed from a 50-mL conical centrifuge tube. The skim fraction was decanted and further subjected to freeze-drying to reduce water content. The resulting 15-mL concentrated coconut lipase was stored at –20 °C for further analysis. Protein concentration was determined using the BCA kit according to the manufacturer's instruction. The developed color was measured at 562 nm by a spectrophotometer (MultiSkan GO, Thermo Scientific).

Enzyme assay. Coconut lipase activity was assayed for its hydrolytic activity against VCO as a substrate (Khor *et al*. 1986). The reaction mixture consisted of 5 g VCO, 2.5 mL n-hexane, 5 mL 100 mM phosphate buffer pH 7.5, and 1 mL of the enzyme. The mixture was incubated

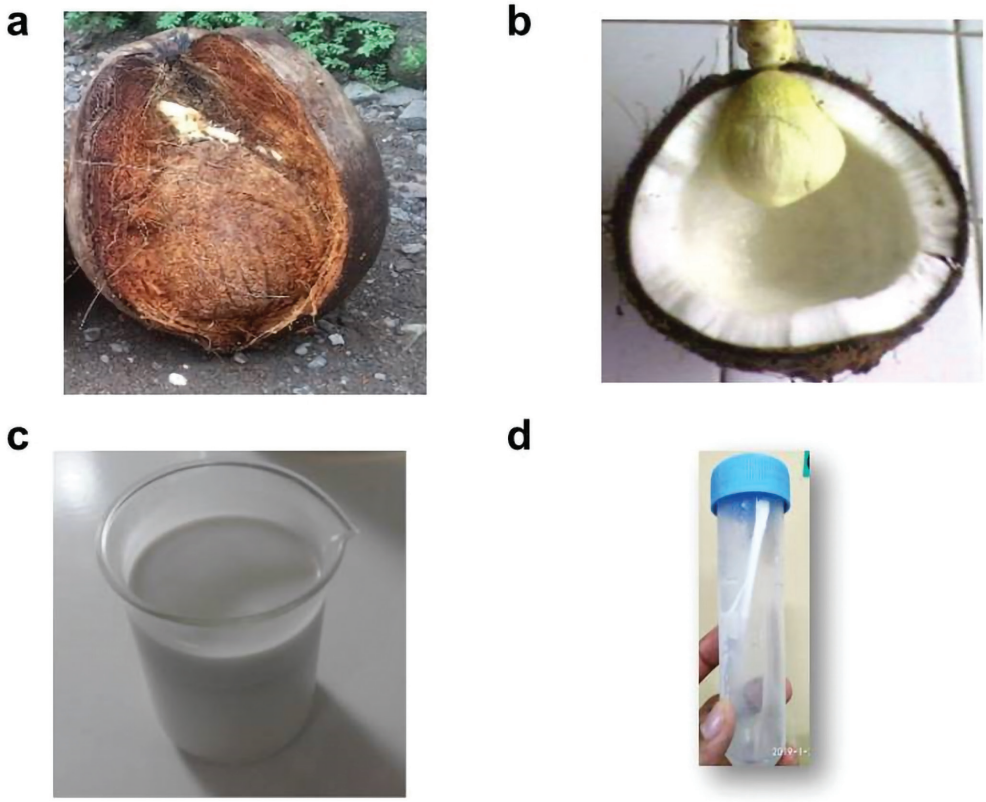

Figure 1. Preparation of coconut lipase from the germinated coconut fruit. a) Coconut shoot appears after a month of germination. b) inside the hard shell, haustorium is developing. Mucilage or coconut flesh was removed and further used as the source of coconut lipase. c) Coconut milk prepared by suspending shredded coconut flesh in 5 mM phosphate buffer, pH 7.0. d) Following centrifugation, the cream fraction was removed. The clear fraction of coconut milk was decanted and stored for electrophoresis and enzyme assays.

in a 35 °C water bath shaker for 45 min and, after this period, 25 mL of acetone/methanol (1:1) was added. The liberated free fatty acids were determined by titration. Sodium hydroxide of 0.01 M was used for the titration following the addition of a few drops of phenolphthalein. Sodium hydroxide was previously standardized against sodium oxalate. Lipase activity was calculated as follows:

$$\text{Lipase activity (U/mL)} = \frac{(V_{sample} - V_{blank})\, x\, [\text{NaOH}]\, x\, 1000}{V_{enzyme\ x\ t}}\ \text{(U/mL)}$$

where:

V_{sample} = titrant volume for sample

V_{blank} = titrant volume for blank

V_{enzyme} = coconut lipase volume

[NaOH] = sodium hydroxide concentration

Coconut lipase activity in the presence of metal ions. Coconut lipase activity was assayed against VCO, as previously described, in the presence of several metal ions. Magnesium, calcium, sodium, potassium, iron, copper, and zinc ions were added to each lipase reaction mixture to a final concentration of 10 mM.

Substrate specificity of coconut lipase. In order to allow kinetics analysis of coconut lipase, a suitable assay condition was first determined. It was performed by hydrolyzing the artificial substrate *p*-nitrophenyl palmitate by serial dilution of coconut lipase. The intensity of the liberated *p*-nitrophenol was measured at 405 nm. Hydrolysis profiles of *p*-nitrophenyl palmitate were recorded every 5 min with lipase dilution range from 1:3,000 to 1:100,000.

For different *p*NP-fatty acids, an 8-min reaction with 1:100,000 dilution of lipase stock was further employed. For each reaction, the *p*NP-fatty acid substrates were prepared as follows: 2 mL of 8 mM *p*NP-fatty acid in n-propanol was added to 18 mL of an emulsifier solution. The emulsifier contained 20 mg of Arabic gum and 41.4 mg sodium deoxycholate in 50 mM Tris-Cl buffer pH 8.0. The substrate solution was kept in the dark before

use. The final concentration of *p*NP-fatty acid in the substrate solution was 0.8 mM. Hydrolysis assay was performed by pre-incubating of 2.7 mL substrate solution at 37 °C for 5 min before the addition of 0.3-mL diluted lipase. The yellow color formation was recorded after 8 min at 405 nm. The coconut lipase specificity was tested against *p*-nitrophenyl butyrate, *p*-nitrophenyl octanoate, *p*-nitrophenyl decanoate, *p*-nitrophenyl dodecanoate, *p*-nitrophenyl myristate, and *p*-nitrophenyl palmitate. One unit activity (U) is defined as micromole(s) of *p*-nitrophenol released upon hydrolysis by 1 mL enzyme at 37 °C under assay conditions (Kanwar *et al*. 2005).

SDS-PAGE and native PAGE. SDS-PAGE was undertaken according to the method initially developed by Laemmli (1970) on 12.5% separating gel and 5% focusing gel. Briefly, a sample containing 50 µg of coconut lipase was precipitated by the addition of an equal volume of cold absolute-ethanol. The resulted precipitate was resuspended in a 5X sample buffer and boiled for 2 min prior to electrophoresis. Electrophoresis was accomplished by applying 150 V of electric current in a mini gel (MiniVe SDS-PAGE apparatus, GEHealthcare) for c.a. 2 h. The resulted gel was stained with Coomassie Brilliant Blue (CBB). For native-PAGE, coconut lipase was subjected to electrophoresis under non-denaturing conditions, *i.e.* by omitting SDS from the gel and running buffer. The sample buffer was also void of 2-mercaptoethanol or dithiothreitol. Ammonium sulfate fractionation was undertaken according to Sana and coworkers (2004). Briefly, ammonium sulfate threshold of 0–30, 30–45, 45–60, 60–75, and 75–90% saturation was added to the protein sample. The excess of salt was removed by dialysis from each fraction. The resulted fractions were subjected to both SDS and native PAGE.

In-gel hydrolysis assay. The activity of lipase subunits was assayed after lipase was separated in 12.5% gel SDS-PAGE. In this case, the sample was not boiled to keep the protein intact. After separation, SDS content was washed by soaking the gel in 50 mM phosphate buffer pH 7.0 and 2.5% (v/v) Triton X-100. It was done with gentle shaking for 30 min. The washing step was repeated twice. The gel loaded with lipase was incubated in a developing solution for 30 min in a dark container to allow hydrolysis to proceed. The developing solution contained alpha-naphthyl palmitate and Fast Blue B salt. Unbound dye was removed by three-time washing in aquadest, 10 min each. The hydrolysis of alpha-naphthyl palmitate corresponded to the lipase subunit activity. The active subunit released a yellow color of alpha-naphthol (Zienkiewicz *et al.* 2014) that appeared on the gel. An identical gel stained by CBB was prepared for comparison.

RESULTS AND DISCUSSION

In this study, lipase was isolated from germinating coconut and its biochemical properties were investigated. Since many biochemical properties of coconut lipase remain unclear, coconut lipase's biochemical characterization is necessary, and the results will facilitate further utilization of coconut lipase.

VCO was used as the substrate for coconut lipase hydrolysis activity instead of using popular olive oil since it offers a more comprehensive composition of fatty acids ester from various chain lengths. VCO has also been investigated in the optimation of many lipases, such as immobilized *Mucor mihei* lipase (Chua *et al*. 2012), *Candida rugosa*, and porcine pancreas lipases (Nguyen *et al*. 2018). The isolated coconut lipase has an activity of 0.56 U/mL corresponding to a specific activity of 0.30 U/mg protein. These results resemble those reported by Su'i and Suprihana (2013).

Figure 2 shows that coconut lipase has a very high esterase activity against artificial substrate *p*-nitrophenyl palmitate. Sample dilution by factors of 6 and 50 thousand times would lead to immediate saturation curves after 5 min of incubation. Sample dilution by a factor of 100 thousand times showed a delayed saturation curve, namely after 20 min of reaction. This dilution factor was used for the specificity assay below since it met the requirement of first-order kinetics in its initial reaction. The high lipase activity from various germinating seeds has been reported (Barros *et al.* 2010) with castor bean (Eastmond 2004), and egusi melon seed (Bege *et al*. 2015) are only a few exceptions as their ungerminated seeds also show significant lipase activity.

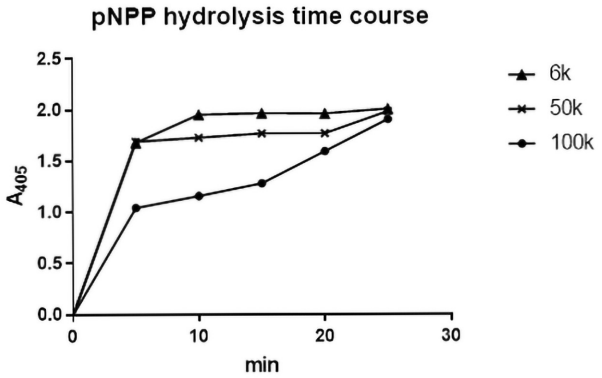

Figure 2. Coconut lipase activity at different dilutions. The activity of coconut lipase of different dilutions was assayed at different time points. The yellow color showed lipase activity as a result of *p*-nitrophenyl palmitate hydrolysis. After ten minutes of reaction, the saturated curve is observed by 6,000 times diluted lipase. For the more diluted lipase, saturation is delayed. In the substrate specificity assay, 8 min of incubation times were chosen, with the sample diluted by 100,000 factors.

Many lipases have their activity altered in the presence of specific metal ions. Here, the effect of several metal ions on the esterase activity of coconut lipase was tested. Figure 3 shows that calcium ions act as coconut lipase activators. A literature survey also suggested that calcium ions activate many plant lipases, such as those from white melon kern (Eze and Ezema 2012). Calcium ion is a well-known activator for different sources of lipases, presumably by stabilizing the three-dimensional structure of lipase during catalysis (Rosenstein and Gotz 2000). On the other hand, Fe^{3+}, Cu^{2+}, Zn^{2+}, and Mg^{2+} – as well as alkali ions K^+ and Na^+ – decreased the esterase activity of coconut lipase (Table 1). It suggests that those ions induced different conformational levels of the lipase that unfavored esterase activity (Hertadi and Widhyastuti 2015), although a deep structural study is necessary to understand the effect of various metal ions. To our knowledge, only a few lipases are inhibited by magnesium ions, such as rice bran (Bhardwaj *et al.* 2001), almond seed (Yesiloglu and Baskurt 2013), and Africa bean seed (Enujiugha *et al.* 2004) lipases (Table 1). Coconut lipase adds a new member to the relatively short list of plant seed lipases inhibited by magnesium ions.

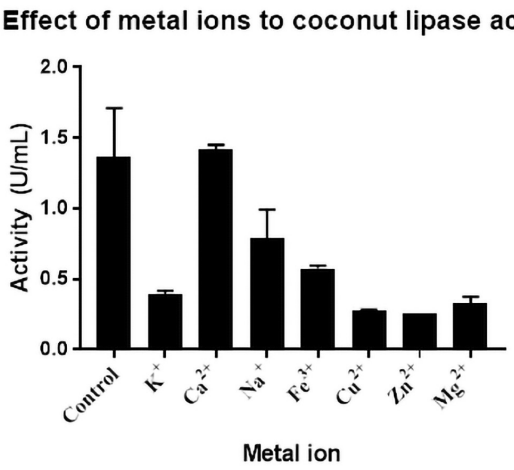

Figure 3. The activity of coconut lipase with the presence of metal ions. The effect of metal ions on coconut lipase activity was assayed by the inclusion of 10 mM of respective metal ions in the assay mixture. The released free fatty acids were titrated by using sodium hydroxide. Control was provided by measuring lipase activity against VCO substrate in the absence of metal ions. All measurements were made in triplicate.

The substrate specificity of coconut lipase was analyzed using various *p*-nitrophenyl fatty acid esters of different chain lengths. Figure 4 shows that *p*-nitrophenyl laurate (C12) gives the highest hydrolysis product in a given time at the initial period of reaction, and the longer fatty acids (C14 *p*-nitrophenyl myristate and C16 *p*-nitrophenyl palmitate) come next. The shorter fatty acids (C4 *p*-nitrophenyl butyrate, C8 *p*-nitrophenyl octanoate, and C10 *p*-nitrophenyl decanoate) give lower hydrolysis products in the same incubation time. Lauric acid (C12) is the predominant fatty acid of coconut that belongs to

Table 1. Properties of some plant-based lipases.

No.	Lipase source	MW (kDa)	Activator	Inhibitor	Reference
1[a]	Rice bran (*Oryza sativa*)	~ 10	Potassium acetate, sodium acetate, NaCl	CHAPS, digitonin, SDS, NP-40, Triton X-100, Mg^{2+}, Mn^{2+}, Cu^{2+}, Cd^{2+}	Barros *et al.* (2010)
	Rice bran lipase II	33	n/a.	n/a	Aizono *et al.* (1976)
	Rice bran	40	n/a	n/a	Kim (2004)
2[b]	Castor bean (*Ricinus communis* L.)	60	Ca^{2+}	p-chloromercuribenzoic, $HgCl_2$	Eastmond (2004)
	Castor bean	n/a	Slightly activated by Na^+, K^+, and dithiothreitol	Mg^{2+}, Ca^{2+}	Muto and Beevers (1974)
	Castor bean	60	Mn^{2+}, Na^+, K^+, Al^{3+} and Li^+	Zn^{2+}, Co^{2+}, Pb^{2+}, Cu^+	Su *et al.* (2010)
3	Linseed (*Linum usitatissimum*)	42	Mg^{2+}, K^+	Triton x-100, Tween 80	Sammour (2005)
4	Almond seed (*Amygdalus communis* L.)	n/a	Ca^{2+}, Fe^{2+}, Mn^{2+}, Co^{2+}, Ba^{2+}	Mg^{2+}, Cu^{2+}, Ni^{2+}	Yesiloglu and Baskurt (2013)
5	Africa bean seed (*Pentachlethra macrophylla* Benth)	n/a	Ca^{2+}	NaCl, $MgCl_2$, EDTA	Enujiugha *et al.* (2004)
6	Sunflower seed (*Helianthus annuus* L.)	40-50	Ca^{2+}, Mg^{2+}	Hg^{2+}, EDTA	Sadeghipour and Bhatla (2003)
7	Canola lipase (*Brassica napus*)	n/a	Ca^{2+}, Bi^{3+}	Fe^{2+}, Fe^{3+}, Zn^{2+}, Hg^{2+}, Cu^{2+}	Sana *et al.* (2004)

MW – molecular weight; n/a – not available; FA – fatty acid; TAG – triacylglycerol
[a]Different reports of lipases from rice bran
[b]Different reports for lipase from these seeds suggest that they have at least two lipases, *i.e.* the acid and alkaline lipase

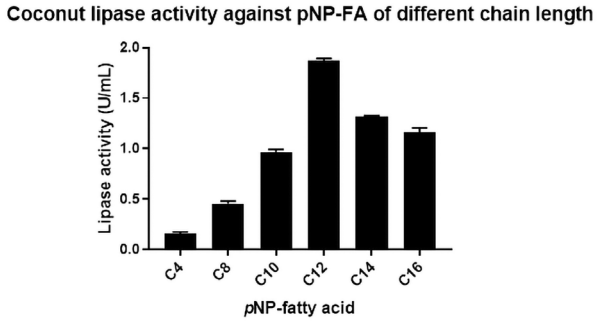

Figure 4. Substrate specificity of coconut lipase. Diluted coconut lipase was allowed to hydrolyze various esters of fatty acids (*p*-nitrophenyl butyrate, *p*-nitrophenyl octanoate, *p*-nitrophenyl decanoate, *p*-nitrophenyl dodecanoate, *p*-nitrophenyl myristate, and *p*-nitrophenyl palmitate) for 8 min of reaction. The released *p*-nitrophenol was measured at 405 nm, and the obtained values were converted to lipase activity. All measurements were made triplicate.

the medium-chain fatty acid (Manohar *et al*. 2019; Dayrit 2014). The complete hydrolysis of VCO by other lipases reported by Chua *et al.* (2012) and Nguyen *et al.* (2018) confirmed that lauric acid is the predominant fatty acid of coconut. Instead of using complete hydrolysis, the kinetics study reported here took advantage of the use of various *p*NP-fatty acid substrates to allow the investigation at the initial period of reaction, from which the fatty acid preference of coconut lipase can easily be determined. The aforementioned result indicates that coconut lipase – in order of preference – hydrolyzes medium, long, and short-chain fatty acid esters.

SDS-PAGE and native-PAGE of coconut lipase are shown in Figure 5. SDS-PAGE electroforegram reveals at least four protein bands of approximately 54 kDa, 32 kDa, 21 kDa, and 15 kDa. However, native-PAGE reveals only a single band of c.a. 130 kDa. After thresholds of ammonium sulfate precipitation, separation of coconut lipase also shows a single complex band in native PAGE for all fractions (Figure 6). Together, these suggest that coconut lipase is a heteromeric enzyme. In humans, hormone-sensitive lipase – an enzyme involves in the mobilization of lipid storage in adipose tissue – has long been shown to be more active in its ~ 160 kDa dimer. It is 40 times more active than the ~ 85 kDa monomer form (Shen *et al.* 2000). A reverse situation is recently reported for the human lipoprotein lipase, whose 55 kDa monomer has similar activity to its 110 kDa homodimer (Beigneux *et al.* 2019). The fact that coconut lipase consists of several subunits and that it is not universal that all subunits of given lipase are functional highlights the need to dissect whether all coconut lipase subunits are active. To address the above question, an in-gel cleavage assay was performed.

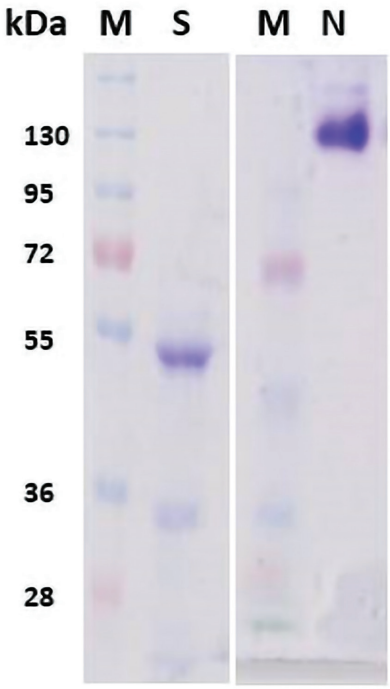

Figure 5. Coconut lipase separation in SDS-PAGE and native-PAGE. Coconut lipase separated on a 10% gel of SDS-PAGE (S) shows different protein bands, *i.e.* 54 kDa, 32 kDa, and 21 kDa. The bands are from a single complex protein of c.a. 130 kDa, as shown in native-PAGE (N). Note: the smallest band of c.a. 15 kDa is not shown here but is obvious on a 12.5% gel (Figures 6 and 8).

In-gel hydrolysis assay allows us to analyze whether a specific protein can hydrolyze fatty acyl ester after the separation of proteins by electrophoresis. An active protein within the gel cleaves the substrate alpha naphthyl palmitate to release a yellow coloring of naphthol (Figure 7), following SDS removal from the gel (Zienkiewicz *et al.* 2015). Figure 8 shows that all coconut lipase subunits can hydrolyze alpha naphthyl palmitate, which indicates that all coconut lipases are active. Two subunits with equal intensity on CBB staining produce different naphthol intensity, demonstrated by the 54 kDa dan 21 kDa subunits (Figure 8). It suggests that the two subunits have a different affinity to alpha naphthyl palmitate, with the latter has a lower affinity. However, this does not nullify the possibility that the 21 kDa subunit has a higher esterase activity for shorter or longer fatty acids. Subashri and coworkers (2018) have identified coconut lipase with a molecular weight between 29–43 kDa , which is comparable to the 32 kDa subunit in the present study. Since Subahsri *et al*. used ester of oleic acid as substrate, one can speculate that the 32 kDa subunit of coconut lipase has a preference for the C18 fatty acid ester. Hence, it is worth testing whether the cleavage of medium-chain and short-chain fatty acids by coconut lipase gives the same

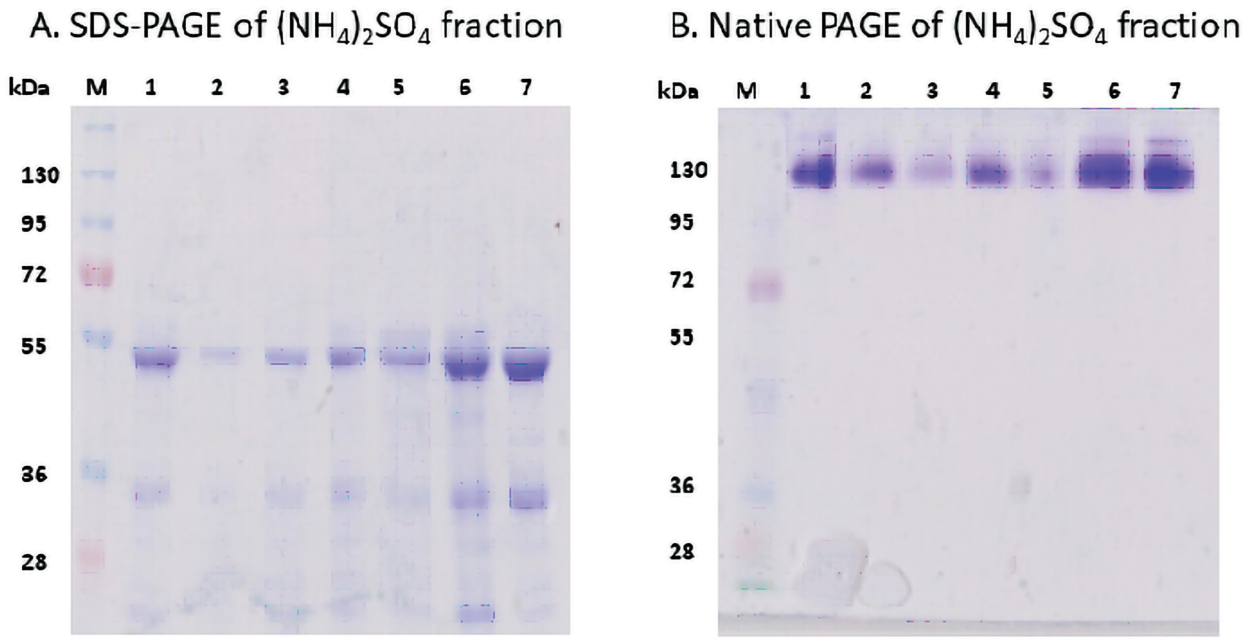

Figure 6. Ammonium sulfate fractions of coconut lipase separated by SDS-PAGE (A) and native PAGE (B). M – protein marker; 1 – crude extract; 2 – fraction 0–15%; 3 – fraction 15–30%; 4 – fraction 30 –45%; 5 – fraction 45–60%; 6 – fraction 60-75%; 7: fraction 75–90%.

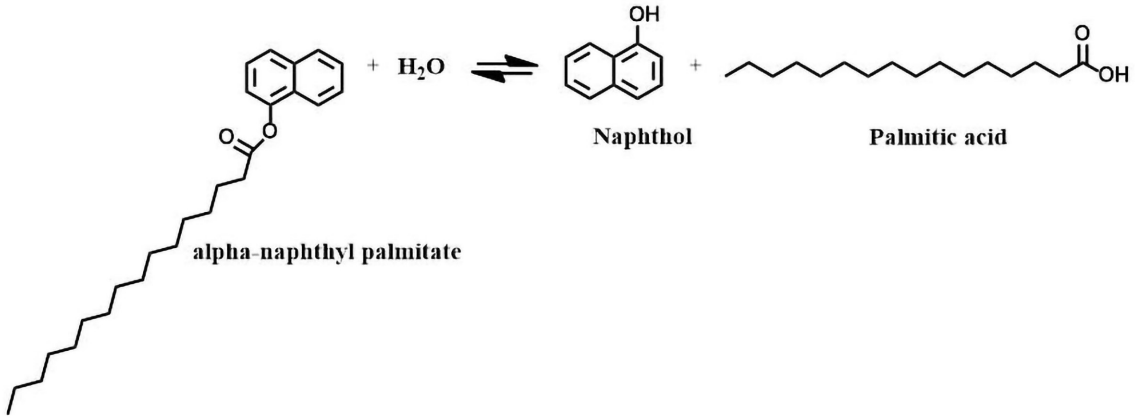

Figure 7. Hydrolysis of alpha-naphthyl palmitate by lipase. An active lipase or esterase cleaves alpha-naphthyl palmitate to release naphthol. The yellow color of naphthol is measured spectrophotometrically at 405 nm.

pattern. There are faint protein bands at c.a. 40 kDa and 45 kDa in addition to the four distinct subunits. We speculate that the two proteins are glycosylated forms of the 32 kDa subunit. Our finding that coconut lipase consists of several active subunits may explain contradictory reports on plant seed lipase activities, such as those from rice *Oryza sativa* lipase (Table 1).

The data presented in this study show that all coconut lipase subunits can cleave fatty acid esters at a different rate of hydrolysis. However, it is worth noting that the regioselectivity and stereoselectivity of coconut lipase remain unclear. To address this issue, the hydrolysis of triacylglycerol substrates of various acyl group lengths will provide the required data. From the present experiment, we expect that coconut lipase has the highest affinity to trilaurin. Hydrolysis study with 1-monolaurin, 1,3-dilaurin, and 2,3-dilaurin substrates will reveal regioselectivity of coconut lipase. Moreover, to obtain details of individual subunits' activity, it is deemed necessary to separate the subunits and test their specificity. Such a study may reveal the contribution of subunits to the coconut lipase as a whole. Furthermore, if cloning and heterologous expression are desired, this can be directed to

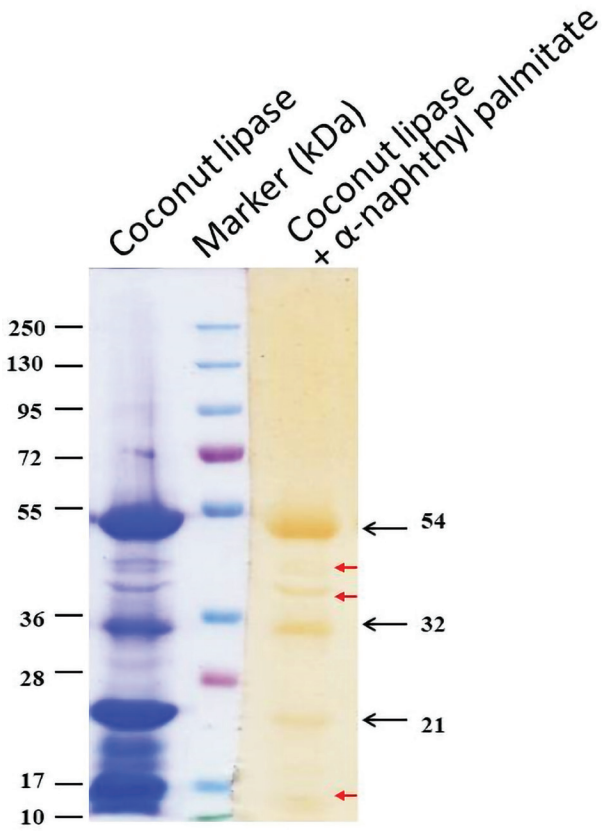

Figure 8. In-gel activity assay of coconut lipase. Coconut lipase is separated in a 12.5% polyacrylamide gel under denatured conditions, except for the boiling step. The gel was cut for CBB staining (left) and an in-gel assay(right). At least four distinct bands are noticed upon CBB staining, including the smallest subunit of c.a. 15 kDa. Additional faint bands are seen above the 32 kDa. The corresponding hydrolysis products by lipase subunits appear as yellow bands. It represents the results of alpha naphthyl palmitate hydrolysis by respective lipase subunits. The pixel density ratio of naphthol to CBB staining for the 54 kDa and the 21 kDa subunits is 77.5% and 43.2%, respectively. Note: several distinct bands above the 32 kDa band (red arrows) are shown to be active in hydrolyzing the alpha naphthyl substrate. It gives a hint that the 32 kDa is glycosylated. We also speculate that a smeary pattern observed in the native gel (Figure 5) indicates the glycosylation of native protein.

the study of individual subunits, especially at the current circumstance when the coconut genome is emerging on the horizon (Xiao *et al.* 2017). Accordingly, biochemical characterization of various subunits (optimum temperature and pH reaction, substrate specificity, metal ions effect, and detergent effect) would provide more detailed information.

CONCLUSION

By using a simple procedure, we have been able to isolate coconut lipase. A direct comparison of SDS-PAGE and native PAGE readily hint that coconut lipase is a complex enzyme. This enzyme consists of four subunits of 54, 32, 21, and 15 kDa. In its complex form, coconut lipase shows the highest preference for lauryl esters. The enzyme is activated by Ca^{2+} ion, whereas Fe^{3+}, Cu^{2+}, Zn^{2+}, Mg^{2+}, K^{+}, and Na^{+} decrease coconut lipase activity. Each subunit can cleave the fatty acid ester bond; hence, this enzyme might be regarded as a cluster of smaller active proteins. Since all coconut lipase subunits are active as esterases, specificity determination of subunits and further biochemical characterization of the subunits are yet to be investigated. We also propose that a similar approach can be applied for the initial study of other plant or seed-based lipases.

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STATEMENT ON CONFLICT OF INTEREST

All authors declare to have no conflict of interest.

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K16 Persetujuan author atas draft kedua 6 Juli 2021

Lalu Rudyat Telly Savalas <telly@unram.ac.id>

Re: 1st Draft of PJS Article Ms 21-049

1 message

Lalu Rudyat Telly Savalas <telly@unram.ac.id> 6 July 2021 at 09:36
To: Philippine Journal of Science <philjournsci@gmail.com>

Dear Mr. Allyster A. Endozo,
Thank you for your email.
I have no further concern regarding our manuscript. So it can now be regarded as final.

Thank you very much and I wish you stay safe and healthy.

Kind regards,
Lalu Rudyat Telly Savalas
University of Mataram
Indonesia

Pada tanggal Sel, 6 Jul 2021 08:37, Philippine Journal of Science <philjournsci@gmail.com> menulis:

> Dear Dr. Savalas,
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> Greetings!
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> Attached below is the third draft of your article entitled, "Biochemical Properties of Coconut (*Cocos nucifera* L.) Lipase" [Ms 21-049] accepted for publication in the Philippine Journal of Science. Kindly review this copy and should you have no further corrections, provide us your approval for printing.
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> We hope to hear from you on the matter within 48 hours after the receipt of this letter. We shall consider this version of your paper as the galley proof for final editorial processing in the absence of a response within the cut-off time.
>
> Thank you very much.
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> Sincerely,
> Mr. ALLYSTER A. ENDOZO
> Managing Editor
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> On Mon, Jul 5, 2021 at 10:46 AM Lalu Rudyat Telly Savalas <telly@unram.ac.id> wrote:
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> > Mr. ALLYSTER A. ENDOZO
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> > Managing Editor
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> > Dear Mr. Allyster A. Endozo,
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