Novel approach to assessing the bioavailability of biopeptide inhibitor of HMG CoA reductase from germinated and ungerminated Kara Kratok (Phaseolus lunatus L.) (#107224)

First submission

Guidance from your Editor

Please submit by **23 Jan 2025** for the benefit of the authors (and your token reward) .

Structure and Criteria
Please read the 'Structure and Criteria' page for guidance.

Custom checks
Make sure you include the custom checks shown below, in your review.

Raw data check
Review the raw data.

Image check
Check that figures and images have not been inappropriately manipulated.

If this article is published your review will be made public. You can choose whether to sign your review. If uploading a PDF please remove any identifiable information (if you want to remain anonymous).

Files

Download and review all files from the materials page.

5 Figure file(s)
1 Table file(s)
1 Raw data file(s)
1 Other file(s)

Custom checks

Cell line checks

- Is the correct provenance of the cell line described?

Vertebrate animal usage checks

- Have you checked the authors ethical approval statement?
- Were the experiments necessary and ethical?
- Have you checked our animal research policies?

Structure and Criteria

Structure your review

The review form is divided into 5 sections. Please consider these when composing your review:

1. **BASIC REPORTING**
2. **EXPERIMENTAL DESIGN**
3. **VALIDITY OF THE FINDINGS**
4. General comments
5. Confidential notes to the editor

You can also annotate this PDF and upload it as part of your review

When ready submit online.

Editorial Criteria

Use these criteria points to structure your review. The full detailed editorial criteria is on your guidance page.

BASIC REPORTING

- Clear, unambiguous, professional English language used throughout.
- Intro & background to show context. Literature well referenced & relevant.
- Structure conforms to PeerJ standards, discipline norm, or improved for clarity.
- Figures are relevant, high quality, well labelled & described.
- Raw data supplied (see PeerJ policy).

EXPERIMENTAL DESIGN

- Original primary research within Scope of the journal.
- Research question well defined, relevant & meaningful. It is stated how the research fills an identified knowledge gap.
- Rigorous investigation performed to a high technical & ethical standard.
- Methods described with sufficient detail & information to replicate.

VALIDITY OF THE FINDINGS

- **Impact and novelty is not assessed.** Meaningful replication encouraged where rationale & benefit to literature is clearly stated.
- All underlying data have been provided; they are robust, statistically sound, & controlled.
- Conclusions are well stated, linked to original research question & limited to supporting results.

Standout reviewing tips

The best reviewers use these techniques

Tip	*Example*
Support criticisms with evidence from the text or from other sources	*Smith et al (J of Methodology, 2005, V3, pp 123) have shown that the analysis you use in Lines 241-250 is not the most appropriate for this situation. Please explain why you used this method.*
Give specific suggestions on how to improve the manuscript	*Your introduction needs more detail. I suggest that you improve the description at lines 57- 86 to provide more justification for your study (specifically, you should expand upon the knowledge gap being filled).*
Comment on language and grammar issues	*The English language should be improved to ensure that an international audience can clearly understand your text. Some examples where the language could be improved include lines 23, 77, 121, 128 – the current phrasing makes comprehension difficult. I suggest you have a colleague who is proficient in English and familiar with the subject matter review your manuscript, or contact a professional editing service.*
Organize by importance of the issues, and number your points	*1. Your most important issue* *2. The next most important item* *3. …* *4. The least important points*
Please provide constructive criticism, and avoid personal opinions	*I thank you for providing the raw data, however your supplemental files need more descriptive metadata identifiers to be useful to future readers. Although your results are compelling, the data analysis should be improved in the following ways: AA, BB, CC*
Comment on strengths (as well as weaknesses) of the manuscript	*I commend the authors for their extensive data set, compiled over many years of detailed fieldwork. In addition, the manuscript is clearly written in professional, unambiguous language. If there is a weakness, it is in the statistical analysis (as I have noted above) which should be improved upon before Acceptance.*

Novel approach to assessing the bioavailability of biopeptide inhibitor of HMG CoA reductase from germinated and ungerminated Kara Kratok (Phaseolus lunatus L.)

Cahyo Budiyanto [1], **Andriati Ningrum** [1], **Agnes Murdiati** [1], **Retno Indrati** [Corresp. 2]

[1] Food and Agricultural Product Technology, Universitas Gadjah Mada, Yogyakarta, Indonesia

[2] Faculty of Agricultural Technology, Universitas Gadjah Mada,, Department of Food and Agricultural Product Technology, Yogyakarta, Yogyakarta, Indonesia

Corresponding Author: Retno Indrati
Email address: indrati@ugm.ac.id

Background: The bioavailability of biopeptide compounds is a development challenge, mainly because of their resistance to the digestion system. This study aims to determine the bioavailability of HMG CoA reductase biopeptide inhibitors from germinated and ungerminated *kara kratok* (*Phaseolus lunatus* L.). **Methods:** Germinated and ungerminated brown *kara kratok* was simulated for digestion enzyme *in vitro* (120 minutes pepsin, 120 minutes pancreatin), followed by the *in-situ* method for absorption. Samples were measured for the absorption percentage, inhibition of HMG CoA reductase, molecular weight (MW), peptide concentration, and hydrolysis degree (%DH). **Results:** The results unequivocally show that germinated kara kratok has the highest absorption (32.42%), and the percentage of HMG CoA reductase inhibition during enzymatic digestion was at 210 minutes (87.51%), MW<10 kDa, peptide concentration 2.39 mg/mL, and %DH 48.90%. These findings suggest that germinated kara kratok is a potent HMG CoA reductase inhibitor with significantly higher bioavailability than ungerminated, thereby establishing its superiority in this context and opening up new possibilities for biopeptide research.

Novel Approach to Assessing the Bioavailability of Biopeptide Inhibitor of HMG CoA Reductase from Germinated and Ungerminated *Kara kratok* (*Phaseolus lunatus* L.)

Cahyo Budiyanto[1], Andriati Ningrum[1], Agnes Murdiati[1], Retno Indrati[1]

[1] Department of Food and Agricultural Product Technology, Faculty of Agricultural Technology, Universitas Gadjah Mada, Jl. Flora No. 1, Bulaksumur, Yogyakarta 55281, Indonesia

Corresponding Author:
Retno Indrati[1]
Department of Food and Agricultural Product Technology, Faculty of Agricultural Technology, Universitas Gadjah Mada, Jl. Flora No. 1, Bulaksumur, Yogyakarta 55281, Indonesia
Email address: indrati@ugm.ac.id

Abstract

Background: The bioavailability of biopeptide compounds is a development challenge, mainly because of their resistance to the digestion system. This study aims to determine the bioavailability of HMG CoA reductase biopeptide inhibitors from germinated and ungerminated *kara kratok* (*Phaseolus lunatus* L.).

Methods: Germinated and ungerminated brown *kara kratok* was simulated for digestion enzyme *in vitro* (120 minutes pepsin, ~~120 minutes~~ pancreatin), followed by the *in-situ* method for absorption. Samples were measured for the absorption percentage, inhibition of HMG CoA reductase, molecular weight (MW), peptide concentration, and hydrolysis degree (%DH).

Results: The results unequivocally show that germinated *kara kratok* has the highest absorption (32.42%), and the percentage of HMG CoA reductase inhibition during enzymatic digestion was at 210 minutes (87.51%), MW<10 kDa, peptide concentration 2.39 mg/mL, and %DH 48.90%. These findings suggest that germinated *kara kratok* is a potent HMG CoA reductase inhibitor with significantly higher bioavailability than ungerminated, thereby establishing its superiority in this context and opening up new possibilities for biopeptide research.

Subjects: Biochemistry, Biotechnology, Food Science and Technology, Legumes
Keywords: Bioavailability, germinated, HMG CoA reductase, in vitro, *Phaseolus lunatus*

Introduction

~~During digestion,~~ the bioactivity of biopeptides at a specified target is crucial. As suggested by Bhandari *et al.* (2020), the differences in bioactivity between various biopeptide compounds indicate their bioavailability, absorption, and susceptibility to breakdown into inactive fragments by physiological digestive enzymes. In this complex process, peptide transporters in the intestine play a key role, facilitating the passage of smaller peptides through digestive enzymes, while oligopeptides are transported passively through hydrophobic areas of epithelial membranes. This intricate process underscores the complexity of our research and the importance of understanding the role of peptide transporters in the absorption of biopeptides.

The bioavailability of macronutrients, carbohydrates, proteins, and fats is usually high, with more than 90% of the amount ingested being absorbed and utilized in the human body (Schönfeldt *et al.*, 2016). Increasing the bioaccessibility and bioavailability of several biopeptide compounds occurs after digestive enzymes hydrolyze them, so process technology is needed, such as encapsulation, which protects the active biopeptide compounds after they enter the body, the process of releasing and degrading digestive enzymes (Indrati, 2021). In the gastrointestinal tract, biopeptides pass the intestinal epithelial barrier before arriving at the organ's target and act its bioactivity (Amigo & Hernández-Ledesma, 2020).

Peptide absorption occurs in two processes, namely, membrane hydrolysis and intracellular hydrolysis. In membrane hydrolysis, peptides with a high affinity for the peptidase found in the brush border membrane ~~will be~~ hydrolyze~~d~~ into shorter peptides. In contrast, peptides resistant to the peptidase ~~will be~~ transfer~~red~~ intact through the small intestinal epithelial cells and then hydrolyzed by the peptidase in the cytoplasm, referred to as intracellular hydrolysis~~.~~ (Xu *et al.*, 2019). According to Miner-Williams *et al.* (2014), bioactive peptide compounds can generally reach the target organ intact because they are resistant to both processes (membrane hydrolysis and intracellular hydrolysis).

Research conducted by Aiello *et al.* (2018) on soybean bioactive peptide compounds IAVPTGVA, LPYP, and IAVPGEVA against Caco-2 cells showed the inefficiency of bioactive peptide compounds during transport in the intestine due to high hydrolysis by brush border enzyme. Marques *et al.* (2018) stated that bioactive peptide compounds with a molecular weight of ≤ 3 kDa from cowpea protein hydrolysate against Caco-2 cells showed that the absorption of the MELNAVSVVHS peptide passed through the cell monolayer membrane. This study aim~~s~~ to get the impact of digestion on bioactivity biopeptide HMG CoA reductase inhibitor from *kara kratok* (*Phaseolus lunatus* L.) via *in vitro* digestion and its bioavailability using the *in-situ* method.

Materials & Methods

Kara kratok beans and chemicals

Kara kratok beans were obtained from the Bondowoso local market, East Java, while white *Sprague Dawley* from PAU Pangan dan Gizi (Food and Nutrition Development Research Center) UGM Yogyakarta. Merck and Sigma Aldrich were chosen for chemical agents and kits that were

used in this study, like o-Phthaldialdehyde (OPA) for peptide concentration measurement and HMG CoA reductase kit (CS-1090) for assessing HMG CoA reductase inhibition.

Preparation of germinated and ungerminated *kara kratok*

Germinated and ungerminated *kara kratok* (*Phaseolus lunatus* L) was prepared by following the Chel-guerrero *et al*. (2012) method with some modifications. Clean *Phaseolus lunatus* L. seeds were soaked for 24 hours in distilled water, continued in 0.2%(w/v) sodium hypochlorite solution for 1 minute, and finally, the bean was cleaned with distilled water three times. Ungerminated bean was obtained after this step, while germinated was obtained from 72 hours of germination in a 16-18°C dark room. This germinated *kara kratok* was dried by cabinet dryer for 8 hours at 50-55°C, then flouring. ~~These modifications were made to ensure the consistency and reliability of our results.~~ Both samples were simulated *in vitro* for gastrointestinal digestion and continued with the *in-situ* absorption method.

Gastrointestinal digestion simulation

The simulation of gastrointestinal digestion was carried out based on Minekus *et al.* (2014) and Moreno *et al.* (2020) methods by *in vitro* simulation using pepsin and pancreatin. Samples of germinated and ungerminated *kara kratok* (*Phaseolus lunatus* L.) were carefully simulated with pepsin and continued with pancreatin enzyme. The total reaction for each sample was 240 minutes, with each enzyme taking 120 minutes and sampling every 30 minutes. The thirty-minute sampling analyzed the peptide concentration, hydrolysis degree (%DH), molecular weight (MW), and the percentage inhibition of HMG CoA reductase.

Ethical statement

The ethical letter no. 00073/04/LPPT/I/2024 from LPPT (Lembaga Penelitian dan Pengujian Terpadu - Integrated Laboratory for Research and Testing) Universitas Gadjah Mada was the basis of the approved experimental protocols and procedures.

Animals Experiment - *In-situ* absorption

The *in-situ* absorption method is ~~carried out~~ based on Xiao et al. (2024) and for sample size calculation with quantitative data in the endpoint follow Charan & Kantharia (2013), using two groups of male healthy *Sprague Dawley* (SD) that obtained from PAU Pangan dan Gizi (Food and Nutrition Development Research Center) UGM Yogyakarta with the body weight 236 – 315 grams (n = 3, we used 6 SD for total animal to minimize the animal usage). Each SD was kept in a cage 20 cm x 20 cm x 24 cm at 22.5°C and RH 50% with the 12-hour light-dark cycle and feed the standard fed diet for rodent, while for drinking use reverse osmosis (RO) water via ad libitum before SD fasted for 24 hours with a normal water intake. Each SD with normal physiologic functions like normal respiratory function was then anesthetized follows the method of Navarro et al. (2021) with ketamine 60 mg/kg bodyweight into the thigh, after SD fainted by ensuring its immobility, dilating pupils, and breathing ceased, then the SD was continued with surgery. A

longitudinal incision midline was done to open the abdomen. Then plastic tubing (4 mm o.d.) was used for cannulation at 10 cm from the jejunum and 10 cm before the cecum for sample collection, followed by perfusion with 0.9% sodium chloride solution with a dosage of 0.2 ml/minute for 120 minutes to flush the retaining food in the digestion tract. Samples were injected with the same flow and time and collected at 30, 60, 90, and 120 minutes (**Fig. 1**). Ungerminated *kara kratok* was used as the control samples (n=3), while germinated ones as the treatment's samples (n=3). The perfusate samples were analyzed for the percentage of absorption via peptide concentration, molecular weight, and the percentage inhibition of HMG CoA reductase. At the end of experiment there is no living SD because all of them were performed euthanasia using ketamine 100 mg/kg body weight as lethal dose via intramuscular injection before burn to ash by incineration method in the incinerator of Food and Nutrition Development Research Center (PAU Pangan dan Gizi) that located out of laboratory. For reporting our animal research, the completed ARRIVE Guidelines checklist is included in the attachment.

Peptide content and degree of hydrolysis

A peptide sample solution of 0.4 mL was added with 3 mL OPA reagent and then incubated for 20 minutes at room temperature. Measurement of these samples used a spectrophotometer (GENESYS 10S) at absorbance λ 340 nm (Agustia *et al*., 2023). Standard curves were set by using tryptophan solution, and %DH was calculated using the equation:

$$\%DH = \frac{(\mathrm{Ntx} - \mathrm{Nt0})}{(\mathrm{Ntot} - \mathrm{Nt0})} x\ 100\%$$

Where Ntx is the sample hydrolysis at x-hour, Nt0 is the sample hydrolysis at 0-hour, and Ntot is the total quantity of the sample hydrolysis for 4 hours at 105°C with 10 mL HCl 6 M.

Molecular weight

Molecular weight was analyzed by following the SDS-PAGE method (Laemmli, 1970) using 5% resolving gel and 13% separating gel (SDS sample buffer containing 0.5M Tris-HCl pH 6.8, 87% glycerol (w/v), 10% SDS (w/v), 0.5% bromophenol blue (w/v) and distilled water was used to dilute the peptide extracts at a ratio of 1:2). Samples solution firstly 4 minutes heating at 100°C, then loaded 20 µl samples or 5µL of standard protein marker into each well. The gel ran at 220V for 60 minutes, continued with 30 minutes soaking in distilled water, and replied ~~thrice~~ 3. The final step was staining with 0.2% Coomassie Brilliant Blue R-250 (containing 50% methanol, 10% acetic acid, and 40% distilled water).

HMG CoA reductase inhibition

The percentage of the HMG CoA reductase inhibition was measured using the Sigma Aldrich CS-1090 kit and followed the Hermanto *et al*. (2020) method with the quantity of each reaction volume mentioned in Table 1. The 200 µL mixtures samples and reagents were measured on 340 nm wavelength for 10 minutes reading. Enzymatic activity was calculated using the equation:

$$\text{Unit per mgP} = \frac{(\Delta \text{ Absorbance 340nm sampel} - \Delta\text{Absorbance 340nm blangko})}{12.44 \text{ x V x } 0.6 \text{ x LP}} x\ TV$$

Description:
12.44 = During reaction need 2 NADPH (the NADPH coefficient at 340 nm is 6.22/mM.cm)
TV = Total reaction volume (1 mL)
V = Enzyme volume used
0.6 = Enzyme concentration in mg-protein (mgP)/mL
LP = Ligh path (1 cm for cuvettes and 0,55 cm for plate)
Then, the equation below is used to calculate the percentage of inhibition:

$$\%\ inhibition = \frac{\text{Enzyme Activity (non} - \text{inhibitor)} - \text{Enzyme Activity (pravastatin or sample)}}{\text{Enzyme Activity (non} - \text{inhibitor)}} x\ 100\%$$

Statistical Analysis

~~SPSS IBM 23 software was used for the data quantitative statistical analysis with significant differences (P<0.05) between treatments.~~ One-way analysis of variance (ANOVA) was used for this statistical analysis if there is any significant difference continued with Duncans' Multiple Range Tests (DMRT).

Results

Peptide content and degree of hydrolysis

The degree of hydrolysis (DH) is used to determine the peptide proportion cleaved from protein hydrolysis (Rutherfurd, 2010). In this study, hydrolysis was carried out to simulate the enzymatic digestion process using the pepsin enzyme for 120 minutes and continued using the pancreatin enzyme for up to 240 minutes. The results showed an incremental degree of hydrolysis (**Fig. 2A**) and peptide concentration (**Fig. 2B**) for both germinated and ungerminated *kara kratok* until 210 minutes and then decreased at the end of digestion (240 minutes), statistically for every 30 minutes of digestion in germinated or ungerminated samples showed a significant difference ($P<0.05$) between these 30 minutes samples. Besides that, germinated *kara kratok* has a higher degree of hydrolysis and peptide concentration than ungerminated. This condition is related to a reduced amount of substrate or maximum protein hydrolysis products. These results are also aligned with Sandoval-Sicairos *et al*. (2020), who studied germinated and ungerminated amaranth on antioxidant activity during gastrointestinal digestion simulation, with the same pattern of incremental until 210 minutes digestion and then reduced at 240 minutes as the activity of pepsin and pancreatin that hydrolyze large protein molecules into small peptides.

Molecular weight

The digestion enzymes of germinated *kara kratok* (*Phaseolus lunatus* L.) get eight fractions higher than those of ungerminated ones. The molecular weight of germinated *kara kratok* was in the range of 6.9 – 38.7 kDa (**Fig. 3A**), while ungerminated *kara kratok* was in the range of 13.5 – 43.2 kDa

(**Fig. 3B**). Both samples, germinated and ungerminated *kara kratok*, the molecular weight's band shows thicker along time than in the beginning form.
Meanwhile, the aim of molecular weight analysis in the perfusate of *in-situ* simulation was to know the unabsorbed peptide in the digestion system, it shows that germinated *kara kratok* (*Phaseolus lunatus* L.) has a thinner band than ungerminated ones (**Fig. 3C**). Both samples show the same molecular weight of the unabsorbed peptide was 15 kDa and 75 kDa.

Inhibition of HMG CoA reductase

The analysis of percentage inhibition of HMG CoA reductase during gastrointestinal enzymatic digestion was purposed to see changes in the peptide ability and its resistance to the digestion enzyme used. It shows that the percentage inhibition of HMG CoA reductase from germinated *kara kratok* (*Phaseolus lunatus* L.) is higher and significantly different ($P<0.05$) than the ungerminated during enzymatic digestion time (**Fig. 4A**). Both germinated and ungerminated *kara kratok* hydrolysate show incremental HMG CoA reductase inhibition during digestion time with a maximum inhibition at 210 minutes. Hydrolysate of germinated *kara kratok* by pepsin and pancreatin also gave a higher percentage of inhibition and was significantly different ($P<0.05$) compared to pravastatin as a control, but not for the ungerminated one because the higher percentage of HMG CoA reductase inhibition from pravastatin only started at 180 minutes. This pattern is in line with the peptide concentration pattern and the formation of low molecular weight proteins results at the end of enzymatic digestion.
Perfusate obtained during *in-situ* absorption was also analyzed for its ability to inhibit HMG CoA reductase, and the results illustrate the peptides that are not absorbed in the digestive tract. The ability to inhibit HMG CoA reductase of peptide in perfusate at the end digestion time from germinated *kara kratok* shows lower than ungerminated ones and statistically significantly different ($P < 0.05$) from each other (**Fig. 4B**). The graph in **Fig. 4B** show reduction of its ability as HMG CoA reductase inhibitor from beginning absorption time to 120 minutes absorption.

Percentage peptide absorption via *in-situ* method

The absorption percentage was calculated from the peptide content difference before and after the sample passed the digestion tract used, a higher value meant higher absorption. The highest concentration of peptides absorbed was in the germinated sample, significantly different ($P<0.05$) from the ungerminated one (**Fig. 5**).

Discussion

Peptide content and degree of hydrolysis

The incremental hydrolysis degree of both germinated and ungerminated *kara kratok* during enzymatic digestion is related to the composition and structure of phaseolin which is known susceptibility to enzymatic hydrolysis (Montoya *et al*., 2008). Pepsin and pancreatin hydrolysis have a specific preference location to cut the peptide, pepsin cuts peptide bonds after phenylalanine, leucine, and glutamic acid, and rarely cuts after histidine and lysine even though it

is close to leucine and phenylalanine (Ahn *et al*., 2014), while pancreatin will hydrolyze aromatic and branched-chain amino acids such as tyrosine, phenylalanine, tryptophan, alanine, and leucine, and cuts all amino acid residues except asparagine, glutamine, arginine, and lysine (Andriamihaja *et al*., 2013; Ratnayani *et al*., 2019). The maximum hydrolysis degree at 120 minutes of pepsin hydrolysis is 23.88% for germinated *kara kratok* and 20.27% for ungerminated, which is also reported by Misquitta *et al*. (2023) in legume hydrolysates using a combination of pepsin and pancreatin generally has %DH in the range of 20 - 46%.

The peptide concentration of germinated *kara kratok* showed an increase starting from the 30th minute of the pepsin hydrolysis phase, while ungerminated started at the 60th minute, due to the storage protein of germinated has changed its shape from folded structure to unfolded, started by carbohydrase activity on cell wall damage continued with a protease that will hydrolyze the storage protein (Halmer, 1975). Ohanenye *et al*. (2021) also found that the microstructure of the cell wall's softening and damage started after 24 to 48 hours of germination. The incremental peptide concentration of germinated and ungerminated *kara kratok* at the 210th minute indicates the formation of a small peptide incremental as enzymatic activity, pepsin initiates the breakdown of proteins into smaller peptides in the stomach that will be able to hydrolyze by pancreatin in the small intestine, pepsin breaks peptide bonds on certain sides that may be resistant to pancreatin hydrolysis (Del Rio *et al*., 2021; Fu *et al*., 2021) in other words pepsin hydrolysis in the early stages of digestion can increase the efficiency of protein hydrolysis by pancreatin enzymes in later stages.

Molecular weight

The difference in the number of fractions formed between germinated and ungerminated *kara kratok* is due to the effect of germination on softens and damages the microstructure of the *kara kratok* seed cell wall, followed by the breakdown of stored protein by pepsin into smaller peptides so it further hydrolyzed by pancreatin. The microstructure of the new cell wall softening and damage began after 24 to 48 hours of germination (Ohanenye *et al*., 2021). According to Atudorei *et al*. (2021), the cell structure of the germinated seeds will undergo continuous changes as indicated by the separation of starch granules from protein but still have a smooth surface due to increased hydrolytic enzyme activity. The thicker density band during 120 minutes of pepsin digestion provides a fairly high increase in short-chain peptides, especially in the germinated samples. Pepsin according to Luo *et al*. (2018) has very active properties since the beginning of digestive hydrolysis, most peptides (50 to 93%) are formed starting at 30 seconds of legume protein digestion, while pancreatin Likittrakulwong *et al*. (2021) reported during *in vitro* digestion of rice protein from paddy rice and germinated paddy rice were completely digested after pancreatin and no protein bands detected. Lower MW is achieved in germinated *kara kratok* than in ungerminated that related to the proteolysis reaction by proteases during germination (Sandoval-Sicairos *et al*., 2020). This phenomenon was aligned with Yuan *et al*. (2019) who reported MW decrease from 52.80 kDa to 14.67 kDa and 12.01 kDa during gastric and intestinal digestion in antioxidant biopeptides from tropical sea cucumbers (*Holothuria leucospilota*). Bera *et al*. (2023) also reported

the comparison peptides from raw chickpeas and chickpea sprouts after going through the oral, gastric, duodenal, and brush border digestion phases, the results showed a doubling of free alpha-amino nitrogen after the seeds sprouted, which reflected an increase in amino acids and oligopeptides, but there was a decrease in the number of larger peptides detected by MS/MS and showed a decrease in the number of peptides that were resistant to all four digestion phases.
The absorption process is influenced by molecular weight, Patel and Mirsa (2011) stated that absorption would generally decrease exponentially at molecular weights above 300 Da, this is aligned with our study in which the molecular weight of the unabsorbed peptide was 15 kDa and 75 kDa as shown by the thickest band on **Fig. 3C**. Perfusate of germinated *kara kratok* (*Phaseolus lunatus* L.) shows a thinner band than ungerminated due to the formation of small peptides and amino acids as the proteolytic actions of endopeptidase and exopeptidase (Smith & Morton, 2010). According to Newstead *et al*. (2011), peptides that can be bound by the PepT1 transporter in peptide absorption are limited to di- and tripeptides. However, it is ~~very~~ difficult for long-chain peptides with peptides more than tetrapeptides because their binding capacity is only on molecules of approximately 13x12x 11 Å. Liu *et al*. (2024) stated that the dipeptide and tetrapeptide with hydrophobic and basic amino acids at the C-terminal can pass the intestinal epithelium. Kiela and Ghishan (2016) stated that dipeptides and tripeptides are very efficiently absorbed in the small intestine, 90% of the absorbed food protein is in the form of amino acids and 10% as dipeptides and tripeptides. Xu *et al*. (2019) reported that the bioavailability of absorbed peptides is influenced by various peptide properties such as size, molecular weight, amino acid sequence, and peptide resistance to enzymatic degradation.

Inhibition of HMG CoA reductase

The percentage of the HMG CoA reductase inhibition from germinated and ungerminated *kara kratok* at the end of enzymatic digestion is higher than pravastatin as the reference in line with the Hermanto *et al*. (2020) report on soy protein isolate using 0.2% papain enzyme at an incubation temperature of 50°C for 3 hours obtained an inhibition value of 95.65%. Shami tree pod extract (*Prosopis cineraria* L.) provides HMG CoA reductase inhibition of up to 78.1% (Jaipal *et al*., 2022), while in legumes it is known that peptides <3 kDa obtained from cowpeas provide a percentage of HMG CoA reductase inhibition of 95.0% higher and significantly different from pravastatin (Silva *et al*., 2018). The ungerminated *kara kratok* gives the lowest HMG CoA reductase inhibition until the end of pepsin digestion for 120 minutes (53.26%) in line with Pak *et al*. (2007) who hydrolyzed soybeans using pepsin with 45% inhibition. Lammi *et al*. (2015) also obtained 17% inhibition of the HMG CoA reductase at a maximum dose of 2.5 mg/ml biopeptide from lupin hydrolysate using pepsin enzyme, but higher inhibition (57%) if using trypsin enzyme at the same concentration. According to Smith and Morton (2010), pancreatic enzymes contain trypsin, chymotrypsin, elastase, and row carboxypeptidases (A and B) that will degrade proteins into small peptides in large quantities and then bond to the HMG CoA reductase enzyme.
During absorption, dipeptides and tripeptides are very efficiently absorbed in the small intestine, 90% of the absorbed food protein is in the form of amino acids and 10% as dipeptides and

tripeptides (Kiela & Ghishan, 2016), while peptides composed of less than six amino acids according to Fan *et al*. (2018) can pass through enterocytes with decreased absorption efficiency due to increasing chain length. Liu *et al*. (2024) stated in their review that the dipeptide and tetrapeptide with hydrophobic and basic amino acids at the C-terminal can pass the intestinal epithelium. Perfusate of germinated *kara kratok* has lower inhibition of HMG CoA reductase than ungerminated meaning germination helps to provide some small peptides and digestion enzymes continue the degradation into amino acids, dipeptide and tripeptide.

Percentage peptide absorption via *in-situ* method

The absorption process is influenced by the molecular weight and size of the peptide compound in the sample, Mehrotra *et al*. (2024) stated that absorption will generally decrease exponentially at molecular weights above 300 Da. Keller (2013) stated that small peptides with a composition of 2 or 3 amino acids can be absorbed directly through the apical membrane of enterocytes lining the small intestine via peptide transporters stimulated by PEPT-1 protons, then these small peptides will be broken down intracellularly into single amino acids. These results are also in line with the peptide concentration and MW of germinated and ungerminated *kara kratok* after *in vitro* digestion simulation which showed that the concentration of peptides from germinated was 2.388 mg/mL while ungerminated was 2.028 mg/mL, the lowest MW fraction of germinated was 6.9 kDa while ungerminated was 13.5 kDa. Bioactive peptide compounds have very low transcellular permeability so they are difficult to absorb into the portal vein through this transcellular pathway, on the other hand, the paracellular route requires very small molecules of less than 500 Da because absorption will go through TJ pores which have sizes ranging between 3 and 10 A° and are full of water. Molecules larger than 500 Da are generally unable to move through these small pores, although TJ can experience increased permeation, which makes the pores larger with a width still less than 20nm (Anderson and Van Itallie, 1995; Suzuki, 2013). This percentage of absorption result is still lower than the research of Pertiwi *et al*. (2020) on raw tempeh and cooked tempeh using the reverse intestinal absorption method with the highest absorption percentage in the jejunum, which was 42.97% in raw tempeh samples and 49.43% in cooked tempeh samples. Indrati (2021) stated that there are four important steps in the effective absorption of bioactive compounds: (a) release from the food matrix; (b) incorporation into bile salt micelles; (c) absorption by epithelial cells; and finally; (d) incorporation into chylomicron secretion into the lymphatic system. Sepulveda and Smith (1974; 1979) stated that the smaller molecular weight of hydrophilic and neutral amino acids will be absorbed more slowly than hydrophobic amino acids with a larger molecular weight, while basic amino acids are absorbed at a moderate rate.

Conclusions

~~In conclusion, the study has revealed that~~ the biopeptide of germinated *kara kratok* exhibits higher bioavailability as an HMG CoA reductase inhibitor and absorption than ungerminated. This is supported by the incremental peptide concentration and degree of hydrolysis that peak at 210 minutes during gastrointestinal simulation, as well as the high formation of lower molecular weight

355 at the end of enzymatic hydrolysis. These promising findings not only inspire further research in
356 this area but also offer hope for the development of more effective biopeptide inhibitors,
357 potentially revolutionizing the field of biochemistry and pharmaceuticals. The study still limited
358 on *in vitro* and *in-situ* method, so further research is needed for human *in vivo*.
359

360 ## Acknowledgements
361 The authors thank the Faculty of Agricultural Technology and LPPT (Lembaga Penelitian dan
362 Pengujian Terpadu - Integrated Laboratory for Research and Testing) and PAU Pangan dan Gizi
363 (Food and Nutrition Development Research Center), Universitas Gadjah Mada, for facilitating this
364 research.
365

366 ## Author Disclosure Statement
367 The authors report there are no competing interests to declare.
368

369 ## Funding
370 This work was supported by the Ministry of Education, Culture, Research, and Technology for
371 research financial support through a postgraduate research scholarship under Grant number
372 048/E5/PG.02.00.PL/2024; 2781/UN1/DITLIT/PT.01.03/2024 from the Directorate of Research,
373 Universitas Gadjah Mada.
374

375 ## References
376 Agustia, F. C., Murdiati, A., Supriyadi, & Indrati, R. (2023). Production of dipeptidyl peptidase-
377 IV inhibitory peptides from germinated jack bean [*Canavalia ensiformis* (L.) DC.] flour.
378 *Preventive Nutrition and Food Science*, *28*(2), 149–159.
379 https://doi.org/10.3746/pnf.2023.28.2.149
380 Ahn, J., Cao, M.-J., Yu, Y. Q., & Engen1, J. R. (2014). Accessing the reproducibility and
381 specificity of pepsin and other aspartic proteases joomi. *Biochimica et Biophysica Acta*,
382 *538*(6), 151–169. https://doi.org/10.1016/j.bbapap.2012.10.003.
383 Aiello, G., Ferruzza, S., Ranaldi, G., Sambuy, Y., Arnoldi, A., Vistoli, G., & Lammi, C. (2018).
384 Behavior of three hypocholesterolemic peptides from soy protein in an intestinal model
385 based on differentiated Caco-2 cell. *Journal of Functional Foods*, *45*(January), 363–370.
386 https://doi.org/10.1016/j.jff.2018.04.023
387 Amigo, L., & Hernández-Ledesma, B. (2020). Current evidence on the bioavailability of food
388 bioactive peptides. *Molecules*, *25*(19). https://doi.org/10.3390/molecules25194479
389 Anderson, J. M., & Van Itallie, C. M. (1995). Tight junctions and the molecular basis for
390 regulation of paracellular permeability. American Journal of Physiology - Gastrointestinal
391 and Liver Physiology, 269(4 32-4), 467–475. https://doi.org/10.1152/ajpgi.1995.269.4.g467
392 Andriamihaja, M., Guillot, A., Svendsen, A., Hagedorn, J., Rakotondratohanina, S., Tomé, D., &
393 Blachier, F. (2013). Comparative efficiency of microbial enzyme preparations versus
394 pankreatin for invitro alimentary protein digestion. *Amino Acids*, *44*(2), 563–572.
395 https://doi.org/10.1007/s00726-012-1373-0

Atudorei, D., Stroe, S. G., & Codină, G. G. (2021). Impact of germination on the microstructural and physicochemical properties of different legume types. *Plants*, *10*(3), 1–19. https://doi.org/10.3390/plants10030592

Bhandari D, Rafiq S, Gat Y, Gat P, Waghmare R, Kumar V. 2020. A review on bioactive peptides: Physiological functions, bioavailability and safety. International Journal of Peptide Research and Therapeutics. 26(1):139-150. https://doi.org/10.1007/s10989-019-09823-5

Bera, I., O'Sullivan, M., Flynn, D., & Shields, D. C. (2023). Relationship between protein digestibility and the proteolysis of legume proteins during seed germination. *Molecules*, 28(7), 3204. https://doi.org/10.3390/molecules28073204

Charan, J., & Kantharia, N. (2013). How to calculate sample size in animal studies?. *Journal of Pharmacology and Pharmacotherapeutics*, 4(4), 303-306. https://doi.org/10.4103/0976-500X.119726

Chel-guerrero, L., Domínguez-magaña, M., Martínez-ayala, A., Dávila-ortiz, G., & Betancur-ancona, D. (2012). Lima bean (*Phaseolus lunatus*) protein hydrolysates with ACE-I inhibitory activity. *Food and Nutrition Sciences*, 3, 511–521. DOI:10.4236/fns.2012.34072

Del Rio, A. R., Keppler, J. K., Boom, R. M., & Janssen, A. E. M. (2021). Protein acidification and hydrolysis by pepsin ensure efficient trypsin-catalyzed hydrolysis. *Food and Function*, *12*(10), 4570–4581. https://doi.org/10.1039/d1fo00413a

Fan, H., Xu, Q., Hong, H., & Wu, J. (2018). Stability and transport of spent hen-derived ACE-inhibitory peptides IWHHT, IWH, and IW in human intestinal Caco-2 cell monolayers. Journal of Agricultural and Food Chemistry, 66(43), 11347-11354. DOI: 10.1021/acs.jafc.8b03956

Fu, Z., Akula, S., Thorpe, M., & Hellman, L. (2021). Marked difference in efficiency of the digestive enzymes pepsin, trypsin, chymotrypsin, and pancreatic elastase to cleave tightly folded proteins. *Biological Chemistry*, *402*(7), 861–867. https://doi.org/10.1515/hsz-2020-0386

Halmer, P. (1975). Enzyme to breakdown lettuce endosperm cell wall during giberellin and light induced germination. *Nature*, *258*, 716–718. https://doi.org/https://doi.org/10.1038/258716a0

Hermanto, S., Octavio, A., Azrifitria, A., & Kusumaningrum, S. (2020). The HMG-CoA reduktase inhibitor activities of soy protein hydrolysates from papain hydrolysis. *Molekul*, *16*(2), 145-155. DOI: http://dx.doi.org/10.20884/1.jm.2021.16.2.724

Indrati, R. (2021). Bioactive Peptides from Legumes and Their Bioavailability. *IntechOpen*, 13. DOI: 10.5772/intechopen.99979

Jaipal, N., Ram, H., Charan, J., Dixit, A., Singh, G., Singh, B. P., Kumar, A., & Panwar, A. (2022). HMG-CoA reduktase inhibition medicated hypocholesterolemic and antiatherosclerotic potential of phytoconstituents of an aqueous pod extract of Prosopis cineraria (L.) Druce: Insilico, invitro, and in vivo studies. EFood, 3(6), 1–16. https://doi.org/10.1002/efd2.42

Keller, J. (2013). Gastrointestinal digestion and absorption. In Encyclopedia of Biological Chemistry: Second Edition (2nd ed.). Elsevier Inc. https://doi.org/10.1016/B978-0-12-378630-2.00106-7

Kiela, P. R., & Ghishan, F. K. (2016). The physiology of intestinal absorption. Best Practice and Research Clinical Gastroenterology, 30(2), 145–159.

https://doi.org/doi:10.1016/j.bpg.2016.02.007.
Laemmli, U. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature Publishing*, *228*, 726–734.
Lammi, Carmen;, Zanoni, C., & Arnoldi, A. (2015). Three peptides from soy glycinin modulate glucose metabolism in human hepatic HepG2 cells. International Journal of Molecular Sciences, 16(11), 27362–27370. https://doi.org/10.3390/ijms161126029
Likittrakulwong, W., Poolprasert, P., & Srikaeo, K. (2021). Effects of extraction methods on protein properties obtained from paddy rice and germinated paddy rice. PeerJ, 9, e11365. https://doi.org/10.7717/peerj.11365
Liu, W., Li, K., Yu, S., Wang, Z., Li, H., & Liu, X. (2024). Alterations in the sequence and bioactivity of food-derived oligopeptides during simulated gastrointestinal digestion and absorption: a review. International Journal of Food Sciences and Nutrition, 75(2), 134-147. http://doi.org/10.1080/09637486.2023.2295224
Luo, X., Yuan, X., Wang, S., Sun, F., Hou, Z., Hu, Q., Zhai, L., Cui, Z., Zou, Y., 2018. Methane production and characteristics of the microbial community in the co-digestion of spent mushroom substrate with dairy manure. Bioresour. Technol. 250, 611–620. doi:10.1016/j.biortech.2017.11.088
Marques, M. R., Maureira, A. D. C., Fontanari, G. G., Pimenta, D. C., Soares-Freitas, R. M., Hirata, M. H., Hirata, R. D. C., & Arêas, J. A. G. (2018). Transport of *cowpea* bean derived peptides and their modulator effects on mRNA expression of cholesterol-related genes in Caco-2 and HepG2 cells. *Food Research International*, *107*(2017), 165–171. https://doi.org/10.1016/j.foodres.2018.01.031
Mehrotra, S., Kalyan, P. B. G., Nayak, P. G., Joseph, A., & Manikkath, J. (2024). Recent progress in the oral delivery of therapeutic peptides and proteins: Overview of pharmaceutical strategies to overcome absorption hurdles. Advanced Pharmaceutical Bulletin, 14(1), 11–33. https://doi.org/10.34172/apb.2024.009
Miner-Williams, W. M., Stevens, B. R., & Moughan, P. J. (2014). Are intact peptides absorbed from the healthy gut in the adult human? *Nutrition Research Reviews*, *27*(2), 308–329. https://doi.org/10.1017/S0954422414000225
Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carrière, F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Egger, L., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U., MacIerzanka, A., MacKie, A., … Brodkorb, A. (2014). A standardised static *invitro* digestion method suiTabel for food-an international consensus. *Food and Function*, *5*(6), 1113–1124. https://doi.org/10.1039/c3fo60702j
Misquitta, S. A., Kshirsagar, D. N., Dange, P. R., Choudhari, V. G., & Kabra, M. M. (2023). Digestibility of proteins in legumes. In Production and Utilization of Legumes-Progress and Prospects. IntechOpen. DOI: 10.5772/intechopen.110372
Montoya, C. A., Gomez, A. S., Lallès, J. P., Souffrant, W. B., Beebe, S., & Leterme, P. (2008). *Invitro* and in vivo protein hydrolysis of beans (Phaseolus vulgaris) genetically modified to express different phaseolin types. *Food Chemistry*, *106*(3), 1225–1233
Moreno, C., Mojica, L., Gonz, E., Mej, D., Mar, R., & Ruiz, C. (2020). Combinations of legume protein hydrolysates synergistically inhibit biological markers associated with adipogenesis. *Foods*, *9*(11), 1678.

Navarro, K. L., Huss, M., Smith, J. C., Sharp, P., Marx, J. O., & Pacharinsak, C. (2021). Mouse anesthesia: the art and science. *ILAR journal*, 62(1-2), 238-273. DOI: 10.1093/ilar/ilab016

Newstead S, Drew D, Cameron AD, Postis VL, Xia X, Fowler PW, Ingram JC, Carpenter EP, Sansom, MSP, McPherson, MJ, et al. 2011. Crystal structure of a prokaryotic homologue of the mammalian oligopeptide–proton symporters, PepT1 and PepT2. The EMBO journal. 30(2):417-426. https://doi.org/10.1038/emboj.2010.309

Ohanenye, I. C., Sun, X., Sarteshnizi, R. A., & Udenigwe, C. C. (2021). Germination alters the microstructure, *invitro* protein digestibility, α-glucosidase and dipeptidyl peptidase-IV inhibitory activities of bioaccessible fraction of pigeon pea (*Cajanus cajan*) seeds. *Legume Science*, *3*(1), 1–12. https://doi.org/10.1002/leg3.79

Pak, V. V., Kim, S. H., Koo, M., Lee, N., Shakhidoyatov, K. M., & Kwon, D. Y. (2007). Peptide design of a competitive inhibitor for HMG-CoA reduktase based on statin structure. Biopolymers, 85(4), 392–406. https://doi.org/10.1002/bip

Patel G, Misra A. 2011. Oral delivery of proteins and peptides: concepts and applications. Challenges in delivery of therapeutic genomics and proteomics. 481-529. https://doi.org/10.1016/B978-0-12-384964-9.00010-4

Pertiwi, M. G. P., Marsono, Y., & Indrati, R. (2020). Invitro gastrointestinal simulation of tempe prepared from koro kratok (Phaseolus lunatus L.) as an angiotensin-converting enzyme inhibitor. Journal of Food Science and Technology, 57(5), 1847–1855. https://doi.org/10.1007/s13197-019-04219-1

Ratnayani, K., Suter, I. K., Antara, N. S., & Putra, I. N. K. (2019). Angiotensin converting enzyme (ACE) inhibitory activity of peptide fraction of germinated pigeon pea (*Cajanus cajan* (L.) Millsp.). *Indonesian Journal of Chemistry*, *19*(4), 900–906. https://doi.org/10.22146/ijc.37513

Rutherfurd SM. 2010. Methodology for determining degree of hydrolysis of proteins in hydrolysates: a review. Journal of AOAC International. 93(5): 1515-1522. https://doi.org/10.1093/jaoac/93.5.1515

Sandoval-Sicairos, E. S., Domínguez-Rodríguez, M., Montoya-Rodríguez, A., Milán-Noris, A. K., Reyes-Moreno, C., & Milán-Carrillo, J. (2020). Phytochemical compounds and antioxidant activity modified by germination and hydrolysis in mexican *Amaranth*. *Plant Foods for Human Nutrition*, *75*(2), 192–199. https://doi.org/10.1007/s11130-020-00798-z

Schönfeldt HC, Pretorius B, Hall N. 2016. Bioavailability of nutrients. Caballero, B, P Finglas and F Toldrá, F (eds) The Encyclopedia of Food and Health. 1:401-406 (2016).

Sepulveda, F. V., & Smith, M. W. (1974). Discrimination between different entry mechanisms for neutral amino acids in rabbit ileal mucosa. Journal Physiol, 129–151.

Sepúlveda, F. V., & Smith, M. W. (1979). Different mechanisms for neutral amino acid uptake by new-born pig colon. The Journal of Physiology, 286(1), 479–490. https://doi.org/10.1113/jphysiol.1979.sp012632

Silva, M. B. de C. e., Souza, C. A. da C., Philadelpho, B. O., Cunha, M. M. N. da, Batista, F. P. R., Silva, J. R. da, Druzian, J. I., Castilho, M. S., Cilli, E. M., & Ferreira, E. S. (2018). Invitro and insilico studies of 3-hydroxy-3-methyl-glutaryl coenzyme A reduktase inhibitory activity of the cowpea Gln-Asp-Phe peptide. Food Chemistry, 259(November

2017), 270–277. https://doi.org/10.1016/j.foodchem.2018.03.132
Smith ME, Morton DG. 2010. Digestion and absorption. The digestive system. 2nd ed. Churchill Livingstone. 129:152
Suzuki, T. (2013). Regulation of intestinal epithelial permeability by tight junctions. Cellular and Molecular Life Sciences, 70(4), 631–659. https://doi.org/10.1007/s00018-012-1070-x
Xiao, Y., Wei, Q., Du, L., Guo, Z., & Li, Y. (2024). *Invitro* evaluation and *insitu* intestinal absorption characterisation of paeoniflorin nanoparticles in a rat model. *RSC Advances*, *14*(31), 22113–22122. https://doi.org/10.1039/d4ra03419h
Xu, Q., Hong, H., Wu, J., & Yan, X. (2019). Bioavailability of bioactive peptides derived from food proteins across the intestinal epithelial membrane: A review. *Trends in Food Science and Technology*, *86*, 399–411. https://doi.org/10.1016/j.tifs.2019.02.050
Yuan Y, Li C, Zheng Q, Wu J, Zhu K, Shen X, Cao J. 2018. Effect of simulated gastrointestinal digestion *in vitro* on the antioxidant activity, molecular weight and microstructure of polysaccharides from a tropical sea cucumber (*Holothuria leucospilota*). Food Hydrocolloids. 89:735-741. https://doi.org/10.1016/j.foodhyd.2018.11.040

Table 1(on next page)

Reaction mixture for inhibition of HMG CoA reductase activity.

Reaction mixture for inhibition of HMG CoA reductase activity.

Table 1. Reaction mixture for inhibition of HMG CoA reductase activity..

Sample	1x Assay Buffer	Pravastatin	NADPH	HMG-CoA	HMG CoA reductase	*Kara Kratok (Phaseolus lunatus*)
Blank	184 µl	-	4 µl	12 µl	-	-
Negative Control	182 µl	-	4 µl	12 µl	2 µl	-
Positive Control	181 µl	1 µl	4 µl	12 µl	2 µl	-
Samples (Biopeptide Candidates)	178 µl	-	4 µl	12 µl	2 µl	10 µl

Figure legends:

Figure 1. Final preparation on *Sprague Dawley* for absorption test with *in-situ method* (n = 3 animals for each sample)

Figure 2. Percentage of hydrolysis degree (A) and peptide concentration (B) from germinated and ungerminated *kara kratok* (*Phaseolus lunatus* L.) during digestion gastrointestinal simulation via in vitro method (n=3, P<0.05 for significant difference).

Figure 3. The molecular weight of (A) ungerminated and (B) germinated *kara kratok*(*Phaseolus lunatus* L.) during gastrointestinal digestion simulation via *in vitro* method – lane M (marker), lane 1-9 (digestion time 0, 30, 60, 90, 120, 150, 180, 210, 240 minutes, (C) biopeptide unabsorbed during *in-situ* method – lane M (marker), lane 2 – 5 (ungerminated sample at 30, 60, 90 and 120 min absorption), lane 6 – 9 (germinated sample at 30, 60, 90 dan 120 min absorption).

Figure 4. Percentage of HMG CoA reductase inhibition from germinated and ungerminated *kara kratok* (*Phaseolus lunatus* L.) versus pravastatin as a drug reference (A) during digestion gastrointestinal simulation via the in vitro method, (B) in perfusate that contains unabsorbed peptide (n=3, P<0.05 for significant difference).

Figure 5. Percentage absorption of germinated and ungerminated *kara kratok* (*Phaseolus lunatus* L.) based on the calculation of peptide concentration before and after the *in-situ* absorption method (n=3, $P<0.05$ for significant difference).

Figure 1

Final preparation on *Sprague Dawley* for absorption test with *in-situ method* (n = 3 animals for each sample)

Final preparation on *Sprague Dawley* for absorption test with *in-situ method* (n = 3 animals for each sample)

Figure 2

Percentage of hydrolysis degree (A) and peptide concentration (B) from germinated and ungerminated *kara kratok* (*Phaseolus lunatus* L.) during digestion gastrointestinal simulation via in vitro method (n=3, P<0.05 for significant difference)

Percentage of hydrolysis degree (A) and peptide concentration (B) from germinated and ungerminated *kara kratok* (*Phaseolus lunatus* L.) during digestion gastrointestinal simulation via in vitro method (n=3, P<0.05 for significant difference)

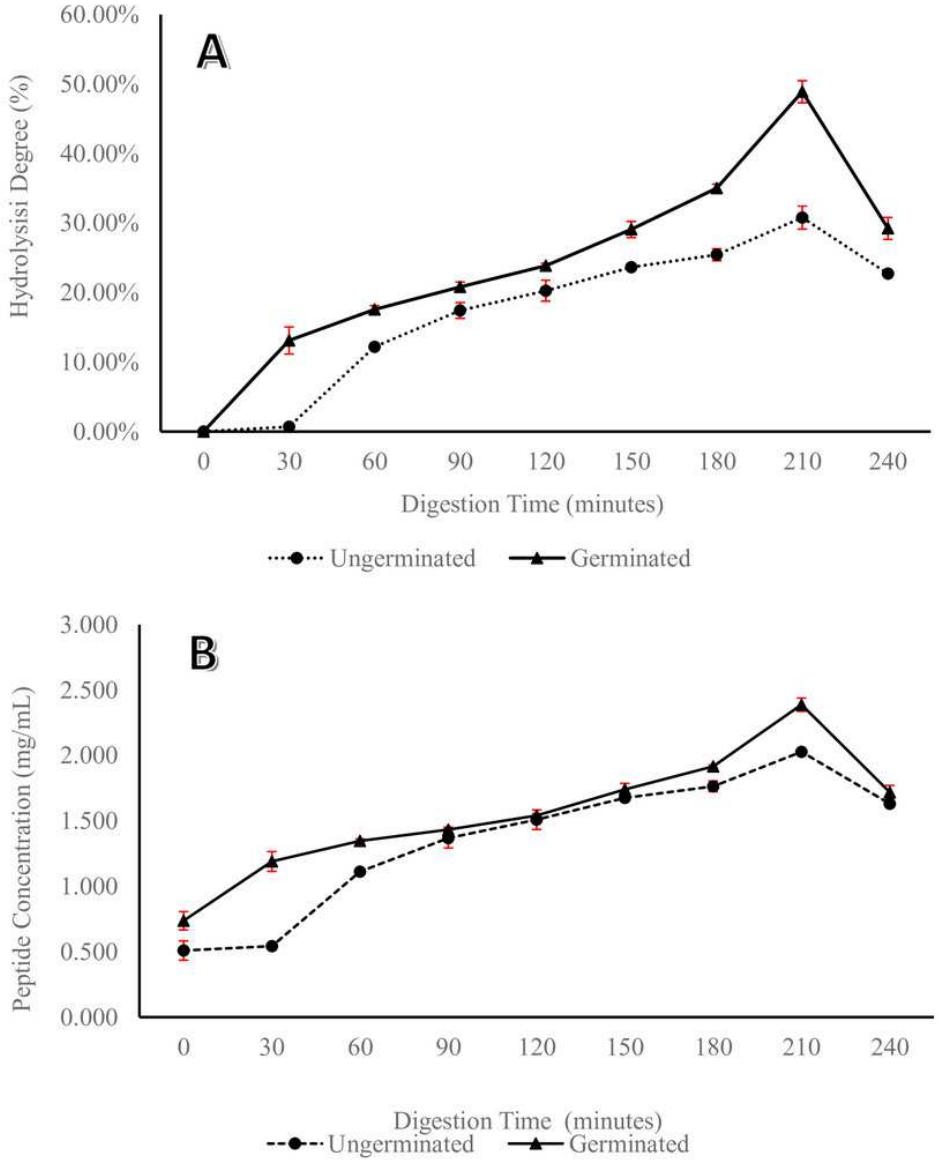

A
Hydrolysisi Degree (%)
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
0
30
60
90
120
150
180
210
240
Digestion Time (minutes)
Ungerminated
Germinated
B
Peptide Concentration (mg/mL)
3.000
2.500
2.000
1.500
1.000
0.500
0.000
0
30
60
90
120
150
180
210
240
Digestion Time (minutes)
Ungerminated
Germinated

Figure 3

The molecular weight of (A) ungerminated and (B) germinated *kara kratok*(*Phaseolus lunatus* L.) during gastrointestinal digestion simulation via *in vitro* method.

The molecular weight of (A) ungerminated and (B) germinated *kara kratok*(*Phaseolus lunatus* L.) during gastrointestinal digestion simulation via *in vitro* method – lane M (marker), lane 1-9 (digestion time 0, 30, 60, 90, 120, 150, 180, 210, 240 minutes, (C) biopeptide unabsorbed during *in-situ* method – lane M (marker), lane 2 – 5 (ungerminated sample at 30, 60, 90 and 120 min absorption), lane 6 – 9 (germinated sample at 30, 60, 90 dan 120 min absorption).

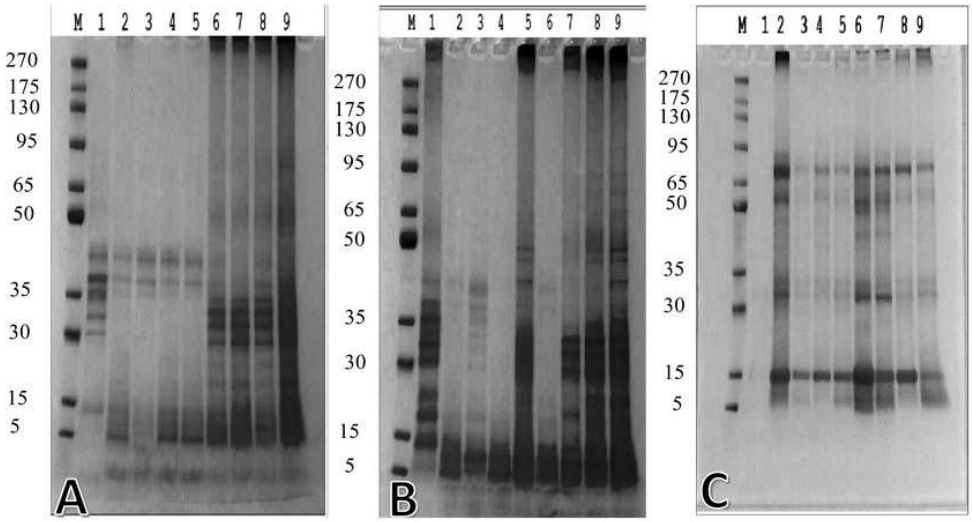
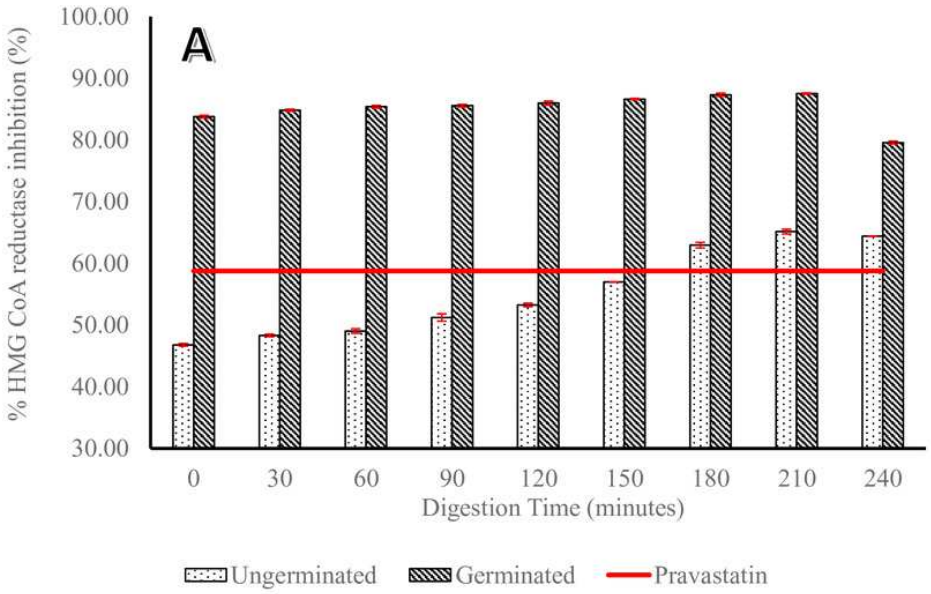

M 1 2 3 4 5 6 7 8 9
270
175
130
95
65
50
35
30
15
5
A
M 1 2 3 4 5 6 7 8 9
270
175
130
95
65
50
35
30
15
5
B
M 1 2 3 4 5 6 7 8 9
270
175
130
95
65
50
35
30
15
5
C

Figure 4

Percentage of HMG CoA reductase inhibition from germinated and ungerminated kara kratok (Phaseolus lunatus L.) versus pravastatin as a drug reference.

Percentage of HMG CoA reductase inhibition from germinated and ungerminated kara kratok (Phaseolus lunatus L.) versus pravastatin as a drug reference (A) during digestion gastrointestinal simulation via the in vitro method, (B) in perfusate that contains unabsorbed peptide (n=3, $P<0.05$ for significant difference).


A
% HMG CoA reductase inhibition (%)
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
0
30
60
90
120
150
180
210
240
Digestion Time (minutes)
Ungerminated
Germinated
Pravastatin

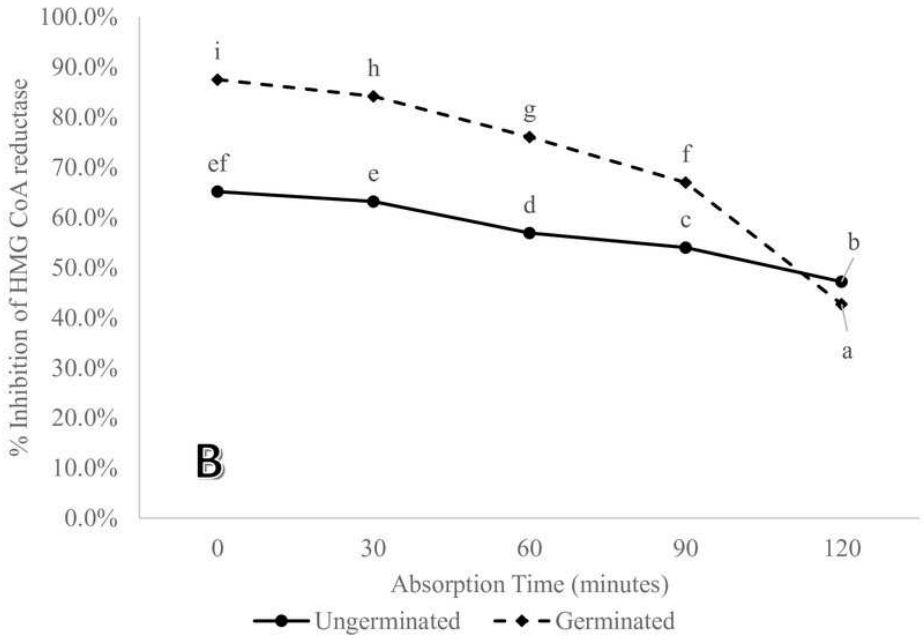
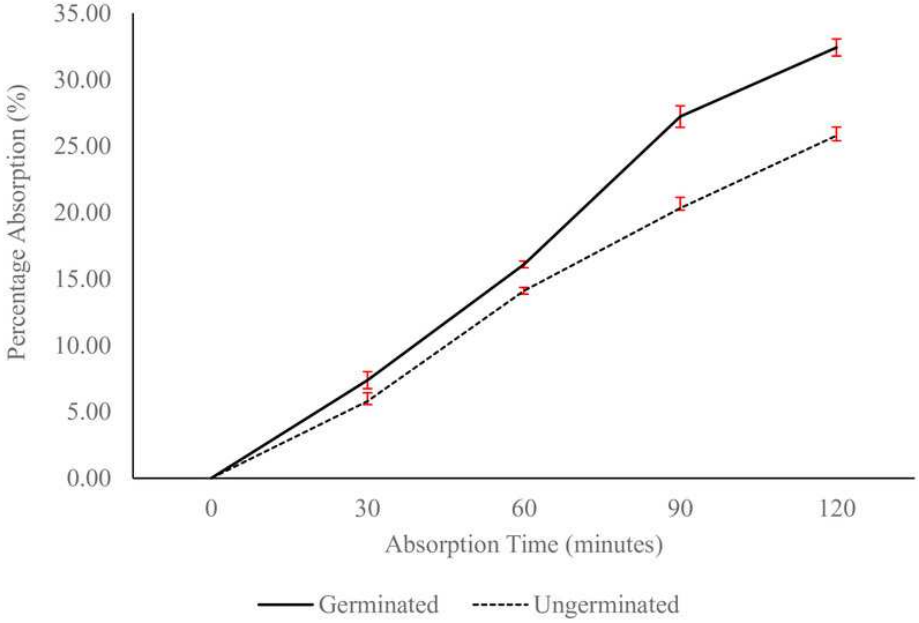

B
% Inhibition of HMG CoA reductase
100.0%
90.0%
80.0%
70.0%
60.0%
50.0%
40.0%
30.0%
20.0%
10.0%
0.0%
i
h
g
f
ef
e
d
c
b
a
0
30
60
90
120
Absorption Time (minutes)
Ungerminated
Germinated

Figure 5

Percentage absorption of germinated and ungerminated kara kratok (Phaseolus lunatus L.) based on the calculation of peptide concentration before and after the in-situ absorption method (n=3, P<0.05 for significant difference).

Percentage absorption of germinated and ungerminated kara kratok (Phaseolus lunatus L.) based on the calculation of peptide concentration before and after the in-situ absorption method (n=3, P<0.05 for significant difference).


35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
Percentage Absorption (%)
0
30
60
90
120
Absorption Time (minutes)
Germinated
Ungerminated