ISSN: 2347-467X, Vol. 12, No. (2) 2024, Pg. 792-804

Current Research in Nutrition and Food Science

www.foodandnutritionjournal.org

In Vitro Antioxidant and Hypocholesterolemic Potency of Melon (*Cucumis Melo* L.) Seed Protein Hydrolysate

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Abstract

Protein hydrolysates have been reported to possess numerous bioactivities. However, research on melon (*Cucumis melo* L.) seed protein (MSP) hydrolysate is limited. This study aimed to analyze the antioxidant and hypocholesterolemic properties of MSP hydrolysate. Protein from melon seed was obtained by conventional alkaline extraction-isoelectric precipitation method. Enzymatic hydrolysis of MSP was carried out using three different proteases: pepsin, thermolysin, and trypsin, with the enzyme-to-substrate (E/S) ratio of 1:50 (w/w). The results showed that all hydrolysates exhibited antioxidant and hypocholesterolemic activity. The thermolysin-digested hydrolysate had significantly greater ($p < 0.05$) radical scavenging properties, while trypsin produced the highest ($p < 0.05$) metal ion chelating activity. At 2 mg/mL, thermolysin-derived MSP hydrolysate showed no significant difference ($p > 0.05$) in HMGR inhibition activity compared to pravastatin. Additionally, the thermolysin hydrolysate had significantly higher ($p < 0.05$) bile acid binding ability than other hydrolysates. Overall, the MSP hydrolysate produced by thermolysin exhibited stronger antioxidant and hypocholesterolemic activities when compared to those produced by pepsin and trypsin, suggesting its potential effectiveness in nutraceutical applications.

Article History
Received: 01 February 2024
Accepted: 26 July 2024

Keywords
Antioxidant;
Cucumis Melo L;
Seed;
Hypocholesterolemic;
Protein Hydrolysate.

Introduction

Hypercholesterolemia or excess blood cholesterol, is one of the risk factors for atherosclerosis, which promotes cardiovascular diseases. Cardiovascular diseases (CVD) lead to high mortality worldwide, with about 18.6 million deaths in 2019.[1] Sources of cholesterol in the human body are from diet and through *de-novo* synthesis in the liver. High levels of blood cholesterol, especially low-density lipoprotein cholesterol (LDL), can cause hypercholesterolemia. This condition is associated with an increased risk of atherosclerosis.[2] There are several strategies to treat

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Doi: https://dx.doi.org/10.12944/CRNFSJ.12.2.24

hypercholesterolemia, including inhibiting 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) activity and using bile acid-binding agents. HMGR is one of the enzymes essential to the synthesis of cholesterol in the liver. Inhibiting HMGR reduces cholesterol synthesis and increases the number of LDL receptors, which helps lower LDL cholesterol in the blood. Bile acid-binding substances reduce bile acid levels in the enterohepatic circulation, leading to lower blood cholesterol as cholesterol is converted to bile acids.[3] Hypercholesterolemia and atherosclerotic risk factors increase the generation of reactive oxygen species (ROS) in the vessel walls, causing oxidative stress.[4]

It is well known that many naturally derived active compounds, including peptides, have great impact on the prevention or treatment of various disorders such as CVD, cancer, and diabetes. Bioactive peptides are potential therapeutic components in the pharmaceutical and biotechnology industries. They exhibit a strong affinity for target molecules, are stable against proteolytic degradation, safe, and multifunctional[5,6] Bioactive peptides can be obtained by the enzymatic hydrolysis process of dietary proteins from plant, marine, and animal sources.[7] Enzymatic hydrolysis is considered more advantageous than the chemical hydrolysis process because it uses mild conditions and is more accurate in the cleavage of peptide bonds.8 The types of enzymes employed in the hydrolysis process determine the diversity of physicochemical and biological properties of hydrolysate proteins and peptides.[9] Each protease enzyme targets specific peptide bonds, producing peptides with varying molecular weights, sizes, and amino acid sequences, resulting in diverse properties.[6] Many studies have demonstrated the various biological activities of hydrolysate protein and peptides, including antioxidant and anti-hypercholesterolemic. Cowpea,[10] hempseed,[9,11] and cumin seed [3,6] are among the plant-derived protein with *in-vitro* antioxidant and hypocholesterolemic capacity.

Melon (*Cucumis melo* L.) is a popular fruit that belongs to the *Cucurbitaceae* family and it is one of the most consumed fruits worldwide with high economic value. However, by-products of the fruit such as seeds, are usually discarded during industrial processing or consumption. In several countries, melon seeds are also consumed after being roasted or are utilized as flavoring agents for traditional dishes and desserts.[12–14] Melon seeds possess a high content of protein (14.91%- 36.3%),[15] however, there is a lack of exploration regarding the biological activities of melon seed protein. Determining the biological effects of melon seeds could increase their value and usage, particularly in the pharmaceutical and food industries.

In vitro studies by Siddeeg *et al*.[16] reported that the protein of *Cucumis melo* var. tibish seed, when sequentially digested with trypsin and pepsin, exhibits antioxidant activity. However, research on the bioactivities of melon (*Cucumis melo* L.) seed protein (MSP) hydrolysate is currently limited. Specifically, no previous reports are available on the hypocholesterolemic activity of MSP hydrolysate. This study aims to investigate the antioxidant and hypocholesterolemic activities of MSP hydrolysate using three different proteases, i.e., pepsin, thermolysin, and trypsin, which are commonly used to produce bioactive peptides. Our findings highlight the potential of MSP hydrolysates as nutraceutical agents, identifying their antioxidant and hypocholesterolemic properties for versatile applications in the food and pharmaceutical sectors.

Material and Method

Material

Melon (*Cucumis melo* L.) fruit was obtained from the local market, and the seeds were removed manually from the fruit. The identification of the plant material was carried out in the Herbarium Depokensis (UIDEP) at the Department of Biology, Indonesia University. Protease enzymes used were pepsin (1:10,000 or 10,000 NFU/mg) from Himedia, trypsin (0.2 Anson units/g) from Himedia, and thermolysin (30-350 units/mg) from Sigma Aldrich.

Preparation of Melon Protein Hydrolysate

Melon seeds were dried at 40 oC and then milled to obtain seed flour. Preparation to obtain protein from melon seed was conducted by conventional alkaline extraction-isoelectric precipitation method as described by Marques *et al*.[10] The flour of the seeds was defatted using soxhlet extraction with n-hexane for 5 h at 60 – 70 °C and the defatted flour was suspended in deionized water with a ratio of 1:10 (w/v). After adding 1 M NaOH to adjust the pH of the mixture to 9.3, it was agitated using a magnetic stirrer at 500 rpm for 2 h at room temperature.

Then the slurry was centrifuged at 5500 ×g at a temperature of 4 °C for 15 min, and the extraction process was carried out once more after the residue was suspended in water. All of the supernatants were mixed and precipitated at pH 4.4 with the addition of 1 M HCl. The precipitate was separated by centrifugation at 5500 ×g at 4 °C for 15 min. The precipitate obtained was melon seed protein isolate, then washed with water and the pH adjusted to 7 by adding 1 M NaOH. The melon seed protein isolate was dried using a freeze-dryer (Buchi Lyovapor L-300). The next step was the hydrolysis process of the freeze-dried MSP, which was conducted with the procedure described by He *et al*.[5] with minor modifications. MSP was dissolved (5% w/v protein basis) in deionized water in a reaction vessel with a stirrer. Then, the samples were digested for 4 h by three different types of enzymes at an enzyme-to-substrate (E/S) ratio of 1:50 (w/w). Temperature and pH were adjusted to the optimum conditions for each enzyme. Trypsin, at 37 °C and pH 8; pepsin, 37 °C and pH 2; and thermolysin, 55 °C and pH 8. During the hydrolysis, the pH of solution was kept constant. Reactions were stopped to inactivate the enzyme by heating the solution in a boiling water bath for about 20 min and then centrifuged at 5500 xg at 4 °C for 30 min. The supernatant was collected as hydrolysate and stored at -20 °C for further analysis. The protein content of the hydrolysate was determined using the Bradford method.[17]

Amino Acid Composition Determination

Analysis of amino acids was performed by acid hydrolysis and using the UPLC system with guidelines according to System Guide of Waters Acquity UPLC H Class and H Class Bio amino Acid Analysis.[18] Each sample of 1 µL was injected into column AccQ.Tag Ultra C18 1.7 µm (2.1 x 100 mm), at 49 °C with PDA detection at 260 nm wavelength. The mobile phase had a flow rate of 0.5 mL/min and the following composition: mobile phase A was AccQ. Tag Ultra amino acid analysis eluent A; mobile phase B was AccQ.Tag Ultra amino acid analysis 10% (in water), mobile phase C was Aquabidest grade HPLC and mobile phase D was AccQ.Tag Ultra amino acid analysis eluent B. While tryptophan (Trp) content was analyzed after alkaline hydrolysis using High Performance Liquid Chromatography (HPLC) in compliance with AOAC official method 988.15. Each sample of 15 µL was injected into column RP-18.5 µm, 250 mm x 4.0 mm, at ambient temperatures. The mobile phase flow rate was 1.5 mL/min, with phase A being 0.0085 M sodium acetate (pH 4) and phase B methanol. The detector was a PDA at 280 nm wavelength.

Antioxidant Activity Measurement

Abts Radical Scavenging Activity Assay

The ABTS (2,2'-azinobis-(3ethylbenzothiazoline)-6-sulfonic acid) radical scavenging properties of hydrolysates were conducted using the procedure from Zhuang *et al*.[19] with slight modifications. The radical cation of ABTS was produced by mixing 5 mL of 7 mM ABTS stock solution with 85 µL of 2.45 mM potassium persulfate. The mixture was incubated at room temperature in the dark for 14–16 h. Then 0.2 M of PBS pH 7.4 was used to dilute the ABTS radical solution until its absorbance was 0.7 at 734 nm. 2 mg/mL of hydrolysate solution was mixed with the diluted ABTS radical solution (1:4). The absorbance of this mixture was recorded at 734 nm using a BioTek Instrument Synergy H1MF microplate spectrophotometer after incubating for 6 min. The percentage of radical inhibition by the sample was calculated using the formula below, with PBS as a blank:

% ABTS• scavenging activity = [(blank absorbance - sample absorbance)/(blank absorbance)] x 100%

Dpph Radical Scavenging Activity Assay

To measure DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging properties, the method by Ben Khaled *et al*.[20] was followed with minor modifications. 500 µL of hydrolysate (9 mg/mL) in 0.1 M phosphate buffer pH 7 was added to 375 µL of 90% ethanol and 125 µL of 0.4 mM DPPH in ethanol. After an hour of incubation at 37 °C, the absorbance was recorded at 517 nm using a BioTek Instrument Synergy H1MF microplate spectrophotometer. The percent inhibition of DPPH radicals by the hydrolysate was calculated using the formula below:

% DPPH• scavenging activity = [(blank absorbance - sample absorbance)/(blank absorbance)] x 100%

Ferrous Ion Chelating Ability Assay

The activity of hydrolysate to chelate ferrous ions was determined by following the method of Yuan *et al*.[21] with slight modifications. 1 mL of hydrolysate (9 mg/mL) in sodium acetate buffer pH 5 was mixed with 100 µL of 2 mM $FeCl_2.4H_2O$, then incubated

at 37 °C for 30 min. After adding 200 µL of 5 mM ferrozine to the mixture, it was incubated for 10 min at room temperature. The absorbance of the sample was read using BioTek Instrument Synergy H1MF microplate spectrophotometer at 562 nm. The chelating activity was calculated using the formula as follows:

% Chelating activity = [(blank absorbance - sample absorbance)/(blank absorbance)] x 100%

Hypocholesterolemic Activity

HMG-CoA Reductase Inhibition Assay

The HMG-CoA reductase (HMGR) inhibition test was conducted using the Sigma-Aldrich® HMG-CoA reductase assay kit CS 1090-1KT. In summary, each hydrolysate sample with NADPH, substrate (HMG-CoA), and buffer was loaded into a 96-well plate. The reactions were initiated by HMG-CoA reductase addition in each well, and the absorbance was read at 340 nm. The specific activity of the HMGR enzyme was interpreted as µmol of oxidized NADPH/min/mgP. The inhibitory capacity of the hydrolysates was determined by reading the decrease in absorbance at 340 nm for 10 min, and this absorbance was directly related to the activity of the enzymes. The results were given as a percentage of the HMGR enzyme's control specific activity without hydrolysate or pravastatin. Pravastatin was a positive control, and the HMGR inhibitory activity of the hydrolysate was calculated as follows:

% Inhibition = [(blank HMGR enzyme activity – HMGR enzyme activity in the precense hydrolysate)/(blank HMGR enzyme activity)] x 100%

Bile Acid Binding Activity

The determination of the bile-acid binding ability was performed using the method of Yoshie-Stark 22. In brief 900 µl of bile acid solution i.e., sodium deoxycholate 2 mM was added to 100 µL of hydrolysate sample (10 mg/mL) in phosphate buffer (0.1 mM pH 7). After incubation at 37 °C for 2 h, each sample was centrifuged at 12000 rpm 4 °C for 10 min. The result supernatant was diluted, and the bile acid content in sample was analyzed following Bile Acid kit MAK309 Sigma Aldrich instructions. All samples were run in duplicate and analyzed fluorescence spectrophotometrically at λex 530 nm and λem 585. The binding activity was determined using the formula as follows:

% Bile acid binding activity = [(bile acid in blank - bile acid in sample)/(bile acid in blank)] x 100%

Statistical Analysis

All tests score are reported as mean ± standard deviation (SD). The one way analysis of variance (ANOVA) test was used in IBM SPSS Statistics version 23 for statistical analysis. A significant difference in means was identified between the samples at $p < 0.05$.

Result and Discussion

Protein Content and Amino Acid Composition

The soluble protein contents of MSP hydrolysate are 2.28 mg/mL, 2.36 mg/mL, and 4.38 mg/mL for pepsin, thermolysin and trypsin-treated hydrolysate respectively (Figure 1). According to Kim *et al*.[23] differences in protein content may arise from the enzymes selected for hydrolysis. Despite the many factors that could affect hydrolysate protein content, the enzyme used for hydrolysis plays a crucial role in determining peptide bond cleavage patterns, which correlate with protein levels in the hydrolysate. Their study[23] demonstrated varying protein content in perilla seed meal protein hydrolysates depending on the enzyme used. In addition, Alashi *et al*.[24] have also reported that canola meal protein hydrolysates from trypsin and chymotrypsin digestion had the highest levels of protein content compared to alcalase, pancreatin, and pepsin hydrolysates.

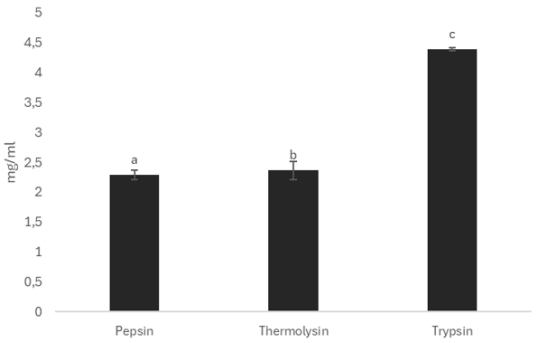

Fig. 1: Protein content of melon seed protein hydrolyasate. Values are expressed as means ± SD. Various letters indicate significant differences between means ($p < 0.05$)

The amino acid compositions of MSP hydrolysates are summarized in Table 1. The amino acids with the highest levels in all samples of hydrolysate are glutamic acid (5050.935-9777.91 mg/Kg sample)

and arginine (6824.28- 8544.495 mg/Kg sample), which is in accordance with the previous study reported by Siddeeg *et al*.[25] Followed by aspartic acid (2441.09- 4903.87 mg/Kg sample), phenylalanine (3071.46- 3590.485 mg/Kg sample), and leucine (2859.185-3970.785 mg/Kg sample). Dash and Gosh[26] reported that major amino acids found in *Cucurbitaceae* seed protein hydrolysate are glutamic acid, aspartic acid, and arginine.

Table 1. Amino acid composition of melon seed protein hydrolysates (mg/Kg sample)

Amino Acids	Pepsin	Thermolysin	Trypsin
Serine	2145.375	3062.72	2279.05
Glutamic acid	5050.935	9777.91	5619.42
Phenylalanine	3071.46	3590.485	3503.54
Isoleucine	1619.44	1852.995	1536.195
Valine	1913.955	2194.295	1760.415
Alanine	1489.95	2524.905	1433.705
Arginine	6824.28	8544.495	8013.415
Glycine	2009.115	2744.35	2118.305
Lysine	868.115	1625.335	794.405
Aspartic acid	2441.09	4903.87	2456.45
Leucine	2859.185	3970.785	2690.685
Tyrosine	1575.545	1857.03	1796.45
Proline	1409.64	2012.825	1367.89
Threonine	1828	2254.13	1927.99
Histidine	1325.24	1389.505	1487.71
Tryptophan	442.44	902.685	493.79
Cystine	564.495	318.495	419.09
Methionine	90.075	119.795	123.095
EAA*	13944.2	17900.01	14317.065
HAA	14593.745	18441.61	14631.08
NCAA	7492.025	14681.78	8070.065
PCAA	8943.925	11559.335	10294.755
AAA	5089.445	6350.2	5793.78

*Essential amino acids (EAA)- histidine, lysine, methionine, leucine, threonine, tryptophan, phenylalanine, isoleucine, valine; Hyrdrophobic amino acid (HAA) – alanine, proline, cysteine, valine, methionine, isoleucine, leucine, tyrosine, and phenylalanine; Negatively charged amino acid (NCAA) – aspartic acid, glutamic acid; Positively charged amino acid (PCAA)- histidine, lysine, arginine; Aromatic amino acid (AAA) – tryptophan, tyrosine, phenylalanine

Protein hydrolysates, obtained through enzymatic hydrolysis, contain amino acids and bioactive peptides, valued for their nutraceutical benefits and critical roles in vital human physiological processes.[27] The composition of amino acid and peptide sequences plays a crucial role in determining the bioactivity of the hydrolysate.[28] Aromatic amino acids such as tyrosine and phenylalanine can donate electrons to convert free radicals into stable molecules, thereby enhancing biological activity.[29] The negatively charged acidic amino acids have excess electrons that can be donated to quenching free radicals. Therefore, they can act as antioxidants.[30,31] Hydrophobic amino acids have the ability to donate protons and electrons, and enhance the lipid solubility of peptides, potentially

increasing their antioxidant activity.[27,29] Furthermore, Maestri *et al*.[32] suggested that hydrophobic bioactive peptides containing leucine, tryptophan, and tyrosine are reported to exhibit better hypocholesterolemic activity. Prados *et al*.[33] revealed that thirty-three peptides identified in the olive seed hydrolysate were found to be rich in hydrophobic amino acids, potentially contributing to their hypolipidemic properties. Several studies have confirmed that peptides from food protein hydrolysates with antioxidant properties exhibit additional biological activities, including hypocholesterolemic effects.[34] In the present study, the hydrolysates from thermolysin digestion have high hydrophobic amino acids (Table 1) such as, leucine, valin , methionine, prolin, and phenylalanine. The hydrolysates also contained more aromatic amino acids, as well as the negatively charged acidic amino acids (Table 1). These results suggest that the hydrolysate produced by thermolysin may have stronger antioxidant and hypocholesterolemic properties compared to those produced by pepsin and trypsin.

Antioxidant Activity

Radical Scavenging Acitivty

ABTS radical scavenging activity of hydrolysate at concentration of 2 mg/mL is shown in Figure 2A. The ABTS radical scavenging method can be used to determine the ability of both lipophilic and hydrophilic molecules to function as antioxidants. In this study, hydrolysate digested with thermolysin exhibited greatest ability against ABTS. The scavenging activity of thermolysin-treated hydrolysate was 47.38 ± 1.51% and significantly different ($p < 0.05$) from trypsin (37.65 ± 0.9%) and pepsin (35.27 ± 1.62%). This result indicates that the type of enzyme used for hydrolysis affected the activity to scavenge ABTS radical. A study by Garcia *et al*.[35] also found that hydrolysates of cherry seeds digested with thermolysin exhibit the highest ABTS radical scavenging activity compared to alcalase and flavorzyme. Zheng *et al*.[36] suggested residues such as tyrosine, tryptophan, or cysteine are required to scavenge ABTS. The mechanism by which antioxidants neutralize ABTS radicals is through hydrogen and electron donors.[28] Amino acids with SH, NH, and OH side chains, such as tyrosine, cysteine, and tryptophan, are considered to play an important role in scavenging ABTS radicals due to the presence of a labile hydrogen atom.[37]

DPPH is a relatively stable free radical that can accept an electron or hydrogen. The DPPH radical scavenging activity of MSP hydrolysate at a concentration of 9 mg/mL is shown in Figure 2B. MSP digested by thermolysin has the strongest capacity to scavenge DPPH radical (21.44 ± 0.37%) followed by pepsin (20.81 ± 0.99%) and trypsin (19.02 ± 0.63%). It was observed no significant difference ($p > 0.05$) between the thermolysin and pepsin-treated hydrolysate against DPPH radical but compared to trypsin-treated hydrolysate was significantly different ($p < 0.05$). Our result is in line with previous work by Alashi *et al*.[24] who reported the canola meal protein hydrolysate of pepsin was more active against DPPH radicals than the hydrolysate treated with trypsin. Moreover, Sun, *et al*.[38] revealed the red algae protein hydrolysate of pepsin has a greater ability to inhibit DPPH radicals compared to trypsin. As reported by Liao *et al*.[37] phenylalanine, proline, histidine and cysteine may serve as DPPH radical scavengers.

Metal (Ferrous) Ion Chelating Activity

Ferrous ions (Fe^{2+}) can act as prooxidants due to being involved in the Fenton and Haber-Weiss reaction which produce hydroxyl radicals that can damage biomolecules such as DNA and protein cellular. In addition, transition metals such as iron and copper stimulate the lipid peroxidation process.[39] Therefore metal chelation activity also relates to the antioxidant properties of bioactive compounds. The ferrous ion chelating activity of MSP hydrolysates is displayed in Figure 3.

The results observed that hydrolysates obtained by three proteases have strong chelating activity toward Fe^{2+} at the concentration of 9 mg/mL. The chelating activity exhibited significant differences ($p < 0.05$) among each three hydrolysates, with trypsin having the most potent activity (70.04 ± 2.40%), followed by thermolysin (56.22 ± 0.18%) and pepsin (25.59 ± 1.40%). In agreement with the current result, several works have also reported that trypsin-treated hydrolysate had the highest activity in binding ferrous ions.[40–42] Trypsin demonstrated a much higher capacity to produce ferrous-chelating peptides of MSP might be due to the specificity of this enzyme to cleavage arginine or lysine residue at the C-terminal of the peptide bond.[43] According to Tian *et al*.,[44] positively charged amino acids such

as lysine, arginine, and histidine have metal-binding ability due to the presence of the nitrogen atom. Moreover, Zhang *et al*.[45] revealed that mung bean peptides chelate ferrous ions mainly through amino, carboxyl, and imidazole groups of amino acids.

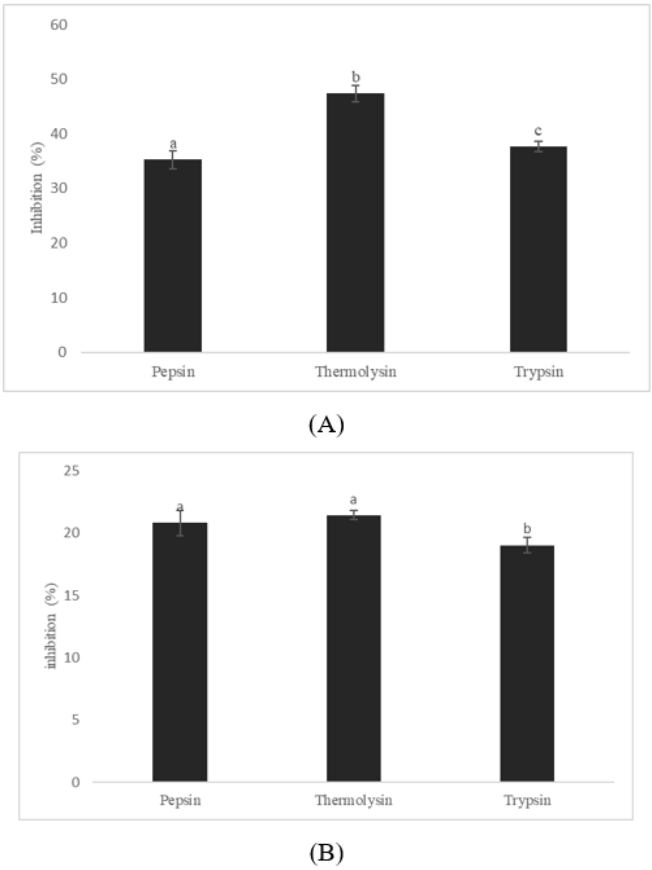

Fig. 2: Free radical scavenging activities of different enzymatic hydrolysates of MSP against ABTS radicals (A) and DPPH radicals (B). All data were presented as the mean ± SD. Significant differences ($p < 0.05$) are indicated by values with distinct letters

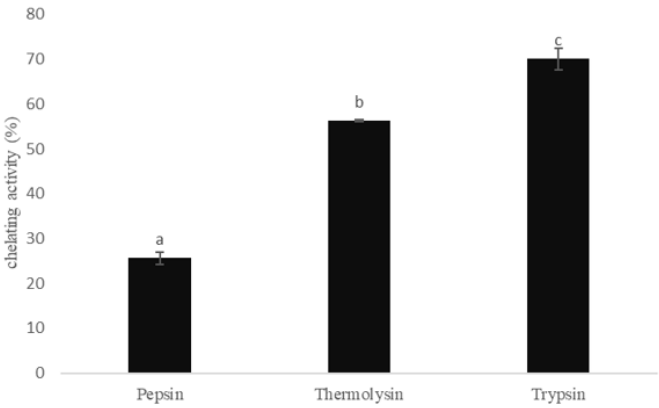

Fig. 3: Ferrous ion chelating capacities of MSP hydrolysates. All data were presented as the mean ± SD. Significant differences ($p < 0.05$) are indicated by values with distinct letters

In general, the hydrolysates from three types of enzymes displayed significant differences ($p < 0.05$) in activity to inhibit free radicals and to chelate metal ions. The findings obtained suggest that antioxidant capacity of MSP hydrolysates depends on the enzyme specificity and activity. Proteases with different specificities will produce peptides with different amino acid compositions, sequence, and molecular weights. Samaranayaka and Li-Chan46 suggested that antioxidant potency of peptides depend on amino acid composition, sequence, and molecular size.

Hypocholesterolemic Activity

HMG CoA Reductase Inhibition

The *in-vitro* investigation of the hypocholesterolemic activity of enzymatic hydrolysates from melon seed protein in this current study involved the HMGR inhibitory activity and bile acid binding capacity assay. Figure 4. shows the activities of HMGR inhibition by the three enzymatic hydrolysates with pravastatin used as a positive control. All the hydrolysates possessed inhibitory activity against HMGR at concentration of 2 mg/mL and are listed in decreasing order: thermolysin (68.25 ± 3.60%), pepsin (57.95 ± 5,11%) and trypsin (44.55 ± 5.99%). The hyrolysate of thermolysin demonstrated most potent inhibitor for HMGR and ANOVA test confirms that there were no significant differences ($p > 0.05$) between the inhibitory activity of thermolysin-treated hydrolysate and control positive pravastatin (81.67 ± 0.65%). While trypsin hydrolysate showed significantly ($p < 0.05$) lower activity compared to thermolysin and pepsin.

In accordance with our current study, several in vitro investigations have also confirmed the inhibitory activity of peptide fractions and hydrolysate from food and plant protein against HMGR. Prados *et al*.[33] demonstrated that olive seed protein hydrolysate from alcalase could inhibit HMGR by 16.8 and 40% at the concentrations of 3.1 mg/mL and 26.7 mg/mL, respectively. Lupin peptide mixture of pepsin and trypsin at a concentration of 2.5 mg/mL1 could reduce HMGR activity by 17 and 61%, respectively, as reported by Lammi, *et al*.[47] One of the inhibitory mechanisms of HMGR activity by peptides is through competitive inhibition of amino acid residues in the catalytic site of HMGR. Interactions between amino acids of the peptide from lupin with the active site of HMGR through electrostatic, hydrogen bond, salt bridge and hydrophobic interactions.[48,49] In this present study, thermolysin and pepsin have a better ability to interfere with HMGR compared to trypsin, which might be due to peptides from trypsin digestion having positively charged amino acids such as arginine or lysine at the C-terminal. These amino acids are less unfit for the active site of HMGR, which is also surrounded by some positively charged residues.[49]

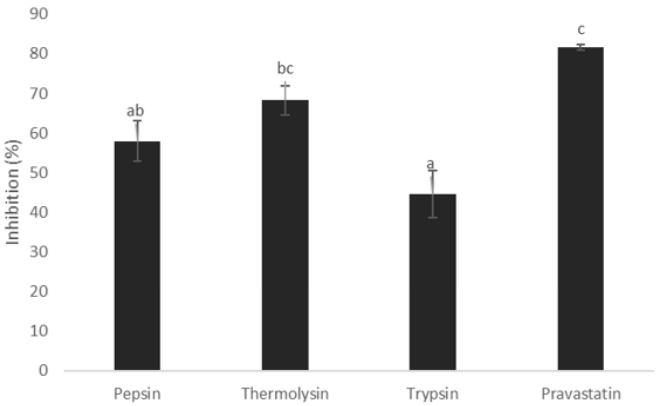

Fig. 4: Percent inhibition of the HMGR by the MSP hydrolysate. The data were shown as the mean ± SD, and different letters mean significant differences ($p < 0.05$).

Bile Acid Binding Capacity

For Figure 5 show sodium deoxycholate binding ability of the hydrolysates at concentration of 10 mg/mL. Among the three hydrolysates, thermolysin exhibited the highest bile acid-binding capacity (19.91 ± 1.43%), followed by pepsin (13.65 ± 0.98%) and trypsin (8.34 ± 0.11%). Our observed result are consistent with Mohan and Udenigwe[50] who

reported that hydrolysate of casein at concentration of 10 mg/mL have 12.8% bile acid binding capacity. Yoshie-Stark and Wäsche[22] reported that de-oiled soybean and its hydrolysate (10 mg/mL) of pepsin and pepsin/pancreatin have sodium deoxycholate binding capacity in a range of 12.6% – 15.0%, while lupin and its hydrolysate was 12.8% – 69.5%. Bile acid is an amphipathic molecule that consists of both a hydrophobic part due to the steroid core with hydrogen and methyl groups and a hydrophilic part with hydroxyl and carboxyl groups. These groups are considered to contribute to binding other molecules.[3] Previous in silico study by Ngoh *et al*.[51] described that most amino acids of peptides derived from pinto bean that interact with bile acid are hydrophobic amino acids such as proline, leucine, glycine, phenylalanine, alanine, and methionine. Moreover, Siow, *et al*[3] reported that amino acids of peptides from cumin seed can bind bile acid molecules through hydrophobic and hydrophilic interactions.

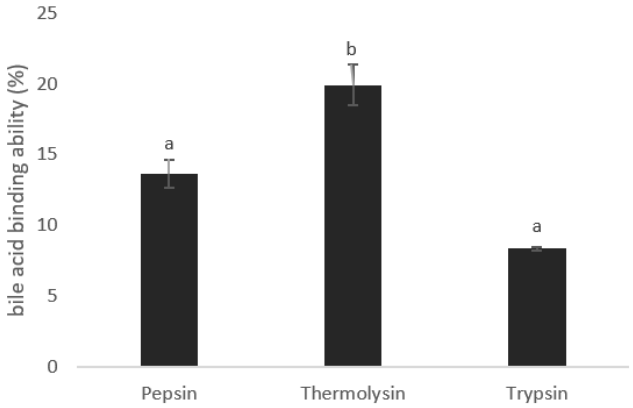

Fig. 5: Sodium deoxycholate binding ability of the MSP hydrolysate. The data were shown as the mean ± SD, and different letters mean significant differences ($p < 0.05$).

Conclusion

In conclusion, this present work displayed that MSP hydrolysates have *in-vitro* antioxidant and hypocholesterolemic properties. The biological capacity of hydrolysates varied depending on the type of protease used for protein digestion. The melon seed hydrolysate obtained using thermolysin enzyme preparation showed better abilities to inhibit HMGR, bind bile acid and scavenge free radicals compared to pepsin and trypsin. The hydrolysate from trypsin digestion has the highest activity in chelating metal ions. These findings suggest that the antioxidant and hypocholesterolemic properties of MSP hydrolysate might be useful in the preparation of nutraceuticals and in the development of pharmaceutical peptide-based products. In addition, melon seed may potentially be used as a raw material to produce bioactive ingredients with health benefits that could contribute to making this underutilized part of fruit more valuable. In future research, it would be beneficial to explore a variety of enzymes to build on the current study. Additionally, conducting in vivo studies will be necessary for improving these findings.

Acknowledgements

The author would like to thank the scholarship providers, namely the Indonesia Funds for Education (LPDP) and Center for Higher Education Funding (BPPT) for their support in carrying out the doctoral program in chemistry.

Funding Source

This research was funded by the scholarship providers, namely the Indonesia Funds for Education (LPDP) and the Center for Higher Education Funding (BPPT), with decree number: 0625/J5.2.3./ BPI.06/10/2021.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Contribution

Deasy Natalia Botutihe: conceptualization, study design, collection of data, analysis of results, manuscript preparation, writing and editing.

Sumi Hudiyono
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Data Availability
Statement Not applicable

Ethics Statement
Not applicable. This study did not involve human or animal participants.

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