The HMG-CoA Reductase Inhibitor Activities of Soy Protein Hydrolysates from Papain Hydrolysis

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ABSTRACT. The search for an HMG-CoA reductase inhibitor agent as a safe and inexpensive alternative treatment for hypercholesterolemia has been carried out using soy protein hydrolysates as one of the bioactive peptide sources. This study was conducted to explore the potency of soy protein hydrolysates as an anti hypercholesterolemia agent by an in vitro assay, through the inhibition capacity of the HMG-CoA (3-hydroxy-3-methyl glutaryl-coenzyme A) reductase enzyme as a key component of cholesterol biosynthesis. Sample preparation started with soy protein isolation through acid precipitation and separated by centrifugation. The samples were analyzed the proximate content and hydrolyzed by papain enzyme at concentration 0.2% (w/v), for 0-6 hours and at 37, 50, and 55 °C. The protein hydrolysates were subsequently evaluated for hydrolysis degree (% DH), hydrolysates profile with SDS-PAGE (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis), and anti-cholesterol assay through HMG-CoA reductase inhibition tests. The sample with the highest inhibition activity was fractionated using gel filtration chromatography (Sephadex G-10) and the molecular weight of fractions was characterized by LCMS QTOF (Liquid Chromatography-Mass Spectrometry Quadrupole Time-of-Flight) for molecular weight determination. The results indicated the optimum hydrolysis conditions of soy protein isolates were obtained at 3 hours incubation, at 50 °C with DH 33.39% and the inhibition value was 95.65% (protein concentration 39.21 μg / mL). LCMS data showed the molecular weight of fractionated peptides were 1514 and 2029 Da. We assumed that both peptides have the same affinity as previous peptides in inhibiting HMG-CoA reductase.

Keywords: HMG-CoA reductase, hypercholesterolemia, papain hydrolysate, soy protein

INTRODUCTION. The presence of high-level cholesterol in the blood is a trigger for clogged arteries (atherosclerosis) and is further implicated in cardiovascular or coronary heart disease. This condition is a global threat and the number one cause of death worldwide. The report from American Heart Association (AHA) in 2017 shows over 17.8 million people die of heart and blood vessel disease, which is 21.1% higher than the data collected ten years ago (Virani et al., 2020). Meanwhile, a reduction in the consumption of fatty foods, performing physical exercises, combined with proper medication helps prevent and treat hypercholesterolemia. Moreover, there is a lack of substantive data to prove the direct relationship between high cholesterol levels and death. This disorder often results in atherosclerosis, stroke, and coronary heart disease (Csonk, Sárközy, Pipicz, Dux & Csont, 2016).

According to previous studies, cardiovascular risks, including coronary heart disease and stroke are increased by hypercholesterolemia (Ramdath Padhi, Sarfaraz, Renwick, & Duncan 2017; (Otvos et al., 2006). Furthermore, with regards to age distribution, cardiovascular is mostly diagnosed in people between 65-74 (3.6%) followed by 75 and above (3.2%), 55-64 (2.1%), and lastly 35-44 years old (1.3%), while according to economic status, this disease is common among the lower (2.1%) and middle-income class (1.6%) (Triallists, 2005).

Moreover, modern drugs, includinglovastatin, pravastatin, gemfibrozil, fenofibrate are used to lower triglyceride and cholesterol levels (Hoie, 2010). However, people prefer to use statins, resulting from the capacity to quickly lower cholesterol levels. According to Hippisley-Cox, & Coupland (2010), therapeutic applications in certain conditions trigger some side effects especially kidney failure, moderate/severe myopathy, cataracts, and liver dysfunction. The recommended dose in hypercholesterolemic patients is between 10 to 40 mg/day, and long-term usage has been implicated in myopathy, kidney failure, and liver damage (Lyons, & Harbinson, 2009), while excess dose and certain conditions increase the risk of developing type 2 diabetes (Huupponen, & Vikari, 2013). Previous studies have shown various herbs or food plants...
capable of reducing cholesterol levels with very minimal side effects.

Currently, there is less exploration of anti-cholesterol sources from natural food plants, where soybeans as one of the few potential resources. The research conducted a few decades ago identified beans as a plant-based food product with various antioxidants (Agaye, 2015) antimicrobial (Vasconcellos, Woiciechowski, Soccol, Mantovani, & Soccol 2014), and antihypertensive potentials (Shimakage, Shinbo & Yamaeda, 2012). Meanwhile, several types are also known to possess natural antioxidant-producing agents rich in phenolic compounds (Scalbert, Johnson, & Saltmarsh, 2005). Also, supplements manufactured from soy grains help to lower cholesterol levels and soy protein can diminish heart disease risks by reducing blood cholesterol or low-density lipoprotein (LDL) levels (Ramdath et al., 2017). The soybean peptides LPYP, IAVPGVEA, and IAVPTGVGA have been reported to effectively activate LDLR-SREBP 2 pathway and increase uptake of LDL in the blood, therefore inhibiting HMG-CoA reductase activity in HepG2 cells (Lammi, Zanoni, & Arnoldi, 2015). Furthermore, consuming diets low in saturated fat and cholesterol, including 25g of soy protein per day is estimated to reduce the risk of heart disease. Dietary upregulation of LDL-R transcription by soybean may be consequent to an enhanced catabolism or a reduced synthesis of intracellular cholesterol. Soy peptides can effectively stimulate LDL-R transcription in the human liver cell line and reduce blood cholesterol level (Cho, Juillerat, & Lee 2008).

The consumption of soy-based products has significantly reduced blood lipid content based on the composition. These include the protein isolates rich in isoflavones, soy fiber, cotyledons, and phospholipids (Anderson, & Hoie, 2005). However, the reduction mechanism resulting from the presence of soy protein remains unclear, hence the need for further investigation. The use of supplements manufactured from soy has produced substantial efficacy and tolerability. Besides, many of these products currently in the market are claimed to lower cholesterol levels, although there is less valid scientific data to support this claim. This study was conducted to explore the potency of soy protein hydrolysates as an anti hypercholesterolemia agent by an in vitro assay, through the inhibition capacity of the HMG-CoA reductase enzyme as a key component of cholesterol biosynthesis.

Hydrolysis was performed using an enzyme: substrate ratio of 0.2% (w/v) with variations in incubation times of 0, 1, 2, 3, 4, 5, and 6 hours at 37, 50, and 55 °C (Mutamimah, Ibrahim, & Trilaksani, 2018). The optimum treatment condition was determined based on dissolved protein content, degree of hydrolysis value, and anti-cholesterol activity. These were evaluated through an in vitro approach, using the enzyme HMG-CoA reductase. This is an important factor in determining cholesterol biosynthesis, especially for the formation of mevalonic acid from HMG-CoA, and also reduces blood cholesterol levels (Lyons & Harbinson, 2009); (Rinto, Putri, & Waktu, 2019). Furthermore, pravastatin was used as a positive control for HMG-CoA reductase inhibitors, which played a key role in suppressing cholesterol synthesis (Murphy, Deplazes, Cranfield, & Garcia, 2020). Consequently, the products’ potentials with bioactive peptides from enzymatic hydrolysis are determined and expected to be applied as an alternative, cheaper, and safer anti-cholesterol agent.

**EXPERIMENTAL SECTION**

**Raw Materials and Chemicals**

The samples used in the study were obtained from supermarkets in the South Jakarta area. BSA (bovine serum albumin) (Sigma Aldrich), hydrochloric acids, PBS (phosphate-buffered saline), trichloro acetic acid (TCA), sodium dodecyl sulfate (SDS), Lowry I and Lowry II solutions, HMG-CoA reductase (Sigma-Aldrich), HMG-CoA, NADPH, pravastatin (Sigma Aldrich), acrylamide solutions (30% T; 2.67 C) (Bio-Rad) bis-acrylamide, resolving buffer (Tris-HCl 1.5M pH 8.8 Bio-Rad); stacking buffer (Tris-HCl 0.5M pH 6.8 Bio-Rad); ammonium peroxosid sulfate (APS) 10%; N, N’,‘-tetramethylethylenediamine (TEMED) (Sigma Aldrich); running buffer Sigma; Coomassie blue R-250 staining solution (Bio-Rad), phospate buffer solution, methanol (MeOH) and acetonitrile (ACN) grade liquid chromatography-mass spectrometry (LC-MS) from Sigma-Aldrich. Formic acid (FA) was obtained from Grüssing (Filsum, Germany), Sephadex G-10; Medium Cas No.9050-68-4 (Sigma-Aldrich).

**Sample Preparation**

These legumes of soybeans were cleaned and blended in cold water, then precipitated with 1 M hydrochloric acid to obtain the hydroxylates. The mixture was further centrifuged at a speed of 12000 rpm for 15 minutes to produce protein precipitates. Therefore, dialysis was performed for 24 hours, before freeze-drying, and storage at 4 °C.

**Proximate Analysis**

The proximate content of soy protein isolates was analyzed the crude protein, crude fat, moisture, and ash contents (AOAC International, 2016).

**Measurement of Protein Content**

The measurement of protein precipitate levels was conducted with the Bradford method using BSA solution as a standard (He et al., 2015). This sample solution vortexed as the Bradford reagent was added and incubated at room temperature for 10 minutes. Furthermore, blue color with a 595 nm wavelength was produced, and the protein content was consequently extrapolated from the standard curve.
using the formula: \( y = ax + b \) where: \( y = \) absorbance, \( x = \) concentration.

**Hydrolysis of Soy Protein Isolates**

The papain enzyme was used in the enzymatic hydrolysis of soy protein isolates. This was performed using a 7.5 pH phosphate buffer at 0.1 M concentration, as well as temperatures of 37, 45, and 50 °C. The ratio of substrate used was 0.2% (w/v) with an incubation time of 0-6 hours (Mutamimah et al., 2018b). Furthermore, 5 mL of the mixture was obtained at intervals of 0, 1, 2, 3, 4, 5, and 6 hours during this process, then the degree of hydrolysis was measured. Subsequently, each hydrolysate mixture and the enzyme were inactivated by adding 0.5 M Tris-HCl with pH 8 and heated at 80 °C for 5 minutes.

**Determination of Hydrolysis Degree**

The degree of hydrolysis was determined using the SN-TCA method (Hoyle & Merritt, 1994). Approximately 5 mL of protein hydrolysate was added with 20 mL TCA 10% (w/v). This mixture was then allowed to settle for 30 minutes and centrifuged at a speed of 12000 rpm for 15 minutes. The supernatant protein content was determined with the Bradford method, and consequently calculated with the following formula:

\[
\text{N HCl} = \frac{\text{TCA dissolved protein 10\%}}{\text{the total protein sample}} \times 100\%
\]

**Anticholesterol Activity Assay** (Liang et al., 2015)

**Reagent Preparation**

The reagent used in this test contained an enzyme solution of HMG-CoA reductase dissolved in 550 µL buffer. This substrate along with NADPH was dissolved in 1.3 mL and 440 µL dH2O, respectively. Subsequently, all diluted reagents and a 10 mM pravastatin inhibitor were stored at -20 °C and room temperature respectively, before use.

**Sample Preparation and Measurement of Inhibition %**

The protein hydrolysate sample was prepared and filtered using a 0.45 µm membrane. Therefore, the filtrate was used as an inhibitor in the HMG-CoA reductase inhibition test with the help of an assay kit, where pravastatin was utilized as a positive control.

The % measurement was performed at an ELISA reader wavelength of 340 nm. Subsequently, reagents were added according to the details in Table 1.

Approximately 200 µL of each mixture was read at a wavelength (340 nm) every minute for 10 minutes. The enzyme activity was then calculated using the equation:

\[
\text{Unit/mg P} = \frac{(\Delta A340/\text{minsample} - \Delta A340/\text{minblank}) \times \text{TV}}{12.44 \times \text{V} \times 0.4 \times \text{LP}}
\]

**Analysis of SDS-PAGE Protein Hydrolysate**

The protein profile for soybean hydrolysates was determined using the SDS-PAGE method with a 7.5-17.5% resolving and 4% stacking gel solution in a buffer of 1.5 M Tris HCl at pH 8.45 (Laemmli, 1970). These samples were denatured with a buffer, including 1% Coomassie brilliant blue, 25% glycerol, 6.8 pH Tris-HCl 1M, 20% SDS, and boiled at 90 °C for 2 minutes where the ratio with protein was 1:1. The electrophoresis device was prepared using a resolving and stacking solution with a concentration of 1.5 M and 0.5 M at pH 8.8 and 6.8, respectively. The resulting sample was termed Tris-HCl. Subsequently, 1.5 % bisacrylamide and 48% acrylamide were added, and the electrophoresis process commenced for 55 minutes at a voltage of 150 volts with a Biorad protein marker, where the range of 7.7-204.0 kDa was compared. Also, 0.1% (w / v) dye solvent was used for protein staining and the process yield was washed using 7.5% acetic acid and 40% methanol solvent.

**Table 1.** Addition of anti-cholesterol activity test reagents

<table>
<thead>
<tr>
<th>Mixture</th>
<th>buffer (µL)</th>
<th>pravastatin (µL)</th>
<th>NADPH (µL)</th>
<th>HMG-CoA (µL)</th>
<th>HMGGR (µL)</th>
<th>Hydrolysate (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>184</td>
<td>-</td>
<td>4</td>
<td>12</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Negative control</td>
<td>182</td>
<td>-</td>
<td>4</td>
<td>12</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Positive control</td>
<td>181</td>
<td>1</td>
<td>4</td>
<td>12</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Sample</td>
<td>172</td>
<td>-</td>
<td>4</td>
<td>12</td>
<td>2</td>
<td>10</td>
</tr>
</tbody>
</table>
Table 2. Proximate content of soybean protein isolates

<table>
<thead>
<tr>
<th>Proximate composition</th>
<th>Soy protein isolate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude protein content</td>
<td>36.59±0.01</td>
</tr>
<tr>
<td>Crude fat content</td>
<td>10.24±0.26</td>
</tr>
<tr>
<td>Moisture content</td>
<td>8.22±0.06</td>
</tr>
<tr>
<td>Ash content</td>
<td>4.26±0.08</td>
</tr>
</tbody>
</table>

Figure 1. Soy protein isolate hydrolysis degree value at 37, 50, and 55 °C hydrolysis temperatures

Separation and Purification of Anti-Cholesterol Peptides from Hydrolysates

The soybean protein hydrolysate with the highest anti-cholesterol activity value of 15 mL was concentrated by freeze-drying. This was consequently fractionated using a G-10 Sephadex column to separate the peptide portion below 3 kDa. The molecular weight was then analyzed using an LCMS/MS QTOF Mass Analyzer (Li, Li, Chang, & Guo 2008).

Peptide Identification using LCMS QTOF Mass Analyzer

A total of 5 μL purified peptides were obtained and filtered using a 0.2 μm syringe filter then injected into the Analyzer. Furthermore, ionization was performed using ESI electrospray ionization mode. The ESI parameters used include Column C-18 with a 1.8 μm x 2.1 x 100 mm size, 0.2 mL/min flow rate, and the acetonitrile mobile phase. The process also utilized a 1: 1 v/v water, source voltage range of 4-50 volts, and a capillary temperature of 50 °C. The molecular weight of peptides was analyzed by mass spectroscopy, while the de-convolution calculations were conducted using ESI-prot online (www.bioprocess.org/esiprot/) (Zhou Guo, Shi, Jiang, & Wang 2016).

RESULTS AND DISCUSSION

Proximate content of Soy Protein Isolates

Table 2 shows the proximate data of soy protein isolates, indicating the presence of crude protein, crude fat, moisture, and ash contents. The proximate analysis results show a crude protein content of <50%. This low level was probably due to the lack of an optimal isolation process for soy protein, and most were assumed to be soluble and not precipitated. This study was performed using an imported variant with a possible difference in chemical composition from the local form. Furthermore, results from the moisture analysis and ash contents indicated a relatively low moisture range for the isolates. The highest value tends to make the microbes grow easier and trigger legume damage with mold. However, total ash content determination was conducted to establish the quality of soybean processing. This parameter represents the presence of important minerals, including organic and inorganic salts where a high amount consequently influenced the enzyme activity in food production.

Hydrolysis of Soy Protein Isolates

The hydrolysis of soy protein isolates was performed using the 0.2% (w/v) papain enzyme with incubation temperatures of 37, 50, and 55 °C.
in thickness to below 10 KDA over time. Furthermore, this outcome indicates the complete breakdown of glycinin and β-conglycinin present in soy protein isolates. However, the bands subsequently reappeared with lower intensity at 5-6 hours. This finding is in line with the results obtained during hydrolysis degree evaluation at 50 °C, where another decline in DH value was recorded 5-6 hours after the optimum condition was reached at 3 hours. Based on the soy protein isolate profile, an extended hydrolysis time using papain enzyme leads to more significant conversions of longer protein structures into shorter peptides.

The soybean protein is known to have a β-conglycinin fraction, consisting of α-β-conglycinin, β-conglycinin, and γ-β-conglycinin. These components possess molecular weights of 80.22, 48.42, and 46.24 kDa, respectively. Particularly, the single protein fraction termed glycinin is characterized by A-glycinin and B-glycinin, with molecular weights of 34-35 and 18-22 KDa, respectively (Barać et al., 2011). This glycinin molecule model is a hexamer comprising of five subunits, including Gly I (A1A B1B), Gly II (A2 B1B), Gly III (A1B B2), Gly IV (A4 B5 B3), and Gly V (A3 B4), where each has two polypeptide components, encompassing acid and base. Meanwhile, β-conglycinin is a glycoprotein present in soybean with a trimeric molecular model and is known to possess 3 subunit types, including the α, α, and β (Wang et al., 2014).

HMG-CoA Reductase Soy Protein Hydrolysate Inhibitory Activity

Figure 3 shows the results of the HMG-CoA reductase inhibitory activity test for soybean hydrolysates. The results showed the highest inhibition activity obtained at 50 °C (95.65%), with a concentration of 39.21 µg/mL. This was followed by treatments at 37 °C (78.29% with concentration of 83.78 µg/mL), and 55 °C (52.37% with concentration of 30.15 µg/mL).

The difference in HMG-CoA reductase inhibition was probably due to the relative difference of the number of peptide fragments produced in each hydrolysate. These phenomena can be seen from the results of the different electrophoretic band separations from each sample (Figure 2). According to Pak et al., (2005) soybeans hydrolysis using pepsin as a protease enzyme can produce anti-cholesterol activity, with a 45% inhibition value (concentration of protein 1 ppm).

Moreover, HMG-CoA reductase is a controlling enzyme in cholesterol biosynthesis (EC 1.1.1.88; NADPH dependent, EC 1.1.1.34) produced from the mevalonate pathway. The introduction of catalysts of this reaction facilitates the conversion of HMG-CoA to mevalonic acid and is considered the first step in cholesterol biosynthesis (Figure 4).
Figure 2. Soy protein hydrolysate electrophoregram (M = Marker, 0 = before hydrolysis, 1-6 = hydrolysis time 1-6 hours)

Figure 3. Percent inhibition of HMG-CoA reductase from soy hydrolysate

Figure 4. The formation reaction of mevalonic acid from HMG-CoA using HMG-CoA reductase enzyme and 2 molecules of NADPH through the transfer of 2 hydride ions. (Son et al., 2013)
The HMG-CoA reductase enzyme functions by catalyzing the HMG-CoA compounds into mevalonic acid, with the help of NADPH, estimated to serve as a precursor in cholesterol formation. Meanwhile, the inhibitors of this enzyme tend to effectively reduce cholesterol biosynthesis and are considered valuable hypolipidemic agents, therefore the drug of choice for atherosclerosis. In addition, pravastatin is a polyketide bioactive component synthesized by the microorganism Bacillus megaterium and is commercially used as one of the drugs to reduce cholesterol biosynthesis. The medication activity is executed by inhibiting the HMG-CoA reductase enzyme, due to the structural characteristics with close resemblance to HMG-CoA. However, the enzyme inhibition in the liver instigates an increase in hepatic LDL (low-density lipoprotein) receptor expression, which reduces plasma LDL cholesterol levels. Therefore, a large number of HMG-CoA reductase inhibitor studies use pravastatin as positive control while treating hypercholesterolemic disorders (McFarland et al., 2014).

Results of Soy Protein Hydrolysate Separation and Molecular Weight Analysis

The soy protein hydrolysate with the highest HMG-CoA reductase inhibitory capacity was purified and separated by gel filtration chromatography (Sephadex G-10). Figure 5 shows the fractionation result, and the 21st fraction reportedly produced the most significant absorption value, with an absorbance of 0.36. This yield is assumed to contain protein/peptide with a smaller molecular weight compared to others. In addition, further analysis was performed for molecular weight using the LCMS QTOF Mass Analyzer, and the results are shown in Figure 6.

![Figure 5. Fractionation results of soy protein hydrolysate (50 °C)](image)

![Figure 6. Chromatogram for the 21st fraction of LCMS QTOF-MS analysis results.](image)
Figure 6 showed the LCMS analysis results of the 21st fraction, and two dominant peaks with retention times of 4.82 and 5.53 minutes were obtained. Figure 7 demonstrated mass spectra with m/z data, while the molecular weight was analyzed using ESIprot online (www.bioprocess.org/esiprot/). Based on the deconvolution results using ESIprot online (www.bioprocess.org/esiprot/), the molecular weights of the peptide compounds were <3 kDa, at 1514 and 2029 Da, respectively. According to Lammi et al., (2015) a research by soybean hydrolysis using pepsin and trypsin enzymes produces peptides weighing 1178 Da and 1177 Da, correspondingly. Therefore, both yields were considered to be of similar mass. Hence, there is a tendency for identical inhibitory potentials against HMG-CoA reductase enzyme activity. Many soy peptides have been identified to lower cholesterol and triglycerides, and to suppress fat synthesis and storage in different experimental systems. LPYPR from the glycinin subunit of the soybean was one of the initial hypcholesterolemic peptides discovered by Yoshikawa et al., (2000). Two other cholesterol-lowering peptides derived from glycinin are IAVPGEVA and IAVPTGVA (Pak, Koo, Lee, Kim, & Kwon, 2005). Similar to LPYP, these peptides were shown to inhibit HMGR activity in cultured HepG2 cells and promote LDL uptake via the LDLR–SREBP2 pathway (Pak, Valeriy, Koo, Kwon, & Yun, 2012). Lammi et al., (2015) uncovered two hypocholesterolemic peptides—YVVNPNDEN and YVVNPDNNE derived from soy conglycinin, which also modulate cholesterol by an identical mechanism. However, further studies still needed to determine the peptide structure is required to ascertain this assertion.

CONCLUSIONS

The inhibitory activity of HMG-CoA reductase by soy protein hydrolysate from hydrolysis using papain enzyme has enormous potential as an anti-cholesterol agent. This product anticipated to serve as a replacement for statin drugs. Therefore, further in vivo testing and studies on peptide interactions with HMG-CoA enzyme is expected to influence future drug development.
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