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Anti-Hypertensive and Anti-Hypercholesterolemic Effects of Protein Hydrolysates from (*Phaseolus vulgaris)* L. in Functional Beverage

Yvonne D. Bolayo[1*], Mary Ann O. Torio[2]

[1]Department of Chemistry, Benguet State University, La Trinidad, 2601 Benguet, Philippines
[2]Institute of Chemistry, University of the Philippines Los Baños, Los Baños, 4030 Laguna, Philippines

ABSTRACT

The study aimed to formulate a functional beverage from common bean seeds, isolate the proteins and evaluate their anti-hypertensive and anti-cholesterolemic properties. White common beans (*Phaseolus vulgaris* L.) mature seeds were used to prepare the beverage. Proteins isolated from the beverage were subjected to digestion with pepsin and combined enzymes including trypsin, thermolysin, and chymotrypsin. The unhydrolyzed beverage and protein hydrolysates were subsequently tested for Angiotensin Converting Enzyme (ACE) inhibition and cholesterol micellar solubility inhibition. The results showed that both unhydrolyzed and hydrolyzed proteins exhibited blood pressure and cholesterol-lowering properties, with high ACE inhibition (77.60%) and cholesterol micellar solubility inhibition (27.38%). The formulated functional beverage from white common bean seeds has potential for preventing hypertension and hypercholesterolemia. This study offers a theoretical foundation for the formulation of functional beverages or bean-based food products by food companies.

Keywords: anti-hypertensive, anti-cholesterolemic, bioactive peptides, common beans, functional beverage

INTRODUCTION

In recent years, studies on functional foods from plant sources have increased dramatically because of their nutritional attributes and beneficial effects on health. Several studies focus on various food-derived proteins as sources of bioactive peptides that exhibit potent pharmacological properties and provide safety profiles to consumers (Antony & Vijayan 2021). Bioactive peptides have multifunctional properties that include immunomodulatory, antibacterial, antihypertensive, anticancer, antioxidative, antilipidic, anti-inflammatory, hypocholesterolemic, and the property of minerals binding (Angeles *et al.* 2021; Manzoor *et al.* 2022). Bound peptides must be released from their protein sources through enzymatic cleavage or fermentation to produce bioactivity (Tadesse & Emire 2020). Digestive enzymes such as trypsin, pepsin, or chymotrypsin produce peptides with varied chain lengths which may directly affect transportation to different tissues in order to elicit a favorable impact on the organism (Jakubczyk *et al.* 2017).

One of the most cultivated legumes in the Philippines particularly in Cordillera Administrative Region (CAR) is common beans. These sources of nutrients and bioactive compounds provide physiological advantages, reducing the susceptibility of developing type 2 diabetes and cardiovascular diseases, preventing various cancers, and managing some metabolic processes (Sparvoli *et al.* 2021). Chronic conditions significantly increase the expense of long-term medical treatment and are among the major causes of death worldwide (Chen *et al.* 2019). Seeds of common beans are rich sources of proteins, a class of macromolecule that serve an important role in the food industry as components which provide nutritional, functional, and sensory qualities to foods (Aryee *et al.* 2018). Bioactive peptides generated from plant proteins can be used for creating innovative functional foods, presenting a feasible alternative to peptides from proteins of animal sources (Lopes *et al.* 2023). Plant-based proteins have been recommended for their nontoxic characteristic, affordability, wide availability, and various biological functions (Fan *et al.* 2022).

[*]**Corresponding Author:** tel: +639503911493, email: y.bolayo@bsu.edu.ph
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Plant-based beverages have gained popularity as an alternative to traditional drinks due to several reasons, such as their health-promoting properties, bioavailability of active ingredients, sensory characteristics, and suitability for consumers with lactose intolerance and dairy milk allergies (Arbach *et al.* 2021; Aydar *et al.* 2023). Legumes are viable solution to a range of food industry challenges since they offer a promising protein source in plant-based diet (Aydar *et al.* 2023). There are various legume and cereal-based beverages in the market. However, for legumes, soybean milk is still dominant and studies on common bean beverages are scarce.

Bean-based beverages offer nutraceutical benefits and regular consumption by consumers should be encouraged. These could introduce a prospective market for health products from a low-cost sustainable source. At present, information on common bean beverage formulations and their nutraceutical properties is limited. The outcome of the current study will serve as a theoretical basis in-vitro for the investigation of innovative food development.

This research sought to formulate a functional beverage from common bean seeds and assess its potential for lowering blood pressure and cholesterol. Specifically, it isolated the protein from the formulated functional beverage, digested the protein, and evaluated the blood pressure and cholesterol-lowering activities of the hydrolyzed and unhydrolyzed proteins isolated from the formulated beverage.

METHODS

Design, location, and time

The in-vitro study was carried out at the laboratory of the Institute of Chemistry, University of the Philippines Los Baños, Philippines from January 2020 to February 2020.

Materials and tools

The common bean seed samples were obtained from local markets in Benguet, Philippines. For the identification process, the complete parts of the fresh plant samples, including the leaves, seeds and pods, were brought to the Museum of Natural History in the University of the Philippines Los Baños where they were identified as *Phaseolus vulgaris* L. Analytical grade chemicals used in the study were purchased from local distributors.

Procedures

Formulation of functional beverage. White common beans (*Phaseolus vulgaris* L.) mature seeds were the main ingredient in the preparation of functional beverage. The blending method with a few modifications was adopted from Afroz *et al.* (2016). About 100 g of dried mature seeds were soaked separately for four hours with 1 liter of warm water containing a small amount of sodium bicarbonate. Sodium carbonate was added to eliminate the bitterness and anti-nutritional factors. After manually removing the seed coats, the seeds were rinsed with distilled water. Dehulled seeds were then placed in a stone mill with 1 liter of hot water poured in. The seeds were ground and then filtered using a filter machine with cheesecloth. The filtrate was boiled over low/medium heat for ten minutes with constant stirring. After adding 45 g of sugar, the mixture was boiled for 2 more minutes. The prepared beverage was cooled to 70^{o}C before mixing one teaspoon of vanilla and was then set aside to cool to approximately 25^{o}C before being stored in a refrigerator set at 2^{o}C .

Isolation of protein from common bean beverage. Precipitation of proteins was conducted following the methodology developed by Hermanto *et al.* (2019), with some modifications. With stirring, 1.0 N NaOH was added to about 450 mL of the beverage to achieve a pH of 8.5. The solution was centrifuged at 5,600 x g for 20 minutes at 4^{o}C . The supernatant layer was decanted and 1.0 NHCl was stirred in to achieve a pH of 4.5. It was further centrifuged for another 20 minutes at 5,600 x g at 4^{o}C . The precipitate was collected and kept in a freezer.

Digestion of isolated protein. In order to imitate the digestion of proteins in human, enzymes pepsin, trypsin and chymotrypsin were employed. Pepsin, an aspartic protease in the stomach functions at optimum pH range between 1 and 2. Subsequently, during the digestion with pancreatic enzymes, such as trypsin, peptide bonds are cleaved after basic amino acids while chymotrypsin acts after aromatic amino acids (Fu *et al.* 2021). Peptides produced by thermolysin exhibited a high antihypertensive potential of peptides (Garcia *et al.* 2016) and was then used to enhance the production of bioactive peptide. The isolated protein sample was digested with pepsin using a 20:1 (v/v) substrate-to-enzyme ratio at pH 2.0 for one hour, and combined with enzymes

(trypsin, thermolysin, and chymotrypsin) to simulate the digestion that occurs in the digestive system except for thermolysin. Then, the mixture was placed in a water bath (Benchmark Scientific SB-12L Shaking Water Bath) at a temperature of 25°C. The digestion process was terminated by immersing the mixture in a boiling water bath for 5 minutes. Then, the mixture was promptly stored in a freezer at -2°C. Prior to sample utilization, the mixtures were separated in a centrifuge at 4°C for 2 minutes at 12,857 x g. The supernatant was collected and loaded onto a gel for subsequent electrophoresis.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970), with slight modifications, using an 8% stacking gel and a 10% resolving gel. The solutions were prepared by diluting with the extraction buffer to obtain the desired concentration. A buffer of 1:1 (v/v) sample to 2x Laemmli was prepared and placed in Eppendorf tubes. The solutions were then mixed using a vortex mixer and heated in a boiling water bath for 5 minutes. Then, the solutions were cooled to approximately 25°C.

About 20 µl of each sample was put into the prepared gel and allowed to migrate down the gel at 90 V using a Biorad Mini-PROTEAN® Tetra cell until the dye front was approximately 0.5 cm from the bottom of the gel. The resolving gel was carefully removed from the plates and placed in a container for staining overnight using Coomassie Brilliant Blue R-250. A solution consisting of 50% methanol and 10% glacial acetic acid was used to destain the gel.

Extraction of Angiotensin-Converting Enzyme (ACE) from pig's lungs. The extraction process was conducted according to the method outlined by Dumandan *et al.* (2014) with modification on solvent and buffer used. The ACE was extracted in the laboratory due to its cost-efficiency. Previous research has shown that it provides reliable results. Initially, the cleaned and minced fresh pig's lungs were suspended in acetone and then homogenized using blender. The resulting mixture was filtered and air dried to evaporate the solvent residue that could interfere with further extraction steps. Subsequently, the dried residue was defatted using hexane to remove the lipids or fats that could contaminate the protein. Thus, a more purified extract was produced. The sample was then air dried to eliminate the remaining solvent that could impede the ACE extraction and its activity. To extract the Angiotensin Converting Enzyme (ACE) from the dried powder, 100 mM sodium borate buffer (pH 8.3) was added and then incubated at 4°C for 3 hours. Afterwards, it was centrifuged at 11,000 x g for 60 min at 4°C. The reddish supernatant obtained was the ACE extract, which was stored at -20°C.

ACE inhibitory activity assay. The assay for ACE inhibitory property was measured based on the method of Dumandan *et al.* (2014) with modifications on buffer and quantity of chemicals used. Assay mixtures for the sample, control, and blank were prepared in three replications. Captopril, an ACE inhibitor drug used in the management of high blood pressure, was used as the positive control. Each sample assay mixture contained 25 µL of 0.1 M sodium phosphate buffer, 25 µL of 0.3 M NaCl, 50 µL of 5 mM Hippuryl-L-Histidyl-Leucine (HHL), and 50 µL of protein hydrolysates. The mixtures were pre-incubated for 5 minutes at 37°C. Meanwhile, the blank also contained similar amounts of solutions in the sample mixtures except that instead of protein hydrolysate, 0.1 M sodium phosphate buffer with volume of 50 µL was introduced. The control contained 25 µL of 0.1 M sodium phosphate buffer and 125 µL of 1 N HCl. To initiate the reaction, 25 µL of ACE extracted from pig's lungs was introduced to all mixtures, excluding the control. Subsequently, the mixtures were placed in shaker for 30 minutes at 37°C. To stop the chemical reaction, about 150 µL of 1 N HCl was added to the sample and blank assay mixtures, while 25 µL of ACE was allowed for the control. Hippuric acid was obtained through extraction process involving vigorous shaking of the mixtures with 750 µl of ethyl acetate using a vortex for 15 seconds, followed by centrifugation for 2 minutes at 3,600 x g. The upper layer was separated and the solvent was allowed to evaporate in a steam bath. A total of 500 µL distilled water was used to dissolve the extracted hippuric acid and the resulting mixture was vigorously mixed using a vortex mixer. The sample absorbance was at 228 nm.

ACE inhibition activity was determined using the following equation:

$$\text{Inhibitory activity (\%)} = \left(\frac{B-A}{B-C}\right) \times 100\%$$

Where A=Absorbance in the presence of both ACE, HHL and inhibitor

B=Absorbance of blank with ACE and HHL but without inhibitor

C=Absorbance of control

The protein concentrations used in the assay were 1.00 mg/mL for crude and 0.25 mg/mL for partially purified fractions. The concentration of the extract that inhibited 50% of the ACE activity was the IC_{50} value, which was determined by measuring the ACE inhibitory activity and derived from the inhibition graph plotted for the concentration of protein hydrolysates.

Cholesterol micellar solubility inhibition. Cholesterol micelles were generated as a model for the micelles found in the human gut by sonicating 10 mM sodium taurocholate, 0.4 M cholesterol, 1 mM oleic acid, and 132 mM NaCl in 15 mM sodium phosphate (pH 7.4). Assessing the amount of cholesterol that remains within micelles when agents that destabilize or displace micelles are present, is a significant basis for determining the efficacy of protein or peptide hydrolysates in cholesterol reduction (Zhang *et al.* 2012).

The cholesterol micellar solubility inhibition was assessed based on the method described by Zhang *et al.* (2012), with modification. Micellar solution was prepared by sonication using Omni Sonic Ruptor 400. The solution contained 10 mM sodium taurocholate, 0.4 mM cholesterol, 1 mM oleic acid, 132 mM NaCl and 15 mM sodium phosphate (pH 7.4). To each 400 µL of the sample, positive control (cholestyramine) and blank, 450 µL of the micellar solution was added. The samples were then incubated at 37°C for 24 hours. The mixtures were centrifuged at 37°C for 30 minutes at 18,845 x g, and supernatant was collected.

One hundred µL of glacial acetic acid was mixed with 80 µL of the supernatant, followed by the addition of 120 µL of a color reagent. The mixtures were then incubated at 37°C for 15 minutes. Standard solutions with varying concentrations from 0 mg/mL to 0.06 mg/mL cholesterol in glacial acetic acid were prepared. The absorbance of each solution was read at 560 nm using a UV-vis spectrophotometer (Thermo Scientific Multiscan GO, Finland). The generated calibration curve was used to determine the amount of cholesterol that remained in the solution. The following equation was used in the calculation of the cholesterol micellar solubility inhibition (Zhang *et al.* 2012):

$$\text{\% Cholesterol solubility inhibition} = [(Co-Cs)/Co]*100\ \%$$

Where: Co=Cholesterol concentration of original micelles

Cs=Cholesterol concentration of micelles with hydrolysates/positive control

Data analysis

All samples were analyzed in three replications. The analyses were repeated and experiments were conducted at least twice. ANOVA (one-way analysis of variance) was utilized to test the significance of differences ($p<0.05$) between conditions followed by Tukey's HSD test post hoc analysis. GraphPad Prism 6 was used for statistical analyses.

RESULTS AND DISCUSSION

Digestion of proteins

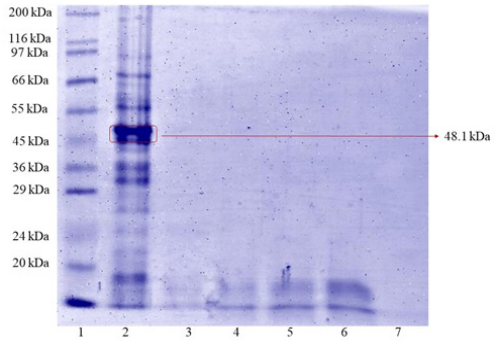
Cleavage specificity of proteolytic enzymes, degree of digestion, and stability of peptides during hydrolysis are crucial in the production of bioactive peptides (Mazorra-Manzano 2018). Liberated peptides may comprise 2 to 20 amino acids, and with small molecular weight, their assimilation capability into the small intestine is enhanced, increasing their ability to elicit physiological responses (Saad *et al.* 2021).

In the study, total protein isolated was digested with pepsin and then followed by a combination of enzymes: trypsin, thermolysin and chymotrypsin, sequentially. Pepsin is an endopeptidase that cleaves peptide linkages on the amino-terminal side of aromatic amino acids like tyrosine, phenylalanine, and tryptophan. Trypsin acts on the carboxyl end of basic amino acids arginine and lysine. In contrast, chymotrypsin acts on the carboxyl terminal of amino acids specifically leucine and aromatic amino acids in peptide chains (Mangussad *et al.* 2021). Thermolysin, a zinc-dependent protease, catalyzes the hydrolysis at the hydrophobic residues including leucine, isoleucine, valine, and phenylalanine (Nielsen *et al.* 2019). This in vitro analysis does not exactly mimic the digestion process of food that occurs in digestive tract of human because interactions with other components and other physiological factors were not included. Thus, the observed result

may not demonstrate the inherent bioactive peptides' stability against proteases present in gastrointestinal tract (Chew *et al.* 2019).

Electrophoretic profile of formulated functional beverage protein. The electrophoretogram from SDS-PAGE analysis of the functional beverage from common bean is illustrated in Figure 1. Densitometric analysis of the unhydrolyzed protein showed multiple bands visible in Lane 2, ranging from 26.8 kDa to 180.6 kDa. The most prominent fraction of protein had a molecular weight of 48.1 kDa, with the highest band volume at 11.4%. This protein band may correspond to a subunit of a phaseolin, a vicilin-like 7S globulin which consists of subunit polypeptides that have molecular weights ranging from 43–54 KDa (Saad *et al.* 2021) and have isoelectric points within pH range of 5.6 to 5.8. The 7S globulin is the most predominant protein in Phaseolus legumes seeds that accounts for the 40%–60 % total protein content (Sathe 2016).

The disappearance of bands corresponding to functional beverage protein and the presence of faint low molecular weight bands (17.1–24.7 kDa) after hydrolysis of functional beverage suggest that the proteins extracted were degraded (Lanes 3–7). After 24 hours of combined enzyme digestion, the bands completely disappeared indicating that peptides were liberated from their parent protein. According to data from Saad *et al.* (2021), phaseolin is resistant to enzymatic hydrolysis, unlike vicilin. In the case of vicilin, the bands completely disappear. Various factors, such as temperature, hydrolysis conditions, and enzymes, have an impact on protein binding and changes in molecular weight.



(1) Protein profile of molecular weight marker; (2) Unhydrolyzed crude protein of functional beverage protein; (3) 1-hour peptic digest: (4) 1-hour combined enzymes digest; (5) 6-hour combined enzymes digest; (6) 12-hour combined enzymes digest, and; (7) 24-hour combined enzymes digest

Figure 1. SDS-PAGE of formulated functional beverage and hydrolysates from *Phaseolus vulgaris* L

Previous research conducted by Tagliazucchi *et al.* (2015) on cooked 'Pinto' beans showed similar findings on the existence of faint protein bands and their absence after digestion with specific enzymes. Cooking of the beans is a thermal treatment that enhanced the degree of in comparison to uncooked beans, making them more susceptible to enzymatic hydrolysis.

ACE inhibitory property of functional beverage. The formulated functional beverage protein hydrolysates of the common bean beverage have exhibited ACE inhibition (Table 1). Significantly, the highest ACE inhibition (77.60%) was obtained at the optimum hydrolysis time of 6 hours, suggesting that the peptides demonstrating ACE inhibitory property were liberated consistently at 6-hour hydrolysis time although compared to the positive control, this was noticeably lower. The findings are consistent with those of Gao *et al.* (2019) who observed that hydrolysis time influences the ACE inhibitory

Table 1. ACE inhibitory property of hydrolyzed and unhydrolyzed crude protein (1.0 mg/mL) of functional beverage formulated frommature seeds of *Phaseolus vulgaris* L.

Crude protein from functional beverage formulated from *Phaseolus vulgaris* L. and its hydrolysates	% ACE inhibition*	Std. Dev
Functional beverage (unhydrolyzed)	55.68^{e}	0.26
Peptic digest (1 hour)	73.65^{c}	0.73
1-hour combined enzymes digest	68.93^{d}	1.97
6-hour combined enzymes digest	77.60^{b}	1.76
12-hour combined enzymes digest	66.82^{d}	0.75
24-hour combined enzymes digest	58.89^{e}	1.73
Captopril (positive control)	85.43^{a}	0.20

*All data represent the means of three measurements. According to Tukey's HSD test, there are significant differences between letter superscripts at $p<0.05$, after conducting a one-way ANOVA with a 95% confidence interval

activity of cottonseed protein digests. They observed that ACE inhibition increased up to the 6th hour and then decreased with extended time. This could be associated to the degradation of peptides and the loss of their bioactivity (Mao *et al.* 2007).

The findings revealed that unhydrolyzed protein beverage exhibited 55.68% ACE which is significantly lower compared to the peptic digest (73.65%) and to the combined enzymes digest ranging from 68.93% to 77.60 at different time intervals. This suggests that in the unhydrolyzed beverage, the peptides remain bound in the protein resulting in reduced efficacy. On the contrary, the utilization of two or more enzymes has been found to improve the effectiveness of hydrolysates as opposed to a single enzyme (Luna-Vital *et al.* 2015).

The finding of this study indicates that 1-hr peptic digest (73.65%) is higher than the result of Hermanto *et al.* (2019), who observed the ACE inhibitory activity of soymilk pepsin hydrolysates ranging from 24.2%–58.3% obtained over a period duration of 0 to 4 hours. However, they observed the highest ACE inhibition activity of 79.31% of soymilk pepsin hydrolysates after 48 hours, which is comparable to the highest value obtained in this study at 6-hour combined digest. Despite the variations in the source of peptides, enzymes, and hydrolysis period, this may imply that the peptides with maximum activity were generated.

Furthermore, the size of peptide components could potentially contribute to variability in the ACE inhibition of protein hydrolysates. This indicates that small-sized peptides present in the protein hydrolysates possess a more bioactive property than the larger peptide components found in unhydrolyzed protein. In a study on black soybean glutelin hydrolysates by Zhang *et al.* (2019), smaller peptides that have less than 3 kDa molecular weight demonstrated a greater ACE inhibitory activity than peptides exceeding 3 kDA in size. The synergistic effect of component peptides plays important role in enhancement of biological activities of peptides (Shao *et al.* 2023). Consequently, the peptides formed during hydrolysis exhibit interactions that result in increased ACE inhibitory activity. Differences in strength of ACE reduction property discussed in previous studies could also be ascribed to the peptide length and proportion of hydrophobic amino acids, peptides molecular weight, and the existence of branched-chain amino acids such as Leucine (L), Isoleucine (I) and Valine (V) (Xu *et al.* 2021; Zheng *et al.* 2020). Although the unhydrolyzed beverage and all hydrolysates did not surpass the ACE suppression of the positive control, captopril, an ACE inhibitor prescribed to control hypertension, this signifies as promising sources of antihypertensive peptides.

ACE Inhibitory IC$_{50}$. The IC_{50} of the processed bean drink hydrolysates is 0.20 mg/ml at 6-hour digestion using combined enzymes. This value for the formulated drink is lower than that of fresh milk (IC_{50}=1.18 mg/mL) observed by Chen *et al.* (2007), indicating a higher ACE inhibitory property of the formulated drink after a 6-hour sequential hydrolysis. This also has a higher value, suggesting lower ACE inhibitory activity in comparison to the value obtained in IC_{50} of processed soya milk with 0.26 µg/mL and 8.75 µg/mL regular soya milk (Tomatsu *et al.* 2013).

ACE is a constituent of Renin-Angiotensin-Aldosterone System (RAAS) and it regulates blood pressure and maintains balance of water and electrolytes in the body (Jakubczyk *et al.* 2017). Angiotensin I, a decapeptide derived from angiotensinogen, is converted to angiotensin II, a potent vasoconstrictor, through the action of ACE. In addition, ACE deactivates bradykinin, a substance with vasodilatory property that also influences aldosterone production. Thus, suppressing this enzyme contributes to the lowering of blood pressure.

Cholesterol micellar solubility inhibition of functional beverage. The micelle generated with sodium taurocholate performs similar mechanisms in the solubilization of cholesterol in human digestion. The cholesterol lowering property of protein could be evaluated by determining the amount of cholesterol that remains in the micelles in the presence of micelle-destabilising or micelle-displacing agent (Zhang *et al.* 2012).

Table 2 shows the cholesterol lowering properties of proteins from the formulated functional beverage. The obtained results reveal that hydrolyzed proteins including pepsin (24.31%) and coupled enzymes (ranging from 19.71%–27.38%) have significantly higher inhibitory effects than unhydrolyzed proteins (14.89%). This suggests that peptides with biological activities should be liberated from

proteins' primary sequences to facilitate their effect. Regardless of the enzyme, hydrolysis greatly enhances functionality (Dent & Maleky 2023).

The 6-hour combined digest of the functional beverage exhibited the highest inhibitory activity (27.38%) for cholesterol micellar solubility; although this was noticeably lower than the control, cholestyramine (60.45%). This could be related to the peptides' low concentration released during digestion. Moreover, the inhibitory activities were lesser than the value reported by Marques *et al.* (2015) in cooked cowpea beans (39.8%), indicating that cooking has enhanced the release of peptides that can suppress cholesterol solubility.

During digestion, dietary cholesterol interacts with bile acids to create micelles. This interaction enhances the solubility and absorption of cholesterol in the intestines. Consequently, formation of micelle can be suppressed to reduce cholesterol (Chen *et al.* 2021). In bovine milk, β-lactoglobulin tryptic hydrolysates (Ile-Ile-Ala-Glu-Lys) were observed to suppress the incorporation of cholesterol into the micelles. This regulates the absorption of cholesterol in the jejunum (Nagaoka *et al.* 2001). Cholesterol not incorporated into the micelles forms aggregates and is unable to be absorbed. As a result, it travels into the colon to be expelled in the feces as organic matter (Zhang *et al.* 2012). This may be because of the composition and amino acid sequence in peptides. For instance, amino acids with hydrophobic properties bind competitively with bile acid and can modify the structure of cholesterol, therefore disrupting cholesterol micelle formation and limiting the absorption of exogenous cholesterol (Chen *et al.* 2021).

Table 2. Cholesterol micellar inhibitory property of hydrolyzed and unhydrolyzed crude protein (0.05 mg/mL) of functional beverage formulated from mature seeds of *Phaseolus vulgaris* L.

Crude protein from functional beverage formulated from *Phaseolus vulgaris* L. and its hydrolysates	% Cholesterol micellar inhibition*	Std. Dev
Functional Beverage (unhydrolyzed)	14.89^{e}	0.76
Peptic Digest (1 hour)	24.31^{bc}	1.00
1-hour combined enzymes digest	21.25^{cd}	2.28
6-hour combined enzymes digest	27.38^{b}	1.65
12-hour combined enzymes digest	19.71^{d}	0.76
24-hour combined enzymes digest	22.12^{cd}	1.00
Cholestyramine (positive control)	60.45^{a}	1.00

*All data are the means of three measurements. Tukey's HSD test with one-way ANOVA at a 95% confidence interval reveals significant differences between letter superscripts of means at $p<0.05$

Notably, the 6-hour combined enzyme demonstrated the highest % cholesterol micellar inhibition compared to the unhydrolyzed beverage and single enzyme peptic digest. This could be related to the combination of enzymes used in digestion which demonstrated synergistic effect enhancing the generation of bioactive peptides (Akbarian *et al.* 2022). Protein digests bioactivities could also be affected by the proteases used, enzyme/substrate ratio, processing conditions, sequences of peptides, and amino acids generated during hydrolysis due to specificity of enzymes (Mangussad *et al.* 2021; Nasri 2017).

The protein hydrolysates produced through enzymatic digestion compete with cholesterol in forming cholesterol micellar mixture and interacting with bile salts. Polar peptides may interact with micellar hydrophilic bile salts and consequently reduce solubility of micellar cholesterol, whereas nonpolar peptides bind with cholesterol, impeding cholesterol solubilization into the micelles (Jiang *et al.* 2020). The disrupted formation of micellar cholesterol solution leads to cholesterol aggregation which could be eliminated from gastrointestinal tract via feces (Upadhyay *et al.* 2021).

CONCLUSION

Phaseolus vulgaris L. could be exploited for the development of functional beverages. The protein hydrolysates from the formulated functional beverage of common bean seeds can potentially prevent hypertension and hypercholesterolemia. Understanding the properties of proteins derived from common beans is essential for incorporating the proteins

as ingredients in the processing of highly nutritious beverages or bean-based food products. Furthermore, in vivo studies are needed to explore more detail mechanism of *Phaseolus vulgaris* L.

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DECLARATION OF CONFLICT OF INTERESTS

The authors declared that they have no conflict of interest.

REFERENCES

Afroz MF, Anjum W, Islam N, Kobir A, Hossain K, Sayed A. 2016. Preparation of soymilk using different methods. Journal of Food and Nutrition Sciences 4(1):11–17. https://doi.org/10.11648/j.jfns.20160401.13

Akbarian, M, Khani, A, Eghbalpour S, Uversky VN. 2022. Bioactive peptides: Synthesis, sources, applications, and proposed mechanisms of action. Int J Mol Sci 23(3):1445. https://doi.org/10.3390/ijms23031445

Angeles JGC, Villanueva JC, Uy LYC, Mercado SMQ, Tsuchiya MCL, Lado JP, Angelia MRN, Bercansil-Clemencia MCM, Estacio MAC, Torio MAO. 2021. Legumes as functional food for cardiovascular disease. Appl Sci 11(12):5475. https://doi.org/10.3390/app11125475

Antony P, Vijayan R. 2021. Bioactive peptides as potential nutraceuticals for diabetes therapy: A comprehensive review. Int J Mol Sci 22(16):9059. https://doi.org/10.3390/ijms22169059

Arbach CT, Alves IA, Serafini MR, Stephani R, Perrone ÍT, de Carvalho da Costa J. 2021. Recent patent applications in beverages enriched with plant proteins. NPJ Science of Food 5(1):28. https://doi.org/10.1038/s41538-021-00112-4

Aydar EF, Mertdinç Z, Demircan E, Çetinkaya SK, Özçelik B. 2023. Kidney bean (*Phaseolus vulgaris* L.) milk substitute as a novel plant-based drink: Fatty acid profile, antioxidant activity, in-vitro phenolic bio-accessibility and sensory characteristics. Innov Food Sci Emerg Technol 83:103254. https://doi.org/10.1016/j.ifset.2022.103254

Aryee ANA, Agyei D, Udenigwe CC. 2018. Impact of processing on the chemistry and functionality of food proteins. In Proteins in food processing. Woodhead Publishing. https://doi.org/10.1016/B978-0-08-100722-8.00003-6 (page 27–45).

Chen GW, Tsai JS, Pan BS. 2007. Purification of angiotensin I-converting enzyme inhibitory peptides and antihypertensive effect of milk produced by protease-facilitated lactic fermentation. Int Dairy J 17(6): 641–647. https://doi.org/10.1016/j.idairyj.2006.07.004

Chen Y, Zhang H, Liu R, Mats L, Zhu H, Pauls KP, Tsao R. 2019. Antioxidant and anti-inflammatory polyphenols and peptides of common bean (*Phaseolus vulga* L.) milk and yogurt in Caco-2 and HT-29 cell models. Journal of Functional Foods 53:125–135. https://doi.org/10.1016/j.jff.2018.12.013

Chen GW, Lin HTV, Huang LW, Lin CH, Lin YH. 2021. Purification and identification of cholesterol micelle formation inhibitory peptides of hydrolysate from high hydrostatic pressure-assisted protease hydrolysis of fermented seabass byproduct. Int J Mol Sci 22(10):5295. https://doi.org/10.3390/ijms22105295

Chew LY, Toh GT, Ismail A. 2019. Application of proteases for the production of bioactive peptides. In Enzymes in food biotechnology 247–261. Academic Press. https://doi.org/10.1016/B978-0-12-813280-7.00015-3

Dent T, Maleky F. 2023. Pulse protein processing: The effect of processing choices and enzymatic hydrolysis on ingredient functionality. Crit Rev Food Sci Nutr 63(29):9914–9925. https://doi.org/10.1080/10408398.2022.2070723

Dumandan NG, Angelica MRN, Belina-Aldemita MD, Torio MAO. 2014. Extraction and characterization of bioactive peptides derived from the hydrolysates of total soluble proteins of pistachio nuts (*Pistacia*

vera L.). Kimika 25(1):1–10. https://doi.org/10.26534/kimika.v25i1.1-10

Fan H, Liu H, Zhang Y, Zhang S, Liu T, Wang D. 2022. Review on plant-derived bioactive peptides: Biological activities, mechanism of action and utilizations in food development. J Foods 2(2):143–159.

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. Biol Chem 402(7) 861–867. https://doi.org/10.1515/hsz-2020-0386

Gao D, Zhang F, Ma Z, Chen S, Ding G, Tian X, Feng R. 2019. Isolation and identification of the Angiotensin-I Converting Enzyme (ACE) inhibitory peptides derived from cottonseed protein: optimization of hydrolysis conditions. Int J Food Prop 22(1):1296–1309. https://doi.org/10.1080/10942912.2019.1640735

García MC, Orellana JM, Marina ML. 2016. Chapter 11 - Novel Applications of Protein by-Products in Biomedicine. In Protein Byproducts. 1st Edition. Cambridge (USA): Academic Press.

Hermanto S, Septiana A, Putera DK, Hatiningsih F, Muawanah A. 2019. ACE inhibitory and antioxidative bioactive peptides derived from hydrolyzed soy milk. Molekul 14(1):56–63. http://dx.doi.org/10.20884/1.jm.2019.14.1.506

Jakubczyk A, Karaś M, Złotek U, Szymanowska U. 2017. Identification of potential inhibitory peptides of enzymes involved in the metabolic syndrome obtained by simulated gastrointestinal digestion of fermented bean (*Phaseolus vulgaris* L.) seeds. Food Res Int 100:489–496. https://doi.org/10.1016/j.foodres.2017.07.046

Jiang X, Pan D, Zhang T, Liu C, Zhang J, Su M, Wu Z, Zeng X, Sun Y, Guo Y. 2020. Novel milk casein–derived peptides decrease cholesterol micellar solubility and cholesterol intestinal absorption in Caco-2 cells. J Dairy Sci 103(5):3924–3936. https://doi.org/10.3168/jds.2019-17586

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227(5259):680–685. https://doi.org/10.1038/227680a0

Lopes C, Ferruccio CA, de Albuquerque Sales AC, Tavares GM, de Castro RJS. 2023. Effects of processing technologies on the antioxidant properties of common bean (*Phaseolus vulgaris* L.) and lentil (*Lens culinaris*) proteins and their hydrolysates. Food Res Int 172:113190. https://doi.org/10.1016/j.foodres.2023.113190

Luna-Vital DA, Mojica L, de Mejía EG, Mendoza S, Loarca-Piña G. 2015. Biological potential of protein hydrolysates and peptides from common bean (*Phaseolus vulgaris* L.): A review. Food Res Int 76:39–50. https://doi.org/10.1016/j.foodres.2014.11.024

Mangussad D, Sucgang AT, Clemencia MCM, Manalo M, Uy LY, Torio MA. 2021. Isolation and characterization of the total protein in 'lakatan' banana (*Musa acuminata* Colla) with bioactive peptides exhibiting antioxidative and antihypertensive activities. Philipp Agric Sci Philipp 104(3).

Manzoor M, Singh J, Gani A. 2022. Exploration of bioactive peptides from various origin as promising nutraceutical treasures: In vitro, in silico and in vivo studies. Food Chem 373:131395. https://doi.org/10.1016/j.foodchem.2021.131395

Mao XY, Ni JR, Sun WL, Hao PP, Fan L. 2007. Value-added utilization of yak milk casein for the production of angiotensin-I-converting enzyme inhibitory peptides. Food Chem 103(4):1282–1287. https://doi.org/10.1016/j.foodchem.2006.10.041

Marques MR, Freitas RAMS, Carlos ACC, Siguemoto ÉS, Fontanari GG, Arêas, JAG. 2015. Peptides from cowpea present antioxidant activity, inhibit cholesterol synthesis and its solubilisation into micelles. Food Chem 168:288–293. https://doi.org/10.1016/j.foodchem.2014.07.049

Mazorra-Manzano MA, Ramírez-Suarez JC, Yada RY. 2018. Plant proteases for bioactive peptides release: A review. Crit Rev Food Sci Nutr 58(13):2147–2163. https://doi.org/10.1080/10408398.2017.1308312

Nagaoka S, Futamura Y, Miwa K, Awano T, Yamauchi K, Kanamaru Y, Tadashi K, Kuwata T. 2001. Identification of novel hypocholesterolemic peptides derived

from bovine milk β-lactoglobulin. Biochem Biophys Res Commun 281(1):11–17.

Nasri M. 2017. Protein hydrolysates and biopeptides: Production, biological activities, and applications in foods and health benefits. A review. Adv Food Nutr Res 81:109–159. https://doi.org/10.1016/bs.afnr.2016.10.003

Nielsen NV, Roedel E, Manna D, Etscheid M, Morth JP, Kanse SM. 2019. Characterization of the enzymatic activity of the serine protease domain of Factor VII Activating Protease (FSAP). Scientific Reports 9(1):18990. https://doi.org/10.1038/s41598-019-55531-x

Saad AM, Sitohy MZ, Ahmed AI, Rabie NA, Amin SA, Aboelenin SM, Soliman MM, El-Saadony MT. 2021. Biochemical and functional characterization of kidney bean protein alcalase-hydrolysates and their preservative action on stored chicken meat. Molecules 26(15):4690. https://doi.org/10.3390/molecules26154690

Sathe SK. 2016. Beans: Overview, reference module in food science. In Encyclopedia of Food Grains. 2nd Edition. Cambridge (USA): Academic Press.

Shao B, Huang X, Xu M, Cheng D, Li X, Li M. 2023. Peptides isolated from black soybean synergistically inhibit the activity of Angiotensin Converting Enzyme (ACE). J Funct Foods 106:105604. https://doi.org/10.1016/j.jff.2023.105604

Sparvoli F, Giofré S, Cominelli E, Avite E, Giuberti G, Luongo D, Predieri S. 2021. Sensory characteristics and nutritional quality of food products made with a biofortified and lectin free common bean (*Phaseolus vulgaris* L.) flour. Nutrients 13(12):4517. https://doi.org/10.3390/nu13124517

Tadesse SA, Emire SA. 2020. Production and processing of antioxidant bioactive peptides: A driving force for the functional food market. Heliyon 6(8).

Tagliazucchi D, Martini S, Bellesia A, Conte A. 2015. Identification of ACE-inhibitory peptides from *Phaseolus vulgaris* after in vitro gastrointestinal digestion. Int J Food Sci Nutr 66(7): 774–782. https://doi.org/10.3109/09637486.2015.1088940

Tomatsu M, Shimakage A, Shinbo M, Yamada S, Takahashi S. 2013. Novel angiotensin I-converting enzyme inhibitory peptides derived from soya milk. Food Chem 136(2):612–616. https://doi.org/10.1016/j.foodchem.2012.08.080

Upadhyay SK, Torio MAO, Lacsamana MS, Diaz MGQ, Angelia MRN, Sucgang AT, Uy LYC. 2021. Introduction of the hypocholesterolemic peptide, LPYPR, to the major storage protein of mung bean [*Vigna radiata* (L.) Wilczek] through site-directed mutagenesis. Int Food Res J 28(3):527–537. https://doi.org/10.47836/ifrj.28.3.12

Wongsa P, Yuenyongrattanakorn K, Pongvachirint W, Auntalarok A. 2022. Improving anti-hypertensive properties of plant-based alternatives to yogurt fortified with rice protein hydrolysate. Heliyon 8(10). https://doi.org/10.1016/j.heliyon.2022.e11087

Xu Z, Wu C, Sun-Waterhouse D, Zhao T, Waterhouse GI, Zhao M, Su G. 2021. Identification of post-digestion Angiotensin-I Converting Enzyme (ACE) inhibitory peptides from soybean protein Isolate: Their production conditions and in silico molecular docking with ACE. Food Chem 345:128855. https://doi.org/10.1016/j.foodchem.2020.128855

Zhang H, Yokoyama WH, Zhang H. 2012. Concentration-dependent displacement of cholesterol in micelles by hydrophobic rice bran protein hydrolysates. J Sci Food Agric 92(7):1395–1401. https://doi.org/10.1002/jsfa.4713

Zhang Y, Zhang Y, Chen P, Shu F, Li K, Qiao L, Chen Z, Wang L. 2019. A novel angiotensin-I converting enzyme inhibitory peptide derived from the glutelin of vinegar soaked black soybean and its antihypertensive effect in spontaneously hypertensive rats. J Biochem 166(3):223–230. https://doi.org/10.1093/jb/mvz029

Zheng Y, Wang X, Zhuang Y, Li Y, Shi P, Tian H, Li X, Chen X. 2020. Isolation of novel ACE-inhibitory peptide from naked oat globulin hydrolysates in silico approach: Molecular docking, in vivo antihypertension and effects on renin and intracellular endothelin-1. J Food Sci 85(4):1328–1337. https://doi.org/10.1111/1750-3841.15115