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Research Article

Antioxidant properties of common bean (*Phaseolus vulgaris*) husk-derived peptides

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Abstract
Protein hydrolysates and plant peptide extracts can become next-generation natural, eco-friendly food supplementary with a wide range of bioactive features. From folk medicine, it is known about the aqueous extract of the common bean (*Phaseolus vulgaris*) and its exceptional treatment features. Various abilities of peptides from *P.vulgaris* have been closely studied in recent years, but another component of "bean pod tea" – husk hydrolysates has not been studied at all. Therefore, this study aimed to obtain and perform a primary analysis of peptides from common bean husks using two methods-acidic hydrolysis and perchloric acid extraction. The first method was based on the hydrolysis of protein-rich plant extracts by acetic acid, allowing to obtain hydrolysis-derived peptides, while extraction by perchloric acid resulted in retrieving of native endogenous peptides. Using Spectrophotometry and size-exclusion chromatography, the study showed that the perchloric acid extraction method allows the extraction of peptides with MW 205-590 Da, which have moderate OH-scavenging activity. Peptides obtained by acidic hydrolysis (192-610 Da) had significantly higher levels of DPPH-scavenging and FRAP activities (14 ± 0,68 % and 27 ± 1,12 % respectively). Therefore, peptides from P. vulgaris bean husk have antioxidant activity, and to elaborate on these findings, the antimicrobial and inhibitory activities of these peptides should be tested in future studies.

Keywords: Antioxidant activity, Bean husk hydrolysate, Peptides, *Phaseolus vulgaris*

INTRODUCTION

In recent decades, close attention has been paid to bioactive peptides as potential novel candidates for therapeutic purposes. Special role in this class of compounds is the peptides from plant sources since they are cheap, animal component-free and do not raise any ethical, religious or socio-cultural questions. Many researches prove the existence of plant-derived peptides that are antimicrobial, antioxidant, angiotensin-potentiating, antidiabetic and have many other activities (Fan *et al*., 2022). Many of these plant sources for bio-

active peptides have been known as medicinal plants in traditional medicine in many cultures. For example, hot aqueous extract of common bean (*Phaseolus vulgaris*) pods (or bean pod tea) is famous in folk medicine mostly for its antidiabetic, but also anti-obesity, antioxidant and many other properties (Helmstädter, 2010; Rivai *et al*., 2020).

Peptides obtained from legumes, especially common beans, are well-characterized, including their biological activities, amino acid sequences, and even potential receptors through *in silico* molecular docking methods (de Fátima Garcia *et al*., 2021; Hu *et al*., 2023). However, the other part of bean pods – pod husks- was not considered a source of peptides because they are inedible to humans. The husks have their pros as cheap, practically free plant material, which may contain interesting compounds with bioactive properties and, therefore, are interesting from the biotechnological point of view (Tassoni *et al*., 2020). Thus, the present research aimed to obtain the peptides from common bean husks and characterize them because of their potential bioactivity. Moreover, the different methods of isolating peptiderom plant material generate various types of peptides (Cruz-Casas *et al*., 2021). The present study aimed to obtain a kind of peptide by acidic hydrolysis and another one by extraction of native non-hydrolyzed peptides present in bean husk cells and compare their characteristics and properties.

MATERIALS AND METHODS

Obtaining bean husk extracts

The husks of common beans (P. vulgaris) grown in one of the fields in the Kyiv region, Ukraine, were used as the starting material in the experiment. About 132 g of dried powdered bean husks were mixed with 1 l of boiling distilled water and left tightly closed for 15 minutes, after which this mixture was cooled, filtered and centrifuged at 1000 g for 10 min. The supernatant was collected and lyophilized, producing nearly 8 grams of freeze-dried material.

Acidic hydrolysis and peptide extraction

Peptide extraction was achieved by adding 250 ml of 1 M acetic acid to 8 g of lyophilized bean husk extract with consequent hydrolysis for 1 hour at constant mixing. After that, the solution was heated to 100 °C, boiled for 1 hour and left to cool to room temperature. Next, the suspension was centrifuged at 5,000 rpm for 45 minutes, and the supernatant was lyophilized. Dialysis procedure was performed to remove non-protein impurities and lyophilized the obtained fraction again.

Extraction of middle-mass molecules

The procedure of extraction of middle-mass molecules (MMMs) or native non-hydrolyzed peptides was conducted according to the protocol described elsewhere (Gunas *et al*., 2023). Aqueous solution of bean husk extract was mixed with 1.2 M $HClO_4$ in 1:1 proportion and left for 30 min at +4°C, and centrifuged at 1,500 g for 15 min afterwards. To obtain the supernatant, a few droplets of 5 M KOH were added to neutralize it to pH 7.0. After 15 minutes at room temperature, the sample solution was centrifuged again and to obtain supernatant, 80-% ethyl alcohol was added in 1:5 proportion, left for 15 minutes at room temperature and centrifuged again with the same parameters as before. The supernatant was diluted with distilled water in 1:1 proportion, frozen and then lyophilized.

Determination of peptide concentration

The concentration of peptides was determined spectrophotometrically at a wavelength of 254 nm against the blank probe containing no peptides. Calculations were performed according to the calibration curve, using various concentrations of N-carboxy- glycyl-glycine and determining its concentration at 254 nm.

Size-exclusion chromatography

Determination of peptide molecular masses was conducted using the size exclusion chromatography method, as described by Rieder *et al. (2021)*.

Examination of antioxidant activities

Total antioxidant activity was measured using 2,2-Diphenyl-1-picrylhydrazyl (DPPH)-reducing possibilities of obtained peptide fractions (Rice-evans *et al*., 1995). Peptide samples were prepared by dissolving 0.5 mg lyophilized peptides in 1 ml of distilled water, 0.05 ml of peptide samples were mixed with 0.2 ml of 1 mM DPPH ethanol solution. The reaction mixture was left at room temperature for 30 minutes in complete darkness, and after that, its optical density was measured Spectrophotometrically at wavelength 517 nm. Blank samples were prepared by mixing peptide samples with corresponding volumes of ethanol solution. Control samples contained an ethanol solution of DPPH and 0.05 ml of distilled water. A 5 mM ascorbic acid solution was used as a reference antioxidant compound.

NO-inactivation activity was measured using Sodium nitroprusside (SNP). 0.1 ml of peptide samples (0.5 mg/ml) was mixed with 0.2 ml of 10mM SNP in PBS buffer (pH=7.4). This mixture was incubated for 120 min in the dark room. Blank samples contained peptide samples and the corresponding volume of PBS. Control samples contained 0.2 ml of 10mM SNP in PBS buffer and 0.1 ml of distilled water. After incubation, from each reaction mixture, 0.1 ml, added 0.1 ml of 10-% Griess reagent was and left in a dark room for 30 minutes. Afterwards, the optical density of samples was measured Spectrophotometrically at wavelength 546 nm.

The OH-scavenging potential of our peptides was examined using the method described by Prieto and colleagues (Prieto *et al*., 1999), and some modifications were made. To 0.05 ml of peptides samples (0.5 mg/

ml), 0.075 ml of 0.066 mM 1,10-phenanthroline was added in sodium-phosphate buffer (pH=7.4), 0.075 ml of 0.066 mM $FeSO_4$ and 0.05 ml of 0.05 % hydrogen peroxide. Reaction mixture was incubated for 1 hour at +37 ^oC, and after that, its optical density was measured at wavelength 546 nm. Control samples contained distilled water instead of peptide solution, and the blank sample contained sodium-phosphate buffer instead of phenanthroline.

The ferric-reducing antioxidant power (FRAP) of peptides was examined according to the method described by Jayaprakasha and colleagues (Jayaprakasha *et al*., 2001). Peptide sample (0.05 ml, 0.5 mg/ml) was mixed with 0.5 ml of 1-% potassium ferrocyanide solution and 0.045 ml of 0.2 M PBS buffer. This mixture was incubated for 20 minutes at +50 ^oC, mixed with 1 ml of 10-% trichloroacetic acid solution and centrifuged for 10 minutes at 3,000 g. To 0.01 ml of supernatant aliquots, an equal volume of distilled water and 0.002 ml of $Fe_2(SO_4)_3$ solution were added, measuring the mixture's optical density at 500 nm afterwards. Control samples contained a corresponding volume of distilled water instead of a peptide solution.

Total antioxidant, NO-inactivating, OH-neutralizing and reducing activities were calculated according to the formula:

$$\text{Antioxidant activity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100; \quad \text{Eq. 1}$$

$A_{control}$– absorption of the control sample, A_{sample}– absorption of peptide sample; all probes' optical densities were measured against the blank sample.

Statistical analysis

All the samples were prepared and measured in triplicates, and the obtained results were expressed as mean ± SEM (M ± m). Differences between two peptide groups were determined using one-way analysis of variances (ANOVA) via OpenEpi online software and considered statistically significant *p<0.05.

RESULTS AND DISCUSSION

Bioactive peptides have been obtained from a huge amount of various plant sources, including agricultural wastes. Protein- and peptide-rich sources include nuts, seeds, and kernel cakes, but other parts of plants are also being investigated for bioactive peptides' presence (Durand *et al*., 2021). The research tried to shed light on bioactive peptides from common bean husks, which had not been described before. Furthermore, the study tried to obtain these peptides by two different methods – acidic hydrolysis, generating acid hydrolysate peptides (AHPs) as products of proteolysis (meaning these peptides aren't present in living cells), and extraction of MMMs, specific peptide fraction, the molecular mass of which does not exceed 5 kDa (Gunas *et al*., 2023), representing native peptides, which are present in living cells and maintain homeostasis functions. Results of the peptide extraction efficiency and yields are shown in Table 1.

According to Table 1, a higher yield of peptides from protein-rich extract of ben husks can be obtained using the MMMs-extraction method – about 87% more peptides retrieved, compared to acid hydrolysis, meaning MMMs extraction protocol is more efficient for this type of plant material. The results of size-exclusion chromatography revealed the number of obtained peptide fractions and their masses - using the acidic hydrolysis method, the study obtained 5 main fractions with the masses 610,65 Da, 436,29 Da, 347,92 Da, 239,05 Da and 192,04 Da. The middle mass-molecule mixture contained 6 fractions: 590,25 Da, 321,41 Da, 300,36 Da, 266,79 Da, 239,97 Da and 205,29 Da. Considering that, on average 1 amino acid weighs 110 Da, the peptides were estimated to contain 2 to 5 amino acid residues. At the same time, fractions with masses lower than 210 kDa can be single amino acids (for example, the mass of Tryptophan is 204.2 Da). Other methods, such as mass-spectrometry, should be used to determine the exact sequence. According to other studies, peptides containing from 2 to 7 amino acid residues possess high bioactive properties; for example, dipeptides from common bean extracts were found to exert ACE-inhibitory, DPP-IV-inhibitory and antioxidant properties both in recent studies and *in silico* modelling (de Fátima Garcia *et al*., 2021).

Moreover, low-molecular-weight peptides can be easily absorbed through the intestinal wall and act as inhibitors of α-amylase and α-glucosidase, enhancers of pancreatic beta-cell proliferation and insulin secretion, which can be helpful for patients with carbohydrate metabolism disturbances.

The next main goal was to examine the antioxidant properties of obtained peptides. Antioxidant activity is quite often found in peptide fractions obtained from

Table 1. Comparative efficiency of *P. vulgaris,* bean husk peptide extraction methods

Husk peptide Extraction method	Volume of extract, ml	Dry-mass of extract, g	Optical density at 254 nm	Peptide concentration in protein-rich extract, mg/ml
AHPs (Method 1)	300±14	4,51±0,2	0,55±0,03	12,15±0,6
MMMs (Method 2)	1130±52 *	7,70±0,4 *	1,03±0,04 *	22,76±1,1 *

* – p < 0,05, comparing to Method 1: Acid hydrolysate peptides (AHPs); Middle-mass molecules (MMMs)

Table 2. Comparison of antioxidant activities of *P. vulgaris,* bean husk peptides, obtained by different methods

Antioxidant activity	AHPs (Method 1)	MMMs (Method 2)	Ascorbic acid (reference compound)
DPPH-scavenging, %	14 ± 0,68	9 ± 0,42*	89 ± 3,96
OH-scavenging, %	7 ± 0,31	18 ± 0,87*	-
NO-inactivating, %	6 ± 0,27	8 ± 0,35	-
FRAP activity, %	27 ± 1,12	16 ± 0,73*	71 ± 3,45

* – p < 0,05, comparing to Method 1: Acid hydrolysate peptides (AHPs); Middle-mass molecules (MMMs)

various plant sources (Olivares-Galván *et al*., 2020), including common bean seeds (Ohara *et al*., 2021). Therefore, it was quite possible that the present peptide fractions also possessed antioxidant abilities. First, to determine whether the peptides have such abilities, total antioxidant activity was analysed using the DPPH-scavenging method. Obtained results are shown in Table 2, and according to them, both peptide fractions have antioxidant potential, not as high as ascorbic acid (strong antioxidant). Yet, it is a solid result, considering results from similar studies. Due to a variety of oxidants, the present study used a couple of methods to describe peptides' antioxidant abilities more precisely. Thus, it was tried to analyze OH-scavenging, NO-inactivating and FRAP activity; the results of the present experiment are shown in Table 2.

According to the present results, NO-inactivating activity in the peptides is not expressed much; both fractions showed nearly equal low potential. Neutralizing of -OH radicals was better achieved by MMMs, with more than 2 times higher activity, compared to AHPs. However, the most promising results are those retrieved from the FRAP activity assay - both MMMs and AHPs showed surprisingly decent activity levels compared to the activity of such strong antioxidants as ascorbic acid.

The DPPH-scavenging activity achieved by the peptides in this experiment was higher than that previously obtained from other plant sources. For example, in one study, the best result of common bean enzymatic hydrolysate was 50.96 ± 1.87 % at peptide concentration 10 mg/ml(Ohara *et al*., 2021). In another study, peptides from bitter beans (*Parkia speciosa*) showed 78.48 ± 3.16% DPPH-scavenging activity, but also at a concentration of 10 mg/ml (Muhialdin *et al*., 2020).

OH-radical is one of the most dangerous reactive oxygen species; it is involved in oxidizing many molecules inside the cell, causing great damage to its integrity (Gülçin, 2012). In present experimental conditions MMMs performed better in OH-neutralizing activity, and compared with the results of other studies, the present study suggests an average level of OH-scavenging activity. For example, 18.79 % OH-scavenging activity of sweet potato protein hydrolysate (0.5 mg/ml) (Zhang *et al*., 2014), and in another study, 50-% OH-scavenging activity was achieved by black soybean peptides at concentration 1,7 mg/ml (Haiwei, 2010).

Reducing activity, or redox-potential, is a compound's ability to donate electrons and reduce oxidant molecules within the cell. In this experiment, the present peptides showed a prominent level of FRAP activity, considering their concentration in a reaction mixture of only 0.5 mg/ml. Significant reducing activity of AHPs is most likely related to their amino acid sequence and mass. Some authors report that higher reducing activity is observed in peptides with higher molecular weight containing Cys, Lys, Ile and Pro in their sequences (Ohashi *et al*., 2015; Sonklin *et al*., 2018).

According to many previous studies, peptides from extracts and hydrolysates of common beans have many bioactive properties. This study shows that the antioxidant potential of P. vulgaris bean husk peptides supports previous findings. Moreover, these very peptides can also have ACE-inhibitory, antimicrobial, and antifungal properties and act as inhibitors of α-amylase and α-glucosidase. To confirm these assumptions, it is suggested that corresponding assays be conducted. Furthermore, determining the amino acid sequence of these peptides will let us predict their potential bioactive sites of action more precisely.

Conclusion

Because of the bioactive properties of peptides from plant sources became an objective of scientific research. From the biotechnological and commercial point of view, the most interesting sources for peptides can be agricultural residues, such as bean husks. The present study obtained crude peptide fractions using two methods- i) acidic hydrolysis and ii) perchloric acid extraction by comparing their composition and antioxidant activities. The perchloric acid extraction method showed a higher yield of peptides with masses 205-590 Da with moderate OH-scavenging and FRAP activities. At the same time, peptides obtained by acidic hydrolysis (610-192 Da) showed better DPPH-scavenging and relatively high FRAP activities, suggesting that both methods work well for the production of peptides with bioactive properties. Amino acid sequence determination assessment of antimicrobial and inhibitory properties of these peptides will lay the basis for future studies.

Conflict of interest

The authors declare that they have no conflict of interest.

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