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Antioxidant and anitoperoxidative effect of polypeptides from common beans (*Phaseolus vulgaris*, cv BRS Pontal)

Efeito antioxidante e anitoperoxidativo dos polipéptidos do feijão comum (*Phaseolus vulgaris*, cv BRS Pontal)

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Brazilian Journal of Development

ABSTRACT
Common beans have high content of protein and can be used as source for obtaining bioactive peptides which are compounds that exhibit an effect on body functions or conditions and may influence human health. This study was undertaken to examine the antioxidant and antiperoxidative potential of naturally-occurring peptides from easy-to-cook (ETC) and hard-to-cook (HTC) beans (*Phaseolus vulgaris*, cv BRS Pontal). The extracted proteins were partially purified using ultrafiltration membranes. Thereafter, the antioxidant activity of the produced fractions was analyzed by DPPH and FRAP methods. The lipid peroxidation was measured by thiobarbituric acid reactive substances (TBARS) analysis. Results evidenced that the antioxidant potential was highly improved after ultrafiltration, especially for those fractions containing molecules with low molecular weight. Peptide fractions (F3-10kDa) from ETC beans exhibited a better ability to inhibit the reactive oxygen species formation when compared to the synthetic antioxidant BHT, which corroborates their antioxidant role and protection against lipid peroxidation. Regarding the peptide fractions from HTC beans, the samples had similar lipid peroxidation to BHT in the tested concentration range (200 to 600μg). The HTC phenomenon did not seem to affect definitively the bioactivity of the bioactive peptides, suggesting that it is possible to use these components as an alternative for the use of the grains affected by the hardening process.

Keywords: *Phaseolus vulgaris*, easy-to-cook, hard-to-cook, bioactive properties

RESUMO
O feijão comum tem um elevado teor de proteínas e pode ser utilizado como fonte para a obtenção de peptídeos bioactivos que são compostos que exibem um efeito sobre as funções ou condições corporais e que podem influenciar a saúde humana. Este estudo foi realizado para examinar o potencial antioxidante e antiperoxidativo dos peptídeos naturais de feijões fáceis de cozer (ETC) e difíceis de cozer (HTC) (Phaseolus vulgaris, cv BRS Pontal). As proteínas extraídas foram parcialmente purificadas utilizando membranas de ultrafiltração. Posteriormente, a actividade antioxidante das fracções produzidas foi analisada pelos métodos DPPH e FRAP. A peroxidação lipídica foi medida por análise de substâncias reactivas ao ácido tiobarbitúrico (TBARS). Os resultados evidenciaram que o potencial antioxidante foi altamente melhorado após a ultrafiltração, especialmente para as fracções que continham moléculas com baixo peso molecular. As fracções de peptídeo (F3-10kDa) dos feijões ETC demonstraram uma melhor capacidade de inibir a formação de espécies de oxigénio reactivas quando comparadas com o antioxidante sintético BHT, o que corrobora o seu papel antioxidante e protecção contra a peroxidação lipídica. Relativamente às fracções de peptídeo do feijão HTC, as amostras apresentavam peroxidação lipídica semelhante ao BHT na gama de concentrações testadas (200 a 600μg). O fenómeno do HTC não parecia afectar definitivamente a bioactividade dos peptídeos bioactivos, sugerindo que é possível utilizar estes componentes como alternativa para a utilização dos grãos afectados pelo processo de endurecimento.

Palavras-chave: *Phaseolus vulgaris*, fácil de cozer, difícil de cozer, propriedades bioactivas

1 INTRODUÇÃO

Free radical formation occurs continuously in the cells during the electron transfer processes in the normal cell metabolism as consequence of both enzymatic and nonenzymatic reactions. However, the concentration of these radicals may increase due to enhanced intracellular generation or the deficiency of antioxidant mechanisms (Silva & Jasiulionis, 2014).

Brazilian Journal of Development

This imbalance between oxidant and antioxidant molecules results in the induction of cellular damage, among them the oxidation lipid from membranes, leading to loss of fluidity and increased permeability, with consequent releasing nutrients and toxic substances from cell to the extracellular space; formation of cytotoxic products, such as malonaldehyde (MDA); and even cell rupture, resulting in cellular apoptosis (Lobo *et al*., 2011). In addition, this redox imbalance may be associated with health disorders which can lead to diseases such as atherosclerosis, cancer, inflammatory and degenerative diseases (Lemes *et al*., 2016).

The redox balance of cells by eliminating free radicals produced in excess and present in the body becomes necessary. For this purpose, antioxidant substances produced by the body or absorbed from the appropriate diet may be used (Zhao *et al*., 2014). In this sense, the discovery of new potentially antioxidant substances, mainly of natural occurrence, has been the focus of several studies, highlighting the bioactive peptides.

Bioactive peptides are compounds that exhibit an effect on body functions or conditions and may influence human health. They differ widely in their amino acid composition, chemical structure and, therefore, in their biological function. These compounds frequently have cholesterol-lowering effects, besides antiprotozoal, antiviral, antithrombotic, antioxidant, antihypertensive and antimicrobial activities, which make them attractive for application to foods and pharmaceuticals (Lemes *et al*., 2016).

Peptides can naturally occur in raw food materials exerting their physiological action directly or in an encrypted form, in which the bioactive molecule is inactive or in latent form within the sequence of the protein, and can be released and activated by proteolysis or several other techniques (Lemes *et al*., 2016).

Naturally occurring peptides can be found in several food sources, especially those with a high protein composition, such as beans (*Phaseolus vulgaris*), in which seeds are found 20-30% protein in dry mass basis (Roterman *et al*., 2017).

Several bioactivities have been identified in peptides from common beans which include anti-inflammatory properties, anticancer, metal chelating, inhibition of angiotensin-converting enzyme, antihypertensive and antioxidant (Luna-Vital *et al*., 2014).

Another important feature of beans is the fact that the overall quality and acceptability of these grains can be affected during inadequate storage at elevated temperatures (≥ 30 ºC) and humidity (≥ 75%) resulting in the phenomenon known as *Hard-to-cook* (HTC), characterized mainly by changes in flavor, texture and increase of cooking time (Batista *et al*., 2010; Siqueira et al., 2016). It is estimated that 500 thousand tons of beans per year are not used as human food due to hardening (Lopes *et al*., 2012).

Brazilian Journal of Development

Therefore, the aim of this study was to investigate the occurrence of naturally-occurring peptides in seeds of common beans (*Phaseolus vulgaris*, cv BRS Pontal), with antioxidant and antiperoxidative property, and evaluate if these activities are affected by the development of the "Hard-to-cook" phenomenon.

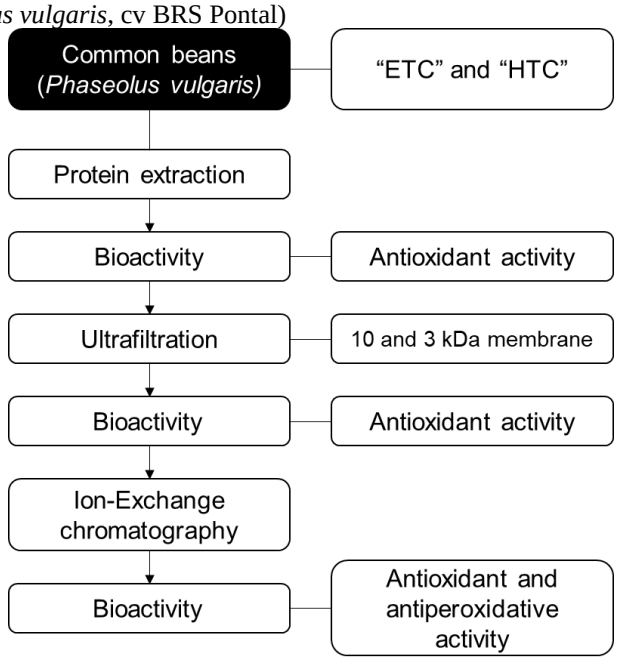
2 MATERIAL AND METHODS

The strategy used to obtain purified peptides with antiperoxidative activity from "ETC" and "HTC" common beans (*Phaseolus vulgaris*, cv BRS Pontal) was based according to the flowchart shown in Figure 1.

2.1 MATERIALS

Seeds of common bean (*Phaseolus vulgaris*, cv BRS Pontal) were provided by EMBRAPA Rice and Beans (Santo Antônio de Goiás, Goiás, Brazil). After removing impurities, the grains were stored at 4ºC for experiments and analysis. The hardening was carried out by incubating the grains at 40ºC ± 2°C and 75% of relative moisture for 120 days (Ribeiro *et al*., 2005). The grains were manually dehulled and the whole beans were ground to produce a flour with a particle size equivalent to 32 mesh. The common bean flour was stored at -20ºC until processing.

Figure 1. Flowchart of obtaining partially purified peptides with vasorelaxant, antiperoxidative and antioxidant activity from common beans (*Phaseolus vulgaris*, cv BRS Pontal)



Brazilian Journal of Development

2.2 PROTEIN EXTRACTION

Proteins and peptides were extracted with acetonitrile, water and formic acid in the proportion of 25:24:1 as described by Mahatmanto *et al*. (2014). 5 mL of solvent were added to 1 g of flour (ETC and HTC bean) and the mixture was stirred at 25ºC for 1 h. After extraction, organic solvents were evaporated and the protein concentrates were frozen and lyophilized for further analysis. The protein content was determined using the Qubit® Protein Assay Kit, following the manufacturer instructions.

2.3 FRACTIONATION BY ULTRAFILTRATION

The ultrafiltration runs were carried out in a stirred dead-end cell with 400 mL using 10 and 3 kDa ultrafiltration membranes (regenerated cellulose Millipore®). The system was operated at 1.5 kgf/cm^2 and 4ºC with a suitable pH (Lemes *et al*., 2014). Fractionation of components was conducted using membranes in a consecutive manner. The protein content and antioxidant activity of the feed, retentate and permeate were assayed for each batch to determine the purification factor and process efficiency. The fractions were named F>10kDa; F3-10kDa and F<3 kDa.

2.4 BIOLOGICAL ACTIVITY

2.4.1 DPPH radical scavenging activity

The determination of DPPH scavenging activity was carried out based on the method described by Brand-Williams, et al (1995). An aliquot of 50 µL of sample was mixed with 200 µL of 150 mmol L^{-1} DPPH radical. The mixture was homogenized and incubated at 25 ºC for 15 min and then the absorbance against blank was measured at 520 nm, using an Epoch microplate spectrophotometer (Biotek Instruments, VT, USA). A Trolox calibration curve was prepared in the range from 0 to 300 mmol L^{-1}, and the inhibition of oxidative process obtained for the sample was interpolated to calculate the concentration in Trolox equivalents (mmol L^{-1} TE mg^{-1} protein).

2.4.2 Ferric reducing antioxidant power (FRAP)

The FRAP assay was performed according to the method described by Benzie and Strain (1996). In the first round, the FRAP reagent was prepared as a mixture of 2.5 mL of 0.01 mol L-1 TPTZ (2,4,6-tris(2-pyridyl)-s-triazine) in 0.04 mol L-1 HCl solution and 2.5 mL of 0.02 mol L-1 FeCl3 in 25 mL of 0.1 mmol L-1 acetate buffer, pH 3.6. An aliquot of 1.3 mL of freshly prepared FRAP reagent was warmed at 37 ºC for 10 min and mixed with 50 µL of sample and 150 µL of deionized water. The mixture was incubated at 37 ºC for 30 min and the absorbance was determined

at 595 nm. In the FRAP assay, the antioxidant potential of the sample was determined from a standard curve plotted using Trolox. FRAP values were expressed as mmol L-1 TE per mg of protein.

2.4.3 Lipid peroxidation

Lipid peroxidation activity was determined according to the method described by Liu & Ng (2000). The rat brain was dissected and homogenized with a homogenizer in ice-cold phosphate buffer (50 mM, pH 7.4) to produce a 1/10 (w/v) homogenate. The homogenate was centrifuged at 10,000 *g* for 15 min at 4°C. The supernatant was used as liposome for an in vitro lipid peroxidation assay. The ability of peptides in the fraction F3-10kDa to inhibit lipid peroxidation was studied by incubating rat brain homogenates treated with 10 µM hydrogen peroxide and different concentrations from 200 to 600 µg of F3-10kDa. Hydrogen peroxide induced lipid peroxidation in the rat brain homogenates. Lipid peroxides reacted with thiobarbituric acid to form a pink product measurable at 532 nm. The difference between the control and peptides fraction was measured by the decrease in thiobarbituric acid reacting substances formation (TBARS), reflecting reduced hydroxyl radical-induced lipid peroxidation. Butylated hydroxytoluene (BHT) was used as the reference standard for comparison.

2.5 STATISTICAL ANALYSIS

The statistical analyses were performed using *Graphpad Prism*® 5.0 software (GraphPad Software, Inc.) and the data were expressed as mean ± standard deviation. Analysis of variance (one-way ANOVA) followed by t-Student test were used to compare the results. Differences were considered significant when $p<0.05$.

3 RESULTS AND DISCUSSION

3.1 PROTEIN EXTRACTION

The crude extract obtained from ETC and HTC beans presented a protein content of 0.89 ± 0.03 and 0.97 ± 0.06 mg mL^{-1}, respectively (Table 1). These values are lower than those obtained by Batista et al. (2010) and León et al. (2007), since these authors used methods for extraction of total hydrophilic proteins. Because of its medium to high polarity, the combination of acetonitrile, water and formic acid in the solvent solution is capable of extracting proteins and peptide compounds with different degrees of hydrophobicity, with the presence of residues loaded on its surface (Carrasco-Castilla et al., 2012). In addition, the resulting extractive solution simultaneously alters the solvation layer and the isoelectric point of proteins and peptides. The acetonitrile decreases the dielectric constant of the extractive solution, which alters the polarity of the extract, causing the increase of the

Brazilian Journal of Development

solubility of proteins and peptides with lower polarity (Rendina, 1971). Although this method did not present higher protein and peptide content in relation to the other commonly used protein extraction methods, it is effective for the extraction of peptides with bioactivity (Huang et al., 2005; Kessel & Ben-Tal, 2018).

Table 1 - Protein content and antioxidant activity determined in the crude extract and fractions from ETC and HTC beans

Samples	Protein content (mg mL^{-1})[1]		Antioxidant activity (mmol L^{-1} TE mg^{-1} protein)[1]			
			DPPH assay		FRAP assay	
	ETC	HTC	ETC	HTC	ETC	HTC
Crude extract	0.89±0.03^{b}	0.97±0.06^{a}	17.8±0.20^{a}	15.2±1.08^{b}	11.3±0.57^{a}	6.2±0.24^{b}
Fractions						
F>10kDa	3.9±0.04^{a}	4.3±0.01^{a}	0.0±0.0^{a}	0.0±0.0^{a}	3.7±0.29^{a}	3.3±0.22^{a}
F<10kDa	0.49±0.04^{a}	0.71±0.07^{b}	22.2±0.09^{a}	19.2±0.19^{b}	62.0±1.59^{a}	48.1±3.15^{b}
F3-10kDa	2.1±0.14^{a}	1.25±0.14^{b}	18.1±0.1^{b}	20.8±0.1^{a}	158.6±0.0^{b}	200.3±0.0^{a}
F<3kDa	0.83±0.04^{a}	0.79±0.01^{a}	20.3±0.1^{a}	19.0±0.2^{b}	140.7±2.8^{a}	134.1±0.4^{b}

1 The means were compared by t-student test and considered significantly different when $p<0.05$.

3.2 ANTIOXIDANT ACTIVITY

The DPPH and FRAP methods were used to evaluate the antioxidant activity of the extracted proteins. As can be seen in Table 1, despite the higher content of proteins extracted from HTC beans, the hardening process negatively affected their antioxidant activity, being observed a reduction of about 15% in the antioxidant potential against DPPH and 45% for the antioxidant activity by FRAP method.

The fractions obtained by ultrafiltration, especially those with components with low molecular weight, presented high antioxidant activity. The peptides from the fraction F<3kDa showed an antioxidant activity varying from of 134 to 141 mmol L^{-1} TE mg^{-1} protein, whereas the fraction with molecular weight between 3 and 10kDa had an antioxidant activity ranging from 159 to 200 mmol L^{-1} TE mg^{-1} protein (Table 1).

Regardless the method used to determine the antioxidant activity, the fractions obtained by ultrafiltration showed higher antioxidant activity than the crude extract. This result can be due to the reduction of steric hindrance caused by the presence of bulky proteins in the crude extract and consequent exposure of electron-dense amino acid side chain groups, such as polar or charged moieties, which are also related to antioxidant activity (Silva et al., 2017). These results also corroborate the idea that antioxidant activity is determined mainly by the presence of peptides extracted from the beans.

It is known that the methods for determination of antioxidant activity have different stereo-selectivity radical, where amino acids and peptides found in the sample are able to react and stabilize different radicals (Zhu et al. 2008). It is also known that the methods have different stoichiometric

Brazilian Journal of Development

reactions that occur among the antioxidant compounds found in the protein source and the radicals DPPH and Fe^{3+}, depending on the nature of the radicals and their solubility and diffusivity in the system (Zhu et al., 2008; Lemes et al., 2016).

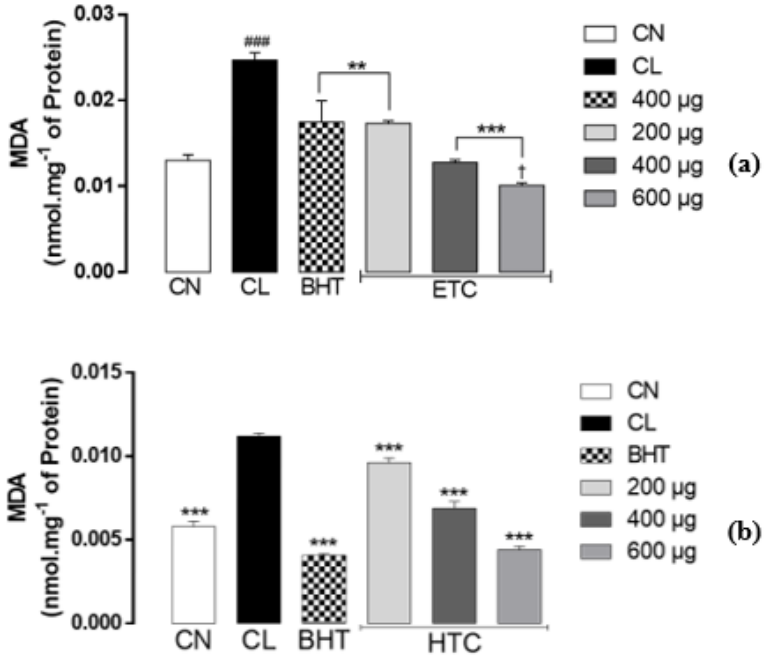
Other factors that directly interfere on the protein and peptides extraction and consequently in the antioxidant activity may be related to the difference observed in the values obtained, such as the solvent molecular structures, their functional groups, and the type of surface amino acids in the protein and peptides structure (Ramakrishnan et al., 2016; Ungcharoenwiwat and H-Kittikun, 2015).

It is important to note that the amount of protein extracted seems not to be responsible for the antioxidant activity, but the type of amino acid obtained, which act as donors of protons to stabilize free radicals (Mahatmanto et al., 2014). Another important factor for the antioxidant activity is related to the operational conditions applied to isolate these molecules and also the structure of the obtained peptides (Vernaza et al., 2012; Hernandez-Ledesma et al., 2009).

3.2 LIPID PEROXIDATION

Considering that the fractions F3-10KDa showed best results for antioxidant activity, they were chosen for the evaluation of the antiperoxidative evaluations. The lipid peroxidation was measured by thiobarbituric acid reactive substances (TBARS) analysis. The TBARS method has been widely used to determine the degree of lipid peroxidation inhibition. The Figure 2 shows the influence of adding peptide fractions from ETC and HTC common beans on the lipid oxidation.

Figure 2. Malondialdehyde (MDA) levels from lipid peroxidation in rat brains using peptides fractions from "ETC" (a) and "HTC" (b) common beans; (a) ### p<0.001 vs. CN (vehicle); ** p<0.01 vs. CL (hydrogen peroxide); *** p<0.001 vs. CL; † p<0.05 vs. BHT; (b) p<0.001 vs. CL.



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The results of the present study, mainly from ETC and HTC fractions, show that peptides fraction present ability to inhibit the MDA formation. Regarding to the fraction F3-10KDa from ETC fraction, the results revealed that using 200 µg of peptide the total amount of MDA formation was similar to the control using butylated hydroxytoluene (BHT). In addition, increases in the concentration of F3-10kDa led to the reduction in the formation of MDA (Figure 2a). On the other hand, the fraction F3-10kDa from HTC beans (Figure 2b) presented similar lipid peroxidation activity to BHT independent of concentration used in this test (200 to 600 µg).

It is known that uncontrolled lipid peroxidation is involved in the occurrence of numerous chronic diseases because it causes damage to biomolecules such as amino acids, proteins and DNA. Therefore, the elimination of free radicals, mainly hydroxyl, is probably one of the most effective alternatives in the defense of living organisms against these diseases (Je *et al*., 2009).

It has been reported that in some situations there is an increase in the production of free radicals that exceeds the repair capacity of the available antioxidant defenses, culminating in an increase of lipid peroxidation levels (Mandelker, 2008). Thus, the organs and endogenous systems accumulate aggressive reactive species, which may be involved in many diseases, such as diabetes, hypertension, arteriosclerosis, arthritis and Parkinson's (Pham-Huy et al., 2008; Kumar et al., 2015).

These polypeptide fractions from common beans presented potential protection against the oxidative effect, which may be associated with the fact that these substances act as antioxidants, protecting the membrane lipids against the oxidative effect caused by free radicals. Furthermore, the antioxidant action is associated with the presence of hydrophobic amino acids as phenylalanine and glycine in the peptide sequence, which contributes to the peroxidation inhibition, increasing the solubility of the peptide in lipids, contributing to a better interaction with free radicals (Rajapakse *et al*., 2005).

This suggests the importance of the discovery of alternative molecules that inhibit the process of lipid peroxidation and extends the future application of bioactive peptides as a potential ingredient in food preservation and also as an inhibitor for some diseases as atherosclerosis, cancer, inflammatory and degenerative diseases.

The “Hard-to-cook” phenomenon did not seem to affect definitively the bioactivity of the bioactive peptides present in beans, suggesting that it is possible to use these components as an alternative for the use of the grains affected by the hardening process.

4 CONCLUSIONS

This study highlighted the antioxidant and antiperoxidative properties of different peptide fractions from ETC and HTC beans. We have confirmed the occurrence of naturally-occurring

peptides in seeds of common beans and these peptide fractions showed antioxidant activity and, therefore, have great potential as natural antioxidants. Additionally, it was verified that peptide fractions from ETC beans presented ability to inhibit the formation of reactive oxygen species such as malondialdehyde better than the synthetic antioxidant BHT. Regarding the HTC sample, was verified similar lipid peroxidation activity to BHT in the concentration range used in the test (200 to 600 μg). The "Hard-to-cook" phenomenon did not seem to affect definitively the bioactivity of the bioactive peptides, suggesting that it is possible to use these components as an alternative for the use of the grains affected by the hardening process.

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