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Potential Iron and Copper Chelating Activity of Naturally Occurring Peptides and Protein Fractions from Common Bean (*Phaseolus Vulgaris*)

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Abstract

The peptides found in the common bean (*Phaseolus vulgaris*) have been the target of many studies since these peptides exhibit biological activities, including the ability to react with metals, and thus acting as agent chelating. The chelating n complex formed between a peptide and a metal are frequently more stable in the gastrointestinal tract than the mineral in the free form. Therefore, this study aimed to evaluate the chelating capacity and stability of natural peptides obtained from common bean flour (*Phaseolus vulgaris*). In this study, the flour obtained from freshly harvested beans (ETC), hardened beans (HTC) and hardened autoclaved beans (AUT) were evaluated as a source of bioactive peptides. Protein extracts were produced using acetonitrile/water/formic acid. The extracts were tested for iron and copper chelating and the peptide-metal complex were submitted to simulated gastric digestion. Results showed that the bioactivity is related to the presence of peptides, with molecular weight less than 10 kDa. The peptide-metal complexes were stable under simulated gastric digestion, suggesting these complexes may be promising for nutraceutical purpose.

Keywords: Chelating activity; Chelating capacity; Gastric stability; Natural peptides

Abbreviations: HTC: Hard-to-Cook; ETC: Easy-to-Cook; ANOVA: Analysis of Variance.

Introduction

Recently the food industry has devoted attention to functional foods. The research in this area focuses on natural products rich in bioactive compounds. The bean fits this requirement, since it presents polyphenols, resistant starch, oligosaccharides and bioactive peptides [1]. Several bioactivities have been related to peptides, such as protease and amylase, inhibitors, biomarkers, as well as molecules with therapeutic and medicinal purposes [2].

Among the peptide's bioactivities, the ability to react with metals, thus acting as chelating agent is very important, since the peptide-metal complexes are frequently more stable in the gastrointestinal conditions than the metal in its free form. In addition, the chelating prevents the reaction of metals with other food components and the formation of insoluble metal complexes, which normally present low bioavailable. In this sense, the peptide-metal complexes may play an important role improving the nutritional quality of foods, and consequently enhancing the health of individuals [3,4].

In this study, the seeds of (*Phaseolus vulgaris*) *cv.* BRS-Perola were used as a source of protein fractions and naturally occurring bioactive peptides. Seed of freshly harvested (ETC), hardened (HTC) and hardened autoclaved (AUT) beans were used to produce copper- and iron-peptide complexes. Finally, the stability of the peptide-metal complexes was evaluated in a simulation of the gastric conditions.

Materials and Methods

Material

The material used in this study was provided by the Brazilian Agricultural Research Corporation (EMBRAPA) rice and beans, Santo Antônio de Goiás, Goiás.

After harvest, the beans were separated in two groups: the control group also designated as "easy-to-cook" (ETC), was stored under refrigeration, at 5°C. For the hardening of beans was used the methodology described by Ribeiro, et al. [5]. The grains were stored in glass vials for 120 days, at 40°C and 75% relative humidity. These grains were designated as "hard-to-cook" (HTC). After hardening procedure, part of the HTC grains was submitted to autoclaving: the HTC grains were put in glass jars capped with foil perforated to allow water vapor penetration. Autoclaving was carried out for 15 min at 120°C and 121 kgf/cm^2. These graisn were designated as AUT.

Extractions of Peptides and Protein Fractions

ETC, HTC and AUT grains were manually dehulled, milled (500 µm) and the produced flour was stored at 4°C.

Extract of protein fractions were done by adding 1 g of the respective flour to 5 mL of a solution containing a mixture of acetonitrile, water and formic acid in proportion to 25:24:1. The samples were subjected to stirring for 1 hour at room temperature. Then, the samples were centrifuged for 5 minutes at room temperature. The collected supernatants were concentrated in an Eppendorf Vacufuge Concentrator hub and then lyophilized and stored at room temperature.

Peptide Fractionation

In order to better investigate the origin of the chelating activity, protein extracts were subjected to ultrafiltration in porous membrane ("cut-off" 10 kDa) (Amico Bioseparations). The ultrafiltration was conducted under nitrogen gas pressure (50 kgf/cm^2). The filtrate with molecular weight less than 10 kDa was used as source of peptides (F<10). The residue retained in the membrane with molecular weight higher than 10 kDa was resuspended in buffer solution and used as source of proteins (F>10).

Copper Chelating Activity

Copper chelating activity was assayed according to the methodology of Carrasco-Castilla, et al. [6] with modifications. Prior to the assay, a curve varying the concentrations of F>10 and F<10 was done. Briefly: the assay was carried out by adding 200 µL of sodium phosphate buffer 50 mmol L-1, pH 6.0, containing 10 µg of copper and 50 µL of the F>10 (10 µg of protein) or F<10 kDa (5 µg of protein). The reaction was left to proceed for 10 min. Then, 5 µL 4.0 mmol L^{-1} of piracatecol Violet were added to reaction and the readings were done in microplate spectrophotometer (EPOCH) at a wavelength of 632 nm. EDTA (ethylenediamine tetra acetic acid) was used as positive control and a negative control test was done in the absence of sample.

The percentage of chelating activity was determined as follows:
Copper chelating % = (Abs positive control – Abs sample / Abs positive control) x 100

Iron Chelating Activity

The iron chelating activity was tested according to the methodology of Carrasco-Castilla, et al. [7] with adaptations. Prior to the assay, a curve varying the concentrations of F>10 and F<10 kDa was done. Briefly: the assay was carried out by adding 180 µl of 100 mmol L^{-1} sodium acetate buffer, pH 4.9; 60 µL of a solution of iron chloride tetrahydrate (containing 1.12 mg Fe^{2+}) and 50 µL of the F>10 (10 µg of protein) or F<10 kDa (5 µg of protein). The assay was left to occur for 30 minutes and then 10 µL of a 40 mmol L-1 ferrozine solution was added to the reaction. After 5 min incubation at room

temperature, the reactions were read at microplate spectrophotometer (EPOCH) at 560 nm. EDTA was used as positive control and the negative control was done in the absence of a sample. To determine the percentage of chelating activity, the following equation was used:

Iron chelating activity % = (Abs positive control – Abs sample / Abs positive control) x 100

Preparation of Peptide-Copper Complex

The peptide-copper complex was prepared according to the methodology described by Carrasco-Castilla, et al. [7] with adaptations. To 8.0 mL of 50 mmol L^{-1} sodium phosphate buffer solution, pH 6.0 containing 10 µg of copper salt were added 2.0 mL of the sample (F>10 or F<10), containing 60 µg of protein. The mixture was incubated under stirring at room temperature for 1 hour, and every 15 min the pH was checked to keep it constant at pH 6.0. Subsequently the mixture was centrifuged for 20 min at 5000 rpm. The supernatant was lyophilized and stored at room temperature.

Preparation of Peptide-Iron Complex

The preparation of the peptide-iron complex was done according to the methodology described by Carrasco-Castilla, et al. [7] with adaptations. To 2.0 mL of iron chloride tetrahydrate solution were added 1.6 mL of sample (F>10 or F<10) containing 60 µg of protein and 6.4 mL of 100 mmol L^{-1} sodium acetate buffer solution, pH 4.9. The assay was incubated under stirring at room temperature for 1 hour, and every 15 min the pH was checked to keep it constant at pH 4.9. Subsequently the mixture was centrifuged for 20 min at 5000 rpm. The supernatant was lyophilized and stored at room temperature.

Stability of Peptide-Metalic Complexes to Gastric Conditions

To evaluate the stability of the peptide-metal complexes under simulated gastric conditions, the methodology described by Silva [8] with adaptations was used. In these tests, 2.0 mg of the peptide-metal complex was mixed with 1.9 mL of gastric fluid (NaCl 35 mmol L^{-1}, pH 2.0). The mixture was incubated for 15 min at 37°C and then, 100 µL of pepsin solution (1.0 mg mL^{-1}) was added to the assay. The reaction was incubated under orbital shaking for 1 hour at 37°C. Subsequently, the assay was adjusted to pH 4.9 with 1.0 mol L^{-1} NaOH for iron-peptide complex, and to pH 6.0 for the peptide-copper complex assay. After that, the samples were centrifuged for 20 min at 5000 rpm. The content of free copper or free iron was measured in the supernatant were determined by the colorimetric methods of piracatecol violet and ferrozine, respectively, as described by Carrasco-Castilla, et al. [7].

Statistical Analysis

The tests were conducted using a completely randomized design. The experiments were carried out in triplicate, with repetitions, and the results were expressed as mean and standard deviation. Data were subjected to analysis of variance (ANOVA) and Tukey's test for comparison between the averages. The program used was the Statistica 10.0 (Stat Soft Inc., Tulsa, Ok, USA), with a significance level of 95%.

Results and Discussions

Chelating Activity

The chelating activity is based on the interaction between an electron donor group situated on the surface of the protein or peptide and a metal ion, to produce biologically stable coordination complexes [8].

The results for copper and iron chelating activity can be observed in table 1.

Chelating activity		ETC	HTC (%)	AUT
Fe2+	F>10	30^{bB} ±0.01	30^{bB} ±0.05	40^{aB} ±0.12
	F<10	51^{bA} ±0.05	51^{bA} ±0.05	82^{aA} ±0.02
Cu2+	F>10	33^{cB} ±0,03	64^{bB} ±0,03	96^{aB} ±0.00
	F<10	72^{cA} ±0.03	90^{bA} ±0.01	97^{aA} ±0.03

Table 1: Copper and iron chelating of activity of bean's peptide and protein fractions.

Results expressed as average of three determinations ± standard deviation. In the same column, data followed by same capital letters in the columns and lowercase in the lines, do not differ significantly (p >0.05).

As can be observed, the F<10 (peptides) presented higher chelating activity for both ions. The values in the table correspond to a chelating activity obtained with 5 µg of F<10, whereas the activity obtained with F>10 (proteins) correspond to 10 µg of this sample. In this sense, the F<10 presented chelating activity around 3-fold higher than F>10, confirming the potential of naturally occurring peptides from beans as natural source of organic molecules for production metal-complexes.

Regarding the bean's samples, it is interesting to observe that hardening phenomenon does not interfere in the efficiency for iron chelating, which was the same in ETC and HTC. However, the chelating activity of the F<10 from AUT beans was almost 60% higher than in the ETC and HTC, implying that this thermal/pressure treatment changes the structure of the grain's proteins, either hydrolyzing proteins and consequently increasing the amount of peptides available to chelate iron, or changing the peptide's structure to expose chelating points in their surface.

In the opposite, regarding the copper chelating activity, it is possible to affirm that an improvement of around 20% was obtained due to the hardening of the grains, since the values increased from 72% in ETC to 90% in HTC beans. The thermal/pressure treatment of autoclaving had lower impact in the copper chelating activity than that observed for iron chelating activity, with an improvement of less than 10%.

Results expressed as average of three determinations ± standard deviation. Data followed by same capital letters in the columns and lowercase in the lines, do not differ significantly ($p > 0.05$).

The presence of chelating activity for iron and copper in F<10 explained by Ashmead [9], who claims that the formation of an iron-peptide complex is needed to stabilize of the electron demand and the charge of the metal ion. Another aspect that to be considered when studying chelating activities is the amino acids composition of peptide. The presence of specific amino acids which show affinity for iron and copper is required for complex formation. The histidine and cysteine are amino acids that show the highest affinity to iron and copper due to the imidazole ring and thiol group, followed by carboxylic groups of glutamic and aspartic acids. The OH-groups of serine or threonine also represents metal ion binding sites [8,10].

Chelating Capacity Copper and Iron

In order to determine the chelating capacity of the F<10 from ETC, HTC and AUT, increasing concentrations of F<10 were left to react with fixed concentrations of 10 µg of copper and 1.12 mg of iron. Results are shown at Figure 1 (A-iron and B-copper).

The figure shows that affinity of peptides is higher for copper than to iron, except AUT. From these results, it was decided that 60 µg of F<10 should be the amount used to produce the peptide-metal complexes, since this amount assured that peptides will be in excess and did not limit the reaction rate or efficiency.

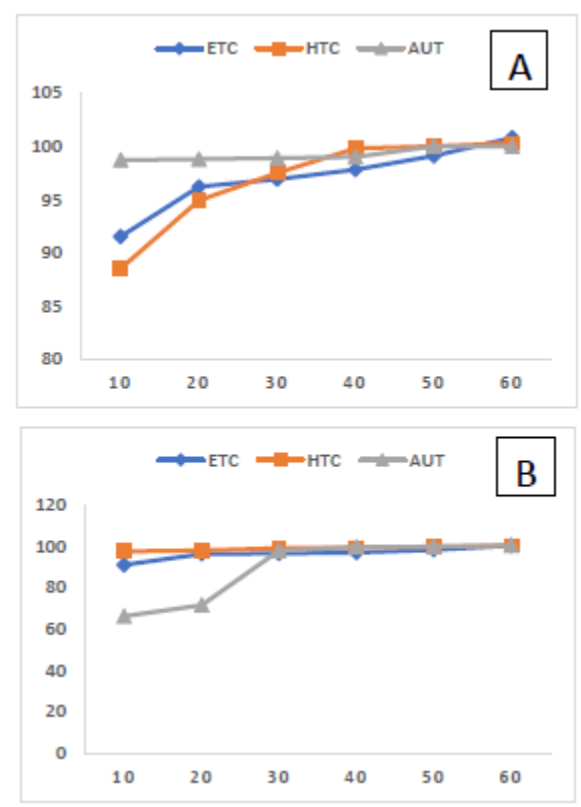

Figure 1: Affinity of peptides is higher for copper than to iron, except AUT.

According to Ashmead [9], the absorption of peptide-metal complexes with molecular mass between 200 and 400 Daltons is faster than the free metal ions. This author report that peptide-metal are resistant to hydrolysis in the intestinal mucosa, remaining unchanged until complete absorption. After absorption the complex releases iron to bind to ferritin or transferrin. Similar results were reported by Eckert, et al. [11], using a hexapeptide-iron complex.

Stability of Peptide-Metallic Complex Gastric Conditions

Our body lacks a physiological mechanism to eliminate the excess of iron and copper. Therefore, the absorption of iron and copper is generally regulated to avoid the

accumulation of these ions [12]. One of the barriers to the uptake of metal ions is the maintenance of the ion in the digestive tract. The main point of control is the stomach due to the combined action of the enzyme pepsin and the extremely acidic pH of this compartment.

The first step to ensure the absorption of iron and copper from the body is to test the gastric stability of the chelates during the passage through the gastrointestinal tract. Peptide-metal complexes produced with F<10 from beans ETC, HTC and AUT were submitted to simulated gastric digestion, in order to assess the stability of the $Fe^{2\pm}$ peptide and $Cu^{2\pm}$ peptide complexes. Results of this test are shown in table 2.

Peptide-metal complex	Chelating Activity	ETC	HTC (%)	AUT
Fe^{2+}	Initial	98.6Aa ±0.09	97.8Ab ±0.07	95.2Ac ±0.07
	NaCl pH 2.0	95.2Ba ±0.20	91.7Bb ±0.30	89.8Bc ±0.30
	pH 2.0/pepsin	90.3Ca ±0.40	90.0Cab ±0.50	89.2Bb ±0.30
Cu^{2+}	Initial	90.8Ab ±0.54	87.4Ac ±0.76	93.2Aa ±0.09
	NaCl pH 2.0	82.6Bb ±0.84	79.1Bc ±1.09	88.1Ba ±3.43
	pH 2.0/pepsin	76.5Cc ±0.17	78.3Bb ±0.86	83.5Ca ±0.61

Table 2: Stability of iron-peptide and copper-peptide complexes after simulated gastric conditions.

Results expressed as average of three determinations ± standard deviation. Data followed by same capital letters in the columns and lowercase in the lines, do not differ significantly (p >0.05).

As shown in table 2, both iron and copper-peptide complexes presented very good stability during simulated gastric digestion. This implies that the bound between ions and peptide is very stable, since they resist to the acid treatment without release of the metal ion. Other relevant aspect is the stability of the copper and iron-peptide complex submitted to enzymatic treatment. Pepsin is an aspartic protease able to quickly break peptidic bound from the carboxyl side of the amino acids phenylalanine, tryptophan and tyrosine. The stability of the peptide-metal complexes may reflect the lack of these amino acids in the peptide structure.

Note that comparing the three types of beans, the more stable iron-peptide complex was obtained with ETC beans, whereas for copper the more stable complex was obtained with AUT beans.

Stability of copper-peptide complex AUT bean is explained by the presence of certain amino acids (histidine, serine, methionine, aspartate, glutamate, and tryptophan) on the surface of the molecule, which may have been exposed after the autoclaving treatment. Such amino acids exhibit high affinity for copper ions, resulting in very strong peptide-copper bound [13]. The results obtained for the iron-peptide complex with ETC, HTC and AUT beans were superior to those found by Silva [8] using whey peptides or beans proteins obtained by Durak, et al. [14]. When these authors analyzed the stability of iron-peptide complex under simulated gastric conditions they found 40,1% - 59% stability.

These results demonstrated that the peptide-metallic complexes formed with bean peptides from ETC, HTC and AUT are resistant to gastric conditions. This resistance reflects that the kind of bond formed is strong and that the peptide probably lacks sites for pepsin digestion [15]. Another factor that contributes to the stability of the binding in the metal-peptide complex is the number of links of the ligand to the metal ion, either copper or iron. The higher the number of linkages, the higher is the stability of the complex [16].

Conclusion

The results found in this study show that beans are rich source of naturally to explore their bioactivity. A single step of ultrafiltraion was enough to produce a pool of peptides with very high metal chelating activity. Moreover, the peptide-metal chelating complexes were resistant to simulated gastric digestion, remaining active and therefore available for intestinal absorption. The results of this study also showed that thermal/pressure treatment such as autoclaving may be valuable to increase the number of sites for metal chelating activity in peptide structure.

Compliance with Ethical Standards

This work has been submitted to publication with the agreement of all authors. The authors declare that they have no conflict of interest

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