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Anti-nutritional factors and digestibility of protein in *Cayocar brasiliense* seeds

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Abstract

The Brazilian Cerrado presents a wide variety of natural products, including the Pequi (*Caryocar brasiliense*). The important factor that determines the dietary and nutritional ideality of protein is degestitibility. This work aims to evaluate the protein digestibility of Pequi seeds and the presence of anti-nutritional factors. The protein Pequi almonds were extracted, toasted and untoasted. Evaluation digestibility was structurally characterized by SDS-PAGE 15%, which can be used to analyze simulated gastric fluid (SFG) and digestion in intestinal fluid (SFI) in the presence of pepsin and trypsin/chymotrypsin. The extract from Pequi almonds showed inhibitory activity and was not detected hemagglutination. The intensity of the bands according to 2S albumins, after heat treatment, did not decrease in comparison to the condition native, significantly. In the crisp crude extract, which was incubated under SGF and SIF, the intensity of the corresponding bands at 8 and 3 kDa was resistant to SGF and indigestible after 4 hours under SIF. The characterization of Pequi almonds demonstrated that the product of the crude extract has anti-nutritional factors, which were confirmed by evaluating digestibility.

Keywords: *Caryocar brasiliense*; almonds; *in vitro* digestibility; 2S albumin; peptidase inhibitor; lectins.

Practical Application: The study of anti-nutritional factors identification is very important because of its inactivation on food industry and it can also be used to avoid allergy due to them.

1 Introduction

Animal and vegetable proteins obtained from food are very important source of amino acids, especially the essentials. It is clear that is necessary to multiply the offering of proteins while there is a fast increase in the world population. Those from the vegetal origins have an important significance because it is indispensable food source in development countries (World Health Organization, 2007).

Pequi is one of the native species of the Cerrado, widely used by the population because of its high nutritional value, variety of nutrients and especially socioeconomic value (Kerr et al., 2007; Pinto et al., 2009; Baeta, 2013). The fruit is formed by almonds and hoopoes, in which the first is responsible for the extraction of proteins. The presence of proteins in almond can be related to the source of plant reserves may have from 10 to 40% by dry weight, as it should be necessary to ensure that the protein reserves for the plant development stages (Lima et al., 2007; Costa, 2012).

Protein source has important nutritional value when there are adequate compositions in terms of total amino acids, essential amino acids, digestibility, method of preparation, anti-nutritional factors and toxicity. Despite its nutritional availability, as a product of plant origin, Pequi can have anti-nutritional properties, synthesized by the fruits, for their own defense. Thus the diet and nutritional quality of the protein could be defined by digestibility (Pompeu et al., 2014; Silva et al., 2015a; Maradini-Filho et al., 2017).

The anti-nutritional factors present in food may reduce the bioavailability of nutrients and also cause harmful effects to human health, for example lectins, 2S albumins and peptidase inhibitors. Some plants kernels have proteins associated with the defense mechanism against insects and microorganisms (Oliveira et al., 2014) and when these vegetables are eaten, these proteins are considered toxic and antinutritional (Gonçalves et al., 2016).

Peptidase inhibitors are endemic diffused in vegetables and is able to inhibit some enzymes, among which trypsin and chymotrypsin (Oliva et al., 2011; Silva et al., 2015a, b; Pesoti et al., 2015; Pompeu et al., 2016). The adverse health effects have been detected in experimental animals and it can be seen that occurred pancreatic hypertrophy, decreased growth and weight reduction in animals (Grela, 1996; Clemente et al., 2015).

Lectins can act as non-nutritional factors with the capacity to combine with sugars and glycoconjugates, inducing erythrocyte agglutination (Silva et al., 2007; Van Damme, 2014). The consumption of power by lectins, can lead to gross changes of various animal organs and even lead to hyperplasia and hypertrophy of the pancreas and intestine and may lead to various adverse health effects, by decreasing the absorption of nutrients (Seena & Sridhar, 2004; Pompeu et al., 2015). This is because lectin glycosylated binds to receptors present on intestinal cells and thus interferes with the processes of digestion, absorption and utilization of nutrients (Vasconcelos & Oliveira, 2004).

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2S Albumins may differ in chains, but converge on features to be structurally homologous, they are classified as compact globular proteins with arrangement and distribution of conserved disulfide bridges. 2S albumins Isoforms are composed by two disulfide bonds linked a large subunit (9 kDa) and a small subunit (3-4 kDa) (Shewry et al., 1995; Pantoja-Uceda et al., 2003; Koppelman et al., 2005; Hsiao et al., 2006; Lule et al., 2015). Moreover, this class of protein showed a broad spectrum function that has been described as a new trypsin inhibitor (Mandal et al., 2002). Additionally, the immune system is sensible to the most food allergens that are stable and have the capacity to reach the intestinal mucosa and than release an IgE-mediated allergic response (Dimitrijevic et al., 2010; Kumar et al., 2014; De Angelis et al., 2017).

The SDS-PAGE technique has been generally used to conduct the analysis of the gastric fluid (SFG), in the presence of pepsin, and the intestinal fluid (SFI) in the presence of trypsin/chymotrypsin. In addition, *in vitro* digestibility of protein after heat treatment can be measured, probably due to denaturation of peptidase inhibitors, 2S albumins and lectins, inactivating the antinutritional factors (Pompeu et al., 2014; Lang et al., 2015; Silva et al., 2015a).

One of the consequences of low level of digestibility is the hydrogen bonding, hydrophobic interactions and disulfide bonds that stabilized the variety of globular structures of food proteins. The role of anti-nutritional factors in food is little known in Caryocaraceae family, and in addition to the need for knowledge of suitable methods for the inactivation of these in the food industry, the study to evaluate the influence on digestibility of proteins and amino acids availability is required (Damiani et al., 2013).

This study aims to evaluate the protein digestibility of seeds Pequi (*Caryocar brasiliense*) to determine the presence of peptidase inhibitor, 2S albumin and lectin using Osborne method and heat treatment to simulate digestion.

2 Materials and methods

2.1 Chemicals

Bovine serum albumin (BSA), bovine pancreatic trypsin, α-chymotrypsin and bovine pancreatic, NR-benzoyl-DL-arginine-p-nitroanilide (BApNA) and N-benzoyl-L-tyrosine p-nitroanilide (BTpNA) were purchased from Sigma (St. Louis, MO, USA) and acrylamide, bis-acrylamide, dithiothreitol (DTT), standard molecular weight marker and other electrophoresis reagents. The seeds of *Caryocar brasiliense* were collected at the Federal University of São João del Rei (UFSJ).

2.2 Protein extraction

The crude extract was obtained according to the method of Osborne (Osborne, 1924), which toasted and untoasted seeds (50 g) were ground using a power mill by stirring with 1 mM phosphate buffer pH 7.6 for 1 h at 37 °C. After the crude extract was centrifuged at 3.600 x g for 15 min at 37 °C, using distilled water to dialysis for 48 h at 4 °C and then lyophilized.

2.3 Determination of protein concentration

The protein assay was determined using the Bradford method (Bradford, 1976), assuming that an A_{760} of 1.0 corresponded to a protein concentration of 1 mg/mL using BSA as standard.

2.4 Peptide inhibition assays

The trypsin inhibitor activity assay used 50 mL of crude extract (1 mg/mL) incubated with 50 mL of trypsin (0.33 mg/mL of 1 mM HCl) and 250 µL of 0.1 M of Tris-HCl buffer pH 8.0, during 10 min at 37 °C. After, 1.0 mL of BAPNA solution (0.4 mg/mL BAPNA 0.1 M Tris-HCl buffer pH 8.0) was mixed and incubated for 20 min at 37 °C. The reaction was stopped with 500 µL of 30% acetic acid solution according to Cruz et al. (2013). The amount of inhibitor required for 50% inhibition by trypsin using BAPNA as the substrate was defined as one unit. This experiment was performed in triplicate and the results were plotted as mean ± standard deviation (SD).

2.5 Hemaggutionation assay

The Pequi extract was subjected to a hemagglutination assay, as proposed by Pompeu et al. (2015). These trials were conducted using B and O human bloods in the presence and absence of trypsin in 96-well plate. To characterize the minimal concentration able to erythrocytes of different types 15 mg/mL of crude extract was diluted in series in the 96-well plate. These assays were performed in the presence of EDTA in CTBS solution (150 mM NaCl, 20 mM Tris-HCl, 5 mM $CaCl_2$). The experiment was performed in triplicate.

2.6 Electrophoresis

The apparent molecular weight was estimated using DTT (Ditiotreitol) for reducing conditions (15% SDS-PAGE) using Bio Rad electrophoresis system (Hercules/USA) according to Laemmli (1970). The amount of 50 mg/mL of crude extract was added to 10 µL of sample buffer and 0.1% Coomassie R-250 bright blue was used to staining proteins.

2.7 Analysis of in vitro digestibility

Three different peptidases: trypsin (E.C. 3.4.21.4, Type III), α-chymotrypsin (E.C. 3.4.21.1, Type II) and Pepsin A (E.C. 3.4.23.1) were used to perform the *in vitro* digestibility using SDS-PAGE. The enzymes were used to simulate gastric fluid (SGF) and intestinal fluid (SIF). The seeds were evaluated under native conditions, thermal denatured (100 °C for 30 min) and toasted (120 °C for 30 min). In SGF 40 µL of the crude extract fraction (24 mg/mL of crude extract diluted in 250 µL of 10 mM phosphate buffer pH 6.8) was mixed with 5 µL of enzyme (6.2 mg/mL pepsin, 50 mM HCl and 0.1 M phosphate buffer containing 0.1 M NaCl pH 7.7) and 55 µL sample buffer. In SIF 40 µL of the crude extract fraction (24 mg/mL of crude extract diluted in 250 µL of 0.1 M phosphate buffer with NaCl pH 7.7) was mixed with 5 µL of enzyme (1 mg/mL of trypsin/chymotrypsin in 0.1 M phosphate buffer with NaCl pH 7.7) and sample buffer. After, 15 µL of the mixed sample was added in different times 0.5, 1, 2, 4 and 8 h according the condition SGF or SIF.

2.8 Statistical analysis

A comparison of mean residual trypsin activity was performed by ANOVA one way (α = 0.05), followed by the Tukey test with 5% confidence limits.

3 Results and discussion

3.1 Hemagglutination activity detection and inhibitory for Protease

In non-leguminous plants of almonds there are some studies that have detected the presence of peptidase inhibitors or hemagglutinating activities. The protein content in the studied *Caryocar brasiliense* species of seeds is shown in Table 1. However, this had low protein content and a lower percentage of the residual activity of trypsin, indicating potentially lower enzyme inhibition (Figure 1).

For detection of hemagglutination activity (AHE) in the total extract of the species under study, they used red blood cells from all blood types, in order to select the one with greater susceptibility to hemagglutination. However, for the *C. brasiliense* extracts AHE not detected over the tested erythrocytes.

As the AHE is the best known property of lectins, this is the most widely used method for the detection of these proteins. The results showed the specificity of lectins in the two tests made with different concentrations, one test done with 4 mg/mL and another made with 8mg/ml, since the hemagglutinating effect caused by these proteins is dependent on the molecular structure of each lectin individually.

Table 1. Concentration of protein in protein extracts from roasted and untoasted Pequi seeds.

Sample	mg proteins/mL
Caryocar brasiliense toasted	0.088
Caryocar brasiliense untoasted	0.167

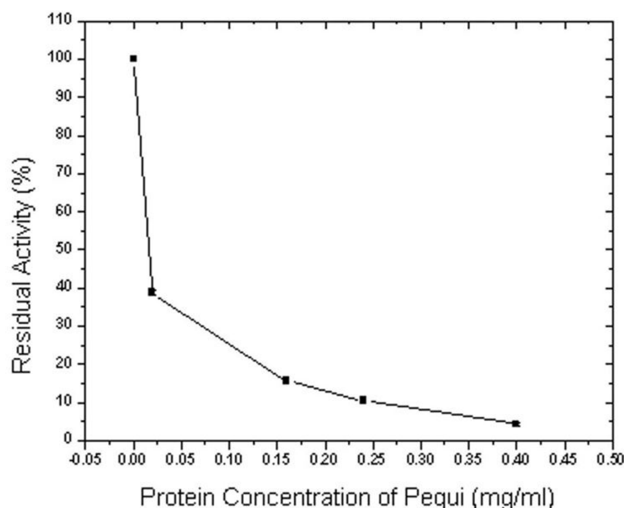

Figure 1. Inhibition of trypsin activity by the crude protein extract of *C. brasiliense* seeds. Hydrolysis of BAPNA by 1 mM bovine trypsin at pH 8.0 at 37 °C, in the presence of increasing concentrations of total extract protein.

Pequi seed extract demonstrated trypsin inhibitory activity. An inhibition curve was performed using the Pequi extract and trypsin showed that lost 60 and 76% of its activity when the molar ratio was 0.05 and 0.1 mg/mL, respectivelly. Proportionally, the 2S albumin Pequi inhibited the same mass of bovine trypsin using the specific substrate, BApNA. Other result presented the same values according to trypsin inhibitor belongs to Kunitz family, such as SKTI from soy seeds (Ribeiro, 2010).

The harms of anti-nutritionals factors such as peptidase inhibitors are reported in animal studies, and observed pancreatic hypertrophy, and some lectins may have toxicity and not others (Pompeu et al., 2014). Lima et al. (2008) found a trypsin inhibitor in jabuticabas, with higher levels observed in seeds, followed by the peel and the entire fruit, with lower levels in the pulp and hemagglutination activity in the peel. Peptidase inhibitors and lectin were observed in almonds bacuri and seeds sapucaia (Barbosa, 2006; Denadai et al., 2007), while Hiane et al. (2006) detected low concentrations of lectins and no peptidase inhibitors in bocaiuva almonds.

3.2 Electrophoresis of isolated fractions of Pequi seeds

There are a few studies about the digestibility of proteins in Caryocaceae family. Pequi seeds were used without toasting, it native or denatured being, and toast for the preparation of protein digestibility *in vitro*. Electrophoresis on polyacrylamide gel was performed to determine the molecular weight and number of protein bands, which is an easy method to implement and low cost (Anema, 2009). Figure 2 shows the amount of protein extracted without heating and roasted crude extract from the delipidated flour 15 g, half of which was used for roast extract and the other half to extract untoasted. The electrophoretic pattern of protein extracts, not roasted and toasted, the species studied showed seeds distribution of protein bands between the molecular weight ranges from about 110 to 3 kDas.

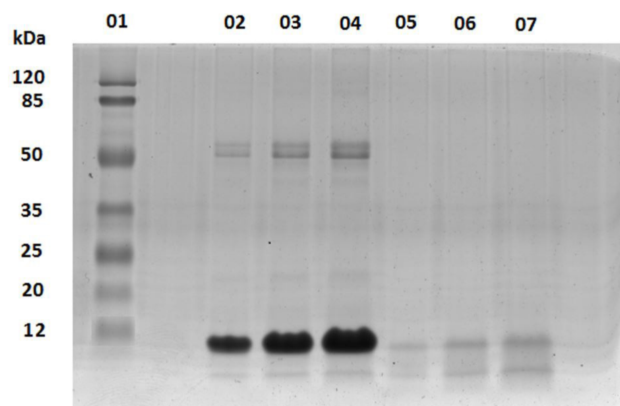

Figure 2. Electrophoretic profile of Pequi protein fractions in 15% SDS-PAGE. Column 1: molecular weight marker; Columns 2, 3 and 4: extract of untoasted at concentrations 0.006 to 0.018 mg; Columns 5, 6 and 7: toasted extract at concentrations of 0.006 to 0.018 mg.

For almond Pequi without toasting, it was observed that the protein content showed bands of 110, 52 and 51 kDa and other minors between 23.11 and 3 kDa. Regarding almonds were subjected to roasting processes it was observed in the electrophoretic profile of proteins, the presence of two strong protein bands with molecular weight of 8 and 3 kDa.

In a study by Hiane et al. (2006), it was observed that the globulin protein bocaiuva almond showed three bands, one above 100 kDa, 66 kDa and another of 60 kDa. In almond sapucaia, Denadai et al. (2007) identified the bands for globulin for 18, 34, 40 and 50 kDa. When some bands were disappeared toast, this fact can probably be attributed to the reactions occurring during the roasting process, such as Maillard complexation with polyphenolic compounds in addition to the possible formation of different polymers. These various reactions that occur during roasting can produce insolubilization and lower extractability of the protein during preparation of the sample to be applied on the gel. The roasted seeds contained low protein quality, which is probably due to its amino acid profile (Freitas & Naves, 2010).

It not was found on the gel electrophoresis SDS-PAGE molecular mass protein Pequi between 30 and 35 kDa that is typical of the structure of lectins (Sharon & Lis, 1995; Devi et al., 2014; Van Damme, 2014). Technically, the protein bands present in the range of 5-25 kDa suggest the presence of inhibitors of serine peptidases type Kunitz, Bowman-Birk and 2S albumin which has been reported in the scientific literature (Carlini & Grossi-de-Sá, 2002; Ruan et al., 2011). The band near the molecular weight of 11 kDas was more abundant in both phases, this being apparently very similar to the protein that shows 40% activity against trypsin, purified from Pequi seed, called 2S albumin (Costa, 2012).

3.3 Seeds of crude extract untoasted simulating gastric fluid (SGF)

In the Figure 3A the pepsin was incubated with the sample of the crude extract. Bands of proteins at 110, 52, 51, 23 and 11 kDa was observed at various times up to 8 hours under the condition of SGF (simulated fluid gastric) in 15% SDS-PAGE. Furthermore, no hydrolysis products were detected. Subsequently denaturation treatment, pepsin easily degrades bands of proteins at 110, 52 and 23 kDa, but one 11 kDa band was resistant and divided into two subassemblies 8 and 3 kDa (Figure 3B).

3.4 Seeds of crude extract untoasted simulating intestinal fluid (SIF)

In the Figure 4A the trypsin/chymotrypsin were incubated with the sample of the crude extract. Bands of proteins at 52 and 51 kDa gradually decreased under the condition of SIF, but 120 and 11 kDa bands were resistant in 15% SDS-PAGE. Furthermore, there hydrolysis products in the range 50 to 20 kDa. After thermal treatment, the band according to 11 kDa (2S Albumin) was not reduced significantly in comparison to native conditions (no heat treatment).

In this study, it was shown that the potential of the allergenic 2S albumin after SGF and SIF digestion was not reduced significantly when compared with other Pequi proteins, which were digested. In addition, some studies have been demonstrated that digestibility is greatly influenced by pH level and temperature. Some rice proteins in SGF conditions were digested faster at pH 1.2 as compared to pH levels of 2.0 and 2.5 as well as the effects of temperature were decisive in accelerating protein digestion (Pompeu et al., 2014; Gámez et al., 2015; He et al., 2015.; Lang et al., 2015).

3.5 Seed extract toasted simulating gastric and intestinal fluid

In the Figure 5A the crude extract sample was toasted, simulating the time to prepare the seed for consumption, and incubated with pepsin. In this condition no more bands of proteins are presented in the gel higher than 20 kDa in comparison with untoasted. The presence of bands around 3 and 8 kDa demonstrated that there are some proteins resistant to these enzymes up to 8 h. In the Figure 5B the crude extract sample was crisp and incubated with trypsin/chymotrypsin. In this condition

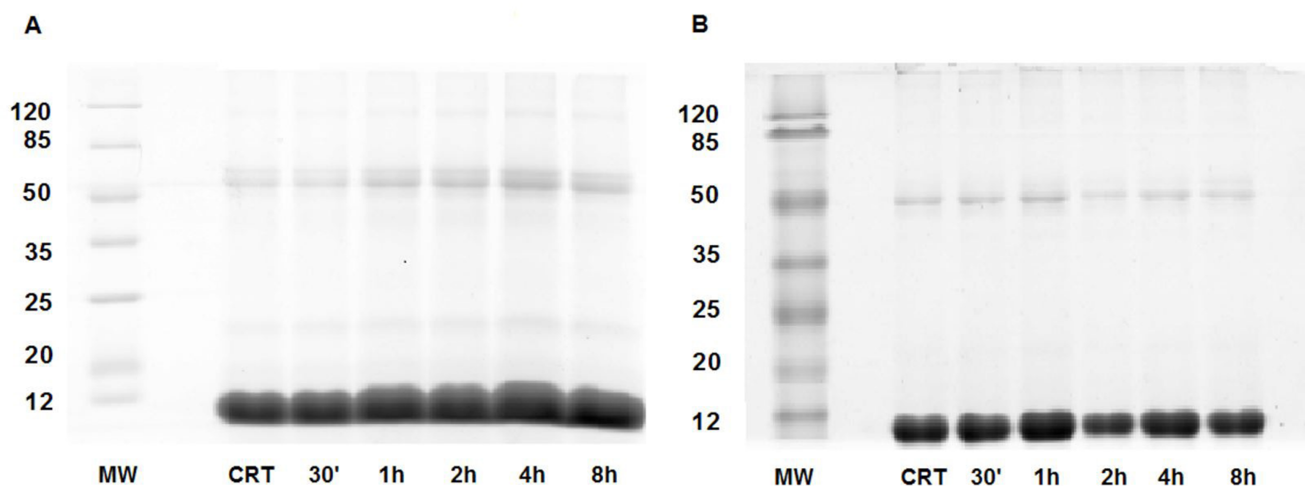

Figure 3. Electrophoretic profile of protein digestibility seed Pequi by Pepsin. (MW) molecular weight marker; time of incubation: 30 min to 8h; (CRT) control; Gel A: native. Gel B: denatured.

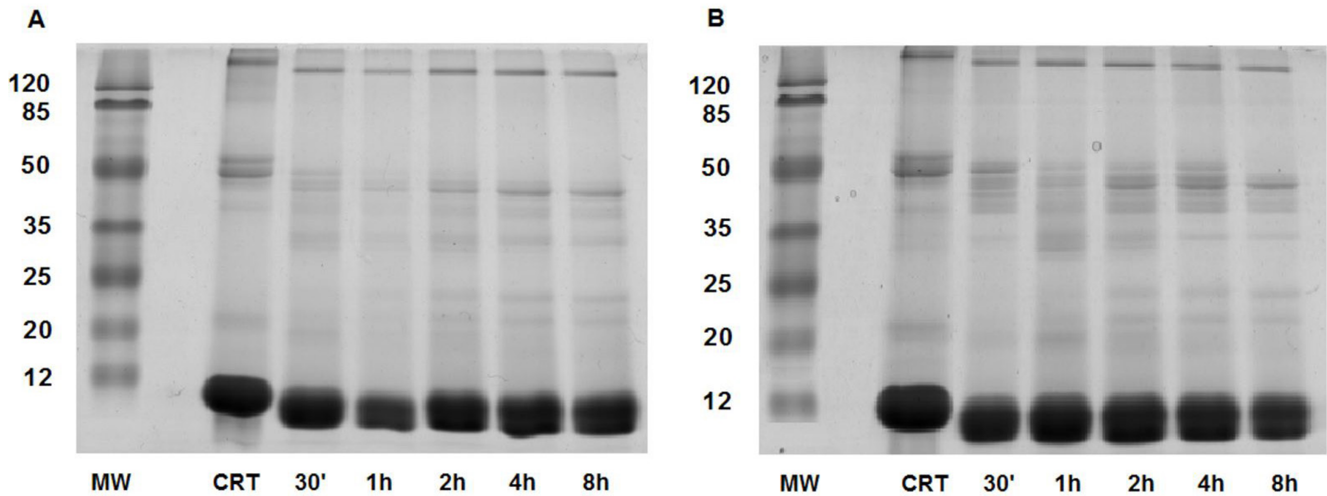

Figure 4. Electrophoretic profile of protein digestibility Pequi seed Trypsin/Chymotrypsin. (MW) molecular weight marker; time of incubation: 30 min to 8h; (CRT) control; Gel A: native. Gel B: denatured.

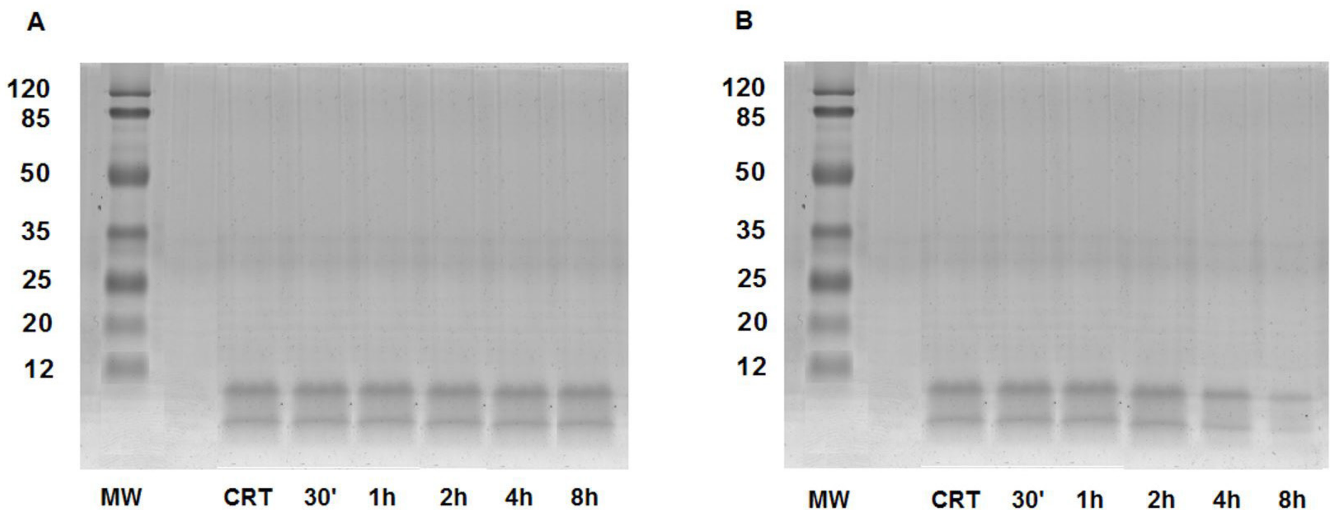

Figure 5. Electrophoretic pattern of protein digestibility in toasted Pequi seeds by pepsin (A) and trypsin/chymotrypsin (B). (MW) molecular weight marker; time of incubation: 30 min to 8h; (CRT) control; Gel A: native. Gel B: denatured.

no more bands of proteins are presented in the gel higher than 20 kDa in comparison with untoasted. The presence of bands around 3 and 8 kDa demonstrated that there are some proteins resistant to these enzymes up to 4 h. The visual analysis of the gels indicated that thermal treatment proteins in the gastric conditions have higher resistance to proteolysis than in intestinal conditions, showing different stabilities related to food allergens.

These resistant proteins have been reported as 2S albumins and are related in various species, including peanut, mustard, Brazil nut, sesame seed and sunflower (Murtagh et al., 2003; Moreno et al., 2005; Vissers et al., 2011; Orruno & Morgan, 2011; Sirvent et al., 2012; Berecz et al., 2013).

It is clear that cysteine residues, in particular their connections intra-chain disulfide, stabilize the three-dimensional structure of the protein independently of its action against the digestive enzymes. However, almost every food allergens have some resistance, which upon reaching the digestive tract in a fragment with immunogenic conformation can cause systemic reactions (Moreno et al., 2005).

The main chain polypeptide and digested fragments are formed by disulfide links, corresponding an undigested protein and stabilized by the interactions of the three-dimensional structure. The high temperatures reached during the roasting process were capable of breaking the disulfide bonds of the protein, by modifying its tertiary structure, presenting the cleavage sites for the action of proteolytic enzymes. This fact has a great correlation with the 2S albumins which present highly stable protein structures thermal denaturation and to proteolysis due to its compact 3D structures stabilized by disulfide bridges. Thus, the digestibility of these proteins is quite important, and these are shown only in SIF digestible toasted extracts after the time of 4h (Murtagh et al., 2003; Vissers et al., 2011).

The stability against the action of digestive enzymes pepsin and trypsin/chymotrypsin from the Pequi seed extract suggests a high concentration of cysteine in its structure, which may contribute to reduce its nutritional quality. The globular structure of 2S albumin has been shown to be more resistant to the action of pepsin, preventing the action of this proteolytic enzyme on the peptide bonds adjacent to the amino acids Met, Leu and aromatics, whereas it was less resistant to the action of trypsin/chymotrypsin on the peptides bonds adjacent to amino acids Arg, Lys and aromatics (Fontana et al., 2004; Singh et al., 2014).

Studies using electrophoresis have shown that native black bean lectins were resistant to both gastric and intestinal digestion (He et al., 2015). On the other hand, whole-grain proteins showed different levels of digestibility in gastric digestion and were susceptible to rapid intestinal digestion (Lang et al., 2015). Alteration in protein structure is evident with heat treatment, independent if it is gastric or intestinal, indicating that the conformational structure plays a primordial role in its resistance to proteolytic degradation (He et al., 2015). During a 60 min reaction period most food allergens are stable under gastric conditions (Grozdanovic et al., 2014; He et al., 2015). However, there is no consensus on which of the conditions allergenic proteins are more susceptible to the action of proteolytic enzymes (Fu et al., 2002).

4 Conclusions

Pequi seeds characterization demonstrated that the product of the crude extract has anti-nutritional factors. The protein of approximately 11 kDa (untoasted) and 8 and 3 kDa (toasted) proved quite similar to the 2S albumins, which are able to bind to intestinal receptors triggering an IgE-mediated allergic response. In toasted conditions the 2S albumin was more stable in gastric conditions and its globular structure contributed for the resistance to action of proteolytic enzymes. The nutritional capacity of protein *Caryocar brasiliense* was evaluate, however the mechanism involved in the digestion of these proteins needs more attention, which is not yet well established.

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