Original Article
Food Science and Technology
ISSN 0101-2061 (Print)
ISSN 1678-457X (Online)
DOI: https://doi.org/10.5327/fst.467

Evaluation of antinutritional factors in the digestibility of proteins from *Amaranthus caudatus* seeds

Daniela Regiane da SILVA[1], Helon Guimarães CORDEIRO[1], Fhelipe de OLIVEIRA[1], Bruno SOARES[1], Vinicius TARABAL[1], Paulo Afonso GRANJEIRO[1], José Antônio da SILVA[1]*

Abstract

With population growth, the need for natural products with a good nutritional balance increases. Digestibility is an important factor that defines the nutritional diet and the quality of proteins. This work aims to evaluate the protein digestibility of Amaranth (*Amaranthus caudatus*) seeds. Amaranthus seed extracts showed inhibitory activity and hemagglutinating activity. In the simulating gastric fluid condition, hydrolysis products were detected, a 70 kDa band, which does not appear in the control. After the heating treatment, pepsin easily digested the 29 kDa lectin bands and a gradual digestion of the 12 and less than 10 kDa bands, probably the protease inhibitors. In the simulating intestinal fluid evaluation, without heating, the bands of 29, 12, and less than 10 kDa showed resistance to digestion. After heat treatment, the intensity of the corresponding bands 12 and those less than 10 kDa gradually decreased compared to the band of 29 kDa, which was digested quickly. Amaranthus seeds showed antinutritional factors, inhibitors, and lectins, demonstrating that at appropriate treatment and increased temperature it can improve more the efficient activities of digestive enzymes. Therefore, this work shows the importance of cooking for better protein absorption of Amaranthus seeds, making them a good source of amino acids.

Keywords: *Amaranthus caudatus*; seeds; in vitro digestibility; lectins; protease inhibitor; 2S albumin.

Practical Application: Amaranthus seeds are an excellent protein source; however, boiling them properly reduces the effects of antinutrients and improves digestibility.

1. INTRODUCTION

In the last decade, the nutritional balance of the population has become a severe challenge due to the increasing demographic ratio for cultivable regions. In order to regulate this relationship, there is a quest for food sources that contain a rich number of nutrients and are climatically malleable, in addition to offering a rich nutritional composition such as essential amino acids, fats, carbs, and vitamins (Food and Agriculture Organization of the United Nations et al. 2023; Williamson et al., 2024). An increasing use of protein-rich vegetables has become an option that may replace animal proteins in the human diet (Canoy et al., 2024; Gueugneau, 2023). However, research has recently focused on vegetable protein's in vitro digestibility (Ashaolu et al., 2025; Bailey et al., 2023; Canoy et al., 2024; Tang et al., 2022).

Amaranthus (*Amaranthus caudatus*) belongs to the Amaranthaceae family and consists of a pseudocereal, heavily cultivated in the Andean region and used by pre-Columbian cultures for centuries. Likewise, for other species of Amaranthus, such cultures are currently gaining popularity due to their genetic variability, agronomic advantages, and excellent nutritional properties, such as a high percentage of proteins with an excellent balance of essential amino acids. Amaranthus a highly efficient and fast growing annual crop, which can prosper under adverse agronomic conditions (Jan et al., 2023; Pulvento et al., 2022). The main focus of some research areas has been the folk claims of their proteins, outlining their pharmaceutical properties and emphasizing their clinical usefulness (Zhu, 2023).

The role of antinutritional factors in foods such as inhibitors, lectins, and 2S albumin is little known in the Amaranthaceae family, and, in addition to the need for knowledge of adequate methods for inactivating these in the food industry, it is necessary to study and assess the influence on protein digestibility and amino acid availability (Ohanenye et al., 2022). Despite its nutritional availability, Amaranthus may have antinutritional properties, synthesized by fruits, for its defense either against pathogens or as an evolutionary remnant for its proliferation. Thus, digestibility is an important factor that defines the nutritional diet and the quality of proteins (Silva, Pompeu, Costa et al., 2015; Silvestrini et al., 2017).

Protease inhibitors are only a part of the compounds related to antinutritional properties. They consist of proteins that interact with proteases, forming stable complexes that block or

Received: Feb. 02, 2025.
Accepted: Mar. 12, 2025.
[1]*Universidade Federal de São João Del Rei, Campus Centro Oeste Dona Lindu, Divinópolis, Minas Gerais, Brazil.*
*Corresponding author: jasilva01@ufsj.edu.br
Conflict of interest: nothing to declare.
Funding: The authors are grateful for the financial support of the Coordination for the Improvement of Higher Education Personnel (CAPES) and the National Council for Scientific and Technological Development (CNPq). The authors are also grateful to the Federal University of São João del Rei.

alter the active sites of enzymes (Mangena, 2022; Silva, Pompeu, Costa et al., 2015; Silva, Pompeu, Smolka et al., 2015). The biological function attributed to the inhibitors of this family is mainly related to the defense of plants against attacks by insects and pathogens (Mangena, 2022; Rodríguez-Sifuentes et al., 2020). Such inhibitors in mammals strongly inhibit the activity of the main pancreatic enzymes (trypsin and chymotrypsin), thus reducing the digestion and absorption of dietary proteins by the formation of indigestible complexes, even in the presence of high amounts of digestive enzymes. It can become a problem from the biochemical point of view, interfering and causing serious physiological dysfunctions (Kårlund et al., 2021; Samtiya et al., 2020).

Lectins are glycoproteins that are able to recognize specific sites in molecules and bind reversibly to monosaccharides and oligosaccharides, without altering the covalent structure of glycosidic bonds, presenting several biological functions (Naser et al., 2024; Silva et al., 2024). Ingesting lectins incorrectly can cause an interaction with the intestinal mucosa, causing inflammation and mucosal damage, which interfere with the absorption of nutrients. In the liver, liver lipidosis and necrosis occur, and, in addition, it causes type I hypersensitivity, which can be local or systemic and direct tissue damage (Kong et al., 2022; Vasconcelos & Oliveira, 2004).

The 2S albumin class received this name based on its sedimentation coefficient. They are water-soluble proteins, widely distributed by both dicot and monocot seeds, being rich in cysteine, arginine, glutamine, and asparagine (Souza, 2020). The isoforms of 2S albumin consist of a small subunit with approximately 3–4 kDa and a large subunit with approximately 9 kDa, joined by disulfide bonds that guarantee excellent stability. These proteins are related as antinutrients due to their allergenic nature (Bueno-Díaz et al., 2022; Katsube-Tanaka & Monshi, 2022; Souza, 2020).

In order to improve the nutritional quality of Amaranthus seeds, different feeding processes have been developed to inactivate or decrease inhibitory activities within limits. Thus, the present study aims to characterize the antinutritional factors of *A. caudatus* seeds in order to inactivate these factors, enabling the consumption and absorption of nutrients surely.

1.1 Relevance of the work

With population development, there is a greater demand for natural products with a high nutritional balance. Amaranth (*Amaranthus caudatus*) seeds stand out because they are an excellent protein source; however, boiling them properly lowers the effects of antinutrients and increases digestion. This study demonstrates the need for boiling for increased protein absorption in Amaranthus seeds, making them a good source of amino acids.

2. MATERIALS AND METHODS

2.1 Materials

Bovine serum albumin (BSA), trypsin, bovine α-chymotrypsin, NR-benzoyl-DL-arginine p-nitroanilide (BAPNA), and N-benzoyl-L-tyrosine p-nitroanilide (BTpNA) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Also, acrylamide, bis-acrylamide, dithiothreitol (DTT), standard molecular weight markers, and other electrophoresis reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). The seeds of *A. caudatus* were purchased from the Municipal Market of Divinópolis-MG. It is noteworthy that the seeds used in this study were identified by a taxonomy specialist from the Department of Botany at UNICAMP (University of Campinas).

2.2 Extraction of proteins

The crude extract was prepared according to Osborne's method (Osborne, 1924), in which untoasted seeds (50 g) were ground using a power mill while stirring with 1 mM phosphate buffer at pH 7.6 for 1 hour at 37°C. The crude extract was centrifuged at 3,600 x g for 15 minutes at 37°C, and the supernatant was dialyzed for 48 hours at 4°C with distilled water. The recovered material was subsequently lyophilized.

2.3 Determination of protein concentration

The protein concentration was calculated using the Bradford technique (Bradford, 1976), which assumed that an A760 nm of 1.0 equated to a protein concentration of 1 mg/mL using BSA as a standard.

2.4 Purification of crude extract from Amaranthus seeds

The crude extract of Amaranthus seeds was submitted to molecular exclusion chromatography on a Shephadex G50 molecular exclusion column previously balanced with AMBIC (ammonium bicarbonate) at pH 7.6 buffer, with a flow rate of 0.25 mL/min and fractions monitored at A280 nm. The fractions were lyophilized and employed in the next procedures.

2.5 Determination of protease inhibitory activity

To determine protease inhibitory activity, 50 mL of extract (1 mg/mL) was incubated with 50 mL of trypsin (0.33 mg/mL of 1 mM HCl) and 250 µL of 0.1 M Tris-HCl buffer pH 8.0 for 10 minutes at 37°C. According to Pompeu et al. (2014), mix 1.0 mL of BAPNA solution (0.4 mg/mL of 0.1 M BAPNA Tris-HCl pH 8.0 buffer) and incubate for 20 minutes at 37 °C before stopping the reaction with 500 µL of 30% acetic acid. The amount of inhibitor required to achieve 50% trypsin inhibition with BAPNA as the substrate. The experiment was repeated three times, and the obtained values were plotted as mean ± SD.

2.6 Determination of hemagglutinating activity

In a 96-well microtiter plate, hemagglutinating experiments were performed with O red blood cells in the presence or absence of trypsin. To determine the minimal concentration of erythrocytes of various types, 6.5 mg/mL inhibitor was serially diluted in a 96-well microtiter plate. Pompeu et al. (2015) reported that these tests were carried out in the presence of EDTA in CTBS solution (150 mM NaCl, 20 mM Tris-HCl, 5 mM $CaCl_2$). The experiment was carried out in triplicate.

2.7 Electrophoresis

According to Laemmli (1970), the apparent molecular weight was determined using DTT (dithiothreitol) under reducing conditions (15% SDS-PAGE) on a Bio-Rad electrophoresis system (Hercules/USA). An amount of 50 mg/mL of crude extract was added to 10 µL of sample buffer, and 0.1% bright blue Coomassie R-250 was used to stain the proteins.

2.8 In vitro *digestibility*

Three peptidases, trypsin (EC 3.4.21.4, Type III), α-chymotrypsin (EC 3.4.21.1, Type II), and pepsin A (EC 3.4.23.1), were utilized to analyze in vitro digestibility using SDS-PAGE. The enzymes were utilized to generate gastric juice (SGF) and fluid (SIF). The seeds were tested in their natural environment before being thermally denatured at 100°C for 30 minutes. In SGF, 40 µL of crude extract fraction (20 mg/mL) was diluted in 250 µL of 10 mM phosphate buffer at pH 6.8 and combined with 5 µL of enzyme (6.2 mg/mL of pepsin, 50 mM HCl, and 0.1 M phosphate buffer containing 0.1 M NaCl, pH 7.7) and 55 µL of sample buffer. In SIF, 40 µL of crude extract fraction (24 mg/mL 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) and sample buffer. Mixed samples of 0.5, 1, 2, 4, and 8 hours were added at different times based on SGF or SIF conditions, totaling 15 µL.

2.9 Statistical analyses

The mean residual trypsin was compared using one-way ANOVA ($\alpha = .05$), followed by the Tukey test. $p < .05$ was employed as statistically significant. *GraphPad Prism 5* was used to conduct statistical studies and plot graphics.

3. RESULTS AND DISCUSSION

In the Amaranthaceae family, there are several studies that have detected the presence of protease inhibitors or hemagglutinating activities by the action of their proteins (Adamcová et al., 2021; Capraro et al., 2021; Dang et al., 2017; Hasan et al., 2021; Resendiz-Otero et al., 2024). However, little is known about their protein digestibility, which may interfere significantly with nutritional health (Kong et al., 2022).

The protein content in the seed of the *A. caudatus* in this study was 0.267 mg/mL. This result was greater than that presented by native proteins from Pequi seeds (*Caryocar brasiliense*) (Silvestrini et al., 2017). Different methods have been used to quantify total protein in plant origin samples. However, few studies have compared the results of such methods. Several factors must be analyzed before choosing a methodology for the quantification of total proteins; however, knowledge of the nature of the sample constituents and their approximate concentrations is essential (Pesoti et al., 2015; Pompeu et al., 2015; Pompeu et al., 2016; Silvestrini et al., 2017). The extraction of vegetable proteins depends on the characteristics of protein solubility, which relate to the hydrophobicity of the amino acid residues and the protein load, in addition to their interaction with the solvent (Lao et al., 2023).

3.1 Partial purification of Amaranthus seeds

The crude extract was then applied to a gel filtration column containing Sephadex G-50 resin. The resulting chromatographic profile revealed the separation of components into three main fractions, as shown in Figure 1. The peaks were analyzed individually for the presence of trypsin inhibitors, lectins, and proteases. Peak I showed hemagglutinating activity, while peak II showed inhibitory activity for trypsin protease, and peak III showed protease activity.

In a gel filtration column containing Sephadex G-50 resin, the proteins are eluted in accordance with the weight of proteins; proteins with the highest molecular weight are eluted first, followed by those with the lowest weight. Lectins, which have a larger molecular weight, were eluted first, as evidenced by strong hemagglutinating activity in peak I, whereas protease inhibitors, which are lower-weight proteins, were eluted later. Accordingly, Pompeu et al. (2015) and Pompeu et al. (2022) eluted *Chenopodium quinoa* lectins in a peak I gel filtration column with Sephadex G-50 resin. Pesoti et al. (2015) eluted protease inhibitors from *C. quinoa* seeds in a peak II filtration column with Sephadex G-50 resin.

3.2 Trypsin inhibition activity and inhibition curve

Figure 2 shows the inhibition curve obtained from peak II. In order to confirm the presence of protease inhibitors in the extract of Amaranthus seeds, an activity test was carried out demonstrating the presence of proteins with inhibitory capacity against trypsin. An inhibition curve was constructed using peak II gel filtration chromatography, elucidating that trypsin lost 50 % of its activity when the molar ratio was 0.1 mg/mL and 90% of its activity when the molar ratio was increased to 0.4 mg/mL. A linear extrapolation to obtain 100% inhibition indicated that the inhibitors of Amaranthus can bind to trypsin, approximately, in a proportion of 1:1 molar ratio. The inhibition curve was confirmed by the Tukey test ($\alpha = .05$).

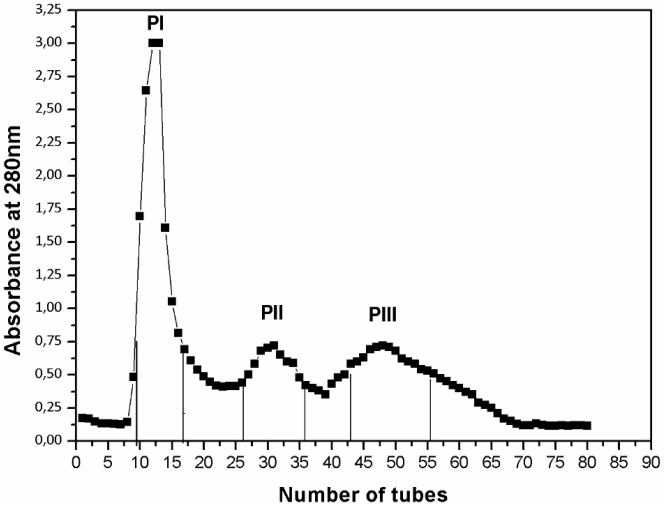

Figure 1. Sephadex G-50 gel chromatography using the fast protein liquid chromatography system.
The column was equilibrated with AMBIC 1 M buffer, under a flow of 0.25 mL/min (5 mL / tube). The numbers correspond to the three fractions obtained.

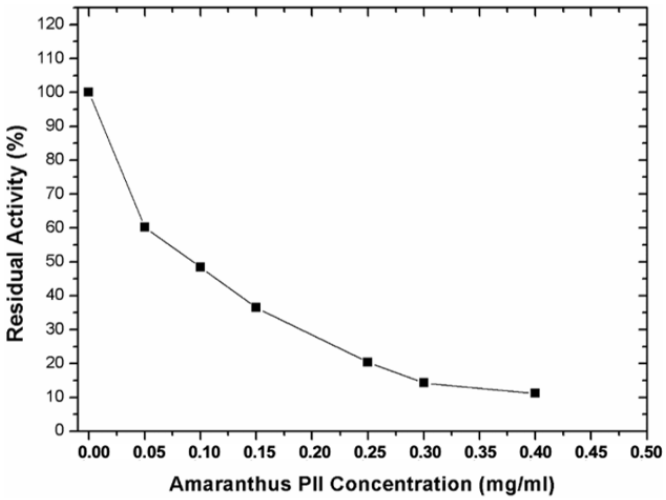

Figure 2. Inhibition curve of bovine trypsin by peak II obtained by gel filtration chromatography. Residual trypsin activity was monitored by DL-BAPNA hydrolysis.

The inhibition curve allows us to evaluate the partial concentration to promote the interaction and formation of the enzyme–inhibitor complex, being similar to the complex formed between enzyme–substrate (Pesoti et al., 2015; Silvestrini et al., 2017). The substrate used in the technique was BAPNA, with the purpose of identifying the specific activity of the inhibitor against trypsin. The rate of change in residual trypsin activity can occur, since the specificity of inhibitors by specific classes of proteases is associated with the composition, the conformation of the reactive site, and the three-dimensional shape of the inhibitor molecule, these factors being essential for efficiency and protein stability (Bobrovs et al., 2022; Krauchenco et al., 2004; Vishvakarma & Mishra, 2022).

3.3 Detection of hemagglutinating activity

To detect hemagglutinating activity (AHE) at peak I derived from the column in gel filtration, red blood cells from the most common blood types were used in order to select the one with the greatest susceptibility to hemagglutination. As AHE is the well-elucidated property of the lectin family, this method has become more widely used for detecting these proteins (Castro et al., 2018; Idries et al., 2024). Peak I of *A. caudatus* demonstrated an AHE over the tested red blood cells that had not been trypsinized to a concentration of 0.020 mg/mL, showing a high protein rate of lectins capable of forming immunological complexes with the most common blood types.

It is common that in plants that have high climatic malleability, as in the study carried out by Arcoverde et al. (2014), about 66 species of plants native to the cerrado (Brazilian savanna) were subjected to hemagglutinating activity. Of the results, 77.7% were positive for hemagglutinating activity, that is, capable of forming immunocomplexes; in addition, 66% of the species showed trypsin inhibitory activity, possibly due to the presence of the protease inhibitor. Studies to identify the presence of protease inhibitors and lectins are performed in different types of plants because many plants show these proteins to defend themselves (Arcoverde et al., 2014). Interestingly, a study undertaken by Feijoo-Coronel et al. (2024) showed antibacterial synthetic peptides derived from a protein with lectin-like properties.

3.4 Electrophoresis of the isolated fractions of the Amaranthuss seeds

SDS-PAGE (polyacrylamide electrophoresis gel with sodium dodecyl sulfate) was used to determine the quantity and molecular weights of the protein fractions, which is a simple and cost-effective approach (Pompeu et al., 2014; Silva, Pompeu, Smolka et al., 2015; Silvestrini et al., 2017). In electrophoresis, you can observe the proteins of the fractions and their molecular weights. Figure 3 shows the amount of protein extracted from the crude extract, reduced with DTT and without reduction. The electrophoretic profile of the unreduced protein extracts from the seeds of the examined species indicated a dispersion of protein bands with molecular weights ranging from roughly 110 to less than 10 kDa. Protein content in crude extracts of Amaranthus seeds treated with DTT showed bands about 110, 29, and 12 kDa, as well as proteins smaller than 10 kDa. In the reduced conditions with DTT, PI hydrolysis products were found, a 70 kDa band that cannot be seen in the control, and a 110 kDa band, most likely from a vicillin subunit. The electrophoretic profile of the seed proteins subjected to heating methods revealed two apparent molecular areas of 30 (lectin) and less than 10 kDa (inhibitor).

It was pointed out in the SDS-PAGE electrophoresis gel of Amaranthus protein fractions with molecular masses ranging from 29 to 35 kDa, which are indicative of lectin structures (Van Damme, 2014). Protein bands ranging from 5 to 25 kDa appear to be consistent with the presence of proteinase inhibitors. Accordingly, Cordeiro et al. (2024) discovered a protease inhibitor from the medicinal plant *Alocasia macrorrhizos* in two bands: 11 and 24 kDa, using SDS-PAGE. The molecular weight band at 12 kDa was the most prevalent in both stages, and it appears to be very similar to proteins with trypsin inhibitory action (trypsin inhibitors), as seen in the protein composition of *Albizia niopoides* seeds investigated by Pompeu et al. (2016).

When preparing the sample to be placed in the gel, several reactions may occur during heating including insolubilization and reduced protein extractability. Furthermore, the likelihood that the collected samples have a large molecular mass, making them impossible to permeate the gel and therefore eliminated from the electrophoretic runs (Carrillo, Gomez-Casati et al., 2017), cannot be ruled out.

3.5 Protein digestibility by electrophoresis

There are several studies on digestibility in legumes, but little is known about the digestibility of the Amarantaceae family. Then, Amaranthus seeds were subjected to heating (denatured) and under native conditions for the elaboration of protein digestibility in vitro. To evaluate the effect of heating on the denaturation of antinutritional protein factors, in vitro digestibility was performed based on SGF (simulating gastric fluid) and SIF (simulating intestinal fluid), monitoring the

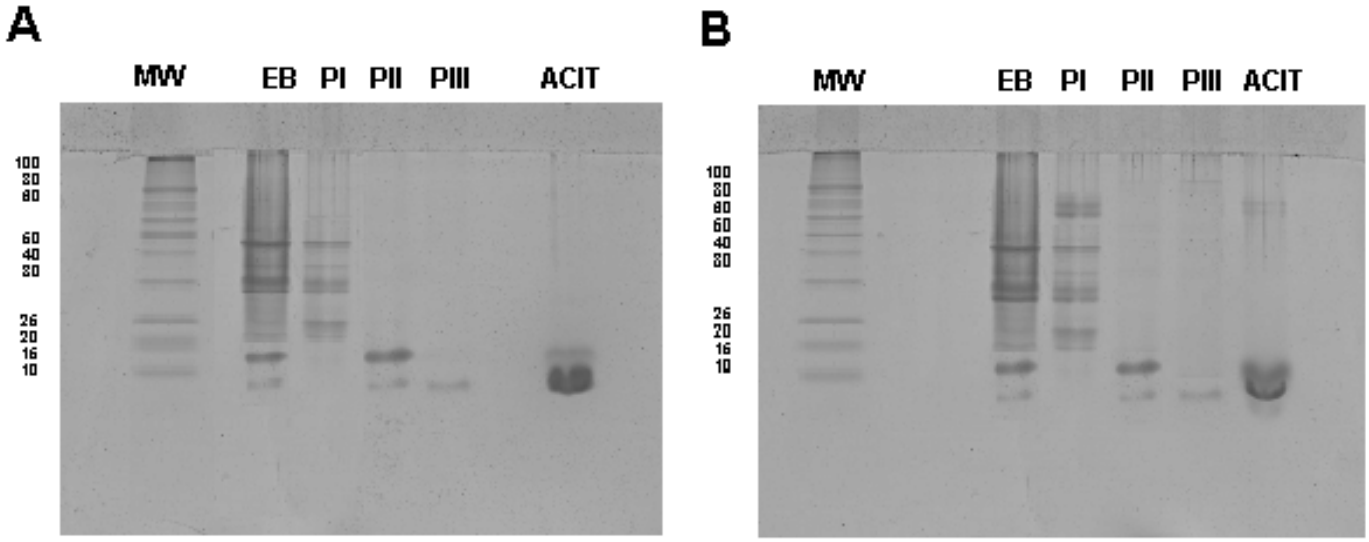

Figure 3. Electrophoretic profile of Amaranthus protein fractions in 15% polyacrylamide-SDS gel. **(A)** No reduction with dithiothreitol and **(B)** Reduced with dithiothreitol.
MW: Molecular weight marker; EB: crude extract; fractions purified by peak 1 exclusion chromatography PI; PII: peak 2; PIII: peak 3; ACIT: trypsin inhibitor.

incubation of fractions with digestive tract enzymes until a time of 8 h, time referring to the complete digestion of food (Lang et al., 2015; Lang et al., 2021; Luz et al., 2024; Pompeu et al., 2014; Silvestrini et al., 2017). A higher rate of digestion in duodenal conditions can be attributed to the combined actions of trypsin and α-chymotrypsin and their specificity of sites of cleavage in proteins, while pepsin is related to the digestion of proteins in gastric conditions (Rawdkuen et al., 2022; Zhang, Wen et al., 2022).

Acidification, hydrolysis, and heat treatment are critical processes in the complete digestion of dietary proteins. These parameters have a substantial impact on protein structures, as well as their resistance to digestion (Bhat et al., 2022; Zhou et al., 2023). Choosing appropriate ways for inactivating or decreasing the antinutritional and allergic components of Amaranthus seeds is highly important. The reactions were designed as a model that simulates physiological conditions seen at various intervals in order to determine whether the percentage of proteins, with potential inhibitor and enzyme activity, were digested and the time required for complete digestion. The samples were separated into two groups: native and denatured, which had been heated at 100°C for 30 min.

3.5.1 Crude seed extract simulating gastric fluid

In the evaluation of SDS-PAGE 15%, a sample of the crude extract was treated with pepsin, and the intensity of the associated bands 70, 29, 26, 12, and less than 10 kDa were seen at various intervals up to 8 h under simulating gastric fluid (SGF) conditions (Figure 4). When the 70 kDa bands were heated, they disappeared, while others dropped in intensity; this finding is most likely related to the occurrence of reactions during the heating process, such as those responsible for breaking bonds between protein subunits. Under native conditions, pepsin did not show significant activity, and it is possible to observe that there was almost no change in the bands over the time of the technique.

A study conducted by Lee et al. (2024) demonstrated that heating plays a crucial role in protein digestion in adult and elderly in vitro digestive models. Accordingly, in the study of Carrillo, Cordoba-Diaz et al. (2017), it was pointed out that after heat treatment, there is a very rapid decline in the 29 and 26 kDa bands in the denatured sample after 30 min, as well as a time-dependent smooth digestion in the 5 and 10 kDa bands (from 4 to 8 h).

3.5.2 Crude seed extract simulating intestinal fluid

In the evaluation using 15% SDS-PAGE, a sample of the crude extract was incubated with trypsin/chymotrypsin (Figure 5), and the intensity of the corresponding bands 29, 26, 12, and less than 10 kDa were not digested by the enzymes without heating. After heat treatment, the intensity of the bands decreased during digestion, significantly higher when compared to the results of digestion in a condition without heat treatment. The 29 and 26 kDa bands, probably lectins, had a good digestion in both gastric and intestinal fluids, suggesting the efficiency of heat treatment.

Changes in protein structure are evident with heat treatment, regardless of whether it is gastric or intestinal, indicating that the conformational structure plays a major role in its resistance to proteolytic degradation (He et al., 2015). According to Zhang, Wang et al. (2022), a study performed on whole soybeans with variable moisture content demonstrated that protein structure contributes to variability of in vitro digestibility.

In the present study, it was demonstrated that, after SGF and simulating intestinal fluid (SIF) digestion, the 12 kDa protein that was not digested by pepsin was both denatured and native. In SIF, the 12 kDa protein starts the digestion process with

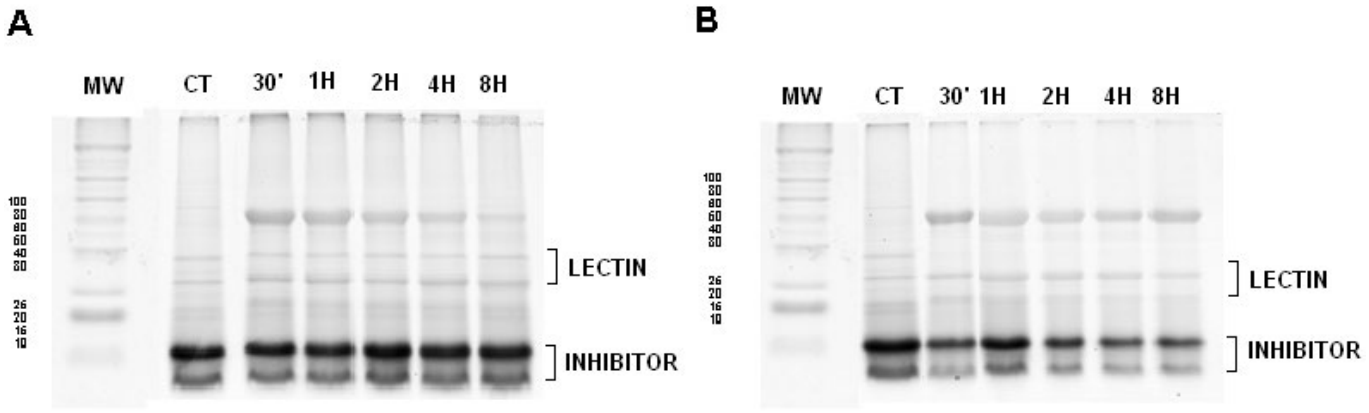

Figure 4. Electrophoretic profile of the protein digestibility of the yellow seed by pepsin.
MW: Molecular weight marker; Incubation times: 30 min to 8 h; CT: control without enzyme; Gel A Native. Denatured B. Gel. The arrows indicate the storage proteins.

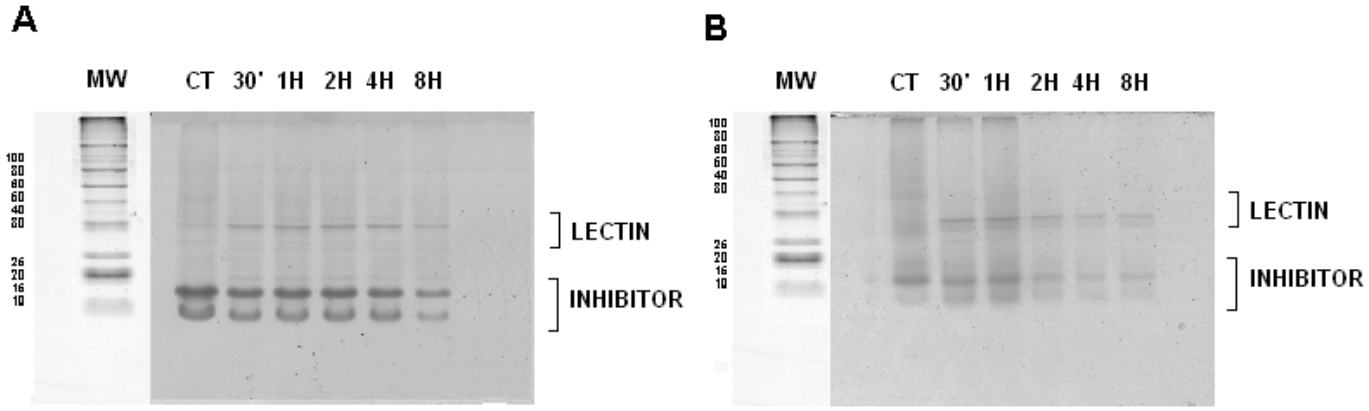

Figure 5. Electrophoretic profile of protein digestibility of the Amaranthus seed by trypsin/chymotrypsin.
MW: Molecular weight marker; Incubation times: 30 min to 8 h; CT: Control without enzyme; Gel A Native. Denatured B. Gel. The arrows indicate the storage proteins.

approximately 2 h in the native state and 1 h in the denatured state. Most food allergens are stable under gastric conditions, giving this band a possible allergenic potential (Sun et al., 2022). Resistant protein fragments larger than 3.5 kDa in the low pepsin activity test may be immunologically important in the context of allergenicity risk assessment for vulnerable groups (Wang et al., 2022).

A study performed on pea showed that pea seeds lacking specific proteins such as lectin, pea albumin 2 (PA2), and trypsin inhibitors (TI) through genetic alterations boosted protein digestibility and had no negative influence on seed protein content or yield (Olías et al., 2023). Legume seed protein is a valuable source of nutrients; however, it is not as digestible as animal protein. Protein modifications during germination often improve proteolysis and digestibility by lowering antinutrient levels. Such knowledge could lead to the development of more digestible food preparations, possibly combining germination with other factors affecting digestibility, such as heating and fermentation (Bera et al., 2023). A study on pigeon pea, a protein-rich legume with low protein digestibility, found that thermal treatments can increase the nutritional quality of pigeon pea protein, making it suitable for use as a new ingredient in the development of healthy diets (Dutta et al., 2024). Our findings showed that the protein content of Amaranthus was significantly reduced, implying that digestion of the ACTI inhibitor and lectin after heating lowered the antinutritional components of Amaranthus seeds.

4. CONCLUSION

The analysis of protein portions of Amaranthus demonstrated the presence of antinutritional factors, in which lectins and trypsin ACT inhibitors stand out. Proteins with a mass less than 10 kDa proved to be quite similar to protease inhibitors, and 29 and 26 kD proteins were similar to lectins. Both showed good stability in gastric and intestinal conditions, requiring a previous heating for a better action of proteolytic enzymes due to their compact structures. Heating methods can diminish and inactivate Amaranthus seeds' antinutritional properties, improving their nutritive and absorptive capabilities.

REFERENCES

Adamcová, A., Laursen, K. H., Ballin, & N. Z. (2021). Lectin Activity in Commonly Consumed Plant-Based Foods: Calling for Method Harmonization and Risk Assessment. *Foods*, *10*(11), Article 2796. https://doi.org/10.3390/foods10112796

Arcoverde, J. H. V., Carvalho, A. S., Neves, F. P. A., Dionízio, B. P., Pontual, E. V., Paiva, P. M. G., Napoleão, T. H., Correia, M. T. S, Silva, M. V., & Carneiro-da-Cunha, M. G. (2014). Screening of

Caatinga plants as sources of lectins and trypsin inhibitors. *Natural Product Research*, *28*(16), 1297–1301. https://doi.org/10.1080/14786419.2014.900497

Ashaolu, T. J., Greff, B., & Varga, L. (2025). The structure–function relationships and techno-functions of β-conglycinin. *Food Chemistry*, *462*, Article 140950. https://doi.org/10.1016/j.foodchem.2024.140950

Bailey, H. M., Fanelli, N. S., & Stein, H. H. (2023). Effect of heat treatment on protein quality of rapeseed protein isolate compared with non-heated rapeseed isolate, soy and whey protein isolates, and rice and pea protein concentrates. *Journal of the Science Food Agriculture*, *103*(14), 7251–7259. https://doi.org/10.1002/jsfa.12809

Bera, I., O'Sullivan, M., Flynn, D. & Shields, D. C. (2023). Relationship between Protein Digestibility and the Proteolysis of Legume Proteins during Seed Germination. *Molecules*, *28*(7), Article 3204. https://doi.org/10.3390/molecules28073204

Bhat, Z. F., Morton, J. D., Bekhit, A. E.-D. A., Kumar, S., & Bhat, H. F. (2022). Non-thermal processing has an impact on the digestibility of the muscle proteins. *Critical Reviews in Food Science and Nutrition*, *62*(28), 7773–7800. https://doi.org/10.1080/10408398.2021.1918629

Bobrovs, R., Basens, E. E., Drunka, L., Kanepe, I., Matisone, S., Velins, K. K., Andrianov, V., Leitis, G., Zelencova-Gopejenko, D., Rasina, D., Jirgensons, A., & Jaudzems, K. (2022). Exploring Aspartic Protease Inhibitor Binding to Design Selective Antimalarials. *Journal of Chemical Information and Modeling*, *62*(13), 3263–3273. https://doi.org/10.1021/acs.jcim.2c00422

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*(1–2), 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Bueno-Díaz, C., Biserni, C., Martín-Pedraza, L., Las Heras, M., Blanco, C., Vázquez-Cortés, S., Fernández-Rivas, M., Batanero, E., Cuesta-Herranz, J., & Villalba, M. (2022). Association Between the Seed Storage Proteins 2S Albumin and 11S Globulin and Severe Allergic Reaction After Flaxseed Intake. *Journal of Investigational Allergology and Clinical Immunology*, *32*(5), 375–382. https://doi.org/10.18176/jiaci.0713

Canoy, T. S., Wiedenbein, E. S., Bredie, W. L. P., Meyer, A. S., Wösten, H. A. B., & Nielsen, D. S. (2024). Solid-State Fermented Plant Foods as New Protein Sources. *Annual Review of Food Science Technology*, *15*(1), 189–210. https://doi.org/10.1146/annurev-food-060721-013526

Capraro, J., Benedetti, S., Heinzl, G. C., Scarafoni, A., & Magni, C. (2021). Bioactivities of Pseudocereal Fractionated Seed Proteins and Derived Peptides Relevant for Maintaining Human Well-Being. *International Journal of Molecular Science*, *22*(7), Article 3543. https://doi.org/10.3390/ijms22073543

Carrillo, C., Cordoba-Diaz, D., Cordoba-Diaz, M., Girbés, T., & Jiménez, P. (2017). Effects of temperature, pH and sugar binding on the structures of lectins ebulin f and SELfd. *Food Chemistry*, *220*, 324–330. https://doi.org/10.1016/j.foodchem.2016.10.007

Carrillo, J. B., Gomez-Casati, D. F., Busi, M. V., & Martín, M. (2017). Development of fast and simple chromogenic methods for glucan phosphatases in-gel activity assays. *Analytical Biochemistry*, *517*, 36–39. https://doi.org/10.1016/j.ab.2016.11.005

Castro, A. H. F., Tavares, H. S., Pereira, S. R. F., Granjeiro, P. A., Silva, J. A., & Galdino, A. S. (2018). Production and characterization of lectin from Bauhinia holophylla (Fabaceae:Cercideae) calli. *Plant cell Tissue and Organ Culture*, *134*, 423–432. https://doi.org/10.1007/s11240-018-1432-7

Cordeiro, I. H., Lima, N. M., Scherrer, E. C., Carli, G. P., Andrade, T. J. A. S., Castro, S. B. R., Oliveira, M. A. L., Alves, C. C. S., & Carli, A. P. (2024). Protease inhibitors characterisation by SDS-PAGE and MALDI-TOF from *Alocasia macrorrhizos* and their modulation of macrophage immune-inflammatory properties. *Natural Product Research*, *38*(19), 3454–3459. https://doi.org/10.1080/14786419.2023.2246278

Dang, L., Rougé, P., & Van Damme, E. J. M. (2017). Amaranthin-Like Proteins with Aerolysin Domains in Plants. *Frontiers in Plant Science*, *8*, Article 1368. https://doi.org/10.3389/fpls.2017.01368

Dutta, M., Dineshkumar, R., Nagesh, C. R., Lakshmi, Y. D., Lekhak, B., Bansal, N., Goswami, S., Kumar, R. R., Kundu, A., Mandal, P. K., Arora, B., Raje, R. S., Mandal, S., Yadav, A., Tyagi, A., Ramesh, S. V., Prashat, G. R., & Vinutha., T. (2024). Exploring protein structural adaptations and polyphenol interactions: Influences on digestibility in pigeon pea dal and whole grains under heat and germination conditions. *Food Chemistry*, *460*(Part 1), Article 140561. https://doi.org/10.1016/j.foodchem.2024.140561

Feijoo-Coronel, M. L., Mendes, B., Ramírez, D., Peña-Varas, C., Monteros-Silva, N. Q. E., Proaño-Bolaños, C., Oliveira, L. C., Lívio, D. F., Silva, J. A., Silva, J. M. S. F., Pereira, M. G. A. G., Rodrigues, M. Q. R. B., Teixeira, M. M., Granjeiro, P. A., Patel, K., Vaiyapuri, S., & Almeida, J. R. (2024). Antibacterial and Antiviral Properties of Chenopodin-Derived Synthetic Peptides. *Antibiotics, 13*(1), Article 78. https://doi.org/10.3390/antibiotics13010078

Food and Agriculture Organization of the United Nations, International Fund for Agricultural Development, United Nations Children's Fund, World Food Programme, & World Health Organization. (2023). *The State of Food Security and Nutrition in the World 2023. Urbanization, agrifood systems transformation and healthy diets across the rural–urban continuum*. FAO. https://doi.org/10.4060/cc3017en

Gueugneau, M. (2023). The value of dietary plant protein in older people. *Current Opinion in Clinical Nutrition and Metabolic Care*, *26*(1), 3–7. https://doi.org/10.1097/mco.0000000000000884

Hasan, I., Rahman, S. N., Islam, M. M., Ghosh, S. K., Mamun, M. R., Uddin, M. B., Shaha, R. K., & Kabir, S. R. (2021). A N-acetyl-D-galactosamine-binding lectin from Amaranthus gangeticus seeds inhibits biofilm formation and Ehrlich ascites carcinoma cell growth in vivo in mice. *International Journal of Biological Macromolecules*, *181*, 928–936. https://doi.org/10.1016/j.ijbiomac.2021.04.052

He, S., Simpson, B. K., Ngadi, M. O., & Ma, Y. (2015). In vitro studies of the digestibility of lectin from black turtle bean (Phaseolus vulgaris). *Food Chemistry*, *173*, 397–404. https://doi.org/10.1016/j.foodchem.2014.10.045

Idries, A. H., Naser, E. H., Dafalla, M. B., Elmubarak, S. A. A., Abdelrahim, Y. E., Abdalrhman, E. A., Alwali, S. M., Ahmed, B. M., Yousef, B. A., Ebrahim, R. M. A., Abdellatif, A. O., Awadallah, A. K. E., Osman, M. E. M., & Konozy, E. H. E. (2024). Biological activity and characterization of leaf and seed lectins from *Terminalia brownii:* Insights into their analgesic and antiulcer properties. *Heliyon*, *10*(20), Article e39351

Jan, N., Hussain, S. Z., Naseer, B., & Bhat, T. A. (2023). Amaranth and quinoa as potential nutraceuticals: A review of anti-nutritional factors, health benefits and their applications in food, medicinal and cosmetic sectors. *Food Chemistry: X*, *18*, Article 100687. https://doi.org/10.1016/j.fochx.2023.100687

Kårlund, A., Paukkonen, I., Gómez-Gallego, C., & Kolehmainen, M. (2021). Intestinal Exposure to Food-Derived Protease Inhibitors: Digestion Physiology- and Gut Health-Related Effects. *Healthcare*, *9*(8), Article 1002. https://doi.org/10.3390/healthcare9081002

Katsube-Tanaka, T., & Monshi, F. I. (2022). Characterization of 2S albumin allergenic proteins for anaphylaxis in common buckwheat. *Food Chemistry: Molecular Sciences*, *5*, Article 100127. https://doi.org/10.1016/j.fochms.2022.100127

Kong, X., Li, Y., & Liu, X. (2022). A review of thermosensitive antinutritional factors in plant-based foods. *Journal of Food Biochemistry*, *46*(9), Article e14199. https://doi.org/10.1111/jfbc.14199

Krauchenco, S., Nagem, R. A. P., Silva, J. A., Marangoni, S., & Polikarpov, I. (2004). Three-dimensional structure of an unusual Kunitz (STI) type trypsin inhibitor from Copaifera langsdorffii. *Biochimie*, *86*(3), 167–172. https://doi.org/10.1016/j.biochi.2004.03.004

Laemmli, U. K. (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

Lang, G.-H., Kagiya, Y., & Kitta, K. (2015). Multiplex comparison of the digestibility of allergenic and non-allergenic proteins in rice grains by in vitro digestion. *Food Chemistry*, *168*, 606–614. https://doi.org/10.1016/j.foodchem.2014.07.089

Lang, Y., Li, B., Gong, E., Shu, C., Si, X., Gao, N., Zhang, W., Cui, H., & Meng, X. (2021). Effects of α-casein and β-casein on the stability, antioxidant activity and bioaccessibility of blueberry anthocyanins with an in vitro simulated digestion. *Food Chemistry*, *334*, Article 127526. https://doi.org/10.1016/j.foodchem.2020.127526

Lao, Y., Ye, Q., Wang, Y., Vongsvivut, J., & Selomulya, C. (2023). Quantifying the effects of pre-roasting on structural and functional properties of yellow pea proteins. *Food Research International*, *172*, Article 113180. https://doi.org/10.1016/j.foodres.2023.113180

Lee, S., Jo, K., Jeong, S.-K. C., Jeon, H., Kim, Y.-J., Choi, Y.-S., & Jung, S. (2024). Heat-induced gelation of egg white proteins depending on heating temperature: Insights into protein structure and digestive behaviors in the elderly in vitro digestion model. *International Journal of Biological Macromolecules, 262*(Part 2), Article 130053. https://doi.org/10.1016/j.ijbiomac.2024.130053

Luz, A. B. S., Medeiros, A. F., Medeiros, G. C. B. S., Piuvezam, G., Passos, T. S., & Morais, A. H. A. (2024). Experimental protocols used to mimic gastrointestinal protein digestion: a systematic review. *Nutrients*, *16*(15), Article 2398. https://doi.org/10.3390/nu16152398

Mangena, P. (2022). Pleiotropic effects of recombinant protease inhibitors in plants. *Frontiers in Plant Science*, *13*, Article 994710. https://doi.org/10.3389/fpls.2022.994710

Naser, E. H., Idries, A. H., Elmubarak, S. A. A., Dafalla, M. B., Abdelrahim, Y. E., Abdalrhman, E. A., Ahmed, B. M., Osman, M. E. M., Awadallah, A. K. E., Ebrahim, R. M. A., Abdellatif, A. O., Saad, H. A., & Konozy, E. H. E. (2024). Isolation, purification, and characterization of lectins from medicinal plant Combretum glutinosum seeds endowed with analgesic and antiulcer properties. *Biochimie*, *227*(Part A), 273–285. https://doi.org/10.1016/j.biochi.2024.08.003

Ohanenye, I. C., Ekezie, F.-G. C., Sarteshnizi, R. A., Boachie, R. T., Emenike, C. U., Sun, X., Nwachukwu, I. D., & Udenigwe, C. C. (2022). Legume Seed Protein Digestibility as Influenced by Traditional and Emerging Physical Processing Technologies. *Foods*, *11*(15), Article 2299. https://doi.org/10.3390/foods11152299

Olías, R., Rayner, T., Clemente, A., & Domoney, C. (2023). Combination of three null mutations affecting seed protein accumulation in pea (Pisum sativum L.) impacts positively on digestibility. *Food Research International*, *169*, Article 112825. https://doi.org/10.1016/j.foodres.2023.112825

Osborne, T. B. (1924). *Monographs on Biochemistry: The vegetable proteins* (2nd ed.). Longmans, Green and Co.

Pesoti, A., Oliveira, B. M., Oliveira, A. C., Pompeu, D. G., Gonçalves, D. B., Marangoni, S., Silva, J. A., & Granjeiro, P. A. (2015). Extraction, purification and characterization of inhibitor of trypsin from Chenopodium quinoa seeds. *Food Science and Technology*, *35*(4), 588–597. https://doi.org/10.1590/1678-457X.6655

Pompeu, D. G., Carvalho, A. S., Costa, O. F., Galdino, A. S., Gonçalves, D. B., Silva, J. A., & Granjeiro, P. A. (2014). Fatores antinutricionais e digestibilidade "in vitro" de folhas de Pereskia aculeata Miller. *BBR - Biochemistry and Biotechnology Reports*, *3*(1), 1–9. https://doi.org/10.5433/2316-5200.2014v3n1p1

Pompeu, D. G., Cordeiro, H. G., Tonelli, F. C. P., Godin, A. M., Melo, I. S. F., Matsui, T. C., Rodrigues, F. F., Silva, J. A., Coelho, M. M., Machado, R. R., & Granjeiro, P. A. (2022). Chenopodin as an anti-inflammatory compound. *Natural Product Research*, *36*(17), 4429–4432. https://doi.org/10.1080/14786419.2021.1980791

Pompeu, D. G., Mattioli, M. A., Ribeiro, R. I. M. A., Gonçalves, D. B., Magalhães, J. T., Marangoni, S., Silva, J. A., & Granjeiro, P. A. (2015). Purification, partial characterization and antimicrobial activity of Lectin from Chenopodium Quinoa seeds. *Food Science and Technology*, *35*(4), 696–703. https://doi.org/10.1590/1678-457X.6823

Pompeu, D.-G., Pompeu, L.-G., Policarpo-Tonelli, F.-C., Moreira-dos-Santos, D., da Silva, J.-A., Granjeiro, P. A., (2016). Extraction, Purification, partial Characterization and Antimicrobial Activity of a Protease Inhibitor from Albizia niopoides seeds. *International Journal of Advanced Scientific Research and Management*, *1*(1), 27–34. https://www.researchgate.net/publication/318531561_EXTRACTION_PURIFICATION_PARTIAL_CHARACTERIZATION_AND_ANTIMICROBIAL_ACTIVITY_OF_A_PROTEASE_INHIBITOR_FROM_Albizia_niopoides_SEEDS

Pulvento, C., Sellami, M. H., & Lavini, A. (2022). Yield and quality of Amaranthus hypochondriacus grain amaranth under drought and salinity at various phenological stages in southern Italy. *Journal of the Science Food and Agriculture*, *102*(12), 5022–5033. https://doi.org/10.1002/jsfa.11088

Rawdkuen, S., D'Amico, S., & Schoenlechner, R. (2022). Physicochemical, Functional, and In Vitro Digestibility of Protein Isolates from Thai and Peru Sacha Inchi (*Plukenetia volubilis* L.) Oil Press-Cakes. *Foods*, *11*(13), Article 1869. https://doi.org/10.3390/foods11131869

Resendiz-Otero, M. F., Bernardino-Nicanor, A., Lugo-Magaña, O., Betanzos-Cabrera, G., González-Cruz, L., Morales-González, J. A., Acosta-García, G., Fernández-Martínez, E., Salazar-Campos, A., & Valadez-Vega, C. (2024). Purification, Structural Characterization, and Bioactivity of *Amaranthus hypochondriacus* Lectin. *Molecules*, *29*(21), Article 5101. https://doi.org/10.3390/molecules29215101

Rodríguez-Sifuentes, L., Marszalek, J. E., Chuck-Hernández, C., & Serna-Saldívar, S. O. (2020). Legumes Protease Inhibitors as Biopesticides and their Defense Mechanisms against Biotic Factors. *International Journal of Molecular Sciences*, *21*(9), Article 3322. https://doi.org/10.3390/ijms21093322

Samtiya, M., Aluko, R. E., & Dhewa, T. (2020). Plant food anti-nutritional factors and their reduction strategies: an overview. *Food Production, Processing and Nutrition, 2*(1), Article 6. https://doi.org/10.1186/s43014-020-0020-5

Silva, J. A., Pompeu, D. G., Costa, O. F., Gonçalves, D. B., Spehar, C. R., Marangoni, S., & Granjeiro, P. A. (2015). The importance of heat against antinutritional factors from Chenopodium quinoa seeds. *Food Science and Technology*, *35*(1), 74–82. https://doi.org/10.1590/1678-457X.6427

Silva, J. A., Pompeu, D. G., Smolka, M. B., Gozzo, F. C., Comar Jr., M., Eberlin, M. N., & Granjeiro, P. A., & Marangoni, S. (2015). Primary Structure of a Trypsin Inhibitor (Copaifera langsdorffii Trypsin Inhibitor-1) Obtained from C. langsdorffii Seeds. *Journal of Biomolecular Techniques*, *26*(3), 90–102. https://doi.org/10.7171/jbt.15-2603-002

Silva, J. K., Veras, A. C. C., Sousa, S. M., Albuquerque, J. S. S., Ribeiro, F. P. B., Lima, N. K. S., Nascimento, L. B. S., Alves, R. R. V., Aires, R. S., Coelho, L. C. B. B., Napoleão, T. H., Paiva, P. M. G., Paixão, A. D., & Vieira, L. D. (2024). The water extract and the lectin WSMoL from the seeds of Moringa oleifera prevent the hypertension onset by decreasing renal oxidative stress. *Anais da Academia Brasileira de Ciências*, *96*(4), Article e20231266. https://doi.org/10.1590/0001-3765202420231266

Silvestrini, V. C., Gonçalves, D. B., Granjeiro, P. A., Silva, J. A. (2017). Anti-nutritional factors and digestibility of protein in Cayocar brasiliense seeds. *Food Science and Technology*, *37*(4), 632–639. https://doi.org/10.1590/1678-457X.28716

Souza, P. F. N. (2020). The forgotten 2S albumin proteins: Importance, structure, and biotechnological application in agriculture and human health. *International Journal of Biological Macromolecules*, *164*, 4638–4649. https://doi.org/10.1016/j.ijbiomac.2020.09.049

Sun, N., Liu, Y., Liu, K., Wang, S., Liu, Q., & Lin, S. (2022). Gastrointestinal fate of food allergens and its relationship with allergenicity. *Comprehensive Reviews in Food Science and Food Safety*, *21*(4), 3376–3404. https://doi.org/10.1111/1541-4337.12989

Tang, J., Wichers, H. J., & Hettinga, K. A. (2022). Heat-induced unfolding facilitates plant protein digestibility during in vitro static infant digestion. *Food Chemistry*, *375*, Article 131878. https://doi.org/10.1016/j.foodchem.2021.131878

Van Damme, E. J. (2014). History of plant lectin research. In J. Hirabayashi (Ed.), *Lectins: Methods and Protocols* (Volum 1200, pp. 3–13). Humana Press. https://doi.org/10.1007/978-1-4939-1292-6_1

Vasconcelos, I. M., & Oliveira, J. T. A. (2004). Antinutritional properties of plant lectins. *Toxicon*, *44*(4), 385–403. https://doi.org/10.1016/j.toxicon.2004.05.005

Vishvakarma, R., & Mishra, A. (2022). Characterization of a Novel Protease Inhibitor from the Edible Mushroom Agaricus bisporus. *Protein and Peptide Letters*, *29*(5), 460–472. https://doi.org/10.2174/0929866529666220405161903

Wang, K., Crevel, R. W. R., & Mills, E. N. C. (2022). Assessing protein digestibility in allergenicity risk assessment: A comparison of in silico and high throughput in vitro gastric digestion assays. *Food and Chemical Toxicology*, *167*, Article 113273. https://doi.org/10.1016/j.fct.2022.113273

Williamson, E., Ross, I. L., Wall, B. T., & Hankamer, B. (2024). Microalgae: potential novel protein for sustainable human nutrition. *Trends in Plant Science*, *29*(3), 370–382. https://doi.org/10.1016/j.tplants.2023.08.006

Zhang, J., Wang, J., Li, M., Guo, S., & Lv, Y. (2022). Effects of heat treatment on protein molecular structure and in vitro digestion in whole soybeans with different moisture content. *Food Research International*, *155*, Article 111115. https://doi.org/10.1016/j.foodres.2022.111115

Zhang, K., Wen, Q., Wang, Y., Li, T., Nie, B., & Zhang, Y. (2022). Study on the in vitro digestion process of green wheat protein: Structure characterization and product analysis. *Food Science and Nutrition*, *10*(10), 3462–3474. https://doi.org/10.1002/fsn3.2947

Zhou, H., Tan, Y., & McClements, D. J. (2023). Applications of the INFOGEST in vitro digestion model to foods: a review. *Annual Review Food Science and Technology*, *14*, 135–156. https://doi.org/10.1146/annurev-food-060721-012235

Zhu, F. (2023). Amaranth proteins and peptides: Biological properties and food uses. *Food Research International*, *164*, Article 112405. https://doi.org/10.1016/j.foodres.2022.112405