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PRODUCTION OPTIMIZATION OF ORA-PRO-NÓBIS PROTEIN HYDROLYSATE FOR FOOD PRODUCTS USE

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ABSTRACT:
Despite the vast biodiversity of edible plant species, humans utilize only about one thousand species. Plants that are unknown, underutilized, and have one or more edible parts are referred to as Non-Conventional Edible Plants (NCEPs). Among them, *Ora-pro-nobis* (OPN) stands out for its high protein content. This study aimed to perform the physicochemical characterization of flour derived from two *Ora-pro-nobis* species, namely *Pereskia aculeata Mill* (PAM) and *Pereskia grandifolia Haw* (PGH). The species were analyzed for moisture, protein, lipids, crude fiber, and ash content. The flour from the species with the highest protein content was subjected to enzymatic hydrolysis. To optimize the production of the protein hydrolysate, a Rotatable Central Composite Design (RCCD) was adopted, evaluating the independent variables time, temperature, and enzyme concentration. The dependent variables analyzed were the degree of hydrolysis and the percentage of free radical scavenging activity. The present study shows that OPN presents high levels of protein, fiber, and minerals, indicating that this plant source has potential to contribute to the diet to meet the recommended daily intake of these nutrients. However, enzymatic hydrolysis with bromelain had no significant effect on the degree of hydrolysis or free radical scavenging percentage, indicating that the model was not predictive for this experiment. Therefore, further studies on the nutritional analyses of *Ora-pro-nobis* are suggested to enhance its potential for developing new products, food enrichment, and formulations as a substitute for proteins and nutrients derived from animal products.

Keywords: *Pereskia aculeata* Mill., *Pereskia grandifolia* Haw., hydrolysis, bromelain.

OTIMIZAÇÃO DA PRODUÇÃO DE HIDROLISADO PROTEICO DE ORA-PRO-NÓBIS PARA INSERÇÃO EM PRODUTOS ALIMENTÍCIOS

RESUMO:
Apesar da vasta biodiversidade de espécies de plantas alimentícias, o homem utiliza apenas cerca de mil espécies. As plantas que não conhecemos, não produzimos, consumimos pouco e que possuem uma ou mais partes comestíveis são denominadas Plantas Alimentícias não Convencionais (PANCs). Dentre elas, destaca-se a Ora-pro-nóbis (OPN), que possui altos valores proteicos. Esse estudo teve como objetivo fazer a caracterização físico-química da farinha proveniente de duas espécies de OPN, sendo estas a *Pereskia aculeata* Mill (PAM) e a *Pereskia grandifolia* Haw (PGH). As espécies foram analisadas quanto ao teor de umidade, proteína, lipídios, fibras brutas e cinzas. A farinha da espécie com maior teor de proteína foi submetida à hidrólise enzimática. Para a otimização da obtenção do hidrolisado proteico foi adotado o delineamento estatístico de composição central rotacional (DCCR), avaliando as variáveis independentes tempo, temperatura e concentração enzimática. Já as variáveis dependentes analisadas foram o grau de hidrólise e o percentual de sequestro de radicais livres. O presente estudo mostra que a OPN apresenta altos

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teores de proteínas, fibras e minerais, evidenciando que essa fonte vegetal tem potencial para contribuir na dieta para atingir as recomendações de ingestão diária destes nutrientes. No entanto, da obtenção do hidrolisado proteico com a enzima bromelina não teve efeito para o percentual de grau de hidrólise e para o percentual de sequestro de radicais livres indicando que o modelo não foi preditivo para este experimento. Então, sugere-se a continuidade de estudos, acerca das análises nutricionais da ora-pro-nóbis, viabilizando o melhoramento do potencial no desenvolvimento de novos produtos, com enriquecimento de alimentos e formulações, como substituto no consumo de proteínas e nutrientes advindos de produtos de origem animal.

Palavras-chave: *Pereskia aculeata* Mill., *Pereskia grandifolia* Haw, hidrólise, bromelina.

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INTRODUCTION

Globally, over 390,000 plant species are known (RBG, 2017), yet despite this enormous biodiversity, humanity uses only approximately one thousand species as food (FAO, 2018). Among these, 15 species predominantly account for about 90% of the global diet (Biondo et al., 2018). Mirroring these global patterns, Brazil possesses a vast biodiversity of plants with significant potential and beneficial properties for human consumption, yet they remain largely underexplored. These are thus called Non-Conventional Edible Plants (NCEP, or PANCs in Portuguese).

According to Kinupp (2007), NCEPs are defined as plants that are not widely known, cultivated, or consumed on a large scale, yet possess one or more edible parts. NCEPs serve to diversify diets, contribute to local and regional economies (Nesbitt, 2010), and are associated with environmental conservation, sustainability, and agroecology.

Among the NCEPs, *Ora-pro-nóbis* (OPN) is particularly noteworthy. Taxonomically, it is classified within the Class Magnoliopsida, Order Caryophyllales, Family Cactaceae, and Genus *Pereskia*, which is considered one of the least evolved genera within the family (Mauseth, 1999). OPN species possess well-developed woody stems, flattened and succulent leaves, and terminal flowers arranged in cymes (Barroso, 1978). The genus comprises 17 species, which can manifest as either foliaceous shrubs or arboreal plants (Edwards & Donoghue, 2006). Originating from temperate and tropical regions of Central and South America, OPN is found throughout Brazil, from the Northeast to the South (Takeiti et al., 2009). Its consumption is most widespread in the state of Minas Gerais (Oliveira et al., 2013; Dias et al., 2005), where it is primarily associated with gastronomic tourism (Kinupp, 2006; Lima Junior et al., 2013).

Studies conducted with *Pereskia aculeata* have identified that the protein content found in its leaves can vary from 17.40%–28.59% (Almeida et al., 2014; Rocha et al., 2008; Takeiti et al., 2009). This range allows it to be considered a high-protein food source, in accordance with Brazilian Health Regulatory Agency Resolution No. 54 (Brasil, 2012).

Zappi et al. (2012) classifies OPN as a non-endemic native plant, describing it as a non-conventional vegetable (Brasil, 2002, 2010a). Despite being a spontaneous plant, it holds potential for sustainable production, both in family farming (Souza et al., 2013) and in dense cultivation, aiming to increase productivity through good agricultural practices (Madeira et al., 2016). OPN is considered easy to cultivate and propagate, needs low water demand, and presents very few phytosanitary problems, such as pest attacks, thus favoring its cultivation (Madeira and Silveira, 2010).

Easily cultivated, managed, and adapted to various soil types (Brasil, 2010b), coupled with its high contents of protein, fiber, vitamins, and minerals, the succulent and edible leaves of *Ora-pro-nóbis* (OPN) have piqued the interest of both the food industry and the general population. This interest extends to its use both as a dehydrated raw material and for direct consumption in various culinary applications, such as salads, stir-fries, soups, omelets, and other cooked preparations.

This interest has led to the publication of studies on OPN's use in various food preparations, aiming to enhance nutritional value. Examples include the development of vegetarian burgers with added OPN leaf proteins (Santos, 2019), the addition of OPN flour to beef burgers (Ziegler et al., 2020), the production of fermented beverages (Pocai, 2016), its incorporation into commonly consumed preparations like pasta dough (Rocha, 2008), bread dough (Martinevski et al., 2011), and cake batter (Paula et al., 2016), as well as the elaboration of extruded corn snacks (Francelin et al., 2021).

Beyond the *in natura* (fresh) and processed consumption of OPN as a protein source, research on other plant sources, such as chia, flaxseed, amaranth, sunflower, cupuaçu, okra, and quinoa seeds, demonstrates enhanced protein potential, biological activity, and amino acid profiles when subjected to enzymatic hydrolysis (Aluko & Monu, 2003; Silva-Sanchez, 2008; Megías et al., 2009; Fritz et al., 2011; Segura-Campos et al., 2013; Silva, 2012; Cruz, 2014; Nascimento, 2015).

Feijoo-Siota (2018) indicates that OPN contains a significant amount of protease. This enzyme is essential for protein digestion and reduction into amino acids, which in living organisms participate in metabolic and cellular signaling pathways (Silva, 2013). Proteases are widely used in the biotechnology industry, accounting for 60% of global commercial use (Li et al., 2016; Fernández-Lucas et al., 2017).

To enhance systemic absorption, plant proteases such as Papain and Bromelain cleave internal peptide bonds within proteins through enzymatic hydrolysis (Pavan et al., 2012; Romanova and Sweedler, 2015). This process converts them into smaller amino acid units (Carreira et al., 2003), which are physiologically superior compared to intact proteins (Smith et al., 1975; Grimble et al., 1986; Ziegler et al., 1999; Shimamura et al., 1999). In the food industry, enzymatic hydrolysis is employed for its nutritional applicability, owing to its technological properties. It is used in the supplementation of products such as cookies, cereal bars, and burgers, adding functional characteristics such as osmotic balance, hypoallergenicity, pleasant taste, and modification of food properties, such as high protein absorbability. This process assists diets for individuals with protein digestion and malabsorption deficiencies, given their high digestibility potential (Guadix et al., 2000; Furlan and Oetterer, 2002; Carreira et al., 2004; Martins, 2005; Moraes & Colla., 2006; Parra, 2009; Elsohaimy et al., 2015).

Enzymatic hydrolysis offers several advantages, including precise control over the degree of hydrolysis, moderate operating conditions, large-scale commercial availability, moderate cost, lower salt content in the final product, and minimal byproduct formation (Mannheim & Cheryan, 1992; Pearce, 1995). This contrasts with chemical hydrolysis, which involves a difficult process to control, yields products with diminished nutritional quality, and can lead to the formation of toxic substances like lysinoalanine (Lahl & Braun, 1994; Clemente, 2000).

Specific fragments derived from enzymatic protein hydrolysis form bioactive peptides due to the specificity of the protease. These peptides generate diverse amino acid sequences that positively impact bodily functions, aiding in health maintenance and influencing various physiological systems by acting as antioxidant agents and reducing oxidative processes in the body (Meisel & Fitzgerald, 2003; Singh et al., 2014).

In addition to its socioeconomic, tourism, and environmental potential, OPN is notable for its nutritional profile, particularly its high content of protein, fiber, minerals, and vitamins. Its extract exhibits a significant quantity of phenolic compounds, indicating high antioxidant activity (Garcia et al., 2019), high digestibility, and a high content of essential amino acids such as lysine (Mercê et al., 2001; Conceição et al., 2014). Furthermore, OPN has not shown toxicity (Silva et al., 2017).

The scientific community shows a clear and growing interest in prospecting plant species, primarily driven by the current scenario of food insecurity caused by exponential population growth and the depletion of natural resources. Thus, optimizing the production of high-protein foods that enable low environmental impact proves to be a nutritional alternative to enrich the population's diet in a responsible and environmentally sustainable manner (Martinelli & Cavalli, 2019).

Despite several studies highlighting relevant characteristics regarding OPN's nutritional and antioxidant potential, coupled with increasing interest from the food industry, it is observed that its full potential has not yet been explored. Specifically, there are no studies in the literature on the optimization of OPN protein hydrolysis using bromelain enzyme (Alves, 2020). Within this context, this study aimed to optimize the production of OPN protein hydrolysate using the commercial enzyme bromelain, preceded by the physicochemical characterization of flour from two OPN species: *Pereskia aculeata* (PAM) and *Pereskia grandifolia* (PGH).

MATERIALS AND METHODS

The raw materials for this study were collected in the municipalities of Palmas and Porto Nacional, in the State of Tocantins, Brazil. Processing and analyses were conducted at the Food Technology Laboratory (Nutrition Course, Nutrition Ambulatory Complex) and the Food Analysis Laboratory (Food Engineering Course), both located at the Federal University of Tocantins (UFT). Figure 1 provides a simplified description of the experimental procedures conducted in this study.

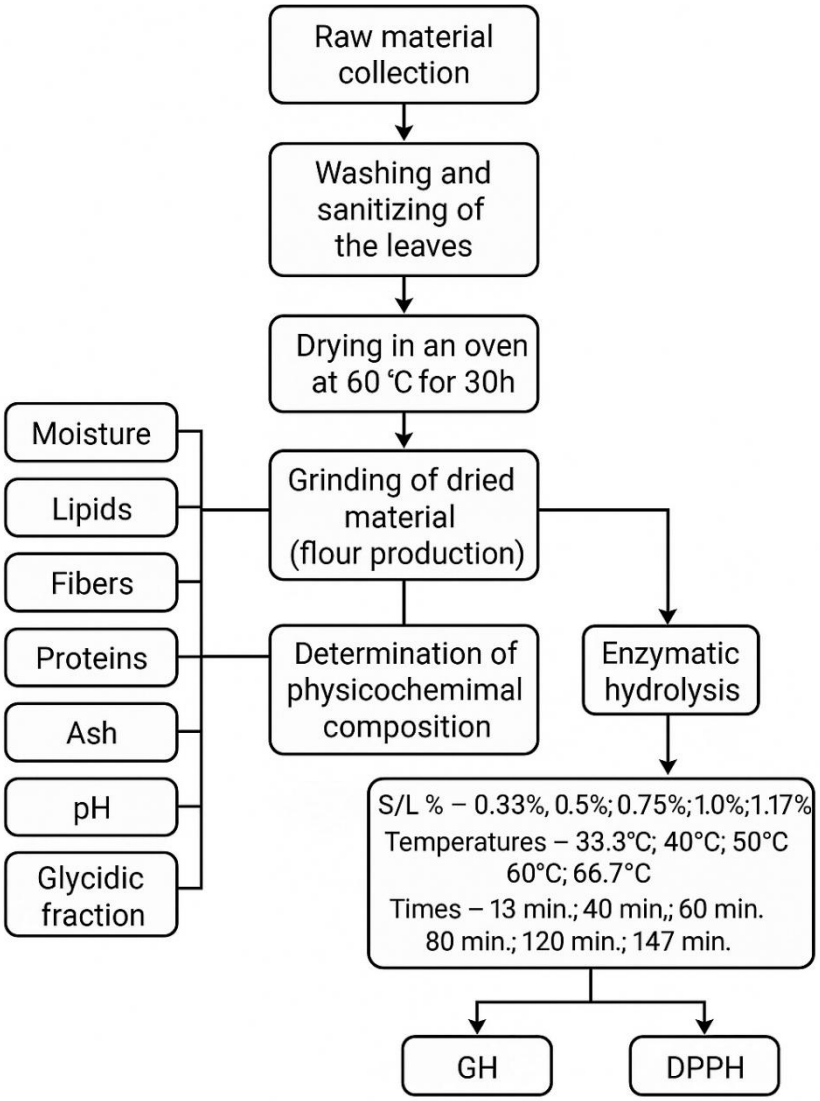

Figure 1. Flowchart of the procedures for physicochemical analyses and enzymatic hydrolysis of Ora-pro-nobis (OPN).

For the development of this research, *Ora-pro-nóbis* (OPN) leaves from the species *Pereskia aculeate Mill* (PAM) (Figure 2) and *Pereskia grandifolia Haw* (PGH) (Figure 3) were collected from different locations. In all collections, leaves were randomly harvested, intact (including blade and petiole), from various parts of the plant, with attention to size, coloration, and absence of injuries.

After collection, the leaves were washed three times with running water and sanitized with hypochlorite (50 ppm). They were then dried at room temperature with the aid of paper towels, packed in low-density polyethylene plastic bags, and stored under refrigeration at 2 ± ºC.

For the preparation of OPN flour, the leaves were dehydrated in a forced-air oven at 60 ºC for 30 hours. They were then ground in a blender until a homogeneous, flour-like granular material was obtained. Three replicates were used for the physicochemical and antioxidant analyses of each species.

Figure 2. *Ora-pro-nobis*, *Pereskia aculeata*: climbing plant (A), leaf pruning (B), flower (C).

Figure 3. *Ora-pro-nobis*, *Pereskia grandifolia*: shrub (A and B), flower (C).

Physicochemical Analyses of *Ora-pro-nóbis* Flour

For moisture determination, approximately 10 g of the whole sample (raw material) and approximately 5 g of the flour sample were weighed on an analytical balance into pre-dried Petri dishes. These were then placed in an oven at 60 °C for about 30 hours until constant weight was achieved. Subsequently, the dried samples in the dishes were weighed, and the dried samples were stored in moisture-free plastic bags. The difference between the initial and final weights corresponded to the moisture content (AOAC, 2000). The analysis was performed in triplicate.

For the determination of OPN ethereal extract, approximately 3 g of the dried sample were weighed into a cellulose thimble, which was then placed in a pre-dried distillation flask (reboiler), and hexane solvent was added to the flask until the sample was submerged. Subsequently, the flask was coupled to the heating mantle of a Soxhlet apparatus at 70 °C, remaining under reflux for approximately 3 hours. After this interval, the thimble was suspended above the hexane level for an additional 3 hours to ensure solvent removal. Following the evaporation of hexane from the flask, it was transferred to an oven at 105 °C until constant weight was achieved (AOAC, 2000).

For protein determination, 100 mg of defatted dry matter, wrapped in filter paper, was transferred to a digestion tube. To this, 600 mg of K_2SO_4, 300 mg of $CuSO_4$, and 5 mL of H_2SO_4 were added. The tube was placed in a digestion block, with the temperature gradually increasing by 50 °C increments until it reached 400 °C and the sample became colorless. Subsequently, the digested sample tube was connected to a Kjeldahl apparatus, and an Erlenmeyer flask containing 10 ml of boric acid was fitted to the condenser outlet. Then, 15 mL of NaOH were added to the appropriate reservoir, slowly

dripping into the previously connected tube. The temperature was activated to boil the water in the boiler, which carried the ammonia into the Erlenmeyer flask containing boric acid. Subsequently, 100 mL of the condensate were collected in the Erlenmeyer flask for titration with HCl until the color changed from green to red. The nitrogen content of the sample was then calculated and converted to protein using a conversion factor of 6.25 (AOAC, 2000).

For ash determination, approximately 2.0 g of a pre-dried sample (dried in an oven) were weighed. The crucible was weighed before and after sample addition to determine the initial sample weight. After weighing, the samples were placed in a muffle furnace at 550 °C for 4 hours until white or light gray ash was obtained. After cooling in a desiccator, the samples were weighed (AOAC, 2000).

Crude fiber content was determined by acid and basic digestion and filtration of the samples contained in a non-woven fabric bag (AOAC, 2000). Meanwhile, the carbohydrate fraction of the samples was determined by difference, according to Equation 1: % Carbohydrate = 100 - [% moisture + % ethereal extract + % protein + % crude fiber + % ash fraction], calculated on a total sample basis. The pH was determined using a digital potentiometer according to the AOAC (1992) method.

OPN Hydrolysate Production and Optimization

Hydrolysate production was carried out according to the methodology proposed by Paiva et al. (2015), with some adaptations. For the optimization of protein hydrolysate production, a Rotatable Central Composite Design (RCCD) was adopted, as described in Table 1.

Table 1. Factors and levels tested for the central composite design with axial points.

Factors	Lower axial point (-1,68)	Lower level (-1)	Intermediate level (0)	Higher level (+1)	Higher axial point (+1,68)
Time	13.00	40.00	80.00	120.00	147.00
Temperature	33.30	40.00	50.00	60.00	66.70
[ ] Enzyme/Substrate (%)	0.33	0.50	0.75	1.00	1.17

[E]:[S] % = Enzyme: substrate ratio (g protein/g protein).

The OPN substrate was weighed and placed in a Falcon tube, then homogenized with distilled water at a 1:10 ratio (OPN solids:water, w/v). The enzyme was added after temperature adjustment, at an enzyme protein to substrate protein ratio. Enzymatic hydrolysis was performed in a water bath under constant agitation, following a complete 2^3 factorial design with three repetitions at the central point. The study considered the degree of hydrolysis and antioxidant activity as dependent variables, taking into account the influence of independent variables (hydrolysis time, temperature, and enzyme concentration), totaling 17 assays, as presented in Table 2. The enzymatic reaction was stopped by boiling at 90 °C for 15 minutes.

After enzyme inactivation, samples were centrifuged at 4000 rpm for 10 minutes to separate the soluble and insoluble fractions. The supernatant was then transferred to Eppendorf tubes and stored in a freezer for subsequent analyses of hydrolysis degree and antioxidant activity.

Table 2. Coded and actual values of the assays for enzymatic hydrolysis using the commercial enzyme bromelain.

Assays	Time (min)		Temperature °C		E/S (%)	
	Cod	Real	Cod	Real	Cod	Real
1	-1	40	-1	40	-1	0.50
2	-1	40	-1	40	1	1.00
3	-1	40	1	60	-1	0.50
4	-1	40	1	60	1	1.00
5	1	120	-1	40	-1	0.50
6	1	120	-1	40	1	1.00
7	1	120	1	60	-1	0.50
8	1	120	1	60	1	1.00
9	-1.68	13.0	0	50	0	0.75
10	1.68	147.0	0	50	0	0.75
11	0	80	-1.68	33.3	0	0.75
12	0	80	1.68	66.7	0	0.75
13	0	80	0	50	-1.68	0.33
14	0	80	0	50	1.68	1.17
15	0	80	0	50	0	0.75
16	0	80	0	50	0	0.75
17	0	80	0	50	0	0.75

E/S (%): Enzyme: substrate ratio (g protein/g protein).

Chemical Analyses of OPN Hydrolysate

For this analysis, the OPA (*o*-phthalaldehyde) reagent was prepared according to Church et al. (1983). For the derivatization reaction, a modified method from Spellman et al. (2003) was employed. Specifically, 10 µl of sample and 120 µl of distilled water were mixed with 1 ml of OPA reagent. This mixture was left to stand at 25 °C for two minutes. Subsequently, absorbance was measured at 340 nm.

For the determination of total antioxidant activity by the DPPH (2,2-diphenyl-1-picrylhydrazyl) method, the soluble fraction of the hydrolysate was used. In each assay, 0.1 mL of sample was added to 3.9 mL of DPPH solution, according to the methodology proposed by Rufino et al. (2007), with some modifications. The percentage of DPPH radical scavenging was calculated relative to the control sample, in which 0.1 mL of distilled water was used with the addition of 3.9 mL of DPPH solution.

Readings were performed after 120 minutes using a spectrophotometer at 515 nm. Results were obtained according to Equation 2: % Radical Scavenging Activity (RSA) = (Ac – Am) × 100 /Ac. Where: Ac = absorbance of the control; Am = absorbance of the sample.

Statistical Analysis

For the physicochemical characterization of the flours, a completely randomized design was adopted, involving two species (*Pereskia aculeata* Mill and *Pereskia grandifolia* Haw), and evaluated using three replicates. For the statistical analysis of these data, Student's t-test with a 95% confidence interval was applied, using SISVAR software (Ferreira et al., 2011).

For the production of OPN protein hydrolysates, a Rotatable Central Composite Design (RCCD) was adopted. The F-test was performed with the application of Analysis of Variance (ANOVA) to determine the level of significance between samples, using STATISTICA 8.0 statistical software.

RESULTS AND DISCUSSION

Physicochemical Composition of Raw Materials

The moisture content of the two OPN species was analyzed in both the leaves and the obtained flour, as explained previously. *Pereskia aculeata* (PAM) showed a higher moisture content than *Pereskia grandifolia* (PGH) in both forms, as demonstrated in Table 3.

Table 3 also shows the expected difference in moisture content when comparing leaves and flour, as fresh OPN leaves naturally contain a large amount of water. However, no statistically significant difference was observed when comparing the moisture content of the two species in either their leaf or flour forms.

Table 3 - Mean values and standard deviation of moisture of OPN leaves and flours. *.

Species	**Leaves**	**OPN flour**
Pereskia aculeata Mill.	88.94±0.05[a]	12.22±0.05[a]
Pereskia grandifolia Haw.	87.77±0.11[a]	11.01±0.11[a]

Mean of triplicates. Different superscript letters in the same column indicate significant differences according to Student's t-test ($p \leq 0.05$).

High moisture values in the leaves (88.94% for PAM and 87.77% for PGH) indicate that OPN contains a large amount of water, which increases the volume of mucilage. This is an important factor, considering that mucilage is rich in polysaccharides and hydrocolloids and is used as an intestinal transit regulator, emollient, and thickener (Ferreira & Mastro, 2019), making OPN important for food consumption and for its use in the cosmetics industry (Monteiro, 2009).

According to the Brazilian Food Guide for the Population, water intake should predominantly come from direct water consumption or from water contained in food and culinary preparations (Brasil, 2014). In this sense, the water content of fresh OPN, when compared to conventional vegetables such as lettuce (95.81%) and kale (91.58%) (Pereira et al., 2015), demonstrates to be a good strategy to meet daily fluid intake goals, promoting the functioning of physiological processes like digestion, absorption, and excretion, and optimizing the composition of body tissues (Krause and Mahan, 2005).

Regarding the moisture content of the flour, the values found were 12.22% for *Pereskia aculeata* (PAM) and 11.01% for *Pereskia grandifolia* (PGH). These values are similar to the 12.46% and 10.94% reported by Almeida (2014) and the 12.04% and 14.41% by Guimarães (2018). The obtained values also indicate that OPN flour complies with Brazilian legislation, which establishes a maximum moisture limit of 15% for whole flours (Brasil, 2005). Moisture levels above this limit can lead to physical instability, microbial growth, and undesirable biochemical changes (Auris et al., 2012). It is worth noting that moisture content is crucial for product quality, commercial value, and shelf-life control (Moreta, 2015).

Regarding crude fiber content, the values found for *Pereskia grandifolia* (PGH) were significantly higher than those found for *Pereskia aculeata* (PAM), as described in Table 4. According to the results, *Pereskia grandifolia* (PGH) showed significantly higher crude fiber values. It is worth noting that the crude fiber values found in OPN species are higher when compared to those found in some conventional vegetables such as broccoli (4.63%), collard greens (3.1%), and spinach (2.1%) (TACO/NEPA, 2011). Studies on five *Pereskia aculeata* matrices reported crude fiber contents ranging from 11.4% to 16.0% (Magalhães, 2011), values similar to those obtained in this study.

Table 4 - Mean values and standard deviation of fiber and ash in OPN flours.*.

Composition	*Pereskia aculeata*	*Pereskia grandifolia*
Fibers	11.57±0.22[b]	14.86±3.15[a]
Ashes	19.54±0.02[a]	20.03±0.21[a]

*Different superscript letters in the same row differ significantly according to Student's t-test ($p \leq 0.05$).

The human body excretes 20 to 30 g of minerals daily and requires immediate replenishment through diet (Franco, 2004). Mineral sources are widely distributed in nature and perform indispensable functions in the human body. The present study indicated that the total ash content in the flour of both OPN species is considerably high. However, they did not differ statistically, revealing that the analyzed species possess high mineral levels.

The average ash content was 19.54% for PAM and 20.03% for PGH. These values are higher than those reported in studies by Almeida (2014), who found 14.81% for PAM and 12.57% for PGH; by Silveira (2015), who reported 16.01% for PGH; and by Santana, who reported 15.23% for PAM.

Kinupp and Barros (2008) reported that no ash contents higher than those of the studied species were found among Non-Conventional Food Plants (PANCs), nor in some conventional vegetables such as iceberg lettuce (9.1%) and broccoli (7.2%). Therefore, plants of the genus *Pereskia* are relevant sources of minerals.

The ash content obtained in this study reveals a great potential for minerals. Other studies have reported high iron content in OPN, with values ranging from 20.56 mg/100 g to 47.81 mg/100 g (Maciel et al., 2021). Therefore, OPN leaves can provide a high iron content, considering the FAO/WHO dietary recommendation for adults of 14 mg day^{-1}.

Thus, the total mineral content, defined by the ash content found in OPN, determines its nutritional value as a key factor of mineral constituents in food and is of great relevance for consumption, considering that *Pereskia aculeata* and *Pereskia grandifolia* species are already consolidated mineral sources in scientific literature (Takeiti et al., 2009).

Regarding the average protein contents, PAM showed significantly higher values (23.32 g/100 g) than those found for PGH (14.01 g/100 g) (Table 5). Some authors reported similar protein values on a dry matter basis for this vegetable. Guimarães (2018) found 18.25 g/100 g for PAM and 24.19 g/100 g for PGH in samples, while Almeida (2014) observed higher contents of 28.99 g/100 g and 32.02 g/100 g for PAM and PGH, respectively.

Table 5 - Mean values and standard deviation of proteins, lipids, and carbohydrates of OPN flours. *.

Composition	*Pereskia aculeata*	*Pereskia grandifolia*
Proteins	23.32±0.93[a]	14.01±1.95[b]
Lipids	2.05±0.07[b]	3.56±0.09[a]
Carbohydrates	45.22±0.74[b]	50.71±1.42[a]

* Different superscript letters in the same row differ significantly according to Student's t-test ($p \leq 0.05$).

Studies report that protein contents in OPN leaves are directly related to the physiological age of the plant, botanical origin, and soil composition, with clayey texture recommended for cultivation (Mazia & Sartor, 2012; Sousa et al., 2014).

According to Regulation No. 75/20 from the Ministry of Health, food can be considered a source of protein when it provides at least 10% of the daily recommended intake per 100 g, and it is considered rich in protein when it provides at least 20% of the

daily recommended intake per 100 g (Brasil, 2020). Therefore, the use of the analyzed species as a protein source is viable in its dry form, where the cactaceae showed a high protein content.

OPN flour can be used to diversify diets, serving as a supplement, especially since proteins are predominantly found with higher percentages in animal-derived products. Thus, the protein source from this non-conventional vegetable is an inexpensive and abundant alternative for consuming these nutrients, particularly for individuals with different dietary habits and low purchasing power (Kinupp & Barros, 2008).

Regarding the average lipid values, significantly higher values were found in the PGH species. Almeida et al. (2014) also reported average values of 5.07 g/100 g and 6.72 g/100 g for PAM and PGH, respectively. Conversely, Rocha et al. (2008) and Takeiti et al. (2009) found higher lipid values in PAM, specifically 3.64 g/100 g and 4.1 g/100 g, respectively. Such differences may occur due to variations in water supply or soil management conditions (Queiroz et al., 2015), whereas seasonality does not influence the lipid fraction (Vargas, 2017). Due to these low lipid values, Rocha et al. (2008) and Rodrigues et al. (2016) suggest the use of OPN in hypocaloric and low-fat diets.

Carbohydrates in this study were determined by subtracting the sum of moisture, lipids, ash, proteins, and crude fiber from 100. PGH showed significantly higher carbohydrate values than PAM, at 50.71% and 45.22%, respectively. These values are considered high when compared to the results obtained by Almeida (2014), which were 29.53% for PAM and 29.86% for PGH. However, they are close to those reported by Vargas et al. (2017) for PAM at 43.04% in winter and 48.47% in summer. In contrast, Zem et al. (2019) found a percentage of 7.17%, a value lower than those reported by the aforementioned authors.

The pH is a relevant parameter for selecting microbial presence and chemical interactions, defining the rigor of industrial treatments and influencing conservation, thus playing a significant role in the chemical processes that occur in foods (Leitão, 1991). The pH values obtained in the present study for OPN samples were 5.92 for PAM and 6.74 for PGH. Guimarães (2013) also found similar pH values in PAM (5.27) and PGH (6.67), while Trennepohl (2016) reported a pH of 4.89 for PAM, which was more acidic compared to the values obtained in this study.

Optimization of Enzymatic Hydrolysis Conditions

Following the physicochemical characterization results of the two OPN species, *Pereskia aculeata* (PAM) was chosen for further analysis to obtain the protein hydrolysate, considering its higher protein content compared to *Pereskia grandifolia* (PGH).

For hydrolysis, the commercial enzyme Bromelain was used. Bromelain is a proteolytic enzyme found in pineapple and other plant species of the Bromeliaceae family (Raml et al., 2018; Rojas et al., 2018), capable of cleaving internal peptide bonds of proteins, thus being classified as an endopeptidase (Romanova & Sweedler, 2015). This capability favors the generation of peptides with different amino acid sequences and positively influences bioactivity (Joana Gil-Chávez et al., 2013).

Table 6 presents the values for Degree of Hydrolysis (DH) and Free Radical Scavenging (FRS), obtained after executing the Rotatable Central Composite Design (RCCD).

Table 6 - Percentage values of degree of hydrolysis (DH%) and antioxidant activity (RSA%) obtained after enzymatic activity of the commercial enzyme bromelain on OPN.*

Assays	Time (min)		Temperature ºC		E/S (%)		DH (%)	RSA (%)
	Cód.	Real	Cód.	Real	Cód.	Real	Bromelain	
1	-1	40	-1	40	-1	0.5	7.76[b]	81.76[a]
2	-1	40	-1	40	1	1.0	2.42[d]	81.93[a]
3	-1	40	1	60	-1	0.5	14.32[a]	81.20[a]
4	-1	40	1	60	1	1.0	4.76[c]	81.01[a]
5	1	120	-1	40	-1	0.5	8.57[b]	81.72[a]
6	1	120	-1	40	1	1.0	2.62[d]	82.29[a]
7	1	120	1	60	-1	0.5	9.64[b]	82.69[a]
8	1	120	1	60	1	1.0	4.02[c]	83.17[a]
9	-1.68	13.0	0	50	0	0.75	3.52[c]	81.52[a]
10	1.68	147.0	0	50	0	0.75	0.81[d]	81.76[a]
11	0	80	-1.68	33.3	0	0.75	13.25[a]	83.19[a]
12	0	80	1.68	66.7	0	0.75	8.74[b]	81.76[a]
13	0	80	0	50	-1.68	0.33	4.75[c]	82.69[a]
14	0	80	0	50	1.68	1.17	8.56[b]	81.08[a]
15	0	80	0	50	0	0.75	4.43[c]	81.64[a]
16	0	80	0	50	0	0.75	4.80[c]	82.29[a]
17	0	80	0	50	0	0.75	6.29[c]	83.37[a]

* Identical letters in the same column do not differ significantly from each other by Tukey's test at 5% significance level ($p \leq 0.05$)

DH is a method that evaluates the percentage of cleaved peptide bonds relative to a protein (Soares et al., 2017). The hydrolysis degree values for OPN, using the Bromelain enzyme, ranged from 0.810% to 14.32%. Assays No. 03 (Time: 40 minutes; Temperature: 60 °C; E/S: 0.5) and No. 11 (Time: 80 minutes; Temperature: 33.3 °C; E/S: 0.75) stood out with the highest DH percentages of 14.32% and 13.25%, respectively.

In food production, DH classification varies according to its applicability: 1% to 10% (low degree) is used to enhance functional properties, while greater than 10% (high degree) is used in foods for special purposes (Benítez et al., 2008). Thus, depending on the adopted conditions, the OPN hydrolysate falls into both categories.

For the antioxidant activity analysis, the DPPH (2,2-diphenyl-1-picrylhydrazyl) method was used, which is one of the most employed methods to evaluate the antioxidant mechanism of action of peptides derived from food proteins (Malomo et al., 2021).

As demonstrated in Table 6, no significant difference was observed among the assays, and OPN exhibited high antioxidant capacity. The Free Radical Scavenging (FRS%) values ranged from 81.52% to 83.3%, indicating a potential to prevent, impede, or reduce disorders caused by excess free radical reactions, such as those in heart diseases, arteriosclerosis, some types of cancer, Alzheimer's, and diabetes (Takao et al., 1994; Valko et al., 2006; Gonenc et al., 2011; Vera-Ramirez et al., 2011).

Jardim et al. (2021) observed positive results for the presence of alkaloid, flavonoid, tannin, and saponin metabolites in low percentages in their OPN analyses, along with the absence of anthraquinone glycosides. Among eighteen phenolics previously identified in studies with OPN extract, Souza (2014) highlights the presence of four important compounds, namely chlorogenic, caffeic, and ferulic acids, which benefit various bodily systems when present in foods.

Hydrolysates obtained from OPN plant peptides can potentially contribute to the field of food technology, as they provide nutritional and functional properties to foods.

Analysis of Variance (ANOVA) for DH and Antioxidant Activity of the Protein Hydrolysate

Table 7 presents the analysis of variance (ANOVA) data for the dependent variables degree of hydrolysis (DH) and antioxidant activity of the PAM protein hydrolysate. The ANOVA results revealed that the factors: reaction time (min), temperature (°C), and enzyme concentration (%), as well as their interactions, did not show a significant effect ($p < 0.05$) on the degree of hydrolysis and antioxidant activity parameters, as presented in Figure 4.

Furthermore, in both cases, the R^2 values were below 0.80, indicating that the experiments did not show good model fits (Halim & Sarbom, 2017). The coefficient of determination found, R^2 = 49% for %DH and 36% for %FRS, indicates that the obtained model is not predictive for explaining protein hydrolysis as a function of the enzyme concentration, time, and temperature used in this experiment.

Table 7. Analysis of Variance (ANOVA) for degree of hydrolysis (DH%) and antioxidant activity (FRS%) in PAM protein hydrolysate using bromelain enzyme.

Dependent Variables	Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F calculated	F tabulated	R^2
Degree of Hydrolysis (%)	Regression	85.51	9	9.50	0.67	3.68	0.49
	Residual	99.15	7	14.16			
	Lack of Fit	97.21	5	19.44			
	Pure Error	1.94	2	0.97			
	Total	194.19	16	12.13			
Antioxidant Activity (%FRS)	Regression	84.97	9	9.44	13.48	3.68	0.36
	Residual	4.87	7	0.70			
	Lack of Fit	3.36	5	0.67			
	Pure Error	1.51	2	0.75			
	Total	7.48	16	0.46			

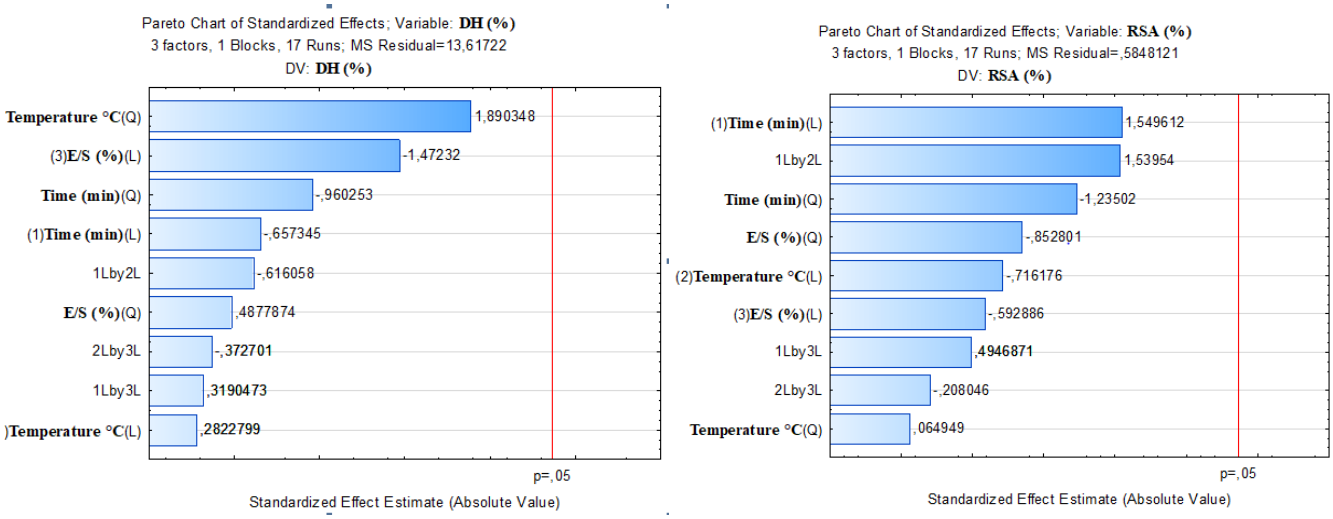

Figure 4. Pareto chart for degree of hydrolysis (DH%) and antioxidant activity (FRS%) in PAM protein hydrolysate using bromelain enzyme.

According to Santos et al. (2021), to achieve good proteolytic hydrolysis performance, factors such as temperature, enzyme concentration, and substrate concentration should be taken into consideration, as they directly influence reaction rate, peptide cleavage, and the efficiency of enzymatic substrate hydrolysis.

Therefore, it is necessary to continue studies on PAM hydrolysis using other enzymes with different pHs, times, and temperatures.

CONCLUSION

The physicochemical characterization analyses of the two OPN species demonstrated that both *Pereskia aculeata* (PAM) and *Pereskia grandifolia* (PGH) flour possess high contents of protein, fiber, and minerals, indicating that this plant source has the potential to be incorporated into the diet to meet daily recommended nutrient intakes. This also allows for greater sustainability and economy, as it is a non-conventional, spontaneous, and low-cost food plant.

Regarding the optimization for obtaining the protein hydrolysate using PAM, it was found that the RCCD using the commercial enzyme bromelain did not yield a predictive model. Therefore, new studies are necessary concerning other commercial enzymes and proteases naturally present in OPN.

Furthermore, there is potential for developing new products, enriching commonly consumed foods, or creating formulations with added protein hydrolysate for special dietary purposes, including vegan diets or as a substitute for proteins and nutrients derived from animal products.

As a final remark, it is suggested that more nutritional analyses be conducted, addressing aspects such as antinutritional factors, in order to better establish its nutritional profile and bioavailability.

Further research is necessary to expand on the present study, which could include evaluating more Ora-pro-nobis species, optimizing hydrolysate production using other proteases, and combining new factors that may influence the hydrolytic process.

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