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Production of thermostable antioxidant activity from chia (*Salvia hispanica*) protein after enzymatic hydrolysis

Obtenção de atividade antioxidante termoestável a partir da proteína da chia (Salvia hispanica) após hidrólise enzimática

Camila Piai Rossi[1], Paulo Henrique Mariano Marfil[1], Carolina Merheb-Dini[1*]

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1. Federal University of Triângulo Mineiro, Institute of Technological and Exact Sciences, Uberaba (MG), Brazil.
*Autor correspondente: carolina.dini@uftm.edu.br

Abstract: Proteases are promising tools for producing protein hydrolysates with bioactive properties. This study aimed to obtain chia protein hydrolysates with antioxidant activity using free and immobilized proteases, characterize the most active hydrolysate under different pH and temperature conditions, and evaluate its stability during simulated gastrointestinal digestion. Enzymatic hydrolysis was performed with commercial enzymes (Corolase; pepsin), and hydrolysates were collected at 15, 30, 60, and 120 min to assess degree of hydrolysis (DH) and antioxidant activity (AA). Free enzymes hydrolyzed chia proteins more extensively, while hydrolysis time did not affect AA, which was detectable after 15 min. Immobilized pepsin produced hydrolysates with the highest AA (65.7%). This activity was stable at acidic pH (70%), increased at 30–70 °C (250%), and retained 63% after simulated digestion. The hydrolysate was incorporated into passion fruit jelly, enhancing AA by 50%. Results highlight chia hydrolysates as promising ingredients for functional foods.

Keywords: Proteases. Immobilization. Alginate Beads. Vegetable Protein. Bioactive Peptides.

Resumo: Proteases apresentam potencial para produzir hidrolisados proteicos com propriedades bioativas. Este estudo teve como objetivo obter hidrolisados de proteína da chia com atividade antioxidante utilizando proteases livres e imobilizadas, caracterizar o hidrolisado mais ativo em diferentes condições de pH e temperatura e avaliar sua estabilidade durante digestão gastrointestinal simulada. A hidrólise enzimática foi realizada com enzimas comerciais (Corolase; pepsina); os hidrolisados foram coletados em 15, 30, 60 e 120 minutos para determinação do grau de hidrólise (GH) e da atividade antioxidante (AA). As enzimas livres promoveram maior extensão de hidrólise, mas o tempo não influenciou a AA, detectável já após 15 minutos. A pepsina imobilizada produziu hidrolisados com maior AA (65,7%), estáveis em pH ácido (70%), com aumento em 30–70 °C (250%) e retenção de 63% após digestão simulada. Incorporado em geleia de maracujá, o hidrolisado elevou a AA em 50%, indicando potencial para aplicação em alimentos funcionais.

Palavras-chave: Proteases. Imobilização. Partículas De Alginato. Proteína Vegetal. Peptídeos Bioativos.

1. Introduction

Considered a superfood, chia (*Salvia hispanica*) is an oilseed native to southern Mexico, and its consumption has increased in recent years due to its nutritional value. The chemical composition of chia seeds consists of approximately 25% protein, 30% lipids—mainly polyunsaturated fatty acids—and 34% fiber (Marineli *et al.*, 2014). Its nutritional value is also associated with several biological activities attributed to peptides generated from the enzymatic hydrolysis of its protein fraction, such as antioxidant, antimicrobial, and antihypertensive activities (Campos *et al.*, 2013; Orona-Tamayo *et al.*, 2015; Coelho *et al.*, 2018; Pablo-Osorio *et al.*, 2019; Villanueva-Lazo *et al.*, 2021; Villanueva-Lazo *et al.*, 2022). These physiological and functional properties depend on the maintenance of the structural and functional integrity of these peptides until they reach their specific sites of action in the organism (Sgarbieri, 2004; Korhonen & Pihlanto, 2006; Phelan *et al.*, 2009; Jao *et al.*, 2012).

The stability of these biological activities in different food matrices, as well as during the digestion process, must be considered when formulating products containing such peptides. The functional potential of new bioactive peptides continues to attract considerable interest from the scientific community, and more recently from consumers, who are increasingly attentive to the role of food proteins in human health beyond basic nutrition. Currently, consumer demand has shifted toward foods that provide health benefits while also contributing to sustainability, positioning plant proteins as key raw materials due to their availability, functionality, and nutritional properties (Guyomarc'h *et al.*, 2021).

To date, no studies have reported the use of immobilized enzymes to produce protein hydrolysates with biological activity from chia, nor studies evaluating the stability of these biological activities to enable technological applications. The use of immobilized enzymes offers advantages compared to soluble enzymes, such as the possibility of biocatalyst reuse, easier separation of the catalyst and reaction products, rapid interruption of reactions, and the potential for continuous processing (Basso & Serban, 2019). Therefore, the objective of this study was to hydrolyze chia proteins using microbial and animal proteases in both free and immobilized forms, quantify and characterize the antioxidant activity of the resulting hydrolysates, and evaluate the biological potential of passion fruit jelly containing these hydrolysates.

2. Material and methods

The enzymes Corolase 7089 and Pepsin were kindly provided by the companies ABEnzymes (Barueri, SP, Brazil) and Bela Vista (Alto Bela Vista, SC, Brazil), respectively. The defatted chia flour, containing 25% protein, was kindly provided by R&S Blumos (Cotia, SP, Brazil).

2.1 Enzyme immobilization by encapsulation

The enzymes were immobilized separately in alginate according to the procedure described by Ferreira and Merheb-Dini (2020). Briefly, the enzyme solution was mixed with a 2% (w/v) sodium alginate solution at a 1:1 ratio. The enzyme–alginate mixture was then added dropwise into a calcium chloride solution (0.2 mol/L) under continuous stirring to form the particles. The particles were washed with distilled water and used for the hydrolysis experiments.

2.2 Enzymatic hydrolysis

A 1% (w/v) chia flour solution was prepared in 0.2 mol/L Britton–Robinson (BR) buffer (pH 2.0 for Pepsin and pH 6.0 for Corolase) and subjected to hydrolysis in 250-mL Erlenmeyer flasks at 40 °C under agitation (120 rpm). Hydrolysis was initiated by adding 20 mL of either free or immobilized enzymes - Corolase with a specific activity of 840 U/g and Pepsin with a specific activity of 1,000 U/g - to 10 mL of the substrate solution. The hydrolysates were collected at different time periods (15, 30,

60 and 120 min), boiled for 10 min to inactivate the enzymes, and used for the evaluation of antioxidant activity and degree of hydrolysis. All hydrolysis experiments were repeated three times.

2.3 Determination of the degree of hydrolysis (DH)

The degree of hydrolysis (DH) was determined according to the method described by Castro and Sato (2014). An aliquot of 0.5 mL of the hydrolysate was mixed with an equal volume of trichloroacetic acid (TCA) solution (0.44 mol/L). The mixture was incubated at room temperature for 30 min and then centrifuged at 5000 rpm for 15 min. The TCA-soluble fraction (0.22 mol/L) and the hydrolysate supernatant (without TCA addition) were both analyzed to determine protein content using the Hartree method (Hartree, 1972), with bovine serum albumin as the standard. The DH, expressed as a percentage, was calculated as the ratio between TCA-soluble protein (0.22 mol/L) and the total protein in the hydrolysate supernatant. All analyses were performed in duplicate.

2.4 Determination of antioxidant activity (AA)

The DPPH radical scavenging activity was determined according to the method described by Bougatef *et al*. (2009). This method is based on an electron transfer reaction in which the antioxidant (AH) or another radical species reduces 2,2-diphenyl-1-picrylhydrazyl (DPPH), a purple radical, forming diphenyl-picryl-hydrazine, which is yellow. This reduction results in a decrease in absorbance that can be monitored spectrophotometrically (Nascimento *et al*., 2011). A volume of 500 µL of the protein hydrolysate sample was mixed with 500 µL of 99.5% ethanol and 0.02% DPPH in 99.5% ethanol. The mixture was kept at room temperature in the dark for 60 min, and the decrease in DPPH absorbance was measured at 517 nm. The DPPH radical scavenging activity (%) was calculated as follows: $AA = (A - B) / A \times 100$, where A = absorbance of the control and B = absorbance of the sample. The control was prepared in the same way, except that distilled water was used instead of the sample. Analyses were performed in duplicate.

2.5 Characterization of antioxidant activity of the hydrolysate

The hydrolysate with the highest antioxidant activity was selected for the stability characterization experiments under different pH, temperature, and gastrointestinal digestion conditions. In all cases, the antioxidant activity of the untreated hydrolysate was considered as 100%.

To assess pH stability, the hydrolysate was mixed (1:1) with 0.1 mol/L Britton–Robinson (BR) buffer solutions adjusted to pH values ranging from 2.0 to 12.0. After one hour, samples were collected to determine residual antioxidant activity, as previously described.

To assess temperature stability, the hydrolysate was incubated at different temperatures (30 to 75 °C, in 5 °C intervals) for one hour. After incubation, samples were collected to determine residual antioxidant activity, as previously described.

To assess stability under gastrointestinal conditions, a digestion simulation was performed according to Aljewicz and Cichosz (2015). Briefly, 1.5 mL of the hydrolysate and 1.6 mL of Pepsin (prepared by dissolving 16 g of Pepsin P-7000, Sigma-Aldrich, in 100 mL of distilled water) were added to 50 mL of water adjusted to pH 2.0 with 1 mol/L HCl, and the mixture was incubated at 37 °C for 2 h under shaking (100 rpm). The pH was then adjusted to 6.8–7.0 using 6% $NaHCO_3$. A pancreatin–bile solution (0.4 g pancreatin and 2.5 g bile, both from Sigma-Aldrich, dissolved in 200 mL of 0.1 mol/L $NaHCO_3$) was prepared, and 15.8 mL of this solution was added to 50 mL of the sample mixture. The system was incubated again at 37 °C for 2 h under shaking (100 rpm). A control simulation was performed by replacing the hydrolysate with water. At the end of the simulations, antioxidant activity was measured as previously described. The digestion simulation experiment was performed in duplicate.

2.6 Application of the hydrolysate: passion fruit jelly production

To evaluate the biological potential of the hydrolysate when incorporated into food formulations, the hydrolysate with the highest antioxidant activity was used as an ingredient in the preparation of passion fruit jelly, and its antioxidant activity was assessed. Fruits were purchased from a local market in Uberaba, MG, Brazil, and in the laboratory, they were cut with a stainless-steel knife; the pulp and seeds were filtered to obtain the juice. Pectin was extracted from the peel by immersion in boiling water for 30 min. The jelly was prepared using pectin, sucrose, and fruit juice in a 3:3:1 ratio, with the addition of 10 mL of the hydrolysate. A control jelly was prepared by replacing the hydrolysate with water. Cooking was performed in a stainless-steel pan with manual stirring until the final soluble solids reached 67 °Brix (Lago *et al*., 2006), measured using a refractometer. The jellies were hot-filled into sterilized glass jars, sealed with metal lids, and immediately cooled in cold water for 15 min. Antioxidant activity was evaluated as previously described, and pH was measured after cooling.

2.7 Immobilized Pepsin reuse

To evaluate the reusability potential of immobilized Pepsin ,the degree of hydrolysis (DH) was determined after three consecutive hydrolysis cycles, using the same particles and a fresh substrate for each cycle. The immobilized Pepsin particles were washed between cycles. Hydrolysis was performed as previously described, for 15 minutes, and the first cycle was considered as 100%.

2.8 Experimental design and statistical analysis

To evaluate the effect of enzyme configuration on the production of hydrolysates with antioxidant activity, a 2 × 4 factorial experiment was conducted in a completely randomized block design. The enzyme factor had two levels (free and immobilized), and the hydrolysis time factor had four levels (15, 30, 60, and 120 min). The hydrolysis runs (1, 2, and 3) were considered as blocks. Analysis of Variance (ANOVA) was used to assess the effect of the enzyme and hydrolysis time on the degree of hydrolysis (DH) and antioxidant activity (AA) of the hydrolysates. Tukey's test was applied to compare means at a 5% significance level using Statistica 7.0 software.

3. Results and discussion

3.1 Enzymatic hydrolysis

The results of the hydrolysis experiments are presented in Tables 1 and 2 and in Figure 1.

Table 1- Effect of treatment and time on the degree of hydrolysis (DH) and antioxidant activity of chia hydrolysates (n=3)

Factors	*P values**			
	Pepsin		Corolase	
	DH	AA	DH	AA
Treatment[a]	0.0000*	0.0082*	0.0054*	0.7139
Time[b]	0.2370	0.1105	0.0222*	0.3874
Treatment x time	0.6807	0.9743	0.0043*	0.5031

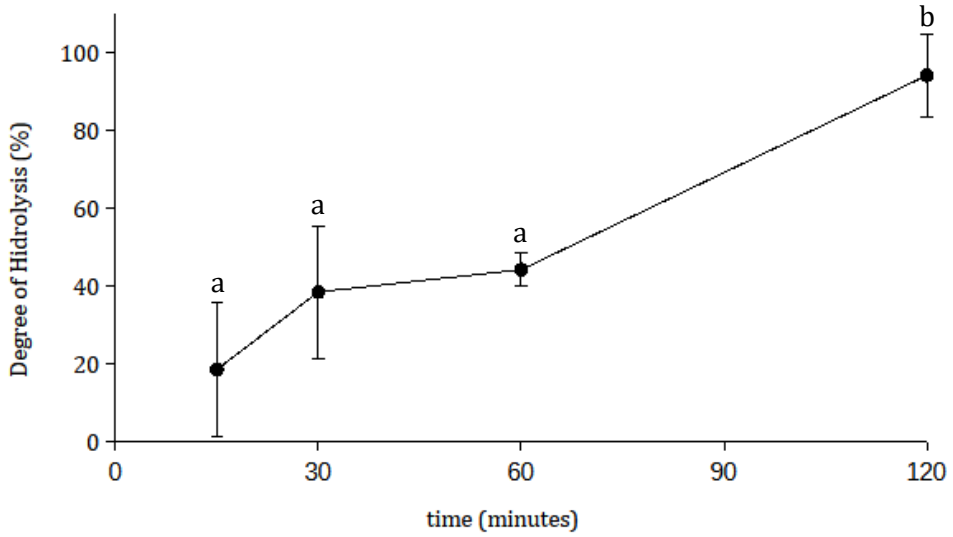
Source: Authors, 2025.
[a] Treatment: Free enzyme or Immobilized enzyme
[b] Time: Hydrolysis time (15, 30, 60, 120 minutes)
* $p<0.05$

Table 2 - Effect of enzyme configuration on the degree of hydrolysis and antioxidant activity of chia hydrolysates (n=3)

Enzymes	DH	AA (%)
Free Pepsin	59,3[a] ± 3,4	49,9[b] ± 6,9
Immobilized Pepsin	17,3[b] ± 3,9	65,7[a] ± 9,2
Free Corolase	48,89[a] ± 32,15	59,5[NS] ± 2,8
Immobilized Corolase	25,50[b] ± 10,30	56,9[NS] ± 13,4

Source: Authors, 2025.
[a, b] means with different letters, for the same analysis and same enzyme, are statistically different (p<0.05)
[NS] Not significant (p>0.05)

Figure 1 - Effect of time on the degree of hydrolysis of chia hydrolysates obtained using free Corolase (n = 3)



Source: Authors, 2025.
[a, b] means with different letters are statistically different (p<0.05)

The ANOVA results presented in Table 1 show that the enzymatic configuration significantly influenced the degree of hydrolysis for both proteases. For pepsin, the treatment effect was significant ($p = 0.0000$), indicating that the free or immobilized form directly affects the extent of protein cleavage. The same behavior was observed for Corolase ($p = 0.0054$), reinforcing that immobilization alters enzyme accessibility to the substrate and/or its catalytic efficiency. In both cases, the free enzymes hydrolyzed chia protein more extensively (Table 2 and Figure 1). For Corolase, the degree of hydrolysis (DH) increased significantly over time when the enzyme was used in the free form (Figure 1). In contrast, for immobilized Corolase, the DH remained constant throughout the hydrolysis period ($p > 0.05$), stabilizing at an average of 25.5% (Table 2).

Pepsin is known to preferentially hydrolyze peptide bonds adjacent to aromatic amino acids such as phenylalanine, tryptophan, and tyrosine (Altun & Cetinus, 2007). However, chia protein contains low levels of these residues and is instead rich in arginine, leucine, valine, and lysine, with phenylalanine as the only aromatic residue present in appreciable amounts (Kulczyński et al., 2019). This compositional characteristic may explain the lack of significant increase in DH during hydrolysis with pepsin, regardless of the enzymatic configuration. Conversely, Corolase, an endopeptidase commonly used to hydrolyze both plant and animal proteins of high molecular weight into low–molecular weight peptides, was able to increase the DH over time. The free form of the enzyme produced extensively hydrolyzed peptides, reaching 94.2% DH after 2 hours (Figure 1).

According to Table 1, the analysis of antioxidant activity showed that the response to the type of treatment varied depending on the enzyme used. For Pepsin, the treatment effect was significant ($p = 0.0082$), indicating that immobilization not only alters the extent of hydrolysis but also influences the release of bioactive peptides. In contrast, for Corolase, the treatment did not affect the antioxidant activity ($p = 0.7139$), suggesting that, in this case, the formation of peptides with antioxidant potential is independent of the enzyme configuration. It was observed that immobilized Pepsin produced higher antioxidant activity compared to its free form (Table 2), regardless of hydrolysis time, which also did not show a significant effect on antioxidant activity ($p = 0.1105$). For Corolase, neither the enzyme configuration nor the hydrolysis time influenced the antioxidant activity of the hydrolysates ($p > 0.05$) (Table 1), resulting in an average value of 58.20% (Table 2).

The chia flour used in this study exhibited 43% antioxidant activity, and according to Table 2, immobilized Pepsin was able to produce hydrolysates that increased this original activity to 65.7%, which corresponds to a 50% enhancement. This increase in antioxidant activity in protein hydrolysates is related to the exposure of antioxidant amino acids from chia protein after hydrolysis (Elias *et al*., 2008), and has also been reported by other authors (Segura-Campos *et al.*, 2013; Villanueva-Lazo *et al.*, 2021). Protein hydrolysates with antioxidant activity were also reported by Castro, Sato (2014) when studying soybean protein isolate hydrolyzed with microbial proteases. The soybean protein isolate exhibited a 7-fold increase in antioxidant activity after hydrolysis.

As shown in Table 2, immobilized Pepsin produced hydrolysates with lower DH but higher AA compared to the free enzyme. A similar behavior was reported by Segura-Campos *et al.* (2013), who observed an inverse relationship between DH and AA: the antioxidant activity of chia protein hydrolysates generated with Alcalase and Flavourzyme decreased as DH increased.

Based on these results, hydrolysis with immobilized Pepsin was selected as the most suitable condition for generating peptides with antioxidant activity. The hydrolysates obtained under this condition were subsequently used for the antioxidant stability assays and for jelly preparation.

3.2 Hydrolysate characterization

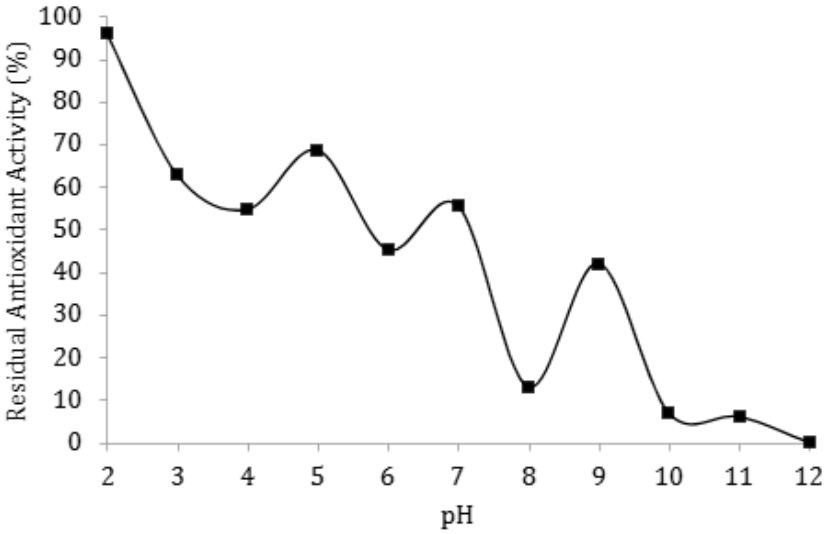
Figures 2 and 3 show the effect of pH and temperature on the stability of the antioxidant activity of the chia protein hydrolysate obtained with immobilized Pepsin.

Analyzing the effect of pH on antioxidant activity (Figure 2), the hydrolysate remained stable under acidic conditions, with activity decreasing as pH increased. Up to pH 5.0, antioxidant activity remained close to 70%.

Figure 3 shows the effect of temperature, and the antioxidant activity increased after incubation at the tested temperatures, indicating that the hydrolysate is thermostable. This behavior suggests the presence of numerous hydrophobic interactions that are strengthened at elevated temperatures (Scheilman, 1997). This finding is highly relevant from a technological standpoint, since it indicates activation of antioxidant activity at temperatures commonly applied in industrial processes, supporting the feasibility of incorporating the hydrolysate into food products.

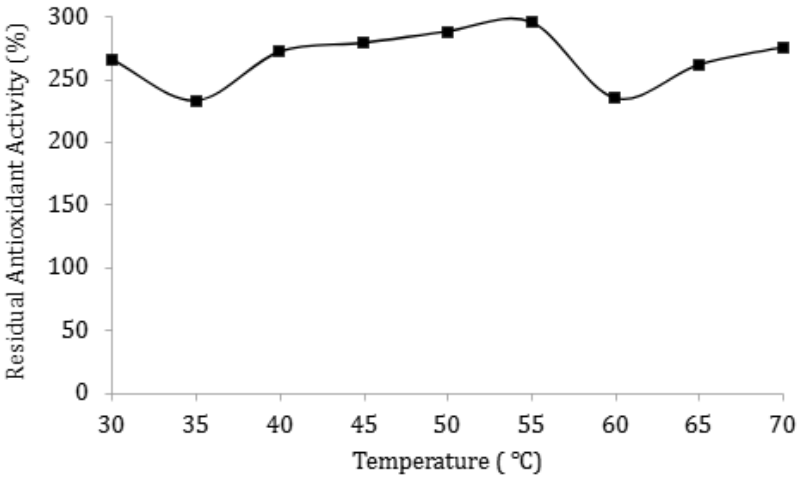
After simulated gastrointestinal digestion, 63% of the antioxidant activity was retained, suggesting high bioavailability and indicating the biological potential of the hydrolysate. The reduction in activity (37%) is likely due to proteolysis by the gastrointestinal enzymes, which modify the peptide profile. Similar behavior was reported by Mazloomi *et al*. (2020), who observed an even greater reduction (58%) of the antioxidant activity of orange seed protein hydrolysates after simulated digestion.

Figure 2 - Effect of pH on the antioxidant activity of the hydrolysate obtained with immobilized Pepsin after 30 minutes of hydrolysis



Source: Authors, 2025.

Figure 3 - Effect of temperature on the antioxidant activity of the hydrolysate obtained with immobilized Pepsin after 30 minutes of hydrolysis



Source: Authors, 2025.

3.3 Jelly production

Given the stability of the hydrolysate under acidic pH and at a wide temperature range, it was decided to apply it to passion fruit jelly. Passion fruit peel, which is typically considered a by-product of the juice industry, contains significant amounts of high-methoxyl pectin with strong gelling capacity (Kulkarni & Vijayanand, 2010; Zeraik *et al.*, 2010). Therefore, passion fruit naturally provides the essential ingredients for jelly production, with the exception of sugar.

The jelly produced with the addition of the hydrolysate showed 62.23% antioxidant activity and a pH of 3.49. The control jelly, produced with the addition of water, exhibited 41.13% antioxidant activity and a pH of 3.57. In other words, incorporating the chia hydrolysate into the passion fruit jelly resulted in a 50% increase in antioxidant activity. The acidic pH of the jellies confirms that the antioxidant activity of the hydrolysate is indeed stable under these conditions.

Segura-Campos *et al.* (2013) studied the hydrolysis of chia using commercial proteases (Alcalase and Flavourzyme) and observed an increase in antioxidant activity in the hydrolysates. However, when these hydrolysates were applied to white bread and carrot cream, no effect was observed in the final product.

3.4 Evaluation of the reuse of immobilized Pepsin

When evaluating the degree of hydrolysis (GH) of the chia hydrolysates using the same alginate particles containing immobilized Pepsin over three consecutive hydrolysis cycles, GH values of 127% in the second cycle and 115% in the third were observed. These results suggest that immobilized Pepsin can be reused without loss of catalytic activity, as it was able to hydrolyze chia in subsequent cycles with similar efficiency. In the study by Ferreira and Merheb-Dini (2020), Corolase immobilized in alginate retained only 60% of its activity after the third cycle, likely due to particle leakage. Considering that one of the main advantages of using immobilized enzymes is the possibility of reuse, the results obtained here are highly promising.

4. Conclusions

The data obtained in this study demonstrate that it was possible to produce protein hydrolysates with antioxidant activity from chia using microbial and animal proteases in both free and immobilized forms. The hydrolysates exhibited degree of hydrolysis ranging from 17.3 to 94.2% and antioxidant activity between 50 and 65.7%.

Immobilized Pepsin showed superior antioxidant activity release, regardless of hydrolysis time. After only 15 minutes of reaction, the hydrolysate already reached 65.7% antioxidant activity. This hydrolysate showed stability of its antioxidant activity under acidic pH conditions and at temperatures up to 70 °C, demonstrating technological potential for application in food formulations. When incorporated into passion fruit jelly, the hydrolysate increased the antioxidant activity of the product by 50%, confirming that its functional activity is preserved in a real food system.

These findings reinforce the importance of selecting the appropriate enzyme and its method of application to produce hydrolysates with functional potential. The originality of this study lies in the novel use of immobilized enzymes to produce chia hydrolysates, combined with the evaluation of their functional stability and application in a model food product. Together, these results highlight the potential of chia hydrolysates as bioactive and sustainable ingredients for the food industry.

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