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Antidiabetic and Antioxidant Potential of Protein Hydrolysates and Peptide Fractions from *Leucaena esculenta*

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ABSTRACT

The increase and prevalence of chronic degenerative pathologies (chronic cellular oxidation) associated with changes in dietary habits has resulted in a demand for pharmacological alternatives that can be incorporated into the food to reduce the incidence of these diseases; where the use of plant peptides (especially those derived from legumes) offer promising results. Enzymatic hydrolysis of legume native protein fraction constitutes a useful technological tool to release bioactive peptides *encrypted* in long polypeptide structures. The legume native protein fraction was isolated by solid/liquid extraction by a isoelectric point methodology. The protein hydrolysates were digested with Alcalase, Flavourzyme and Neutrase proteases and subsequently fractionated by ultrafiltration cartridges. The antioxidant activity was evaluated by the DPPH* and ABTS^{+*} methodologies and the antidiabetic was evaluated by the inhibition of the α-glucosidase and α-amylase. Flavourzyme non fractionated hydrolysates exhibited the higher antioxidant activity (49.74% DPPH* and 96.47% ABTS^{+*}). Neutrase and Flavourzyme fractionated hydrolysates exhibited the higher antioxidant activity (74.95% DPPH* and 74.95% ABTS^{+*} respectively). Alcalase hydrolysates exhibited higher antidiabetic activity (47.07% for amylase and 10.95% for glucosidase). The objective of this study was to evaluate antioxidant and antidiabetic activities of protein hydrolysates fractions isolated from *Leucaena esculenta* ripe seeds.

Key words: Antidiabetic, Antioxidant, *Leucaena esculenta,* Protein hydrolysis.

The increase and prevalence of chronic degenerative pathologies derived from chronic cellular oxidation processes (associated with the increased consumption of meat products, processed flour, colorants and preservatives) has stimulated the search for new molecules with biological activity against cellular oxidation, where the proteins stand out as the most interesting molecules. There are several amino acid sequences (peptides) that exhibit bioactive properties of interest but are normally found in large and complex polypeptide sequences that *inhibit* their biological activity (Arnal *et al.,* 2024). The biological active is correlated to the peptide amino acid composition, residues quantity, residues position in the carbon chain and the spatial structure (Sun *et al*., 2024). In human health, therapeutic products enhanced with peptides are used mainly against the metabolic syndrome, antibacterial and antiviral therapies and especially for developing cell-targeting platforms and improving cell-penetrating properties (Zhu *et al.,* 2019). Peptides are base of dietetic formulas for infant and geriatric products and weight-control/high-energy products (Kehinde and Sharma, 2020; Valenzuela *et al.,* 2022).

The use of animal sources, with the exception of some sub-products of the milk industry, has usually been discarded as a source of bioactive peptides, mainly because of high and sustained demand for these raw products by the food industry (Shabir *et al*., 2023).

Protein from plant sources traditionally has not been considered as an important source of proteins due they often contain allergenic proteins and other anti-nutritional factors (*e.g.* glycinin, trypsin inhibitors, *etc.*); but when vegetal protein is hydrolyzed, antigenic and anti-nutritional behavior decreases significantly (Clemente and Chambers, 2000). Bioactive peptides from legume have usually been described as a chains of 2 to 20 amino acid residues (Acquah *et al.,* 2022), where soybean and chickpea (Castro and Sato, 2014) are the most widely used legumes.

Leucaena esculenta, a legume widely distributed in Mexico and Central America, has been utilized as a protein

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supplement in fodder feeds (Parrota, 1992). The seeds are not commonly used in human nutrition due the presence of mimosine; however, *Leucaena esculenta* seeds can be safely consumed when the seeds are soaking and cooking (Balderas-León *et al*., 2004). Despite this limitation, *Leucaena esculenta* seeds represent a promising nutritional resource due to their high protein content. Nevertheless, the utilization for human nutrition of *Leucaena esculenta* seeds protein remains underexplored.

The aim of this study was to evaluate the antioxidant *in vitro* activity (by DPPH*/ABTS*+ radicals) and antidiabetic activity (α-amylase/α-glucosidase inhibitory activity) from the hydrolyzed protein from *Leucaena esculenta* ripe seeds. Additionally, the study evaluate the correlations between the biological activity and hydrolyzed protein fractions sizes (≤100 kDa, ≤10 kDa and ≤3 kDa).

Collection of plant material

Ripe *Leucaena esculenta* seeds, were collected in Oaxaca, Mexico (16°59′21″N, 96°43′26″W) during 2021. The identification of the species was carried on in the Herbarium Mtra. Graciela Calderón Díaz-Barriga and Dr. Jerzy Rzedowski.

Pre-treatment of plant material

Ripe seeds were cleaned in a commercial solution of 5% (v/v) of sodium hypochlorite. Seeds were reduced to particulate material (<0.149 mm).

Protein isolation

The protein fraction (native fraction) was obtained according to the methodology of Ohara *et al.* (2020).

Protein quantification

The protein quantification was used according to the methodology of Grimsley and Pace (2004).

Enzymatic hydrolysis

Enzymatic hydrolysis was performed according to Hamada (2000). Three proteolytic enzymes were used: i) Alcalase (serine-type endoprotease.); ii) Flavourzyme (mixtures of different endo- and exoprotease) and iii) Neutrase (endoprotease random behavior).

The hydrolysis conditions were kept within the optimal ranges specified by the enzyme supplier (Novozymes). Alcalase: 3 μg^{-1}, pH 6.5, 60°C; Flavourzyme: 500 μg^{-1}, pH 6.0-50°C and Neutrase: 1.5 μg^{-1}, pH 6.0-50°C.

Determination of the degree of hydrolysis

It was determined by the OPA technique according to method of Nielsen *et al.* (2021).

Recovery of protein fractions

Isolation of the protein fractions was performed using Centricon-Plus centrifugal filters of 100 kDa, 10 kDa and 3 kDa mesh sizes. Filters were preconditioned according the manufactures instructions. 2 mL of the protein hydrolysate solution was added to each filter. The filters loaded with the protein samples were centrifuged (3500 × g for 5 min at 25°C) to recover the filtrate. Protein fraction from 100-10 kDa was denominated as 100kDa fraction, the fraction from 10-3 kDa was denominated as 10 kDa fraction and the protein fraction ≤3kDa was denominated as 3 kDa.

Bioactive properties. Assessment of antioxidant activity; DPPH* assay

Antioxidant activity was determined according to the method of Brand-Williams *et al.* (1995).

Bioactive properties. Assessment of antioxidant activity; ABTS+* assay

Antioxidant activity was determined according to the method of Re *et al.* (1999).

Inhibition of α-amylase and α-glucosidase enzymes

The protein sample concentration was diluted with sodium phosphate solution (50 mM at pH 6.9) to obtain a protein content of 30 mg mL^{-1}.

Inhibition of α-amylase and α-glucosidase were evaluated according to the method of Mojica and González (2015). The content of reducing sugars released by α-amylase is determined by the dinitrosalicylic acid method according to Miller (1959).

The polysaccharide acarbose was used as a positive control (25 mg mL^{-1}; human standard therapeutic dose for glucose en blood control).

As negative control of enzymatic activity a solution of 10 mg mL^{-1} of wheat starch was used.

Statistical analysis

Results of each protein sample were analyzed by an analysis of variance (ANOVA) with Tukey's media test (significance level, p<0.050). Correlation analysis was carried out by a multiple linear regression (significance level, p<0.050). Analysis was performed using Statistica ver. 7.0.

Antioxidant capacity

In legumes, the use of proteases (*e.g*., alcalase) is used as a strategy to obtain a higher concentration of peptides/ increase the bioactive activity (Kumar *et al.,* 2022). Peptides of lower molecular size and/or enriched in hydrophobic amino acid have been associated with higher antioxidant potential (Yust *et al.,* 2012; González-Garza *et al.,* 2017).

Our results show that hydrolysis by all the evaluated proteases increase the antioxidant activity (p<0.050). The Flavourzyme hydrolysates exhibit the highest antioxidant activity against the DPPH* and ABTS+* radicals (Table 1). The fraction with a molecular size of 100 kDa, showed the highest ABTS+* radical inhibitory capacity, 74.95% (Fig 1b). This could be attributed to the specific peptide sequences or sequences rich in hydrophobic amino acids.

The hydrolysate fraction from Neutrase with a molecular size of 10 kDa showed the highest DPPH* radical inhibitory capacity, 74.96% (Fig 1a).

However, both for the hydrolysates from Flavourzyme and Neutrase there is no significant correlation (p=0.053) between the size and the antioxidant capacity. Xu *et al*. (2024) suggests that the specific amino acid sequences (*e.g*. histidine, methionine and cysteine-electron donators) may be more significant than peptide size.

It has been proposed that the antioxidant action of peptides is due their action on several signaling pathways: i) Kelch-like ECH-associated protein 1/NFE2-related factor 2/antioxidant response elements, Keap1-Nrf2/ARE (inactivation of the Keap1 Kelch active site); ii) growth factor-β/small mothers against decapentaplegic, TGF-β/SMAD (promoting the formation of collagen in fibroblasts by activating the TGF-β1/SMAD2); iii) AMP-activated protein kinase/Sirtuin/peroxisome proliferator-activated receptor γ coactivator-1α, AMPK/SIRT1/PGC-1α (inhibition mitochondria-mediated apoptosis, improving the mitochondrial function and avoiding the mitochondrial autophagy); iv) phosphatidylinositol 3-kinase/Akt, PI3K/Akt/mTOR (promoting the cytoprotective effects by activating the PI3K/Akt pathway) and v) nuclear factor-κB (inactivation of RelA (P65) and P50 of the NF-κB family) (Feng *et al.,* 2021; Li *et al.,* 2022).

Enzymatic breakdown of polysaccharides (sugar molecules)

α-glucosidase and α-amylase are essential for metabolizing polysaccharides in food. The inhibition of those enzymes causing a lowering post-prandial glucose levels (Elferink *et al.,* 2020).

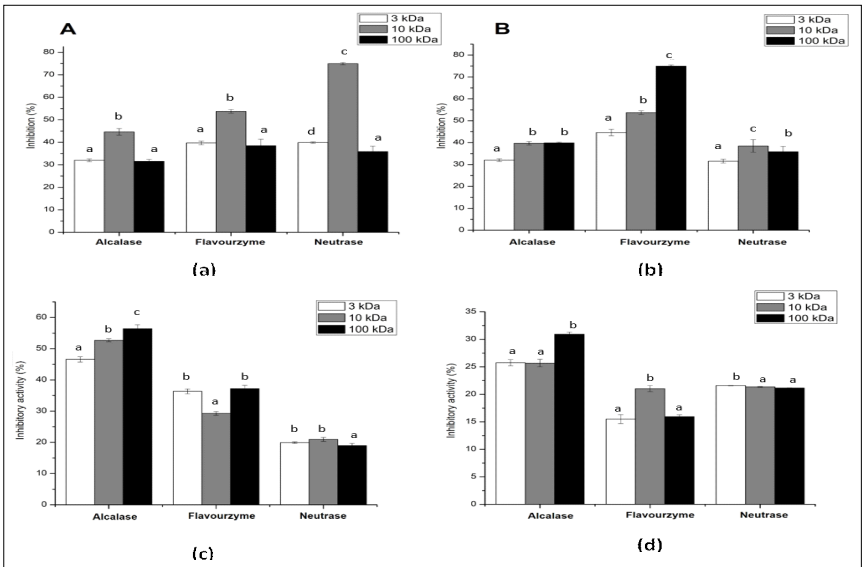
Inhibitory capacity of evaluated protein fractions against the α-glucosidase and α-amylase varied significantly (p<0.050) (Table 1). Hydrolysate fraction form Alcalase with molecular size of 100 kDa, showed the highest α-glucosidase and α-amylase inhibitory capacity, 33.42% and 56.42% respectively (Fig 1c and 1d). Lu *et al*. (2023) propose that peptides with a high concentration of hydrophobic residues effectively inhibit the α-amylase and α-glucosidase.

Protein fractions isolated from legumes exhibit a low inhibition capacity of α-glucosidase and α-amylase (Acquah *et al*., 2022).

Table 1: Antioxidant and antidiabetic behavior.

	Native proteinfraction	Alcalase	Flavourzyme	Neutrase	Acarbose
DPPH*	20.84±0.15^{b}	36.35±0.37^{a}	49.74±0.98^{c}	37.17±0.38^{a}	NA
ABTS⁺*	11.14±0.71^{a}	88.70±0.78^{b}	96.47±0.86^{d}	92.41±0.77^{c}	NA
Glucosidase	3.96± 0.33^{a}	10.95±0.77^{a}	4.94±.0.14^{c}	8.51± 0.26^{d}	65.86±0.14^{e}
Amilase	33.16±0.38^{b}	47.07±1.22^{c}	35.87±0.22^{d}	28.33±0.67^{a}	78.07±0.90^{e}

Different super indexes indicate significant differences (p<0.05) between mean values of the protein fractions evaluated.
NA: The application of this concept is not applicable in this case.



(a) DPPH* radical stabilizing capacity. (b) ABTS⁺* radical stabilizing. (c) α-amilase. (d) α-glucosidase. Results are mean and bars represent the deviation standard of three independent determinations. Different alphabets on the bars represent significance (p<0.05).

Fig 1: Antioxidant and antidiabetic behavior of protein hydrolysates.

In α-amylase, three structural domains with catalytic activity over polysaccharides are observed, were the residues 1-99; 170-404; are described as the main active site for the glycosyl-enzyme intermediate complex (Ramasubbu *et al.,* 2004).

Our results show no significant correlation between the size of any of the hydrolysates evaluated and the inhibition of the enzymatic breakdown reaction of polysaccharides (p>0.050), further suggesting that the inhibitory effect might be more related to the amino acid sequences. Previous studies have reported that α-amylase and α-glucosidase inactivation by peptides is correlated with the concentration of hydrophobic residues (Wei *et al.,* 2022; Fu *et al.,* 2023).

Several authors report a significant increase in the inhibitory activity of α-glucosidase/α-amylase by protein fractions isolated from different legumes after hydrolysis (Rivero-Pino *et al*., 2021; Arnal *et al*., 2024).

CONCLUSION

The use of exo/endo-protease increased antidiabetic and antioxidant activity of protein fraction isolated from *Leucaena esculenta* seeds. Among the proteases evaluated, Flavourzyme treatment showed the highest antioxidant activity. The 100 kDa hydrolysate fraction of each evaluated protease showed the highest bioactive properties.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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