EXTRACTION OF PROTEINS FROM NORTHEASTERN BRAZILIAN PLANTS WITH POTENTIAL FOR PROTEASE INHIBITION

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ABSTRACT

Protease inhibitors are molecules that block the activity of proteases. These enzymes degrade protein, are essential in biological functions, and are used for developing therapies for different diseases, such as viral infections and cancer. These inhibitors have varied protein sources, such as animal tissues, plants, or microorganisms. Legumes from the Fabaceae family may contain protease inhibitors with distinct chemical structures and mechanisms of action. Hence, the interactions between the inhibitors and the respective proteases must be understood. This study aimed to characterize the seeds of *Caesalpinia echinata*, *Prosopis juliflora*, and *Pithecellobium Dulce* in terms of morphology and centesimal composition and to determine the protease inhibition potential of the extracts obtained with ionic liquid-based choline. The physicochemical composition of the seeds was determined using the moisture, fiber, ash, protein, lipid, carbohydrate, and energy contents. The best extraction of protease inhibition was observed using *Prosopis juliflora* and choline bitartrate (10.42 %) as extracting agents in the solid-liquid ratio of 1:5 at 25ºC. The kinetic study shows that 20 min is needed to extract proteins. The *Prosopis juliflora* extract inhibited trypsin by 93.8 ± 0.8%.

Keywords: Proteins, Inhibition, Extraction, Protease.

1 INTRODUCTION

Protease inhibitors are substances that interfere with the action of enzymes responsible for degrading proteins, playing a significant role in treating some diseases, such as HIV/AIDS, hepatitis C, and COVID-19. Inhibiting protease is important because of its role in breaking down proteins in a physiological mechanism, affecting processes such as fertilization, homeostasis, neuronal growth, apoptosis, and immune mechanisms. These inhibitors are found in several plants, widely distributed in the plant kingdom, and comprise around 5 to 10% of the total soluble protein content in dicot and monocot seeds of the main Fabaceae, Poaceae, and Solanaceae families. They act by inhibiting the activity of the protease enzyme, which is essential for the viral replication cycle[1–5]. Legume seeds such as soya, beans, and peas are rich sources of protease inhibitors, particularly trypsin and chymotrypsin inhibitors. Exploring less studied plant species can reveal new bioactive compounds that broaden the understanding of protease inhibitors and their interactions with target proteases and produce inhibitors with greater specificity and efficacy against specific proteases, potentially resulting in more effective therapies with fewer side effects. Research and using other legumes can encourage the conservation of these species and their natural habitats, highlighting their ecological and economic importance[6-8].

The study of the extraction of protease inhibitors from plant seeds involves a series of techniques and specific solvents that help to isolate and purify proteins of interest and to ensure that functional, high-purity proteins are obtained. The choice of solvent involves factors such as the solubility of the proteins, the preservation of biological activity, compatibility with subsequent purification steps, and the safety and practicality of the solvent. Some studies have reported the extraction of proteins from plants using saline solutions, buffer solutions, and organic solvents[1,9-11]. Feitosa et al.[12] maximized the extraction of proteins present in brazilwood seeds using ionic liquid as a solvent, and the extract had the potential to inhibit trypsin activity by 60.8%. This work aims to assess the physicochemical characteristics of leguminous plants from the Fabaceae family (*Caesalpinia echinata*, *Prosopis juliflora*, and *Pithecellobium Dulce*) to verify the nutritional value of their seeds. The protein extraction process will use choline-based ionic liquids to obtain protein extracts, which will be evaluated using an inhibition test. The extract with the highest inhibition potential will be subjected to sample separation by electrophoresis.

2 MATERIAL & METHODS

The seeds of *Caesalpinia echinata* were collected from the gardens of Tiradentes University, *Prosopis juliflora* and *Pithecellobium Dulc* from parks in the city of Aracaju-Sergipe. The material transported to the laboratory was washed with a 200 ppm sodium hypochlorite solution and dried in an oven at 50°C. The seeds were then subjected to morphological characterization by measuring the length, width, and thickness of the whole seeds using a manual caliper. The mass of 100 seeds was obtained from a digital scale with a precision of 10^{-5}g. *Caesalpinia echinata*, *Prosopis juliflora*, and *Pithecellobium Dulce* seeds were crushed to obtain their centesimal composition.

The extraction procedure for obtaining the protein concentrate from legume seeds is based on the study by Feitosa et al.[12], which used the following variables: solid-liquid ratio 1:5 (w/v), temperature of 25ºC, under continuous mechanical agitation of 500 rpm for 60 min, the extracting solvent choline bitartrate [Ch][Bit] at 5.42% (m/v) with pH 7. All extractions will be carried out in triplicates. The protein concentrate was determined according to the Bradford method [13].

The protease inhibitory activity of the protein extract was evaluated using the methodology proposed by Liu [14], using trypsin from bovine pancreas. The reagents included Tris-buffer (50mM, pH 8.2) with 20 mM $CaCl_2$, HCl solution (1 mM, with 5 mM $CaCl_2$), acetic acid solution (30%, v/v), trypsin solution (0.2 mg/mL) and benzoyl-DL-arginine-p-nitroanilide hydrochloride solution (0.4 mg/mL). The procedure took place in test tubes, including sample and blank. After centrifugation, the absorbance at 410 nm was measured to assess trypsin activity in the presence of the extract inhibitors. The test was carried out in a water bath at 37°C, with the reaction starting precisely 10 minutes after adding the trypsin solution and stopping after adding 1 mL of 30% acetic acid solution. The corrected reading of the sample was in the range of 30-70% of trypsin inhibition, as shown in Equation (1).

$$UTI = \frac{[(A_R - A_{RB}) - (A_S - A_{SB})]}{(A_R - A_{RB})} X\ 100 \quad (1)$$

A_{410S} and A_{410R} are the sample and reference absorbances, respectively, for the inhibition determinations. The sample blank (A_{410SB}) and reference blank (A_{410RB}) are prepared by adding the acetic acid solution before the trypsin, *i.e.* reversing the order of the third and fourth reagents in the sequence.

The presence of the protease inhibitor will be monitored by SDS-PAGE [15].

3 RESULTS & DISCUSSION

The seeds were characterized in terms of their size and mass of 100 seeds, respectively: *Caesalpinia echinata* (5.4 ± 2.4 mm long, 4.3 ± 0.1 mm thick and 11.1 ± 1.8 mm wide) and 34,2 ± 0,5 g, *Prosopis juliflora* (0.72 ± 0.05 mm long, 0.23 ± 0.1 mm thick and 0.42 ± 0.2 mm wide) and 4,3 ± 0,1 g, *Pithecellobium dulce* (1.27 ± 0.1 mm long, 0. 33 ± 0.01 mm thick and 0.86 ± 0.1 mm wide) and 17.1 ± 0,2 g, submitted to the centesimal composition process (Table 1).

Table 1 Centesimal composition of *Caesalpinia echinata*, *Prosopis juliflora* and *Pithecellobium Dulce.*

Constituents	*Caesalpinia echinata*	*Prosopis juliflora*	*Pithecellobium dulce*
Moisture (%)	9.21 ± 0.03	12.65 ± 0.25	16.9 ± 0.08
Ash (%)	3.37 ± 0.05	3.12 ± 0.17	2.52 ± 0.11
Lipids (%)	32.61 ± 0.81	1.78 ± 0.06	12.21 ± 0.58
Fiber (%)	0.80 ± 0.08	0.83 ± 0.08	10.17 ± 0.14
Proteins (%)	17.24 ± 1.21	27.58 ± 0.81	18.29 ± 0.84
Carbohydrates (%)	36.77	53.04	39.91
Energy (Kcal/100g)	509.5	338.5	342.69

The best extraction of proteins was observed (solid-liquid ratio 1:5; 25 ºC and [Ch][BIT] at 5.42%) using Prosopis juliflora (3.30 ± 0.02 $mg.mL^{-1}$) corroborating the amount of proteins present in the seed. Doubling the concentration of the ionic liquid (10.42 $mg.L^{-1}$) made it possible to increase the protein concentration to 3.76 ± 0.02 $mg.mL^{-1}$. In This case, trypsin inhibition by the extract reached a value of 93.8% (Table 1).

Table 1 Protein concentration and trypsin inhibition for different biomass and [Ch][Bit] concentration

Condition	Protein Concentration ($mg.mL^{-1}$)	Trypsin Inhibition (%)
Caesalpinia echinata [Ch][Bit] 5.42%	1,88 ± 0,01	60,8 ± 2,3
Pithecellobium dulce Ch][Bit] 5.42%	3,03 ± 0,02	77,7 ± 1,2
Prosopis juliflora Ch][Bit] 5.42%	3,30 ± 0,02	93,8 ± 0,8
Prosopis juliflora Ch][Bit] 10.42%	3,76 ± 0,02	94,2 ± 0,5

The kinetic study demonstrated that 20 minutes is enough for the occurrence to occur (Figure 1a). Studies reported in the literature state that the seeds of these plants are capable of inhibiting trypsin[9,12,17,17]. The protein profile of the *Prosopis juliflora* seed extract showed protein bands with a molecular weight of less than 36 kDa on SDS-PAGE electrophoresis (Figure1b).

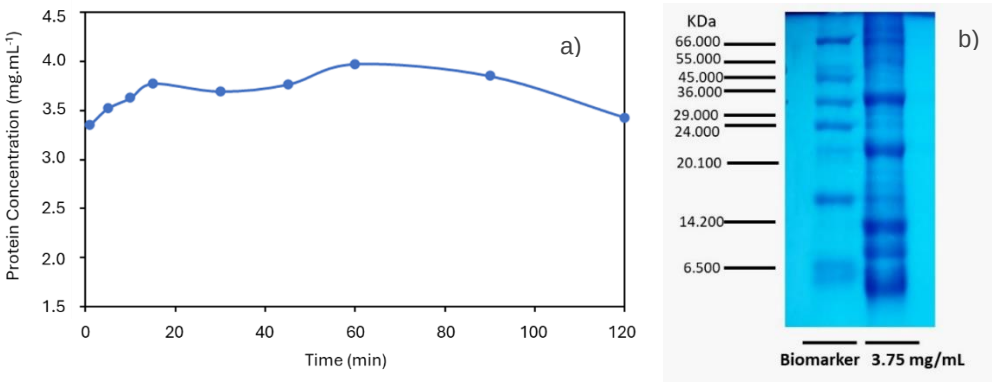

Figure 1 – a) Kinetic study of protein extraction from Prosopis juliflora seeds. b) Coomassie blue stained SDS-PAGE (gel 12 wt%) of the protein extract of *Prosopis juliflora* seed using [Ch][Bit].

4 CONCLUSION

The results of the seed characterizations show small differences in relation to what has been found in the literature, which can be attributed to soil and climate conditions, genetic variability and the degree of ripeness of the seeds. Extraction with choline-based ionic liquids ([Ch][Bit]) extracted a high protein content from the seeds, and the trypsin inhibition test comparatively showed that Prosopis juliflora inhibited trypsin by almost 94%.

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