

Characterization of Novel Trypsin Inhibitor in Raw and Toasted Peanuts Using a Simple Improved Isolation

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Trypsin inhibitors have been described in peanuts and their derived industrialized foods, demonstrating diversity and thermoresistance. Given their most varied applications, these enzymatic protease inhibitors have been isolated and characterized for their potential use as bioinsecticides, herbal medicines, or medicines, but it is not simple. There are still no reports in the literature of the isolation and characterization of trypsin inhibitors in cultivar cavalo rosa (CCR) peanut, a common variety in Brazil. However, there are biological activities related to trypsin inhibitors from peanut-derived products. In this study, we isolated and characterized a novel trypsin inhibitor in CCR peanuts (*Arachis hypogaea* L.) under different processing conditions using a simple improved isolation. Raw and toasted peanut inhibitor was isolated by ammonium sulfate fractionation and trypsin-cyanogen bromide-activated Sepharose[®] 4B (CNBr-Sepharose[®] 4B) chromatography. The inhibitors from raw and toasted peanut were called AhTI1 and AhTI2, respectively, with potent anti-trypsin activity. Activity at different temperatures and pH was evaluated, and both samples were similarly stable under tested conditions. Minimum concentration for inhibition to occur (IC₅₀) was 2.78×10^{-10} M and 2.39×10^{-10} M for AhTI1 and AhTI2, and inhibition constant (K_i) was 3.26×10^{-10} M and 1.54×10^{-10} M, respectively, showing non-competitive reversible kinetics. We concluded that AhTI1 and AhTI2 presented highly specific to trypsin and stable to toasting, different temperatures, and pH ranging. These are important characteristics in the process of developing bioinsecticides or biopharmaceuticals. Thus, this may be an interesting molecule, aiming at its biotechnological application, and it was obtained using a simple and easy isolation process.

Keywords: *Arachis hypogaea* L., protein inhibitors, anti-trypsin activity, chromatography thermoresistance, bioactive protein

Introduction

Among the species of the genus *Arachis* L., *Arachis hypogaea* L. is one of the most economically important oil seed. Popularly known as peanut, it is an herbaceous legume seed, much grown for the production of high-quality vegetable oil and also consumed in both its raw and processed forms [1, 2]. The consumption of peanut of the CCR variety stands out in northeast Brazil and São Paulo state, due to its easy access, being commonly found in markets and supermarkets [3].

Peanut has a high nutritional value in its composition. It has high energy value and is rich in proteins with essential amino acids, carbohydrates, lipids, and fibers and also micronutrients such as calcium, iron, magnesium, zinc, and potassium [1, 4]. In addition, studies show bioactive health activities in peanuts, such as anti-inflammatory, anti-tumoral, anti-oxidant, hypoglycemic, hypolipidemic, anti-platelet aggregation, and satietogenic [2, 5–10].

The action in satiety was mainly attributed to the presence of monounsaturated and polyunsaturated fatty acids, since they are more easily mobilized for β -oxidation when compared to the saturated fatty acids. However, the effect of peanuts on satiety may also be attributed to the presence of a trypsin inhibitor [11, 12]. In fact, there are studies that confirm the presence of various en-

zymatic trypsin inhibitors in different peanut varieties [13–16], as well as in their derived food products, submitted to the most diverse industrial processes [12, 17], demonstrating diversity and thermoresistance of these molecules.

The primary role of inhibitors is to protect plants against phytopathogenic microorganisms. The mechanism for this is not clearly understood, but inhibitors synthesis is believed to hamper cell wall and membrane protein proteolysis. This prevents the action of pathogens and cellular disorganization, inhibiting the growth of transformed cells, which may explain the bio-insecticide action of these molecules [18, 19]. However, also because of their inhibitory action, inhibitors have been positively highlighted in the control of various biological processes, with positive perspectives in medicinal application. They act by regulating or inhibiting the hyperactivity of certain enzymes and are recognized for their heterologous actions as bioactive molecules for health with anti-coagulant, anti-inflammatory, anti-bacterial, anti-fungal, anti-fibrinolytic, and anti-obesity activities [2, 20–25].

In view of this, proteic enzyme inhibitors are increasingly researched for isolation and purification. Thus, aiming at their potential use as bio-insecticides, herbal remedies, or medicines, their biochemical characterization is important. This encompasses determination of three-dimensional structures, inhibitory specificity for classes of peptidases, and mechanisms of inhibition [18, 26–28]. Despite the pressure to reduce costs in the investigation of new

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drugs of natural origin, which demands a high cost for active principle or component purification, the separation, purification, and characterization of natural products are considered the basis of biopharmaceuticals development [29].

Although isolating and characterizing protease inhibitors is not simple, it is the safest and most promising route in bioprospecting of new bioproducts, as well as biopharmaceuticals. Chromatography, especially affinity chromatography, is the dominant technique in the purification of these molecules. This is due to a high efficiency in the separation of similar components in a complex matrix, which is important for defining structural characteristics and specificity to various enzymes [17, 30].

Thermostability, pH (hydrogen ionic potential), and enzymatic kinetics of inhibition are extremely important variables in the area of protein biotechnology [31, 32]. By means of temperature and pH, for example, it is possible to determine the extreme limits of a molecule bioactivity [29]. As for the enzymatic kinetics, it is possible to identify the type of inhibition, inhibition constant (K_i) and concentration of an inhibitor where the response (or binding) is reduced by half (IC_{50}), and this information is essential for the study of potential biopharmaceuticals [30].

The literature has presented the most diverse bioactivities of molecules isolated from peanut and its derived food products [12]. Nevertheless, there are no data concerning the isolation and characterization of trypsin inhibitors from the CCR variety, which is largely consumed in Brazil. Therefore, isolating a novel inhibitor using a simple and easy isolation process of the same variety of peanut and characterizing under different processing conditions will reveal important information, since the processing may interfere directly in molecular bioactivity.

Experimental

Materials. CCR peanuts (*A. hypogaea* L.) were locally purchased. Analytical grade reagents and trypsin-CNBr-Sepharose[®] 4B affinity chromatography were from Sigma (St. Louis, USA) and VETEC Química Fina Ltda (Rio de Janeiro), Brazil. Experiments were done in the Laboratory of Chemistry and Function of Bioactive Proteins in the Department of Biochemistry at Federal University of Rio Grande do Norte state, Brazil (UFRN).

Trypsin Inhibitor Isolation. The total proteins were extracted from raw and toasted peanuts flour in 50 mM Tris-HCl buffer, pH 7.5, at ratio of 1:10 (w/v). The crude extract was sequentially fractionated with ammonium sulfate at 0–30% (F1), 30–60% (F2), and 60–90% (F3) saturation. Approximately 10 mg of F2 (30–60%) to a trypsin-Sepharose 4B affinity column (10 cm × 1.5 cm) was applied. The protein profile was evaluated by spectrophotometry at 280 nm. The degree of purity and the molecular masses were monitored by electrophoresis [33].

Antitrypsin Activity. The anti-trypsin activity of the CE, F1, F2, and F3 was determined in accordance to Kakade et al. (1969) [34]. The aliquot of 20 μ L bovine trypsin solution (0.3 mg/mL 2.5 mM HCL) was pre-incubated with 560 μ L of Tris-HCL buffer 50 mM, pH 7.5, 120 μ L of HCL 2.5 mM. and 100 μ L CE, F1, F2, and F3 for 15 min at 37 °C. Afterwards, the reaction was started by adding 500 μ L of substrate solution (BAPNA, 1.5 mM). Inhibition of trypsin was measured by the residual activity of the enzyme and expressed in relation to the hydrolysis promoted in the absence of the inhibitor (100% of the enzymatic activity), and therefore 0% of antitryptic activity. The inhibition unit represents the difference between the enzymatic activity of the enzyme and the tested material, with 1.0 IU equal to 0.01 nm. Protein isolates with anti-trypsin activity of raw and toasted peanuts were named AhTI1 and AhTI2, respectively.

Stability Determination to Variations in Temperature and pH. Determination of AhTI1 and AhTI2 stability to different

temperature and pH conditions was done as previously established [35]. For the temperature curve, 1.0 mL aliquots of the isolates were incubated for 30 min at 40, 60, 80, and 100 °C and then cooled to 4 °C. For the pH curve (2, 3, 6, 8, 11, 12), aliquots of the same volume of the inhibitors were dialyzed against different buffers for 16 h. Buffer solutions used were as follows: glycine-HCl, pH 2.0–3.0; sodium phosphate, pH 6.0 and 8.0; and glycine-NaOH, pH 11–12, all at the concentration of 100 mM. After 30 min of incubation at 37 °C in the buffers, samples were dialyzed for about 4 h in 20 mM sodium tetraborate, pH 7.5. Aliquots submitted to different temperatures and those submitted to different pHs had, at the end of the treatments, their inhibitory activity for trypsin tested according to methodology described elsewhere [34].

IC₅₀ Determination. To determine the concentrations of AhTI1 and AhTI2 that inhibit 50% of trypsin enzymatic activity (IC_{50}), assays with different concentrations of the inhibitors were performed. Inhibition curves relating the percentage of trypsin inhibition and the concentration of AhTI1 and AhTI2 used in the assays were constructed with increasing concentrations of inhibitor incubated with aliquots of 10 μ L (3 μ g) of trypsin [20].

Inhibition Constant (K_i) Determination. To determine the K_i values of AhTI1 and AhTI2 for trypsin, graphs were constructed, where the abscissa axis (X) corresponded to increasing concentrations of AhTI1 and AhTI2 and the ordinates (Y) axis corresponded to the inverse of the values of the maximum velocity [36]. The anti-trypsin enzyme assay for K_i determination was performed in the presence of two concentrations of benzoyl-DL-arginine-*p*-nitroanilide (BAPNA, 6.25×10^{-4} mol/L and 1.25×10^{-3} mol/L). K_i value was obtained from the intersection of the two lines corresponding to the concentrations of the substrate used. The velocity value was determined by $V = OD \ 405 \text{ nm/h/mL}$.

Statistical Analysis. Data represent at least three independent experiments and were expressed as mean and standard deviations (SDs), unless otherwise noted. Data were analyzed by GraphPad Prism 6.0 software.

Results

Isolation of the Trypsin Inhibitor from Raw and Toasted CCR Peanut. In this study, we observed an inhibitory activity upon trypsin, in both raw and toasted peanuts, in all steps of isolation preceding trypsin-CNBr-Sepharose[®] 4B affinity chromatography. The trypsin inhibitor in raw CCR peanut (AhTI1) presented high (100%) inhibitory activity (Figure 1A) and a specificity that increased from 5.12 IU/mg to 2391.89 IU/mg after isolation. The trypsin inhibitor in toasted CCR peanut (AhTI2) also presented a 100% inhibitory activity upon trypsin (Figure 2A) and a specificity that increased from 20.1 IU/mg to 1703.39 IU/mg after isolation.

We also observed molecular mass using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In samples from raw and toasted peanuts, a predominant molecular mass of about 32 kDa was observed (Figures 1B and 2B).

AhTI1 and AhTI2 Characterization in Different Temperatures and pHs. Figure 3 shows that AhTI1 and AhTI2 are stable at high temperature, exhibiting an optimum activity between the temperatures of 40 °C to 100 °C. In Figure 4, those samples respond differently to pHs ranging from 2 to 11. Reduction in its activity is seen only when exposed to pHs over 11.

AhTI1 and AhTI2 IC₅₀ and K_i . AhTI1 and AhTI2 required concentration for a 50% inhibition on trypsin catalytic activity was 2.78×10^{-10} M and 2.39×10^{-10} M, respectively (Figure 5). According to the titration, the maximum inhibition percentage obtained in the curve was 94% for AhTI1 and 93% for AhTI2.

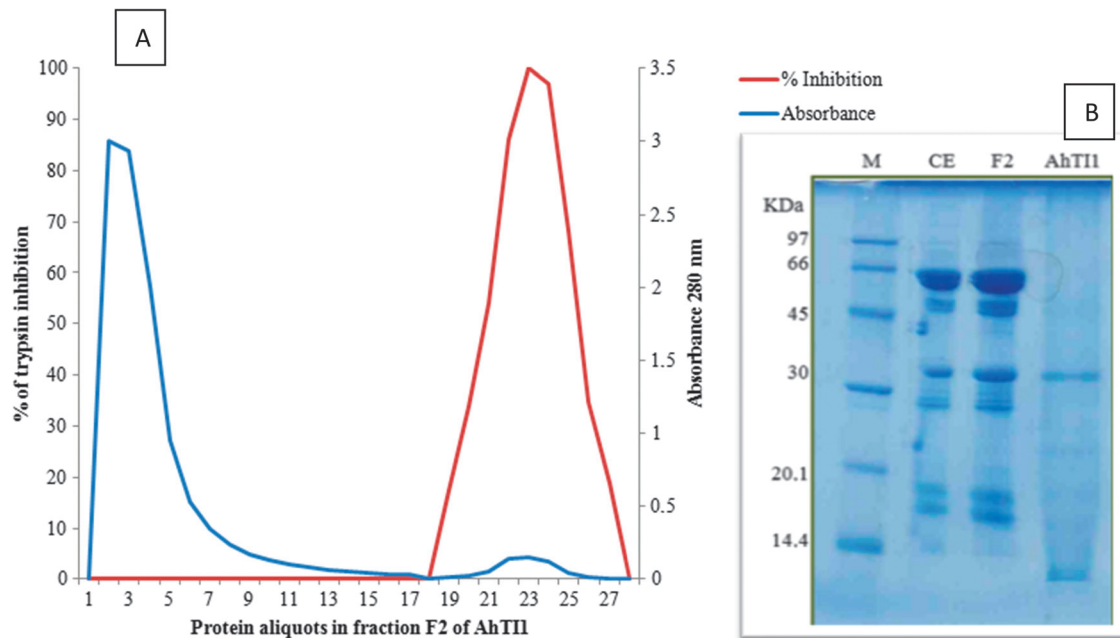


Figure 1. A) Fraction 2 trypsin-cyanogen bromide-activated Sepharose[®] 4B. B) Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE): M, molecular marker; CE, crude extract; F2, fraction F2; AhTI1, raw cultivar cavalo rosa peanut trypsin inhibitor

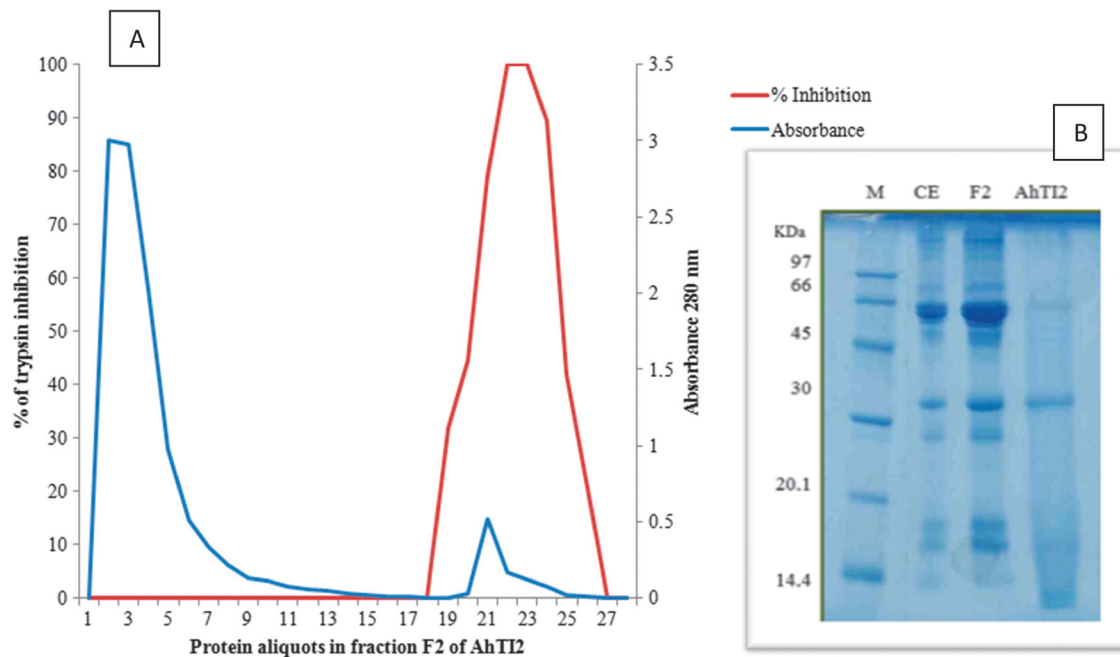


Figure 2. A) Fraction 2 trypsin-cyanogen bromide-activated Sepharose[®] 4B. B) Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE): M, molecular marker; CE, crude extract; F2, fraction F2; AhTI2, toasted cultivar cavalo rosa peanut trypsin inhibitor

The K_i values of AhTI1 and AhTI2 were 3.26×10^{-10} M and 1.54×10^{-10} M, respectively (Figure 6).

Discussion

In peanuts and some of their products, the natural presence of trypsin inhibitors is known. In these cases, probably, the thermoresistance of these inhibitors, presented in industrialized products, explains the fact that this inhibitory activity is still present after the use of heat in its processing, as already well discussed in the literature [17].

In this study, we observed an inhibitory activity upon trypsin, in both raw and toasted peanuts. Although some trypsin inhibitors demonstrate thermolability, this is not the case of the protein herein; once in toasted peanuts, the inhibitory action was still found.

It is known that thermal stability of trypsin inhibitors is determined by several factors: temperature and humidity submitted, size of particles and structural conformation, and processes that could influence the activity of the inhibitor [37].

When comparing the inhibitory activity on trypsin between raw and toasted peanuts commercially sold in Canada, the United Kingdom, and the USA, toasted peanuts showed a 3.6-fold increase in the inhibition percent [38]. Thus, applying heat to food cannot be generalized as a process to reduce the activity of trypsin inhibitors, and this applies to the CCR peanut variety.

To isolate using a simple improved isolation, composed for only three steps and verify the specificity of inhibitors, the protein extraction, the ammonium sulfate precipitation, and the trypsin-CNBr-Sepharose[®] 4B affinity chromatography were performed, and the inhibitory activity on trypsin was

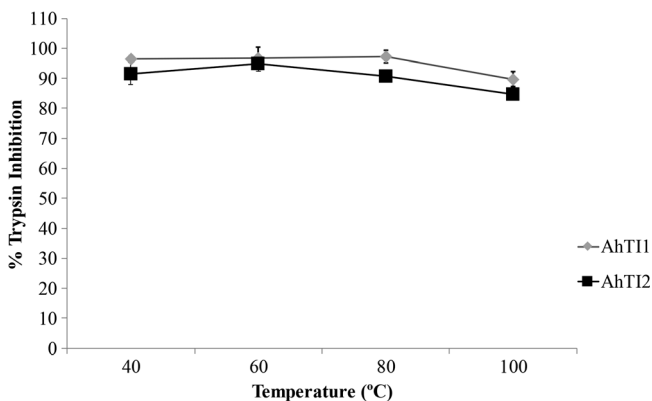


Figure 3. Stability in different temperatures of protein isolates with anti-trypsin activity of raw (AhTI1) and toasted (AhTI2) cultivar cavalo rosa peanuts. One milliliter aliquots of protein isolates were incubated for 30 min at different temperatures (40 to 100 °C) and further tested in a trypsin inhibition assay with BApNa 1.25×10^{-3} mol/L as a protein substrate

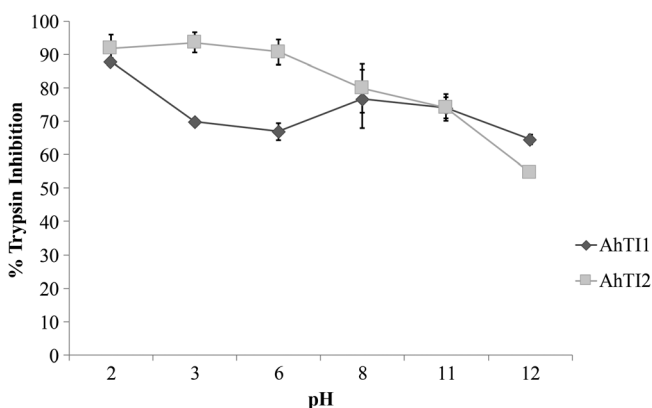


Figure 4. Stability in different pHs of protein isolates with anti-trypsin activity of raw (AhTI1) and toasted (AhTI2) cultivar cavalo rosa peanuts. One milliliter aliquots of protein isolates were incubated for 30 min at different pHs (2 to 12) and further tested in a trypsin inhibition assay with BApNa 1.25×10^{-3} mol/L as a protein substrate

then observed. The trypsin inhibitor in raw CCR peanut (AhTI1) presented high inhibitory activity and a specificity that increased during the isolation. We also observed molecular mass using SDS-PAGE. In samples from raw and toasted peanuts, a predominant molecular mass of about 32 kDa was observed.

Serine type protease inhibitors are classified according to their molecular mass; those with high molecular weight are named Kunitz (approximately 20 kDa), and those with low-weight,

Bowman-Birk (6 to 10 kDa)[39]. Nevertheless, although AhTI1 and AhTI2 presented a high molecular weight, better characterization is needed to classify them, preferably using sequencing techniques.

When comparing SDS-PAGE gels for AhTI1 and AhTI2, we hypothesized that they were the same inhibitor in both raw and toasted peanuts, but other studies need to be performed to confirm this supposition. Other studies in peanuts and its products have found a trypsin inhibitor of 33 kDa in peanut sweet and Japanese peanut [12, 17]. In sweet potatoes, a trypsin inhibitor of 33 kDa (SPTI) was also found [40]. However, the isolation of a trypsin inhibitor in CCR peanuts resistant to toasting, with this molecular mass (32 kDa) isolated, has not been documented in the literature yet.

To better characterize the isolated trypsin inhibitors, optimal action temperatures and pHs of both AhTI1 and AhTI2 were determined. Thus, reinforcing the hypothesis above, the trypsin inhibitors isolated from raw and toasted peanuts presented similar results when exposed to different temperatures and pHs. It is known that most trypsin inhibitors are resistant to heat, extreme pH, and the action of some proteolytic enzymes [41].

Results from Figures 4 and 5 demonstrate that AhTI1 and AhTI2 are stable at high temperature, which exhibits optimum activity between the temperatures of 40 °C to 100 °C and pHs ranging from 2 to 11. Reduction in its activity is seen only when exposed to pHs over 11 (Figure 4). This stability is probably due to the presence of disulfide bonds between their polypeptide chains [17, 37].

The determination of thermostability and the ideal pH in the characterization of potential biopharmaceuticals is extremely important for biotechnology [31]. Thus, the resistance to a huge variation of temperatures and pHs, an advantage against the most varied conditions required for enzymatic reactions, and the inhibitory action upon trypsin may put this inhibitor as a biopharmaceutical candidate.

Considering that IC_{50} is the concentration required to induce 50% of the maximum response, AhTI1 and AhTI2 required very low concentrations for a 50% inhibition on trypsin catalytic activity. This test is important for molecules that are biopharmaceutical candidates, once it may help determining the minimum dose or concentration necessary to obtain the effect of the molecule.

Likewise, K_i is an inhibition constant that refers to the concentration of the molecule to exert its bioactivity, allowing the establishment of the molecule kinetics [32]. Hence, taking data altogether, we confirmed that AhTI1 and AhTI2 are the same inhibitor. Considering its kinetics, it is a non-competitive inhibitor. Observing IC_{50} and K_i values of Kunitz type inhibitors, the inhibitor we studied has higher affinity for trypsin than the

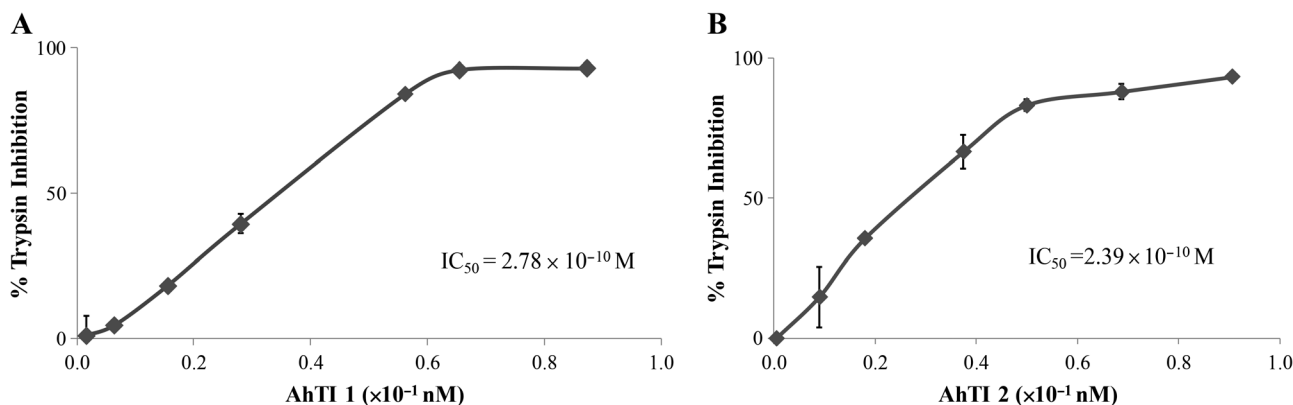


Figure 5. IC_{50} determination of protein isolates with anti-trypsin activity of raw (AhTI1) and toasted (AhTI2) cultivar cavalo rosa peanuts. A) Titration curve using increasing amounts of AhTI1 (0.01, 0.06, 0.15, 0.28, 0.56, 0.65, 0.88 nM) and a fixed concentration of trypsin (10 μ L, 0.3 mg/mL). B) Titration curve using increasing amounts of AhTI2 (0.01, 0.09, 0.18, 0.37, 0.5, 0.68, 0.9 nM) and a fixed concentration of trypsin (10 μ L, 0.3 mg/mL)

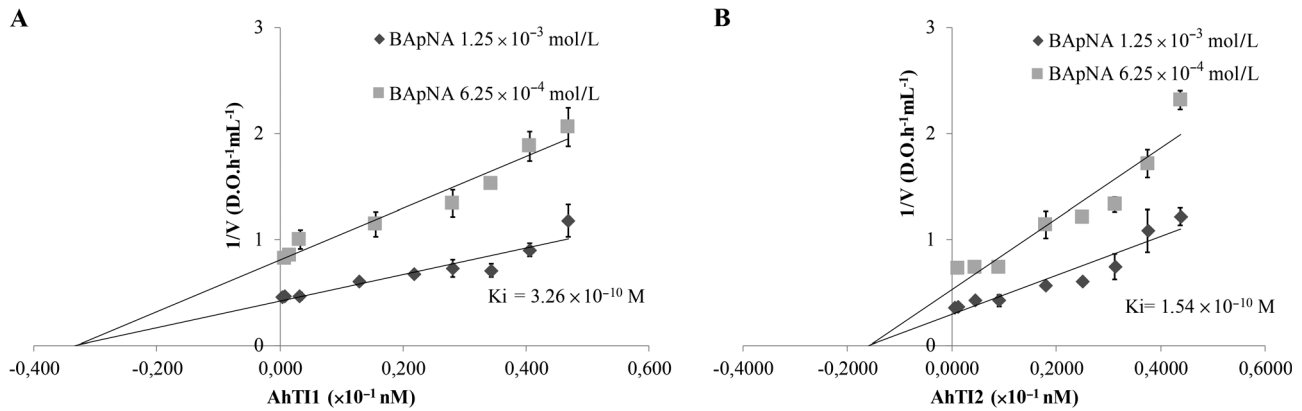


Figure 6. The inhibition constant (K_i) upon trypsin of protein isolates of raw (AhTI1) and toasted (AhTI2) cultivar cavalo rosa peanuts was determined as proposed by Dixon et al. (1979). The K_i value was obtained from the intercept points of the two molarities used of BApNA substrate (1.25×10^{-3} mol/L and 6.25×10^{-4} mol/L). The velocity value was determined by $V = OD\ 405\ \text{nm/h/mL}$. A) AhTI1 K_i . B) AhTI2 K_i

inhibitors of *Erythrina variegata* seeds (IC_{50} of 2.0×10^{-8} and K_i of 5.2×10^{-6} M), *Archidendrom ellipticum* seeds (K_i of 2.4×10^{-10} M), and also *Caesalpinia bonduc* seeds (K_i of 2.46×10^{-10} M) [42, 43]. K_i values found in our study are similar to those found for *Inga laurina* and *Dimorphandra mollis* seeds [44, 45]. Still, according to the literature, there are five protease inhibitors in peanuts, with K_i values between 1.4 and 4.4×10^{-7} M14. Thus, compared to the K_i values of AhTI1 and AhTI2, these present higher affinity for trypsin.

In addition, the non-competitive reversible enzyme inhibition, which is how the studied inhibitor acts against trypsin, is characterized by reversible binding of the inhibitors to the enzyme at the substrate binding site [46]. This type of inhibition is independent of substrate concentration, other than competitive reversible enzyme inhibition. Thus, the effect is not reversed by increasing the substrate concentration, and therefore, the inhibition mechanism is not dependent on substrate and inhibitor concentrations. This is an interesting feature, once it is known that many drugs used routinely are based on specific non-competitive inhibition of enzymes [47].

Conclusion

Our data support that, using a simple and easy isolation process, it was possible to obtain isolated protein isolates from raw and toasted CCR peanuts and contain the same molecule with inhibitory action upon trypsin. For this inhibitor, toasting is not sufficient to decrease or inactivate its anti-trypsin activity. Moreover, characterization revealed that this inhibitor is stable to extremes of temperature and pH and shows high affinity for trypsin and reversible non-competitive kinetics. Given the properties and health claims attributed to enzymatic inhibitors with bioactive activities, related mainly to their power to inhibit or modulate proteolytic enzymes, this puts this molecule as a candidate for biotechnology.

Abbreviations

CNBr-Sepharose [®] 4B	bromide-activated Sepharose [®] 4B
AhTI1	anti-trypsin activity of raw
AhTI2	anti-trypsin activity of toasted
K_i	inhibition constant
IC_{50}	minimum concentration for inhibition to occur
BApNA	benzoyl-DL-arginyl- <i>p</i> -nitroanilida
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

Conflict of Interest

The authors declare no conflicts of interest.

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