Extraction, purification and characterization of inhibitor of trypsin from *Chenopodium quinoa* seeds

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**Abstract**

A novel trypsin inhibitor of protease (CqTI) was purified from *Chenopodium quinoa* seeds. The optimal extracting solvent was 0.1M NaCl pH 6.8 (p < 0.05). The extraction time of 5h and 90 °C was optimum for the recovery of the trypsin inhibitor from *C. quinoa* seeds. The purification occurred in gel-filtration and reverse phase chromatography. CqTI presented active against commercial bovine trypsin and chymotrypsin and had a specific activity of 5,033.00 (TIU/mg), which was purified to 333.5-fold. The extent of purification was determined by SDS-PAGE. CqTI had an apparent molecular weight of approximately 12KDa and two bands in reduced conditions as determined by Tricine-SDS-PAGE. MALDI-TOF showed two peaks in 4,246.5 and 7,908.18m/z. CqTI presented high levels of essential amino acids. N-terminal amino acid sequence of this protein did not show similarity to any known protease inhibitor. Its activity was stable over a pH range (2-12), temperatures range (20-100 °C) and reducing agents.

**Keywords:** purification; characterization; inhibitor of trypsin; *Chenopodium quinoa*; seeds.

**Practical Application:** The purification and characterization of this novel trypsin inhibitor allow its study for a diversity of applications such as antibacterial, anticancer, anti-inflammatory and biotechnology and its further use as drugs for human diseases.

1 Introduction

Proteinase inhibitors (PIs) have important mechanism that plants produce to offer protection against predators or from infection by pathogens. They are widely distributed in the plant kingdom and the plant families Leguminoseae, Solanaceae and Graminaceae are known to be rich in these inhibitors (Nakahata et al., 2011). The PIs are small regulatory proteins generally present in high concentration and distributed in 10 families or more determined according to the primary structure, that is, the active amino acid in their reactive site, which are serine-, cysteine-, aspartic-, and metallo-PIs (Losso, 2008). In specially, plant serine-PIs are grouped into Bovine pancreatic trypsin inhibitor (Kunitz), Pancreatic secretory trypsin inhibitor (Kazal), Streptomyces subtilisin inhibitor, Soybean trypsin inhibitor (Kunitz), Soybean proteinase inhibitor (Bowman-Birk), Potato I inhibitor, Potato II inhibitor, Ascaris trypsin inhibitor and Other families (Laskowski & Kato, 1980).

The most popular classes of PIs are Kunitz and BBI (Bowman-Birk). The Kunitz inhibitors family is usually 18-24kDa heterogeneous proteins consisting of a number of iso-inhibitors, with two disulfide linkages and a single trypsin reactive site (defined by an arginine residue) in one of the protein loops. The BBI are small cysteine-rich proteins in size (8-20kDa), with seven disulfide linkages, high cysteine content, and two independent reactive sites for trypsin and chymotrypsin, soluble between pH 1.5 and 12 and in dry state or in 0.02% aqueous solution is stable at 100 °C or at 105 °C for 10 min, respectively (Losso, 2008). These classes of proteins has been studied due to its application in the treatment of different pathologies as cancer (Zhang et al., 2011), antifungal activity (Kim et al., 2005), inflammation and coagulation (Machado et al., 2013) or as bioinsecticides (Rai et al., 2010), demonstrating its importance as new therapeutic agents for the pharmaceutical industry. Despite there are abundantly studies of PIs in leguminous plants, little is known in cereals, such as amaranth (Tamir et al., 1996); buckwheat (Park et al., 1997) and buckwheat Seeds (Tsybina et al., 2001).

The *Chenopodium quinoa*, a pseudo-cereal, is original cultivated in Colombia, Peru, Bolivia, Equator, Chile and Argentina in variety latitudes and altitudes. Due to its potential food source and South America limited production, *C. quinoa* has being introduced in Europe, North America, Africa and Asia (Bhargava et al., 2006). This has contributed for its popularization, especially in the developing countries, as alternative food, which had to its high nutritional importance. Quinoa has high concentration of essential aminoacids and high content of calcium, magnesium, iron, copperand and zinc, and are rich in vitamins, as α-carotene and niacin (Vega-Gálvez et al., 2010). Especially, chemical composition revealed the potential of *C. quinoa* seeds as a valuable ingredient in the preparation of cereal foods of improved nutritional characteristics (Stikic et al., 2012), but heat is necessary to degradation of antinutritional factors for...
human consumption (Silva et al., 2015a). In Brazil, the C. quinoa was introduced in the decade of 1990, as part of an effort to diversification of production systems in the cerrado (Savannah), the Brazilian hotspot (Spehar & Santos, 2002). The purpose of this work was extraction, purification and characterization of the protease inhibitors isolated from Chenopodium quinoa seeds.

2 Materials and methods

2.1 Chemicals

BSA (bovine serum albumin), bovine pancreatic trypsin, bovine pancreatic a-chymotrypsin, papain, a-amylase, casein, BTPNA (N-Benzoyl-L-tyrosine p-nitroanilide) and BApNA (N-R-benzoyl-DL-arginine-p-nitroanilide) were purchased from Sigma (St. Louis, MO, USA), as were molecular weight markers standard, acrylamide, bis-acrylamide, DTT (Dithiothreitol) and other electrophoresis reagents. TNBS (2,4,6-trinitrobenzenesulfonic acid), TFA (Trifluoroacetic acid) were purchased from Merck (Darmstadt, Germany). The chromatography supports were from GE Healthcare Life Sciences (Uppsala, Sweden). All other chemicals and reagents used were of analytical grade.

2.2 Preparation of seed flour

The C. quinoa seeds were courtesy from Carlos Roberto Spehar, researcher at University of Brasilia. The seeds were washed and crushed using a blender to obtain flour. The seeds (50g) were ground in an electric mill and the seed flour obtained it was used in the next step.

2.3 Effect of extraction media on trypsin inhibitor extraction

Different extracting solvent including distilled water, 0.1M NaCl, 0.15M NaCl, 0.30M NaCl, 0.01M NaOH and 0.02M NaOH were used to extract protease inhibitor according Klomklao et al. (2011). The seed flour was added to the medium at a ratio of solvent mass to feed mass of 5:1 (S/F) and shaken for 1h at 150rpm at room temperature. The extract was recovered by centrifuging at 10,000g for 30min. The trypsin inhibitory activity and protein content in the supernatant were determined and the specific inhibitory activity obtained using different media were compared. The extracting solvent used for solubilizing the trypsin inhibitor, which was able to extract the trypsin inhibitor with the highest specific trypsin inhibitory activity, was selected for further steps.

2.4 Effect of extraction time on trypsin inhibitor extraction

Seed flour was mixed with 0.1M NaCl in a ratio of 5:1 (S/F) and, then, shaken for 1, 2, 3, 4, and 5h. At designated time, the mixture was centrifuged at 10,000g for 30min and the supernatants were subjected to the determination of the trypsin inhibitory activity and protein content. The specific trypsin inhibitory activities were then calculated. The extracting time rendering the highest specific trypsin inhibitory activity was chosen for further study.

2.5 Effect of extraction temperature on trypsin inhibitor extraction

Seed flour extract in 0.1M NaCl and shaken for 5h was heated at different temperatures (room, 37, 60, 70, 80, 90 and 100 °C) for 10min and then cooled with ice water. To remove the heat coagulated debris, the extracts were centrifuged at 10,000g for 10min at 4 °C. The activity and specific activity of the trypsin inhibitor in the supernatant was measured. The heat treatment, which gave the supernatant with the highest specific activity, was chosen for further study.

2.6 Purification of trypsin inhibitor from Chenopodium quinoa seeds

The heat-treated extract was subsequently submitted at different ammonium sulfate precipitation (0-40% and 40-90% saturation) and the protein content and trypsin inhibitory activity was analysed. The highest specific activity of the fractions was selected and lyophilized.

The lyophilized was dissolved in AMBIC (Ammonium bicarbonate) buffer 1M and applied to an exclusion molecular Sephadex G-50 (1.0 × 50cm, eluted with AMBIC buffer 0.2M) equilibrated with the same buffer, with a flow rate of 0.5mL/min. The single peak of antitryptic activity was collected and dialyzed against distilled water and lyophilized. The G-50 column fraction was then separated by reverse-phase HPLC, as described by Macedo et al. (2007), on a Hypersil Gold C18 (300 × 4mm) (Thermo Scientific) that was previously equilibrated with 0.1% (v/v) TFA (solvent A), followed by a 50min linear gradient from 0 to 100% (v/v) of 66% (v/v) acetonitrile in 0.1% (v/v) TFA (solvent B) with a flow rate of 0.5mL/min. Proteins were detected by monitoring absorbance at 280nm.

2.8 Determination of protease inhibitory activity

The inhibitory activity against proteases was determined by measuring the remaining hydrolytic activity toward specific synthetic substrates after pre-incubation with CqTI (Silva et al., 2015a). The inhibitory activity against chymotrypsin and trypsin was determined toward the substrate BTPNA and BApNA, respectively, in Tris (2-Amino-2-hydroxymethyl-propane-1,3-diol) buffer pH 8.0 at 37 °C in 30min. The reaction was stopped with 500μL acetic acid 30% (v/v), and the substrate hydrolysis followed by absorbance at 405nm. All assays were done in triplicate. One trypsin unit (TU) was arbitrarily defined as an increase of 0.01 absorbance units at 405nm per ml of reaction medium. Results were expressed as units of trypsin inhibitory (TI) per mg protein, one inhibitory unit responsible for the inhibition of one unit of trypsin.

2.9 Protein determination

Protein contents were determined using Coomassie blue staining by Bradford method (Bradford, 1976) or by absorbance at 280nm. BSA (1mg/ml) was used a standard.
2.10 Tricine Sodium Dodecyl Sulfate Polyacrylamide Gel (SDS-PAGE)

Tricine SDS-PAGE was carried out according to the procedure of Schägger & von Jagow (1987) with some modifications. The separating gel, spacer gel, and stacking gel were well prepared and solidified. For supernatant, the highest protein concentration sample was diluted to 1mg/mL. Precipitate samples were treated with the same method as supernatant. Then 0.5mL of diluted sample was mixed with 0.5mL of sample buffer [0.05M Tris–HCl at pH 6.8, 4% (w/v) SDS, 12% (v/v) glycerol, 0.01% (w/v) bromophenol blue, and 2% (v/v) 2-ME (or no for nonreducing)] effectively and heated for 3 min in a boiling water bath, then centrifuged at 10000 rpm for 3min. Then 10μL or 20μL of each sample was loaded up to a well. Tricine SDS-PAGE was performed at 30V for 1h, and then up to 100V. After electrophoresis, the gels were stationed for 2h in stationary liquid [45.4% (v/v) methanol, 9.2% (v/v) glacial acetic acid] and then stained for 2h using 0.1% (w/v) Coomassie Brilliant Blue R-250. After staining, the gels were destained using a 10% (v/v) acetic acid solution until a clear background was obtained. The proteins used as molecular weight standards for Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) were phosphorylase b (94kDa), bovine serum albumin (66kDa), ovalbumin (44kDa), bovine anhydrase carbonic (30kDa), inhibitor of trypsin (20kDa), lysozyme (14.kDa).

2.11 Mass spectrometry analysis

Samples were mixed with α-cyan matrix (CHCA) in the ratio of 1:1 and aliquots of 1μL were applied in the reading plate of the mass spectrometer Voyager TM Biopectrometry (Applied Biosystems). Operating in reflector positive mode, the detection of ions was achieved by the associated mass espectrometric techniques matrix-assisted laser desorption/ionization and time-of-flight (MALDI/TOF MS). Calibration was done externally with a mixture of four standard proteins with their respective mass/charge ratio.

2.12 Amino acid analysis

Amino acid analysis was performed in a Pico-Tag amino acid analyzer (Water System), as described by Heinrikson & Meredith (1984). One nmol of CqTI was hydrolyzed in 6M HCl and 1% phenol at 106 °C for 24h. The hydrolysates were reacted with 20ml of fresh derivatization solution methanol/triethylamine/water/phenylisothio-cyanate, 7:1:1:1, (v/v) for 1h at room temperature. After pre-column derivatization, phenylthiocarbamyl-amino acids were identified by comparing their reverse phase high-performance liquid chromatogram (HPLC) retention times to those of standard PTC-amino acids (Pierce). Cysteine residues were quantified as cysteic acid.

2.13 N-terminal sequence determination

Initially the native inhibitor was reduced and modified with 4-vinylpyrididine and two chains were separated by HPLC. The sequence of first 13 and 10 amino acids each subunits from N-terminal end of CqTI was determined by Edman degradation method (Edman et al., 1950) using an 477A - Applied Biosystems. 500pmoles purified CqTI was electrophoresed on SDS-PAGE (15–4%) and was electrophoretically transferred to a polyvinyl difluoride (PVDF) membrane (Mini Trans-Bolt Electrophoretic Transfer Cell, Bio Rad). The membrane was stained with CBB R-250, destained and washed thoroughly to remove any bound glycine to the membrane. The lanes of CqTI band on the membrane was cut and loaded on to the protein sequencer. Percent homology of N-terminal amino acid sequence of CqTI with other known sequences of proteins was assessed using protein sequence database, SWISS-PROT by all the relevant BLAST programs, i.e. NCBI-protein–protein blast (blastp), NCBI-blast- search for short nearly exact matches, and NCBI-protein query vs. translated database (tblastn). Further the search was done in protein sequence database, UNIPROT using the BLAST program WU-blastp and WU-tblastn.

2.14 Stability studies

In stability studies, effect of pH, temperature and DTT was examined on inhibitory activity of CqTI against trypsin. In pH stability, trypsin inhibition assays under a range of pH (2–12) conditions were performed to assess the pH stability and pH optima of CqTI. Purified CqTI (50μg) was incubated with buffers of pH 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 to a final concentration of 1mg/ml. The buffers used were 0.1M each of glycine–HCl buffers (pH 2, 3), acetate buffers (pH 4, 5), phosphate buffers (pH 6, 7), Tris–HCl buffers (pH 8, 9) and glycine–NaOH buffers (pH 10, 11, 12). After incubation of 30min, the trypsin inhibitory activity was determined at pH 8.0. In thermal stability studies, purified protein (50μg) in 50mM Tris–HCl buffer pH 8.0 was incubated at various temperatures (20-100 °C) for 30min. After incubation, all the samples were kept on ice for 15min and then centrifuged. Supernatants were taken for the estimation of residual trypsin inhibitory activity and measured using BAPNA as substrate at 37 °C. The effect of DTT on inhibitory activity was determined by incubating purified CqTI (50μg) with DTT at final concentrations of 1, 10 and 100μM for 15, 30, 60 and 120min at 37 °C. Adding twice the amount of iodoacetamide to each DTT concentration stopped the reaction and then the residual trypsin inhibitory activity was measured. All experiments were done in triplicate and the results are the mean of three assays.

2.15 Statistic analyses

Mean pairwise comparisons residual activity of trypsin and chymotrypsin was carried out by setting a General Linear Model ANOVA (α = 0.05), followed by Tukey Method and 95% Confidence.

3. Results and discussion

3.1 Extraction of trypsin inhibitor from C. quinoa seeds

We used various extracting solvent to extract the trypsin inhibitor from Chenopodium quinoa seeds (Table 1). The better extractant was NaCl 0.1M (p < 0.05) with 26.07 (± 0.019) and 12.18 (± 0.0094) as highest trypsin inhibitor activity and specific trypsin inhibitor, respectively. From the results, NaCl
Specific Inhibitory Activity

12.18±0.0094
23.46±0.053
25.90±0.026
8.56±0.0089
2.73±0.031

Total IU
23.43±0.013
2.14±0.020
1.93±0.034
2.19±0.012
2.0±0.020
3.0±0.027
25.90±0.026

Table 1. Effect of extractants on the recovery of trypsin inhibitor from C. quinoa seeds. Mean ± SD from triplicate determinations.

<table>
<thead>
<tr>
<th>Extracting Solvent*</th>
<th>Total IU</th>
<th>Total Protein (mg)</th>
<th>Specific Inhibitory Activity (Units/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>14.68±0.050a</td>
<td>2.73±0.031a</td>
<td>5.38±0.012a</td>
</tr>
<tr>
<td>NaCl 0.1M</td>
<td>26.07±0.019b</td>
<td>2.14±0.020b</td>
<td>12.18±0.0094b</td>
</tr>
<tr>
<td>NaCl 0.15M</td>
<td>23.43±0.013c</td>
<td>1.93±0.034c</td>
<td>12.14±0.018bc</td>
</tr>
<tr>
<td>NaCl 0.3M</td>
<td>23.46±0.053d</td>
<td>2.19±0.012d</td>
<td>10.71±0.0059d</td>
</tr>
<tr>
<td>NaOH 0.02M</td>
<td>26.10±0.009e</td>
<td>3.0±0.027e</td>
<td>8.56±0.0089e</td>
</tr>
<tr>
<td>NaOH 0.01M</td>
<td>25.90±0.026f</td>
<td>3.29±0.014f</td>
<td>7.87±0.0044f</td>
</tr>
</tbody>
</table>

*The different letters in the same column denote the significant differences (ANOVA, p<0.05). *The seed flour was shaken in different media at ambient temperature for 1h and trypsin inhibitor was analyzed using BAPNA as substrate.
3.5 Amino acid composition

The study of the amino acid composition demonstrated a high concentration of essential amino acids, lysine and valine (R aliphatic nonpolar group) in CqTI (Table 3). The quantity of histidine, lysine and leucine demonstrated better similarity with legumes species (Silva et al., 2001; Machado et al., 2013). The concentration of Glu, Gli and Arg was better than the concentration in cereal and legume (Tamir et al., 1996; Silva et al., 2001). The presence of four cysteine residues was similarly that observer for the trypsin inhibitor (BWI-2c) from buckwheat (Fagopyrum esculentum) (Tsybina et al., 2001). This pool of amino acids of quinoa was found close to the standards of the FAO/WHO for high-quality protein (Ruales & Nair, 1992).

3.6 N-terminal amino acid sequence

The amino acid sequencing of each subunit revealed that CqTI has two different sequences AFQNTGKQAWNTG and AREDPYTWSW, which were not homologous to any other

Figure 1. Effect of extraction time on the specific trypsin inhibitory activity (a) and trypsin inhibitory activity (b) of C. quinoa seed extract. Effect of heat treatment at different temperatures on the trypsin inhibitory activity (c), and specific trypsin inhibitory activity (d) of C. quinoa seed extract. There was a significant difference between the times evaluated (ANOVA, p <0.05).
Figure 2. (a) Gel filtration of the CqTi on Superdex G-50 on FPLC system. (b) Elution profiles for RP-HPLC analysis of *C. quinoa* trypsin inhibitor (CqTi). (c) Sodium dodecyl sulphate polyacrylamide gel electrophoresis of proteinase inhibitor from *C. quinoa* seed and different fractions under reducing and nonreducing condition. MW: molecular weight standard; TR2: Sephadex G-50 fraction; B: B fraction from RP-HPLC, C: C fraction from RP-HPLC, D: D fraction from RP-HPLC, C + DTT: C fraction from RP-HPLC + DTT.

Table 2. Purification of trypsin inhibitors from *C. quinoa* seeds.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Mg prot/g seed</th>
<th>TUI/g seed</th>
<th>TUI/mg prot</th>
<th>Purification fold</th>
<th>Protein Yield (%)</th>
<th>Activity Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>53.291</td>
<td>804.28</td>
<td>15.09</td>
<td>1</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Sephadex G50</td>
<td>0.145</td>
<td>588.14</td>
<td>4.056</td>
<td>268</td>
<td>0.27</td>
<td>73.2</td>
</tr>
<tr>
<td>C18</td>
<td>0.0684</td>
<td>344.27</td>
<td>5.033</td>
<td>333.5</td>
<td>0.13</td>
<td>42.8</td>
</tr>
</tbody>
</table>
Inhibitor of protease from Chenopodium quinoa

**Figure 3.** Mass spectrophotometric profile of CqTI showing peaks corresponding to the charged ions [M + H]+ and [M + 2H+/2] with its respective mass/charge ratio (analyzed on MALDI-TOF). The principal peaks correspondent for 4.2 and 7.9kDa molecular masses.

**Table 3.** Amino acid composition of CqTI from *C. quinoa* in comparison with Bowman-Birk LSI-1 (Rocco et al., 2011) and TDI-I Kunitz (Silva et al., 2001).

<table>
<thead>
<tr>
<th>Essentials AA</th>
<th>CqTI</th>
</tr>
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<tbody>
<tr>
<td>His</td>
<td>3</td>
</tr>
<tr>
<td>Thr</td>
<td>5</td>
</tr>
<tr>
<td>Val</td>
<td>6</td>
</tr>
<tr>
<td>Met</td>
<td>2</td>
</tr>
<tr>
<td>Ile</td>
<td>3</td>
</tr>
<tr>
<td>Leu</td>
<td>4</td>
</tr>
<tr>
<td>Phe</td>
<td>2</td>
</tr>
<tr>
<td>Lys</td>
<td>10</td>
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<table>
<thead>
<tr>
<th>Non-Essentials</th>
<th>CqTI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>5</td>
</tr>
<tr>
<td>Ser</td>
<td>6</td>
</tr>
<tr>
<td>Gly</td>
<td>23</td>
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<tr>
<td>Arg</td>
<td>10</td>
</tr>
<tr>
<td>Ala</td>
<td>4</td>
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<tr>
<td>Pro</td>
<td>7</td>
</tr>
<tr>
<td>Tyr</td>
<td>3</td>
</tr>
<tr>
<td>Cys</td>
<td>4</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Aml</th>
<th>TDI-I</th>
<th>Non-Essentials</th>
<th>CqTI</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>3</td>
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</tr>
<tr>
<td>ND</td>
<td>2</td>
<td>5</td>
<td>11</td>
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<td>3</td>
</tr>
<tr>
<td>4</td>
<td>1/2</td>
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</tr>
</tbody>
</table>

*Residues are expressed as number of residues per mol of protein. A Correction factor was applied for serine (5%), Threonine (10%) and Tyrosine (10%) for losses during hydrolysis; nd, not determined.*
known sequence of inhibitor of trypsin and chymotrypsin, which makes it a unique protein. No similarity in N-terminal sequences with other classes of inhibitor of protease were observed (Park et al., 1997; Tsybina et al., 2001).

3.7 Inhibition curve, pH, Temperature and DTT Stability

CqTI showed trypsin and chymotrypsin activities (Figure 4a), however the inhibitory activity was higher against trypsin than chymotrypsin. Trypsin lost 65% of its activity when the molar ratio of CqTI to trypsin was 0.75 and further lost ~75% of its activity when the molar ratio was increased to 1.0. In contrast, CqTI showed no obvious stoichiometry with chymotrypsin. The study of the effects of pH (2-10) and temperature (20-100 °C) revealed the high stability of the inhibitor against trypsin. In pH stability studies, CqTI was highly stable under conditions in the pH range 2-10 for 1h (Figure 4b). The inhibitory activity of CqTI was maintained at temperatures of up to 40 °C for 30min. Above 40 °C, the inhibitory activity decreased slightly, but maintained 70% activity at up to 100 °C for 30min (Figure 4c). The effect of DTT (1, 10 and 100μM) on CqTI activity was studied (Figure 4d). When CqTI was incubated with 1μM of

![Figure 4](image-url)

**Figure 4.** (a) Titration of bovine trypsin with CqTI. Enzyme concentration were 1.9x10⁻⁷ M. After 20 min of incubation, the residual enzyme activity was measured at 37 °C with BAPNA as substrate. Each point is the average of three assays. (b) Stability of CqTI a pH stability of SSTI, after incubation at the indicated pH for 1h at 37 °C. (c) Temperature stability of the inhibitory activity of CqTI after incubation for 30 min at the indicated temperatures; c effect of DTT on the stability of SSTI. (d) The inhibitor was treated with different final concentrations (1, 10 and 100 μM) of DTT for 15–120 min at 37 °C. The residual trypsin inhibitory activity was measured using BAPNA as substrate. Values are mean ± SD of triplicate measurements. There was significant difference between the treatments (ANOVA, p<0.05).
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DTT, trypptic inhibitory activity was affected in up to 2h (40%). When incubated with 10μM of DTT after 2h the inhibitory activity was ~15%, proving that the influence of the reduced reagent was slow. Using 100μM of DTT, the inhibitory effect of CqTI remained 15% after 15min of pre-incubation, but no activity after 1h of pre-incubation was detected.

Further studies demonstrated acting of PIs on trypsin and chymotrypsin simultaneously (Xavier-Filho & Ventura, 1998; Tamir et al., 1996; Tsybina et al., 2001; Prasad et al., 2010b). A 1:1 molar ratio with trypsin by stoichiometric studies was concorded well with other studies (Prasad et al., 2010b; Pompeu et al., 2014). The pH stability in large range is common in PIs. Our study is in concordance with PIs purified from cereals (Tamir et al., 1996; Tsybina et al., 2001). The temperature stability showed the same results demonstrated for SSI from S. saponaria (Macedo et al., 2011) and RgPI from C. cajan Seeds (Prasad et al., 2010b). On the other hand, the trypsin inhibitory activity of PRTI was maintained only 20% above 80 °C (Chaudhary et al., 2008). The effect of DTT in CqTI occured in lower levels in comparison with other studies (Prasad et al., 2010b; Macedo et al., 2011). The functional stability of PIs in the presence of various denaturants such as temperature, pH, and reducing agents has been explained for the presence of sulfhydryl groups (Macedo et al., 2011). Moreover, PIs with tertiary structure, as dimeric, presented more stability (Macedo et al., 2011; Silva et al., 2015b).

4 Conclusions

The methodology to extraction and purification demonstrated three isoforms of inhibitor of protease. The amino acid composition determined high concentration of essential amino acids being similar with legume and better than other cereals. The mass spectrometry showed two subunits with molecular weight of 4.2 and 7.9KDA. It is not possible classify CqTI as the specific PI family since N-terminal sequence of subunits found showed no homology. Its activity was stable over a pH (2-12), temperature (20-100 °C) and reducing agents.

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References


