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Purification and primary structure determination of two Bowman–Birk type trypsin isoinhibitors from *Cratylia mollis* seeds

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Abstract

Two Bowman-Birk type trypsin inhibitors ($CmTI_1$ and $CmTI_2$) were purified from *Cratylia mollis* seeds by acetone precipitation, ion exchange, gel filtration and reverse-phase chromatography. $CmTI_1$ and $CmTI_2$, with 77 and 78 amino acid residues, respectively, were sequenced in their entirety and show a high structural similarity to Bowman-Birk inhibitors from other Leguminosae. The putative reactive sites of $CmTI_1$ are a lysine residue at position 22 and a tyrosine residue at position 49. Different reactive sites, as identified by their alignment with related inhibitors, were found for $CmTI_2$: lysine at position 22 and leucine at position 49. The dissociation constant K_i of the complex with trypsin is 1.4 nM. The apparent molecular mass is 17 kDa without DDT and 11 kDa with reducing agent and heating.

Keywords: *Cratylia mollis*; Leguminosae; Bowman-Birk inhibitor purification; Primary sequence; Trypsin inhibitor

1. Introduction

Plant serine proteinase inhibitors are divided into certain protein groups according to primary structure homology, position of the reactive sites and number or location of disulphide bonds (Park et al., 2000; Silva et al., 2001). Bowman-Birk inhibitors (BBIs) from dicotyledonous seeds are comprised of a cysteine-rich polypeptide chain which is bridged by seven conserved disulphide bonds, a fact that probably explains the high degree of stability that BBIs maintain in the presence of denaturing agents and heat (Singh and Appu, 2002).

Dicotyledonous BBIs contain two functional inhibitory domains within disulphide-linked loops each composed of 7 amino acid residues (Mello et al., 2003). Characteristic amino acid residues of the inhibitory regions determine their inhibition specificity, e.g., for trypsin or chymotrypsin (Singh and Appu, 2002). Comparisons among BBIs from different species showed that the first N-terminally located reactive site is more conserved than the second C-terminally located one: Lysine is normally the P_1 residue of the first site, whereas at the second P_1 site there may be present amino acids of different structures (Tanaka et al., 1997). Alignment analysis showed that the N-terminal amino acid of these inhibitors is usually serine, and that the N- and C-terminal regions are highly hydrophilic (Wu and Whitaker, 1991; Prakash et al., 1996). C- and N-terminal extensions determine the number of amino acid residues of *Vigna unguiculata* and *Pisum sativum* BBIs (Mello et al., 2003).

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The interaction between BBIs and their target enzymes follows a tight-binding inhibitory mechanism. Synthetic peptides derived from the BBI reactive site loop retain this inhibitory ability and were used to study interaction between BBIs and enzymes (Scarpi et al., 2002), stability towards proteolytic hydrolysis (Gariani and Leatherbarrow, 1997) as well as tools for pharmacological studies (Dittmann et al., 2001). Analysis of the BBIs chymase interaction showed that the complex did not dissociate in SDS–PAGE under non-reducing conditions (Ware et al., 1997).

Leguminosae seeds contain multiple BBIs differing in the number of amino acid residues (Park et al., 2000) and hydrophobicity (Wu and Whitaker, 1991). The isoinhibitors of these BBIs may vary due to differences in genetic polymorphism or to post-translational protein modification (Quillien et al., 1997). In soybean, the soybean cultivar determines the number of BBI isoforms present with trypsin inhibitory activity (Gladysheva et al., 2000).

Trypsin inhibitors have been isolated by affinity chromatography through the interaction inhibitor reactive site and matrix (Silva et al., 2001) or by their glycosylated moiety using lectins (Paiva et al., 2003). However, for inhibitor structure analysis ion exchange and reverse-phase chromatography were used aiming to avoid undesirable inhibitor modification by enzyme matrix (Shibata et al., 1986).

The biotechnological potential of BBIs has been intensely investigated. These inhibitors are thought to play important roles in plant defense mechanisms against insects (Birk, 1996) and can be used for biological control. The potential of serine protease activity during tumour development has stimulated various lines of research. For example, Chu et al. (1997) investigated the proteolytic activities of transformed cells inhibited by BBIs. Garcia-Gasca et al. (2002) analysed the action of *Phaseolus acutifolius* BBI on in vitro cell proliferation and cell adhesion of transformed cells, and Kennedy and Wan (2002) evaluated the anticarcinogenic activity of soybean BBIs on prostate cancer cells.

BBI detection in Leguminosae seeds is important for the evaluation of the nutritive value of unheated seeds (Hossain and Becker, 2002). *Cratylia mollis*, or camaratu bean, is a legume that is native to the semi-arid Northeast region of Brazil and is highly resistant to desiccation. The seeds have been an alternative source of nutrition in livestock feed, contributing to regional development and a better quality of life for the local inhabitants. In this study, we describe the purification process of two Bowman-Birk type trypsin isoinhibitors derived from *C. mollis* seeds, $CmTI_1$ and $CmTI_2$ as well as their properties and amino acid sequences.

2. Experimental

2.1. Proteases, markers and columns

Bovine trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), DL-Bz-Arg-pNan and Suc-Phe-pNan were purchased from Sigma Chemical Company (St. Louis, MO, USA), porcine pancreatic elastase (EC 3.4.21.35) and MeO-Suc-Ala-Ala-Pro-Val-pNan from Calbiochem (San Diego, CA, USA). Molecular mass markers (carbonic anhydrase, β-lactoglobulin, lysozyme and ovalbumin) were also from Sigma. DEAE-Sephacel and Superdex 75 (FPLC) columns were from Pharmacia Fine Chemicals (Uppsala, Sweden). Ultrasphere and Vydac C_4 columns (15.0 cm × 0.46 mm) as well as Aquapore RP300 C (7–8 μm) and LiChrospher 100 RP (8.5 μm) columns were from Applied Biosystems (Foster City, CA, USA).

2.2. Crude extract

Mature seeds, manually harvested from wild plants in the Northeast region of Brazil (near the city of Ibimirim, in the state of Pernambuco), were milled to a fine powder. The meal (60 g) was then homogenised in a blender with 0.15 M NaCl (600 ml), centrifuged at 5000*g*, and the supernatant was maintained at 60 °C for 15 min. This crude extract was taken as starting material. The proteins in the crude extract were precipitated using acetone–H_2O (4:1) at 4 °C. The sediment separated by centrifugation was vacuum dried and dissolved in 0.05 M Tris–HCl buffer, pH 8 (acetone fraction). Protein concentration was according to Lowry et al. (1951). The initial specific activity of crude extract may not represent a real value due to eventual loss of protein but was considered as the initial activity of the isolation procedure.

2.3. Ion exchange chromatography

The acetone fraction (165 mg proteins) was applied to a DEAE-Sephacel column (1.5 × 9.0 cm) using an FPLC system equilibrated with 0.05 M Tris–HCl buffer, pH 8 (1.5 ml/min flow rate). After extensive washing with equilibrium buffer, the inhibitor was eluted with a linear gradient of NaCl (0–0.6 M in 88 min). Spectrophotometry at 280 nm was used to follow protein elution, and inhibitory activity was determined by trypsin inhibition using DL-Bz-Arg-pNan as substrate (see below). Fractions with trypsin inhibitory activity were pooled, dialysed and concentrated under nitrogen atmosphere using a membrane to exclude the M_r 3000 range.

2.4. Gel filtration chromatography

Any protein with inhibitory activity (2.2 mg) was submitted to gel filtration on a Superdex 75 column (1.0 × 25 cm) using an FPLC system equilibrated with

0.05 M Tris–HCl buffer, pH 8, containing 0.5 M NaCl (0.5 ml/min flow rate). Protein content and inhibitory activity were measured as described above.

2.5. Reverse-phase chromatography

The gel-filtered CmTI inhibitor (20 μg) was applied onto a C_4 reversed-phase column using an HPLC system equilibrated with 0.1% (v/v) CF_3CO_2H acid (TFA) in water. Separation was achieved using an CH_3CN gradient (0–80%, 120 min) in 0.1% (v/v) TFA. The eluted protein was used for amino acid sequence determination.

2.6. SDS electrophoresis

The CmTI molecular mass was assessed by electrophoresis on 15% SDS–polyacrylamide gels as described by Laemmli (1970) at reducing or non-reducing conditions. CmTI was reduced with 1.3 or 2.6 M DTT and heated to 100 °C for 15 or 45 min. Protein was detected by silver staining (Heuskoven and Dernick, 1985).

2.7. Structure determination

For CmTI sequence determination, peptides were obtained from the C_4-column protein peak. After reduction and carboxymethylation, proteins were subjected to Aquapore RP300 C (7–8 μm column) HPLC system and from enzymatic digestion (chymotrypsin and endoproteases Lys-C, Glu-C, Asp-N) as well as CNBr cleavage (Friedman et al., 1970). The peptides in the mixtures were separated using the same HPLC system described above and their sequences determined by automated Edman degradation on a 477A Applied Biosystems protein sequencer.

2.8. Mass spectroscopy

Purified inhibitor was infused into an atmospheric-pressure ionization source fitted to a tandem quadruple instrument AP III (Sciex) as previously described (Covey et al., 1988; Mann, 1990).

2.9. Sequence comparison

The database from the Max-Planck-Institute for Biochemistry at Martinsried, Germany, was accessed using the Lipmann and Pearson fast protein-searching algorithm, FASTP (Lipmann and Pearson, 1985). Alignments were optimised using CLUSTAL (Higgins and Sharp, 1988).

2.10. Active site and inhibitor titration

Trypsin active site concentration was determined by NPGB titration (Sampaio et al., 1984). Inhibitor concentration was determined by titration with trypsin assuming an equimolecular binding between enzyme and inhibitor.

2.11. Trypsin inhibition and K_i determination

The equilibrium dissociation constant (K_i) and the inhibitor concentration were determined for trypsin through pre-incubation of the enzyme with increasing concentrations of the inhibitor at 37 °C in 0.1 M Tris–HCl, pH 8.0, in a final volume of 200 μl. Residual activity was subsequently measured using 4 mM Bz-Arg-pNan as substrate.

2.12. Inhibition of other serine proteinases

After pre-incubation with CmTI, inhibition of chymotrypsin and porcine pancreatic elastase was determined by measuring the remaining hydrolytic activity towards 10 mM Suc-Phe-pNan or 5 mM MeO-Suc-Ala-Ala-Pro-Val-pNan, respectively. Enzymes or enzyme-inhibitor mixtures (20 μl) were added to a solution containing substrate in 0.05 M Tris–HCl buffer, pH 8.0, in a final volume of 200 μl at 37 °C. The reaction was monitored for 15–30 min and stopped by addition of 50 μl of 30% acetic acid and substrate hydrolysis was followed by absorbance at 405 nm in an ELISA reader.

2.13. Temperature and pH stability

CmTI was maintained in 0.15 M NaCl at 20, 37, 60 and 100 °C. After 30 min, the inhibitor solutions were incubated for 15 min with trypsin at pH 8.0. Subsequently, the residual trypsin activity was measured with 4 mM DL-Bz-Arg-pNan as substrate. The pH effect on CmTI activity was determined through pre-incubation of CmTI for 30 min at 37 °C in buffers with pH values ranging from 2.0 to 10.0. The pH was subsequently adjusted to 8.0, and trypsin inhibition was assayed as described above.

3. Results

3.1. Inhibitor purification

The results of the isolation procedure are summarised in Table 1. Trypsin inhibition was only detected after the *C. mollis* seed extract had been heated. This treatment abolished the endogenous DL-Bz-Arg-pNan hydrolyzing activity. Acetone fractionation was efficient in concentrating the trypsin inhibitor but did not increase biological activity (Table 1).

Samples with trypsin inhibitory activity were subjected to DEAE-Sephacel chromatographic column

Table 1
Purification of CmTI from *Cratylia mollis* seeds

Preparation	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (*x* fold)	Yield (%)
Crude extract	1200	6.6	0.0055	1	100
Acetone fractionation	976	5.6	0.0057	–	85
DEAE-Sephacel fraction	280	2.8	0.01	1.7	42
Superdex 75 fraction	19	2.0	0.10	18	30

The protein was measured according to Lowry et al. (1951); inhibitory activity was determined by trypsin inhibition assay whereby 1 U = 1 mg of inhibitor; specific activity means mg of inhibitor/mg of protein; the purification factor was determined via the specific activity values; yield was expressed as percentage of isolated inhibitor (Tanaka et al., 1997). The initial specific activity of crude extract was considered as the initial activity of the isolation procedure.

where the inhibitor was 1.7-fold purified with a yield of 42% (Table 1). The pooled inhibitor material was subsequently loaded onto a Superdex-75 column and activity was recovered in only one peak (Fig. 1).

3.2. Electrophoretic analysis

The purity of CmTI was analysed using polyacrylamide gel electrophoresis. A single band (M_r 17 kDa) was obtained, indicating that CmTI was homogeneous (inset 1A). After 1.3 M dithiothreitol treatment and heating for 15 min, the inhibitor showed the same protein migration behavior; however, a faint band of 11 kDa was also detected at 2.6 M DTT. The increased heating time (45 min) resulted in 11 kDa as main peptide.

3.3. Temperature and pH stability

Analysis of the temperature and pH effect revealed that CmTI is remarkably stable. Inhibitory activity was not affected within the pH 2–10 range and temperatures of 20, 37, 60 and 100 °C did not decrease its effect on trypsin.

3.4. Stoichiometry of trypsin inhibition

Bovine trypsin was inhibited by CmTI and the dissociation constant of the complex was measured by determination of the enzyme's residual activity after incubation with the inhibitor. A K_i value of 1.4×10^{-9} M (Fig. 2) was calculated using the equation described by Morrison for slow tight binding. We assayed also the inhibitory effect of CmTI on chymotrypsin and porcine pancreatic elastase but no inhibition was detected.

3.5. Primary structure determination

CmTI was loaded onto a C_4 reversed-phase column and the eluted protein peak was used to determine the primary structure. Peptides were obtained from the C_4 column through reduction followed by enzymatic cleav-

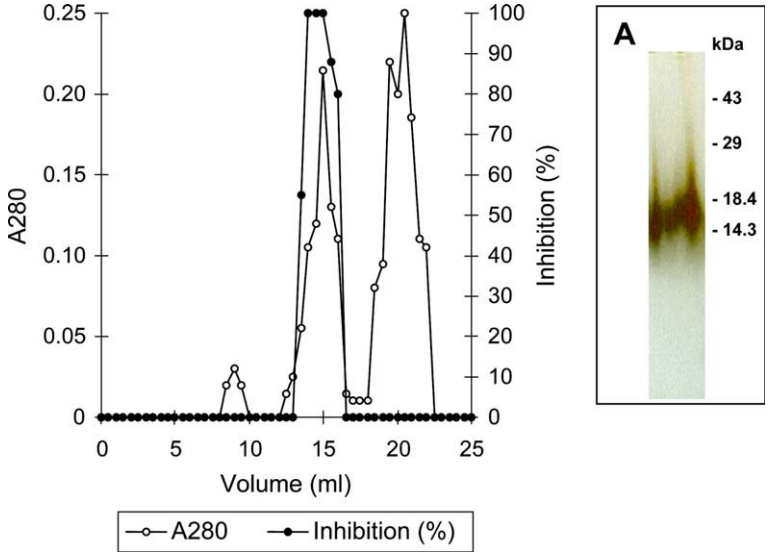

Fig. 1. Gel filtration on Superdex 75 column (FPLC). Protein (2.2 mg) was subjected to Superdex 75 CC (1.0 × 25 cm) equilibrated (0.5 mL/min) with 0.05 M Tris–HCl buffer, pH 8.0. The inset A shows SDS–PAGE of the native (not reduced) inhibitor purified by gel filtration on Superdex 75.

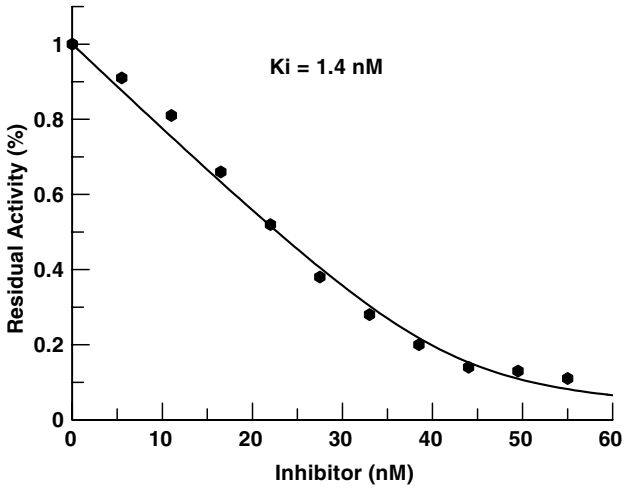

Fig. 2. Trypsin inhibition by CmTI. Bovine trypsin (0.42 μM) pre-incubated (10 min, 37 °C, 0.1 M Tris–HCl buffer, pH 8.0) with increasing amounts of CmTI eluted from Superdex 75 column. The residual trypsin activity was assayed with DL-Bz-Arg-pNan (4 mM) as substrate as described in Section 2.

age, CNBr cleavage, and modification with 4-vinyl pyridine. The resulting proteins were identified as $CmTI_1$ and $CmTI_2$ (Fig. 3).

The amino acid sequences of $CmTI_1$ and $CmTI_2$ were determined by automated sequence analysis resulting in polypeptides with 77 and 78 amino acid residues, respectively (Fig. 4). Both isoinhibitors showed highest structural similarity to the Bowman-Birk inhibitors from other Leguminosae (Fig. 4) and high homology with each other, but differed in the reactive site of the C-terminally located inhibitor domain. The putative reactive site of $CmTI_1$ is a lysine residue at position 22 and a tyrosine at position 49, whereas $CmTI_2$ shows besides lysine at position 22 a leucine at position 49 (identified by alignment to related inhibitors). The molecular mass of $CmTI_1$, as determined by mass spectrometry, was 8555.5 (elementary composition: $\mathbf{C}_{335}\,\mathbf{H}_{520}\mathbf{N}_{101}\mathbf{O}_{128}\mathbf{S}_{17}$; $pI = 4.6$). The molecular mass of $CmTI_2$ was 8625.5 (elementary composition: $\mathbf{C}_{341}\mathbf{H}_{530}\mathbf{N}_{99}\mathbf{O}_{133}\mathbf{S}_{15}$; $pI = 4.2$).

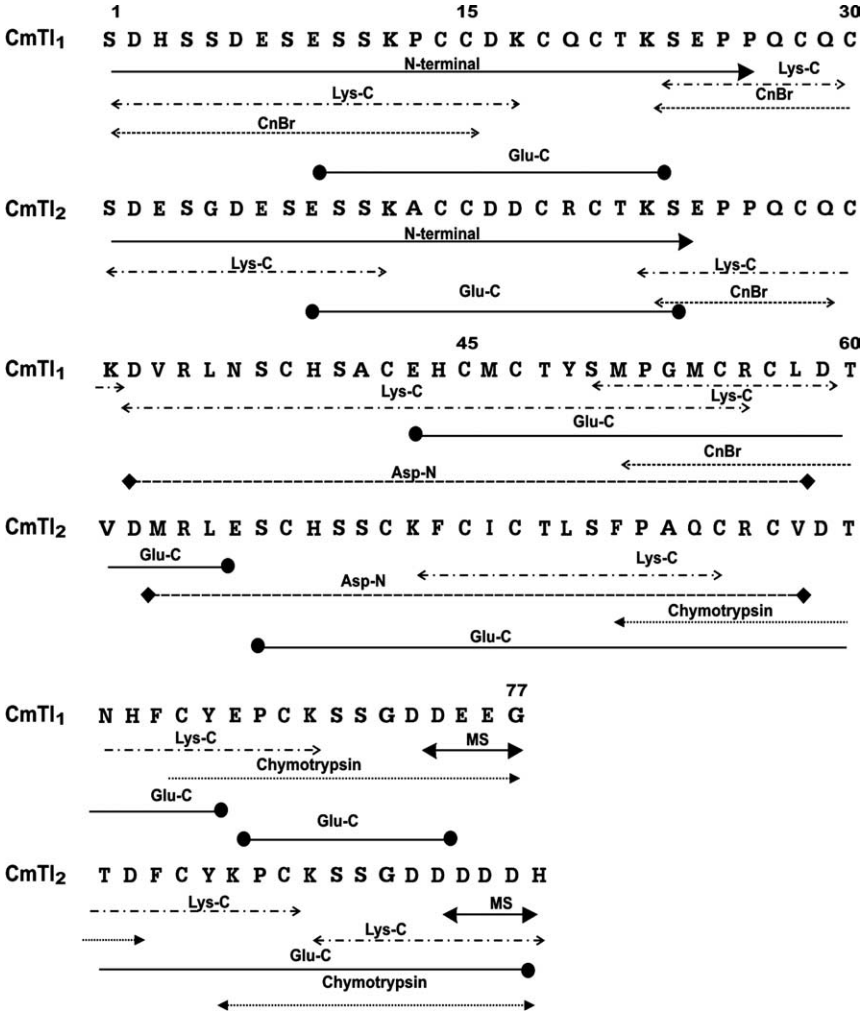

Fig. 3. Amino acid sequences of $CmTI_1$ and $CmTI_2$. The peptides obtained after fragmentation by enzyme digestion with chymotrypsin and endoprotease Lys-C, Glu-C, Asp-N or by cyanogen bromide are indicated. MS, mass spectroscopy.

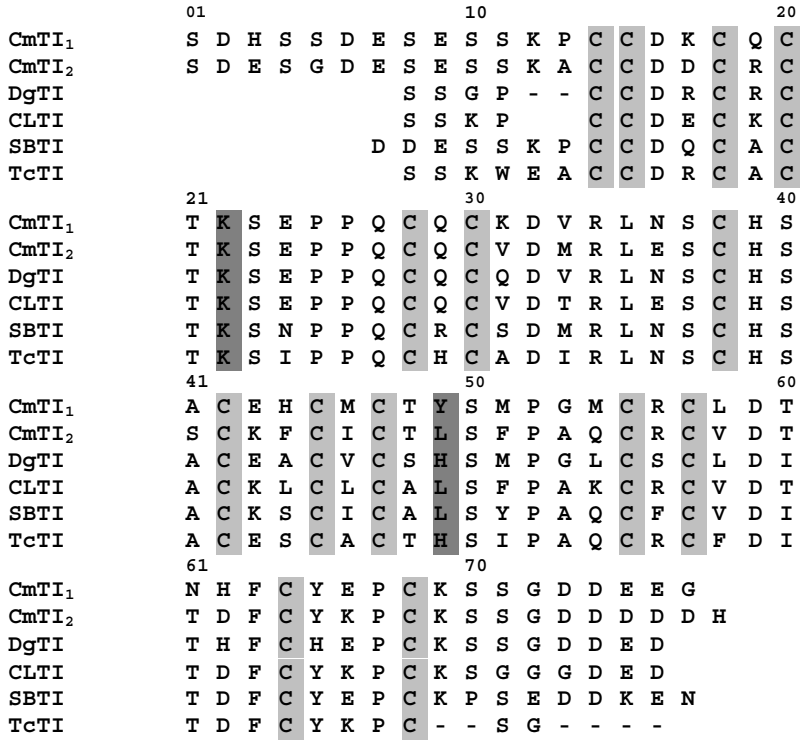

Fig. 4. Amino acid sequences of $CmTI_1$ and $CmTI_2$ and other structurally homolog Bowman-Birk type inhibitors. DgTI (Bueno et al., 1999); CLTI-*Canavalia lineata* (Odani and Ikenaka, 1972); SBTI-*Glycine max* (Lourenço et al., 1989) and TcTI (Tanaka et al., 1997). Amino acids at the P_1 positions of the reactive sites are in white lettering on a gray background and cysteine residues are shaded in gray.

4. Discussion

The inhibitor (CmTI) material isolated by gel filtration chromatography contains two isoforms with high affinity for bovine trypsin (1.4×10^{-9} M). In contrast to other structurally related inhibitors, CmTI neither inhibited chymotrypsin nor porcine pancreatic elastase (Tanaka et al., 1996; Deveraj and Manjunatha, 1999). Its high specificity for trypsin is comparable to that of *Dioclea glabra* trypsin inhibitor, DgTI (Bueno et al., 1999).

CmTI is remarkably stable over a wide pH range and unusually heat-resistant (data not shown). The large number of cysteine residues that form disulfide bonds render them structurally stable (Singh and Appu, 2002). Comparison of the molecular mass of CmTI (M_r 17,000 Da determined by SDS–PAGE) with the molecular masses determined by sequence of $CmTI_1$ (8554.5 Da) and $CmTI_2$ (8624.4 Da) indicates a molecular association of inhibitor isoforms, a common characteristic of a Bowman Birk inhibitor (Tanaka et al., 1997; de la Sierra et al., 1999). The different migration obtained (11 kDa polypeptide) under SDS–PAGE and 2.6 M DTT could be explained by disulfide bond of different stabilities in the inhibitor structure. In fact, a model has been proposed to localize labile and more stable disulfide bonds (Biewenga and van Run, 1992). The molecular masses (determined by mass spectrometry) of $CmTI_1$ (8555.5) and $CmTI_2$ (8625.5) are close to the calculated masses from amino acid sequences but post-translational modifications to the isolated proteins need to be evaluated.

The protein sequences established showed clearly that $CmTI_1$ and $CmTI_2$ are structurally related to the Bowman Birk inhibitor family. In particular, the 14 cysteine residues and the lysine residue in P_1 position of the N-terminally located reactive site are in conserved positions previously described for other Bowman-Birk inhibitors. The second putative reactive site has a tyrosine in $CmTI_1$ at P_1 position and a leucine in $CmTI_2$; however, CmTI preparation containing both inhibitors was able to inhibit only trypsin.

A remarkable difference between CmTI inhibitors and known Bowman-Birk inhibitors is that CmTI inhibitors are rich in acidic amino acids, which suggests that these inhibitors are more closely related to DgTI (Bueno et al., 1999). Also lack of chymotrypsin inhibition by CmTI makes it functionally more similar to DgTI and less similar to *Torresea cearensis*, TcTI, which inhibits chymotrypsin with a K_i of 5.0×10^{-8} M (Tanaka et al., 1997) probably due to a strong negatively charged carboxyl portion that interferes with the chymotrypsin interaction.

Bowman-Birk inhibitors are classified according to their structural features and inhibitory characteristics. Isolated from dicotyledons, they are doubled-headed

proteins. Inhibitors isolated from monocotyledons are classified as Bowman-Birk type I (M_r 16,000 proteins and two reactive sites) or Bowman-Birk type II inhibitors (M_r 8,000 proteins with only one reactive site).

The reactive sites are formed by a consensus sequence **C-T-P_1-S-X-P-P-Q-C** (X being any amino acid residue) with lysine, arginine or serine at P_1 position of the first reactive site being effective for trypsin inhibition. At the second reactive site, lysine, arginine, phenylalanine, tyrosine or leucine can occupy the P_1 position (Prakash et al., 1996). The lack of a second reactive site in monocotyledon Bowman-Birk type II inhibitors may be due to the loss of the Cys^{10}-Cys^{11} disulphide bridge that connects the second inhibitory loop to the first inhibitor domain or to the loss of some conserved residues thus resulting in specific trypsin inhibitors (Lin et al., 1993). This explanation serves as well for $CmTI_1$ and $CmTI_2$ as it did for DgTI isolated from dicotyledons. The sequence **C-T-K-S-E-P-P-Q-C** (8 conserved residues and appropriate P_1 residue) comprises the first reactive sites of both $CmTI_1$ and $CmTI_2$. However, the second reactive site sequences **C-T-Y-S-M-P-**G-M-**C** (of $CmTI_1$) and **C-T-**L-**S-**F-A-Q-M-**C** (of $CmTI_2$) present only 5 and 4 conserved residues, respectively, and have different residues in P_1 position (Y or L). This variability may be responsible for the poor activity of the second reactive site of these inhibitors, allowing specificities similar to those of Bowman-Birk type II inhibitors. But they have not lost the disulphide bridge, as occurs in some monocotyledonous inhibitors that lack cysteine residues in conservative positions (Prakash et al., 1996).

Trypsin activity was already detected in *C. mollis* seeds (data not shown); the presence of CmTI in the same vegetal tissue may minimize digestion of peptides and proteins in quiescent seeds.

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