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Research article

A trypsin and chymotrypsin inhibitor from *Caesalpinia bonduc* seeds: Isolation, partial characterization and insecticidal properties

Arindam Bhattacharyya [a,b,*], Shruti Rai [b,c], C.R. Babu [b]

[a] *Department of Botany, University of Delhi, Delhi 110 007, India*
[b] *Centre for Environmental Management of Degraded Ecosystems, University of Delhi, Delhi 110 007, India*
[c] *School of Biotechnology, Guru Gobind Singh Indraprastha University, Kashmere Gate, Delhi 110 006, India*

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Abstract

Evolution of proteinase inhibitor diversity in leguminous plants of tropical rainforests is under immense pressure from the regular upregulation of proteolytic machinery of their pests. The present study illustrates the isolation and bioinsecticidal potency of a serine proteinase inhibitor from the seeds of *Caesalpinia bonduc* (CbTI), inhabiting Great Nicobar Island, India. Following initial fractionation by ammonium sulfate precipitation, CbTI was purified to homogeneity by ion exchange, gel filtration and trypsin affinity chromatography. SDS–PAGE of gel filtrated CbTI showed a couple of proteins CbTI-1 (~16 kDa) and CbTI-2 (20 kDa) under non-reducing conditions, which subsequent to trypsin affinity chromatography yielded only CbTI-2. Both Native PAGE as well as iso-electric focusing showed 2 iso-inhibitors of CbTI-2 (p*I* values of 5.35 and 4.6). CbTI exhibited tolerance to extremes of temperatures (0–60 °C) and pH (1–12). A 1:1 stoichiometric ratio was noted during CbTI-2–trypsin complex formation, which was absent on binding with chymotrypsin. Further, SDS–PAGE analysis also showed that CbTI-1 has affinity only towards chymotrypsin, whereas both trypsin and chymotrypsin formed complexes with CbTI-2. Dixon plot analysis of CbTI-2 yielded inhibition constants (K_i) of 2.75×10^{-10} M and 0.95×10^{-10} M against trypsin and chymotrypsin activity respectively. Preliminary investigations on the toxicological nature of CbTI revealed it to be a promising bioinsecticidal candidate.

Keywords: Bioinsecticide; *Caesalpinia bonduc*; Chymotrypsin; Isoinhibitors; Proteinase inhibitor; Stoichiometry; Trypsin

1. Introduction

Multiple trade-offs direct plant–herbivore interaction in tropical forests, and thrust the foundation of the theory of phytochemical co-evolution. Trade-offs, both for the benefactor as well as the beneficiary serve as the major motor behind the production of toxins in plants and evolution of disarming mechanisms in herbivores. Thus, through time, plants have turned more toxic in response to which herbivores have undertaken antagonistic adaptive measures.

Over the years proteinase inhibitors (PIs) have been established as an effective natural protective system of plants and their evolutionary relationships with insect herbivores have also been well-documented. Inhibitors of proteolytic enzymes are ubiquitously distributed among all living organisms [1,2]. Primarily, these proteins form an integral part of plant arsenal against herbivores and combats proteinases (single/several) of pests and pathogens [3–9]. PIs influence insect growth and development by causing amino acid deficiency, and eventually effect their death either by inhibition of gut proteinases or due to a massive overproduction of the digestive enzymes, reducing the availability of essential amino acids for the production of other proteins [10–14].

Currently, 59 distinct families of proteinase inhibitors have been recognized [7,15]. They are grouped mainly as serine, cysteine, aspartic or metallo proteinase inhibitors. However,

* Corresponding author. Present address: Malaria Research Group, International Centre for Genetic Engineering and Biotechnology (ICGEB), Aruna Asaf Ali Marg, New Delhi 110067, India. Tel.: +91 11 26189358; fax: +91 11 26189170.
E-mail address: ady1111@rediffmail.com (A. Bhattacharyya).

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of all the PI groups maximum focus has been on the characterization of serine PIs. Most of the serine PIs are widespread in the seeds of the three Leguminosae subfamilies, Mimosoideae, Caesalpinioideae and Papilionoideae [10,16]. Most of these inhibitors bind to cognate enzymes according to a common substrate-like canonical mechanism [17] forming stable complexes with target proteinases, and blocking, altering or abolishing access to the enzyme active site. Among the serine PIs, the Kunitz trypsin inhibitor superfamily [18] has generated much prominence for its specific activity against trypsin-like serine proteinases, with no inhibition of other proteinase classes [19]. The stability of proteinase-PI complex is largely brought about by intramolecular interactions viz. disulfide bond, hydrogen bond, and hydrophobic interaction, involving the primary binding loop (reactive site loop) structure [18,20]. Based on the molecular masses, cysteine content and number of reactive sites, these inhibitors have been categorized into two families of inhibitors, the Kunitz Type and the Bowman Birk Type. The latter type inhibitors are usually 8-kDa proteins with seven disulfide bridges whereas the Kunitz type inhibitors are 20-kDa proteins with just two disulfide linkages [13,16].

The distribution of these two families of serine PIs in the seeds of Leguminosae has been implicated to be in coherence with the legume plant evolution. In fact, Kunitz type inhibitors are more common in the seeds of highly primitive Mimosoideae and primitive Caesalpinioideae, in comparison to the recently evolved Papilionoideae which frequently shows the presence of Bowman Birk inhibitors [21].

Because of the potential effects of their peculiar binding properties *in vivo* and *in vitro*, this group of proteins can be sustainably harnessed to derive maximum benefits for human needs. *Caesalpinia bonduc* (L.) Roxb. is a commonly occurring scandent shrub along the coastal areas of Great Nicobar Island, Andaman and Nicobar Islands, India. Incidences of insect outbreak either on the pods or the seeds were sparse. In order to understand the magnitude of interaction of the inhibitor with bovine trypsin and chymotrypsin as well as midgut proteases from the polyphagous insect *Spodoptera litura*, we report here the isolation, partial purification and characterization of a trypsin and chymotrypsin inhibitor from the seeds of *C. bonduc* (CbTI). The inhibitor thus purified was used for a comprehensive study of its biochemical traits.

2. Materials and methods

2.1. Materials

Caesalpinia bonduc (L.) Roxb. seeds were manually harvested from the coastal forests of Great Nicobar Island, Andaman and Nicobar Islands, India. Superdex S-75, Trypsin–Sepharose CL 4B and molecular weight markers for electrophoresis were from Amersham Biosciences; DEAE-52 cellulose was from Whatman (UK); acrylamide and methylene bis-acrylamide were from Sigma (St. Louis, MO, USA); TAME and STI were products of Merck, Germany. All other chemicals used were of analytical grade.

2.2. Isolation and purification of CbTI

Initially, 55 g of *Caesalpinia bonduc* seeds were mechanically scarified by making a cut in the hilar region and then soaked in 0.15 N NaCl solutions for 24 h. Following removal of testa, the cotyledons were soaked overnight in the same solution. The swollen embryonic cotyledons were then homogenized with 350 ml of saline Tris buffer (20 mM Tris, pH 8.0; 0.15 M NaCl) containing 1 mM sodium metabisulfite at 4 °C using a pre-chilled pestle and mortar. The homogenate was filtered through a chilled four-fold muslin cloth. After centrifugation for 15 min at 12,000 × *g* at 4 °C, the supernatant (crude extract) was precipitated with ammonium sulfate at concentrations of 0–30% and 30–65%. These two fractions were then dialyzed against cold deionized water, lyophilized and submitted to proteinase inhibitory assays. The 30–65% ammonium sulfate precipitated fraction showing high antitryptic activity was applied to an anion exchanger column DEAE 52, pre-equilibrated with 20 mM Tris buffer, pH 8.0. Fractions (5 ml) were collected using a step gradient (0.1–0.5 M) of NaCl at a flow rate of 0.5 ml/min. The peak (P1) obtained was pooled, dialyzed, lyophilized, and applied (1.0 mg/ml) to a Superdex S-75 column pre-equilibrated with 0.1 M Tris–HCl buffer (pH 8.0) containing 0.1 M NaCl, for gel filtration chromatography. Fractions (0.5 ml) from the protein peak were collected at a flow rate of 20 ml/h. Proteinase inhibitory activities were checked and fractions with maximum inhibitory activity were dialyzed, concentrated and loaded onto a trypsin Sepharose CL-4B column (25 × 1.5 cm) pre-equilibrated with 0.1 M Tris–HCl buffer (pH 8.0), 0.1 M NaCl. The retained proteins were eluted with 1 mM HCl solution at flow rate of 30 ml/h and dialyzed against cold deionized water. The antitryptic peak (CbTI) was pooled and concentrated for further analyses.

2.3. Proteinase inhibition studies

Following Birk [22], assays for trypsin and chymotrypsin inhibitions were carried out by estimating the remaining esterolytic activity of trypsin and chymotrypsin towards the substrate TAME (Tosyl-Arginyl-Methyl Ester hydrochloride) and BTEE (n-Benzoyl Tyrosine Ethyl Ester) respectively. Both the proteinases were pre-incubated with CbTI for 5 min at 25 °C, in 1 ml Tris buffer (46 mM) containing 11.5 mM $CaCl_2$, pH 8.1, prior to the reaction. One trypsin or chymotrypsin unit is defined as 1 μmol of substrate hydrolyzed per minute of reaction. One inhibition unit is defined as unit of enzyme inhibited. Specific activity is defined as trypsin inhibition units (TIU) per absorbance unit, at 280 nm (A_{280}), of the inhibitor.

2.4. Estimation of proteins

Protein content was measured by Coomassie blue staining according to the procedure of Bradford [23] as well as from the absorbance at 280 nm. BSA (1 mg/ml) was used as a protein standard.

2.5. Reduction and S-alkylation

Reduction of CbTI (5 mg) was done with 0.1 M dithiothreitol and using iodoacetic acid it was *S*-carboxymethylated following the procedure outlined by Crestfield et al. [24]. Subsequent to desalting, CbTI was redissolved in 0.05 M Tris buffer pH 8.0, and separated on Superdex S75, equilibrated with the same buffer.

2.6. SDS–PAGE and native-PAGE profiles

SDS–PAGE (15%) of purified CbTI was carried out as described by Laemmli [25], at room temperature. For both SDS–PAGE and Native-PAGE the protein samples were pretreated with Laemmli's buffer but for the latter Laemmli's buffer were excluded of SDS and β-mercaptoethanol. The gels were stained by Coomassie brilliant blue R250. Protein molecular weight markers (Amersham Biosciences), in a range of 14–116 kDa, were used.

2.7. Isoelectric focusing

Iso-inhibitors of CbTI were determined by performing isoelectric focusing using a Phast System apparatus from Pharmacia LKB Biotechnology. Phast Gel IEF 3–9, which operates in the pH range 3–9, and Ampholine polyacrylamide gel plates from Pharmacia (pH range 3.5–9.5) were used together with a Pharmacia broad-range p*I* calibration kit containing proteins with various isoelectric points ranging from 3 to 10.

2.8. Kinetics of inhibitory activity of CbTI

A Dixon plot [26] analysis was employed to determine the constants of inhibition for bovine trypsin and chymotrypsin by preincubating the respective enzymes with increasing concentrations of CbTI. Enzyme inhibition was carried out at two different substrate concentrations ($[S_1]$ and $[S_2]$). Typically, TAME (0.005 mM and 0.01 mM) and BTEE (0.53 mM and 1.07 mM) were used for trypsin and chymotrypsin activity respectively. Samples were prepared to achieve inhibitor concentrations (nM) of: 0.4, 0.5, 0.66 and 1. The initial slope ν was determined for each inhibitor concentration. Dixon plots were generated using the reciprocal velocity ($1/\nu$) versus [PI], for each substrate concentration, $[S_1]$ and $[S_2]$. Intersection of the two regression lines for each [S] respectively, yielded the K_i (Inhibition constant).

In addition, the formation of proteinase-PI complex and the cross interference of each of the proteinase towards the complex formation by the other was also studied using SDS–PAGE. Both trypsin and chymotrypsin were incubated with CbTI individually, as well as with previously formed complexes of either of the proteinases with CbTI in a buffer (0.05 M Tris–HCl; 0.001 M $CaCl_2$, pH 8.0) at 37 °C for 30 min and then electrophoresed on SDS–PAGE.

2.9. Biological properties of CbTI

The temperature optima of purified CbTI was determined by its antitryptic activity, under varying temperatures (5–95 °C, with an increment of 10 °C) in 0.05 M Tris–HCl buffer, pH 8.0. Two incubation periods of 30 min and 60 min duration were used for each temperature.

Antitrypsin assays under a range of pH (1–12) conditions were used to assess the pH optima of CbTI. Purified CbTI was mixed with buffers of pH 1, 3, 5, 6, 9 and 12 respectively in the ratio of 1:1 (v/v), and incubated for 3 h. The buffers used were 0.02 M each of KCl–HCl (pH 0.5–1.5), glycine–HCl (pH 2.0–3.5), sodium phosphate (pH 6–7.5), Tris–HCl (pH 8.0–10.0) and glycine–NaOH (pH 10.5–13.0). Three replicates were maintained for each temperature and pH condition.

Stability of CbTI on storage at −20 °C for over 1 year was determined by monthly assessment of its trypsin inhibitory potency.

2.10. Isolation of larval gut proteinases

Spodoptera litura were collected from the Division of Entomology, Indian Agricultural Research Institute (IARI) and reared on standard artificial diet [27]. Fifth instar larval proteinases were obtained after dissection and extraction of the guts. The guts were surgically removed from the animal and placed into 40 μl of 1 mM HCl solution. Gut tissue was stirred and centrifuged at 12,000 × *g* at 4 °C for 10 min. The supernatants were then recovered and used for in vitro assays. Total protein content was measured as described previously [42].

2.11. CbTI inhibitory assay against proteinase extracts from insect

CbTI effects on the proteolytic activity of midgut extracts were measured both by using X-ray film [28] as well as using the esterolytic substrate TAME as outlined earlier [32].

Small strips of the X-ray films having a coating of gelatin on it were used as substrate for assessing the inhibitory activity of CbTI. 30 μl of the sample containing 15 μl each of the enzyme (1 mg/ml) and the inhibitor of variable concentration in Azocoll buffer (0.05 M Tris–HCl containing 1 mM $CaCl_2$, pH 7.8) were mixed and loaded on to a 11.0 cm × 2.5 cm strip of the X-Omat XK-5 X-ray films (Kodak Chemical Corp.). A negative control containing only Azocoll buffer and a positive control containing enzyme fraction only, were also maintained. The strips were then incubated at 42 °C for 1 h and subsequently washed under tap water for 1 min. Proteolysis of the gelatin coating on the film by the enzyme results in the presence of cleared zones on the strip and absence of such zones was taken as a measure of proteinase inhibitor activity.

For a typical enzymatic reaction, the crude enzyme extract was diluted in the ratio of 1:12.5 using 46 mM Tris buffer containing 11.5 mM $CaCl_2$ (pH 8.1). The inhibitory potential of CbTI and SKTI (as a reference standard for Kunitz inhibitors)

were monitored, after mixing variable concentrations of the crude enzyme extract with each of the two inhibitors respectively. As a control only the crude gut extract (without any inhibitor) was used. Bovine trypsin was used as a reference proteinase. Five replicates for all the tests were maintained.

3. Results

3.1. Initial extraction and purification

C. bonduc (55 g of 0.15 N NaCl swollen cotyledons) were homogenized as described in Section 2. The total protein content in the crude extract was ~1.35 g. The trypsin inhibitor designated as CbTI showed a specific activity of ~0.7 TIU/A_{280} (Table 1). Two saturation levels (0–30% and 30–65%) of ammonium sulfate precipitation were used for partial purification of crude CbTI. Both the protein fractions showed good antitryptic activity but the 31–65% fraction was preferred for subsequent studies due to poor yield of the 0–30% fraction. Constituting ~20% of the crude proteins, the 30–65% fraction was then subjected to DEAE-52 cellulose ion exchange chromatography. A major peak (0.2 M NaCl) was accompanied by three smaller ones (0.3, 0.4 and 0.5 M NaCl) respectively (Fig. 1). All the protein peaks exhibited trypsin inhibitory activity but only 0.2 M NaCl protein fraction was further processed owing to higher trypsin inhibition (Table 1, Fig. 1 inset). Ion exchange chromatography yielded a purification factor of 3.7% corresponding to a specific activity of 2.6 TIU/A_{280} (Table 1). The 0.2 M NaCl fraction was then applied to gel filtration on Superdex S-75. The fractions with maximum inhibitory activity were dialyzed and lyophilized respectively.

Two peaks (P1 and P2) were obtained following gel filtration chromatography (Fig. 2A). The first peak (P1: fractions 2–12) was sharp in contrast to a broad trailing peak (P2: fractions 18–40). Surprisingly, proteins collected from peak P1 were totally devoid of any antitrypsin activity. However, proteins from peak P2 (constituting just 0.27% of the total proteins) demonstrated ~85% trypsin inhibitory activity (Fig. 2B, Table 1). Protein fractions constituting P2 were finally purified on trypsin-Sepharose CL-4B. A single protein peak showing >90% inhibition of bovine trypsin was obtained. The details of purification procedure of CbTI are shown in Table 1.

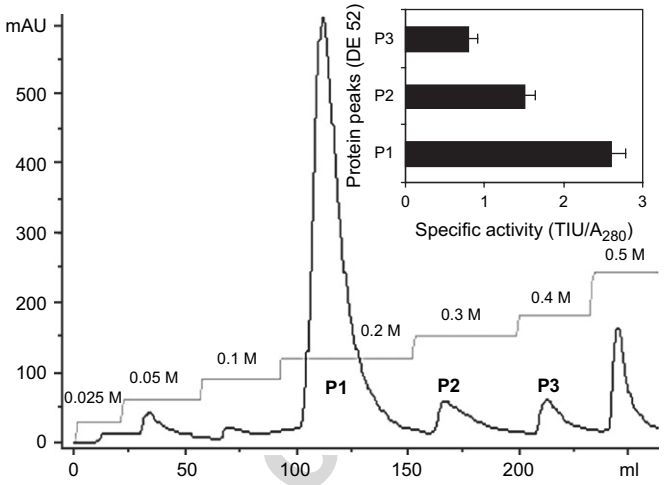

Fig. 1. Anion exchange chromatography, on DEAE-52 cellulose, of the 31–65% ammonium sulfate fractionated proteins of *C. bonduc* seed extract. Column dimensions: 2.5 × 20 cm; starting buffer: 20 mM Tris–HCl (pH 8). Protein elution was performed using NaCl (0.05–0.5 M) in a step-gradient fashion. The line cutting across the graph indicated the length and duration of a particular molar concentration of NaCl applied. Three major peaks P1 (0.2 M NaCl), P2 (0.3 M NaCl) and P3 (0.4 M NaCl) were obtained with variable antitryptic activity. Inset: Assessment of the trypsin inhibitory effects of ion exchanged inhibitor peaks P1, P2 and P3 against bovine trypsin. Fractions corresponding to peak P1 showed the maximum specific activity towards trypsin inhibition.

3.2. Molecular properties of CbTI

Both the GPC purified (peak P2) and the affinity-purified inhibitor was analyzed by SDS–PAGE with and without β-mercaptoethanol to examine both the molecular weight as well as the purity of the inhibitor. Following GPC, CbTI showed two bands designated as CbTI-1 (~16 kDa) and CbTI-2 (20 kDa) under non-reducing conditions. On reduction and alkylation CbTI-2 resolved into two bands of ~14 and 6 kDa (Fig. 2A inset). Subsequent to affinity purification only CbTI-2 was obtained which showed a single band of ~20 kDa under

Table 1
Purification chart of a trypsin inhibitor from *Caesalpinia bonduc* (CbTI)

Step/Characteristics		Total protein, mg	% Inhibition	Specific activity, TIU/A_{280}	% Recovery	Purification factor
Crude inhibitor		1352.1	70.6	0.7	100	1.00
Ammonium sulfate precipitation	0–30%	86.4	62.7	nd	6.4	nd
	31–65%	263.2	75.6	1.3	19.4	1.9
DEAE cellulose chromatography[a]	0.05 M	12.3	23.0	0.3	0.9	–
	0.1 M	12.0	17.7	0.2	0.9	–
	0.2 M	30.9	85.2	2.6	2.3	2.0
	0.3 M	52.3	78.6	1.5	3.9	3.7
	0.4 M	23.5	41.9	0.8	1.7	–
	0.5 M	15.2	27.0	0.7	1.1	–
Superdex S-75 gel filtration		5.7	88.4	6.5	0.4	9.3
Trypsin–Sepharose CL 4B		3.6	90.4	8.0	0.3	11.4

One trypsin or chymotrypsin unit is defined as 1 μmol of substrate hydrolyzed per minute of reaction. One inhibition unit is defined as unit of enzyme inhibited. Specific activity is defined as inhibition units per absorbance unit, at 280 nm, of the inhibitor. nd, not done; –, insignificant; TIU, trypsin inhibitory units; A_{280}, absorbance at 280 nm.

[a] NaCl gradient (0.05–0.5 M) used to elute the inhibitory protein during ion-exchange chromatography on DEAE-52 cellulose.

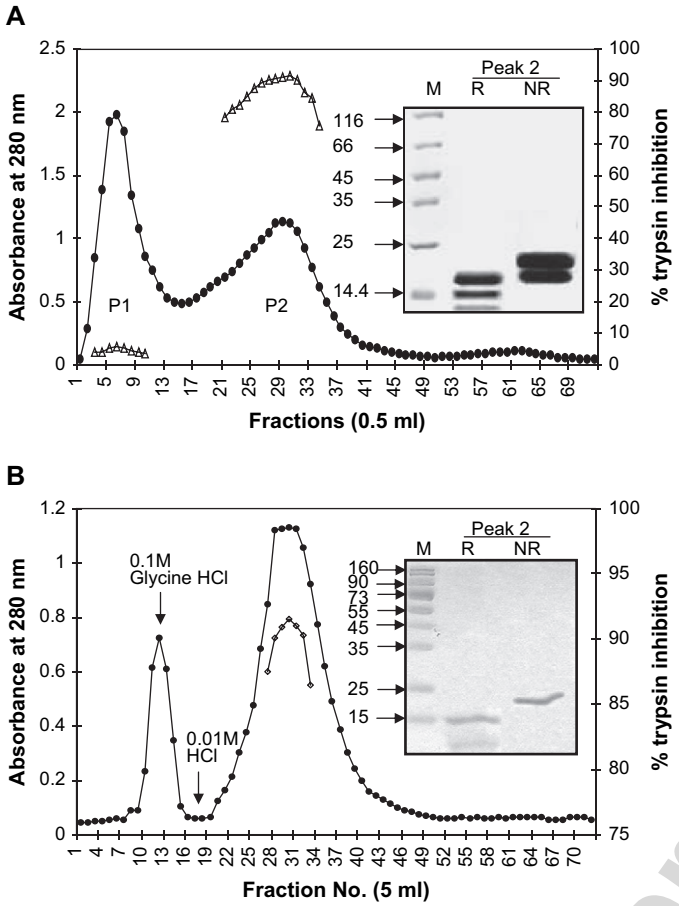

Fig. 2. (A) Gel-filtration chromatography and SDS–PAGE analyses of CbTI. Anion exchanged fractions (fractions from peak P1) of CbTI were pooled, lyophilized and loaded on a Superdex S-75 gel-filtration column; monitored at 280 nm (●) and assayed against trypsin (△). CbTI resolves into two peaks; peak P1 (fractions 3–10) showed negligible trypsin inhibitory activity and peak P2 (fractions 20–40) possessed maximum antitryptic activity. Inset: SDS–PAGE profile of CbTI. Following reduction (R) and alkylation, CbTI showed three bands of 6, 14 and 20 kDa (lane 2); non-reducing (NR) form of CbTI showed a couple of bands: ∼16 and 20 kDa (lane 3) and molecular weight markers (lane 1). (B) Affinity chromatography on Trypsin–Sepharose CL 4B of gel filtrated (fractions from peak P2) of CbTI. The column was equilibrated with 0.1 M Tris–HCl buffer and 0.1 M NaCl, pH 8.0; the retained peak was eluted with 0.01 M HCl solution, monitored at 280 nm (●) and assayed against trypsin (◇). Inset: SDS–PAGE at 12% of purified CbTI, stained with Coomassie Blue. Affinity purified CbTI showed two bands of ∼6 and 14 kDa on being reduced (R) and alkylated (lane 2); a single band of ∼20 kDa under non-reducing (NR) conditions (lane 3) and protein molecular weight markers (lane 1).

non-reducing conditions. On reducing and alkylating, it resolved into two subunits of ∼14 kDa and ∼6 kDa (Fig. 2B inset).

CbTI-2 exhibited two closely occurring bands in native gel electrophoresis, indicating two isoforms (Fig. 3A). Similarly, isoelectric focusing also revealed a couple of isoinhibitors CbTI-2A and CbTI-2B possessing acidic p*I*s of 5.35 and 4.6, respectively (Fig. 3B).

3.3. Thermal tolerance and pH stability of CbTI

CbTI showed high trypsin inhibitory activity between 25 °C to 55 °C. Beyond 55 °C there was a gradual decline in

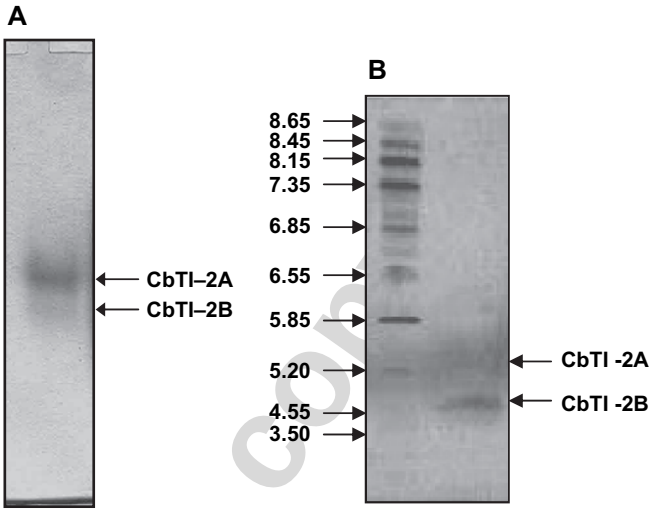

Fig. 3. Native PAGE and Isoelectric focusing of affinity purified CbTI. (A) Native PAGE profile of CbTI showing two bands representing two isoinhibitors differing minutely in the *e*/*m* ratio. (B) Isoelectric focusing analysis of CbTI on Ampholine polyacrylamide gel plates (pH range 3.5–9.5, Pharmacia) over a broad-range p*I* calibration kit containing proteins with various isoelectric points ranging from 3 to 10. Lane 1, marker; lane 2, purified CbTI showing two isoinhibitors with p*I* at 5.35 and 4.6.

the inhibitory activity, which was profound following a heat treatment of both 30 min as well as 60 min at 75 °C (Fig. 4A). A total loss of activity was observed on heating the inhibitor at 95 °C, regardless of the duration.

Displaying wide pH amplitude, CbTI is highly stable under conditions ranging from highly acidic to highly alkaline. In fact, the inhibitor maintained over 95% of its inhibitory activity through a pH gradient of 1–12 (Fig. 4B).

Upon being stored at −20 °C, CbTI remained stable for the first 5 months after its purification, retaining over 90% of its inhibitory potency. However, following the initial 5 months, there was a steady fall in its inhibitory activity with an annual loss of ∼27% (data not shown).

3.4. Kinetic properties of CbTI activity

Primarily, CbTI-2 is a strong trypsin inhibitor albeit possessing good antichymotryptic activity. Incidentally, it showed ∼1.5-fold higher inhibitory activity towards bovine trypsin in comparison to that of chymotrypsin (Fig. 5A). In fact, the titration curve for inhibition of trypsin and chymotrypsin by increasing amounts of CbTI-2 remained linear up to ∼90% and ∼65% inhibition, respectively. Interestingly, a 1:1 stoichiometry was observed for the inhibition of trypsin which was absent in case of chymotrypsin (Fig. 5A). Inhibition constants of 2.75×10^{-10} M and 0.95×10^{-10} M were obtained for trypsin and chymotrypsin inhibition respectively using Dixon plot analysis (Fig. 5B,C).

SDS–PAGE was employed to assess the stability and patterns of CbTI–trypsin and CbTI–chymotrypsin complexes (Fig. 6). A variety of combinations involving 1:1 molar ratios of CbTI-1 and 2 (3 μg) with trypsin and chymotrypsin respectively, as well as competition among trypsin and chymotrypsin

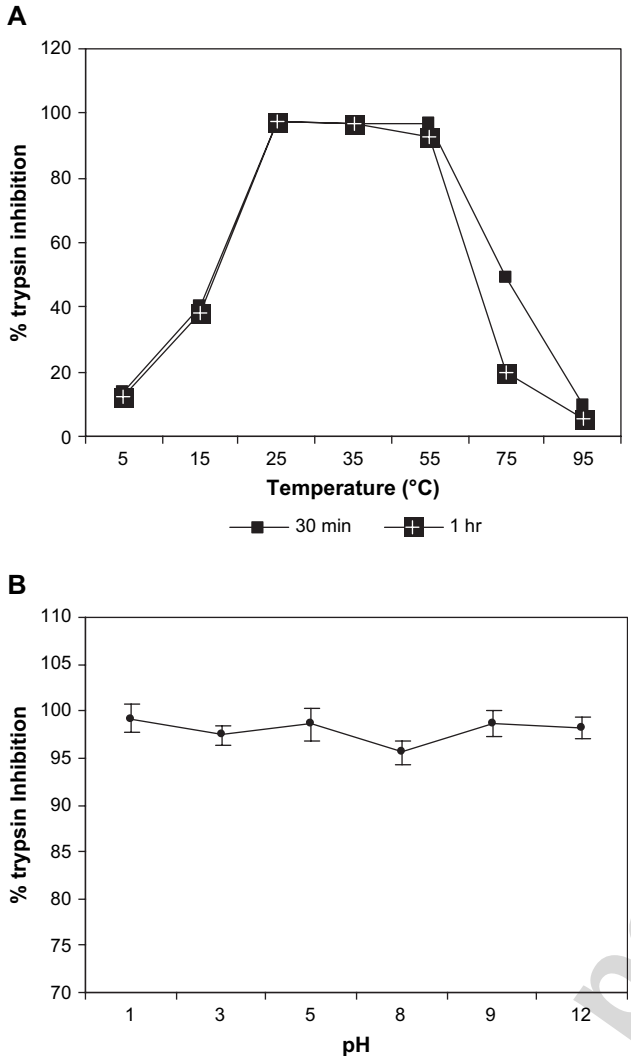

Fig. 4. Effect of pH and temperature on the activity and stability of affinity purified CbTI. (A) CbTI was incubated in 0.05 M Tris–HCl buffer, pH 8.0 at the required temperatures for 30 min (closed squares) and 60 min (crossed squares) and trypsin inhibitory activity was measured as described in Section 2. (B) CbTI was pre-incubated in a buffer of the required pH for 3 h (closed circles) and trypsin inhibitory activity was measured as described in Section 2.

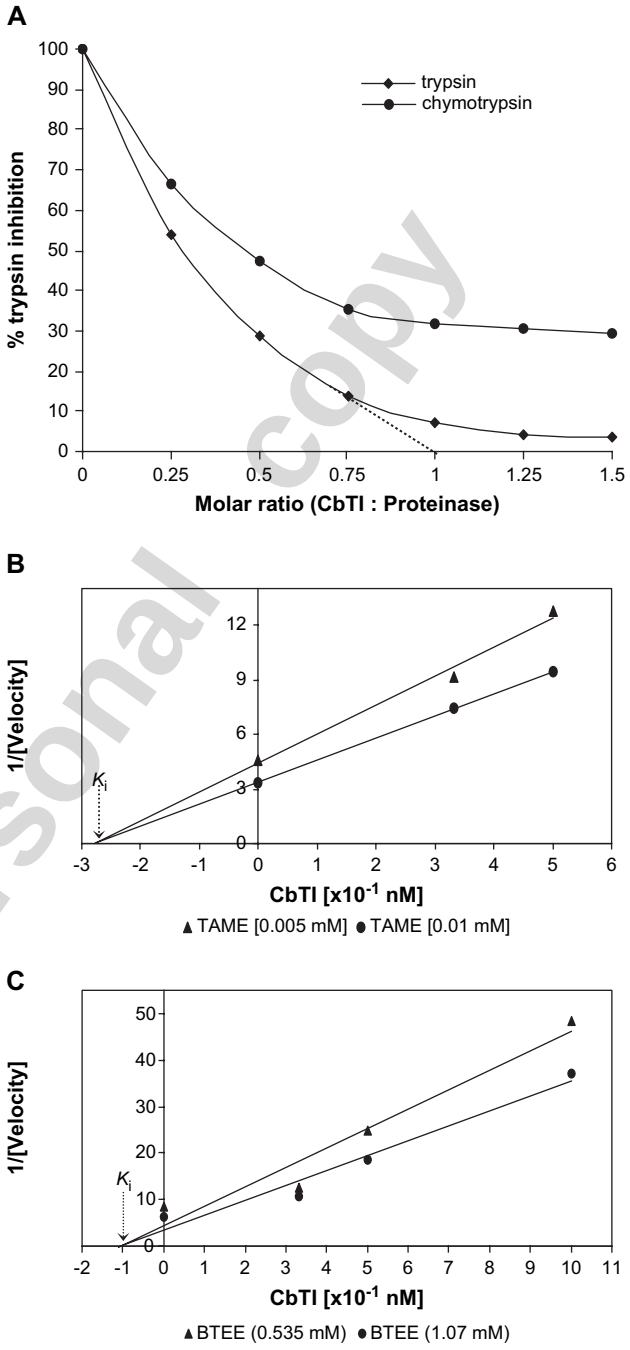

Fig. 5. Inhibition kinetics of trypsin and chymotrypsin activity by purified CbTI. Inhibitory effect of increasing amounts of CbTI against bovine trypsin (◆) and α-chymotrypsin (●) at pH 8.0 (A) Dixon plot of data obtained by varying CbTI against trypsin [$K_i = 2.75 \times 10^{-10}$ M] (B) and chymotrypsin [0.95×10^{-10} M] (C). Increasing concentrations of CbTI were used trypsin and chymotrypsin assays in the presence of at two different concentrations of TAME and BTEE, respectively. The reciprocals of velocity were plotted against the CbTI concentration, and the K_i value was obtained from the intercepts of two lines at two concentrations of substrate.

for the binding to both forms of CbTI were carried out, and subsequently analyzed by SDS–PAGE. In case of CbTI–trypsin interaction, four major bands were noted—a couple of which (~38 and 45 kDa) represented the complex formation between trypsin–CbTI-1 and trypsin–CbTI-2, respectively (Fig. 6). The bands at ~16 and 20 kDa represented free CbTI-1 and unreacted trypsin. In presence of chymotrypsin however, complex (~40 kDa) formation is noted only with CbTI-1. Also, it appeared that CbTI-2 undergoes proteolysis in presence of chymotrypsin (Fig. 6). Interestingly, extraneous addition of chymotrypsin to reaction mixtures containing CbTI and trypsin led to proteolysis of the CbTI-2–trypsin complex and instead, there was a formation of a CbTI-1–chymotrypsin (~40 kDa) complex. Expectedly, any free CbTI-2 was degraded and only free trypsin was visible (Fig. 6). Conversely, experiments in which trypsin was added to reaction mixtures of CbTI and chymotrypsin, there was no effect on a previously formed CbTI-1–chymotrypsin (40 kDa) complex. Electrophoretic patterns for a mixture of trypsin and chymotrypsin incubated with CbTI showed resemblance to the SDS–PAGE profile of the tests in which trypsin was added to reaction mixtures of CbTI and chymotrypsin (Fig. 6).

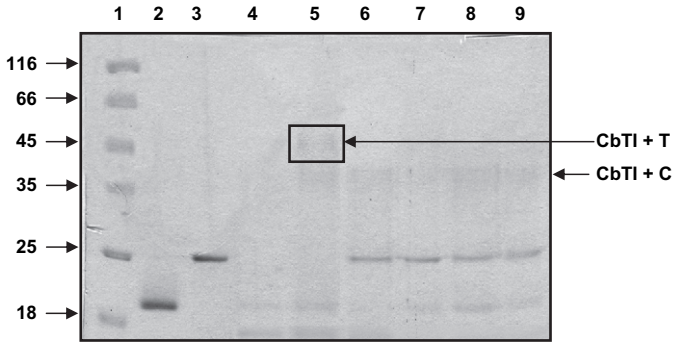

Fig. 6. SDS–PAGE analyses of gel filtrated CbTI following treatment with serine proteinases in a buffer (0.05 M Tris–HCl; 0.001 M $CaCl_2$, pH 8.0), incubated at 37 °C for 30 min. Typically, trypsin (T) and chymotrypsin (C) were incubated with CbTI (Cb; 5 μg) (1:1 molar ratio) in various combinations, and subsequently run on a SDS–PAGE. Complex formation between CbTI–trypsin and CbTI–chymotrypsin were observed. Lane 1, molecular weight standards; lane 2, bovine trypsin; lane 3, bovine chymotrypsin; lane 4, CbTI; lane 5, Cb + T (1:1); lane 6, Cb + C (1:1); lane 7, [Cb + T] + C (1:1); lane 8, [Cb + C] + T (1:1); lane 9, Cb + [T + C] (1:1).

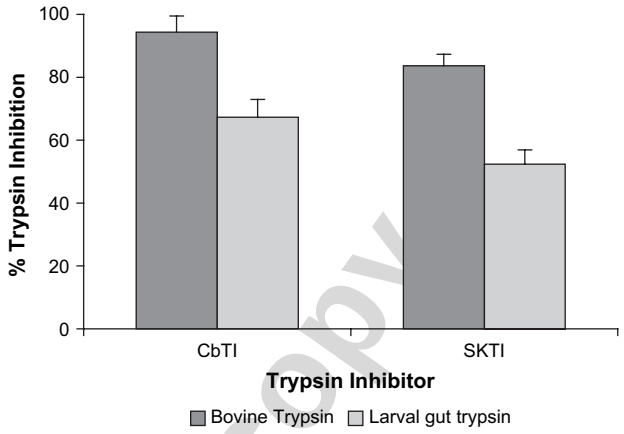

Fig. 7. Effect of CbTI on the bovine and larval gut trypsin activity of *Spodoptera litura*. CbTI protein was tested for potential inhibitory activity against bovine trypsin as well as larval gut trypsin like activity using the substrate TAME. Equimolar ratio of inhibitor:enzyme led to complete inhibition of bovine trypsin as well as gut trypsin like activity. SKTI was used as a reference standard. Five replicates were maintained for each measurement.

3.5. Effect of the inhibitor on insect gut proteinases

The efficacy of CbTI was checked against *Spodoptera litura* gut proteinases by assessing the inhibition of larval gut trypsin like activity. The pH of the gut extract was observed to be highly alkaline (>10.8). Initial experiments on the gut proteinases–CbTI interaction were executed using unused and unprocessed X-ray films. Larval proteinases of fifth instar larvae gut proteinases showed digestion of the gelatin coating in form of small holes on the X-ray film strip. Following interaction with CbTI, the activity of gut proteinases was terminated and expectedly, no holes were visible on the X-ray film strip (data not shown). Importantly, dosage of CbTI required for inhibition of gut proteolytic activity was ∼1.5-fold lower than that of SKTI. These results were further confirmed by trypsin inhibition assays using the esterolytic substrate TAME. On incubating the crude larval gut trypsin with CbTI, there was ∼ 68% reduction in the gut tryptic activity. Comparatively however, bovine trypsin was ∼25% more susceptible to CbTI than larval gut trypsin (Fig. 7). A comparison between the antitryptic capabilities of CbTI and SKTI showed that inhibition of Spodoptera gut trypsin by CbTI exceeded that of SKTI by ∼15% (Fig. 7).

4. Discussion

Serine proteinase inhibitors and their binding to cognate proteinases have been extremely well characterized and in particular, the Kunitz family trypsin inhibitors from a variety of plant sources have received special attention over the years [2,7,16–18]. Several such Kunitz PIs have been shown to impart protection to host plants by having detrimental effects on the growth and development of their insect pests [29–32]. The present study provides a detailed account of the purification and, biochemical and biological characterization of a related proteinase inhibitor from the seeds of *Caesalpinia bonduc*, growing at coastal forests of Great Nicobar Island, India. The seeds of *Caesalpinia bonduc* are protein rich and, have been implicated as a remedy of diuretic, for diabetes and for hypertension.

Two forms of CbTI (CbTI-1 and CbTI-2) were obtained following gel filtration. Subsequent to affinity purification, only CbTI-2 corresponding to a molecular mass of 20 kDa was obtained. This is consistent with the molecular weight of other Kunitz trypsin inhibitors [21,33–38].

Native gel electrophoresis indicated the presence of two isoforms which were further confirmed by isoelectric focusing revealing a couple of isoinhibitors. Presence of isoinhibitors and an acidic p*I* are common features of Kunitz inhibitors [16]. The physiological relevance of these isoinhibitors is still to be understood clearly, but leguminous plants endowed with such inhibitors seems to gain protection against herbivory [16,32].

The broad temperature tolerance and high pH amplitude of CbTI is in good agreement with the previously reported chemical characteristics of several Kunitz PIs, namely DmTI [21], AeTI [32], AcTI [33], EcTI [34], PdTI [39] and DmTI-II [40]. Intramolecular disulfide linkages have been implicated in imparting high intrinsic stability to PIs to overcome physiologically stressful conditions [38].

The kinetics of trypsin and chymotrypsin inhibition indicates that CbTI is inclined more towards trypsin than chymotrypsin. Serine proteinase inhibitors belonging to the Caesalpinioideae family vary in their specificity of inhibitory activity. For instance, the Kunitz trypsin inhibitor from *Delonix regia* [35] showed inhibition only against trypsin whereas *Peltophorum dubium* trypsin inhibitor [39] showed high trypsin and low chymotrypsin inhibitory properties. Inhibition of trypsin and chymotrypsin by increasing amounts of CbTI showed a titration curve reflecting a slow-tight binding mechanism for both the proteinases. Similar non-linear titration curves showing 1:1 binding stoichiometry and low inhibition constants are a regular hallmark of legume Kunitz trypsin inhibitors and have been

particularly well documented, namely *Leucaena leucocephala* (LlTI) [13], *Dimorphandra mollis* (DmTI) [21], *Archidendron ellipticum* (AeTI) [32], *Enterolobium contortisiliquum* (EcTI) [34] and *Adenanthera pavonia* (ApTI) [41].

SDS–PAGE-based analysis of the molecular interactions between trypsin, chymotrypsin and CbTI showed that both CbTI-1 and CbTI-2 possessed different binding avidities for the two proteinases. CbTI-2 exhibited a strong affinity for trypsin, forming a complex of ~45 kDa whereas CbTI-1 not only displayed a binding preference for chymotrypsin, but also showed a weak binding with trypsin. This weak binding was however disintegrated in presence of chymotrypsin and thus, only a complex with chymotrypsin was formed. Notably, presence of CbTI-2 previously exposed to chymotrypsin prevented the formation of any CbTI–trypsin complex, even under trypsin superfluous conditions. So, only free trypsin was noted on SDS–PAGE. Similar interference of free trypsin to previously formed complexes of inhibitor and chymotrypsin, and free chymotrypsin to previously formed complexes of inhibitor and trypsin were also observed in case of a proteinase inhibitor from *Bauhinia purpurea* [42].

Our studies on the effects of CbTI on insect gut proteinases reflect that CbTI is a powerful antifeedant of insect herbivores. The deleterious effects of CbTI on larval gut proteinases of *S. litura* were similar to previously observed results with proteinase inhibitors from other leguminous plants [43–47]. An in-depth analysis of the diverse biological activities, such as toxicity, sequestration, and unpalatability of CbTI on the growth and development of different crop pests, needs to be carried out to confirm the biotechnological potential of CbTI as an agent against phytophagous insects.

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