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# Recombinant Cystatin-Like Thiol Proteinase Inhibitor (rCTPI) of Cicer arietinum (Chickpea): A potential source of Bio-pesticide.

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**Abstract:**

*Cystatins are thiol proteinase inhibitors ubiquitously present in many plants as well as animal tissues. In the present study, a cystatin like a thiol proteinase inhibitor (rCTPI) gene was isolated from chickpea plant and cloned in pET28a vector. The recombinant vector with CTPI gene was expressed and recombinant CTPI proteins was isolated and purified. The purified rCTPI was physiologically active at pH 7.5 and temperature range of 35-37$^0$ C. Larvicidal activity of rCTPI were determined with a potential application as bio-pesticide.*

**Keywords:** *Cicer arietinum* **(**Chickpea); Cystatin Thiol proteinase inhibitor; Bio-pesticide; Cloning

## Introduction

Proteinase inhibitors that are found endogenously are protein molecules that specifically inhibit the function of proteinases. They are mainly non-competitive inhibitors of proteinases [1]. Generally plant proteinase inhibitors are small proteins with a molecular mass less than 50 kDa. However, dimeric and tetrameric inhibitors have been isolated from tomato and potato [2, 3]. They have been isolated from solanaceae, leguminaceae and graminaceae families [4]. These inhibitors are highly selective in function. Cells possess these inhibitors to regulate the enormous hydrolytic ability of proteolytic enzymes that are otherwise essential for the survival of organisms [5]. The major proteases of the lysosomal pathway of protein degradation are cathepsins which are naturally controlled by cysteine proteinase inhibitors.

Natural inhibitors of cysteine proteinases include the members of cystatin superfamily comprising of stefins, cystatins, and kininogens [6, 7]. Cystatins constitute a powerful regulatory system for endogenous cysteine proteinases, which may otherwise cause uncontrolled proteolysis and tissue damage. They form potent, reversible, non-covalent competitive inhibitors of cysteine proteinases of the papain superfamily [9, 10]. It has been shown that members of this superfamily interact directly with the active site cleft of papain at three regions of the mature cystatin. The unique properties and a wide range of physiological functions of thiol proteinase inhibitors are remarkable and demand attention. Phytocystatins or plant Cystatins, the 4$^{th}$ family of this inhibitor

superfamily, plant homologs of animal cysteine proteinase inhibitors [13]. They have been identified in both monocotyledonous [8,14,15] and dicotyledonous [16,17] plants. They are particularly well characterized in crops such as rice [8, 14], maize [15] and soybean [16,17]. Cystatin has been successfully isolated in the plants as Rice [8,19,20], Soybean [21,22], Sugarcane [23], Maize [24] and Kiwi fruit [25]. Phytocystatins have also been isolated from plants such as cowpea, potato, and carrot [26-28]. Thiol proteases present in the plant system perform a variety of functions, and their regulation is performed by phytocystatins. They are important in a variety of ways, including their role in storage proteins [29], as regulators of endogenous proteolytic activity [30] and as participants in the mechanism of programmed plant cell death [31]. Furthermore, proteinase inhibitors are expressed in abiotic stress [33] and in plant defence processes against insect attacks [34]. Phytocystatins present in cereal seeds like rice and maize have been used to prevent certain types of cancer [35]. Thus, Cysteine Protease Inhibitors (CPIs) have been known to be present in a variety of seeds of plants, and have been intensively studied as useful tools for potential utilization in pharmacology and agriculture.

The present communication describes the cloning, expression, purification and characterization of cystatin-like thiol proteinase inhibitor (CTPI) of chickpea (*Cicer arietinum*) an important legume of the family Fabaceae. The potential role of rCTPI as bio-pesticide against Helicoperva Larva has also evaluated on two common crop pest.

## Material and methods

### Chemicals

Enzymes, substrates, Sephacryl S-100 HR, anti-rabbit alkaline phosphatase (conjugate) and p-nitrophenyl phosphate were obtained from Sigma (St. Louis, MO, USA). Casein, Ethylene Diamine Tetra Acetate (EDTA), Tri Carboxylic Acid (TCA), L-cysteine, and electrophoresis reagents were purchased from SRL (Mumbai, India). Medium molecular weight markers from Genei, India Limited. All other chemicals used were of highest purity grade available commercially.

### Isolation of Plant DNA

Chick Pea Leaves from one-month-old field grown plants were harvested and brought on ice to laboratory. The leaves were thoroughly washed with tap water and rinsed with distilled water, blot dried and weighed. The leaves were either stored at –70˚C or used directly for extraction. Around 0.5 g of leaf tissue was placed in a mortar and homogenized with 2 ml of extraction buffer. The extraction buffer (pH 8.0) consisted of 100 mM Tris, 20 mM EDTA, 0.5 M NaCl, 7 M Urea, 0.1% β-mercaptoethanol and 2% SDS. Long fibres of the tissue were retained back after crushing and the homogenate was transferred to a 2 ml-microfuge tube. An equal volume of phenol:

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chloroform :Isoamlyalcohol ( 25:24:1) was added to the tubes and mixed well by gently shaking the tubes. The tubes were centrifuged at room temperature for 10 min at 15,000 rpm. The upper aqueous phase was collected in a new tube and an equal volume of chloroform: Isoamlyalcohol (24:1) was added and mixed. The upper aqueous phase obtained after centrifuging at room temperature for 10 min at 15,000 rpm was transferred to a new tube. The DNA was precipitated from the solution by adding 0.1 volume of 3 M Sodium acetate pH 7.0 and 0.7 volume of Isopropanol. After 15 min of incubation at room temperature the tubes were centrifuged at 4ºC for 15 min at 15,000 rpm. The DNA pellet was washed twice with 70% ethanol and then very briefly with 100% ethanol and air dried. The DNA was dissolved in TE (Tris-Cl 10 mM pH 8.0, EDTA 1 mM). To remove RNA 5 µl of DNAse free RNAse A (10 mg/ml) was added to the DNA.

**Estimation of DNA**

The amount of DNA in the RNAse treated mixture was estimated by measuring the fluorescence emission using the dye Hoechst. Since the dye binds specifically to double strand DNA, the presence of small fragments of degraded RNA in the sample did not interfere with the measurements. The fluorescence intensity was measured at emission maximum of 546 nm using the fluorimeter Hoefer DyNA Quant 200. The amount of DNA was estimated by comparing the emission intensity obtained using a known amount of standard DNA (100 µg/ml) (provided by Amersham) which acts as the reference. The DNA was also observed on 2% agarose gel by electrophoresis using 1X TBE buffer. The DNA fragments were stained with ethidium bromide and viewed under UV light and subsequently photographed. The quantity of the DNA was estimated against a marker (λHindIII, 100 ng/µl) which was also loaded in the gel along with the DNA. The DNA was further used for PCR.

**PCR amplification and cloning of CTPI**

Based on the nucleotide sequence of the protein-encoding region of the CTPI gene primers were designed to clone CTPI in the vectors pET28a (+) (Novagen) with a histidine tag at the amino terminus. The CTPI ORF was amplified by PCR from genomic DNA of *Cicer arietinun* using sense (5'-TTTTAAGCTTAAGTCACTCACAATTAAC AACCCG -3') and an antisense (5' TTTTGGATCCAGAGGTAACAGTAGAAC AAATGAAT - 3') primers and DNA polymerase (Fermentas). PCR was performed in a 25 ml reaction mixture containing 0.2 mM each dNTPs, 2.0 mM MgCl2 1.0 mM each primer, 1 ng of *Cicer arietinun* genomic DNA and 1.0 U DNA polymerase with Taq buffer. The conditions used to amplify the CTPI gene were hot start at $95^0$C for 5 min, followed by the 30 cycles of denaturation at 94 $^0$C for 1

min, annealing at 60 $^0$C for 45 s and elongation at 72 $^0$C for 1.0 min and a final extension at 72 $^0$C for 10 min. The ~1.0 kb PCR products of CTPI were observed, on 1.0% agarose gel electrophoresis. CTPI amplified PCR products were double digested with BamHI and HindIII, purified with gel extraction kit (Qiagen), and cloned into BamHI and EcoRI double digested pET28a(+). The ligated mixture was transformed in competent *Escherichia coli* DH5α cells (Novagen) which produced the pET-28a (+)-CTPI constructs. The insert and ORF orientation of constructs were confirmed by colony PCR and restriction digestion. Construct plasmids were isolated using Qiagen Miniprep Kit, according to manufacturer's instructions. Three independent plasmids were sequenced to verify the gene sequence of the clones. The pET- 28a(+)-CTPI constructs were transformed into competent *E. coli* BL21 (DE3) (Novagen Inc., Madison, WI) cells by heat shock at 42 $^0$ C for 45 s followed by 2 min on ice, and the cells were grown at 37 $^0$ C on Luria Bertani (LB) agar medium in the presence of 50 mg/ml kanamycin and ampicillin, respectively.

**Expression and purification of recombinant CTPI**

Expression of the recombinant CTPI proteins in *E. coli* BL21 (DE3) cells was optimized to get maximum expression in the soluble fraction. For r CTPI -His, 5 ml overnight culture was used to inoculate 500ml fresh LB-Kanamycin medium and cultured at 37 $^0$C with shaking at 200 rpm. When the $A_{600}$ reached between 0.5 and 0.6, 1 mM IPTG was added to induce protein expression and culture continued to grow for 4 h at 37 $^0$C. The *E. coli* cells were harvested by centrifugation at 5000g for 10 min at 4 $^0$C, washed with PBS (pH 7.2), and suspended in lysis buffer, (50 mM Tris-HCl pH 8.0, 300 mM NaCl, and 0.1% Triton X-100), 100 mg/ml lysozyme, and 1.0mMphenyl methyl sulfonyl fluoride (PMSF). The cell suspension was incubated at room temperature for 30 min, sonicated on ice and centrifuged at 14,000g for 20 min at 4 $^0$C. The supernatant was mixed with pre-equilibrated 1.5 ml slurry of nickel-nitrilotriacetic acid (Ni2+-NTA) and incubated for 2 h at 4 $^0$C with gentle shaking. The resin was divided into three 10 ml disposable columns (Bio-Rad), washed with 5X column volumes of lysis buffer containing 10X 50 mM imidazole and eluted with lysis buffer containing 100 mM, 200 mM and 300 mM imidazole, as described previously. The integrity and purity of the rCTPI proteins were confirmed by 10% SDS-PAGE analysis and Coomassie Brilliant Blue R-250 staining. The eluted fractions were dialyzed twice against a 300 fold volume of 50 mM Tris-Cl (pH 8.0), 150 mM NaCl supplemented with 10% glycerol, overnight at 4 $^0$C.

**Protein estimation**

Protein concentration was determined by Lowry's method using bovine serum albumin

(BSA) as standard. The absorbance was read at 660 nm.

**Assay of thiol proteinase inhibitory activity**

The inhibitory activity of CPC was assessed by its ability to inhibit the caseinolytic activity of papain towards casein by the method of Kunitz [35]. Apart from this, inhibitory effects of CPC on proteolytic activity of ficin, bromelain, trypsin, and chymotrypsin were also measured using casein as substrate.

**Thiol group estimation**

For the determination of thiol groups of CPC, the procedure given by Ellman [26] was employed, using DTNB (5,5'-dithiobis-2-nitrobenzoic acid) reagent, and molar extinction coefficient of 13,600 M-1 cm-1. Cysteine was used as a standard.

**pH stability**

Fifty microgram aliquots of the inhibitor were incubated with buffers of different pH values like 50 mM Tris-glycine buffer (pH 3.0 and 4.0), 50 mM sodium acetate buffer (pH 5.0 and 6.0), 50 mM sodium phosphate buffer (pH 7.0 and 8.0), and Tris-HCl buffer (pH 9.0 and 10.0) for 30 min at 37°C. These differentially pH incubated inhibitor samples were used for determination of remaining % inhibitory activity as described in the section of the assay of proteinase inhibitory activity.

**Thermal stability**

(a) Aliquots (50 µg) of inhibitor in 50 mM sodium phosphate buffer (pH 7.5) were incubated at various temperatures (10- 90°C) for 30 min. These samples were rapidly cooled in ice cold water bath and checked for residual activity against 50 µg of papain. (b) Fifty micrograms of the inhibitor at 90°C were incubated for different time intervals (30-130 minutes), rapidly cooled, and residual inhibitory activity measured against papain.

**Larvicidal effect of CTPI against Helicoperva Larva**

The standard larvicide Temephos (Abate ®) was used at a rate of 0.025 µg/µL. Chemicals were prepared and it was distributed in four 100 ml capacity plastic bowls (replicates) 50 ml per bowl. The untreated water control was prepared with the same previous method. All concentrations of the rCTPI and the standard larvicide Temephos were evaluated for mosquito larvicidal activity according to WHO (1969) method. 20 of 3$^{rd}$ instar larvae were placed in each bowl (replicate). The exposure period was 24 hours during which no food was offered to the larvae. Mortality was recorded after the 24 hours, by counting the completely dead or moribund larvae together with the larvae that failed to reach the surface of the solution.

**Result**

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**Cloning, expression and purification of CTPI**

The rCTPI genes were amplified from genomic DNA of *Cicer arietinum* (Figure_1) and the resulting 1083-bp fragments were cloned into pET-28a (+) vectors giving the plasmids pET-28a-CTPI (Figure_2 A and B). The rCTPI ORF encode for proteins having 361 amino acids with predicted molecular weight of ~41 kDa.

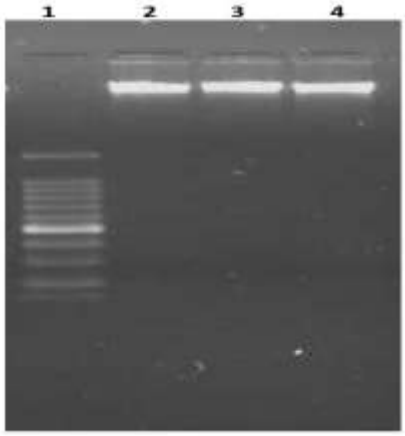


**Figure_1: DNA isolation from *Cicer arietinum* leaf extract.** Lane 1: 100 bp DNA ladder; Lane 2,3 and 4: Isolated DNA from leaves of three different *Cicer arietinum* Plant .

The rCTPI-His proteins were expressed in *E. coli* BL21 (DE3) and purified to homogeneity using Ni2+-NTA agarose resin by affinity chromatography. The rCTPI-His expression was higher in soluble form as compared to rCTPI-His and a high protein yield of ~6.0 mg/L of *E. coli* culture was obtained after purification.

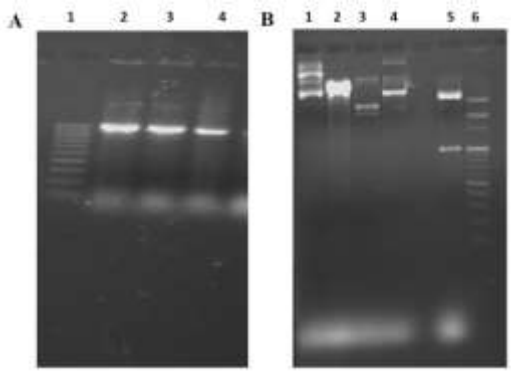


**Figure_2: PCR amplification and restriction digestion**

**A.** Lane 1: 100bp DNA ladder; Lane 2, 3 and 4: Full length gene PCR of CTPI gene.

**B.** Lane 1: plasmid pET28a; Lane 2: Single digested plasmid pET28a; Lane 3: Plasmid pET28b; Lane 4 : recombinant CTPI-pET28a; Lane 5: Double digestion of recombinant plasmid showing insert and vector; Lane 6: DNA ladder.

The purified rCTPI-His eluted at 100 X 200 mM imidazole and a single homogenous band at ~41 kDa (Figure_ 3A, lanes2, 5 and 6) was observed on SDS-PAGE and confirmed with western blot using anti HIS abtibody. Notably The rCTPI-His expression was high in soluble form and a protein yield of ~10 mg/L of E. coli culture was obtained after purification.

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The purified rCTPI-His eluted at 200 mM imidazole and a single homogenous band at ~43 kDa (Figure_ 3B, lane 2, 3 and 4) was observed on SDS-PAGE.

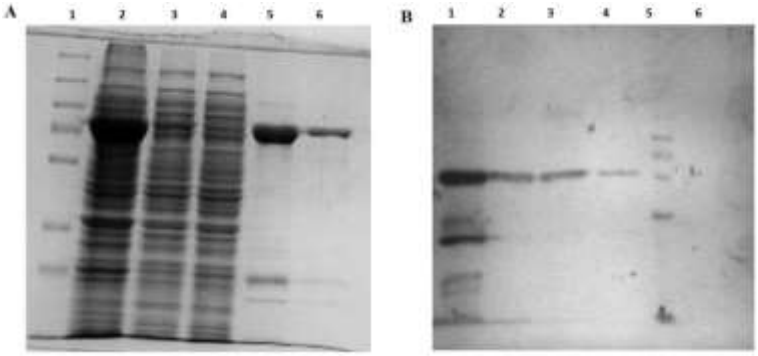

**Figure_3: Expression and purification of rCTPI**

**A.** SDS –PAGE: Lane 1: LMW protein ladder; Lane 2: IPTG induced BL-21 cell with rCTPI expression; Lane 3 and 4: IPTG uninduced BL-21.; Lane 5 and 6: Elution1 and elution 2 after purification.

**B.** Western Blot: Lane 1: Flow through sample; Lane 2, 3 and 4: Elution1, elution 2 and elution 3 respectively after purification; Lane 5: LMW protein ladder; Lane 6: Wash fraction.

**Stability to pH and temperature**

Effect of pH on the thiol proteinase inhibitory activity of CPC was examined at various pH values.

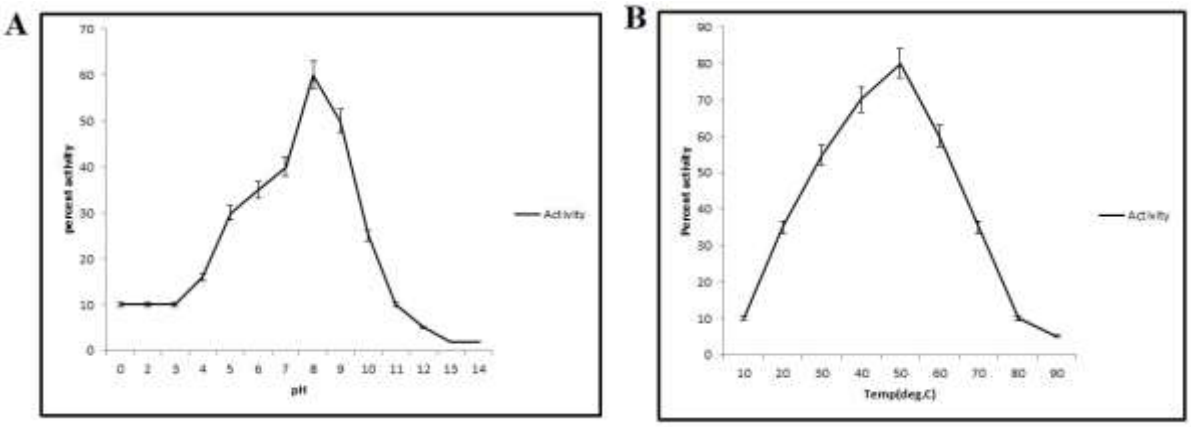


**Figure_4: Effect of pH (A) and Temperature (B) on activity of rCTPI.**

Figure 4 A shows that the inhibitor is stable in the pH range 6-7 and has maximum activity at pH 7.5. Stability of CPTI was investigated as a function of temperature between 10°C and 90°C in 50 mM sodium phosphate buffer pH 7.5, by means of inhibitory activity assay. As can be seen, CPC remained maximally active

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within a temperature range of 40-60°C (Figure_4 B).

***Helicoperva* Larvicidal activity rCTPI**

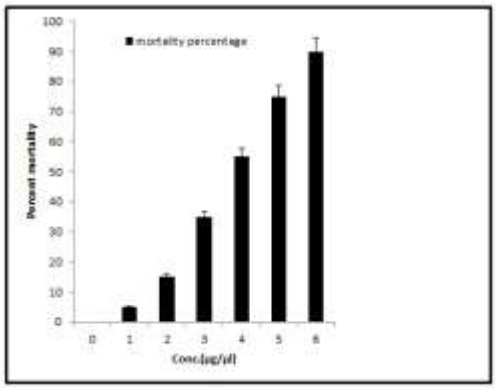


**Figure_5: Larvicidal activity of CTPI against larva of *Helicoperva* pest**

The mortality of larva progressively increased with increasing concentrations of rCTPI and showed 90 % mortality at 6 µg/µl rCTPI concentrations (Figure_5).

**Discussion and Conclusion**

Cystatins are a group of cysteine proteinase inhibitors (CPIs) or thiol proteinase inhibitors (TPIs) belonging to the superfamily of evolutionary, structurally and functionally related proteins involved in the inhibition of papain and related cysteine proteinases like ficin and bromelain. Most of the evidences indicate that the proteolytic activity of both endogenous and exogenous cysteine proteinases is primarily regulated by this cystatin super family. These inhibitors (cystatins) are ubiquitous in organisms, ranging from viruses to bacteria, plants, and mammals. Cystatins in plant systems perform a variety of functions. They are expressed in abiotic stress and in plant defense processes against insect attacks. Phytocystatins present in some cereal seeds has been reported to prevent certain types of cancer. These CPIs are present in seeds of all plants and are involved in the mobilization of protein reserves, plant senescence etc by providing a powerful regulatory role. These have been isolated in a variety of plant sources some of which have been already discussed in the introduction section. However, isolation and characterization of cystatin from chick pea is reported for the first time through this communication. It was found to be specific for cysteine proteases since no inhibitory activity against aspartic (pepsin) and serine proteinases (chymotrypsin and trypsin) was detected.

Thus, in the present work, a cysteine thiol proteinase inhibitor (CTPI) gene was cloned, expressed and purified. Analysis of the influence of pH on the activity of CPC reveals that the inhibitor remains fairly active in the pH range of 6.0-8.0 with maximum activity around pH 7.5 (Figure 4). CPC also exhibited

stability in a wide temperature range of 40-60°C (Figure 4). High stability of the purified CPC in broad temperature and pH ranges is in good agreement with other cystatins like *Phaseolus mungo* (black gram) cystatins [26], goat brain cystatin [27], stefin A and B [28] etc.

Pesticides play significant roles in agriculture and public health programmes. However, increased use and abuse of pesticides have caused great environmental and public health concerns. The need of bio pesticide is of utmost important. The mortality of larva progressively increased with increasing concentrations of rCTPI and showed 90 % mortality at 6 µg/µl rCTPI concentration. These results suggest further studies are needed to determine the mode of action, toxicity, and stability and to study their impacts on human health.

**Conflict of Interest**

The authors confirm that this article content has no conflicts of interest.

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