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# Cloning, expression and mutational studies of a trypsin inhibitor that retains activity even after cyanogen bromide digestion

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ABSTRACT

A winged bean trypsin inhibitor (WbTI-2) of molecular mass ~20 kDa, has been cloned and expressed in *Escherichia coli* with full activity like the one from seed protein. It completely inhibits trypsin at an enzyme:inhibitor molar ratio of 1:2. PCR with cDNA and genomic DNA using same primers produced about 550 base pair product, which indicated it to be an intronless gene. Through site-directed mutagenesis, the Arg64 has been confirmed as the P1 residue. For the presence of five methionine residues in WbTI-2, cyanogen bromide (CNBr) digestion was carried out. Out of three fragments the one (about 65% of original size) containing the reactive site loop retained 50% activity.



## Introduction

Protease inhibitors are regulatory proteins controlling proteolytic events in all living organism [1]. In plants, protease inhibitors are mainly found as constitutive storage proteins. It is a well established fact that they play an important role in the defense of plants against insect attack and their expression levels are increased in injured tissues [2]. The majority of protease inhibitors studied in plants belong to three main families, viz., solanaceae, leguminosae and gramineae. The leguminous plants contain two major types of serine protease inhibitors namely Bowman-Birk type and Kunitz type. Winged bean [*Psophocarpus tetragonolobus*] is a tropical legume, with a high content of serine protease inhibitors. Winged bean seeds have been reported to contain at least three Bowman-Birk type inhibitors and nine Kunitz type inhibitors [3,4]. The initial report on purification of protease inhibitors was from the psophocarpin B fraction of winged bean seeds by Kortt [5,6]. Following that winged bean trypsin, chymotrypsin and trypsin–chymotrypsin inhibitors, which belong to the Kunitz family, were purified from seeds and their interactions with proteases were studied in our lab [7]. The complete 3-D structure of a winged bean chymotrypsin inhibitor was solved by us in collaboration [8–10], whereas the cloning and expression of the chymotrypsin inhibitor was carried out earlier from our lab [11]. Recently single step purification, cloning, expression of a trypsin/chymotrypsin inhibitor (WbCTI) has been reported from our lab [12,13]. Through site-directed mutagenesis we have converted it to a strict chymotrypsin inhibitor and a trypsin inhibitor indicating the specific involvement of P1 and P2 residues with these dual activities [14]. It thus appeared to us that such inhibitors might withstand manipulation to a great extent and may be a small portion of it takes part in activity.

For several decades, the Kunitz soybean trypsin inhibitors have served as one of the model systems for the study of protein structure and protease-inhibitor interaction [15]. The major common structural element of the serine protease inhibitors is the primary contact region, in other words the reactive site loop (RSL[1]), by which the inhibitors interact with their cognate proteases. For several trypsin inhibitors it was reported that there are strong preferences for specific amino acids at different positions of a twelve residue stretch around the reactive site loop [16]. In comparison to the Kunitz type with respect to the size, the Bowman-Birk type inhibitors consist of only 60–70 amino acids, but equally potent as the Kunitz type. A sunflower trypsin inhibitor (SFTI-1) consisting of only fourteen amino acids showed trypsin inhibition [17]. Large number of synthetic peptides mimicking the Bowman-Birk type

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[1] *Abbreviations used:* RSL, reactive site loop; WbTI-2, winged bean trypsin inhibitor; TOFMS, time-of-flight mass spectroscopy; TAME, *N*-α-p-Tosyl-L-Arginine Methyl Ester.

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reactive site loop with trypsin inhibitory property were also reported [18]. However, there is no report of such peptide fragments of Kunitz type inhibitor with relevant activity.

In this paper, we report the cloning, expression, and mutation of a Kunitz type trypsin inhibitor, WbTI-2. Moreover, through CNBr digestion we have aimed at finding out smaller fragments that will retain the structural recognition elements, antigenicity as well as the inhibitory property.

## Materials and methods

### Materials

T/A cloning kit from MBI Fermentas (USA) was used in cloning and sequencing genes whereas pTrc99A, a high expression vector from Amersham Pharmacia Biotech (USA) was used in the expression of rWbTI-2. LB (Luria–Bertani) broth was from Himedia (India). All primers (custom made), Protease inhibitor cocktail (for use with bacterial cell extract), trypsin, TAME (*N*-α-p-Tosyl-L-Arginine Methyl Ester), TRI reagent (single-step total RNA isolation reagent), IPTG (isopropyl β-D-thiogalactopyranoside), X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside), Freund's complete and incomplete adjuvant were purchased from Sigma–Aldrich (USA). CNBr was from SRL (India). Superscript-II Reverse Transcriptase kit was purchased from Invitrogen (USA). Restriction enzymes and other reagents used in molecular biology techniques were from MBI Fermentas (USA). Affiprep Hz Hydrazide support was purchased from BioRad (USA). Quick Change Site-Directed Mutagenesis kit was purchased from Stratagene (USA). Antibody against WbTI-2 was raised in our laboratory. All the other reagents used were of analytical grade.

Winged bean (*Psophocarpus tetragonolobus*) plants were grown in the institute garden, Kolkata, India.

Animals were maintained and handled according to the institutional guidelines. The institutional animal ethics committee approved the experimental protocols and care of animals was taken according to CPCSEA guidelines.

### Isolation of genomic DNA from winged bean

Dry winged bean seeds were imbibed overnight and germinated on moist filter paper at 25 °C under sterile condition. After 12 day from when the seed coat cracked, the shoots were collected and stored in liquid nitrogen. Then genomic DNA was purified from developing shoots as described by Shure [19].

### Preparation of total RNA from winged bean and cDNA synthesis

Total RNA was purified from cotyledons of 24–26 day old pods using TRI reagent solution, according to vender's protocol. About 1.8 μg of total RNA was obtained from one gram of tissue. cDNA was prepared with 5 μg total RNA using Superscript-II Reverse Transcriptase kit following vender's protocol. In this step oligo-dT12-18 was used as primer.

### PCR amplification and cloning of the WbTI-2 gene

Genomic DNA and cDNA were PCR amplified with degenerate forward and reverse primers having *Eco*R1 and *Sal*1 restriction enzyme sites, respectively. The two primers were forward 5′-GCGAATTCGARGARYTNGTHGAYG-3′ and reverse 5′-CCGTCGAC**TTA**TTTTTTTTTTTTTTTTT-3′, where R represents A or G; Y represents C or T; H represents A or C or T and N represents A or C or G or T. Degenerate forward primer was designed on the basis of N-terminal amino acid sequence of WbTI-2 [20]. The restriction enzyme sites are underlined and the stop codon on the reverse primer is represented in bold. Genomic DNA and cDNA were used as template for amplification for 35 cycles under the following cycling conditions: 45 s at 94 °C, 45 s at 55 °C, and 60 s at 72 °C. After completion of 35 cycles, the reaction was continued for another 10 min at 72 °C.

The purified PCR product was then digested with *Eco*R1 and *Sal*1 and ligated to T/A vector as per vendor's protocol. *Escherichia coli* DH5α cells were transformed with the recombinant plasmid by $CaCl_2$ method [21]. The insert was sequenced from Bangalore Genei Pvt. Ltd., India (presently Merck-Millipore).

### Expression of WbTI-2 in E. coli

From the above determined gene sequence unique primers were designed with *Eco*R1 and *Sal*1sites on forward and reverse primers, respectively, and the gene was ligated to expression vector pTrc99A, that provided the start codon ATG upstream to *Eco*R1 site. The resulting pTrc-WbTI-2 construct was transformed into *E. coli* BL21 (DE3), grown in LB medium, and was selected on X-gal/IPTG LB plates containing ampicillin. For expression of recombinant protein, overnight grown *E. coli* containing pTrc-WbTI-2 culture was used to inoculate fresh medium and grown at 37 °C until the spectrophotometric reading at 600 nm was about 0.6. The gene was over expressed under varying concentrations of IPTG (0.1–0.6 mM) [11] and the culture incubated for 5 h at 37 °C. Same amount of cells were pelleted down for each IPTG concentration, suspended and boiled with 1× SDS PAGE loading buffer (250 mM Tris–HCl, pH 6.8, 10% SDS and 40% glycerol with 5% bromophenol blue) containing 20% β-mercaptoethanol and were used for SDS–PAGE [22] and western blot analysis using rabbit anti-WbTI-2 antibody [27].

### Immunization of rabbits using experimental protein

Winged bean trypsin inhibitor was purified from seed as described in an earlier report from our lab [7]. 200 μg of homogeneous preparation of seed purified winged bean trypsin inhibitor (WbTI-2), emulsified in complete Freund's adjuvant were used to immunize two rabbits by subcutaneous injections under the arms. Before immunization, about 5 ml of blood was collected from rabbit's ear vein to prepaper the preimmune sera. Second injection, prepared like the first one, was given at 20 days interval. After about 10 days of the second injection, blood was again collected from rabbit's ear and tested for the titre value. Subsequent booster doses, 200 μg each, prepared in incomplete Freund's adjuvant were given at a frequency of about 20 days of the second injection. A total of about 0.8 mg protein was injected per rabbit. For collection of WbTI-2 antibodies, rabbits were bled through their ear vein after about 10 days of completion of immunization. The blood collected was allowed to coagulate at 37 °C for release of the sera. The sera were then centrifuged at 800*g* for five minutes to precipitate down the erythrocytes. The clear supernatant was collected and decomplemented at 55 °C for fortyfive minutes and then stored at −20 °C for future use.

### Preparation of antibody for immunoaffinity column

Sera was obtained from the blood collected from rabbits in batches and were decomplemented as mentioned earlier and pooled together and given a 70% ammonium sulfate cut with constant stirring. The stirring was continued for 1 h after complete addition at 4 °C. Immunoglobulins were recovered after centrifugation at 5000*g* for twenty minutes as pellet and the supernatant was discarded. The pellet was dissolved in a minimum volume of phosphate buffer saline, pH 7.4, and dialyzed extensively against the