

Impact of oxidizing, reducing, and stabilizing agents on the inhibitory properties of *Cyamopsis tetragonoloba* trypsin inhibitor

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ABSTRACT

Antinutritional factors in plants diminish the digestive action of stomach enzymes and reduce the accessible supplement for assimilation in the gut. Isolation of a trypsin-chymotrypsin inhibitor from *Cyamopsis tetragonoloba* seeds is being reported in this paper. The inhibitor was purified by DEAE-cellulose chromatography followed by gel permeation on Sephadex G-75. The inhibitor was found to be of 11 kDa on denaturing polyacrylamide gel electrophoresis and possess the highest inhibitory action at pH 7.5 and 37°C. The isolated inhibitor was found unusually stable at high temperatures and microwave heating. Freshly prepared inhibitor exhibits around 71% inhibition on incubation for 30 min in the temperature range from 20°C to 100°C. The inhibitory action was influenced in the presence of oxidizing and reducing agents. This inhibitor was stable in the presence of oxidizers dimethyl sulfoxide, hydrogen peroxide, and sodium hypochlorite. The effect of stabilizers shows that CaCl₂ and glycerol support enhancement of the inhibitory activity. The inhibitor was also checked for antimicrobial activities. Inhibitor did not show any antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella*, *Pseudomonas*, *Salmonella typhi*, and *Proteus*.

1. INTRODUCTION

Protease inhibitors are characteristic immunity-related proteins amassed in seeds and tubers of plants belonging to *Leguminosae*, *Gramineae*, and *Solanaceae* family [1]. Two major families of protease inhibitors found in plants are Bowman-Birk inhibitor and Kunitz inhibitor. Kunitz inhibitor is normally 8–22 kDa proteins, with two disulfide linkages at the solitary binding site for trypsin [2]. These inhibitors have moderate heat stability. The members from the Kunitz family are serine protease inhibitors but may also inhibit aspartate proteases and cysteine proteases. Kunitz family inhibitors dissociate with great difficulty from a tight complex formed with their target proteases [3]. Bowman-Birk inhibitors are 8–12 kDa molecular weight proteins [4] that could hinder the action of serine proteases. These inhibitors are commonly double headed in nature with two similar domains. Each domain contains a different binding site for the target proteases. These Bowman-Birk inhibitor's binding site interact with target protease independent of each other, and the proteases might be of the same or distinctive type [5]. The active site in Bowman-Birk inhibitors is structured by the presence of conserved seven disulfide

bonds, which has two independent heat-stable receptive destinations for trypsin and chymotrypsin. These Bowman-Birk inhibitors are studied vastly because of due to their different applications, as antimicrobial agents [6], bioinsecticides [7], and as anticancer agents [8].

Cyamopsis tetragonoloba (Fabaceae), commonly called cluster bean, is cultivated in semi-arid areas throughout India due to its drought-resistant nature. The major part of yield is consumed as food. Different pharmacological applications that are exhibited by *C. tetragonoloba* includes antimicrobial [9], anti-asthmatic, and anti-inflammatory [10]. The crop is used as home remedies for stomach distress and treatment of gastric disturbances [11]. The present study reports for the first time the isolation of inhibitor from *C. tetragonoloba* and characterization of its inhibitory activity and stability under various conditions.

2. METHODOLOGY

2.1. Purification of Protease Inhibitor

C. tetragonoloba seeds certified to be of Serit Soumya-700 variety, (Rajasthan) India were obtained from authorized seed vendor. The *C. tetragonoloba* inhibitor was isolated by a method essentially described earlier [12]. Briefly, the seeds were soaked, homogenized, heated at 80°C for 30 min, brought to room temperature, and was made to 30–80% ammonium sulfate. The precipitate was run on DEAE chromatography followed by gel permeation chromatography on dextran based G-75 and the molecular weight detected by denaturing gel electrophoresis.

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The purified sample aliquotes were than pooled and put away at -20°C until utilized for additional biochemical investigation.

2.2. Protease Inhibitor Test

The inhibition of proteolytic activity of trypsin and chymotrypsin by purified inhibitor was determined as described by Norioka *et al.* [13]. Inhibitor (20 μg) was added to 20 μg of trypsin or 30 μg of chymotrypsin in 200 μl of 0.01 M Tris-HCl (pH 7.5) containing 0.02 M CaCl_2 . The mixture was incubated for 1 h at 37°C and the reaction was stopped by adding 30% acetic acid. The leftover catalytic activity was estimated by optical density measurement at 410 nm of enzyme-specific substrates, 1 mM BAPNA (benzyl arginine para nitro anilide) for trypsin, and 5 mM N-succinyl- L-phenylalanine-p-nitroanilide (n-SPNA) for chymotrypsin. The action of a catalyst without an inhibitor was taken as 100%.

2.3. Effect of Duration of Incubation and Temperature on Inhibitor Activity

To study the time required by the inhibitor from *C. tetragonoloba* to inhibit the enzyme completely, the enzymes and inhibitor were mixed and incubated for different times (10–80 min), and residual inhibitory activity was analyzed using specific enzyme-substrate and reading the absorbance at 410 nm.

2.4. Effect of Stabilizers on the Inhibitors Thermostability

For the thermal stability analysis, the freshly purified inhibitor was heated at different temperatures (20– 100°C) for 30 min, and inhibitory activity was checked by utilizing the standard test technique. The impact of added substances on the thermostability of inhibitor was investigated at 80°C . Stabilizers, namely, BSA (1%), glycerol (10%), CaCl_2 (10 mM), glycine (1 M), Urea (10 mM), starch (1%), and casein (1%) were added in the inhibitor. Samples were incubated for 3 h at 80°C in the presence of additives and later checked for protease inhibitory activity by standard assay method.

2.5. Effect of Microwave Treatment on Inhibitory Potential

The impact of microwave (Samsung 800 watts full power) treatment on *C. tetragonoloba* inhibitor was determined by heating the inhibitor in the microwave at its full power (800 Watt) for various time spans (30 s, 1 min, 2 min, 5 min, 10 min, 20 min, and 30 min) and remaining inhibitory action was estimated by standard test technique as depicted previously.

2.6. Impact of Oxidizing and Reducing Agents on the Inhibitor

The impact of oxidizing reagents such as dimethyl sulfoxide, hydrogen peroxide (H_2O_2), and sodium hypochlorite (NaOCl) at range from 1% to 5% (v/v) and reducing agents dithiothreitol (DTT) at range from 0.2% to 3% (v/v) on the protease inhibitor were determined. For that, the inhibitor was incubated with the reagents for 30 min separately, followed by the determination of residual inhibitory activity as depicted previously.

3. RESULTS AND DISCUSSION

3.1. Isolation and Purification of *C. tetragonoloba* Inhibitors

We utilized different isolation buffers with varying pH to extricate the trypsin inhibitor from *C. tetragonoloba* [Table 1]. The best extraction buffer was phosphate buffer (0.01 M, pH 7.5) as the most elevated trypsin inhibitory action was obtained in the buffer. Hence, phosphate

buffer (0.01 M, pH 7.5) was picked as the extraction buffer for the trypsin inhibitor. After ammonium sulfate precipitation followed by dialysis of crushed crude, the protein was loaded on DEAE ion-exchange chromatography column (15.5×2.5 cm). The fractions from the DEAE-cellulose segment were pooled, lyophilized, and applied on Sephadex G-75 column (50.5×2.5 cm) for further purification [Figure 1]. The trypsin inhibitory action was observed in the solitary elution peak (47 ml-85 ml part of G-75). The active fractions were pooled and analyzed through 14% sodium dodecyl sulfate-polyacrylamide gel electrophoresis [Figure 1]. The protease inhibitor was observed as a single band of an apparent molecular weight of about 11 kDa. Here, it was also observed that after the DEAE purification, the yield of protein is 0.1 mg/ml with 24.13% fold purification showing 100% inhibitory activity.

3.2. Protease Inhibitory Activity of Isolated Inhibitors

The effect of *C. tetragonoloba* inhibitor on the activity of trypsin, chymotrypsin using 1mM BAPNA, and 5 mM n-SPNA, as a substrate, respectively, was seen [Figure 2]. Results indicate that the inhibitor inhibits trypsin ($95 \pm 1\%$ inhibition) more effectively than chymotrypsin ($80 \pm 2\%$ inhibition). Hence, it may be concluded that the isolated protein is a serine protease inhibitor.

3.3. Impact of Incubation Period on Inhibitory Activity

To study the optimum time required by the isolated inhibitor to inhibit the trypsin completely, the enzyme and inhibitor were mixed and

Table 1: Protein and inhibitor contents of dialyzed extracts in different buffer solution

Extraction buffer	Protein (mg/ml)	Protease inhibitory activity (%)
Citrate phosphate buffer (0.025 M, pH 4.5)	1.026	47
Sodium phosphate buffer (0.05 M, pH 6.8)	1.885	53
Sodium phosphate buffer (0.01 M, pH 7.5)	1.889	92
Tris acetate buffer (0.05 M, pH 9.5)	2.197	65

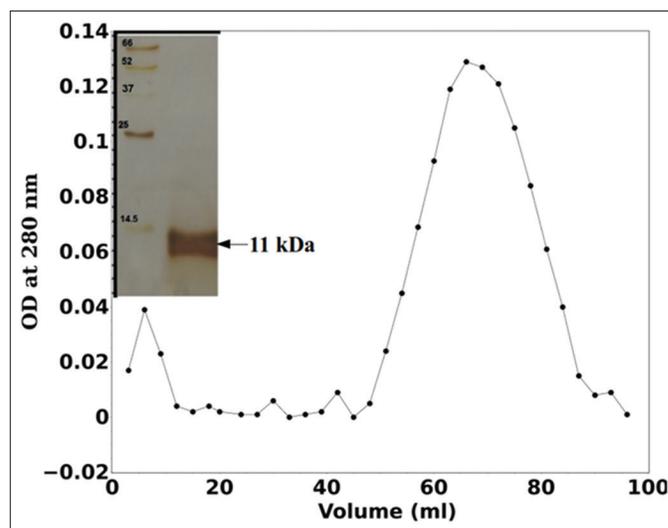


Figure 1: Purification of *Cyamopsis tetragonoloba* inhibitor: Active bound protein fraction from DEAE-cellulose were pooled, lyophilized, and applied on dextran based gel permeation G-75 column (50.5×2.5 cm). Inset: SDS-PAGE (14%) of G-75 eluted protein. The gel was stained with silver staining method

incubated for different time intervals from 10 to 80 min. The residual inhibitory activity was analyzed [Figure 3] to determine the optimum time. This result indicates that at 60 min of incubation, trypsin was inhibited completely. On the basis, 60 min incubation was selected for further experiments. Here, we also found that after 70 min of incubation, inhibitory activity decreased. At 80 min and 90 min, it shows 80% and 50% inhibitory activity, respectively.

3.4. The Impact of Temperature on Inhibitory Action of Freshly Isolated Inhibitors

The isolated inhibitor protein exhibited around 71% of its activity when incubated for 30 min at the different temperature range from 20 to 100°C. Moreover, over 65% of its inhibitory action was found to be stable at all these temperatures even after 30 min (data not shown). Such elevated thermostability indicates that this protein has high inherent stability and this sort of condition was earlier reported for Bowman–Birk type of trypsin inhibitors [14].

3.5. The Impact of Protein Stabilizers on Inhibitory Activity

Strength to withstand high temperature is considered the most significant element for the biotechnological utilizations of proteins and is desired to build the protein efficiency [9]. Some osmolytes, namely, amino acids, polyols, and salts are reported in having the capacity to counteract the heat-induced denaturation of protein and provide protection against heat-induced denaturation [15]. The effect of various components (such as starch, glycerol, CaCl₂, casein, glycine, and BSA) as a stabilizer on the thermal stability of the *C. tetragonoloba* inhibitor was analyzed at 80°C. The results are as shown in Figure 4. When compared with control (60% inhibition, in the absence of stabilizer), two of the stabilizers significantly increased the inhibition by the protease inhibitor. At 80°C presence of stabilizer CaCl₂ (75 ± 2% inhibition) and glycerol (67 ± 3) increases the inhibition, whereas casein (60 ± 1) and starch (56 ± 2) had no effect, urea (24 ± 3) and BSA (~35% inhibition) were shown to decrease the inhibition of inhibitor at 80°C.

3.6. Impact of Oxidizing and Reducing Agents on Inhibitory Properties

Oxidizing agents were analyzed for their effect on the inhibitory activity of *C. tetragonoloba* inhibitor [Table 2]. It was found that with the increasing concentration of the oxidizing agent, inhibitory activity increased up to 25% in case of NaOCl and 10% in H₂O₂ in comparison to control. These results indicate that *C. tetragonoloba* inhibitor is antioxidant in nature. The impact of reducing agent on the inhibitor was also determined [Table 2].

The thermostability of Bowman–Birk inhibitors has been attributed to a high number of disulfide bonds. Due to this reason, the impact of DTT on a decrease in the inhibitory action was measured. There was a decrease in the inhibitory action against trypsin (48%) at 0.2% DTT [Table 2]. Further, results show that as the concentration of DTT increased from 0.2% to 3%, the inhibitory action of inhibitor remarkably declined (30% against trypsin). The decline in inhibitory action might be result of the breakage of intramolecular disulfide bonds at the receptive site loop of inhibitor, which provides the functional stability to the inhibitors.

3.7. Impact of Microwave Heating on Inhibitor

The microwave heating was done for different time periods at power 800watt. The result is shown in Figure 5. It was found that there was

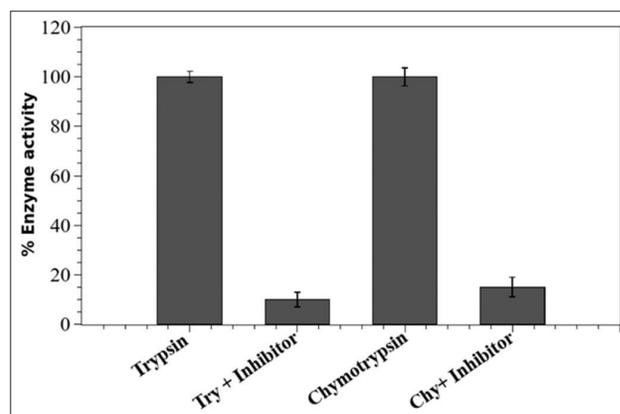


Figure 2: Inhibitory activity of inhibitor against enzymes: A standardized amount of trypsin (20 µg) and chymotrypsin (30 µg) was mixed with inhibitor and the remaining protease activity was determined using appropriate substrates benzyl-arginine-p-nitro-anilide and N-succinyl-L-phenylalanine-P-nitroanilide, respectively

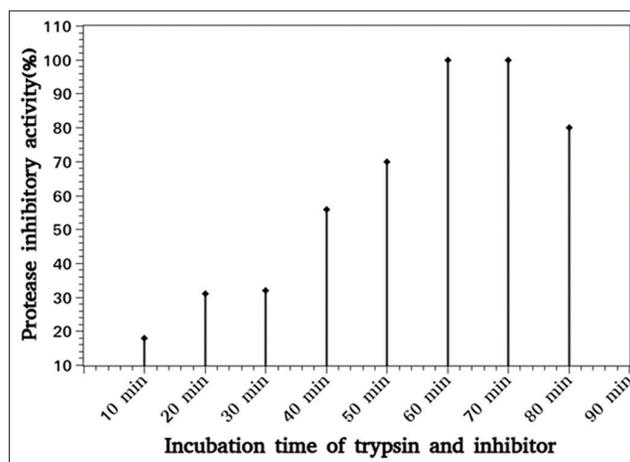


Figure 3: Effect of trypsin and inhibitor preincubation time on residual trypsin activity: Inhibitor was added to trypsin and incubated for different time intervals followed by addition of BAPNA for a specific time to measure the residual trypsin activity

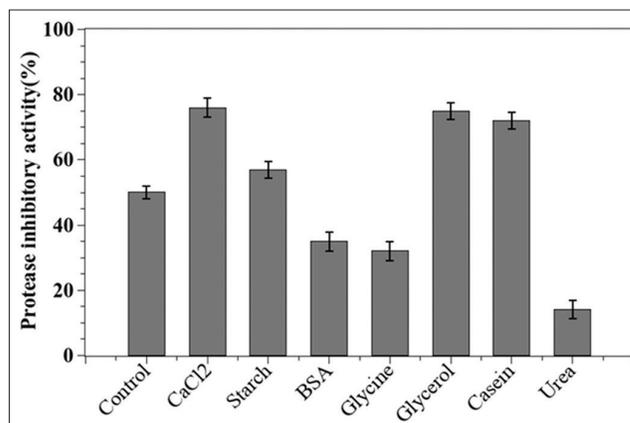


Figure 4: Effect of various stabilizing agents on incubation of inhibitor at 80°C: The protease inhibitory activity of inhibitor was checked by utilizing the standard test technique. After 3 h of incubation at 80°C with different thermo stabilizing agents. Data expressed as mean ± SD of identical triplicate experiment

Table 2: Impact of oxidizing and reducing agents on *Cyamopsis tetragonoloba* inhibitor. Data expressed as mean \pm SD of identical triplicate experiment

Chemical	Concentrations (%)	Residual activity (%)
None	1	80%
Oxidizing agents	2	90 \pm 2.1
Hydrogen peroxide (H ₂ O ₂)	3	89 \pm 1.95
	5	85 \pm 2.2 80 \pm 1.5
Sodium hypochlorite (NaOCl)	1	87 \pm 1.23
	2	91 \pm 2.2
	3	92 \pm 1.95
	5	93 \pm 2.3
DMSO	1	80 \pm 2.1
	2	82 \pm 1.85
	3	82 \pm 2.2
	5	80 \pm 1.5
Reducing agent	0.2	49 \pm 1.2
DTT	0.5	48 \pm 2.1
	0.8	43 \pm 2.12
	1	40 \pm 1.98
	2	39 \pm 1.36
	3	32 \pm 1.4

DMSO: Dimethyl sulfoxide, DTT: Dithiothreitol

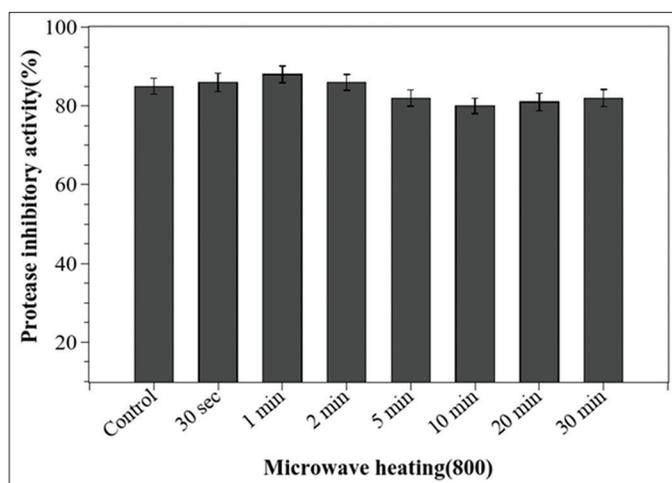


Figure 5: Effect of microwave heating on the *Cyamopsis tetragonoloba* inhibitor: The purified *Cyamopsis tetragonoloba* inhibitor was heated in a microwave at full power (800 watts) for different time intervals and residual inhibitory activity was measured by standard assay method

no loss of trypsin inhibitory activity even after heating for 30 min with all microwave exposed inhibitors showing above 80% of inhibition similar to that of control, indicating that this inhibitor is highly thermostable.

4. CONCLUSION

This current work emphasizes the nature of inhibitor from seeds of *C. tetragonoloba*. The isolated inhibitor was reported to inhibit trypsin and chymotrypsin. The SDS-PAGE demonstrated that the molecular weight of the inhibitor was 11 kDa. The action of *C. tetragonoloba* inhibitor was steady over temperature 20–100°C and could be diminished by DTT, indicating it to be an inhibitor from Bowman-Birk family. The stability of the active protein in the presence of 5% of oxidizing agents represents its antioxidant nature.

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6. CONFLICT OF INTEREST

Authors declared that they do not have any conflicts of interest.

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