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PURIFICATION AND CHARACTERIZATION OF A POTENT TRYPSIN INHIBITOR (CGTI) FROM *CLEOME GYNANDRA* L. SEEDS

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ABSTRACT

A trypsin inhibitor from *Cleome gynandra* seeds (CGTI) was extracted and purified to apparent homogeneity following ammonium sulphate fractionation and Trypsin-Affigel-15 affinity chromatography with a 13.11-fold purification and 14.87 % yield. CGTI was very active against bovine trypsin; on SDS-PAGE, it gave an apparent molecular weight of 14.6 kDa. With a neutral sugar content of 1.5% and no free sulfhydryl groups, it was found to be a glycoprotein. Antitryptic activity of CGTI remained unchanged over a broad range of temperatures (0-80ºC) and pH (3-12) and is relatively stable in the presence of 8M urea, 1% SDS, 6 M Guanidine hydrochloride and PNGase F for 24 h at room temperature. The kinetic analysis demonstrated a non-competitive form of inhibition with a Ki value of 0.40 ± 0.08 nM against bovine pancreatic trypsin, indicating potent inhibitory activity. The trypsin inhibitory activity of CGTI was lost after modification of amino groups with acetic anhydride, suggesting that CGTA is a lysine-active site inhibitor.

Keywords *Cleome gynandra*, antitryptic activity, trypsin inhibitor, glycoprotein, Trypsin-Affigel

1. INTRODUCTION

Protease inhibitors, are proteins or peptides, can hinder the catalytic functions of proteolytic enzymes. They are commonly found in both nature and plants and are widely spread across storage organs such as tubers, leaves, flowers, fruits and seeds [1-3].The classification of protease inhibitors is essentially based on the characteristics of their active sites and the reaction mechanism they employ. They are categorized as serine, cysteine, aspartic and metalloproteinase inhibitors. Serpin proteins that inhibit serine proteinases have been obtained, purified, and studied from several plant sources [4-8]. The inhibitors have a molecular mass that ranges from 4 to 85 kDa, with the bulk falling within the 8-20 kDa range [9]. Of all the serpins, trypsin inhibitors have been the subject of the most extensive research [10-11].

Protease inhibitors are recognized for their ability to modulate internal proteolytic activities. Their importance lies in regulating protease-mediated processes such as intracellular protein breakdown, transcription, cell cycle, cell invasion, and apoptosis. This has sparked interest in understanding their physiological roles [12]. In addition, protease inhibitors provide a defense function in plants by safeguarding them from the intrusion of insects, fungus, and other harmful microorganisms [13]. The pharmacological and medical fields have explored the possibilities of using these inhibitors as therapeutic medicines for treating various diseases. The inhibitors have been shown to possess anti-carcinogenic capabilities through studies conducted in vitro and in vivo model systems and human trials [14,15]. *Cleome gynandra* Linn, sometimes called Cat's whiskers or spider plant, is a member of the *Capparaceae* family (previously *Capparidaceae*) and falls within the subfamily *Cleomoideae*. The seeds possess antihelminthic properties and are consumed to facilitate the elimination of roundworms. The seeds possess piscicidal activities and have carminative and antiseptic effects.The seed's protein content ranges from 17.9% to 31.4%, and the powdered seed is utilized in culinary applications. The present study concentrates explicitly on isolating and purifying trypsin inhibitor from the seeds of *Cleome gynandra* besides studying physico-chemical and kinetic properties.

2. METHODS

Plant Material

Cleome gynandra linn plants with pods of consistent size were collected in Paderu, Visakhapatnam. The pods were collected when they were mature, and the seeds were taken out of the pods to extract and purify the trypsin inhibitor.

Purification of *Cleome gynandra* trypsin inhibitor (CGTI)

200 ml of 0.1 M sodium phosphate buffer, pH 7.6, was used to homogenize 20 g of seeds. The extract was subjected to centrifugation at 5,600 rpm for 15 min at 4ºC and supernatant was collected. The resulting extract (185ml) was treated with four volumes of ice cold acetone for one hour to eliminate lipids and centrifuged at 2,500 rpm for 15 minutes at 4°C. The precipitate was resuspended in 185 ml of 0.1 M phosphate buffer, Ph 7.6. The extract was then subjected to boiling at 60ºC for 10 min, rapidly cooled using ice, and was subjected to centrifugation at 5,600 rpm for 15 min at 4ºC. Ammonium sulfate was added to the supernatant with continuous stirring at 4ºC to achieve 60% saturation. The mixture was kept at 4ºC overnight. The pellet obtained after centrifugation was dissolved in 0.1 M sodium phosphate buffer, pH 7.6 and was then subjected to dialysis using the same buffer. Upon subjecting to DEAE-cellulose column chromatography, the flow-through fractions revealed protein fractions exhibiting antitrypsin activity. The fractions were pooled, concentrated, purified by dialysis using distilled water at 4ºC, and then freeze-dried. The resulting protein yield was 95.6 mg. A comparable pattern was obtained when the dialyzed material was treated with cation exchange column chromatography employing CM cellulose. Upon subjecting the sample (95.6 mg) to gel permeation chromatography on a Sephadex G-150 column, protein fractions with trypsin inhibitory activity were seen in the unretarded fractions. Analogous outcomes were likewise achieved when the protein sample underwent gel permeation chromatography using Sephadex G-100, Sephadex G-200, and DEAE-Sephadex A 25-120 columns, or even in the presence of dissociating solvents like 6M guanidine hydrochloride or 8M urea. The protein sample was subsequently submitted to Affinity chromatography using trypsin–Affigel-15.

Preparation of Trypsin-Affigel:

The preparation of Trypsin-Affigel involved the coupling of commercially available trypsin (Bovine pancreatic 3×crystallized) to Affigel-15 (Bio-Rad labs) at 4°C. The 86.3 mg sample was dissolved in 0.1M phosphate buffer, pH of 7.6. It was then treated with CHAPSO, a non-denaturing zwitterionic detergent at a concentration of 0.1% for a duration of 12 h at 4ºC. After that, the sample was loaded onto a Trypsin-Affigel column measuring 1.5 × 10 cm. Before loading, the column was prepared with a 25 mM Tris-HCl buffer, pH 7.4, having 0.15 M NaCl. The protein that was not bound was rinsed using 25 mM Tris-HCl buffer, pH 7.4. The protein that was retained onto the column was then eluted using 0.1-0.3 N HCl solution containing 0.15 M NaCl. Fractions

of 2 ml were collected at a flow rate of 12 ml/h, and the protein was measured by measuring its absorbance at 280 nm. The fractions were tested for their trypsin inhibitory activity using Nα-Benzoyl-DL-arginine p-nitroanilide hydrochloride (BAPNA) as the substrate.

Determination of protein content

The Lowry method [16] was employed to quantify protein, using bovine serum albumin as the standard.

Polyacrylamide Gel Electrophoresis (PAGE)

As per Davis's technique, the polyacrylamide gel electrophoresis (PAGE) was conducted using slab gels at a pH of 8.3 [17]. The visualization of proteins was achieved by employing Coomassie brilliant blue.

Specific staining of Trypsin inhibitor

According to Filho and Moreira's method, the inhibitor was visualized in acrylamide gels [18].

Determination of molecular weight of trypsin inhibitor

The molecular weight of the inhibitor was determined using SDS-PAGE, following the method developed by Laemmli [19] and gel permeation chromatography on the Sephadex G-200 column.

Determination of trypsin and trypsin inhibitory activity

The activity of trypsin was measured using BAPNA (α-N-benzoyl-DL-arginine-p-nitoanilide HCl) as the substrate, following the method described by Kakade [20]. To measure trypsin inhibitory activity, trypsin (30 µg) was incubated at 37°C for 10 min with aliquots of the inhibitor. residual trypsin activity was then used to indicate the inhibitory activity.

One enzyme unit is defined as an increase in 0.01 absorbance unit at 410 nm for trypsin under the assay conditions. One enzyme inhibitory unit is defined as the number of enzyme units inhibited under these conditions.

Determination of carbohydrate content

The phenol-sulphuric acid method of Dubois [21], was employed to quantify the amount of neutral sugars in the inhibitor. D-mannose (5 mg/100ml water) was the reference standard. The modified Morgan-Elson method [22] used N-acetyl D-glucosamine (5mg/100ml water) as the reference standard for quantifying the hexosamine content of the inhibitors.

Quantification of thiol groups

The free thiol groups in the inhibitor were calculated using the procedures outlined by Ellman [23] and Habeeb [24], which involved the use of 5,5'dithiobis-(2-nitrobenzoic acid) (DTNB).

Effect of protein denaturing conditions/agents

Effect of pH

The inhibitor was dissolved in an appropriate buffer (10 mM) to create a solution with a 1 mg/ml concentration.This solution was then kept at a temperature of 5°C for 24 hours to evaluate the pH stability of the inhibitor. Aliqiots of the inhibitor were diluted using phosphate buffer, pH 7.6 to assess trypsin inhibitory activity.

Effect of Temperature

Three 3 ml samples, each containing a 100 µg/mL solution of the inhibitor in 0.1 M sodium phosphate buffer, pH 7.6, were individually incubated for ten minutes at different temperatures in a water bath. After the heat treatment, the solutions were quickly chilled in ice, and the trypsin-inhibiting activity was measured using suitable aliquots.

Effect of denaturants

To determine the stability of the inhibitor, 1 mg/ml solution of the inhibitor was prepared in 0.1 M sodium phosphate buffer (pH 7.6) with 1% SDS, 8 M urea, and 6 M guanidine hydrochloride. After that, the solution was placed in an incubator and kept at 5°C for 24 h. The buffer was employed to dilute the solutions, and a suitable aliquot of this solution was taken to assess the inhibitory activity of trypsin. The control assay mixture had the same amount of denaturant as in the diluted inhibitor solution.

Treatment with PNGase F (Peptide-N-Glycosidase F)

To assess the effect of deglycosylation on the trypsin inhibitory activity of CGTI, solution containing 100 µg of CGTI in 1 ml of 0.25M sodium phosphate buffer, pH 8.6 with 20 mM EDTA was subjected to incubation with 5µl of PNGase F (2000 units) for a duration of 18 h at 37ºC. Proteinase inhibitory activity was assayed after enzyme treated inhibitor was dialyzed against PBS. The inhibitor, handled identically, but without adding PNGase F, was used as a control.

Kinetic studies of CGTI

The mechanism of trypsin inhibition by CGTI was investigated by exposing trypsin to different concentrations of BAPNA, both with and without the presence of the inhibitor. An amount of substrate ranging from 0.8 to 5.0 µmol was mixed with 30 µg of trypsin and incubated for 10 min at 37°C. Following the incubation period, the reaction was halted by adding 1 ml of 30% acetic acid. The solution's absorbance was measured at 410 nm compared to a blank

that did not contain trypsin. The experiment was conducted again by adding 10 µg, 15 µg, and 20 µg of the inhibitor to the reaction mixture.

Chemical modification of the inhibitor

Modification of amino groups by Acetylation

According to the method of Rice et al. [25], acetylation was done using acetic anhydride.

Modification of arginyl residues

The inhibitor's arginyl residues were chemically changed with 1, 2-cyclohexanedione, following the procedure described by Liu et al. [26].

3. RESULTS AND DISCUSSION

Purification of Trypsin inhibitor

The trypsin inhibitor from *Cleome gynandra* seeds was isolated to a state of apparent homogeneity by using ammonium sulphate fractionation and affinity chromatography. The trypsin inhibitor failed to attach to either the anion or cation exchanger, even when the pH of the buffers was adjusted by one unit. Furthermore, it was observed in the void volume when subjected to gel exclusion on Sephadex columns. The inhibitor's inability to bind to these two columns may be attributed to its low overall charge. CGTI, when analyzed by gel permeation chromatography on Sephadex G-150, did not interact with the beads and eluted directly off the column. Comparable findings have also been achieved with Sephadex G-100, Sephadex G-200, and DEAE-Sephadex A 25-120 columns. To visualize the protein distribution on the gels, the material underwent native polyacrylamide gel electrophoresis (PAGE) using a 12% slab gel with a pH of 8.3. The proteins that displayed trypsin inhibitory activity were observed to be located at the uppermost part of the gel. When analyzed using SDS-PAGE on a gradient gel (5-15%), the sample exhibited several bands, whether with or without 2-mercaptoethanol [Figure 1]. The lower band, which showed a positive response to specific staining, confirmed the presence of a trypsin inhibitor. The fact that the other bands did not react positively to particular staining suggests that they are neither the polymers of CGTI nor formed out of it through monomer-dimer-trimer equilibrium. CHAPSO was more effective in dissociating the trypsin inhibitor from the complex. CHAPSO detergent effectively disrupted the non-covalent bond between CGTI and its related proteins, allowing purification.

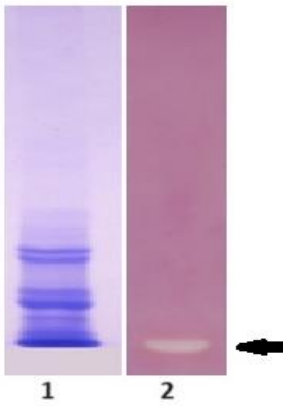

Figure - 1: SDS-Polyacrylamide gel electrophoretic pattern at pH 8.3 in 5-15% gradient slab gel.
1. Coomassie blue staining of ammonium sulphate fraction
2. Specific staining of ammonium sulphate fraction

The protein sample treated with CHAPSO was ultimately passed over the Trypsin-Affigel column, and the resulting elution profile is depicted in Figure 2. When treated with CHAPSO, the trypsin inhibitor protein exhibited a high affinity for trypsin and was then eluted using a 0.3 N HCl solution. The inhibitor ultimately produced an yield of 15%. CGTI was determined to be homogeneous based on the criteria of native PAGE and gel exclusion.

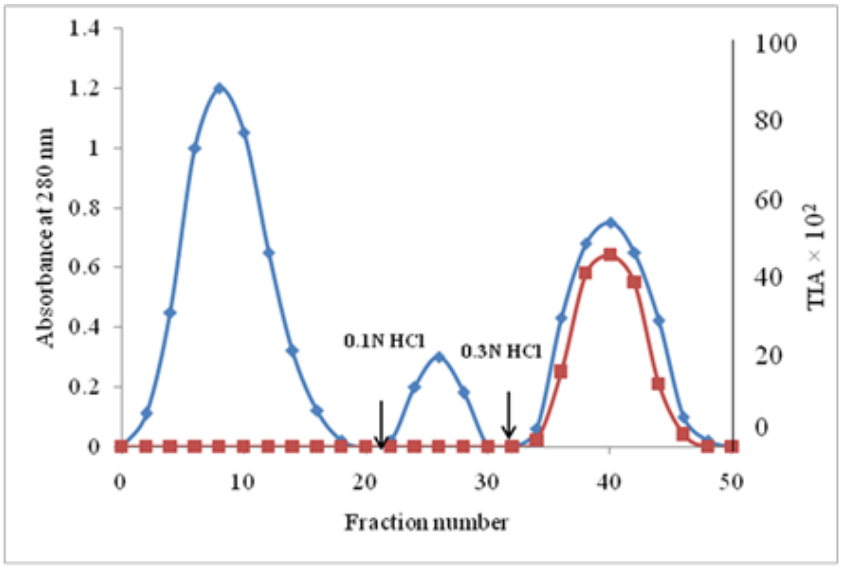

Figure- 2: Affinity chromatography on Trypsin-Affigel of Sephadex G- 150 preparation.
Protein was monitored by absorbance at 280 nm (♦------♦)
Trypsin inhibitory activity (■-------■)

The affinity chromatography technique, using trypsin attached to a solid support, has been widely used to purify trypsin inhibitors. This approach is characterized by its rapidity and ability to enhance the concentration of the inhibitor during the purifying process. Multiple protease inhibitors have been obtained and purified using trypsin-sepharose affinity chromatography [27-30].

Table 1 displays the recoveries and relative purity for a standard purification process using 20g of seeds at each step. A total of 15.2 mg of the inhibitor was recovered using this technique, resulting in a final yield of approximately 14.87 mg for the trypsin inhibitor.

Table 1 Summary of purification of proteinase inhibitor from seeds of *Cleome gynandra* (20 g of seeds)

Preparation	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Yeild %	Fold* Purification
			TIU x10^3	TIA x10^2		
Crude extract	200	1246.4	365.80	2.93	100	1
Acetone precipitation	185	1126.5	344.4	3.05	96.88	1.04
Heat treated extract	175	484.5	294.40	6.06	80.48	2.06
60% Ammonium sulphate	50	145.2	226.60	15.60	61.94	5.32
DEAE – cellulose	135	95.6	165.3	17.29	45.18	5.90
Sephadex G-150	38	86.3	158.1	18.31	43.22	6.24
Trypsin – Affi gel	10	15.2	58.4	38.42	14.87	13.11

*Yield and fold purification were calculated based on TIU and TIA, respectively
TIU - Trypsin inhibitory units
TIA -Trypsin inhibitory activity

SDS-PAGE analysis (Figure 3) revealed that the molecular weight of CGTI is 14.6 kDa. The plot of the (V_e/V_o) versus log molecular weight for calibrating proteins revealed a molecular weight of 14.8 kDa for CGTI, as depicted in Figure 4.

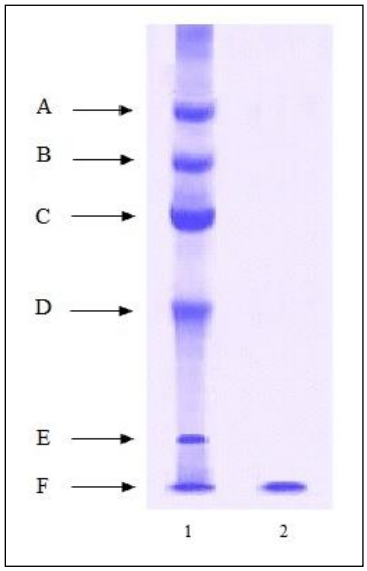

Figure-3 Molecular weight determination of trypsin inhibitors by SDE-PAGE on 5-20% gradient slab gel

(1) Standard proteins - (A) Phosphorylase b, 97 kDa, (B) Bovine serum albumin, 67 kDa, (C) Ovalbumin, 44 kDa, (D) Chymotrypsinogen A, 25 kDa, (E) Soybean trypsin inhibitor, 20.1 kDa, (F) Lysozyme, 14 kDa
(2) CGTI kept at 100°C for 2 min with SDS and 2-mercaptoethanol.

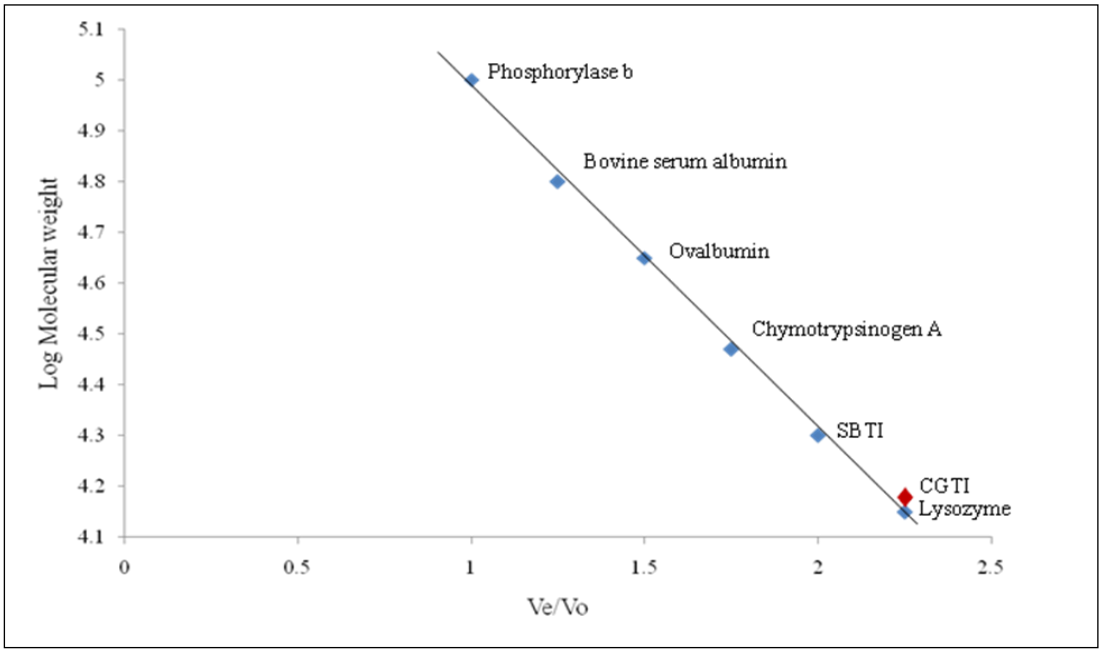

Figure- 4 Molecular weight determination of CGTI by gel filtration Sephadex G-200

Plot of elution volume against log molecular weight of standard proteins (♦) and CGTI (♦)

CGTI was identified as a glycoprotein containing approximately 1.5% carbohydrate, as shown in Table 2. Trypsin inhibitors from the seeds of *Echinodorus paniculatus, Swatzia pickellii, Peltophorum dubium* and *Abelmoschus moschatus* are also reported to be glycoproteins [31-33, 7].

Experiments were conducted to determine the presence of any unbound sulfhydryl groups in the inhibitor. The data displayed in Table 3 demonstrate the lack of unbound sulfhydryl groups in CGTI. The number of unbound thiol groups in ovalbumin and 2-mercaptoethanol was determined. The ovalbumin molecule contained 4 moles of sulfhydryl (-SH) groups, while the 2-mercaptoethanol molecule contained 1 mole of -SH group, as per the given information.

Table 2: Estimation of the carbohydrate content of CGTI

Type of sugar	mg sugar /g protein
Neutral sugar	1.5 ± 0.25
Amino sugars	0

Table 3: **Estimation of free thiol groups in CGTI by DTNB method**

Protein	Yield of thiol groups
2-mercaptoethonal	1.04
Ovalbumin	4.07
0.5 mg CGTI	0.00
1mg CGTI	0.00

*Yield expressed as the number of moles of free –SH groups per mole of the compound.

Stability of the purified inhibitor following various treatments

The purified inhibitor exhibited exceptional stability, maintaining its integrity even when exposed to temperatures as high as 80°C for 10 min. When the incubation period was prolonged by 20 min

at a temperature of 90°C, activity was reduced by 40%. After being subjected to a boiling water bath for 10 min, there was a 70% reduction in activity, and complete activity loss occurred by 20 min. The inhibitor's activity was lost entirely when autoclaved at a 1.04 kg/cm² pressure for 10 minutes, as indicated in Table 4.

Table 4 Effect of heat treatment on *Cleome gynandra* trypsin inhibitor

Temperature °C	Time (min)	Trypsin Inhibitory activity remaining* (%)
25	10	100
37	10	98
50	10	100
60	10	100
70	10	100
80	10	98
90	10	70
90	20	60
100	10	60
100	20	30
121**	10	0

* Inhibitory activity at 25°C was taken as 100%
** Autoclaving at 1.04 kg/cm² pressure TIU – Trypsin inhibitory units

The CGTI was exposed to buffers with different pH levels ranging from 3 to 12 and its trypsin inhibitory activity was measured at a pH of 7.6. The trypsin inhibitory activity of CGTI remained unaffected under both alkaline and acidic environments, as demonstrated in Table 5.

Table 5 Effect of pH on *Cleome gynandra* trypsin inhibitor

pH	Name of the buffer	TIU/mg of CGTI x 10^2
3	Glycine-HCl	38.24
5	Sodium citrate	38.08
7	Sodium Phosphate	38.42
9	Tris-HCL	38.40
12	Glycine-NaOH	38.12

TIU – Trypsin inhibitory units
CGTI was incubated for 24 h at 4°C in the respective buffers and assayed for TIA.
BAPNA was used as the substrate for trypsin.

Upon examination, it was shown that the inhibitor exhibited considerable stability in the presence of urea. Furthermore, exposure to this inhibitor for 24 hours had no discernible impact on its antitryptic activity, as seen in Table 6. The treatment of the inhibitor with a solution containing 1% SDS and guanidine hydrochloride did not reduce trypsin inhibitor activity.

The CGTI sample was subjected to treatment with PNGase F for 18 hours and subsequently, analyzed to determine the impact of sugars on its trypsin inhibitory action. The inhibitor without glycosylation has maintained its ability to inhibit trypsin, as seen in Table 6. The data suggest that carbohydrates may not be necessary for the trypsin-inhibitory activity of CGTI.

Table 6 Effect of 8M urea, 1% SDS and 6M guanidine hydrochloride on CGTI

Denaturants	**TIU/mg of CGTI x 10^2**
Control	38.42
8M urea	37.72
1 % SDS	37.70
6M Guanidine hydrochloride	37.74
6M Guanidine hydrochloride (at 100ºC for 2 min)	37.08
PNGase F	37.46

TIU – Trypsin inhibitory units
CGTI was incubated for 24 h at room temperature (29°C) in 8 M urea, 1% SDS, 6 M Guanidine hydrochloride, PNGase F and assayed for TIA. BAPNA was used as the substrate for trypsin.

CGTI does not possess any free thiol groups, which may be attributed to their participation in creating disulphide bridges. Protease inhibitors are notable for their exceptional stability. CGTI exhibited stability properties comparable to protease inhibitors produced from various plant sources . [34-35, 7]. The inhibitors' minimal cysteine composition disproves the hypothesis that robust intrapeptide crosslinking is responsible for the inhibitors' stability. However, the relatively small size of the protein may play a role in its capacity to regenerate the standard active form after

denaturation or the formation of hydrophobic solid connections that create a central core. This could explain why the inhibitor is exceptionally stable.

CGTI has exhibited non-competitive type of inhibition. The Ki value of CGTI was determined to be 4.0 x10-10 M [Figure 5].

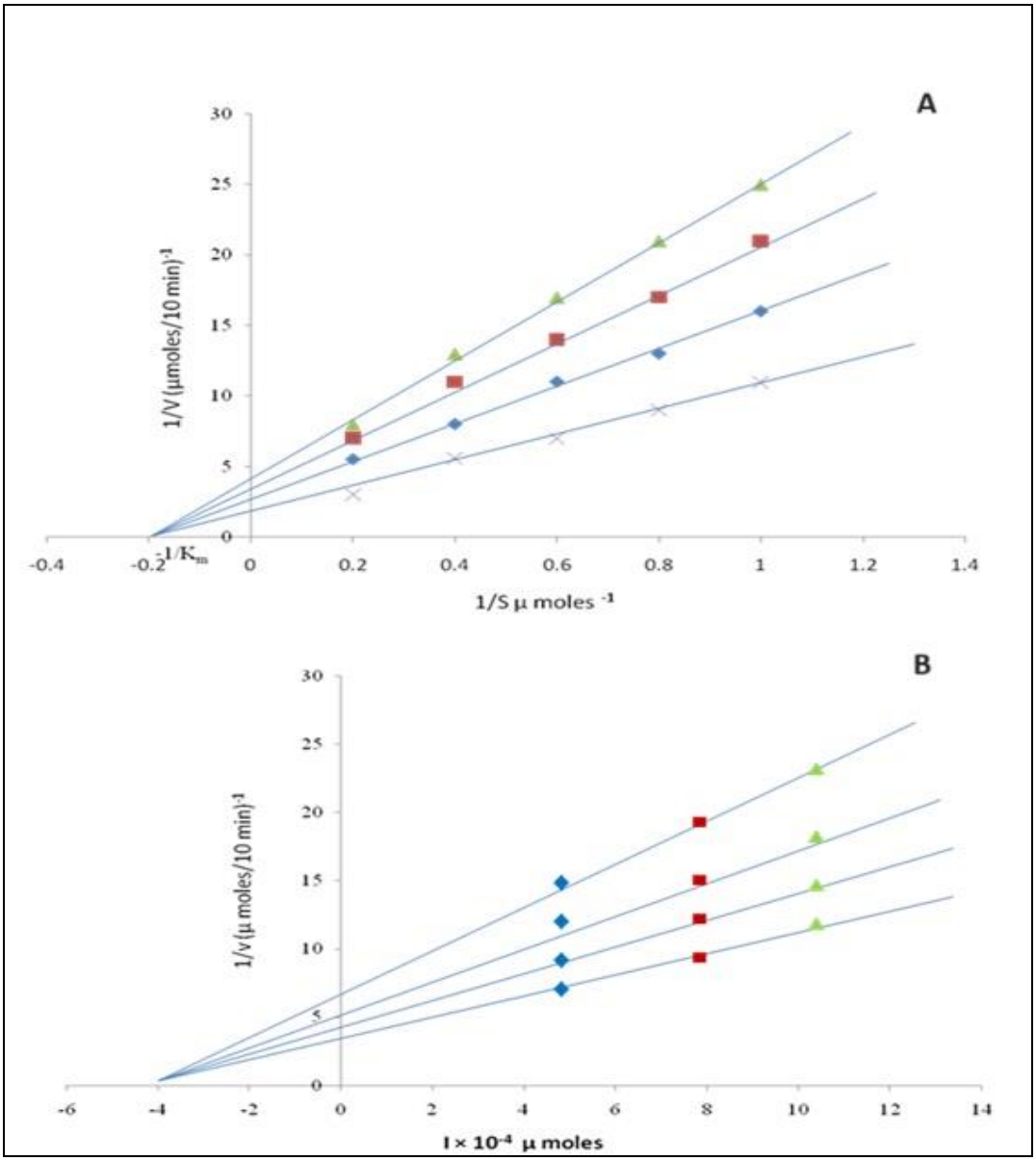

Figure-5 : Mode of inhibition of trypsin activity by CGTI

(A) Lineweaver-Burk plot

(B) Dixon plot

Inhibition of amidolytic activity of trypsin by CGTI was done by Incubating 30 µg of trypsin and BAPNA solution (0.8 – 5 µmol)

With the reaction system containing 10, 15 and 20µg of CGTI.

(- × -) Without CGTI (- ♦ -) With 10 µg of CGTI

(- ■ -) With 15 µg of CGTI (- ▲ -) With 20 µg of CGTI

The low K_i value indicates a high affinity of trypsin towards CGTI. Most inhibitors, including those derived from faba bean, *Bauhinia bauhinioides*, and Inga laurina, exhibited non-competitive inhibition kinetics [36-38].

Table 7 illustrates the impact of altering arginyl residues on the trypsin-inhibitory activity of CGTI. The trypsin inhibitory activity of CGTI showed a slight reduction of 7.8% when its arginine residue(s) were modified. This implies that the arginyl residues of the inhibitor do not play a role in the inhibition of trypsin caused by CGTI.

Table 7: Effect of modification of arginine on the inhibitory activity of CGTI against trypsin

Concentration of Cyclohexanedione	TIU/mg of inhibitor $\times 10^2$
Control	29.79
1 mM	29.70
5mM	27.4

Arginyl residues of CGTI were chemically modified with 1, 2– cyclohexanedione.
Trypsin inhibitory activity of the modified CGTI was assayed using BAPNA as the substrate of trypsin.

The degree of Acetylation and trypsin inhibitory activity of CGTI were evaluated after modifying the free amino groups in the inhibitor through treatment with acetic anhydride. According to Figure 6, a 10% acetylation led to a 20% decrease in the trypsin inhibitory efficacy of CGTI.

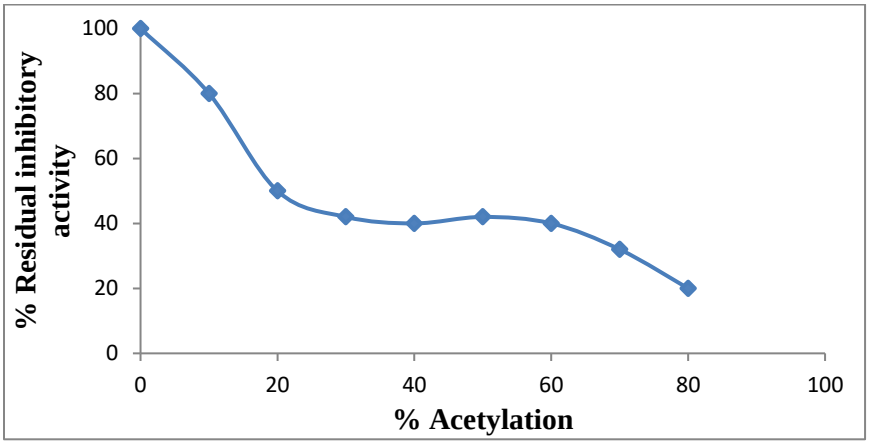

Figure- 6: Loss of inhibitory activity of CGTI on Acetylation with acetic anhydride

When acetylation was raised from 20% to 60%, there was a notable decrease (60%) in the trypsin-inhibitory activity of CGTI. Despite the excessive use of acetic anhydride, around 80% of the amino groups in the inhibitor were acetylated, leading to a reduction of around 80% in its trypsin inhibitory action. Cancelling CGTI's trypsin inhibitory activity following Acetylation implies that the inhibitor's ability to inhibit depends on one or more amino groups.

The presence of lysine residues in CGTI is essential for its inhibitory effect against trypsin, as evidenced by modifying the inhibitor's functional groups using particular reagents. This finding suggests that lysine may be situated at the active site of the inhibitor. Protease inhibitors from the seeds of *Vigna unguiculata, Phaseolus mungo, Inga laurina*, have lysine in the active site [39-40, 38]. The results of the investigation of inhibitory specificity of CGTI have shown it to be a serpin with narrow specificity being very active against bovine trypsin. CGTI is a unique protease inhibitor associated with other proteins in their native form. By CHAPSO treatment, the inhibitor was successfully separated from the assemblage before purification using the affinity column.

4. CONCLUSION

The process of affinity chromatography using Trypsin – Affigel 15 and ammonium sulphate fractionation was employed to isolate and purify a very effective trypsin inhibitor from the seeds of *Cleome gynandra*, achieving a state of apparent homogeneity. The molecular weight of CGTI was determined to be 14.8 kDa. The inhibitor exhibited high efficacy against bovine trypsin. The CGTI had a sugar content of 1.5% and was identified as a glycoprotein without free sulfhydryl groups. The trypsin-inhibiting effect of CGTI remained unchanged throughout a wide range of pH values (3–12) and temperatures (0–80 ºC). The antitrypsin activity of the inhibitor was unaffected after being treated with 8M urea and 1% SDS for 24 hours at room temperature. The kinetic analysis revealed a non-competitive type of inhibition towards Bovine pancreatic trypsin and found to be a lysine-active site inhibitor.

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Authors' contributions

All authors made substantial contributions to the conception and design, acquisition of data, analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit the work to the current journal; gave final approval of the version to be published; and agreed to take responsibility for all aspects of the work.

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