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Original Communication

Plasmin inhibitor from *Sapindus trifoliatus* - Its action on plasmin and other serine proteases

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ABSTRACT

Serine proteases and their inhibitors play an important role in an umpteen number of biological processes and hence are extensively studied in the past few decades. Purified proteolytic inhibitor from *Sapindus trifoliatus* was found to inhibit trypsin, plasmin and pronase of the class of serine proteases. Proteolytic enzymes like chymotrypsin, elastase, subtilisin (serine proteases), papain (Thiol protease), pepsin (acidic protease) and thermolysin (Metallo protease) were not affected by Soap Nut Trypsin Inhibitor (SNTI). Class specificity and group specificity exhibited by SNTI can be a positive outcome for targeted therapy. The enzymes were affected by the inhibitor in a stoichiometric manner and the enzyme substrate complex has no effect on the reaction. SNTI strongly interacted with trypsin and plasmin and showed a residual activity of 20% and 50%, respectively. 20 µg of SNTI is capable of inactivating 100 µg of plasmin. An IC_{50} value of 19.6 µg/mL of purified SNTI acounted for 50% inhibition of plasmin.

KEYWORDS: serine protease inhibitor, *Sapindus trifoliatus*, SNTI, plasmin, inhibitory concentration, residual plasmin activity.

INTRODUCTION

Metabolic activities at cellular level are orchestrated by the activity of proteins. Downregulation and upregulation of their activity, concentration and state is crucial in maintaining life. Proteases, the enzymes that hydrolyze proteins are therefore critical elements of the genome. 2-4% of genes in a typical genome encode proteolytic enzymes [1]. The human degradome consists of nearly 1449 proteases and homologues, of which 399 are serine proteases (MEROPS release 10.0) [2]. A delicate balance of activation and deactivation mechanisms of proteases is required at every point of regulation, i.e., at translation, transcription of protease gene, activation of zymogen etc. Undesired proteolysis leads to various diseased states. In certain diseases like emphysema, tumor invasion, gingivitis and other inflammatory infections the damage of tissue is due to uncontrolled proteases [3]. Some serine proteases such as elastase, collagenase, and cathepsin G are known to be involved in degradation of fibrous proteins like elastin and collagen and ruin the extracellular matrix [4]. Components of the plasminogen-plasmin system participate in a wide variety of physiologic and pathologic processes, including tumor growth, invasion and metastasis, through their effect on angiogenesis and cell migration [5].

If the specificity of proteases is known with respect to their amino acid residues, there can be a probability to inhibit those enzymes that are involved in pathological processes. Inhibitors with prospective inhibitory potential can be developed as new therapeutic agents. Hence, the extracts of protease inhibitors (PIs) from various sources are the main key in developing non-toxic drugs [3]. Protease inhibitor specificity is very helpful in targeting some of the proteases which are known

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to cause pathogenesis in human *viz.*, hepatitis, pancreatitis, cancer, arthritis, AIDS, emphysema, high blood pressure, thrombosis, muscular dystrophy etc. A good number of proteases are potential drug targets or are versatile molecules of consideration as diagnostic and prognostic biomarkers [6].

Protease inhibitors inhibit the enzymatic degradation of insulin and hence are widely used along with insulin to increase the absorption. There are several reports that suggest that in clinical trials, when protease inhibitors and insulin are administered together it had showed better hypoglycemic effects when compared to patients who were administered only with insulin. Nevertheless, their role in insulin therapy still remains uncertain. To understand their positive effect in insulin therapy more clinical studies in larger settings are required [7].

In diabetic nephropathy, mesangial matrix accumulation is associated with reduction in activity of matrix metalloproteinase (MMP)-2, MMP-9 and plasmin. Hyperglycemia in diabetes up-regulates megsin, a serine protease inhibitor, which in turn inhibits plasmin and MMP activities suggesting the accumulation of mesangial matrix [8].

Plasmin, one of the most powerful and reactive serine protease, is involved in various biological processes, including thrombolysis, embryo development and cancer progression. Plasmin activity is tightly controlled by the activation of its precursor plasminogen, which is restricted by time and space as well as through inhibition of active plasmin by its plentiful natural inhibitors. By targeting the plasminogen activating system and upregulation of specific components of the plasminogen activation cascade, such as pro-uPA, uPAR and plasminogen receptors, cancerous cells can increase the generation of plasmin which in turn, alters the extracellular tumor microenvironment which allows cancer progression. For the past few decades, cancer research is centered on extracellular matrix (ECM) degradation, cytokines, growth factors from stroma and matrix metalloproteinase zymogens activation. In the recent past sufficient attention is focused on the ability of plasmin to produce functionally significant hydrolyzed products which induce signal transduction across the membranes [9].

A trypsin inhibitor – SNTI was purified and characterized from the family *Sapindaceae* [10]. Herein is a humble endeavor to investigate the inhibitory potential of a novel inhibitor against plasmin enzyme as it is generated *de novo* at the interface between cross-talking cancer and host cells. In the present study, the inhibitory potential of SNTI was explored against different proteolytic enzymes. *In vitro* studies are carried out to analyze the efficacy of SNTI on plasmin activity and on other proteases which play a major role in various disease pathways [11].

MATERIALS AND METHODS

Sapindus trifoliatus trees bearing soap nuts were selected from Horticulture Research Station, Pandirimamidi, Rampachodavaram, Andhra Pradesh (India). Ripe fruits were collected from selected trees and seeds were removed and preserved for extraction of protein. Purification of the inhibitor was carried out according to the method of Gandreddi *et al*. [10].

Specificity of the inhibitor

Estimation of trypsin activity

Trypsin activity was estimated using casein and BAPNA as the substrates. Trypsin was dissolved in 1 mM HCl containing 20 mM $CaCl_2$ to obtain a concentration of 100 μg per ml.

Caseinolytic activity

Caseinolytic activity of trypsin was determined by the casein digestion method according to Kakade *et al*. [12]. To 3 ml of trypsin solution (containing up to 50 μg) in 100 mM sodium phosphate buffer, pH 7.6, 2 ml of 1% casein solution in phosphate buffer, pH 7.6 was added. After incubation at 37 °C for 20 min, the reaction was stopped by addition of 5 ml of 5% trichloroacetic acid (TCA). The precipitate was filtered after standing for one hour at room temperature and the absorbance of the filtrate was read at 280 nm in Hitachi spectrophotometer against a blank prepared by adding casein solution to the incubation mixture after addition of TCA

Amidolytic activity

Amidolytic activity of trypsin was determined by the method of Kakade *et al*. using BAPNA as the substrate [12]. The substrate solution was prepared by dissolving 30 mg of BAPNA in 2 ml of dimethyl sulfoxide and the solution was made up to 100 ml

with 50 mM Tris-HCl buffer, pH 8.2 containing 20 mM $CaCl_2$. 30 µg of trypsin in 2 ml distilled water was incubated with 7 ml of BAPNA at 37 ºC for 10 min and the reaction was stopped by the addition of one ml of 30% acetic acid. The absorbance of the above solution was measured at 410 nm against a blank prepared as above except that 2 ml of distilled water replaced trypsin solution.

Estimation of trypsin inhibitory activity

For the assay of trypsin inhibitory activity, 30 µg of trypsin was pre-incubated at 37 °C for 10 min with aliquots of the inhibitor extract and then the residual trypsin activity was determined. The decrease in enzyme activity was taken as an index of the inhibitory activity. Suitable controls were included to correct for the presence of endogenous protease activity in the seed extracts.

Units: One trypsin unit (TU) is arbitrarily defined as an increase in 0.01 absorbance unit at 280 nm per 10 ml reaction mixture, in the case of the caseinolytic method, and at 410 nm under the assay conditions of the amidolytic method. One trypsin inhibitory unit (TIU) is defined as the number of trypsin units (TU) inhibited under the same assay conditions [12]. One unit of BAPNAase activity is defined as an increase in 0.01 absorbance unit at 410 nm under the conditions of assay.

Estimation of plasmin activity

To study plasmin activity, 1 ml of reagent buffer (1.361 mg/ml solution of Potassium Phosphate, with 9.94 mg/ml Sodium Phosphate, with 18.3 mg/ml Lysine, in purified water; adjust pH to 7.5 at 37 ºC with 1N NaOH) and 1 ml of VALY (3.58 mg of 6.5 mM D-Val-Leu-Lys-p-Nitroanilide Dihydrochloride Solution- VALY, pH 7.8 at 25 ºC in reagent buffer) were mixed by inversion and then incubated at 37 ºC using a suitable thermostatted spectrophotometer for 3-5 min. Then 100 µL of plasmin (prepare a 0.025-0.10 Unit/ml solution in cold purified water was added and immediately mixed by inversion and the increase in absorbance at 405 nm for approximately 10 min was recorded. The ΔA405 nm/minute over a five-minutes interval for both the Test and the Blank was obtained.

$$\text{Units/ml enzyme} = \frac{(\Delta A405nm/min Test - \Delta A405nm/min Blank)\ (1.35)\ (df)}{(10.5)\ (0.1)}$$

where,

1.35 = Total volume (in milliliters) of assay
df = Dilution Factor
10.5 = Micromolar extinction coefficient for p-Nitroanilide at 405 nm
0.1 = Volume (in milliliters) of enzyme used

Units: One unit will produce one µmole of p-Nitroanilide from D-Val-Leu-Lys-p-Nitroanilide at pH 7.5 at 37 ºC.

Estimation of plasmin inhibitory activity

To study plasmin inhibitory activity, plasmin was pre-incubated with SNTI for 10 min and further estimations were carried out according to the methodology followed for the estimation of plasmin activity. Also, the residual plasmin activity was estimated.

Estimation of chymotrypsin activity

Chymotrypsin activity was estimated using casein and N-acetyl-L-tyrosine ethyl ester (ATEE) as the substrates. Chymotrypsin was dissolved in 1 mM HCl containing 20 mM $CaCl_2$ to obtain a concentration of 100 µg per ml.

Caseinolytic activity

Caseinolytic activity of chymotrypsin was determined by the method of Sumati and Pattabhiraman [13]. To 3 ml of the chymotrypsin solution (containing up to 40 µg of the enzyme) in 100 mM sodium phosphate buffer, pH 7.6, 2 ml of 1% casein solution in phosphate buffer, pH 7.6 was added. After incubation at 37 ºC for 10 min, the reaction was stopped by addition of 5 ml of 5% TCA. The precipitate was filtered after standing for one hour at room temperature and the absorbance of the filtrate was read at 280 nm in Hitachi spectrophotometer against a blank prepared by adding casein solution to the incubation mixture after addition of TCA

Esterolytic activity

Esterolytic activity of chymotrypsin was assayed by the method of Prabhu and Pattabhiraman using N-acetyl-L-tyrosine ethyl ester (ATEE) as the substrate [14]. Chymotrypsin was dissolved in 1 mM HCl containing 20 mM $CaCl_2$ to obtain a concentration of 25 µg per ml. The substrate solution was prepared by dissolving 45 mg of ATEE in 2 ml of methanol and the solution was made up to 20 ml with 100 mM sodium phosphate buffer, pH 7.5.

Aliquots of chymotrypsin solution containing 0.2-2.0 µg of the enzyme in one ml of 100 mM phosphate buffer, pH 7.5 was incubated with one ml of ATEE solution at 37 ºC for 10 min and the reaction was stopped by addition of 5 ml of ethyl acetate. 0.5 ml of the aqueous layer was assayed for N-acetyl-L-tyrosine by the method of Lowry *et al*. [15].

Estimation of chymotrypsin inhibitory activity

For the assay of chymotrypsin inhibitory activity, 30 µg of chymotrypsin was pre -incubated at 37 ºC for 10 min with aliquots of the inhibitor extract and then the residual enzyme activity was determined as described above. The decrease in the proteolytic/ esterolytic activity of the enzyme was taken as an index of the inhibitory activity. Suitable controls were included to correct for the presence of endogenous protease activity.

Estimation of elastase inhibitory activity

Elastolytic activity of elastase was assayed according to the method of Naughton and Sanger using elastin Congo red as the substrate [16]. The method is based on measuring the release of the dye from elastin congo red by elastase at 495 nm. Elastin congo red (2 mg) was suspended in 1.8 ml of 0.2 M Tris-HCl buffer, pH 8.8 and was pre-incubated at 37 ºC for 30 min. Then 0.2 ml of the enzyme solution containing 50 µg elastase was added and further incubated at 37 ºC for 30min. The reaction was terminated by adding 2 ml of 0.5 M phosphate buffer, pH 6.0. The solution was centrifuged, and the absorbance of the supernatant was measured at 495 nm against a blank prepared by adding enzyme solution to the incubation mixture after the addition of 0.5 M phosphate buffer, pH 6.0. For the assay of inhibitory activity, 25 µg of the enzyme was pre-incubated with aliquots of the inhibitor extract and the residual enzyme activity was assayed as described above.

Estimation of pronase inhibitory activity

Amidolytic activity of pronase was estimated using BAPNA as the substrate by the same method described for the activity of trypsin [12]. For inhibitory activity the assay mixture contained varying amounts of the inhibitor and suitable aliquots of the enzyme in 2 ml of water. After pre-incubation for 10 min at 37 ºC, the residual enzyme activity was measured as described earlier.

Estimation of subtilisin inhibitory activity

Caseinolytic activity of subtilisin was assayed according to the method of Vaidyanathan and Virupaksha [17]. To 2 ml of enzyme (10-50 µg) in 100 mM phosphate buffer, pH 7.6 containing 0.15 M NaCl (PBS), 2 ml of 2% casein solution in PBS was added and incubated at 37 ºC for 10 min and the reaction was stopped by addition of 3 ml of 5% TCA. The precipitate was filtered after standing for one hour at room temperature and the absorbance of the filtrate was read at 280 nm in Hitachi spectrophotometer against a blank prepared by adding casein solution to the incubation mixture after addition of TCA. Inhibitory activity assay was carried out by incubating 50 µg of enzyme with aliquots of the inhibitor extract prior to the addition of the substrate.

Estimation of papain inhibitory activity

The proteolytic activity of papain was assayed using casein as the substrate [18]. The enzyme was activated with 50 mM cysteine and 20 mM EDTA. One ml of papain (50 µg) in 100 mM Tris-HCl buffer, pH 8.0 containing 20mM EDTA and 50mM cysteine was added to 1ml of 1% casein solution in Tris-HCl buffer, pH 8.0 and incubated at 37 ºC for 10 min. The reaction was terminated by adding 3 ml of 5% TCA. The precipitate was filtered after standing for one hour at room temperature and the absorbance of the filtrate was read at 280 nm in Hitachi spectrophotometer against a blank prepared by adding casein solution to the incubation mixture after addition of TCA. Inhibitory activity assay was carried out by incubating 20 µg of the enzyme with aliquots of the inhibitor extract prior to the addition of the substrate.

Estimation of pepsin inhibitory activity

The activity of pepsin was estimated by the method of Anson using denatured hemoglobin as the substrate [19]. One ml of 1 mM HCl containing 30 µg of pepsin was incubated with 1 ml of hemoglobin solution (20 mg/ml in 0.06 N HCl) at 37 ºC for 10 min and the reaction was arrested by adding 2 ml of 10% TCA. The solution was filtered and the increase in absorbance over the controls at 280 nm was taken as an index of proteolysis. For the inhibitory activity assay, 25 µg of pepsin was incubated with various aliquots of the inhibitor extract in 1 ml of 1 mM HCl for 10 min before the addition of hemoglobin solution.

Estimation of thermolysin inhibitory activity

Caseinolytic activity of thermolysin was assayed according to the method of Matsubara [20].

Thermolysin solution: Thermolysin crystals (10 mg) were suspended in 4 ml of 0.01 M Tris-HCl buffer, pH 8.0 containing 0.01 M $CaCl_2$ in an ice-bath and dissolved in 0.2 N NaOH (1.0-1.5 ml). The pH of the solution was immediately adjusted to 8.0 with 0.2 N acetic acid (1.0-1.5 ml) and the solution was stored at -10 ºC until use.

Thermolysin solutions, appropriately diluted with 0.01 M Tris-HCl buffer, pH 8.0, were incubated at 35 ºC with 1 ml of 2% casein solution for 10 min. The reaction was terminated by adding 2 ml of 5% TCA, and the precipitate was filtered after standing for 30 min at room temperature and the absorbance of the filtrate was read at 280 nm in Hitachi spectrophotometer against a blank. The blank was prepared by mixing the casein solution with TCA prior to the addition of the enzyme solution. For the assay of thermolysin inhibitory activity, 25 µg of thermolysin was incubated with aliquots of the inhibitor extract for 10 min and the residual thermolysin activity was assayed as described above.

Units for chymotrypsin, elastase, pronase, subtilisin, papain, pepsin, and thermolysin: One enzyme unit is defined as an increase in 0.01 absorbance units over the controls under assay conditions. One enzyme inhibitory unit is defined as the number of enzyme units inhibited under the same assay conditions.

Effect of SNTI on plasmin activity

Effect of SNTI on the serine protease plasmin was assessed using Plasiminogen (PLG) Human ELISA Kit (Abcam life sciences, Cambridge Science Park, Cambridge, UK) conferring to the manufacturer's guidelines. The assay was performed in a plasminogen-specific antibody-coated 96 well plate. 50 µl of plasminogen and 10 µl of inhibitor (Crude, purified SNTI) was added to wells, incubated for one hour and these were compared with other known natural inhibitors. Then, the wells were washed with 200 µl of 1Xwash buffer manually five times. The plate was inverted and decanted each time. 50 µl of 1X Biotinylated Plasminogen antibody was added and incubated for one hour. Then the wells were washed five times manually with 200 µl of 1Xwash buffer. Then, 50 µl of 1X SP conjugate was added and incubated for 30 min. The microplate reader was turned on and the programme was set up. Washings were repeated as mentioned above. Then 50 µl of chromogen substrate was added and incubated for 15 min/until the blue color developed. To stop the reaction 50 µl of stop solution was added and the color changed from blue to yellow. The absorbance was then read at 450 nm. The mean value of the triplicate readings for each standard and sample was calculated by plotting the calibration curve. IC_{50} values were also determined to assess the minimum inhibitory concentrations.

Effect of temperature on inhibition of plasmin by SNTI

To study the effect of temperature, 3.5 ml aliquots of plasmin-SNTI complex were exposed to different temperatures (30, 40, 50, 60, 70, 80, 90 and 100 ºC) in a water-bath for 10 min. After cooling on ice, the residual inhibitory activity was determined by the standard assay using suitable aliquots of the heat-treated extracts.

Effect of inhibitor concentration on plasmin

To study the effect of inhibitor concentration, plasmin was pre-incubated with various amounts of inhibitor at 37 ºC for 45 min and then assayed for caseinolytic activity.

Interaction of SNTI with inactive trypsinogen

30 µg of trypsin was treated with BAPNA in the presence of varying amounts of trypsinogen with and without SNTI and trypsin inhibitory and trypsin activities were determined, respectively.

Kinetic studies

The nature of inhibition of trypsin by SNTI was studied by incubating trypsin with varying concentrations of BAPNA in the presence and absence of the inhibitor. Substrate (0.8-5.0 µmole) was incubated with 30 µg of trypsin for 10 min at 37 ºC. After incubation, the reaction was arrested by adding 0.5 ml of 30% acetic acid. The absorbance of the solution was read at 410 nm against a blank incubation sample without trypsin. The assay was repeated in the presence of 2.5, 5.0 and 7.5 µg of SNTI in the reaction mixture.

Stoichiometric analysis of inhibitor-enzyme complex

The trypsin inhibitor complex was isolated by gel filtration on sephadex G-150 at 4 to 6 ºC. First an elution volume of trypsin alone and the inhibitor alone was determined. Sephadex G-150 column was equilibrated with 0.1 M phosphate buffer, pH 7.6. Trypsin (10 mg) or the inhibitor (2 mg) in 1 ml of phosphate buffer, pH 7.6 was applied on to the column (1.6 x 94 cm) at 4-6 ºC. The column was run with phosphate buffer, pH 7.6 at a flow rate of 10 ml/hour. Fractions of 1.5 ml were collected, and the absorbance of the fractions was measured at 280 nm.

For the isolation of the trypsin-SNTI complex, a mixture of the inhibitor (2 mg) and trypsin (10 mg) in 1 ml of 0.1 M phosphate buffer, pH 7.6 was allowed to stand at room temperature for 15 min. The amount of trypsin present was about 50% in excess of the inhibitor on an activity basis. This solution was then chromatographed on the sephadex G-150 column previously equilibrated with 0.1 M phosphate buffer, pH 7.6. The column was eluted with the same buffer and the absorbance of fractions (1.5 ml) was measured at 280 nm. The trypsin inhibitory activities in the fractions were determined.

RESULTS AND DISCUSSION

SNTI was purified to about 95% homogeneity according to the procedure adopted by Gandreddi *et al*. [10]. The class and group specificity of the inhibitor was investigated using standard protocols.

Specificity of inhibitor

The specificity of SNTI was tested for its inhibitory capacity against several proteases and is presented in Table 1. SNTI was tested against bovine trypsin using both the amide (BAPNA) and casein as the substrates. The inhibition pattern of the inhibitor towards amidolytic activity of bovine trypsin was assayed using BAPNA as the substrate. The percentage residual activity and inhibition were calculated. The inhibition pattern of the inhibitor was observed to be up to 80% (Figure 1).

30 µg of trypsin was incubated with varying amounts of SNTI for 10 min at 37 ºC. The percentage residual enzyme activity was assayed using BAPNA as the substrate. The concentration of the inhibitor required to cause 50% inhibition of the enzyme activity was determined from the graph.

On extrapolation, it was found that 12 µg of the inhibitor can totally inhibit amidase activity of 30 µg of trypsin. Activity of the SNTI against chymotrypsin, plasmin, elastase, pronase (*Streptomyces griseus* protease), subtilisin, papain, pepsin and thermolysin was tested. Except plasmin and pronase, rest of the enzymes was not affected by SNTI. The serine proteases trypsin, plasmin and pronase were inhibited by SNTI.

In the present study protein was extracted from the seeds of *Sapindus trifoliatus* to evaluate its antitryptic potential. The inhibitory effect was studied by measuring the variation in caseinolytic activity and expressing in terms of percent inhibition. Both the caseinolytic and amidolytic activities of

Table 1. Action of inhibitor on several proteolytic enzymes.

S. No	Proteolytic enzyme	Class	Inhibitory effect
1	Trypsin	Serine protease	+
2	Plasmin	Serine protease	+
3	Chymotrypsin	Serine protease	-
4	Elastase	Serine protease	-
5	Pronase	Serine protease	+
6	Subtilisin	Serine protease	-
7	Papain	Thiol protease	-
8	Pepsin	Acidic protease	-
9	Thermolysin	Metallo protease	-

+, activity present; -, activity absent

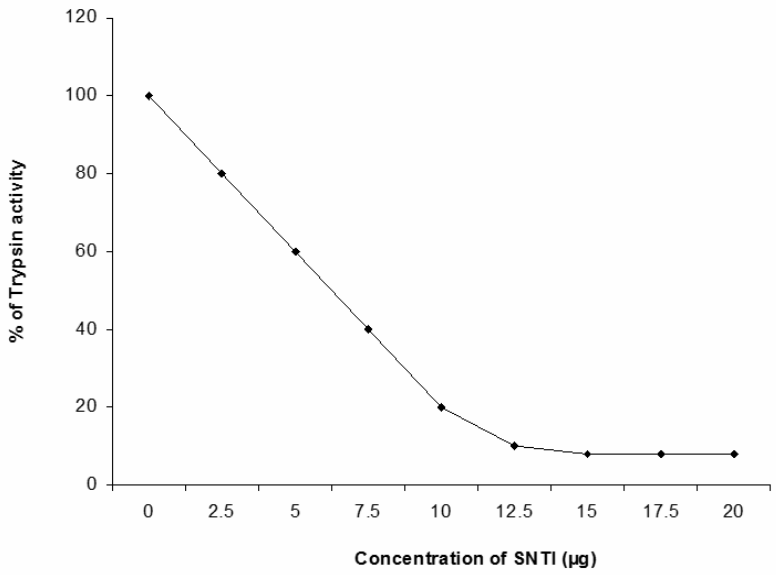

Figure 1. Activity of SNTI towards bovine trypsin.

trypsin were inhibited by SNTI. SNTI specifically inhibited serine proteases trypsin, plasmin and pronase and it had no effect on thiol, acidic and metalloproteases. The result of the investigation of the inhibitory specificity of SNTI has shown it to be a serpine that is strongly active against bovine trypsin and plasmin. SNTI was ineffective against other proteases such as papain (thiol), pepsin (carboxyl) and thermolysin (metallo). Majority of plant protease inhibitors isolated so far have been found to be specific for serine proteases and there are some reports that these inhibitors inhibit other classes of proteases [21]. However, there are reports of plant protease inhibitors inhibiting other classes of proteases. The trypsin/chymotrypsin inhibitor from broad beans inhibits the sulphydryl enzyme papain [22]. Serine protease inhibitors such as barley subtilisin inhibitor [23] and wheat germ protease K inhibitor [24] are found to be active against α-amylases. The human LEKTI has 15 domains and inhibits plasmin, trypsin, elastase, subtilisin A and cathepsin G [25].

Some potent inhibitors of trypsin are inactive or only weakly active against chymotrypsin [26] and vice-versa [27]. But in certain cases, they have been found to inhibit a range of other serine proteases such as elastase [28], thrombin [22], Plasmin and Kallikrein [29]. However, the enzyme pepsin and papain belonging to the class of carboxyl and thiol-proteases are not inhibited by SNTI. SNTI showed no activity against the bacterial proteases such as subtilisin and thermolysin.

Interaction of SNTI with inactive trypsinogen

It is observed from Figure 2 that net activity of trypsin is not altered in the presence of trypsinogen. Similarly, trypsinogen did not have any effect on the inhibitory activity of SNTI towards trypsin even when it was present at 5-6-fold weight excess. A catalytically active enzyme is necessary for the formation of trypsin-inhibitor complex with SNTI.

Stoichiometry

SNTI was treated with excess trypsin and the mixture was preincubated at 37 ºC for 15 min. This mixture when applied on to a column of Sephadex G-150 at 5 ºC gave rise to two distinct A_{280} absorbing peaks (Figure 3). Peak I had an elution volume of 20 ml which is lower than free SNTI 35 ml. The binary complex of trypsin-SNTI did not show any trypsin activity or trypsin inhibitory activity. The molecular weight calculated for trypsin-SNTI complex in Peak I based on the calibration curve for standard proteins gave a value of 68.9 kDa. This would mean a mole/mole interaction of SNTI with trypsin. Peak II was small and represented uncomplexed SNTI with corresponding trypsin inhibitory activity. The trypsin left over after the

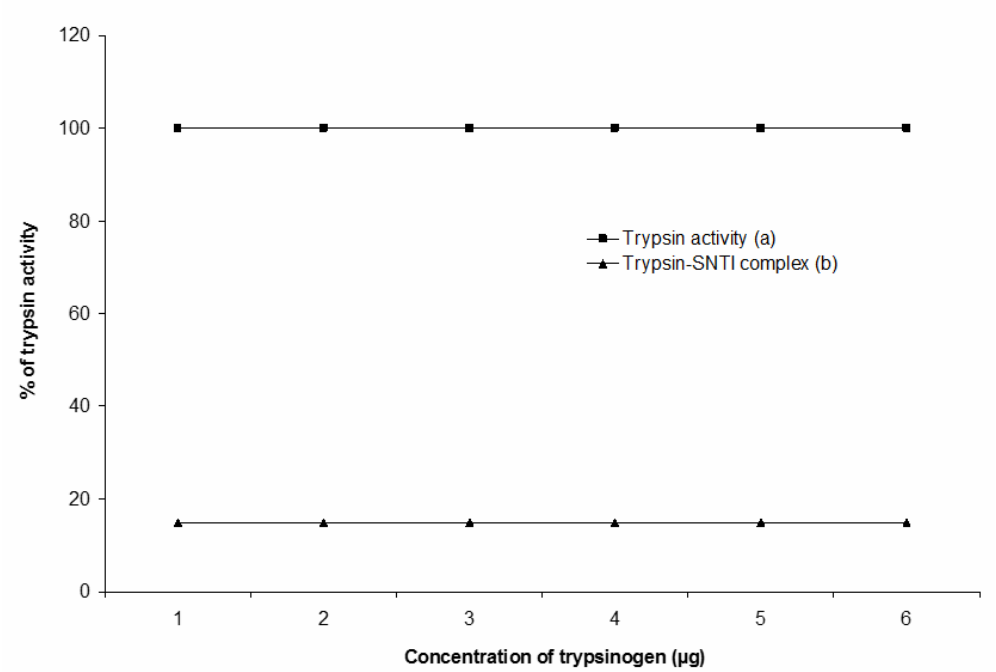

Figure 2. Influence of inactive trypsinogen on trypsin and trypsin-SNTI complex. a) 30 µg of trypsin is treated with BAPNA in the presence of varying amounts of trypsinogen. b) Trypsin inhibitory activity of SNTI in the presence of varying amounts of trypsinogen.

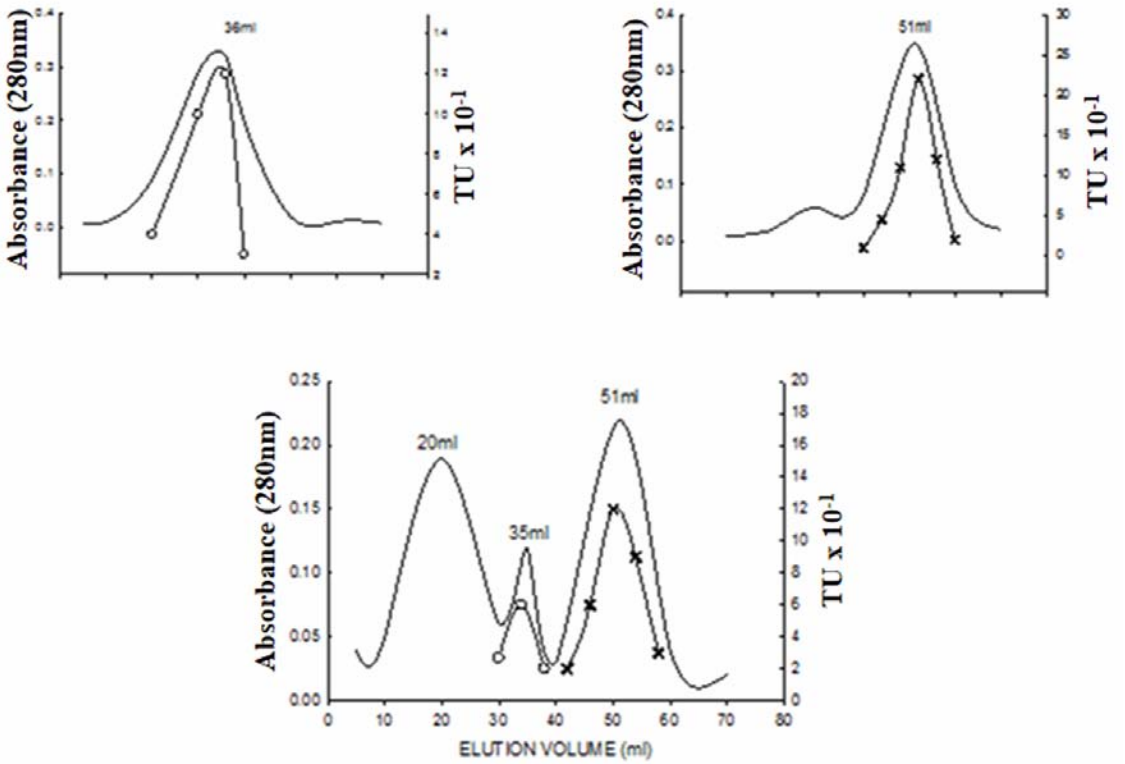

Figure 3. Chromatographic elution patterns of SNTI, trypsin and trypsin-SNTI complex. SNTI, trypsin and trypsin-SNTI complex elution patterns on Sephadex G-150 column; Protein was monitored at 280 nm (–).

enzyme inhibitor complex formation was eluted out (Peak III) with a corresponding elution volume of 51 ml.

Regarding the mechanism of action, SNTI has shown non-competitive type of inhibition. Although a few like soybean trypsin inhibitor has shown competitive type of inhibition, majority of the inhibitors follow non-competitive inhibition kinetics [30]. Jack fruit seed protease inhibitor isolated by Annapurna *et al.* [31] also showed non-competitive enzyme inhibition but the one isolated by Bhat and Pattabiraman exhibited uncompetitive inhibition [32]. The Ki value of SNTI was found to be 0.75 ± 0.05 x 10-10 M. The low Ki value indicates high affinity of SNTI towards trypsin. The formation of stable trypsin inhibitor complex has been demonstrated by Sephadex G-150 gel filtration studies. The results obtained suggest that the inhibitor binds to trypsin in a 1:1 molar ratio. SNTI is a mono-headed inhibitor with a site for trypsin. Double-headed inhibitors with overlapping or non-overlapping binding sites have been reported from plant sources [33, 34]. SNTI was purified and characterized and it was found to possess low ki value thereby having very high affinity towards trypsin. Further studies on its amino acid sequence, structure, phylogenetic relationship and its application in regulating trypsin dependent activities in biological systems need to be carried out.

Effect of inhibitor on Plasmin

Total plasmin activity in control was observed to be 99.97%. Different natural inhibitors along with SNTI at 20 µg concentration were tested against plasmin (Figure 4). The results indicate maximum percentage of inhibition by *Ocimum* extracts (78.87%), followed by crude SNTI (74.44%), purified SNTI (51.02%), *Phyllanthus niruri* (47.92%), nerium (41.53%), SBTI-a std. trypsin inhibitor (35.44%), *Thuja occidentalis* (16.21%) and *Vinca rosea* (13.41%). Interestingly, it was observed that there was a difference of about 23% between the crude extract and pure SNTI. The crude extract exhibited similar activity as that of *Ocimum*. Purified SNTI showed inhibition of about 51% at 20 µg concentration higher than all other extracts next to *Ocimum*.

From the plasmin assay the percentage inhibition of all the natural inhibitors were obtained. By regression analysis IC_{50} was determined to predict the efficacy of natural inhibitors. IC_{50} determines the potency of drug by measuring the concentration essential to inhibit about 50% of activity in *in vitro* conditions. The highest efficacy was identified for *Ocimum* extracts (12.77 µg/mL), followed by crude SNTI (13.51 µg/mL), purified SNTI (19.6 µg/mL) *P.neruri* (20.86 µg/mL), nerium (24.07 µg/mL), SBTI (28.21 µg/mL), thuja (61.69 µg/mL) and

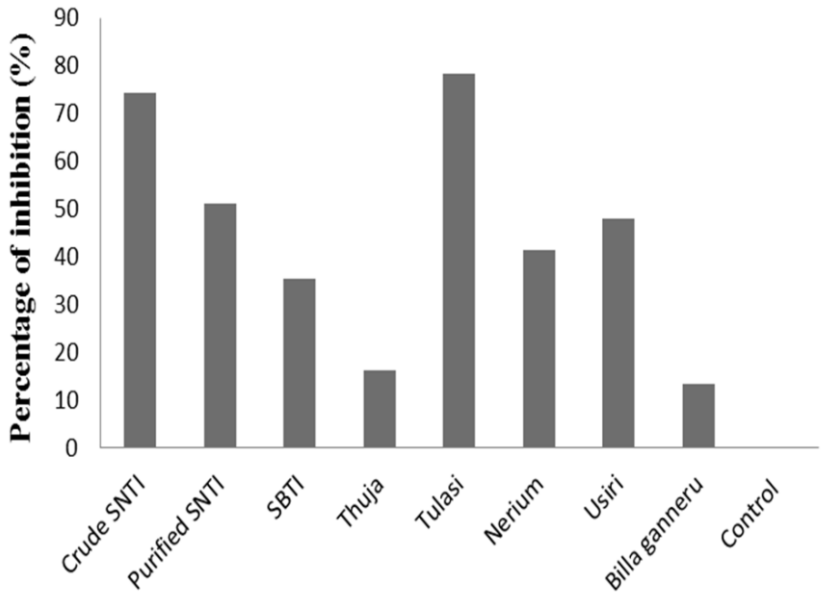

Figure 4. Percentage inhibition of SNTI & different plant protease inhibitors on plasmin.

V. rosea (74.57 µg/mL). These results suggest that at a concentration of 19.6 µg/mL of purified SNTI about 50% of inhibition is observed (Figure 5).

Effect of temperature and inhibitor concentration on plasmin activity

The kinetics of plasmin inhibition with respect to temperature and inhibitor concentrations is shown in Figures 6 and 7. It is evident from these figures that the inhibition reaction is temperature-dependent and shows no deviation from linearity with increasing amounts of inhibitor. With increase in temperature there was decrease in activity. In the absence of inhibitor at 80 ºC plasmin exhibited 33% activity. In the presence of inhibitor less than 30% residual plasmin activity was observed with increase of temperature above 50 ºC. The residual activity was almost zero at 70 ºC (Figure 6). In the presence of increasing amounts of inhibitor there was a steady increase of inhibition with maximum inhibition at 20 µg concentration. The results obtained after incubation of plasmin with various amounts of inhibitor at 37 ºC for 45 min correlate with the results obtained with IC_{50} values. On the basis of our findings 20 µg of SNTI is capable of inactivating 100 µg of plasmin (Figure 6).

Earlier it was thought that direct inhibition of plasmin-mediated proteolysis was a second choice in anticancer therapeutic strategies. Most of the research was targeted on PA system. However, little progress was achieved in translating the findings from bench to bed [35, 36]. Specific transmembrane proteins are triggered by proteolysis by uPA-activated plasmin, signaling the vicious cycle of events leading to crosstalk between the tumor and host cells. This proteolysis-induced signaling can be choked upstream of the signaling cascade, instead of gross inactivation of enzymes. Specifically, designed antimetastatic tools targeting discrete transmembrane and plasma membrane-associated molecules, which are activated by limited plasmin cleavage [37-39], can dramatically hinder tumor cells from accomplishing various steps of metastatic cascade and eventually decrease dissemination and colonization in tissues [9].

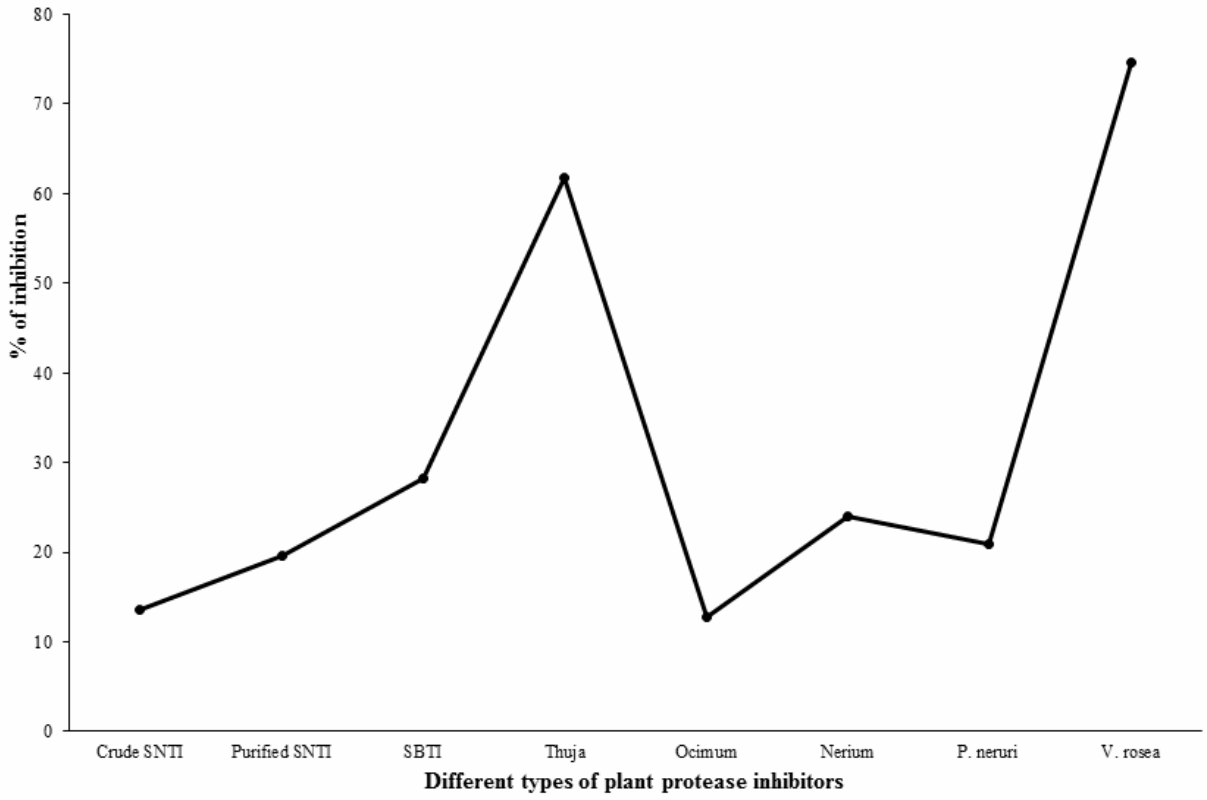

Figure 5. IC_{50} of SNTI & different plant protease inhibitors against plasmin.

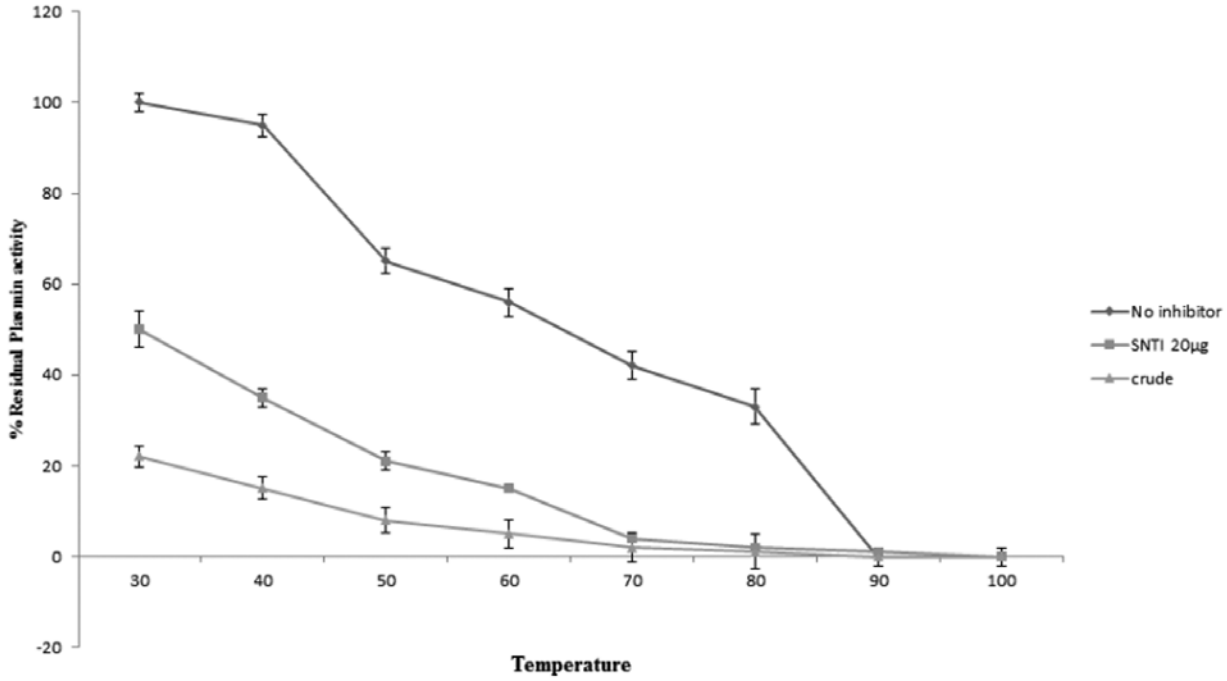

Figure 6. Inhibition of plasmin by SNTI as a function of temperature. Plasmin was pre-incubated with the inhibitor at various temperatures and assayed for anilide activity.

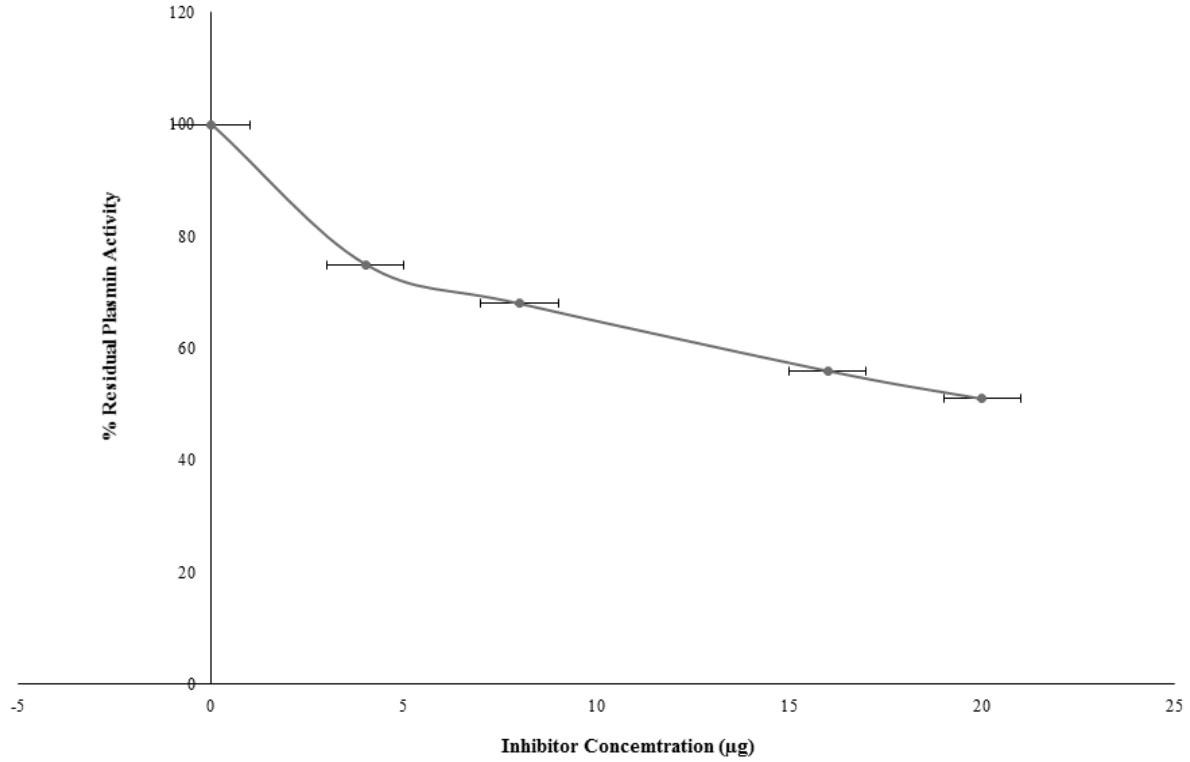

Figure 7. Inhibition of plasmin by SNTI as a function of inhibitor concentration. Plasmin was pre-incubated with various amounts of inhibitor at 37 ºC for 45min and then assayed for anilide activity.

CONCLUSION

Protease inhibitors play a major role in the different regulatory reactions and control the different pathogenic processes of human diseases such as arthritis, pancreatitis, hepatitis, cancer, AIDS, thrombosis, emphysema, blood pressure, muscular dystrophy etc. Serpin family is the largest and the most widespread super family of PIs. Currently over 60 distinct families of protease inhibitors have been recognized. These inhibitors are reversibly interacting with enzyme targets, forming stable complexes by influencing their catalytic activities in either competitive or non-competitive ways. The capability of inhibiting the proteins that are responsible for many dreadful diseases have proved protese inhibitor as the most effective agent in curing diseases and hence these protein inhibitors need to be studied well and further provide an excellent area of research. Further analysis of structures, protein-protein interactions and various biological activities of SNTI on different proteases of diverse biological origins need to be carried out to confirm the biotechnological potential of SNTI as a bio-control agent and its therapeutic potentials. Compromises between increased complexity, pharmacokinetic profiles and drug affordability will challenge biochemists to find new general methods for the simple creation of new inhibitors, which are potent, selective and bioavailable or to find better methods for efficient delivery of protein inhibitors against proteases. We hope that this endeavor can help to stimulate new efforts towards achieving such goals.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest

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