Isolation of a serine Kunitz trypsin inhibitor from leaves of *Terminalia arjuna*

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A serine Kunitz protease inhibitor was isolated from the semi-mature leaves of *Terminalia arjuna*, a host plant for *Antheraea mylitta*, using ammonium sulphate fractionation, gel permeation chromatography and trypsin–sepharose affinity chromatography. A 29-fold purification of *T. arjuna* Trypsin Inhibitor (TaTI) with a yield recovery of 3.2% was achieved. The purified protease inhibitor (TaTI) was resolved into a single protein band corresponding to molecular weight of 19.0 kDa on 12% SDS–PAGE under non-reducing conditions, whereas an additional band of 21.5 kDa was observed when the same fraction was resolved on SDS–PAGE under reducing conditions in the presence of 2-mercaptoethanol. TaTI inhibited both trypsin and chymotrypsin, but showed higher affinity for trypsin compared to chymotrypsin. However, it is more effective on bovine trypsin than midgut trypsin of tasar silkworm. TaTI retains its activity over a wide range of temperatures (0–100°C) and pH (2.0–8.0), with pH optimum of 8.0. These observations indicate that TaTI is not only specific to tasar silkworm but also to bovine serine proteases. Hence it can be considered as a generalist protease inhibitor.

Keywords: *Antheraea mylitta*, midgut trypsin, serine Kunitz trypsin inhibitor, *Terminalia arjuna*.

PLANTS and their insect herbivores are constantly at chemical war, as the plants have to overcome herbivore pressure and herbivores have to overcome the host plant's chemical defences[1]. Protease inhibitors (PIs) are one of the chemical defences widely used by plants against their herbivores. In plants, these PIs act as anti-metabolic proteins, which interfere with the digestive process of insects. Being one of the key proteins with proven inhibitory activity against insect pests, plant PIs offer an effective option of plant genes for the development of transgenics with resistance against insect pests. Plant PIs are known to inhibit all the four classes of proteolytic enzymes, i.e. serine, cysteine, aspartic and metallo proteases. Serine protease inhibitors are best characterized among all and have been grouped into (i) low molecular weight inhibitors with molecular weight ranging from 8 to 9 kDa, represented by the Bowman–Birk family of inhibitors, and (ii) high molecular weight inhibitors with molecular weight ranging from 21 to 22 kDa, represented by the Kunitz family of inhibitors[2].

Plant PIs are generally small proteins that have mainly been described as occurring in storage tissues, such as tubers and seeds, but they have also been found in the aerial parts of plants[3]. They are also induced in plants in response to injury or attack by insects or pathogens[4].

The PIs of serine proteases are widespread and have been reported from various plant families[5]. However, there is no report of PI from the family Combretaceae, comprising genera that serve as host plant to the tasar silk worm. *Terminalia arjuna*, family Combretaceae, is one of the primary host plants of tasar silkworm, *Antheraea mylitta*. In the present work, we report the isolation and characterization of a serine Kunitz trypsin inhibitor from leaves of *T. arjuna*. This PI from a different family of plants can be effectively used for the development of transgenic plants in other families for acquiring resistance against insect pests. Further, the specificity of this PI towards tasar silkworm larval midgut proteases provides an opportunity to develop more effective rearing strategy for tasar silkworm.

T. arjuna leaves and tasar silkworm midgut samples were obtained from Central Tasar Research and Training Institute (CTRTI), Ranchi, India.

Semi-mature leaves (100 g) of *T. arjuna* were homogenized in 500 ml of homogenizing buffer (100 mM Tris-HCl, pH 8.1 containing 1 mM EDTA, 20 mM β-mercaptoethanol, 5 mM $Na_2S_2O_5$ and 20% glycerol). The homogenate obtained was centrifuged at 11,000 rpm for 15 min. The supernatant obtained was subjected to ammonium sulphate [$(NH_4)_2SO_4$] fractionation. The $(NH_4)_2SO_4$ fraction (20–50%) showing maximum trypsin inhibition activity was subjected to gel-permeation chromatography on Sephadex G-50 column (25 × 3 cm) equilibrated with equilibration buffer (50 mM Tris-HCl buffer, pH 8.0 containing 500 mM NaCl) at a flow rate of 20 ml/h. After the void volume, fractions (5 ml each) were collected. Fractions showing trypsin inhibition activity were pooled, dialysed and lyophilized. The lyophilized fraction was dissolved in minimum quantity of equilibration buffer containing 8 mM β-mercaptoethanol and subjected to trypsin–sepharose affinity column (2 × 6.5 cm) equilibrated with the equilibration buffer. The sample was allowed to bind at a flow rate of 1 ml/3 min and the unbound fraction collected was discarded. The column was washed with ten bed volumes of equilibration buffer. Finally the bound trypsin inhibitor was eluted with 100 mM HCl containing 750 mM NaCl at a flow rate of 15 ml/h. The active fractions were pooled, dialysed against 20 mM Tris-Cl buffer, pH 8.0 and lyophilized.

Trypsin and chymotrypsin inhibition assays of *T. arjuna* Trypsin Inhibitor (TaTI) were carried out using the substrates TAME (Tosyl-Arginyl-Methyl Ester hydrochloride) and BTEE (*n*-Benzoyl-Tyrosine-Ethyl Ester)

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Table 1. Different steps of purification of TaTI

Step	Total volume (ml)	Total activity (U)	% T inhibition (25 µg protein)	Total protein (mg)	Specific activity (U/mg)	Yield (% recovery)	Fold purification
Crude inhibitor extract from leaves	500	924.0	76.4	700.0	1.32	100.0	–
20–50% $(NH_4)_2SO_4$ saturation fraction	10	500.0	79.1	51.0	9.8	54.1	7.4
Gel filtration chromatography	5	95.4	84.6	4.5	21.2	10.3	16.1
Trypsin affinity chromatography	1	29.9	89.5	0.78	38.4	3.2	29.0

T, trypsin.

respectively, following the procedure outlined by Birk[6]. Trypsin and chymotrypsin (1 mg/ml each) were prepared in 1 mM HCl, separately. Next, 25 µl of trypsin and 30 µl of chymotrypsin solutions prepared above were pre-incubated with known amount of TaTI in 1 ml of 46 mM Tris buffer (pH 8.1) containing 11.5 mM $CaCl_2$, separately.

The substrate solutions (1.04 M TAME in 46 mM Tris-HCl buffer, pH 8.1 and 1 M BTEE in 50% methanol after mixing in a ratio 1 : 1 with 46 mM Tris-HCl buffer, pH 8.1 as substrates for trypsin and chymotrypsin respectively) were prepared and the reactions were carried out in a Gilford response UV–VIS spectrophotometer, set at 247 nm for trypsin and 256 nm for chymotrypsin. The biological activity of TaTI was expressed in terms of % inhibition of trypsin and chymotrypsin.

The active trypsin–sepharose fraction was subjected to 12% SDS–PAGE under non-reducing (in the absence of 2-mercaptoethanol) and reducing (in the presence of 2-mercaptoethanol) conditions. Fractions obtained at various stages of purification of TaTI were also subjected to SDS–PAGE. The electrophoresed gels were silver-stained.

The thermal stability of the purified TaTI was determined by incubating it at various temperatures (4, 20, 30, 37, 40, 50, 60, 80, 90 and 100°C) for 45 min. After incubation, the trypsin inhibition activity of TaTI was assayed at 25°C, as described earlier. Three replicates were maintained for each treatment. At 100°C, the inhibitor was also incubated for 1 and 2 h before performing the trypsin inhibition assay.

To assess the pH stability of TaTI, purified TaTI was incubated with various buffers with pH 2, 3, 5, 6, 7, 8, 9, 10, 11 and 12, separately for 45 min at 25°C. The activity of TaTI was then assayed, as mentioned earlier. Three replicates were maintained for each treatment.

The proteases present in the midgut of the fifth instar larvae were extracted following the method of Lee and Anstee[7]. Ammonium sulphate (0–80%) was added at 4°C to the aqueous fraction obtained after the above extraction to precipitate the proteins. The precipitated protein was dissolved in minimum quantity of 20 mM Tris-Cl containing 20 mM β-mercaptoethanol and was designated as crude midgut protease (CMP) fraction after dialysis. Trypsin and chymotrypsin activities were assayed in this fraction following Birk[6].

In order to determine the inhibitory effect of TaTI on midgut protease activity, CMP (1 : 10 dilution) showing activity equivalent to 25 µg bovine trypsin was incubated with different concentrations of purified TaTI for 5 min at 25°C. After incubation, the sample mixture was assayed for trypsin and chymotrypsin activity, as mentioned above. Inhibition of bovine trypsin and chymotrypsin by TaTI was assayed as standard reference.

One of the important defence strategies found in plants to combat predators involves PIs which are in particular effective against phytophagous insects and microorganisms[8]. The defensive capabilities of plant PIs rely on inhibition of proteases present in the insect gut or secreted by microorganisms, causing a reduction in the availability of amino acids necessary for their growth and development[5]. According to our previous report, *A. mylitta* has evolved differential feeding behaviour in response to the defence mechanism of host plants[9]. The ability of *T. arjuna* leaf extract to inhibit the mid-gut trypsin activity suggested the presence of anti-herbivore compounds in the leaves. The present study was undertaken to further characterize the anti-herbivore compound from leaves of *T. arjuna*.

TaTI was purified from the semi-mature leaves of *T. arjuna*. Although young leaves contained high concentration of trypsin inhibition activity[9], semi-mature leaves were used for purification because of less tannin present in semi-mature leaves than in young leaves. The PI was purified following ammonium sulphate fractionation, gel permeation and trypsin–sepharose affinity chromatography (Table 1).

The crude protease inhibitor is heterogeneous, as evident by the fact that 0–20 and 20–50% ammonium sulphate saturated fraction showed 20–35 and 75–90% inhibition of trypsin respectively. Fractionation of more active 20–50% ammonium sulphate fraction on SDS–PAGE resolved into protein bands ranging from lower than 14 kDa to more than 43 kDa (Figure 1 *a*). Further purification of 20–50% saturated ammonium sulphate precipitated fraction by the sephadex G-50 gel permeation chromatography removed phenolic activity from the fraction and also resulted in higher yields, but it was still heterogeneous as evident by the appearance of more than one protein band on SDS–PAGE upon resolution of the active peak on the gels (Figure 1 *a*). The active sephadex G-50 peak was subjected to trypsin–sepharose affinity chromatography for further purification of TaTI. This resulted in 3.24% yield with 29-fold purification of TaTI (Table 1). The low

yield of purified TaTI compared to protease inhibitors obtained from other plant species[10,11], may be due to interferences from high levels of phenols and mucilaginous polysaccharides during purification.

This trypsin–sepharose affinity fraction of TaTI, when subjected to 12% SDS–PAGE, resolved into a single protein band corresponding to molecular weight of 19.0 kDa under non-reducing conditions. However, under reducing conditions, an additional band of 21.5 kDa was detected (Figure 1 *b*). The appearance of an additional band under reducing conditions may be due to disruption of intrapolypeptide disulphide linkages by 2-mercaptoethanol, resulting in an expanded structure of TaTI of lower electrophoretic mobility. TaTI appears to be close to the trypsin inhibitor from *Prosopis juliflora*[12] and thaumatin-PR-like inhibitor of the Kunitz group with respect to molecular weight[2]. Therefore, it may be presumed that TaTI also belongs to the Kunitz group of inhibitors.

The pH optimum for TaTI was 8.0 and the activity was nearly the same over the pH range of 2.0–8.0, but declined rapidly with increase in pH from 8.0 (Figure 2). The pH of the midgut of larval instars varies among different insect species and is specific in relation to its food-plant species. For example, the pH of midgut fluid of beetles is highly acidic (3.0), whereas for most of the caterpillars it is highly alkaline (12)[13]. The protease inhibitor of host-plant species is functional only when its pH optimum falls within the range of pH values of midgut fluids of its herbivore. This suggests that TaTI is not only specific for larvae of tasar silkworms, but also to other phytophagus lepidopteron insects.

The TaTI showed stability over a temperature range of 4 to 60°C, with a slight decrease in activity from 75 to 100°C (Figure 3). The ability of TaTI to retain its activity over a temperature range of 4–100°C and being active for 15 min at 100°C indicate that TaTI is tolerant to extreme temperatures. Oat trypsin inhibitor[14] and inhibitors isolated from cabbage[15] were also stable over a wide range of temperature (0–100°C). The ability of TaTI to retain its activity over a wide range of temperature (0–100°C) and pH (2.0–8.0) with pH optimum of 8.0, makes it a novel inhibitor.

To understand the role of TaTI in herbivory and to assess the specificity of TaTI to the midgut proteases of tasar silkworm, midgut proteases of fifth instar larvae were characterized. The midgut fluid had pH of 9.2, which is close to the pH optimum of TaTI. The midgut fluid showed both trypsin and chymotrypsin activities, but per cent trypsin activity was predominant. The chymotrypsin activity was 18–25% compared to 100% of trypsin activity (Table 2). Similar results have been obtained in other lepidopteron larvae[16].

Inhibition assays using crude midgut protease and purified TaTI from leaves of *T. arjuna* demonstrated that TaTI inhibited both midgut trypsin and midgut chy-

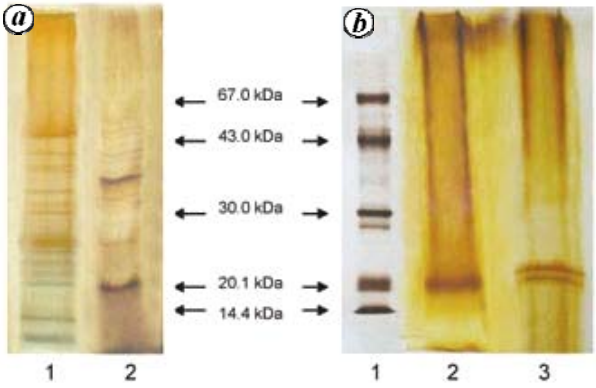

Figure 1. Silver stained SDS–PAGE slab gels (12%) showing resolved protein bands. ***a***, Lane 1, 20–50% $(NH_4)_2SO_4$ saturation precipitated fraction; lane 2, Sephadex G-50 active fraction. ***b***, Lane 1, Molecular weight marker proteins; lane 2, Trypsin–sepharose fraction under non-reducing conditions, and lane 3, Trypsin–sepharose fraction under reducing conditions.

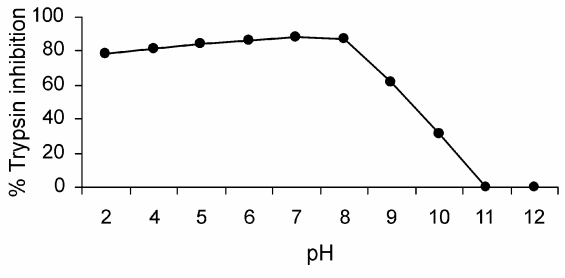

Figure 2. Effect of pH on TaTI activity. Purified TaTI was incubated at various pH values for 45 min at 25°C. After incubation, TaTI was assayed for trypsin inhibition activity.

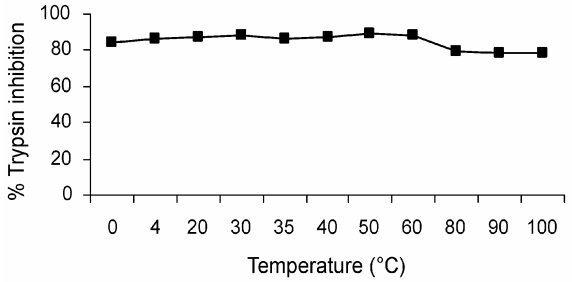

Figure 3. Thermal stability of purified TaTI. TaTI isolated from leaves of *Terminalia arjuna* was incubated at various temperatures for 45 min. After incubation, trypsin inhibition activity of TaTI was determined at 25°C.

Table 2. Comparative midgut trypsin and chymotrypsin activities

Midgut protease	% Protease activity
Trypsin	100
Chymotrypsin	18–25

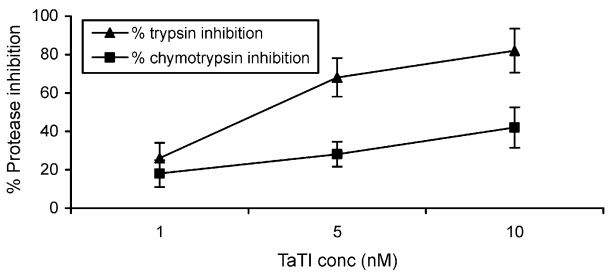

Figure 4. Inhibitory activity of TaTI against midgut proteases of *Antheraea mylitta*. Different concentrations of purified TaTI were incubated with crude midgut protease fraction showing protease activity equivalent to 25 µg of bovine trypsin at 25°C for 5 min. Trypsin and chymotrypsin activities were performed in this mixture. TaTI activity was plotted as % protease inhibition.

motrypsin, but had higher affinity to trypsin compared to chymotrypsin (Figure 4) This suggests the specificity of TaTI to tasar silkworm midgut proteases, which had higher trypsin activity compared to chymotrypsin activity. Activity profiles similar to TaTI have also been reported for PI from other sources[17].

Our previous observation shows that per cent inhibition of bovine trypsin by TaTI was markedly higher than that of midgut trypsin[9]. This indicates that insect serine midgut proteases, which are active at alkaline pH, are sturdier compared to bovine proteases. They are probably coevolved in response to the protease inhibitors which they came across during herbivory. Pests such as *Helicoverpa* spp. frequently feed on plants expressing protease inhibitors, because their digestive system is resistant to the presence of protease inhibitors[18].

These observations suggest that TaTI is not only specific to tasar silkworm, but can also act as a generalist PI that can be exploited for developing transgenics with insecticidal property. Based on knowledge of PIs and their role in various metabolic pathways, many transgenic crop plants expressing plant PI genes have been created and many more inhibitors are also being isolated with divergent modes of action against different pest species[5]. A further detailed study of TaTI can divulge an effective mode of integrated pest management strategy. Besides, understanding the adaptive defensive responses by both plants and their herbivores is one of the critical parameters in the management of insect pest and enhancement of tasar silk production.

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