Acta Biochim Biophys Sin, 2015, 47(5), 376–382
doi: 10.1093/abbs/gmv022
Advance Access Publication Date: 6 April 2015
Original Article

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Purification and characterization of a trypsin inhibitor from the seeds of *Artocarpus heterophyllus* Lam.

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Received 12 December 2014; Accepted 2 February 2015

Abstract

A proteinaceous inhibitor against trypsin was isolated from the seeds of *Artocarpus heterophyllus* Lam. by successive ammonium sulfate precipitation, ion-exchange, and gel-filtration chromatography. The trypsin inhibitor, named as AHLTI (*A. heterophyllus* Lam. trypsin inhibitor), consisted of a single polypeptide chain with a molecular weight of 28.5 kDa, which was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and gel-filtration chromatography. The N-terminal sequence of AHLTI was DEPPSELDAS, which showed no similarity to other known trypsin inhibitor sequence. AHLTI completely inhibited bovine trypsin at a molar ratio of 1:2 (AHLTI:trypsin) analyzed by native polyacrylamide gel electrophoresis, inhibition activity assay, and gel-filtration chromatography. Moreover, kinetic enzymatic studies were carried out to understand the inhibition mechanism of AHLTI against trypsin. Results showed that AHLTI was a competitive inhibitor with an equilibrium dissociation constant (K_i) of 3.7×10^{-8} M. However, AHLTI showed weak inhibitory activity toward chymotrypsin and elastase. AHLTI was stable over a broad range of pH 4–8 and temperature 20–80°C. The reduction agent, dithiothreitol, had no obvious effect on AHLTI. The trypsin inhibition assays of AHLTI toward digestive enzymes from insect pest guts *in vitro* demonstrated that AHLTI was effective against enzymes from *Locusta migratoria manilensis* (Meyen). These results suggested that AHLTI might be a novel trypsin inhibitor from *A. heterophyllus* Lam. belonging to Kunitz family, and play an important role in protecting from insect pest.

Key words: *Artocarpus heterophyllus* Lam., Kunitz-type inhibitor, purification, characterization

Introduction

The majority of proteases can be used as catalysts for the hydrolysis of polypeptide bonds selectively, and their ability to regulate a great deal of other physiological processes has been widely studied. As research continues, some disadvantages such as some human and plant diseases coexisting with the numerous benefits of proteases have been identified. Therefore, for the reduction of their harmful effects, proteases have to be strictly controlled and the protease inhibitors are being taken into consideration [1,2]. Protease inhibitors are widely distributed in plants, animals, and microorganisms, which have the ability to target enzymes through different mechanisms leading to inhibition of the catalytic activity of enzymes completely or partly. Protease inhibitors are promising therapeutic reagents in the treatment of diseases such as tumor, parasitic, fungal, bacterial, and viral infections

[1,3]. They also contribute to the defense mechanisms of plant against insects, pests, and pathogens [4,5].

Protease inhibitors are generally classified according to the types of proteases that they target. The four major subcategories of proteases, serine, cysteine, aspartic, and metalloproteases, are defined by the active amino acid in the reaction center [6]. Serine proteases are the largest subgroup of known protease family and their natural protein inhibitors have been extensively studied [7,8]. Serine protease inhibitors are essential for lots of physiological processes, such as blood coagulation, cell apoptosis, and inflammation [9], which have been categorized into at least 18 families based on the primary and three-dimensional structures of the protease inhibitors, as well as the inhibitory mechanisms [2]. Among the families of serine protease inhibitors, the Kunitz superfamily inhibitors have been paid more attention because of their various bioactivities, such as suppressing ovarian cancer cell invasion by blocking urokinase upregulation [10], and inhibiting the blood coagulation factor Xa to affect physiological function [11]. Kunitz inhibitors also make a contribution to the plant defense system against pests and pathogenic fungus as other protease inhibitors do [5,12].

The *Artocarpus* is widely used in medicine, agriculture, and industry. The anticipated therapeutical value of *Artocarpus* has been recognized, including its antibacterial, antimalarial, antitubercular, antiviral, and antidiabetic activities. The jackfruit (*Artocarpus heterophyllus* Lam.), a member of *Artocarpus*, is well known as tropical fruit that is widely grown in South-East Asian region. Much attention is being paid to its potential pharmaceutical value. In traditional folk medicine, both aerial and underground parts of the jackfruit trees have been utilized as traditional medicines to treat diseases. For instance, seeds can be used in the treatment for cooling tonic and pectorial, roots for diarrhea and fever, leaves for activating milk and vermifuge, and the latex mixing with vinegar for healing of abscesses, snakebite, and glandular swellings [13]. The jackfruit has been found to restrict the diseases, such as inflammation and diabetes mellitus because of the useful proanthocyanidin, flavonoid, and phenolic compounds [14,15].

Most studies of jackfruit seeds focused on the starch and jacalin [13,16], while protease inhibitors from this kind of seeds attracted less attention. In this paper, a novel trypsin inhibitor, named as AHLTI (*A. heterophyllus* Lam. trypsin inhibitor), was purified from the seeds of *A. heterophyllus* Lam. and the properties were characterized.

Materials and Methods

Materials

Seeds of *A. heterophyllus* Lam. were collected from Haikou, China. N_{α}-Benzoyl-L-arginine 4-nitroanilide hydrochloride (BApNA), *N*-benzoyl-L-tyrosine ethyl ester (BTEE), *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide ($SucAla_3$-*p*NA), bovine pancreatic trypsin, bovine pancreatic chymotrypsin, and elastase were purchased from Sigma-Aldrich (St Louis, USA). Q-Sepharose (high performance) was obtained from GE Healthcare (Wisconsin, USA) and the high performance liquid chromatography (HPLC) column, BioSep-SEC-s2000, was purchased from Phenomenex (Torrance, USA). Molecular weight markers were from Thermo (Massachusetts, USA). All other reagents used were of analytical or HPLC grade.

Purification of AHLTI

Artocarpus heterophyllus Lam. seeds (200 g) free of the outer covering were ground with ice-cold 50 mM Tris–HCl, pH 7.8, for 15 min. The homogenate was centrifuged at 12,000 *g* for 30 min at 4°C. The supernatants were collected and centrifuged at 12,000 *g* for 30 min after addition of saturated ammonium sulfate solution drop-wise to a final concentration of 30% with stirring. Then, the pellet was discarded. Saturated ammonium sulfate solution was added to supernatant to a final concentration of 80%, the solution was centrifuged and the pellet was collected. The pellet was re-suspended in water, dialyzed against 50 mM Tris–HCl, pH 7.8, lyophilized, and stored at −70°C until use.

The lyophilized sample (300 mg) was dissolved and dialyzed against 50 mM Tris–HCl, pH 7.8, and applied to a Q-Sepharose column (2.6 cm × 30 cm) pre-equilibrated with the same buffer. The elution was performed with the same buffer at the flow rate of 5 ml/min at 4°C for 8 h. Then, a linear gradient elution was carried out with 0–0.8 M NaCl. The absorbance of eluted fractions was detected at 280 nm. The fractions with trypsin inhibitory activity were pooled and dried by lyophilization. The lyophilized fractions was dissolved, dialyzed against distilled water (18 h) through 3500 Da cut-off membrane, and centrifuged at 12,000 *g* for 15 min. The supernatant was then purified by HPLC using a gel-filtration chromatography (BioSep-SEC-s2000) and eluted with 50 mM phosphate buffer, pH 7.0, at the flow rate of 1 ml/min. The absorbance of eluent was monitored at 215 nm. The substance at the peak of antitryptic activity was collected and named as AHLTI. The protein concentration was measured by using a protein assay kit (Bio-Rad, Hercules, USA) using bovine serum albumin as a standard.

Polyacrylamide gel electrophoresis

For sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), samples were heated for 5 min at 100°C in loading buffer with 2.5% SDS and 1.5% dithiothreitol (DTT) (under reducing conditions) or incubated with loading buffer with 2.5% SDS alone (under non-reducing conditions) at room temperature. For native-PAGE, samples were mixed with loading buffer without SDS and DTT. The gel was stained with 0.1% Coomassie brilliant blue R-250.

N-terminal amino acid sequence determination

N-terminal amino acid sequence of AHLTI was analyzed by automated Edman degradation (model 492C; Applied Biosystems, Foster City, USA).

Trypsin inhibition assay and enzyme kinetics

The inhibitory activity of AHLTI was examined according to the method of Erlanger [17] with minor modification. BApNA was used as chromogenic substrate. Briefly, AHLTI (2 μM) was incubated with 30 μl of trypsin (8 μM) at different molar ratios of 0:1, 1:10, 1:5, 3:10, 2:5, 1:2, and 3:5 (AHLTI:trypsin) for 10 min at 25°C. Then, 500 μl of 0.2 mM BApNA and 350 μl of 50 mM Tris–HCl/20 mM $CaCl_2$ (pH 8.2) were added and adjusted to 1 ml with distilled water. The enzymatic activity was measured by continuously monitoring the absorbance of *p*-nitroaniline at 410 nm for 2 min. The constant of inhibition (K_i) for bovine trypsin was determined by the method of Dixon [18]. A volume of 30 μl of trypsin (8 μM) was incubated with AHLTI (2 μM) at different AHLTI final concentrations (20, 40, 60, and 80 nM) in the reaction system at 25°C for 10 min. The enzymatic activities were measured at the substrate concentrations of 0.2, 0.4, 0.6, and 0.8 mM, respectively. Experiments were performed as described earlier. The K_i value was calculated by plotting the reciprocal velocity ($1/v$) vs. inhibitor concentration.

Preparation of AHLTI and trypsin complex

The combination of AHLTI and trypsin was carried out according to the method of Christensen *et al.* [19]. AHLTI (20 μM) was incubated with bovine trypsin at molar ratios of 1:0, 1:0.5, 1:0.75, 1:1, 1:1.5, 1:2, or 0:1 (AHLTI:trypsin) for 10 min at 25°C. Then, the mixture was subject to 15% native-PAGE.

Protein molecular mass determination of the complex

The molecular mass of AHLTI–trypsin complex was determined by HPLC. AHLTI (20 μM) was incubated with trypsin at different molar ratios with 1:0, 0:1, and 1:2 (AHLTI:trypsin) at 25°C for 10 min. The mixture was loaded on a BioSep-SEC-s2000 gel-filtration column (7.8 mm × 300 mm) and eluted with 50 mM phosphate buffer, pH 7.0, at the flow rate of 1 ml/min. The elution volume was used to calculate the molecular mass by comparing with other standard proteins: ribonuclease A (13.7 kDa), β-lactoglobulin (35 kDa), and immunoglobulin G (156 kDa). The apparent molecular mass of AHLTI and complex (AHLTI–trypsin) was estimated.

Determination of the inhibitory activity of AHLTI toward other proteases

Chymotrypsin inhibitory assays were performed by measuring the hydrolytic activity of BTEE. A volume of 20 μl of different concentrations of AHLTI (0.2–40 μM) was incubated with 66 μl chymotrypsin (0.4 μM). After 10 min of incubation at 25°C, 1.42 ml of 80 mM Tris–HCl (pH 7.8), 1.40 ml of BTEE (1.18 mM), and 0.08 ml of $CaCl_2$ (2 M) were added. The absorbance of reaction mixture was monitored at 256 nm for 2 min. In order to determine the inhibitory activity toward elastase, 33 μl of enzyme (0.4 μM) in 100 mM Tris–HCl, pH 8.0, was pre-incubated with 20 μl of different concentration of AHLTI (0.2–40 μM) for 10 min at 25°C. The optical density was recorded at 410 nm for 2 min after addition of 2.7 ml of 100 mM Tris–HCl (pH 8.0), and 0.2 ml $SucAla_3$-*p*NA (4.4 mM).

Determination of the thermal, pH, and chemical stability of AHLTI

The effect of temperature on AHLTI stability was determined by the residual activity of AHLTI after being heated for 30 min at various temperatures (20–100°C) and cooled to 4°C. The trypsin inhibitory activities of heated AHLTI were detected according to the method described earlier.

In pH stability studies, AHLTI was mixed with the same volume of various buffers of different pH: sodium citrate (pH 2–4), sodium acetate (pH 4–5), sodium phosphate (pH 6–7), Tris–HCl (pH 7–8), and sodium bicarbonate (pH 9–10). After incubation for 30 min at 25°C, the inhibitory activity against trypsin was measured at pH 8.2 as described earlier.

To measure the chemical stability of AHLTI, DTT was incubated with AHLTI at a final concentration of 1, 10, and 100 mM for 15–120 min at 25°C. Iodoacetamide (2, 20, or 200 mM) was added to terminate the reaction, and then the residual inhibitory activity was assayed. All experiments were performed in triplicate, and the results were presented as the mean of three assays. The standard deviation was <10%.

In vitro trypsin inhibition assays of AHLTI toward digestive enzymes from insect pests

Locusta migratoria manilensis (Meyen) and *Drosophila melanogaster* (fruit fly) were obtained from Haikou, China. The digestive enzymes were collected from adult insect after dissection and extraction of the guts. The guts were ground with ice-cold iso-osmotic saline (150 mM NaCl) solution and the homogenates were centrifuged at 12,000 *g* for 20 min at 4°C. The supernatants were stored for *in vitro* trypsin inhibition assays. The measurements of the trypsin inhibition were carried out as described earlier, except that trypsin was replaced by digestive enzymes. A volume of 30 μl of digestive enzymes (5.5 mg/ml) was incubated with different amounts of AHLTI (0.05 mg/ml) for 10 min at 25°C, and then the mixture was added to the reaction system.

Results

Purification of AHLTI

AHLTI was purified by three-step separate procedure from seeds of *A. heterophyllus* Lam. Some impurity proteins in crude extraction were removed by ammonium sulfate methods. The trypsin inhibitory activity components were precipitated with 30%–80% ammonium sulfate, then loaded on ion-exchange chromatography. Four peaks could be observed (**Supplementary Fig. S1**), and the peak IV containing trypsin inhibitor was then subject to gel-filtration chromatography after concentration. The fractions of peak I, displaying antitrypsin activity, were collected (**Supplementary Fig. S2**). **Figure 1A** shows the

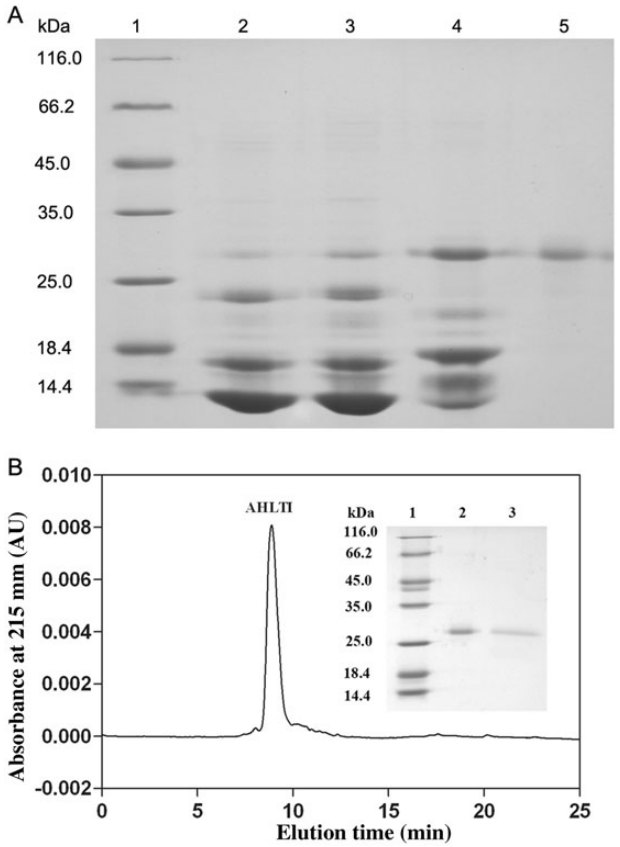

Figure 1. Purification profile of a trypsin inhibitor from *A. heterophyllus* Lam. by chromatographic procedures (A) Fifteen percent SDS–PAGE showing all fractions obtained during purification. Lane 1, molecular mass markers; lane 2, crude extract; lane 3, the fraction precipitated with 30%–80% ammonium sulfate; lane 4, the peak IV after loaded on ion-exchange chromatography; lane 5, AHLTI purified by gel-filtration chromatography (HPLC). (B) The pooled peak I (AHLTI) from BioSep-SEC-s2000 was concentrated and applied to HPLC to confirm the purity. The insert was 15% SDS–PAGE, which was stained with Coomassie brilliant blue R-250. Lane 1, molecular mass markers; lane 2, reduced AHLTI; lane 3, non-reduced AHLTI.

Table 1. Summary for the purification of AHLTI from *A. heterophyllus* Lam. seeds

Purification step	Total protein (mg)	Specific activity (IU mg^{-1})	Total activity (IU)	Purification (fold)	Yield (%)
Crude extract	1346.56	36.95	49755.39	1.00	100
Ammonium sulfate precipitate	308.49	138.29	42661.08	3.74	85.74
Q-Sepharose	25.82	1415.00	36535.30	38.29	73.43
HPLC (gel filtration)	1.65	2143.47	3536.73	58.01	7.11

One trypsin protein inhibitor unit (1 IU) was defined as inhibitor amount that decreased the absorbance at 410 nm by 0.01 OD in the trypsin inhibition assays.

Table 2. Comparison of the N-terminal amino acid sequence of AHLTI with other Kunitz-type inhibitors

Name	Sequence									
AHLTI	D	E	P	P	**S**	E	**L**	D	A	S
PmTKI (*P. moniliformis* seeds) [5]	K	E	L	L	D	A	D	G	D	L
PFTI (*P. foliolosa* seeds) [12]	F	E	L	K	D	M	E	G	D	I
DMTI-II (*D. mollis* seeds) [20]	L	V	Y	D	**S**	D	G	F	P	L
PPTI (*P. parviflora* seeds) [21]	A	P	L	E	D	S	**L**	A	A	K

SDS–PAGE analysis of samples from each purified step. The purity of the fractions of peak I was detected by HPLC and SDS–PAGE under both reducing and non-reducing conditions (**Fig. 1B**). AHLTI was a single peak in HPLC and a single band in the gel under both conditions. Moreover, purification procedures resulted in a purification of a 58.01-fold with a 7.11% yield (Table 1).

Characterization of AHLTI

The results of SDS–PAGE showed that the purified AHLTI was a single band under both reducing and non-reducing conditions, suggesting that AHLTI consists of a single polypeptide chain (**Fig. 1B**). The N-terminal sequence is determined to be DEPPSELDAS. The sequence was submitted to the NCBI-Blast search system for automatic alignment. No similar sequence was found when compared with the targeted N-terminal sequence (Table 2). In order to explore the mechanism of AHLTI inhibition toward trypsin, the method of Dixon was used to analyze the data. The competitive inhibition mode and the K_i (3.7×10^{-8} M) were determined by the crossing point of a family of intersecting lines, acquired by plotting $1/v$ according inhibitor concentration at each substrate concentration, which converged above *X*-axis (**Fig. 2**).

AHLTI was incubated at 25°C for 10 min with trypsin at molar ratios 0:1, 1:10, 1:5, 3:10, 2:5, 1:2, and 3:5 (AHLTI:trypsin). After incubation, the trypsin inhibitory activities of the mixtures were evaluated with BApNA. The trypsin was completely inhibited when the molar ratio reached 1:2 (AHLTI:trypsin) (**Fig. 3**). Meanwhile, AHLTI–trypsin mixtures were analyzed by 15% native-PAGE. As shown in **Fig. 4**, AHLTI and trypsin formed two complexes, the middle complex and the complete complex. AHLTI combined with trypsin to form the middle complexes when the molar ratio was 1:0.5 (AHLTI:trypsin). With the increase of trypsin, the middle complex transformed into the complete complex from the molar ratio of 1:1 (AHLTI:trypsin). At the molar ratio of 1:2 (AHLTI:trypsin), AHLTI and trypsin formed complete complex totally. Based on gel filtration, the molecular mass of AHILTI determined by BioSep-SEC-s2000 was 28.5 kDa. AHLTI, incubated with trypsin at a 1:2 (AHLTI:trypsin) molar ratio, transformed into complete complex with a molecular mass of ~76.5 kDa (**Fig. 5**), which corresponded to the sum of masses of one AHLTI and two trypsin molecules. These results suggested that one AHLTI molecule can inhibit a maximum of two trypsin molecules.

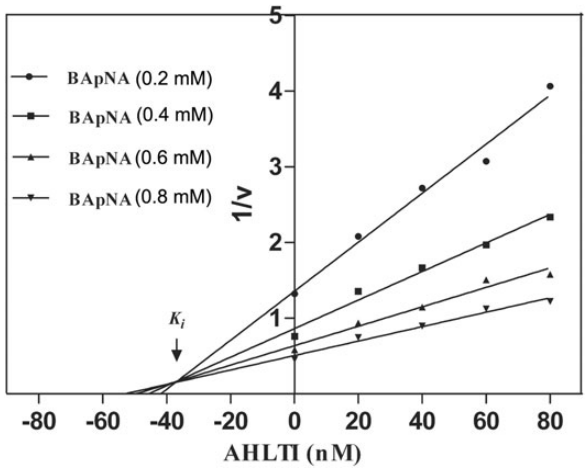

Figure 2. Kinetic analysis of trypsin inhibition activity of AHLTI The inhibition constant K_i was determined by Dixon plot at four different concentrations of BApNA (0.2, 0.4, 0.6, and 0.8 mM). The crossing point of a family of intersecting lines, acquired by plotting $1/v$ according to inhibitor concentration at four substrate concentrations which lies at $-K_i$ and the competitive mode inhibition of AHLTI can be observed from the plot directly. Results are means of three measurements.

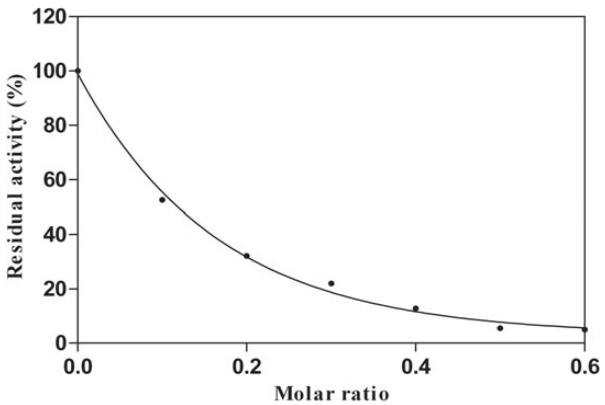

Figure 3. Trypsin inhibitory activity of AHLTI AHLTI was incubated with trypsin at different molar ratios from 0:1, 1:10, 1:5, 3:10, 2:5, 1:2, and 3:5 (AHLTI:trypsin). The residual trypsin activity reduced with the increasing concentration of the inhibitor at a fixed trypsin concentration using BApNA as substrate. All experiments were done three times. The data showed the average of three experiments.

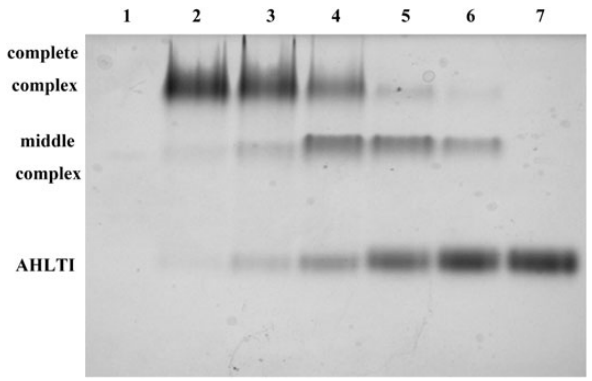

Figure 4. Native-PAGE analysis of the AHLTI–trypsin complexes AHLTI (A) dissolved in 50 mM Tris–HCl, pH 7.8, was incubated with a range of bovine trypsin (T) with molar ratios from 1:0, 1:0.5, 1:0.75, 1:1, 1:1.5 to 1:2 and 0:1 (AHLTI:trypsin) for 10 min at 25°C. Two kinds of complexes, the complete and middle complexes, were observed from the stained gel. Lane 1, trypsin; lane 2, A + T (1:2); lane 3, A + T (1:1.5); lane 4, A + T (1:1); lane 5, A + T (1:0.75); lane 6, A + T (1:0.5); lane 7, AHLTI.

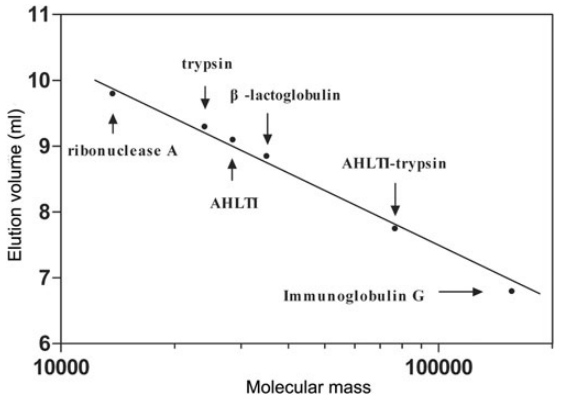

Figure 5. Molecular mass determination of AHLTI and AHLTI–trypsin complex on a gel-filtration chromatography AHLTI, trypsin, and AHLTI–trypsin (1:2) was loaded on the gel-filtration chromatography (HPLC) individually, using ribonuclease A (13.7 kDa), β-lactoglobulin (35 kDa), and immunoglobulin G (156 kDa) as standard proteins to calculate molecular mass.

AHLTI was able to inhibit chymotrypsin (15% inhibition) and elastase (6.2% inhibition) weakly compared with complete inhibition toward trypsin (100%).

Stability of inhibitory activity of AHLTI

The stability of inhibitory activity of AHLTI against trypsin was detected under different conditions. The thermal stability studies showed that the inhibitory activity was stable at temperature range from 20 to 80°C with <30% in activity. However, there was ~75% decrease at 100°C (**Fig. 6A**). Pre-incubation of AHLTI at the pH range of 2.0–10.0 for 30 min had a slight effect on its inhibitory activity. Even at extreme conditions (pH 2–3 or 9–10), the inhibitory activity of AHLTI retained 60% (**Fig. 6B**). Furthermore, the activity was also measured after incubation with different concentrations of DTT. It was found that AHLTI was not sensitive to reducing agent, and only lost ~15% activity when treated with the 100 mM DTT for 2 h (**Fig. 6C**).

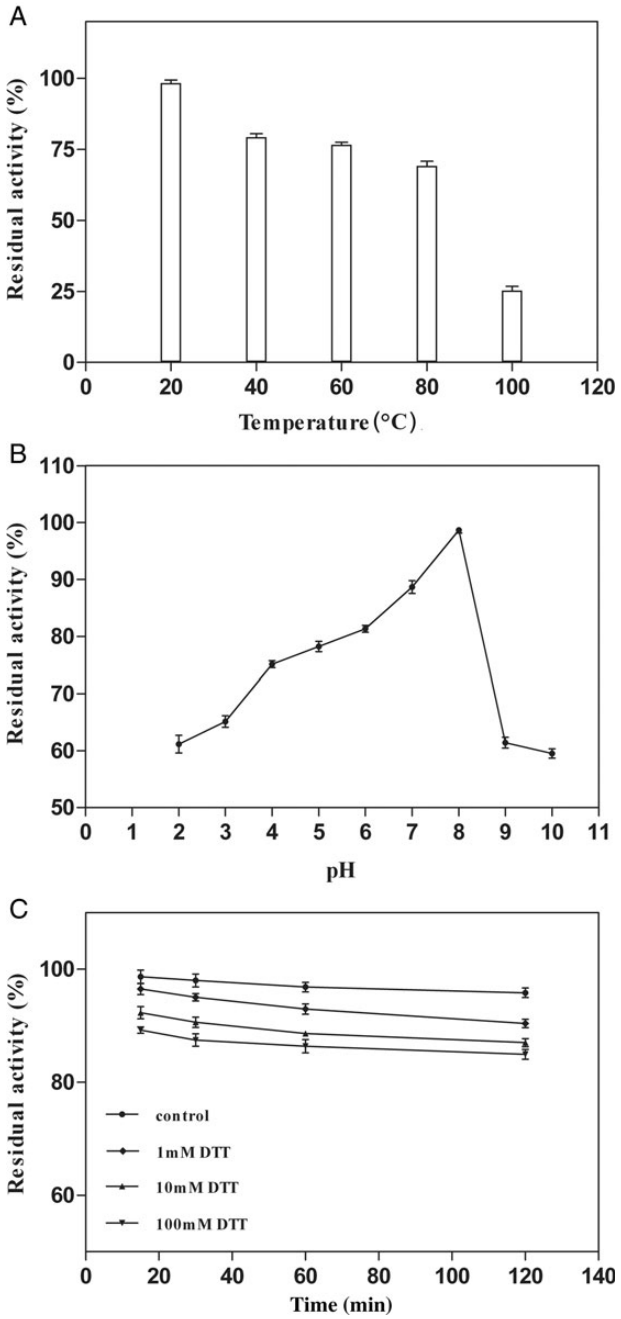

Figure 6. Stability of AHLTI (A) Thermal stability of AHLTI inhibitory activity was carried out after incubation for 30 min at a range of temperature (20–100°C). (B) pH stability was evaluated after mixing with indicated pH buffers (2–10), followed by incubation for 30 min at 25°C. (C) Effect of DTT on the chemical stability of AHLTI was tested after incubation for 15–120 min at 25°C at different final DTT concentrations. The reaction was terminated by addition of iodoacetamide (2-fold molar excess relative to DTT). The residual trypsin inhibitory activity was measured using BApNA as substrate. All experiments were done three times. The standard deviation was <10%.

Inhibitory activity of AHLTI toward digestive enzymes from insect pests

Digestive enzymes from the guts of *L. migratoria manilensis* (Meyen) and *D. melanogaster* (fruit fly) were used for trypsin inhibition assays of AHLTI *in vitro*. A volume of 20 μl of AHLTI (0.05 mg/ml) showed

effective inhibition against 30 μl digestive enzymes of *L. migratoria manilensis* (5.5 mg/ml), but it showed little inhibition toward *D. melanogaster*.

Discussion

It has been reported that the jackfruit *A. heterophyllus* Lam., an evergreen tree, produces higher yield than any other fruit tree species and bears the largest known edible fruit (average weight of 10 kg). The size of jackfruit seeds is extremely large. There are ~100–500 seeds in the fruit representing 8–15% of the total fruit weight [22,23].

Kunitz proteinase inhibitors are well-studied serine protease inhibitors, which can protect plants by inhibiting digestive enzymes of insect pest. Kunitz inhibitors consist of one or two polypeptide chains with the molecular masses within the range of 18–34 kDa [5,12,20,24]. In this study, a novel trypsin inhibitor from jackfruit seeds was successfully purified and characterized. The purification process was effective to obtain the trypsin inhibitor AHLTI. The purified protein showed a single peak in HPLC and a single band in SDS–PAGE under both reducing and non-reducing conditions. AHLTI was a single polypeptide chain protein with the molecular mass of 28.5 kDa determined by HPLC and SDS–PAGE, which was similar to that of Kunitz-type inhibitor. Kunitz inhibitors have been purified from *Crotalaria pallida* seeds and sweet potato [24,25]. The amino acid of N-terminal sequence of AHLTI was DEPPSELDAS, which was submitted to the NCBI-BLAST search system for alignment analysis. There was little homology between the N-terminal sequences of AHLTI and other trypsin inhibitors, suggesting that AHLTI might be a novel trypsin inhibitor from *A. heterophyllus* Lam. However, the structure of AHLTI remains to be determined.

The inhibitory activities of Kunitz-type protease inhibitors obtained from a variety of organisms were not the same. Some inhibitors of this family coming from *Psophocarpus tetragonolobus* (L) Dc and *Bauhinia rufa* can weakly inhibit trypsin, but are effective for chymotrypsin or elastase [26,27]. A few Kunitz-type inhibitors from *Peltophorum dubium*, *Crotalaria paulina*, and *Poecilanthe parviflora* only inhibit trypsin and have no inhibition activity toward chymotrypsin and elastase [21,28,29]. However, some Kunitz inhibitors not only exhibit potent inhibitory activity toward trypsin but also inhibit other proteases, such as chymotrypsin, elastase, and papain [5,12,20]. Specificity experiment of AHLTI suggested that this inhibitor can specifically inhibit trypsin and weakly inhibit chymotrypsin and elastase, which was similar to CpaTI, a trypsin inhibitor from *C. pallida* seeds [24]. The inhibition kinetics revealed that AHLTI was a competitive inhibitor of trypsin, similar to other inhibitors such as PmTKI and PRTI from the seeds of *Piptadenia moniliformis* and *Putranjiva roxburghii* [5,30]. The K_i for trypsin was found to be 3.7×10^{-8} M, which obviously demonstrated that AHLTI was a potent inhibitor. The similar low K_i values of 1.5×10^{-8} and 4×10^{-8} M had been reported for trypsin inhibitors from the seeds of *P. moniliformis* and *Plathymenia foliolosa*, respectively [5,12].

Results from stability assays indicated that trypsin form complex with AHLTI completely at the molar ratio of 1:2 (AHLTI:trypsin). Gel-filtration chromatography was also used to confirm the number of reactive sites. AHLTI and trypsin (1:2) formed a ternary complex with a molecular mass of 76.5 kDa. This result confirmed that AHLTI has two reactive sites, which was consistent with the results observed from native-PAGE and inhibition activity assay. Most members of Kunitz-type inhibitors have one reactive site [12,20], but some have more reactive sites [24,31].

The stability of AHLTI was analyzed under different experimental conditions. The factors, including temperature, pH, and reducing agent, were investigated. AHLTI showed a slight decrease in the inhibition activity when AHLTI was pre-incubated for 30 min at temperature between 20 and 80°C. When heated to 100°C, 75% of the activity was lost. The thermal stability has been reported for DMTI-II and PRTI from the seeds of *Dimorphandra mollis* and *P. roxburghii* [20,30]. Moreover, the inhibitory activity was not sensitive to pH over the range from 4.0 to 8.0, except at extreme pH condition. The inhibition activity of PFTI under different pH values was similar to that of AHLTI, while PmTKI and PRTI were stable at various pH values from 2 to 12 [5,12,30]. AHLTI was not sensitive to DTT and only a slight decrease in activity was observed when AHLTI was incubated with 100 mM DTT for 2 h. This was in contrary to most inhibitors such as PFTI and KPHTI, which lost inhibition activity at high DTT concentrations [30,32]. However, similar result had been reported for Kunitz-type inhibitors such as DMTI-II and PRTI which were not sensitive to DTT [20,30].

Based on the sequence analysis, the Kunitz inhibitors were stable proteins with low cysteine content. And it was reported that the disulfide bridges in some of Kunitz inhibitors, such as *Erythrina* trypsin inhibitor (ETI), were far apart from the active site residues involved in protease binding observed from the crystal structure. The reactive loop in ETI is stabilized by hydrogen bonds, which is in contrast to most inhibitors constrained by secondary structural elements or disulfide bridges [33]. So we deduced that AHLTI has active sites far away from the disulfide bridges or it has low cysteine content. More investigation on AHLTI is necessary to determine its structure and properties.

The trypsin inhibition assays toward digestive enzymes from insect pest guts *in vitro* showed that AHLTI was effective against trypsin-like enzymes from *L. migratoria manilensis* (Meyen). Similar Kunitz inhibitors, such as PmTKI and PFTI, have been reported to be effective to inhibit the digestive enzymes from insect pests [5,12].

In conclusion, a novel competitive trypsin inhibitor has been isolated from the seeds of *A. heterophyllus* Lam. It is a single chain protein with molecular mass of ~28.5 kDa. Based on the properties of Kunitz inhibitors, the purified AHLTI may belong to Kunitz family of inhibitors. The K_i value of 3.7×10^{-8} M indicates a high affinity between the enzyme and the inhibitor. According to the results from polyacrylamide gel electrophoresis, inhibition activity assay toward trypsin and gel-filtration chromatography, the AHLTI molecule has two reactive sites and can inhibit a maximum of two trypsin molecules. The stability of AHLTI is apparently not related to the presence of disulfide bridges. AHLTI also has effective inhibition activity toward trypsin-like enzymes from *L. migratoria manilensis* (Meyen).

Supplementary Data

Supplementary data is available at *ABBS* online.

Funding

This work was supported by the grants from the Middle-west Universities Comprehensive Stress Increased Engineering Program of China (No. ZXBJH-XK007), and the Graduate Student Innovation Center Construction Project of College of Marine Science, Hainan University.

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