UNIVERSITY GRANTS COMMISSION
BAHADUR SHAH ZAFAR MARG
NEW DELHI – 110 002
Final Report of the work done on the Major Research Project

1.	Project report No. 1st/2nd/3rd/Final	Final
2.	UGC Reference No	F. No. 39-238/2010 (SR)
3.	Period of report:	From: 1-02-2011 to 31-01-2014
4.	Title of research project	"Identification, purification, structure and function characterization of Lipid transfer proteins in fenugreek seeds (*Trigonella foenum-graceum*)"
5.	(a) Name of the Principal Investigator	Dr. Lalitha Guruprasad Professor
	(b) Department and University/College where work has progressed	School of Chemistry University of Hyderabad Hyderabad 500 046 Andhra Pradesh India
6.	Effective date of starting of the project	1st February 2011

7. Grant approved and expenditure incurred during the period of the report (1st February 2011 – 31st January 2014)

(a) Total amount approved:	11,10, 000/-
(b) Total expenditure	10, 61,930/-
(c) Report of the work done:	**Detailed Report enclosed (*Annexure-V*)**

(d) Brief objective of the project:
To purify and characterize low molecular weight lipid transfer proteins from fenugreek seeds. To study the binding of various ligands to proteins and using biophysical methods for functional characterization, and their structural characterization using CD spectroscopy.

(e) Work done so far and results achieved and publications, if any, resulting from the work (Give details of the papers and names of the journals in which it has been published or accepted for publication):
Detailed Report Enclosed (*Annexure-V*)

(f) Has the progress been according to original plan of work and toward achieving the objective, if not, state reasons:
The progress has been in accordance to original plan of work.

(g) Please indicate the difficulties, if any, experienced in implementing the project;
The progress of the project has been satisfactory and no difficulties were encountered in implementation of the project.

(h) If project has not been completed, please indicate the approximate time by which it is likely to be completed. A summary of the work done for the period (Annual basis) may please be sent to the Commission on a separate sheet:

The work is complete and its objectives are achieved. Summary of the work done for the period 1st February 2011 to 31st January 2014 has been enclosed. (*Annexure-V*)

(i) Any other information which would help in evaluation of work done on the project. At the completion of the project, the first report should indicate the output, such as (a) Manpower trained (b) Ph.D. awarded (c) Publication of results (d) other impact, if any

(a) One Ph. D student is working on this research project. The results of this work will be a part of the PhD thesis of the student. (b) The student is yet to submit thesis. (c) One publication "Purification and characterization of a stable kunitz trypsin inhibitor from Trigonella foenum-graecum (fenugreek) seeds. Rajender Oddepally, Gopi Sriram, Lalitha Guruprasad, Phytochemistry, 96, 26–36 (2013)." is in print. (d) Low molecular weight fenugreek seed proteins were purified using chromatography tools and assayed by SDS-PAGE and two dimensional electrophoresis. The low molecular basic proteins were submitted to proteomics analysis. Biophysical and biochemical analyses establish their roles as defensing and serine protease inhibitors.

SIGNATURE OF THE PRINCIPAL INVESTIGATOR **FINANCE OFFICER** **REGISTRAR**

Annexure-II

UNIVERSITY GRANTS COMMISSION
BAHADUR SHAH ZAFAR MARG
NEW DELHI – 110 002

STATEMENT OF EXPENDITURE IN RESPECT OF MAJOR RESEARCH PROJECT

1.	Name of Principal Investigator	Prof. Lalitha Guruprasad
2.	Department of University/College	School of Chemistry University of Hyderabad Hyderabad 500 046
3.	UGC approval No. and Date	F. No. 39-238/2010 (SR)
4.	Title of the Research Project	"Identification, purification, structure and function characterization of Lipid transfer proteins in fenugreek seeds(trigonella foenum-graceum)"
5.	Effective date of starting the project	1st February 2011
6.	(a) Period of Expenditure:	From 1st February 2011 to 31st January 2014
	(b) Details of Expenditure	

S. No.	Item	Amount Approved For 3 years Rs.	Amount Released in the 1st Installment Rs.	Expenditure Incurred (1st February 2011 to 31st March 2011) Rs.	Expenditure Incurred (1st April 2011 to 31st March 2012) Rs.	Expenditure Incurred (1st April 2012 to 31st March 2013) Rs.	Expenditure Incurred (1st April 2013 to 31st January 2014) Rs.	Total
i.	Books & Journals	Nil	4,00,000/-	Nil	Nil	Nil	Nil	Nil
ii.	Equipment	4,00,000/-		8,244/-	4,05,835/-	Nil	Nil	4,14,079
iii.	Contingency	1,50,000/-	3,85,000/-	Nil	Nil	Nil	1,27,282/-	1,27,282
iv.	Field Work/Travel (Details given in the proforma at Annexure-III)	50,000/-		Nil	Nil	43,711	Nil	43,711
v.	Hiring Services	Nil		Nil	Nil	Nil	Nil	Nil
vi.	Chemicals & Glassware	4,50,000/-		35,481/-	1,90,011/-	2,044/-	1,89,322/-	4,16,858
vii.	Overhead charges	60,000/-		60,000/-	Nil	Nil	Nil	60,000
vii.	Any other items (Please specify)	Nil		Nil	Nil	Nil	Nil	Nil
viii.	Total	11,10,000	7,85,000	1,03,725	5,95,846	45,755	3,16,604	10,61,930

(c) Staff

Date of Appointment: Not Applicable

S. No.	Expenditure Incurred	From	To	Amount Approved for 3 years (Rs.)	Expenditure Incurred
1.	Honorarium to PI (Retired Teachers) Rs. 10,000/p.m.	--	--	--	--
2.	Project Associate Fellowship @ Rs. 8,000/- p.m.	--	--	--	--
3.	Project Fellow consolidated salary @ Rs. 8000/p.m. (Fellowship is enhanced to @ Rs. 16,000/- p.m. with effect from 1st May 2010)				

1.	It is certified that the appointment(s) have been made in accordance with the terms and conditions laid down by the Commission.
2.	If as a result of checks or audit objective, some irregularity is noticed, later date, action will be taken to refund, adjust or regularize the objected amounts.
3.	Payment @ revised rates shall be made with arrears on the availability of additional funds.
4.	The Utilization Certificate at Annexure IV

SIGNATURE OF
PRINCIPAL INVESTIGATOR **FINANCE OFFICE** **REGISTRAR**

Annexure-III

UNIVERSITY GRANTS COMMISSION
BAHADUR SHAH ZAFAR MARG
NEW DELHI – 110 002

STATEMENT OF EXPENDITURE INCURRED ON FIELD WORK/TRAVEL TO UGC FOR MID-TERM REVIEW AND CONFERENCES

Name of the Principal Investigator: Dr. Lalitha Guruprasad

Name of the Place visited	Duration of the Visit		Mode of Journey	Expenditure Incurred (Rs.)
	From	To		
UGC, New Delhi	5th November, 2012	5th November, 2012	Air	19,571/-
NIPER, CHANDIGARH	27th March 2013-	28th March 2013 ----	Air	22,745/-
SRM Chennai	25th March 2013	25th March 2013	Taxi	1395/- (local travel within Hyderabad only)
Total				43,711/-

Certified that the above expenditure is in accordance with the UGC norms for Major Research Project.

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Annexure-IV

UNIVERSITY GRANTS COMMISSION
BAHADUR SHAH ZAFAR MARG
NEW DELHI – 110 002

Utilization Certificate

Certified that out of the approved Rs. **11,10,000/-,** an amount of **Rs. 7,85,000** was received from the University Grants Commission under the scheme of support for Major Research Project entitled "Identification, purification, structure and function characterization of Lipid transfer proteins in fenugreek seeds (trigonella foenum-graceum)" vide UGC letter F. No. **39-238/2010 (SR)** during the year **2011-2012.** A sum of **Rs. 10,61,930/-** has been utilized upto 31st January 2014 for the purpose for which it was sanctioned and in accordance with the terms and conditions laid down by the University Grants Commission, and leaving a **deficit** of **Rs. 2,76,930/-. This deficit amount of Rs Rs. 2,76,930/- should be returned to the account of the University of Hyderabad**.

SIGNATURE OF THE PRINCIPAL INVESTIGATOR **FINANCE OFFICER** **REGISTRAR**

Annexure V

Progress of work Dr. Lalitha Guruprasad, School of Chemistry, University of Hyderabad, Hyderabad, India
UGC project F. No. 39-238/2010 (SR)
Project title: Identification, purification, structure and function characterization of lipid transfer proteins in fenugreek sseds (Trigonella foenum-graceum)
Dated : 20th April 2014

Purification and characterization of low molecular weight proteins from *Trigonella foenum-graecum* (fenugreek) seeds

Abstract:

Defensin-like a novel antifungal peptide (Tf-AFP), with a molecular mass of 10.3 kDa and Kunitz trypsin inhibitor (TfgKTI) with a molecular mass of 20 kDa were purified from the seeds of *Trigonella foenum-graecum* (TfgKTI) belonging to fabaceae family by ammonium sulphate precipitation, cation exchange, gel filtration, hydrophobic chromatography and RP-HPLC. Mass spectroscopy analysis revealed the intact mass of purified defensin-like antifungal peptide as 10321.469 Da and intact mass of purified inhibitor is 19842.154 Da. MALDI-TOF-MS and Peptide Mass Fingerprinting (PMF) analysis of Tf-AFP showed high homology to plant defensins and other antifungal proteins in database search and TfgKTI showed sequence similarity with Kunitz trypsin inhibitor. Two-dimensional electrophoresis (2D-PAGE) revealed that Tf-AFP is basic in nature with pI value (8.8) and absence of isoforms and TfgKTI showed presence of four isoinhibitors (pI values of 5.1, 5.4, 5.7 and 6.1). The isolated peptide Tf-AFP inhibited growth of fungal species such as *Fusarium oxysporum*, *Fusarium solani*, and *Rhizoctonia solani*. Kinetic studies of TfgKTI showed that the protein is a competitive inhibitor and has high binding affinity with trypsin (K_i $3.01x10^{-9}$ M) and chymotrypsin (K_i $0.52x10^{-9}$ M). The TfgKTI retained the inhibitory activity over a broad range of pH (3–10), temperature (37–90 °C) and salt concentration (up to 3.5%). Far-UV circular dichroism and fluorescence measurements revealed that TfgKTI and Tf-AFP retain structure over a broad range of pH (3–10), temperature (37–90 °C) but Tf-AFP also retain structure in presence of reducing agent (10 mM DTT), in which TfgKTI lost structure as well activity.

Materials and Methods

Fenugreek seeds were collected locally. Chromatography support materials and 2D-PAGE reagents were purchased from GE Healthcare. The Luna C18 HPLC column was purchased from Phenomenex. Trypsin from bovine pancreas, Nα-Benzoyl-L-Arginine Ethyl Ester (BAEE), bovine pancreatic α-chymotrypsin and N-Benzoyl-L-Tyrosine Ethyl Ester (BTEE), were purchased from Sigma-Aldrich. SDS-PAGE molecular weight markers were purchased from Fermentas and Genetix. Stirred Ultrafiltration Cell and 3 kDa cutoff membranes were purchased from Millipore. All other reagents were of analytical or HPLC grade.

Purification of Tf-AFP and TfgKTI

T. foenum-graecum seeds (30 grams) free of integument were ground and defatted by washing with hexane for 1 hour followed by four parts (w/v) of $CHCl_3$+EtOH (2:1 ratio) for 30 min. The flour was filtered through whatman filter paper and kept for overnight air-drying. Defatted flour was then extracted using extraction buffer (25 mM sodium phosphate buffer pH 6.5, 50 mM NaCl, 4 mM EDTA, 0.01% azide) 1:10 (w/v) for 36 hrs at 4 ºC. Homogenate was filtered through muslin cloth and filtrate was centrifuged at 12000 rpm for 20 min at 4 ºC. The supernatant was designated as crude extract (CE). The CE was first fractionated by ammonium sulphate precipitation. It was treated with ammonium sulphate to 20% saturation. Ammonium sulphate was added to the supernatant to obtain 60% saturation and stirred at 4 °C for 2 hrs. After centrifugation at 12,000 rpm for 30 min at 4 °C, the supernatant was discarded while the pellet was dissolved in 20 mM phosphate buffer (pH 6.2), dialyzed against same buffer at 4 °C with several changes in 3.5 kDa cutoff dialysis membrane (Spectra/Por6), and applied on XK16/20 column of SP-Sepharose using Fast Protein Liquid Chromatography (FPLC-AKTA Prime Plus) system, previously equilibrated with the equilibration buffer (20 mM phosphate buffer, pH 6.2). Following the elution of unabsorbed proteins, absorbed proteins were desorbed with a linear gradient of NaCl (0-1 M) in the same buffer at a constant flow of 24 ml/hr. The absorbance was monitored at 280 nm. The cation ion exchange fractions SP3 pooled, dialyzed against extraction buffer at 4 °C for 2 hrs and concentrated by Stirred Ultrafiltration Cell using the membrane with pore size of 3 kDa. Further fractionation was carried out by size exclusion chromatography on XK 16/100 column of Sephadex G-50 pre-equilibrated with extraction buffer. Fractions (5 ml) from the protein peaks were collected at a constant flow of 12 ml/hr. The fractions S2 pooled, concentrated and dialyzed against 50 mM

phosphate buffer pH 7.0, containing 1M $(NH_4)_2SO_4$ then applied to XK16/20 hydrophobic column of Octyl Sepharose CL-4B previously equilibrated with the same buffer. Elution was carried out with a gradient of 50 mM phosphate buffer pH 7.0 (100-30% in 70 ml; 30 % 10 ml; 30-0% in 10 ml). Fractions (5 ml) were collected at a constant flow of 18 ml/hr.

The fractions P1 showing high antifungal activity (Tf-AFP) and P2 showing high protease inhibitory activity (TfgKTI) were subjected to reverse-phase high-performance liquid chromatography (RP-HPLC) on C18 column (Phenomenex, 1 cm x 250 cm; 5 µm particle; 100 A° pores) using HPLC system (Shimadzu) separately. The column was pre-equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA) and then Tf-AFP eluted with a linear gradient of acetonitrile (0-30% in 20 min; 30-50% in 40 min; 50-70% in 20 min; 70-100% in 1 min) in 0.1% TFA, at a constant flow of 2 ml/min. TfgKTI was eluted on a linear gradient of acetonitrile (5min 0%; 0-60% in 60min; 60-80% in 5 min; 80-100% in 5min) in 0.1% TFA, at a constant flow of 180 ml/hr. The eluent was monitored at 220 nm. The peaks were collected manually and dialyzed with 20 mM phosphate buffer with several changes and then used for assays.

Protein quantification

Protein concentration was estimated by the Bradford method (**Bradford, 1976**) with bovine serum albumin as the standard (1 mg/ml).

Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and native-PAGE (13%T, 4%C) was performed by the method of **Laemmli, 1970**. The protein samples were treated with Laemmli's buffer for both SDS-PAGE and native-PAGE. However, for native-PAGE, SDS and DTT were excluded from Laemmli's buffer. Samples for SDS-PAGE were incubated for 10 min at 100 °C before loading onto the gel along with a low molecular weight range of protein markers for comparison. Samples for native-PAGE were incubated at room temperature for 30 min before they were loaded on the gel. Protein bands were visualized by either to 0.1% of Coomassie Brilliant Blue R-250 (CBBR) or silver staining methods. Briefly, after completion of electrophoresis, the gel was left in fixing solution (50% methanol, 12% acetic acid, 0.074% formaldehyde) for one hour, followed by washing with 50% ethanol (2 x 30 min). After pretreatment with sodium thiosulfate for one minute and washing with deionized water three times for 5 min, the gel was immersed in silver nitrate solution (0.2% silver nitrate, 0.1% formaldehyde)

and gently agitated. After 30 min, the silver nitrate solution was removed and gel was incubated in developer (6% Sodium carbonate, 0.074% formaldehyde) until the bands appeared (1 min). The developer was then poured off and the gel was immersed in deionized water containing 12% acetic acid solution.

Molecular mass determination by Mass spectrometry

Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF/MS) was used to analyze the purity and absolute molecular mass of TfgKTI using Autoflex III mass spectrometer (Bruker Daltonics). Spectra were recorded in the positive-ion linear mode of operation with accelerating voltage of 20 kV, a grid voltage of 86% and a delay time of 200 ns. Samples were prepared by mixing equal volumes of 0.1% TFA (aq.), acetonitrile (1:1), and 1 µg of purified protein solution. 2 µl of the above sample was mixed with 2 µl of freshly prepared α-cyano-4-hydroxycinnamic acid (CHCA) matrix in 50% acetonitrile and 1% TFA (1:1), and 1 µl was spotted on target plate.

Proteomic analysis: In-gel digestion and MALDI-TOF-MS analysis

In-gel digestion and matrix assisted laser desorption/ionization time of flight mass spectrometric (MALDI-TOF-MS) analysis was conducted with a MALDI-TOF/TOF mass spectrometer (Bruker Autoflex III smartbeam, Bruker Daltonics, Bremen, Germany) according to the method described by **Shevchenko et al., 1996** with slight modifications. Coomassie R-250 stained 1-D SDS gel band were manually excised from three reproducible gels. The excised gel pieces were destained with 100 µl of 50% acetonitrile (ACN) in 25 mM ammonium bicarbonate (NH_4HCO_3) for five times. Thereafter, the gel pieces were treated with 10 mM DTT in 25 mM NH_4HCO_3 and incubated at 56 °C for 1 hr. This was followed by treatment with 55 mM iodoacetamide in 25 mM NH_4HCO_3 for 45 min at room temperature (25 ± 2 °C), washed with 25 mM NH_4HCO_3 and ACN, dried in speed vac and rehydrated in 20 µl of 25 mM NH_4HCO_3 solution containing 12.5 ng/µl trypsin (sequencing grade, Promega, Wisconsin, USA). The above mixture was incubated on ice for 10 min and kept overnight for digestion at 37 °C. After digestion, it was centrifuged for 10 min and the supernatant was collected in a fresh eppendorf tube. The gel pieces were re-extracted with 50 µl of 1% trifluoroacetic acid (TFA) and ACN (1:1) for 15 min with frequent vortexing. The supernatants were pooled together and dried using speed vac and were reconstituted in 5 µl of 1:1 ACN and 1% TFA. 2 µl of the above sample was mixed with 2 µl of

freshly prepared α-cyano-4-hydroxycinnamic acid (CHCA) matrix in 50% ACN and 1% TFA (1:1) and 1 µl was spotted on target plate. The samples were analyzed using a MALDI-TOF/TOF mass spectrometer with the following parameters. Parent ion masses were measured in the reflection/delayed extraction mode with an accelerating voltage of 20 kV and a grid voltage of 86%. For data processing, flex analysis software was used.

Protein identification: Peptide Mass Fingerprinting analysis

Protein identification was performed by database searches (PMF) using MASCOT program (http:// www.matrixscience.com) employing Biotools software (Bruker Daltonics). The similarity search for mass values was done with existing digests and sequence information from NCBInr and SwissProt database, and the taxonomic category was set to Viridiplantae (green plants). The other search parameters were: fixed modification of carbamidomethyl (C), variable modification of oxidation (M), enzyme trypsin, peptide charge of 1^{+1}, monoisotropic and missed cleavage-1. According to the MASCOT probability analysis ($P<0.05$), only significant hits were accepted for protein identification.

Two-dimensional electrophoresis

For the identification of isoforms, purified TfgKTI was first separated by isoelectric focusing in the Ettan IPGphor 3 system with 3–10 IPG strips (GE Healthcare). The rehydration buffer had 7 M urea, 2 M thiourea, 4% (w/v) 3-[3-cholamidoproplyl (dimethylammonio)]-1-propanesul- phonate (CHAPS), 1 M DTT, 1% bromophenol blue and 0.05% (v/v) IPG buffer 3–10 (GE Healthcare) and the IEF conditions were 300 V for 40 min, followed by gradient to 1000 V in 27 min, gradient to 5000 V in 80 min and 5000 V for 15 min at 20 °C. The IPG strips were equilibrated for 15 min in a buffer solution containing 1 M Tris–HCl pH 8.8, 6 M urea, 30% (v/v) glycerol and 2% (w/v) SDS (**Gorg et al, 2000**). The second dimension was performed on polyacrylamide gels of 13% T and 4% C (**Laemmli, 1970**).

CD spectroscopy

Circular dichroism measurements were carried out on a Jasco J-810 spectropolarimeter equipped with a peltier-type temperature controller and a thermostated cell holder, interfaced with a thermostatic bath. Far-UV CD spectra (190–260 nm) were recorded in 1 cm path length quartz cell at a protein concentration of 5µM. Each CD spectrum was the accumulation of three scans at 50

nm/min scanning speed, with 1 nm slit width, 1 s response time and 0.5 nm data pitch. The effect of temperature on conformational stability of Tf-AFP was determined by measuring CD spectra at temperatures ranging from 20 to 100 °C. CD spectra were background corrected. The secondary structure analysis was performed using CDNN software.

Fluorescence spectroscopy

Fluorescence measurements were performed with Fluoromax-4 Spectrofluorometer with 3 nm band width for both excitation and emission monochromators. The emission spectra of the protein solutions (in 10 mM Phosphate buffer, pH 6.5) were recorded at 25 °C in the region 310–400 nm having an absorbance of less than 0.1 at 280 nm after exciting at 295 nm for TfgKTI and 280 nm for Tf-AFP. The temperature of the cell was maintained at 25 °C by thermostat. Reaction mixtures with protein absent were used to correct base line. For fluorescence quenching studies Protein solution have absorbance less than 0.1 was prepared in 10 mM Phosphate buffer (pH 6.5) without and with DTT (10 mM final concentration). For denatured protein, the protein was incubated with 6 M Gdn-HCl overnight at room temperature. Fluorescence intensity was recorded with progressive addition of small aliquots of 5 M quencher stocks each time after 2 min (allowing for equilibration). The final quencher concentration attained in each case was 0.3 M. The potassium iodide stock solution contained 0.2 mM sodium thiosulphate to prevent the formation of triiodide (I_3^-). Since acrylamide had intrinsic absorption at 280 nm, the inner filter effect was corrected using the equation.

$$F_{corr} = F_{abs}. 10^{A/2} \qquad (1)$$

A is the increase in absorbance at the centre of the cuvette by the addition of acrylamide. Since KI had no significant absorption at excitation wavelength, no correction was necessary.

Trypsin and Chymotrypsin inhibitory activity of TfgKTI

Trypsin inhibitory assays were carried out by using a spectrophotometric method with some modifications (**Fang et al., 2010**). Fixed amount of trypsin (0.21 µM) in 1 ml of assay buffer (50 mM phosphate pH 7.6) were incubated with increasing amount of TfgKTI at 25 °C for 10 min. After incubation, 250 µM of Nα-Benzoyl-L-Arginine Ethyl Ester (BAEE) was added, then the residual enzyme activity was measured by monitoring at 253 nm on Shimadzu UV-1800

spectrophotometer. Chymotrypsin inhibitory activity was assessed by incubating chymotrypsin (0.1 µM) with suitable quantities of TfgKTI in 1 ml of assay buffer (50 mM phosphate pH 7.6) for 15 min at 25 °C, and thereafter with addition of 250 µM of N-Benzoyl-L-Tyrosine Ethyl Ester (BTEE) the residual enzyme activity was measured by monitoring at 256 nm. One trypsin or chymotrypsin unit is defined as 1 µmol of substrate hydrolyzed per minute of reaction. One inhibition unit is defined as unit of enzyme inhibited.

K_i determination of TfgKTI

Kinetic studies over a range of TfgKTI concentrations were performed to determine the inhibition constant (K_i) from Dixon plot using BAEE as a substrate for trypsin and BTEE as a substrate for chymotrypsin (**Dixon, 1953**). Studies were performed by adding a varying range of TfgKTI concentrations to a fixed amount of trypsin (final concentration was 1.26 nM) and chymotrypsin (1 nM) at different substrate concentrations of 0.25 mM and 0.75 mM. All the reactions were performed as described earlier. The reciprocal velocity ($1/v_0$) Vs inhibitor concentrations, for a substrate concentration were plotted (Dixon plots).

Stability of TfgKTI

The thermal stability of TfgKTI (1 mg/ml) was tested by incubation in the assay buffer (50 mM phosphate buffer, pH 7.6) for 30 min at various temperatures (37–100 °C). After incubation, all the samples were kept on ice for 15 min before testing for residual inhibitory activity. Residual inhibitory activity was measured in assay buffer, as described above.

The effect of pH on TfgKTI stability was evaluated by measuring the residual activity after incubation in the range pH (3–10) for 30 min at room temperature. Different buffers used included Mcllvaine buffers (0.2 M Na-phosphate and 0.1 M Na-citrate) for pH 3.0– 7.0 and 0.1 M glycine-NaOH for pH 8.0–10.0. Residual inhibitory activity was measured in assay buffer, as described above.

To test the stability of protein to the added salt, purified TfgKTI was incubated at room temperature for 30 min in the presence of NaCl ranging from 0% to 3.5% (w/v) in 50 mM phosphate buffer. The resultant solution was tested for residual inhibitory activity of trypsin in assay buffer as described above.

The effect of DTT on TfgKTI stability was evaluated by measuring the residual activity after incubation with DTT in different time intervals at room temperature in 50 mM phosphate buffer.

Statistical analysis

All experiments and analyses were carried out at least in triplicates. The mean and standard deviations were calculated. Statistical evaluation was undertaken by analysis of variance (ANOVA) followed by Bonferroni's test for multiple comparisons. $P \leq 0.05$ was considered statistically significant.

Antifungal assay

Antifungal assay was carried out in Petri dishes containing about 20 ml of potato dextrose agar. For the assay, a piece of agar containing frontal mycelia of the test fungus was placed in the center of the plate. The plates were incubated for 6 hr at 24 °C in the case of *Rhizoctonia solani*. However, the plates were incubated for 24 hr for the initiation of fungal growth in the case of *Fusarium oxysporum* and *Fusarium solani*. After this first incubation period, sterile paper discs (3 MM, Whatman) were placed at a distance of 0.5 cm around the frontal mycelia. Different concentrations of the protein sample were added to each disk. The plates were incubated at 24 °C for approximately 36-48 hrs until mycelial growth had enveloped peripheral discs containing control buffer (20 mM phosphate buffer, pH 6.5) and had formed crescent of inhibition around the disc containing an effective concentration of antifungal agent.

The effect of ionic strength on antifungal activity was carried out by adding 50 mM NaCl to the Tf-AFP protein sample.

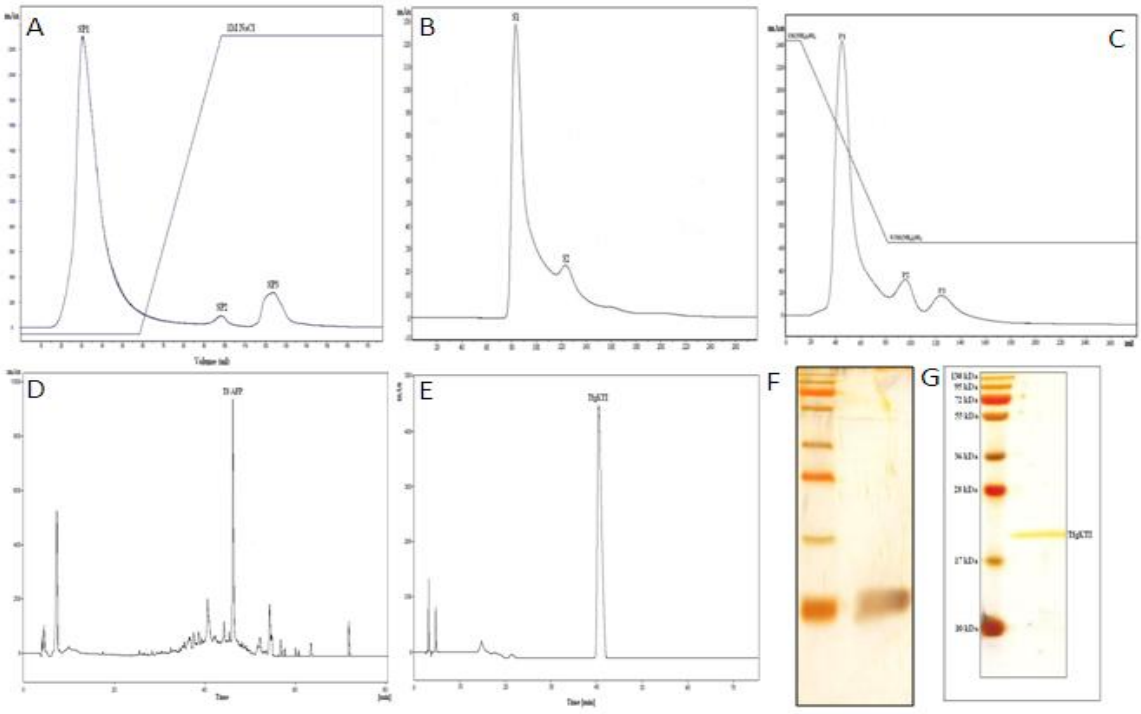
Differential scanning calorimetry of TfgKTI

DSC measurements were performed on a MicroCal VP DSC ultrasensitive differential scanning microcalorimeter (MicroCal LLC, Northampton, MA, USA). Samples were degassed before loading into cells. Experiments were carried out in presence and absence of DTT in buffer (20 mM phosphate, pH 6.2). Buffer scans were subtracted from the thermograms corresponding to the protein samples before further analysis. Data were analyzed by Origin software provided by the DSC manufacturer.

Results and Discussion

Purification of TfgKTI and Tf-AFP

TfgKTI and Tf-AFP were purified by extraction in extraction buffer (25 mM sodium phosphate buffer pH 6.5, 50 mM NaCl, 4 mM EDTA, 0.01% azide), followed by ammonium sulphate precipitation and combination of different chromatographic techniques. The ammonium sulphate precipitated (20–60%) soluble protein fraction from flour of *T. foenum-graecum* seeds crude extract, was chromatographed on cation exchange column (SP-Sepharose) equilibrated with 20 mM phosphate buffer, pH 6.2 (**Fig. A**). Under these conditions, most of the proteins were eluted during the washing step with the equilibration buffer (peak SP1). The fractions eluted with the saline buffer (peak SP3) pooled, concentrated and resolved by size exclusion chromatography on Sephadex G-50 into two peaks, S1 and S2 (**Fig. B**). Afterwards, the fractions (peak S2) were pooled and further purified through hydrophobic column on Octyl Sepharose CL-4B (**Fig. C**). The fraction P1 showing highest antifungal activity was compared to other two peaks (P2, P3). The fraction P1 was finally purified by RP-HPLC. Antifungal peptide was eluted in 43% acetonitrile in RP-HPLC, named as Tf-AFP (**Fig. D** and **F**). The yield of Tf-AFP is 3.5 mg/100 g seeds. The fraction P2 showing single band in SDS-PAGE contained trypsin inhibition with a specific activity of 7.65×10^3 TIU/mg and a 10.34-fold purification (**Fig. G**). It was also analyzed by reverse phase HPLC (**Fig. E**) to confirm its purity.


A
B
C
D
E
F
G

Summary of purification procedure of **TfgKTI** from 30 g of dried fenugreek seeds.

Stage	Total protein (mg)	Specific activity (TIUx10^3/mg)[a]	Total activity (TIU x10^4)[b]	Recovery (%)[c]	Yield (%)[d]	Purification fold[e]
crude extract	2159.20±4.2	0.74±0.02	159.65±4.6	100.00	100.00	1.00
Ammonium sulphate precipitate	1392.86±6.16	0.86±0.06	119.77±8.8	75.02	6.51	1.16
Cation exchange chromatography	182.00±1.44	3.40±0.11	61.82±1.96	38.72	8.43	4.6
Gel filtration chromatography	6.57±0.22	6.41±0.11	4.21±0.71	2.64	0.31	8.66
Hydrophobic chromatography	3.46±0.22	7.65±0.02	2.65±0.07	1.66	0.16	10.34

[a] Specific activity (trypsin inhibitory activity) of TfgKTI was tested by using BAEE as substrate.
[b] Total activity = specific activity × total protein.
[c] Recovery = (total activity of the fraction / total activity of crude extract)×100%.
[d] yield = (total protein of the fraction / total protein of crude extract)×100%.
[e] Purification fold = specific activity of the fraction / specific activity of crude extract.
* The significant differences ($P \leq 0.05$). ** Mean ± SD from triplicate determinations.

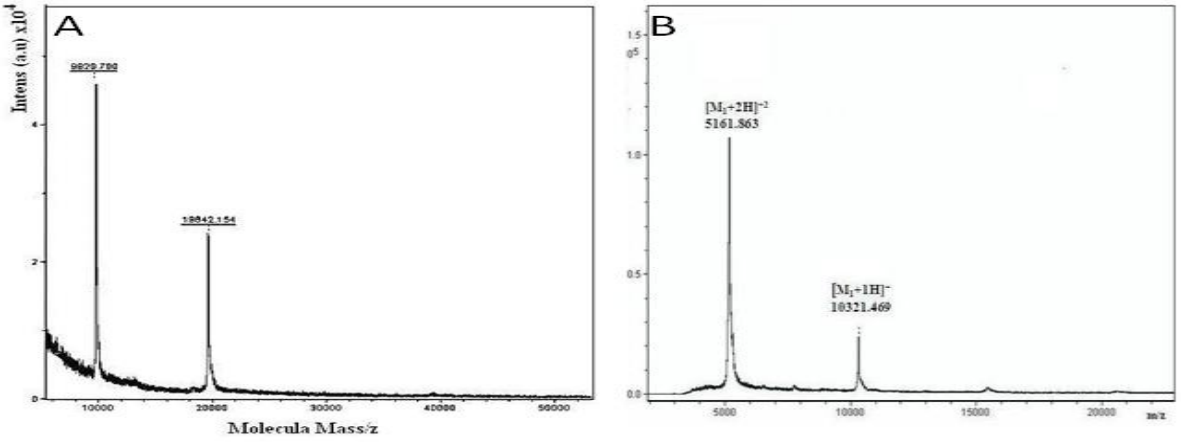
Yields of various chromatographic fractions obtained at different purification stages of **Tf-AFP** from 100 grams of dried seeds.

Purification stage	**Total protein (mg)**	**Yield[a] (%)**
crude extract	3598.6±4.20	100.00
Ammonium sulfate precipitate	2321.4±6.16	64.50
Ion exchange	303.0±1.44	8.40
Gel filtration fraction	10.9±0.22	0.30
Hydrophobic column fraction	5.8±0.22	0.16
RP-HPLC	3.5±0.03	0.10

[a] yield = (total protein of the fraction / total protein of crude extract) × 100%.

Molecular mass determination of TfgKTI and Tf-AFP

TfgKTI showed Molecular mass 19842.154 Da and Tf-AFP showed Molecular mass 10321.469 Da (**Fig A** and **B**).



MALDI-TOF-MS analysis

The Peptide Mass Fingerprinting (PMF) spectra of the 1-D SDS-PAGE gel bands of TfgKTI and Tf-AFP was acquired by MALDI-TOF-MS using R-cyano-4-hydroxycinnamic acid as the matrix are shown in **Fig. A** and **B**. Furthermore, analyses by the MASCOT search program in SwissProt database suggested TfgKTI sequence similarity with the reported Kunitz trypsin inhibitor from *Albizia kalkora*. The matched peptides of TfgKTI covered 90% of Kunitz trypsin inhibitor alpha chain fragments (ITRA_ALBKA) with score 40 (**Table A**).The observed monoisotopic mass of tryptically digested peptides obtained from Tf-AFP by MALDI–TOF-MS and their position with respect to matched protein sequence are presented in the **Table B**. The matched peptides of Tf-AFP covered 58% of defensin [*Trigonella foenum-graecum*/ gi31324677], 48% of antifungal protein precursor [*Medicago sativa* / gi11762086], putative defensin 1.5 precursor [*Medicago sativa*/ gi56267921] and 48% of defensin [*Medicago truncatula*/ gi37362318].

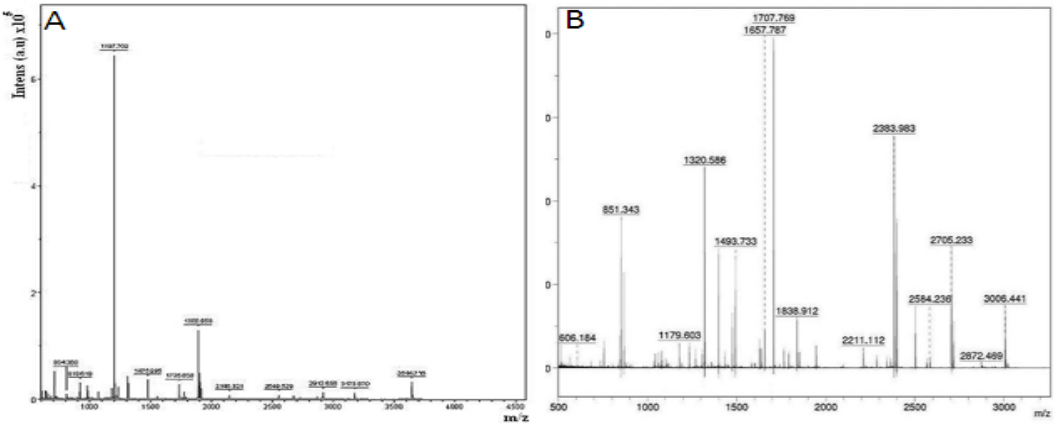


Table A

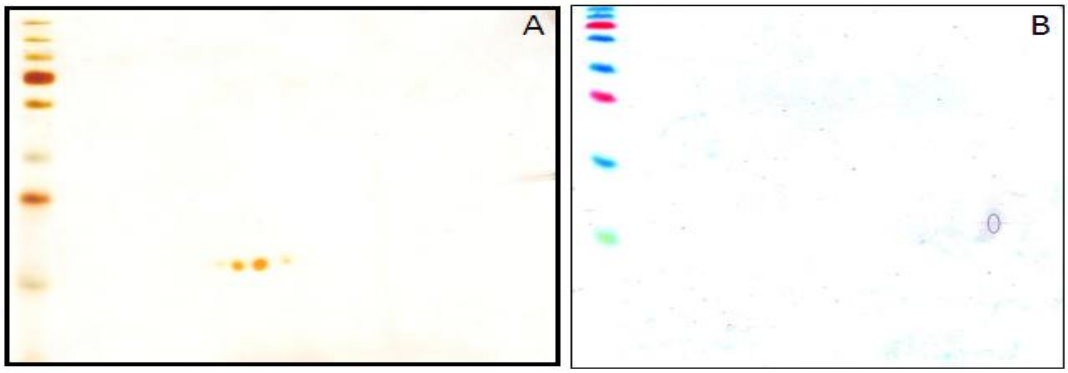
Fragment location	Mass[a] (expt)	Mass (calc)	Observed mass	Resulting peptide
2-12	1228.7007	1228.6299	1229.7080	K.ELLDADGDILR.N
13-38	2679.3247	2679.3228	2680.3320	R.NGGPAYPGLMPGVERDLPASGWGLPR.R
40-50	1218.6637	1218.5551	1219.6710	R.TGDESCPLNVK.A

Table B

Protein name/accession number	Theoretical pI	Score	Peptide mass[a] (Da)	Position	Matched peptides
Defensin [*Trigonella foenum-graecum*]/ 31324677	**8.1**	**85**	754.3187	66 - 70	CWCTK
			755.3525	53 - 59	EHAVSGR
			868.3736	60 - 65	CRDDFR
			1269.5867	29 - 38	TCENLADKYR
			1627.6235	39 - 52	GPCFSGCDTHCTTK
			1946.8048	37 - 52	YRGPCFSGCDTHCTTK
			2364.0171	39 - 59	GPCFSGCDTHCTTKEHAVSGR
Antifungal protein precursor [*Medicago sativa*]/ 11762086	**8.1**	**53**	754.3187	66 - 70	CWCTK
			868.3736	60 - 65	CRDDFR
			1269.5867	29 - 38	TCENLADKYR
			1627.6235	39 - 52	GPCFSGCDTHCTTK
			1946.8048	37 - 52	YRGPCFSGCDTHCTTK
Putative defensin 1.5 precursor [*Medicago sativa*]/ 56267921	**8.1**	**53**	754.3187	66 - 70	CWCTK
			868.3736	60 - 65	CRDDFR
			1269.5867	29 - 38	TCENLADKYR
			627.6235	39 - 52	GPCFSGCDTHCTTK
			946.8048	37 - 52	YRGPCFSGCDTHCTTK
Defensin [*Medicago truncatula*]/ 37362318	**8.09**	**53**	754.3187	66 - 70	CWCTK
			868.3736	60 - 65	CRDDFR
			1269.5867	29 - 38	TCENLADKYR
			1627.6235	39 - 52	GPCFSGCDTHCTTK
			1946.8048	37 - 52	YRGPCFSGCDTHCTTK

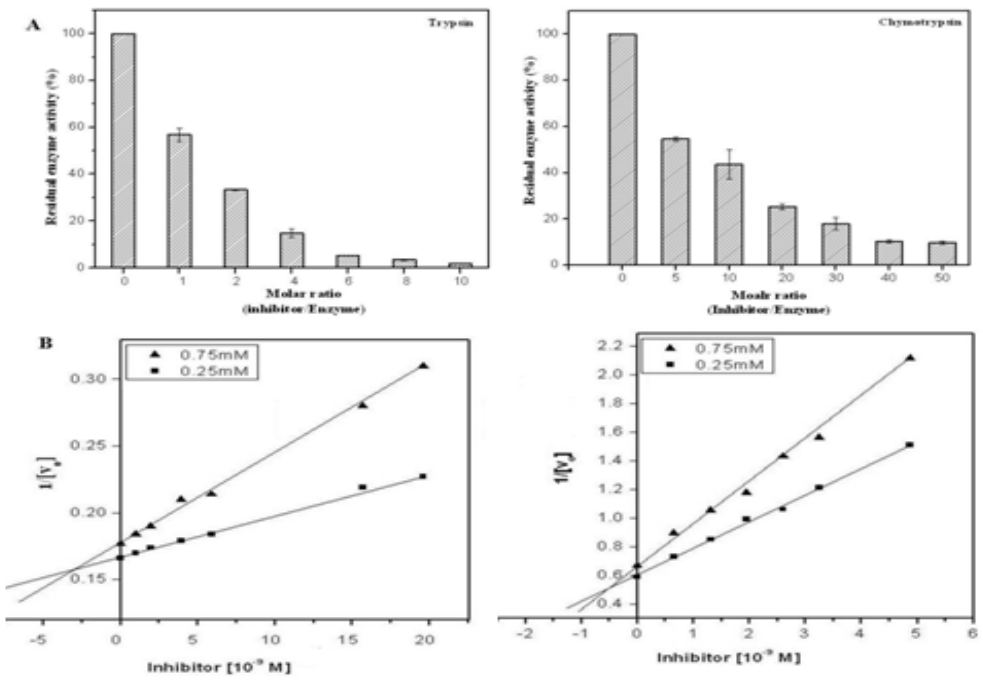
Two-dimensional electrophoresis (2-D) of TfgKTI and Tf-AFP

RP-HPLC showed only one clean peak for TfgKTI but 2-D electrophoresis exhibited four protein isoforms, indicating the presence of four isoinhibitors with separate pI values of 5.1, 5.4, 5.7 and 6.1 (**Fig. A**). Two isoinhibitors having pI 5.4 and 5.7 represent ~90% in total contribution of inhibitor. An acidic nature and the presence of isoinhibitors are common characteristics of Kunitz trypsin inhibitors. The isoelectric point (pI) of the Tf-AFP was determined as 8.8, based on the linear pH range 3–10 of isoelectric focusing electrophoresis conducted by two-dimensional electrophoresis shown in **Fig. B**. The results of basic pI demonstrated that the newly reported peptide has a basic character and a single spot showing absence of isoforms.



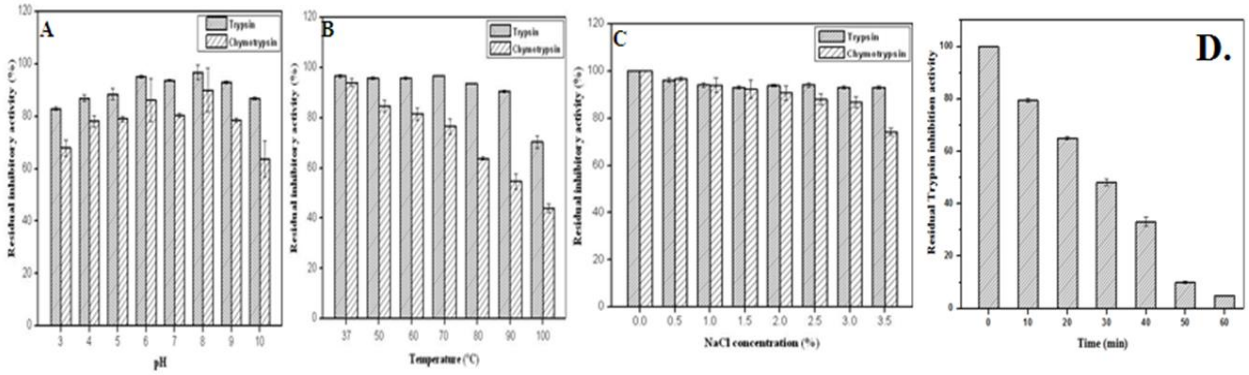
TfgKTI inhibitory properties and Ki determination

The inhibitory activity of TfgKTI against trypsin and chymotrypsin was measured with the increase in the concentration of the inhibitor, using BAEE and BTEE as substrates (**Fig. A**), respectively. The inhibition constant (K_i) value and mode of inhibition of TfgKTI were determined from Dixon plots (**Fig. B**). The analysis of Dixon plots showed that the TfgKTI is a competitive inhibitor with inhibition constant for trypsin was 3.01×10^{-9} M and chymotrypsin was 0.52×10^{-9} M, suggesting a high affinity of the inhibitor for trypsin as well as chymotrypsin.



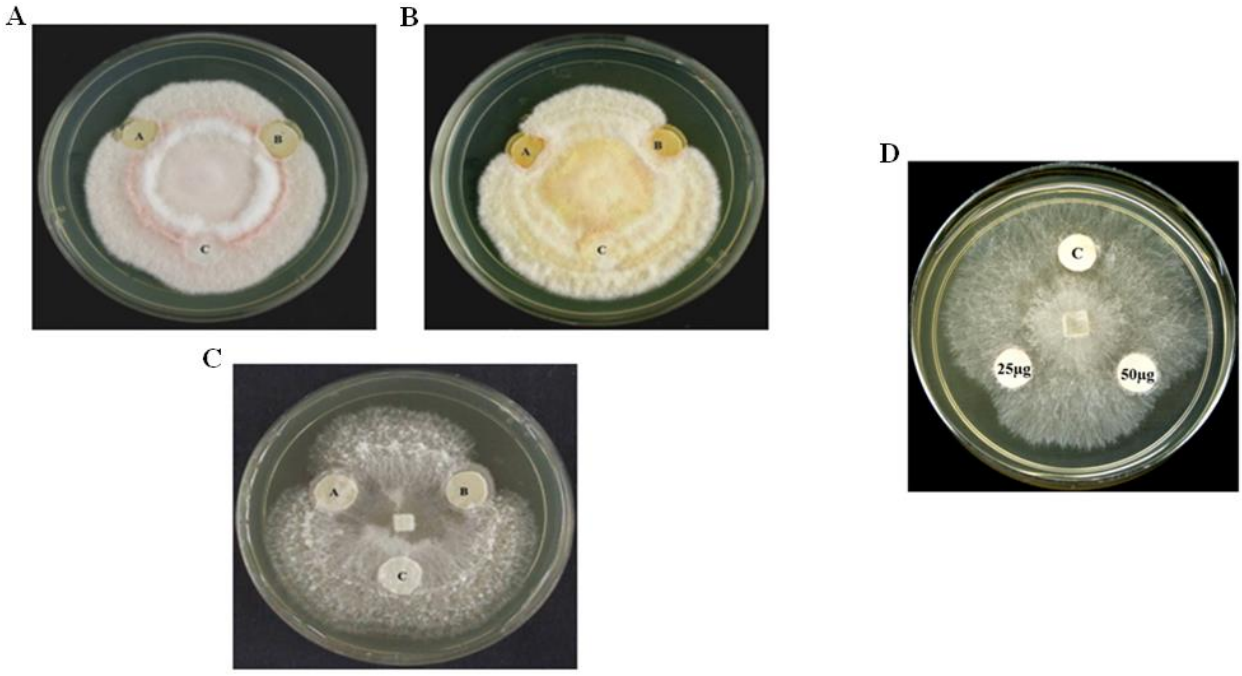
Stability of inhibitory activity of TfgKTI against trypsin and chymotrypsin

The study of the effect of temperature on the TfgKTI stability showed that the trypsin inhibitory activity retained about 90% up to 90 °C but chymotrypsin inhibitory activity retained only ~50% (**Fig. A**). Endurance to high temperature is generally associated with high disulfide content. Pre-incubation of the inhibitor in the pH range of 3.0-10.0 for 30 min shows trivial effect on protease inhibition. Results demonstrated that the inhibitor loses its stability in extreme conditions of pH, remaining active at pH 6.0-8.0 but loss of inhibition activity towards chymotrypsin is greater than trypsin (**Fig. B**). The effect of NaCl on the inhibitory activity of purified trypsin inhibitor is depicted in **Fig. C**. No marked changes in the relative inhibitory activity were found when NaCl was added up to 3.5%, TfgKTI showed high salt stability. In presence of 10mM DTT TfgKTI lost its activity within 60 mins **(Fig. D).** TfgKTI retained over 90% trypsin inhibition upon storage at 4 °C for over a period of six months (data not shown).



Biological activities of Tf-AFP

The antifungal activity of Tf-AFP against fungal species is illustrated in below figure a, b and c. It can be seen that the peptide showed strong antifungal activity towards *Fusarium oxysporum* (**Fig. A**), *Fusarium solani* (**Fig. B**) and *Rhizoctonia solani* (**Fig. C**) but the antifungal activity of Tf-AFP was completely inhibited when 50 mM NaCl was added to the assay buffer. However, it lacks inhibitory activity towards protease enzymes that include trypsin as well as chymotrypsin (data not shown). The antifungal activity of TfgKTI against *Fusarium oxysporum* (**Fig. D**).



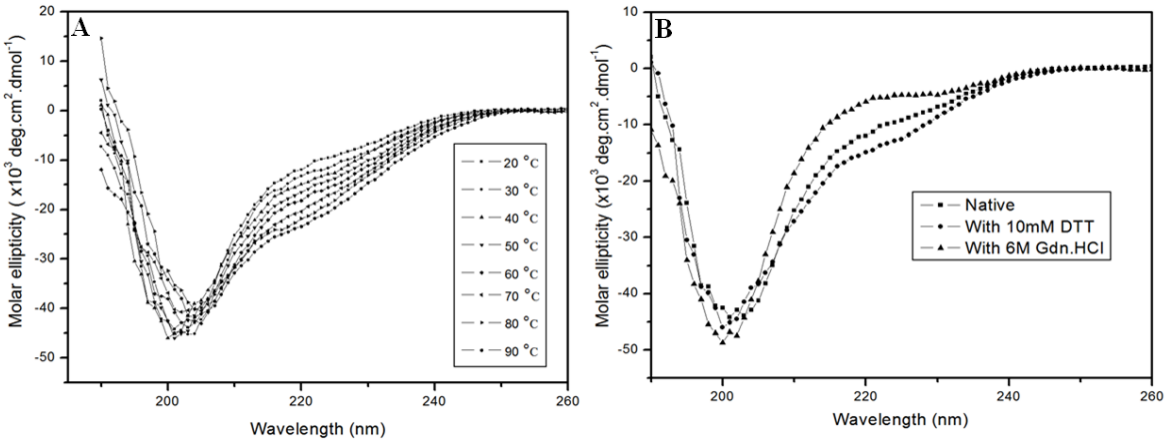
CD Spectroscopic analysis of TfgKTI

Circular Dichroism (CD) spectra of the native TfgKTI (**Fig. A**) exhibited a positive maximum at 235 nm, assigned to disulfide bridge and aromatic side chain contributions, and a minimum at 202 nm, due to contribution of the β-sheet and disordered elements typical of βII class proteins (**Sreerama and Woody, 2003**). Deconvolution of the CD spectra, performed using the CDNN-software shows a high content of β-sheet (39%), unordered structures (48%) and low α-helix content (13%). Based on these results we can deduce that the secondary structure of TfgKTI is predominantly composed of β-sheets and unordered structures with slight helical content, as described for other Kunitz inhibitors (**Chaudhary et al., 2012**). This unordered structure gives flexibility to KTIs to inhibit enzymes of different classes. The TfgKTI retained the back bone

protein folding with no significant change in CD spectra up to 90 °C (**Fig. B**) and wide range of pHs (**Fig. C**) but lost tertiary structure and protein folding in presence of 10 mM DTT within 1 hour (**Fig. D**).

CD Spectroscopic analysis of Tf-AFP

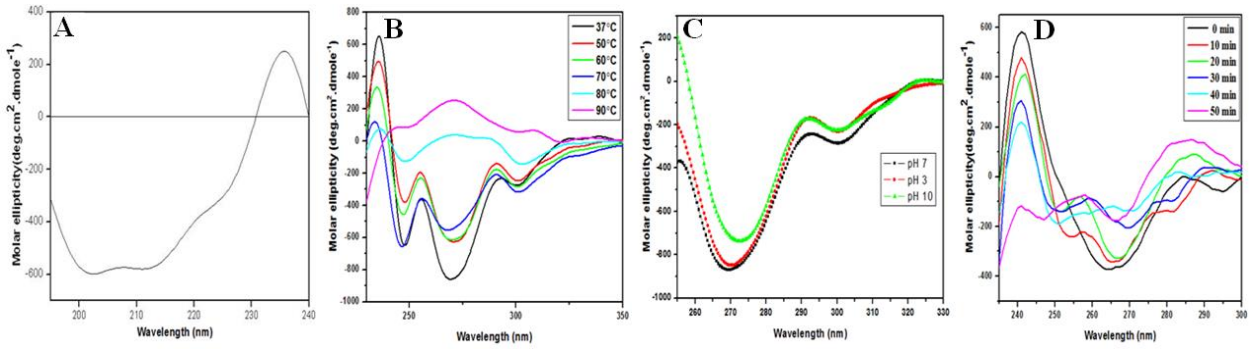
Far-UV CD spectroscopy studies (260–190 nm wavelength range) were carried out to analyse the secondary structure and conformational stability of Tf-AFP at different temperatures from 20 to 90 °C (**Fig. A**). Analysis of CD spectra of native Tf-AFP revealed that high content of β-sheet (42%), low content of α-helix (27%), β-turn (24%) and unordered structure (7%). The CD spectrum exhibited a positive band at 190 nm, corresponding to the formation of antiparallel β-sheet structure. CD studies at increasing temperature demonstrated the thermo stability of Tf-AFP structure. The Tf-AFP retained the back bone protein folding with no significant change in CD spectra up to 90 °C. Tf-AFP also retained back bone protein folding in presence of 10 mM DTT and 6 M Gdn-HCl (**Fig. B**).



Fluorescence studies of TfgKTI

Fluorescence spectra of protein in native, reduced and in presence of denaturant are shown in **Fig. A**. Native Tf-AFP exhibits an emission maximum at (λ_{max}) 339 nm, shifted to higher wavelength (red shift) was observed by reducing with 10mM DTT and in presence of 6 M Gdn-HCl. These results indicate that breaking the disulfide bonds alter in protein structure. Denaturation of TfgKTI with 6M Gdn-HCl showed under native (**Fig.** B) and reduced protein (**Fig. D**). Refolding

of denatured TfgKTI under native condition observed (**Fig. C**) but TfgKTI unable refold once reduced with 10mM DTT.



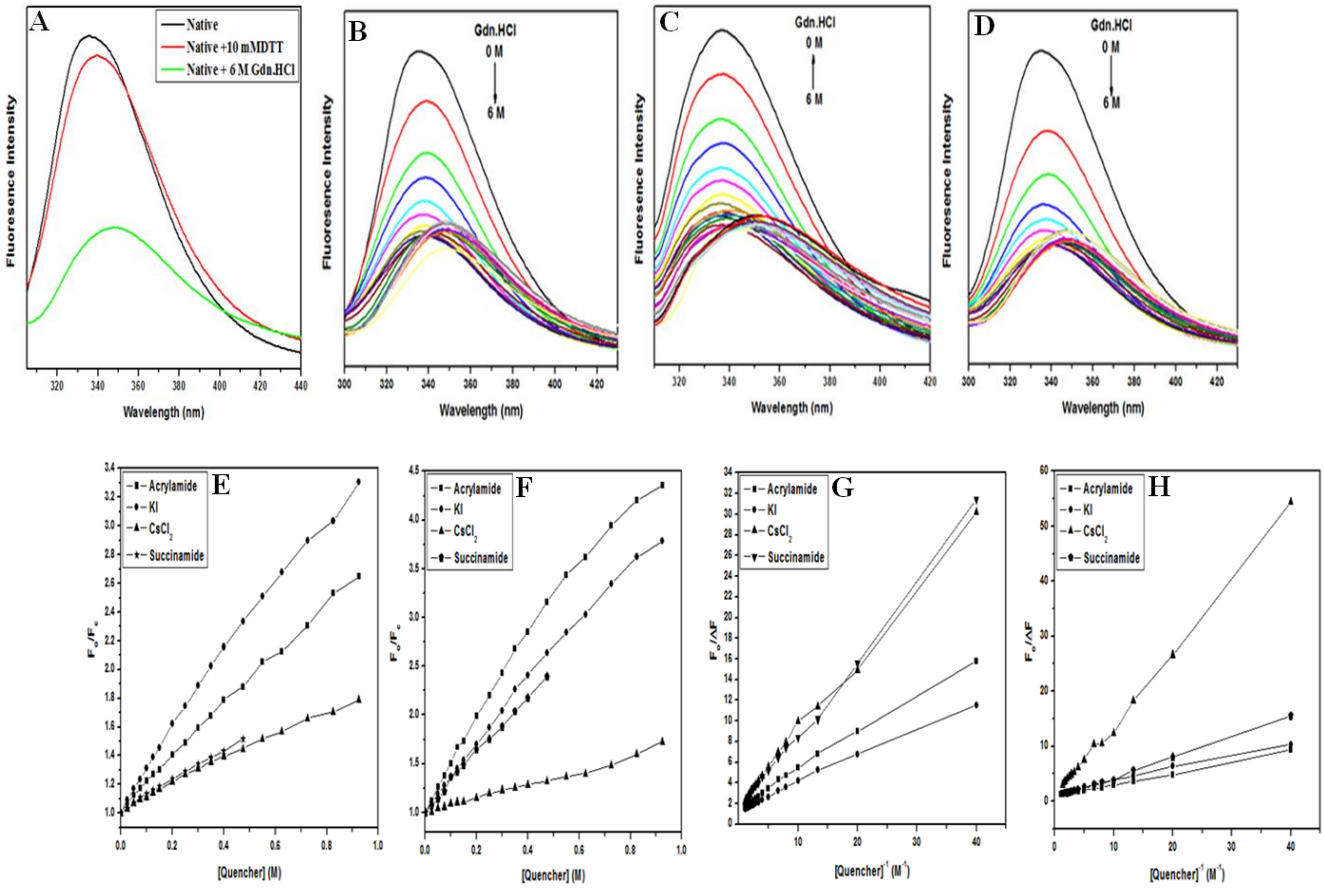
Quenching experiments on native, reduced and denatured Tf-AFP were carried out using different concentrations of acrylamide and succinamide (neutral quencher) and iodide and $CsCl_2$ (ionic quencher). The degree quenching achieved in each case, at a resultant quencher concentration of 0.3 M, shown in below **Table.**

The quenching data was analysed by using Stern–Volmer equation (2) as well as by the modified Stern–Volmer equation (3).

$$F_o/F_c = 1 + K_{sv}[Q] \qquad (2)$$

$$F_o/\Delta F = (f_a)^{-1} + (K_a f_a)^{-1}[Q]^{-1} \qquad (3)$$

Where F_o and F_c are the respective fluorescence intensities, corrected for dilution, in the absence and presence of quencher, respectively, [Q] is the resultant quencher concentration, K_{sv} is the Stern–Volmer quenching constant for given quencher, f_a refers to the fraction of the total fluorescence that is accessible to the quencher and K_a is the corresponding quenching constant. Slopes of Stern–Volmer plots yield K_{sv} values (Eq. 2), whereas the slopes of modified Stern–Volmer plots give $(K_a f_a)^{-1}$ and their ordinate give values of $1/f_a$ (Eq. 3). The values obtained are listed in below Table. The quenching profiles obtained for the native, reduced condition with four quenchers follow a linear dependence on the quencher concentration. Stern–Volmer plots obtained with quenchers under native (**Fig. E)** and reduced conditions **(Fig. F). T**he modified Stern–Volmer plots obtained with quenchers under native (**Fig. G)** and reduced conditions **(Fig. H)**. Trivial changes observed in Quenching constants K_{sv}, K_a and the fraction of total fluorescence that is accessible to the quencher (f_a) under reduced and denatured condition.



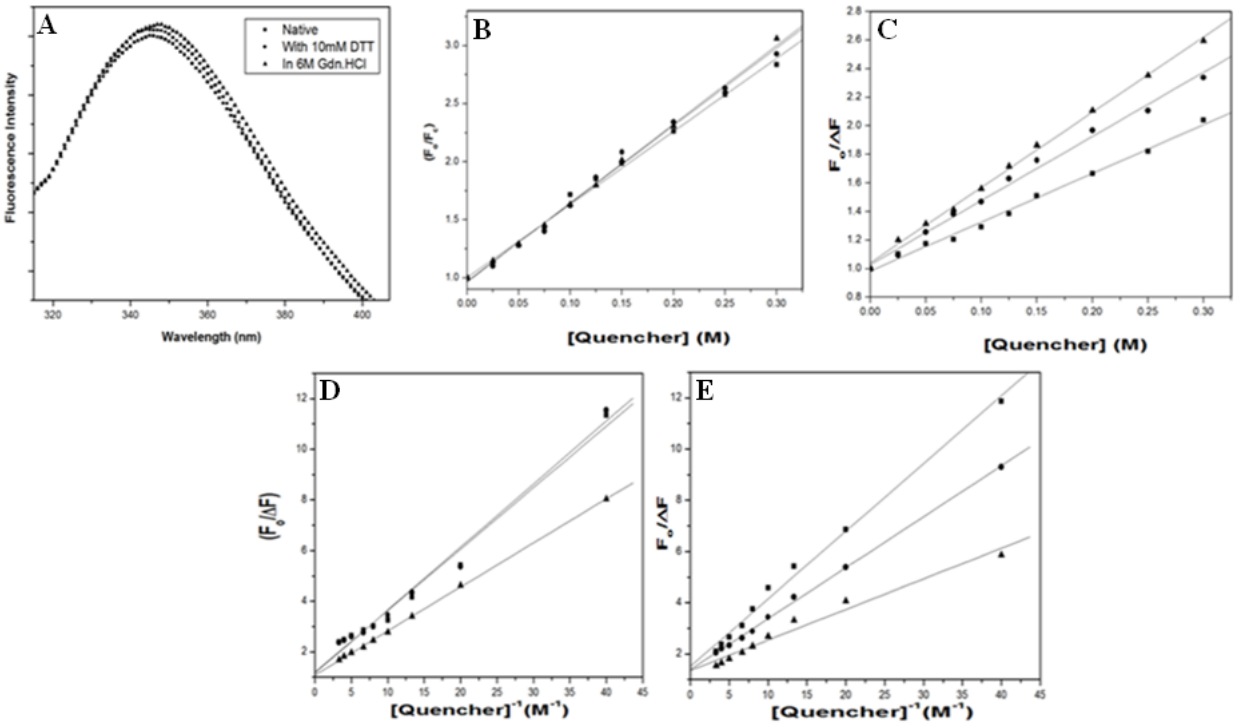
Sample description	Quenching (%)	K_{sv} (M^{-1})	f_a	K_a (M^{-1})
Acrylamide				
Native	64.77	6.30	0.83	5.91
With 10 mM DTT	65.85	6.70	0.85	5.97
Iodide ion (I^-)				
Native	50.99	3.41	0.66	5.70
With 10 mM DTT	57.22	4.49	0.71	6.89
Succinamide				
Native	32.66	1.04	0.68	2.75
With 10 mM DTT	55.79	2.82	0.77	3.5
$CsCl_2$				
Native	30.92	0.85	0.55	2.57
With 10 mM DTT	24.32	0.67	0.52	1.46

Fluorescence studies of Tf-AFP

Fluorescence spectra of protein in native, reduced and in presence of denaturant are shown in **Fig. A**. Native Tf-AFP exhibits an emission maximum at 345 nm, no shift was observed by reducing with 10 mM DTT and trivial red shift observed in presence of 6 M Gdn-HCl. These results indicate that breaking the disulfide bonds or chemical denaturation does not lead to any significant change in tyrosine emission reveals that no alter in protein structure. Quenching experiments on native, reduced and denatured Tf-AFP were carried out using different concentrations of acrylamide (neutral quencher) and iodide (ionic quencher).The intrinsic fluorescence of protein in all conditions quenched by both quenchers without any change in emission wavelength maximum (λ_{max}) at 345 nm. The degree quenching achieved in each case, at a resultant quencher concentration of 0.3 M, shown in **Table.**

The values obtained are listed in below **Table**. The quenching profiles obtained for the native, reduced and denatured condition with acrylamide and iodide ion follow a linear dependence on the quencher concentration. Stern–Volmer plots obtained with acrylamide (**Fig. B**) and iodide (**Fig. C**) under native, reduced and denatured conditions. The modified Stern–Volmer plots obtained with acrylamide (**Fig. D**) and iodide (**Fig. E**) under native, reduced and denatured conditions.. Trivial changes observed in Quenching constants K_{sv}, K_a and the fraction of total fluorescence that is accessible to the quencher (f_a) under reduced and denatured condition.

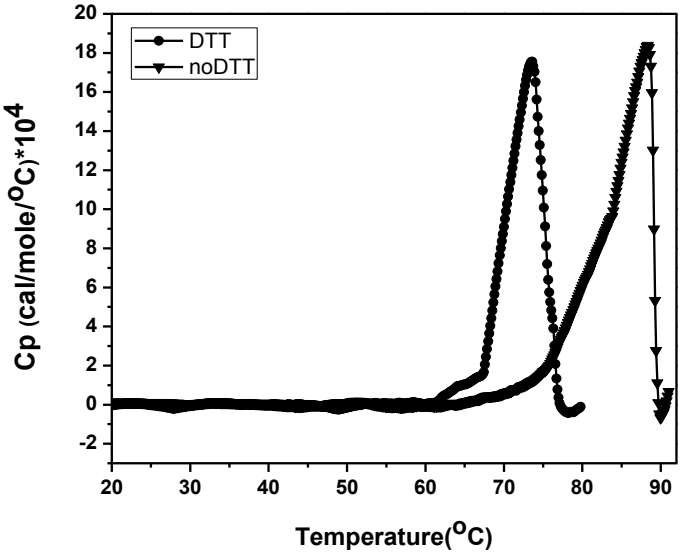
The λ_{max} value of 345 in the fluorescence emission spectrum of Tf-AFP in native, denaturant free solution (pH 6.5) indicates that the Tyr residues of protein molecule are located in a relatively hydrophobic environment. The fluorescence decay profile obtained for reduced by DTT and in presence of 6 M Gdn-HCl remains unaltered to the native protein, indicate do not lead to major changes in environment of tyrosine residues. These results, which are consistent with CD spectral data as well as the quenching experiments, indicate that do not lead any conformational changes resulting from DTT or Gdn-HCl in solution. Between two quenchers used, acrylamide was the effective, quenching 64.77% of the total intrinsic fluorescence of the native protein, whereas ionic quencher iodide quenched 50.99% of total available fluorescence, indicates Tyr residues relatively in a hydrophobic environment. Trivial change in quenching percentage, Stern–Volmer constants and the fractional accessibility (f_a) observed for both quenchers in both reduced and denatured conditions, supports the stability of protein under denaturation conditions.



Sample description	Quenching (%)	K_{sv} (M^{-1})	f_a	K_a (M^{-1})
Acrylamide				
Native	64.77	6.30	0.83	5.91
With 10 mM DTT	65.85	6.70	0.85	5.97
In 6 M Gdn-HCl	61.48	6.79	0.92	6.23
Iodide ion (I^-)				
Native	50.99	3.41	0.66	5.70
With 10 mM DTT	57.22	4.49	0.71	6.89
In 6 M Gdn-HCl	61.48	5.26	0.74	11.29

Differential scanning calorimetry of TfgKTI

In this study, the thermal stability of TfgKTI has been investigated under native and reduced conditions by using high-sensitivity DSC measurements. Below figure shows a typical DSC thermogram of TfgKTI corrected for buffer base line at pH 6.2 and at a scan rate of 30 K h^{-1} along with the fit of the transition data to a non-two-state model. The results obtained, summarized in Table. The Tm values decreased under reducing condition (73.72 °C) compared with native state (87.47 °C).



	T_{m1}	ΔH_{C1} (k cal)	ΔH_{V1} (k cal)	$\Delta H_{C1}/\Delta H_{V1}$	T_{m2}	ΔH_{C2} (k cal)	ΔH_{V2} (k cal)	$\Delta H_{C2}/\Delta H_{V2}$
TI (DTT)	73.72	437.7	284.6	1.53	71.04	552.3	177.3	3.11
TI (noDTT)	87.47	504.1	307.9	1.63	83.01	869.6	104.9	8.3

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