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Isolation, Purification, and Characterization of a Stable Defensin-Like Antifungal Peptide from *Trigonella foenum-graecum* (Fenugreek) Seeds

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Abstract—A novel defensin-like antifungal peptide (Tf-AFP) with molecular mass of 10.3 kDa was isolated from seeds of *Trigonella foenum-graecum* (fenugreek) by ammonium sulfate precipitation, cation-exchange, gel-filtration, hydrophobic chromatography, and RP-HPLC. Mass spectroscopic analysis revealed the intact mass of the purified antifungal peptide as 10321.5 Da and high similarity to plant defensins and other antifungal proteins in database search. 2D-PAGE showed p*I* value to be 8.8 and absence of isoforms. Isolated Tf-AFP inhibited growth of fungal species such as *Fusarium oxysporum*, *Fusarium solani*, and *Rhizoctonia solani*. The antifungal activity was inhibited in the presence of 50 mM NaCl. Circular dichroism analysis demonstrated that the protein is rich in β-sheet structure and highly stable over a wide range of temperatures. Surprisingly, reduction of disulfide bridges and chemical denaturation did not produce large changes in secondary structure as judged by circular dichroism as well as by fluorescence spectroscopy.

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Key words: chromatographic method, defensin-like antifungal peptide, MALDI-TOF-MS, biochemical and biophysical studies, *Trigonella foenum-graecum*

Plants produce a wide array of antimicrobial compounds to protect against attack by pathogens. Antimicrobial peptides are small, mostly basic, cysteine rich peptides that range in size from 2-11 kDa and have been classified into different groups based on their three-dimensional structures [1]. Antimicrobial peptides are pivotal components of innate immunity, constituting an ancient defense mechanism found in diverse organisms. Expression of genes of antimicrobial peptides is promising for engineering of crop protection. The search for novel antimicrobial peptides provides new candidate genes for enhancement of plant resistance to diseases.

A large number of structurally and functionally different antifungal protein defensins, PR-1 proteins, (1,3)β-glucanases, cyclophilin-like proteins, ribosome-inactivating proteins, glycine/histidine-rich proteins, killer proteins (killer toxins), thaumatin-like proteins, protease inhibitors, lipid-transfer proteins (LTPs), chitinases, chitin-binding proteins, and other proteins are elaborated by plants for protection against fungal pathogens. These proteins have been named based on their mechanism of action, structure, or similarity to a known "type" protein [2]. Among these, defensins and defensin-like proteins represent a unique family of antimicrobial peptides present in both animals and plants.

Defensins and defensin-like proteins are relatively abundant in seed tissues to protect seed from soil fungi and thus enhance seedling survival rate. These proteins have also been identified in other plant tissues. Plant defensins and defensin-like proteins exhibit diverse biological activities *in vitro*: antifungal, antibacterial, insecticidal, enzyme inhibitory activity, antiproliferative activity, and blocking of ion channels and protein synthesis [3]. The mode of action of plant defensins has been broadly studied, but the molecular mechanisms are still poorly understood. They are believed to interact with specific sphingolipids on fungal cell membrane and integrate into the phospholipids bilayer of the membrane resulting in fungal membrane permeabilization and destabilization. For some members of the family, penetration through the membrane into the cytoplasm and interaction with intracellular targets has been demonstrated [4].

Fenugreek is an annual herb belonging to the family Fabaceae. The seeds have also been reported to improve

Abbreviations: CHCA, α-cyano-4-hydroxycinnamic acid; Tf-AFP, *Trigonella foenum-graecum* antifungal peptide.

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diabetic conditions, exhibit antioxidant properties, and control plasma cholesterol level [5]. In view of the economic importance of antifungal proteins and peptides in combating fungal invasion, which can bring about massive destruction of agricultural crops, the aim of this study was to purify and characterize a novel antifungal peptide from *Trigonella foenum-graecum* (fenugreek) seeds, and to ascertain the type of antifungal protein group to which it belongs.

MATERIALS AND METHODS

Materials. Seeds of fenugreek were purchased locally. Chromatographic supports and 2D-PAGE reagents were purchased from GE Healthcare (GB). The Luna C18 HPLC column was purchased from Phenomenex (USA). SDS-PAGE molecular weight markers were purchased from Fermentas (Germany) and Genetix (India). Stirred ultrafiltration cell and 3-kDa cutoff membranes were purchased from Millipore (India). All other reagents of analytical or HPLC grade were purchased from Sigma-Aldrich (USA).

Isolation and purification of *T. foenum-graecum* antifungal peptide (Tf-AFP). *Trigonella foenum-graecum* seeds (100 g) free of seed coat were ground, and the flour was defatted by washing with hexane followed by four parts (w/v) of $CHCl_3$–EtOH (2 : 1) for 2 h. Defatted flour was then extracted with 25 mM sodium phosphate buffer, pH 6.5, containing 50 mM NaCl, 4 mM EDTA, and 0.01% azide (1 : 10 w/v) for 36 h at 4°C. The homogenate was filtered through muslin cloth, and the filtrate was centrifuged at 17,226*g* for 20 min at 4°C. The supernatant was designated as crude extract. The crude extract sample was first fractionated by ammonium sulfate precipitation, in which the crude solution was treated with ammonium sulfate to 20% saturation. The resulting supernatant was then adjusted to 70% saturated ammonium sulfate. After centrifugation at 17,226*g* for 30 min at 4°C, the supernatant was discarded, and the precipitate was collected and dissolved in 20 mM sodium phosphate buffer (pH 6.2), dialyzed against same buffer with several changes using 3.5 kDa cutoff dialysis membrane (Spectra/Por6), and applied on XK 16/20 CM-Sepharose using Fast Protein Liquid Chromatography (FPLC-AKTA Prime Plus; GE Healthcare Life Sciences, Sweden) system, previously equilibrated with 20 mM sodium phosphate buffer, pH 6.2. Following the elution of non-absorbed proteins, absorbed proteins were eluted with a linear gradient of NaCl (0-1 M) in the same buffer at a constant flow of 0.4 ml/min. The absorbance was monitored at 280 nm. The absorbed fraction (CM2) demonstrating antifungal activity was pooled, dialyzed against 25 mM sodium phosphate buffer, pH 6.5, at 4°C overnight, and concentrated in a stirred ultrafiltration cell using membrane with pore size of 3 kDa. This fraction was then applied to size-exclusion chromatography on XK 16/100 column of Sephadex G-50 pre-equilibrated with 25 mM sodium phosphate buffer, pH 6.5. Fractions (3 ml) from the protein peaks were collected at constant flow of 0.3 ml/min. The fractions (S3) showing antifungal activity were pooled, concentrated, and dialyzed against 50 mM sodium phosphate buffer, pH 7.0, containing 1 M $(NH_4)_2SO_4$ and then applied to an XK 16/20 hydrophobic column of Octyl-Sepharose CL-4B pre-equilibrated with the same buffer. The column was eluted with a gradient of the buffer and $(NH_4)_2SO_4$, pH 7.0 (10 ml of 50 mM buffer, 70 ml of 50-15 mM buffer). Fractions (5 ml) were collected at constant flow of 0.5 ml/min. The fractions showing high antifungal activity (P1) were subjected to reverse-phase high-performance liquid chromatography (RP-HPLC) on a C18 column (Phenomenex; 10 × 250 mm; 5 μm particle; 100 Å pores) using a HPLC system (Shimadzu, Japan). The column was pre-equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA) and then eluted with a linear gradient of acetonitrile (0-30% for 20 min, 30-50% for 40 min, 50-70% for 20 min, 70-100% for 1 min) in 0.1% TFA, at a constant flow of 2 ml/min. The eluent was monitored at 220 nm, and the peaks were collected manually and dialyzed against 20 mM sodium phosphate buffer, pH 6.5, with several changes, and then used for assays.

Protein quantification, SDS-PAGE, and two-dimensional electrophoresis (2D-PAGE). Protein concentration was determined by a dye-binding method [6] using bovine serum albumin as the standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (15% acrylamide, 4% methylene bis-acrylamide) was performed by the method of Laemmli [7]. Protein bands were visualized by either 0.1% Coomassie Brilliant Blue R-250 or the silver staining method [8]. For the identification of p*I* value, purified Tf-AFP was first separated by isoelectric focusing in an 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-cholamidopropyl (dimethylammonio)]-1-propanesulfonate (CHAPS), 1 M DTT, 1% bromophenol blue, and 0.05% (v/v) IPG buffer 3-10 (GE Healthcare). The isoelectric focusing (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 buffer solution containing 1 M Tris-HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, and 2% (w/v) SDS [9]. The second dimension was performed on polyacrylamide gels of the same composition [7].

Molecular mass determination by mass spectrometry. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) was performed using an Autoflex III mass spectrometer (Bruker Daltonics, Germany). Spectra were recorded in the positive-ion linear mode of operation with accelerating voltage of 20 kV, grid voltage of 86%, and delay time of

200 ns. Samples were prepared by mixing equal volumes of 0.1% TFA, acetonitrile (1 : 1), and the protein solution. A 2-μl portion 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 the 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) according to the method described by Shevchenko et al. [10]. Coomassie R-250 stained 1-D SDS gel bands were manually excised from three reproducible gels. The excised gel pieces were destained with five 100-μl portions of 50% acetonitrile in 25 mM ammonium bicarbonate (NH_4HCO_3). Thereafter, the gel pieces were treated with 10 mM DTT in 25 mM NH_4HCO_3 and incubated at 56°C for 1 h. This was followed by treatment with 55 mM iodoacetamide in 25 mM NH_4HCO_3 for 45 min at room temperature (25 ± 2°C), washing with 25 mM NH_4HCO_3 and acetonitrile, and drying in a vacuum concentrator, and rehydration in 20 μl of 25 mM NH_4HCO_3 solution containing 12.5 ng/μl trypsin (sequencing grade; Promega, USA). This mixture was incubated on ice for 10 min and kept overnight for digestion at 37°C. After digestion, a short spin for 10 min was given and the supernatant was collected in a fresh Eppendorf tube. The gel pieces were re-extracted with 50 μl 1% TFA and acetonitrile (1 : 1) for 15 min with frequent vortexing. The supernatants were pooled, dried using a speed vacuum concentrator, and then reconstituted in 5 μl of acetonitrile and 1% TFA (1 : 1). An aliquot (2 μl) of the above sample was mixed with 2 μl of freshly prepared CHCA matrix in 50% acetonitrile and 1% TFA (1 : 1), and 1 μl was spotted on the 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, a grid voltage of 86%. Flex Analysis software was used for data processing.

Proteins were identified by database searches (PMF, peptide mass fingerprinting) using the 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 the NCBInr and SwissProt databases. 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}, monoisotopic, and missed cleavage – 1. According to the MASCOT probability analysis ($P < 0.05$), only significant hits were accepted for protein identification.

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 h at 24°C in the case of *Rhizoctonia solani*. However, the plates were incubated for 24 h 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; GE Healthcare Life Sciences) were placed at a distance of 0.5 cm around the frontal mycelia. Different concentrations of the Tf-AFP protein sample were added to each disk. The plates were incubated at 24°C for approximately 36-48 h until mycelial growth had enveloped peripheral discs containing control buffer (20 mM sodium phosphate buffer, pH 6.5) and had formed a crescent of inhibition around the disc containing an effective concentration of antifungal agent. The effect of ionic strength on antifungal activity was assayed by adding 50 mM NaCl to the Tf-AFP protein sample.

CD spectroscopy. Circular dichroism was measured using a Jasco J-810 spectropolarimeter equipped with a Peltier-type temperature controller and a thermostatted cell holder interfaced with a thermostatic bath. Far-UV CD spectra (190-260 nm) were recorded in a 1-cm path-length quartz cell at protein concentration of 5 μM. 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. The CD spectra were background corrected. Secondary structure analysis was calculated using CDNN software.

Fluorescence spectroscopy. Fluorescence was measured with a Fluoromax-4 spectrofluorometer with 3-nm bandwidth 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 280 nm. The temperature of the cell was maintained at 25°C. Reaction mixtures with protein absent were used to correct for baseline. For fluorescence quenching studies, protein solution with absorbance less than 0.1 was prepared in 10 mM sodium phosphate buffer (pH 6.5) without and with DTT (10 mM final concentration). For denatured Tf-AFP, 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 (acrylamide or potassium iodide), 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 thiosulfate to prevent the formation of triiodide (I_3^-). Since acrylamide has intrinsic absorption at 280 nm,

the inner-filter effect was corrected using the equation $F_{corr} = 10^{A/2}F$, where A is the increase in absorbance at the center of the cuvette upon acrylamide addition. Since KI had no significant absorption at the excitation wavelength, no correction was necessary.

RESULTS AND DISCUSSION

Isolation and purification of Tf-AFP. A detailed study of the antimicrobial proteins from a defined source could provide important information on how plants build their winning strategy through evolution and assist our design strategy for plant disease control. In *Arabidopsis thaliana*, at least 13 putative plant defensin genes are present, encoding 11 different plant defensins. Two additional genes appear to encode proteins containing a plant defensin domain [11]. Previous work indicated that in some tested plant samples there are multiple antimicrobial proteins, and it has been shown that there is possible synergy among the different purified antimicrobial proteins [12]. We believe that our effort should provide further information on the array of antifungal proteins used by the plants to protect themselves from pathogen attacks from the environment.

In the present study, the antifungal peptide Tf-AFP was purified by ammonium-sulfate precipitation and cation-exchange, gel-filtration, and hydrophobic chromatographies and RP-HPLC. The purification procedure used is simpler and efficient for separating antifungal peptide from other bioactive proteins such as protease inhibitors and lectins. The ammonium sulfate precipitated (20-70%) soluble protein fraction from *T. foenum-graecum* seed flour crude extract was chromatographed on a cation-exchange column (CM-Sepharose) equilibrated with 20 mM phosphate buffer, pH 6.2. Under these conditions, most proteins were eluted with the equilibration buffer (peak CM1). The fractions eluted with saline buffer (peak CM2) contained antifungal activity (Fig. 1a). Tf-AFP was adsorbed on the cation exchanger, like many

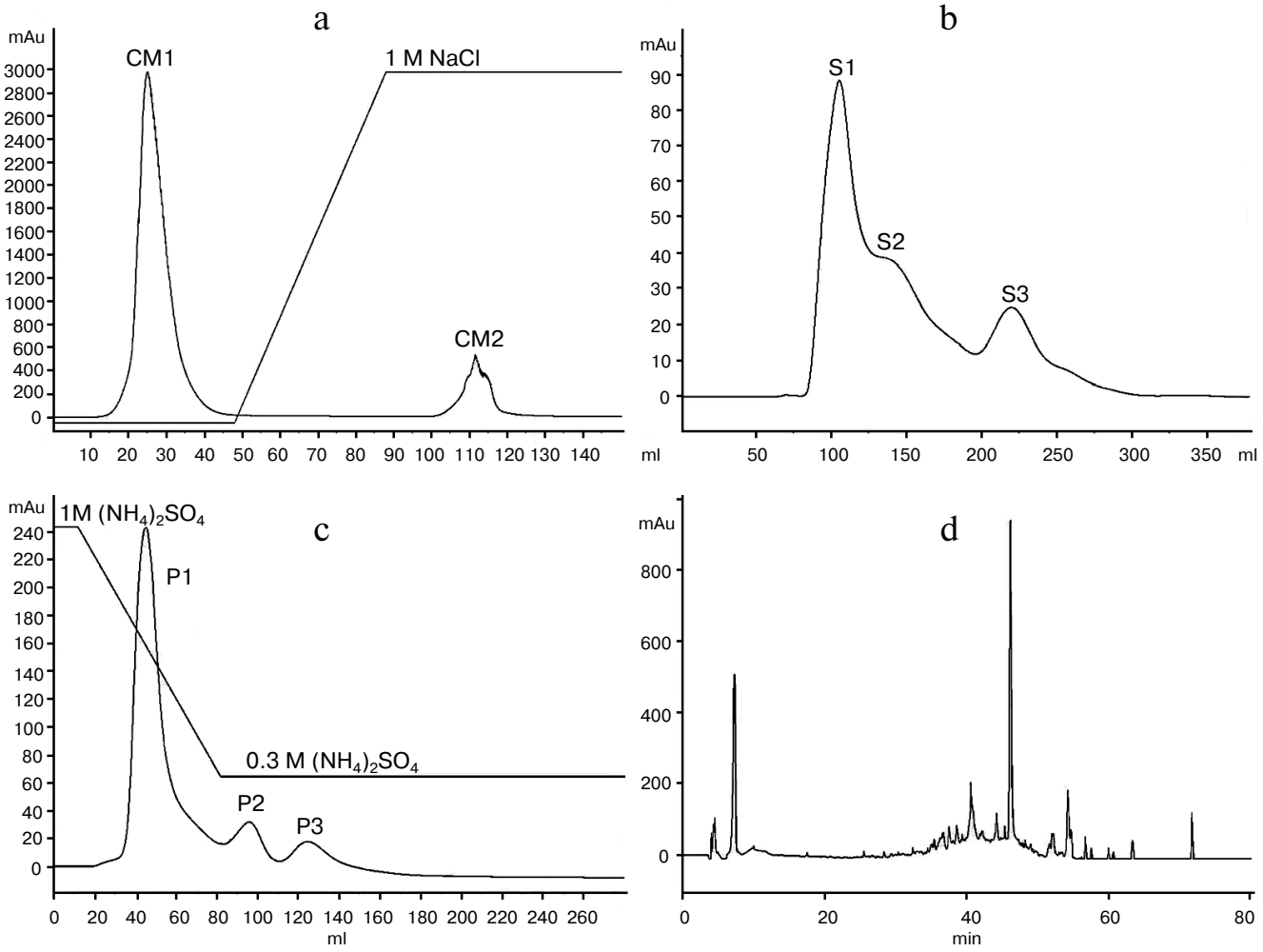

Fig. 1. Elution profiles for fenugreek seed extracts during cation-exchange (a), gel-filtration (b), Octyl-Sepharose CL-4B (c), and RP-HPLC (d) chromatographies.

Table 1. Purification of Tf-AFP from 100 g of dried seeds

Purification stage	Total protein, mg	Yield, %
Crude extract	3600	100
Ammonium sulfate precipitate	2320	64.5
Ion exchange	303	8.4
Gel filtration	10.9	0.30
Hydrophobic chromatography	5.8	0.16
RP-HPLC	3.5	0.10

other antifungal proteins [13]. Peak CM2 fractions were pooled, dialyzed against 25 mM sodium phosphate buffer, pH 6.5, and separated by size-exclusion chromatography on Sephadex G-50 into three peaks – S1, S2, and S3 (Fig. 1b). Antifungal activity was highest in the smallest fraction S3. Afterwards, the fractions of peak S3 were pooled, concentrated, and further resolved on a hydrophobic column of Octyl-Sepharose CL-4B (Fig. 1c). Fraction P1, showing highest antifungal activity, was compared to the two other peaks (P2 and P3). Fraction P1 was finally purified by RP-HPLC. The antifungal peptide was eluted in 43% acetonitrile in RP-HPLC and named as Tf-AFP (Fig. 1d). The yield of Tf-AFP was 3.5 mg/100 g seeds (Table 1). The yield of purified Tf-AFP is 0.1% of total seed protein. In radish, antifungal proteins (Rs-AFPs) represent only 0.5% of the total soluble proteins in seeds. This quantity of protein is sufficient to suppress fungal growth in soil [14].

Molecular mass and p*I* determination. The molecular mass of the isolated antifungal peptide Tf-AFP was determined by SDS-PAGE and mass spectrometry. RP-HPLC-purified Tf-AFP showed a unique band on SDS-PAGE (Fig. 2a) with a molecular mass of approximately 10 kDa, which is in accordance with the results of mass spectrometry showing the molecular mass for singly charged $[M + 1H]^{+1}$ molecular ion at m/z 10321.5 Da and the doubly charged $[M + 2H]^{+2}$ molecular ion at m/z 5161.9 Da (Fig. 3a). The isoelectric point (p*I*) of the peptide was determined as 8.8, based on linear pH range 3-10 of isoelectric focusing electrophoresis conducted by two-dimen-

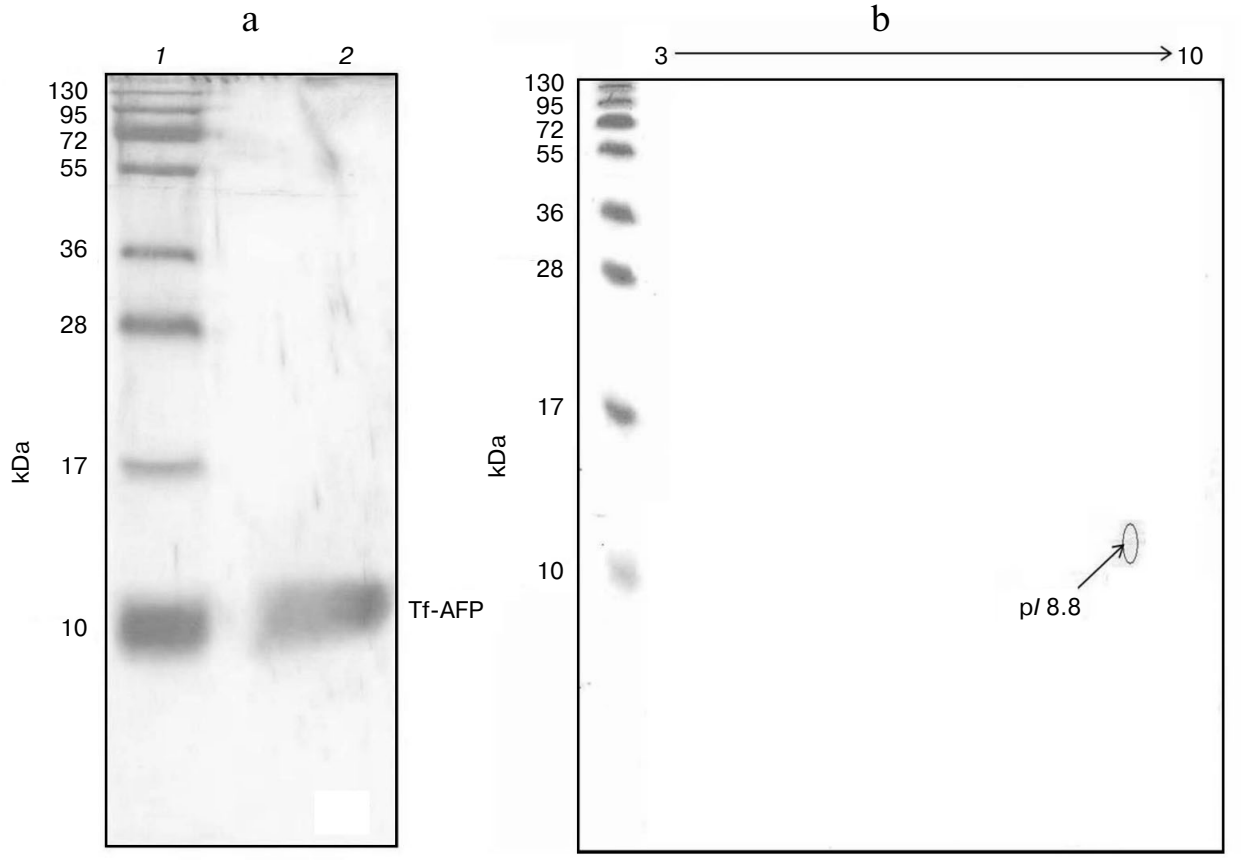

Fig. 2. a) SDS-PAGE profile of Tf-AFP. Lanes: *1*) molecular mass markers; *2*) RP-HPLC-purified Tf-AFP. b) Coomassie stained 2D-gel electrophoresis gel map of RP-HPLC-purified Tf-AFP. IEF was performed on linear pH 3-10 IPG strips.

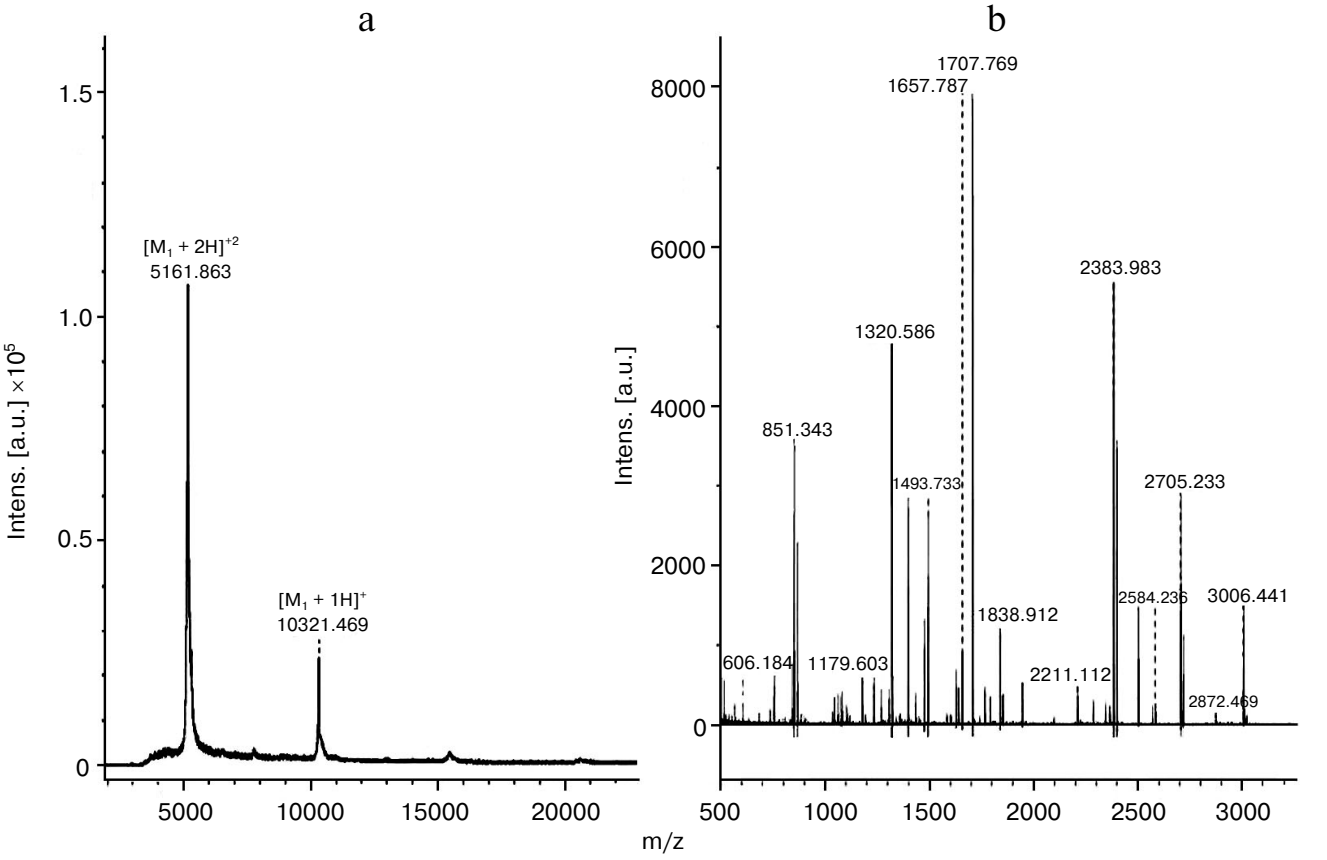

Fig. 3. a) Determination of Tf-AFP molecular mass by MALDI-TOF-MS spectrometry. b) MALDI-TOF-MS spectra of peptides generated from tryptically digested Tf-AFP.

sional electrophoresis shown in Fig. 2b. The results of basic p*I* demonstrated that the newly reported peptide has basic character and a single spot showing absence of isoforms. The extinction coefficient of Tf-AFP of 1% aqueous solution at 280 nm was 46.4 $M^{-1}\cdot cm^{-1}$. These results closely resemble the earlier reported antifungal proteins [15, 16].

MALDI-TOF-MS analysis. The peptide mass fingerprinting (PMF) of the 1-D SDS-PAGE gel bands of purified Tf-AFP were acquired by MALDI-TOF-MS using CHCA as the matrix. The MALDI-TOF-MS spectrum was generated from in-gel trypsin digestion (Fig. 3b). Furthermore, analyses by the MASCOT search program in the NCBInr database suggested sequence highly similar with defensins, antifungal protein precursor, and putative defensin 1.5 precursor. The observed monoisotopic mass of tryptically digested peptides obtained by MALDI-TOF-MS and their position with respect to matched protein sequence are presented in Table 2. The matched peptides of Tf-AFP covered 58% of defensin (*Trigonella foenum-graecum*, gi31324677) with score 85, 48% of antifungal protein precursor (*Medicago sativa*, gi11762086), putative defensin 1.5 precursor (*Medicago sativa*, gi56267921), and 48% of defensin (*Medicago truncatula*, gi37362318) with score 53. This Tf-AFP showed greater p*I* value than previously reported defensins of this source indicating Tf-AFP has a high positive charge. Increased positive charge increases the antifungal activity of defensins in radish seeds [17].

Antifungal activity of Tf-AFP. Overexpression of wasabi defensin (WT1) in rice, potato, and orchid resulted in increased resistance against *Magnaporthe grisea*, *Erwinia carotovora*, and *Botrytis cinerea* [18]. Defensin TvD1 from the wild legume *Tephrosia villosa* exhibited antifungal and anti-insect properties upon expression in transgenic tobacco plants. Similarly, a mustard defensin enhanced the resistance of transgenic tobacco and peanut plants against some fungal pathogens [19]. The antifungal proteins like plant defensins are also useful as antifungal therapeutics in humans [20]. The isolated Tf-AFP displayed antifungal activity against fungal species is illustrated in Fig. 4. It can be seen that the peptide showed strong antifungal activity towards *Fusarium oxysporum* (Fig. 4a), *Fusarium solani* (Fig. 4b), and *Rhizoctonia solani* (Fig. 4c). The antifungal activities of Tf-AFP greatly decreased when 50 mM NaCl was added to the assay buffer. This phenomenon was also observed in other types of defensins. High ionic strength interferes with the interaction of the positively charged defensin with the

Table 2. Summary of matched peptides of Tf-AFP analyzed by MALDI-TOF-MS

Protein name/ accession number	Theoretical p*I*	Score	Peptide mass, Da	Position	Matched peptides
Defensin (*Trigonella foenum-graecum*, 31324677)	8.1	85	754.32	66-70	CWCTK
			755.35	53-59	EHAVSGR
			868.37	60-65	CRDDFR
			1269.59	29-38	TCENLADKYR
			1627.62	39-52	GPCFSGCDTHCTTK
			1946.8	37-52	YRGPCFSGCDTHCTTK
			2364.0	39-59	GPCFSGCDTHCTTKEHAVSGR
Antifungal protein precursor (*Medicago sativa*, 11762086)	8.1	53	754.32	66-70	CWCTK
			868.37	60-65	CRDDFR
			1269.59	29-38	TCENLADKYR
			1627.62	39-52	GPCFSGCDTHCTTK
			1946.81	37-52	YRGPCFSGCDTHCTTK
Putative defensin 1.5 precursor (*Medicago sativa*, 56267921)	8.1	53	754.32	66-70	CWCTK
			868.37	60-65	CRDDFR
			1269.59	29-38	TCENLADKYR
			1627.62	39-52	GPCFSGCDTHCTTK
			1946.80	37-52	YRGPCFSGCDTHCTTK
Defensin (*Medicago truncatula*, 37362318)	8.09	53	754.32	66-70	CWCTK
			868.37	60-65	CRDDFR
			1269.59	29-38	TCENLADKYR
			1627.62	39-52	GPCFSGCDTHCTTK
			1946.81	37-52	YRGPCFSGCDTHCTTK

Table 3. Summary of parameters obtained from Stern–Volmer and modified Stern–Volmer analysis of the intrinsic fluorescence quenching with acrylamide and iodide quenchers of *T. foenum-graecum* seed Tf-AFP

Sample	Quencher	Quenching, %	K_{sv}, M^{-1}	f_a	K_a, M^{-1}
Native Tf-AFP	acrylamide	64.8	6.30	0.83	5.91
+ 10 mM DTT	–"–	65.8	6.70	0.85	5.97
+ 6 M Gdn-HCl	–"–	61.5	6.79	0.92	6.23
Native Tf-AFP	iodide	51.0	3.41	0.66	5.70
+ 10 mM DTT	–"–	57.2	4.49	0.71	6.89
+ 6 M Gdn-HCl	–"–	61.5	5.26	0.74	11.3

negative microbial membrane surface resulting in loss of antifungal activity [21]. Tf-AFP does not show any protease inhibition activity towards trypsin as well as chymotrypsin like some other antifungal proteins.

Secondary structural analysis. Far-UV CD spectroscopy studies (260-190 nm wavelength range) were carried out to analyze the secondary structure and conformational stability of Tf-AFP at different temperatures from 20 to 100°C (Fig. 5a). The solution structure of Rs-AFP1 was determined by nuclear magnetic resonance, which revealed a compact globular structure comprised of three antiparallel β-sheets and one α-helix that is stabi-

lized by a structural motif composed of disulfide bridges [22]. Similar results were obtained for Tf-AFP, which shows high content of antiparallel 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 temperatures in the range 20-90°C demonstrated the thermostability of the Tf-AFP structure. Tf-AFP retained the backbone protein folding with no significant change in CD spectra up to 90°C. In plant defensins, four disulfide bridges are formed by eight Cys residues, which are likely to play an important role in structure stabilization [23]. However, reducing disulfide bridges of Tf-AFP retained the secondary structure with no significant change in CD spectra. Tf-AFP thus has unusual stability towards reducing agent DTT (10 mM) as well as denaturant Gdn-HCl (6 M) (Fig. 5b).

Fluorescence studies. Intrinsic fluorescence of proteins is valuable for investigating conformational and structural changes that occur in a protein under a variety of conditions [24]. Fluorescence spectra of the protein in native and reduced states and in presence of the denaturant are shown in Fig. 6. The λ_{max} value of 345 nm 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. Shift of the λ_{max} was not observed upon reducing with 10 mM DTT, and only a slight red shift was observed in the presence of 6 M Gdn-

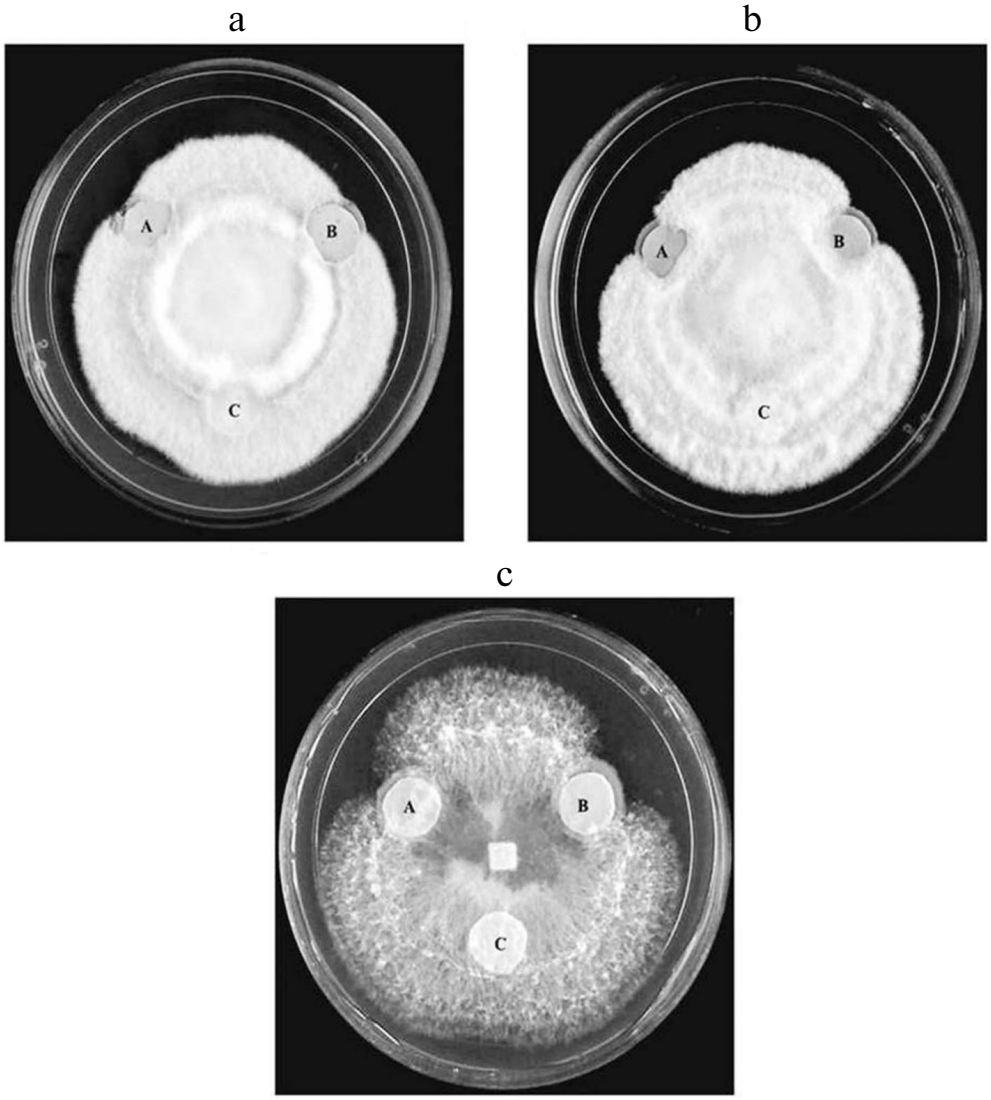

Fig. 4. Inhibitory activity of purified antifungal peptide on mycelial growth of *Fusarium oxysporum* (a), *Fusarium solani* (b), and *Rhizoctonia solani* (c). The samples were as follows: A – 25 μg of Tf-AFP in buffer (20 mM sodium phosphate, pH 6.5); B – 50 μg of Tf-AFP in the same buffer; C – buffer only.

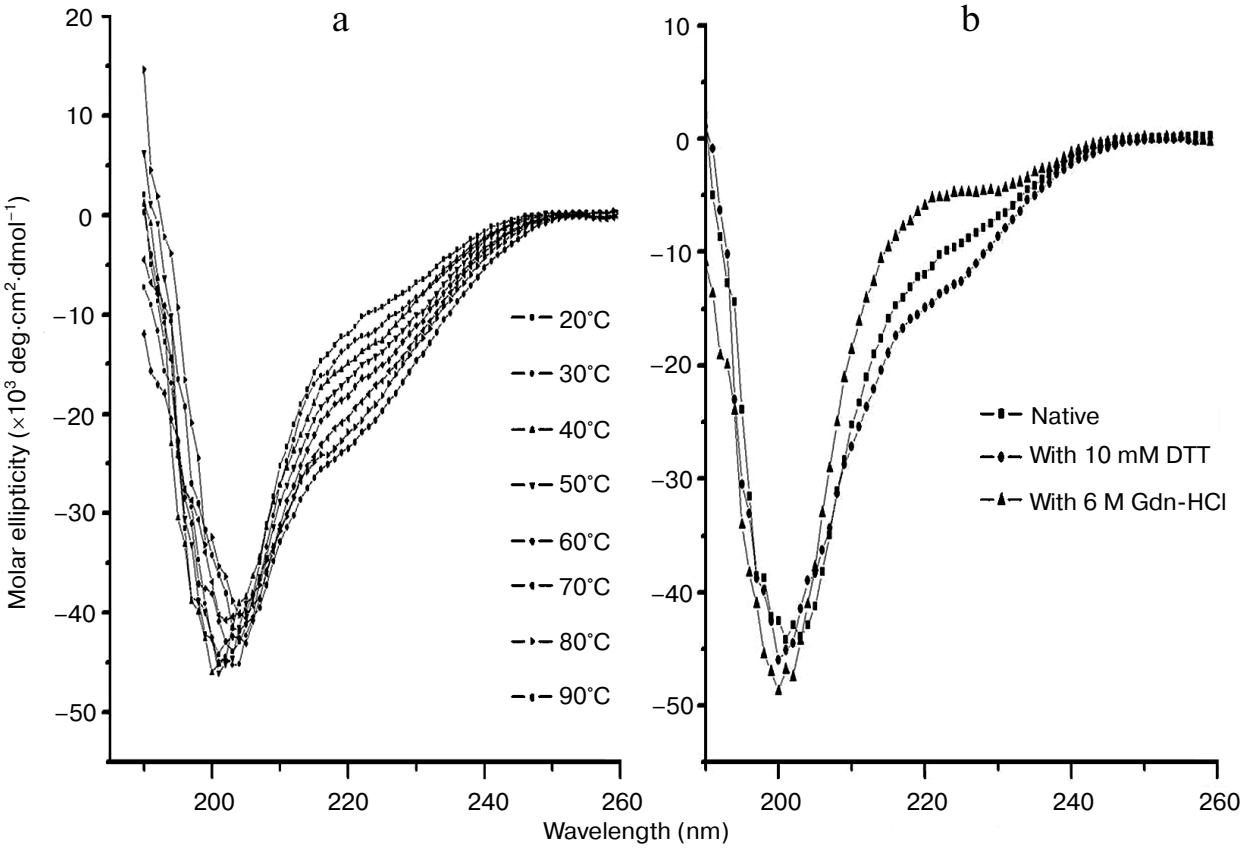

Fig. 5. a) Far-UV CD scans of Tf-AFP at temperatures ranging from 22-90°C. Each sample was incubated at the respective temperature for 10 min, and then spectra were recorded. b) Far-UV CD spectra of native, reduced, and denatured Tf-AFP.

HCl. These results indicate that breaking the disulfide bonds or chemical denaturation does not lead to significant change in tyrosine emission and, hence, 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 the protein under all conditions was quenched by both quenchers without any change in λ_{max}. The quenching degrees achieved in each case at quencher concentration of 0.3 M are shown in Table 3. Of the two quenchers used, acrylamide was more effective (64.8% quenching) than the ionic quencher iodide (51.0% quenching), which indicates that Tyr residues are in a relatively hydrophobic environment.

The quenching data were analyzed using the Stern–Volmer equation (1) and the modified Stern–Volmer equation (2) [25]:

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

$$F_o/\Delta F = (f_a)^{-1} + (K_a f_a)^{-1}[Q]^{-1}, \quad (2)$$

where F_o and F_c are the fluorescence intensities corrected for dilution in the absence and presence of quencher, respectively; [Q] is quencher concentration; K_{sv} is the Stern–Volmer quenching constant for a given quencher; f_a refers to the fraction of the total fluorescence that is accessible to the quencher, and K_a is the corresponding

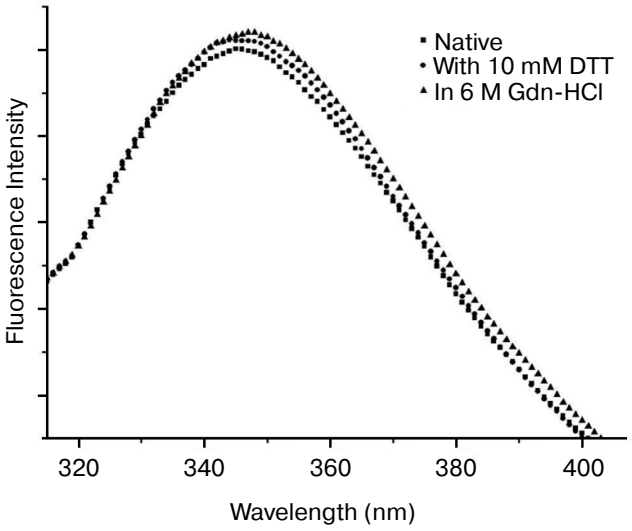

Fig. 6. Fluorescence spectra of Tf-AFP in the native state, reduced with 10 mM DTT, or denatured with 6 M Gdn-HCl.

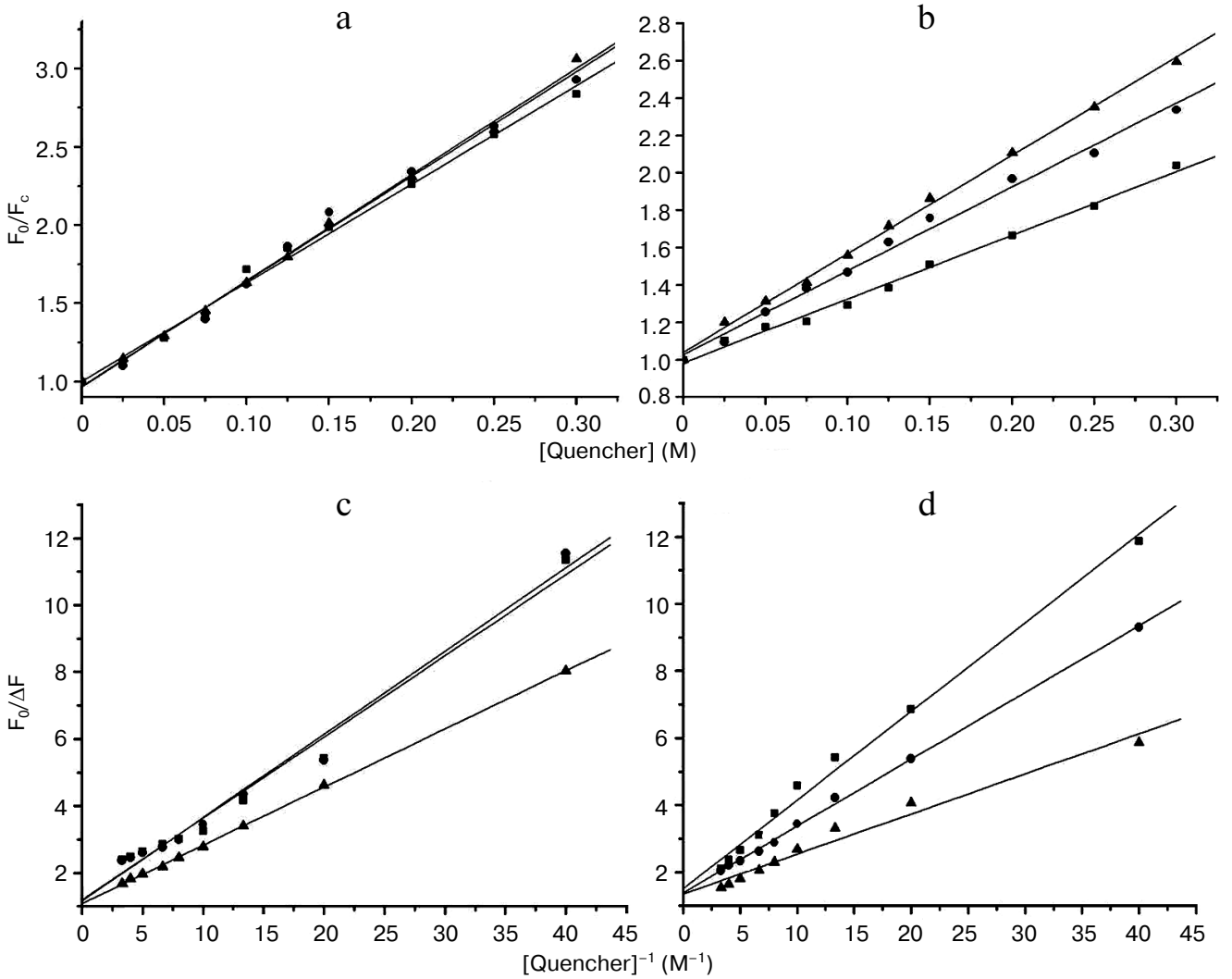

Fig. 7. Stern–Volmer plots of fluorescence quenching data for Tf-AFP with acrylamide (a) and iodide ion (b). Modified Stern–Volmer plots of fluorescence quenching data for Tf-AFP with acrylamide (c) and iodide ion (d). Symbols: native protein (squares), protein reduced with 10 mM DTT (circles), protein denatured with 6 M Gdn-HCl (triangles).

quenching constant. The slopes of the Stern–Volmer plots yielded K_{sv} values (Eq. (1)), whereas the slopes of modified Stern–Volmer plots gave $(K_a f_a)^{-1}$ and their ordinate gave $1/f_a$ values (Eq. (2)). These values are listed in Table 3. The quenching profiles obtained for the native, reduced, and denatured condition with acrylamide and iodide ion follow a linear dependence on quencher concentration. Stern–Volmer plots obtained with two quenchers are shown in Fig. 7 (a and b) and the modified Stern–Volmer plots obtained with two quenchers are shown in Fig. 7 (c and d). Minor changes were observed in the 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. These results, which are consistent with the CD spectral data, indicated no significant conformational changes induced by DTT or Gdn-HCl in solution.

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