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Trypsin Inhibitor from Edible Mushroom *Pleurotus floridanus* Active against Proteases of Microbial Origin

P. P. Manzur Ali · K. Sapna · K. R. Rekha Mol · Sarita G Bhat · M. Chandrasekaran · K. K Elyas

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Abstract Protease inhibitors can be versatile tools mainly in the fields of medicine, agriculture and food preservative applications. Fungi have been recognized as sources of protease inhibitors, although there are only few such reports on mushrooms. This work reports the purification and characterization of a trypsin inhibitor from the fruiting body of edible mushroom *Pleurotus floridanus* (PfTI) and its effect on the activity of microbial proteases. The protease inhibitor was purified up to 35-fold by DEAE-Sepharose ion exchange column, trypsin-Sepharose column and Sephadex G100 column. The isoelectric point of the inhibitor was 4.4, and its molecular mass was calculated as 37 kDa by SDS-PAGE and 38.3 kDa by MALDI-TOF. Inhibitory activity confirmation was by dot-blot analysis and zymographic activity staining. The specificity of the inhibitor toward trypsin was with *K*i of 1.043×10^{-10} M. The inhibitor was thermostable up to 90 °C with maximal stability at 30 °C, active over a pH range of 4–10 against proteases from *Aspergillus oryzae*, *Bacillus licheniformis*, *Bacillus* sp. and *Bacillus amyloliquefaciens*. Results indicate the possibility of utilization of protease inhibitor from *P. floridanus* against serine proteases.

Keywords Trypsin inhibitor · *Pleurotus floridanus* · Purification · *K*i · Thermostable

Introduction

Protease inhibitors are found abundantly in numerous plants, animals and microorganisms [1], owing their significance to their application in the study of enzyme structures, reaction mechanisms

P. P. M. Ali · K. Sapna · K. R. R. Mol · S. G. Bhat · M. Chandrasekaran
Department of Biotechnology, Cochin University of Science and Technology, Cochin 682022 Kerala, India

P. P. M. Ali (✉)
Department of Biotechnology, MES College, Marampally, Aluva 683107 Kerala, India
e-mail: manzur@mesmarampally.org

M. Chandrasekaran
Department of Botany and Microbiology, College of Science, King Saud University, PB No. 2455, Riyadh 11451, Saudi Arabia

K. K. Elyas
Department of Biotechnology, Calicut University, Malappuram 673635 Kerala, India

and also their utilization in pharmacology and agriculture [2–4]. Recognized as defense proteins, they control protease activities of vital importance, regulating proteolytic processes involved in tissue proteins mobilization and in the processing of proteins precursors. Several specific and selective protease inhibitors serve as powerful tools for inactivating target proteases in pathogenic processes of human diseases such as emphysema, arthritis, pancreatitis, thrombosis, high blood pressure, muscular dystrophy, cancer, AIDS as well as bacterial, viral and parasitic diseases [5, 6]. Protease inhibitors can be used in drug design to specifically inhibit proteases essential in the life cycle of organisms that cause mortal diseases such as malaria, AIDS and cancer, thereby impeding proliferation [7, 8]. They can also be employed as effective protective tools against insects, especially lepidopteran larvae, as they block digestion of proteins in the gut dependent predominantly on serine proteases for digestion of plant material [9]. Food spoilage by microorganisms is yet another area of global concern, where an estimated 25 % of all foods produced is lost post-harvest owing to microbial activity [10]. Natural protease inhibitors could be an effective means to prevent proteolysis due to endogenous tissue proteases and exogenous microbial proteases during food processing and preservation, thereby extending the shelf life of protein foods including milk, meat and seafood [11]. The reports on inhibitor in fungi are limited to the yeast inhibitors of endogenous proteases A and B [12, 13], low molecular inhibitors of *Pleurotus ostreatus* [14], serine protease inhibitor from *Lentinus edodes* [15], proteinase inhibitor from *Trametes versicolor* [16], trypsin specific inhibitors from *Clitocybe nebularis*, CnSPIs [17] and Cospin (PIC1) from *Coprinopsis cinerea* [18]. Structural and mechanistic studies of mycocypins, clitocypins and macrocypins, a group of cysteine protease inhibitors isolated from the mushrooms *C. nebularis* and *Macrolepiota procera*, are also reported [19].

Edible and medicinal mushrooms produce several nutritional and physiologically beneficial compounds including polysaccharides and proteins without overt cytotoxicity, thereby representing a resource for new natural drugs. Attempts have been made previously to exploit mushrooms and their metabolites to treat a range of human ailments. About 40 species of *Pleurotus*, commonly called oyster mushrooms, are found in temperate and tropical parts of the world [20]. Commercial cultivation of *Pleurotus floridanus* is carried out in many parts of the world, including India. *P. ostreatus* reportedly yield low molecular weight inhibitors [14]. The present communication describes isolation, purification and evaluation of inhibitory properties of a protein inhibitor (PfTI) from the fruiting body of *P. floridanus*. Protease inhibitors have not been reported previously from this edible mushroom.

Materials and Methods

Source of Mushroom

Edible mushroom *P. floridanus* obtained from Kerala Agriculture University, Trichur, India was used in the present study. The fruiting body of mushrooms was aseptically transferred to an ice box and transported. Stock cultures were maintained at −70 °C. The mushroom was identified based on its molecular characterization using ITS1 and ITS4 primers followed by sequencing and BLAST search.

Extraction and Recovery of Protease Inhibitor

Frozen *P. floridanus* fruiting bodies (250 g) were homogenized with 0.01 M phosphate buffer pH 7.5 (500 mL) in a blender for 5 min. The homogenate was centrifuged at 9,000*g* (Sigma,

Germany) at 4 °C for 15 min, and the clear supernatant obtained was used for the assay of protease inhibitor activity, protein content and inhibitor purification as described below.

Assay of Protease Inhibitor Activity Using BAPNA and Casein as Reaction Substrates

Presence of protease inhibitor in the extract or purified samples of mushroom was determined by assaying residual activity of *N*-tosyl-L-phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (EC 3.4.21.4) from Sigma-Aldrich, India using Hammerstein casein (SRL, India) and α-*N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA, Sigma-Aldrich, India) substrates.

Protease inhibitor activity against trypsin was assayed according to the method described by Kunitz with slight modifications [21]. Hundred microlitres of trypsin (0.1 mg/mL) was pre-incubated with suitable dilution of *P. floridanus* crude extract and purified inhibitor preparations at 37 °C for 15 min and made up to 500 μL with 0.01 M phosphate buffer pH 7.5. To this mixture, 100 μL of 1 % Hammerstein casein prepared in 0.1 M phosphate buffer (pH 7.5) was added and incubated at 37 °C for 30 min. The reaction was terminated by the addition of equal volume of 0.44 M trichloroacetic acid (TCA) solution. The reaction mixture was centrifuged (Sigma, Germany) at 9,000*g* for 15 min. The absorbance of the clear supernatant was measured at 280 nm in UV–Visible spectrophotometer (Shimadzu, Japan) against appropriate blanks. The TCA soluble peptide fractions of casein formed by the action of trypsin in the presence and absence of inhibitor were quantified by comparing with a standard curve, which is generated by known quantities of tyrosine in micromoles. One unit of trypsin activity was defined as the amount of enzyme that liberated 1 μmol of tyrosine per millilitre of the reaction mixture per minute under the assay conditions. One unit of protease inhibitor activity (U) was defined as the decrease in trypsin activity by one unit. For easy computation and understanding, the protease inhibitor activity was expressed in terms of percent inhibition. Appropriate blanks for the enzyme, inhibitor and the substrate were also included in the assay along with the test.

Protease inhibitor activity of *P. floridanus* crude extract and purified inhibitor preparations were also measured using the synthetic substrate BAPNA [22]. Three 75 μL of the protease inhibitor diluted with 0.01 M phosphate buffer (pH 7.5) were incubated with 25 μL of 0.1 mg/mL trypsin in phosphate buffer pH 7.5 for 10 min at 37 °C. Then, 50 μL of 2 mM freshly prepared BAPNA was added and incubated at 37 °C for 30 min. The reaction was stopped by the addition of 500 μL of 30 % acetic acid. The optical absorbance of *p*-nitroaniline released by the reaction was read at 410 nm. The difference in absorbance was calculated by assaying trypsin activity in the absence and presence of inhibitor. One unit of inhibitory activity (U) was defined as the amount of inhibitor needed to inhibit the release of 1 μmol of *p*-nitroaniline per millilitre per minute at pH 7.5 and at 37 °C.

Protein Estimation

Protein content was determined according to Bradford [23] using bovine serum albumin (BSA) as the standard, and the concentration was expressed in mg/mL.

Purification of Protease Inhibitor

The crude extract was fractionated by 30–60 % ammonium sulphate [$(NH_4)_2SO_4$] saturation [24], followed by dialysis against phosphate buffer (0.01 M, pH 7.5), and the partially pure protein was dissolved in 0.01 M phosphate buffer (pH 7.5). Five millilitres (20 mg) of this crude inhibitor preparation was applied to a DEAE-Sepharose column (25×1.5 cm) pre-

equilibrated with 0.01 M phosphate buffer (pH 7.5) in an FPLC system (Bio-Rad, USA). The bound fraction was eluted by a step gradient of 0–100 %, 0.5 M NaCl in 0.01 M phosphate buffer with a flow rate of 1 mL/min. The resultant peaks were monitored for trypsin inhibitor activity. The active fraction was pooled, dialysed against 0.01 M phosphate buffer (pH 7.5) for 4 h and concentrated using amicon UF-10 kDa membrane (Millipore, USA). Further purification was by trypsin affinity column (15×1 cm) prepared by binding trypsin on cyanogen bromide activated sepharose (Sigma-Aldrich, India). The concentrated ion exchange fraction (3 mg) was loaded to trypsin affinity column equilibrated with 0.01 M phosphate buffer (pH 7.5), and the bound protein was eluted using 0.5 M NaCl in 0.01 M HCl with a flow rate of 0.5 mL/min. The dialysed active fraction (1 mg) was again loaded on to Sephadex G-100 gel filtration column (75×1.5 cm) pre-equilibrated with 0.01 M phosphate buffer (pH 7.5) to get a homogenous inhibitor protein. The fractions were collected with a flow rate of 1 mL/min. Yield and fold of purifications were calculated.

HPLC Analysis

The purified active fraction after gel filtration (20 μL, 0.1 mg/mL) was subjected to reversed-phase HPLC (Schimadzu LC 2010) using Phenomenex C18 HPLC column (22.5 mm ID×250 mm length) at a flow rate of 1 mL/min with 100 % solvent A (0.1 % trifluoroacetic acid (TFA) in water) for 10 min and a linear gradient (0–100 %) of solvent B (0.09 % TFA in 60 % acetonitrile) over 60 min. Proteins were detected by monitoring the absorbance at 220 nm.

SDS-PAGE

The purified protease inhibitor was subjected to electrophoresis by tris-glycine SDS-PAGE on a 16 % polyacrylamide gel [25] with 4 % stacking gel in a vertical slab electrophoresis apparatus (Mini-PROTEAN Tetra cell, Bio-Rad, USA) and silver stained. Low-range SDS-PAGE molecular weight markers of Bio-Rad were used.

Mass by MALDI-TOF

Intact molecular mass of the purified inhibitor was determined by MALDI-TOF (ABI 4800) after desalting the sample with ZipTip-C18 (Millipore, Billerica, MA).

Isoelectric Point

Isoelectric point (pI) of the inhibitor protein was determined by isoelectric focusing (Bio-Rad, USA). Immobilized pH gradient (IPG) strip of pH 3-10 (Bio-Rad, USA) was used according to the instructions of the manufacturer and then stained in coomassie brilliant blue.

Activity Staining

Inhibitory activity of the purified protease inhibitor was visualized by the method of Uriel and Berges [26] with slight modification. After Native PAGE (8 %), the gel was incubated in freshly prepared solution of trypsin (0.04 mg/mL) in 0.1 M phosphate buffer pH 7.5 at 37 °C for 30 min. Inhibitory activity was visualized by staining the gel with Fast Blue (Sigma-Aldrich, India) solution containing *N*-acetyl-DL-phenylalanine-β-naphthyl ester (Sigma-Aldrich, India).

Dot-blot Analysis

Dot-blot analysis was performed [27] to determine the protease inhibitory activity of PfTI obtained after gel filtration chromatography. Three microlitres (0.1 mg/mL) of protease inhibitor was mixed with 3 μL trypsin (0.1 mg/mL) and spotted on a strip of X-ray film. Soya bean trypsin inhibitor (0.1 mg/mL) was used as positive control and trypsin as negative control for protease inhibition. Inference was made by observing the zone of hydrolysis indicating degradation of gelatin by trypsin. In the absence of the inhibitor, a clear zone is formed at the site of trypsin application on the X-ray film.

Peptide Mass Fingerprinting

Peptide mass fingerprinting (PMF) by MALDI-TOF and analysis of protein with MASCOT search tool in Swiss-Prot database evolved as an excellent tool to differentiate proteins with very similar physicochemical and functional properties. The PfTI was reduced, alkylated with iodoacetamide and trypsin digested. Peptides were extracted according to standard techniques and were analysed by MALDI-TOF-TOF mass spectrometer using a 4800 Proteomics Analyzer [Applied Biosystems]. Spectra were analysed to identify protein of interest using MASCOT sequence matching software [Matrix Science] with Ludwig NR Database.

*K*i of the Inhibition

Kinetics of trypsin inhibition by protease inhibitor was studied with different concentrations of inhibitor. Using the enzyme rate data, a double reciprocal plot was prepared and analysed to determine whether the nature of protease inhibition is competitive, uncompetitive, or non-competitive. An aliquot of 1-nM trypsin alone was pre-incubated in 0.1 M phosphate buffer (pH 7.5) for 5 min at 37 °C. Fractions of 500 μL of different concentrations (0.014, 0.07 and 0.27 nM) of purified protease inhibitor were pre-incubated with aliquots of 100 μL of 1-nM trypsin for 5 min at 37 °C. Later, the pre-incubated mixtures were added separately to 0.05 to 0.4 mM BAPNA solution and incubated at 37 °C for 10 min. After incubation, the reaction was arrested by adding 400 μL of 30 % (v/v) acetic acid. Lineweaver–Burk $1/v$ versus 1/[s] was plotted, the apparent *K*m (*K*m′) and maximum velocity (V_{max}) were calculated for each concentration of inhibitor and secondary plot was plotted by taking $1/V$max versus [I] to determine dissociation constant of the inhibitor (*K*i) [28].

Stoichiometry of Protease-Protease Inhibitor Interaction

One nanomolar trypsin in 100 μL of 0.1 M phosphate buffer pH 7.5 was pre-incubated with different amounts of purified protease inhibitor (0.05–1.0 nM) at 37 °C for 10 min, and the residual protease activity was estimated using BAPNA as substrate.

pH Stability

The stability of protease inhibitor over a range of pH was determined by performing the inhibitor activity assay at pH 7.5, after incubating the protease inhibitor in different buffers with pH ranging from 2 to 12 for 4 h, at 4 °C. Ten microlitres (0.1 mg/mL) of purified inhibitor was incubated with 40 μL of different buffer systems, which included 0.1 M KCl–HCl buffer (pH 2), 0.1 M citrate buffer (pH 4–6), 0.1 M phosphate buffer (pH 7), 0.1 M Tris–HCl buffer (pH 8–9), 0.1 M borax/NaOH (pH 10), 0.1 M disodium hydrogen phosphate/NaOH (pH 11)

and 0.1 M potassium chloride/NaOH (pH 12). After incubation, sample was assayed (measured after return to native conditions) for protease inhibitory activity using BAPNA as substrate.

Effect of Temperature on Inhibitor Stability

Temperature stability of inhibitor was determined by incubating purified protease inhibitor at temperatures ranging from 4 to 100 °C for 60 min. The protease inhibitor activity of each sample was assayed (measured after return to native conditions) as described in section 2.3.2.

Evaluation of Protease Inhibitor Activity against Commercial and Microbial Proteases

The activities of purified inhibitor against proteases derived from different microbial sources were evaluated. Ten microlitres of purified inhibitor (~0.1 mg/mL) was pre-incubated with 50 μL (0.1 mg/mL) of proteases derived from different microbial sources and performed caseinolytic assay as described in section 2.3.1. Proteases obtained from *Aspergillus oryzae* (P6110, Sigma-Aldrich), *Bacillus licheniformis* (EC 3.4.21.14), *Bacillus* sp. (P0029, Sigma-Aldrich), esperase and *Bacillus amyloliquefaciens* (P1236, Sigma-Aldrich) were used for the study. The protease inhibitory activity was also tested against elastase, chymotrypsin, proteinase K and thermolysin (all the proteases are from Sigma-Aldrich, India).

Results

The identity of the mushroom was confirmed by ribotyping, and the sequence was deposited in NCBI GenBank with accession number GU7210580. The trypsin inhibitor from *P. floridanus*, PfTI was purified to homogeneity by $(NH_4)_2SO_4$ precipitation, DEAE-Sepharose chromatography, trypsin affinity chromatography and gel filtration chromatography. The yield and fold of purification of protease inhibitor extracted are summarized in Table 1. PfTI precipitated at 30–60 % $(NH_4)_2SO_4$ saturation, but without observable activity at 0–30 % and 60–90 % $(NH_4)_2SO_4$ saturations. The fold of purification of protease inhibitor obtained by $(NH_4)_2SO_4$ precipitation, DEAE-Sepharose chromatography, trypsin affinity chromatography and gel filtration was 1.63, 3.61, 21.5 and 35.25, respectively. Though affinity chromatography is an

Table 1 Yield of protein, yield of PfTI activity and fold of purification in comparison with crude extract

Purification step	Volume (mL)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield of protein (%)	Yield of activity (%)	Fold of purification
Crude extract	500	2,342	7,450	3.18	100[a]	100[a]	1[a]
(NH4)2SO4	30	94.92	493	5.19	21.05	6.617	1.63
DEAE-Sepharose (peak V)	1.2	1.836	21.1	11.49	0.0784	0.283	3.61
Trypsin affinity	0.6	0.202	13.83	68.47	0.0086	0.186	21.5
Gel filtration (peak V)	0.45	0.1	11.21	112.1	0.00427	0.15	35.25

[a] Values taken arbitrarily

efficient method to purify inhibitor, the homogenous inhibitory protein was obtained by gel filtration chromatography with a resultant increase in purification fold up to 35.25 and a specific inhibitory activity of 112.1 U/mg protein. The net yield of protease inhibitor was 0.5 μg/g of mushroom. The purity of the PfTI was further refined by reversed-phase HPLC analysis.

The SDS-PAGE of the purified PfTI indicated the homogeneity of the inhibitor protein. Single polypeptide band with a molecular mass of 37 kDa in the reductive SDS-PAGE (Fig. 1a) testifies to the purity of the fraction. Furthermore, the molecular mass was confirmed to be 38.334 kDa by MALDI-TOF (Fig. 1b). In the present study, isoelectric point of the inhibitory protein was determined to be 4.4.

Activity staining of the purified inhibitor PfTI evaluated on native-PAGE and visualization of a clear band on a coloured background confirmed its inhibitory activity (Fig. 1c). Dot-blot analysis on an X-ray film also indicated that the PfTI blocked the gelatin hydrolysis by trypsin as did soya bean trypsin inhibitor (the control), shown by comparing the clearing zone formed by trypsin activity and a reduction zone size due to the presence of inhibitor.

Protein identification and differentiation by peptide mass fingerprinting (PMF) have been an excellent tool to differentiate proteins with very similar physicochemical and functional properties. Peptide mass fingerprint of protease inhibitor isolated from *P. floridanus* analysed with the MASCOT search tool in Swiss-Prot database did not match any of the inhibitors.

The inhibitory constant *K*i of the inhibitor was calculated as 1.043×10^{-10} M from the secondary plot shown in Fig. 2. Data obtained from the kinetic studies performed with trypsin indicated that the protein PfTI molecule has a reversible mechanism of action. A Lineweaver–Burk curve, $1/v$ versus 1/[s], was plotted to study the pattern of inhibition (competitive, uncompetitive or non-competitive). It was observed that identical concentration of trypsin (1 nM) pre-incubated with enzyme buffer alone and with different concentrations of inhibitor

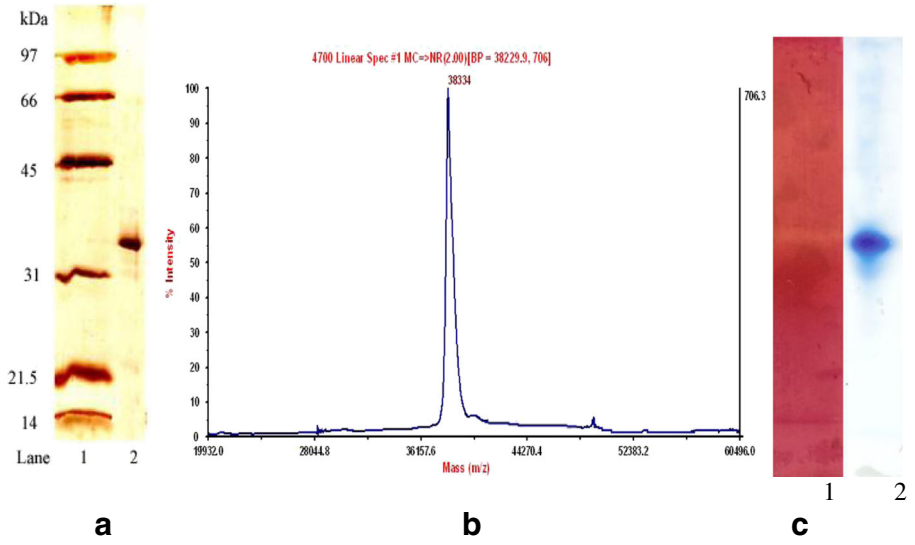

Fig. 1 **a** The inhibitory protein was analysed by SDS-PAGE (16 %) under standard denaturing conditions. Five micrograms of the sample was loaded and silver stained. *Lanes*: *1*. Standard markers, *2*. Purified inhibitor. **b** MALDI-TOF profile of the purified inhibitor. **c** Activity staining of purified protease inhibitor (Uriel and Berges) in 8 % Native-PAGE. *Lanes*: *1*. Activity stained inhibitor, *2*. Inhibitor stained with coomassie

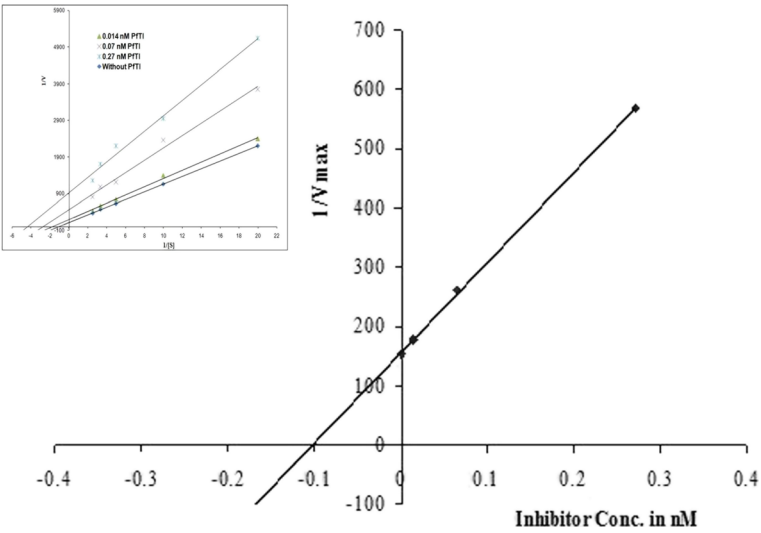

Fig. 2 Secondary plot of protease inhibitor. A secondary plot was drawn by $1/V_{max}$ versus concentrations of inhibitor studied. The X-intercept gives the − *K*i value, and from that the dissociation constant (*K*i) was calculated. The values of $1/V_{max}$ derived from the double reciprocal plot are plotted against the relevant concentrations of protease inhibitor in order to derive the *K*i value for PfTI (*inset*). The rates of each reaction in the presence of various concentrations PfTI as indicated were calculated, and the reciprocal values of these velocities are plotted against reciprocal concentrations of trypsin as a Lineweaver–Burk plot

(0.014, 0.07 and 0.27 nM) yielded different *K*m and *V*max for various concentrations of substrate (BAPNA) ranging from 0.05 to 0.4 mM. Inhibition of substrate hydrolysis occurred at a very low concentration of protease inhibitor, and *K*i was calculated from the secondary plot as 1.043×10^{-10} M under the assay conditions. Extrapolation to zero protease activity (100 % inhibition) corresponding to 1 nM of inhibitor suggests that 1 nM trypsin is completely inhibited by 1 nM of the inhibitor. The amount of inhibitor required for 50 % inhibition (IC_{50}) of trypsin calculated from the graph was 0.5 nM.

The protease inhibitor PfTI showed stability over a wide range of pH. From Fig. 3a, it was observed that PfTI showed considerable protease stability over a pH range of 4–10, although it was most stabile at pH 8. At high alkaline conditions of pH 11–12 and high acidic conditions of pH 2–3, PfTI was not stable.

Similarly when evaluated for temperature stability, the protease inhibitor PfTI demonstrated considerable stability over a range of temperature up to 90 °C (Fig. 3b). Maximal stability of the PfTI was observed at temperatures around 30–40 °C. Whereas, the PfTI recorded a gradual decrease in stability without considerable difference after pre-incubation at 50–90 °C for 60 min and a complete loss of activity at 100 °C. In the present study, the temperature recorded for maximal stability of PfTI was in the range of 30–40 °C. Thermal inactivation of the PfTI was due to loss of stability at temperatures above 90 °C.

Inhibitory activity of PfTI against six different industrially important proteases was evaluated and the results presented in Fig. 4. It was inferred that the PfTI could completely inhibit the commercially available protease esperase compared to a significant level of inhibition of protease of *A. oryzae*, *B. licheniformis*, *Bacillus* sp. and *B. amyloliquefaciens*.

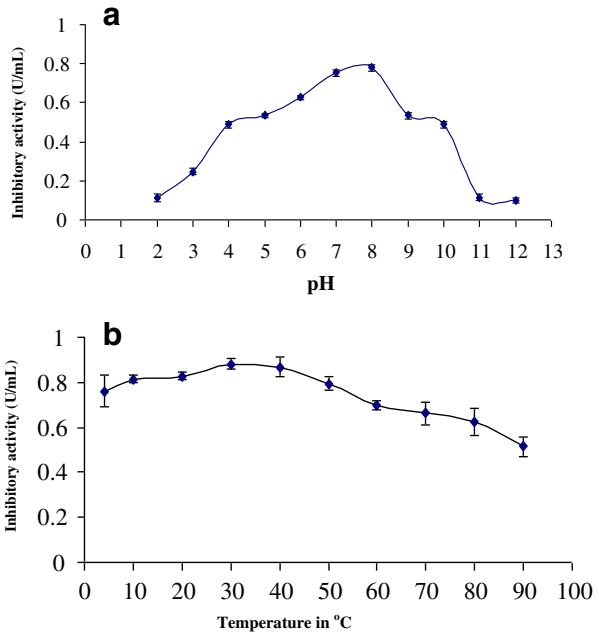

Fig. 3 **a** Effect of pH on inhibitory activity of protease inhibitor. PfTI was incubated at pH 2–12 for 4 h at 4 °C in different buffer systems, and inhibitory activity was measured after return to native condition. **b** Effect of temperature on protease inhibitor stability. Protease inhibitor was incubated at different temperatures ranging from 4 to 90 °C for 1 h, and residual protease inhibitory activity of each sample was determined after return to native condition (experiments conducted in triplicate and values given with standard deviation)

Discussion

Protease inhibitors are in demand in medicine as biocontrol agents in agriculture and food preservative applications. Microbes as source of protease inhibitors are being recognized, and not much literature is available on mushrooms as source of protease inhibitors. In this context, the present study reports the isolation, purification and evaluation of inhibitory activity of trypsin specific inhibitor from the fruiting bodies of edible mushroom *P. floridanus*.

It may be noted that the molecular mass of PfTI was different from that of protease inhibitors isolated from the basidiomycetes *C. nebularis* [17] and *L. edodes* [15] with 16.3 and 15.99 kDa, respectively. Protease inhibitors show acidic and alkaline pI with respect to the source and type of inhibitor; the pI of the PfTI was different from the inhibitors reported. Inhibitors of proteinase B, IB1 and IB2 purified from *Saccharomyces cerevisiae* showed relatively basic isoelectric points of 8.0 for IB1 and 7.0 for IB2 [29], and pI of serine protease inhibitors from *C. nebularis* CnSPIs were 4.8 and 5.2 [17]. The peptide mass fingerprint of PfTI did not match with any of the inhibitor in the database, showing its novelty. The mass spectra obtained after tryptic digestion (peptide mass fingerprint) of inhibitor isolated from *Solanum tuberosum* cv. Desirée analysed with the 'MASCOT search tool' also did not match any of the inhibitors of other plants [30].

The *K*i of PfTI is in sub-nanomolar range and quite similar to the protease inhibitors from mushroom *C. nebularis* [17] and *L. edodes* [15]. The *K*i value of serine protease inhibitor from *C. nebularis*, CnSPIs, for the inhibition of trypsin was 3.1 nM [17]. Similarly, lentinus proteinase inhibitor purified from *L. edodes* showed an apparent dissociation constant of

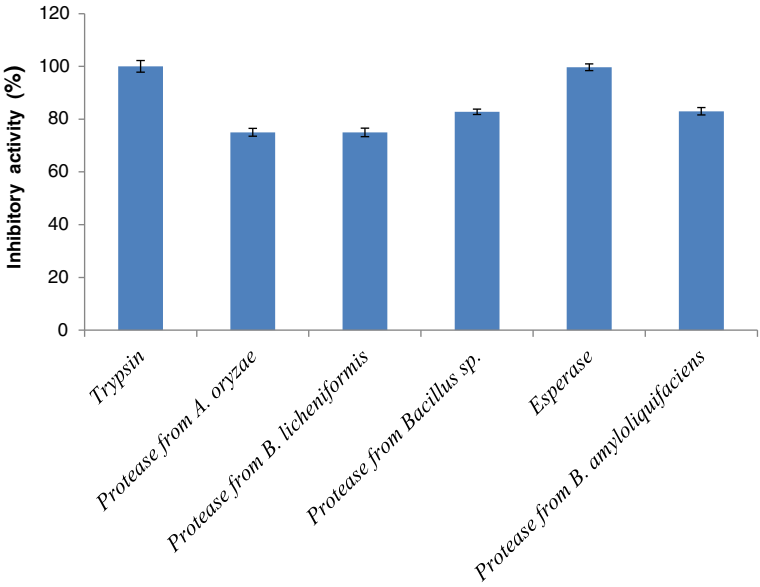

Fig. 4 Inhibitory activity of PfTI against proteases of microbial sources. Protease inhibitor activity was determined by caseinolytic assay and expressed in terms of percent inhibition (experiments conducted in triplicate and values given with standard deviation)

3.5×10^{-10} M [15]. The low *K*i values indicated a relatively high affinity of the inhibitor for the enzyme. Protease inhibitors from plants and microorganisms are characterized by either a reversible or irreversible mechanism [31]. Kinetic studies of trypsin by PfTI revealed that it had a reversible mechanism of action. The kinetic studies of PfTI also revealed that trypsin inactivation occurs by uncompetitive inhibition during which the affinity of the enzyme (*K*m) and *V*max undergoes change. The stoichiometry of trypsin-inhibitor interaction is similar to other trypsin inhibitors. Titration of trypsin with protease inhibitor from *C. nebularis*, inhibitor purified from *L. edodes* and serine proteinase inhibitor from the leguminous plant seeds of *Archidendron ellipticum* (AeTI) inhibited trypsin in the stoichiometric ratio of 1:1 [15, 17, 32].

Under strong acidic or alkaline conditions, the protein inhibitors get denatured, and as a consequence they lose their activity partially or completely. Reports presume that intramolecular disulfide bridges are responsible for the functional stability of the inhibitor in the presence of physical and chemical denaturants such as temperature, pH and reducing agents [33]. Serine and cysteine protease inhibitors isolated from basidiomycete as well as inhibitors of proteinase B from *S. cerevisiae* contain no sulfide bridges, and many of them are pH and/or thermo-resistant [12, 14, 15, 17–19, 34]. It is possible that PfTI shares a disulfide bond-independent mechanism of stability. pH–activity profiles of the gut lumen of the red flour beetle, *Tribolium castaneum*, revealed the presence of proteinases with acidic (pH 4–5) and alkaline (pH 8.5–11) optima. The substrate BAPNA preferentially hydrolysed at the alkaline pH optima suggested trypsin-like proteinases [35]. Protease inhibitors targeting proteases of different insect pests have shown anti-feedent properties. The alkaline gut of lepedopteran and dipteran larvae primarily relies on serine proteases like trypsin and chymotrypsin for the digestion of plant material, whereas cysteine proteases predominate in Hemiptera, Coleoptera and Thysanoptera [36]. Hence, the stability of PfTI in the alkaline range of pH signifies its possible use as

biopesticide, as one of the criteria to withstand the highly alkaline conditions of insect's gut flora is complied with.

Thermal stability studies indicate that the protease inhibitor has high intrinsic stability in its native state, which gives a high degree of thermal stability. Three actinomycetes strains producing alkaline protease inhibitors API-I, API-II and API-III, respectively, exhibited different properties in their molecular nature and in their pH and temperature stabilities [37]. Protease inhibitor (PISC-2002) isolated from culture supernatants of *Streptomyces chromofuscus* was stable over pH (2–10) and at high temperatures (80 °C/30 min), mainly attributed to the presence of proline and a high content of hydrophobic amino acids [38]. The high thermal and pH stabilities of PfTI suggested its applications in various industries. Enhancement of thermal stability is a desirable trait for most of the biotechnological applications of proteins and for their commercial exploitation [37], as it increases the efficiency of proteins and is therefore one of the essential requirements.

The activity spectrum indicated that the interactions of inhibitor with different proteases are a common and generally accepted mechanism as the inhibitory activities were almost similar. It may be noted that the commercial chymotrypsin, thermolysin, elastase and proteinase K were not markedly inhibited by PfTI, which may be due to the lack of binding site for PfTI.

The results clearly indicate that PfTI has high inhibitory activity against the bacterial proteases and protease of *A. oryzae*. Inappropriate proteolysis has been found to have a major role in cancer as well as cardiovascular, inflammatory, neurodegenerative, bacterial, viral and parasitic diseases. Excessive proteolysis can be prevented by blocking the appropriate proteases; this area is widely explored by pharmaceutical companies [6]. Applications of serine protease inhibitors isoforms from *Acacia plumosa* Lowe seeds (ApTI) as both an anticoagulant and an inhibitor of phytopathogenic fungi growth have been studied. It was reported that the antifungal action of ApTI can be associated with inhibition of some serine proteases liberated in the medium by phytopathogenic fungi [39]. The results obtained for the inhibition studies against the commercial enzymes indicate potential therapeutic and agronomic utility of PfTI [40].

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