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ORIGINAL ARTICLE

Cystatin like thiol proteinase inhibitor from pancreas of *Capra hircus*: purification and detailed biochemical characterization

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Abstract A thiol proteinase inhibitor from *Capra hircus* (goat) pancreas (PTPI) isolated by ammonium sulphate precipitation (20–80%) and gel filtration chromatography on Sephacryl S-100HR, with 20.4% yield and 500-fold purification, gave molecular mass of 44 kDa determined by its electrophoretic and gel filtration behavior, respectively. The stokes radius, diffusion and sedimentation coefficients of PTPI were 27.3 Å, 7.87×10^{-7} cm^2 s^{-1} and 3.83 s, respectively. It was stable in pH range 3–10 and up to 70°C (critical temperature, $E_a = 21$ kJ mol^{-1}). Kinetic analysis revealed reversible and competitive mode of inhibition with PTPI showing the highest inhibitory efficiency against papain ($K_i = 5.88$ nM). The partial amino acid sequence analysis showed that it shared good homology with bovine parotid and skin cystatin C. PTPI possessed 17.18% α helical content assessed by CD spectroscopy. The hydropathy plot of first 24 residues suggested that most amino acids of this stretch might be in the hydrophobic core of the protein.

Keywords Goat pancreas cystatin · Kinetics of inhibition · Amino acid sequence · Mammalian cystatins · Characterization

Introduction

Proteolytic enzymes are essential for the survival of all kinds of organisms, and are encoded for by approximately 2% of all the genes (Barrett et al. 2001). Despite their vital functions, these proteases are potentially very damaging in living systems. Cells therefore have several distinct mechanisms to check their enormous hydrolytic potential, important among which are the interactions of the proteases with inhibitors. The major proteases of the lysosomal pathway of protein degradation, cathepsins, are naturally regulated by cystatins. Cystatins (thiol protease inhibitors, TPI), reversible competitive inhibitors of thiol dependent (cysteine) proteases, are widely distributed in animals and plants and are also reported in some lower eukaryotes. Generally, they are classified into three distinct families based on their sequence homology, presence of disulphide bonds and molecular mass (Abrahamson et al. 2003; Turk et al. 1997). Family I—the stefins (mammalian cystatin A and B and bovine stefin C) are cytosolic proteins of about 100-residue single chains, with no disulphide bonds or carbohydrates (Turk et al. 1997). Family II—the cystatins (chicken cystatin) are primarily extracellular and each has a single chain of about 120 amino acids with two disulphide bonds and no carbohydrates (Bode et al. 1988). Family III—the kininogens are blood plasma glycoproteins and have several cystatin like domains (Ohkubo et al. 1984).

Physiologically important cystatins are known to act as defensive agents against bacteria, viruses, and plant eating insects (Ylonen et al. 1999). They can also regulate cell death, antigen presentation and their expression is altered in malignant processes (Ylonen et al. 1999). Cystatins have been purified and characterized from various sources including goat kidney (Zehra et al. 2005), goat brain (Sumbul and Bano 2006), sheep plasma (Shahid et al. 2005), human placenta (Rashid et al. 2006), human spleen (Jarvinen and Rinnie 1982), human liver (Green et al. 1984), and amyloid fibrils (Cohen et al. 1983), etc. The present investigation aimed at understanding the functioning of thiol dependent protease inhibitors in pancreas, the

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capital of proteolytic power. Role of cathepsins in mediating exocrine and endocrine functions of pancreas is already established, e.g. cathepsin B—in conversion of trypsinogen to trypsin (Otto and Riesenkonig 1975), cathepsin B and H in formation of insulin from proinsulin (Ansorge et al. 1977). It is also established that the uncontrolled activation of zymogenic pancreatic secretion by cathepsins lead to pancreatitis (Halangk et al. 2000) and this knowledge prompted an investigation on the natural regulation of cysteine protease activity in pancreas. We describe here a cysteine proteinase inhibitor from goat pancreas and its purification, properties and partial amino acid sequence. It was found to bear similarities with inhibitors of the cystatin superfamily.

Materials and methods

Materials

Enzymes, substrates, Sephacryl S100-HR, electrophoresis reagents, ethylene diammine tetra acetate (EDTA), L-cysteine, anti-rabbit alkaline phosphatase (conjugate) and *p*-nitrophenyl phosphate were obtained from Sigma (ST. Louis, MO). Medium molecular weight markers, Freund's complete and incomplete adjuvants were from Genei, India Limited. All other chemicals used were of highest purity grade available commercially.

Methods

Purification of TPI from goat pancreas

TPI was isolated by a modification of the method reported earlier by Green et al. (1984). Fresh pancreatic tissue (100 g) homogenized in 50 mM sodium phosphate buffer (200 ml), pH 7.5 containing 0.15 M sodium chloride (NaCl), 3 mM ethylene diammine tetra acetic acid and 2% *n*-butanol was centrifuged at 5,000 rpm for 15 min at 4°C; the supernatant was adjusted to pH 11.0 by 3 M sodium hydroxide (NaOH) and incubated for 30 min at 4°C. The pH was then brought back to 7.5 with glacial acetic acid. After centrifugation at 8,000 rpm for 30 min at 4°C the supernatant was treated with equal volume acetone at 4°C with stirring, over a period of 10 min. The precipitate was then removed by centrifugation (at 8,000 rpm for 20 min at 4°C), and discarded. A further portion of acetone equal in volume to the first was added to the supernatant as before. The precipitate was collected by centrifugation at 11,000 rpm for 30 min at 4°C and the supernatant discarded. The precipitate was dissolved in minimum amount of 50 mM sodium phosphate buffer (pH 7.5) and was fractionated between 20 and 80% ammonium sulphate saturation. The precipitated protein procured by centrifugation at 11,000 rpm for 30 min at 4°C was dissolved in 50 mM sodium phosphate buffer pH 7.5 and dialyzed thrice against 100 ml volumes of the same buffer containing 0.15 M NaCl. The sample was subjected to gel filtration chromatography on Sephacryl S-100 HR column (60 × 1.7 cm) equilibrated with 50 mM sodium phosphate buffer, pH 7.5. The flow rate of the column was 15 ml h^{-1}. A single protein peak with papain inhibitory activity was obtained corresponding to the goat pancreas thiol protease inhibitor (PTPI).

Assay of thiol proteinase inhibitory activity The inhibitory activity of TPI was assessed by its ability to inhibit the caesinolytic activity of papain by the method of Kunitz (1947). Inhibitory effects of PTPI on ficin, bromelain, trypsin and chymotrypsin were measured using casein as substrate. Protein in all the samples obtained was estimated by the method of Lowry et al. (1951).

Electrophoresis

To check the homogeneity of the purified preparations native and SDS PAGE in the presence and absence of 2-mercaptoethanol was conducted by the method of Laemmli (1970). 7.5% T native electrophoresis and 12.5% T SDS PAGE were run. The gels were stained with 0.1% Coomassie brilliant blue for native PAGE and silver staining was performed for SDS-PAGE (Nesternko et al. 1994).

Molecular mass determination

The molecular mass of the inhibitor was determined by running marker proteins along with the inhibitor on SDS PAGE. The molecular weight of the inhibitor under native conditions was determined by passing various marker proteins along with the inhibitor on Sephacryl S-100 column.

Determination of stokes radius and diffusion coefficient

Stokes radius (r) was determined by method of Andrews (1964) and Laurent and Killander (1964) using Sephacryl S100-HR column (60 × 1.7 cm) calibrated with proteins of known stokes radii.

Carbohydrate and thiol group estimation

The carbohydrate content was determined by the method of Dubois et al. (1956) using glucose as a standard and thiol groups were estimated by the method of Ellman (1969) using dithionitro benzoate (DTNB) and molar extinction coefficient of 13,600 M^{-1} cm^{-1}.

pH and thermal stability

(a) The pH and thermal stability of the purified inhibitor was estimated by procedure reported earlier by Rashid et al. (2006).

(b) The temperature dependence of PTPI fluorescence was determined at pH 7.5 in 50 mM sodium phosphate buffer. The samples were kept for 30 min at the desired temperature prior to the measurement, to ensure attainment of thermal equilibration. The results were analyzed according to the Arrhenius equation (Gally and Edelman 1962):

$$\ln\left(Q^{-1}-1\right) = -E_a/RT + \ln k$$

where Q is the emission quantum yield, E_a (J mol^{-1}) the activation energy of the radiation less deactivation of the singlet excited state, and R and k are constants.

Immunological properties

Antiserum was raised in rabbits by injecting 300 µg of electrophoretically purified inhibitor emulsified in Freund's complete adjuvant. Immunodiffusion was performed as reported earlier (Rashid et al. 2006). Cross reactivity was checked by the same technique. Lung and brain (Khan and Bano 2009; Sumbul and Bano 2006) cystatins used were also isolated from goat in our laboratory.

Direct binding *ELISA*: The generation of antigen specific antibody was measured in the sera of PTPI immunized rabbits by the technique of direct binding ELISA as reported earlier by Rashid et al. (2006).

Antibacterial activity

Antibacterial activity of cystatin was analyzed by determination of inhibition zone diameter as reported by Zehra et al. (2005). Different concentrations of purified inhibitor were soaked in Whatman discs and placed carefully on the bacterial lawns. The plates were incubated at 37°C overnight. Finally, the zones of inhibition obtained were measured. Strains used were Gram negative strains available, *E. coli*, *Streptococcus* mutants.

Active site titration and kinetics of inhibition

(a) Titrations of activated papain with PTPI for the determination of binding stoichiometries were monitored by changes in the fluorescence emission intensity accompanying the interactions (Björk and Ylinenjärvi 1990). The titrations were done at enzyme concentrations of 0.1–2 µM, and the fluorescence was monitored in the region of maximum change (345–350 nm). Stoichiometry was obtained by non-linear least square regression of the titrations curve with the SIGMA-PLOT9.0 computer software program.

(b) K_i determinations were carried out by lowering the enzymes and inhibitor concentrations to obtain a non-linearity of dose-response curves. Papain, ficin and bromelain were used at a concentration of 0.06 µM to react with inhibitor in varying concentrations from 0.01 to 0.24 µM. Residual activity was measured by the method of Kunitz (1947) using casein as substrate. Four different substrate concentrations were used 0.5 Km, 1 Km, 2 Km and 3 Km and with Km = 2.4 mM. The results were analyzed by the procedure reported earlier (Rashid et al. 2006).

For the dissociation rate constant, K_{-1}, the condition for maximal association between the proteinase and inhibitor was achieved before the reaction was shifted towards dissociation by adding excess substrate, which also binds the entire free enzyme. Substrate induced dissociation was monitored with an identical enzyme–inhibitor complex incubated for 30 min at 37°C. Excess substrate (6% casein) was added to the mixture for different time periods, which was then assayed for enzymatic activity. K_{-1} and half-life of the complexes are related by the equation

$$t_{1/2} = 0.693/K_{-1}.$$

Association rate constant, K_{+1}, was determined using the relation

$K_{+1} = K_{-1}/K_i$ (Abrahamson et al. 1986).

N-terminal analysis

N-terminal sequencing was performed on a Shimadzu PPSQ-21 sequencer by Edman degradation (Edman and Begg 1967). The highly purified protein was transferred to the PVDF membrane for the analysis.

Hydropathy plot

The hydropathy profiles were calculated using the mean segment approach, i.e. determining the average hydropathy within a segment length of four residues, the segment being advanced one residue at a time from the sequence of N-terminal 24 residues (Kyte and Doolittle 1982).

Spectral analysis

(a) An ultraviolet absorption difference spectrum was measured for PTPI (2.66 µM) on a Cintra 10 spectrophotometer. Each spectrum was the average of at least four scans.

(b) Fluorescence measurements were performed at 25 ± 1°C on a Shimadzu spectrofluorimeter model

RF-540 equipped with a data recorder DR-3. The excitation wavelength was 280 nm for total protein fluorescence. The slits were set at 5 nm for excitation and emission. The path length of the sample was 1 cm and emission wavelength range was 300–400 nm. The protein concentration used was 2 μM. Each spectrum was the average of at least four scans.

(c) Circular dichroism spectra of the inhibitor were acquired in the far ultraviolet region (200–250 nm) were acquired in a Jasco Spectropolarimeter, model J-720, equipped with a microcomputer, with protein concentration of about 0.2 g l^{-1} and cells having a pathlength of 0.1 cm.

Results

Purification of the inhibitor

The progress of a typical purification is summarized in Table 1. The initial homogenate contained free cathepsins, so presumably the inhibitors were entirely complexed. The alkali treatment destroyed the lysosomal cysteine proteases liberating the inhibitors in assayable form. A large amount of inactive proteins was precipitated during the readjustment to pH 7.5, and could be removed by centrifugation. Fractionation of the soluble material with acetone decreased the total amount of protein. Further, with ammonium sulphate precipitation (20-80%) undesirable protein content was removed. The protein precipitate thus obtained was dialyzed, Whatman paper filtered and chromatographed on sephacryl S-100HR column (Fig. 1). A single peak giving significant papain inhibition was obtained. The fractions corresponding to the peak were pooled and were lyophilized for further analyses. The procedure provided a fold purification of 500 and percent yield of 20.4.

Homogeneity of the purified PTPI

As observed in Fig. 1, the inhibitor eluted as a single symmetric peak with constant specific activity suggesting a homogenous preparation. In addition, the preparation did not inhibit bovine trypsin, chymotrypsin or pepsin. Physical evidence for homogeneity was further provided by gel electrophoresis under non-denaturing conditions. The electrophoretic pattern of PTPI is shown in Fig. 2, lane e. The inhibitor moved as a single band.

Properties of PTPI

In SDS-polyacrylamide gel electrophoresis (with and without reduction), PTPI migrated as two bands with different mobilities suggesting double subunit structure with subunits held together by non-covalent forces (Fig. 3, lane a, b). The molecular weight of the purified PTPI was determined by gel filtration on sephacryl S-100HR column. The molecular weight of the inhibitor was estimated to be 44,000. It was further confirmed by using SDS-PAGE under reducing and non-reducing conditions (Fig. 3). The molecular weight obtained was 44,000 (without reduction) and 47,194 (with reduction).

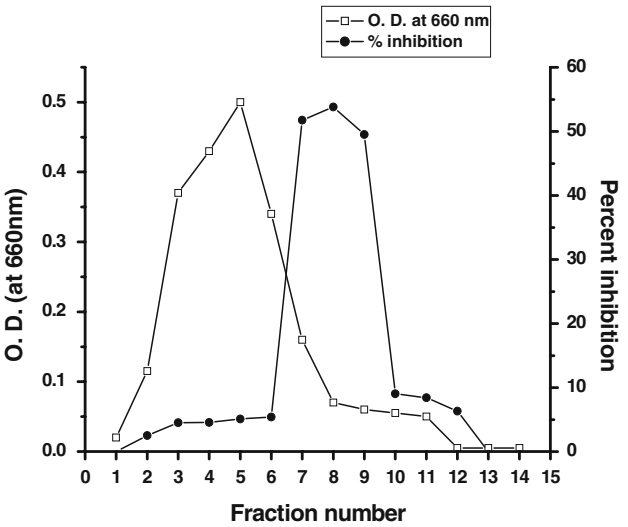

Fig. 1 Gel filtration of PTPI on a column of Sephacryl S-100HR—the precipitate obtained from 20 to 80% ammonium sulphate saturation (after acetone fractionation) was subjected to gel filtration at a flow rate of 15 ml h^{-1}. Fractions of 5 ml were collected and monitored by inhibition of caseinolytic activity of papain. Fractions 7, 8, 9 were pooled for further studies

Table 1 Results of a typical purification of PTPI

Step	Volume (ml)	Total protein (mg)	Total units[a]	Specific activity (Units/mg protein)	Fold purification	Percent yield
Crude	200	8,000	106	0.013		100
Alkaline treatment	100	2,899	76	0.026	2	71.6
Acetone fractionation	13	254.05	62.39	0.245	18.84	58.8
Ammonium sulphate cut (20–80%)	10	147.7	54.8	0.371	28.53	51.6
Sephacryl S-100 HR	15	3.34	21.66	6.485	498.84	20.4

[a] One unit of enzyme inhibitory activity is defined as the amount of inhibitor bringing about 0.001 change in OD ml^{-1} min^{-1}

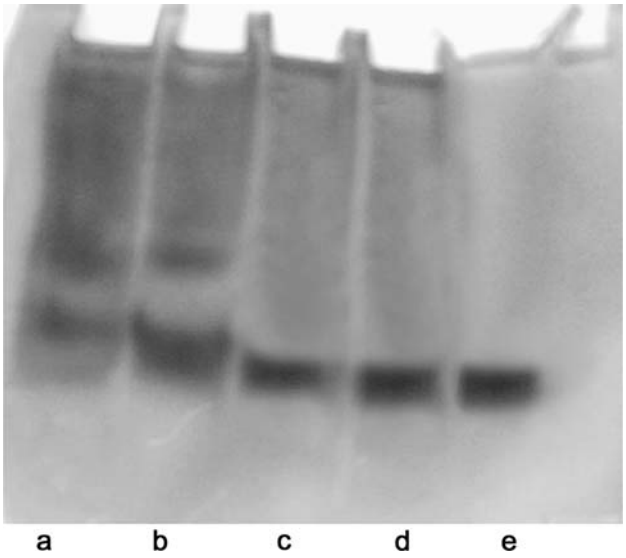

Fig. 2 Gel electrophoresis of PTPI during various stages of purification. Electrophoresis was performed on 7.5% gel. *Lane a* contained 60 μg pancreas homogenate, *lane b* contained 60 μg homogenate after alkaline treatment, *lane c* is 60 μg fraction obtained after acetone treatment, *lane d* is 60 μg dialyzed fraction after ammonium sulphate fractionation, *lane e* is 60 μg pancreatic cystatin after Sephacryl S-100 gel filtration

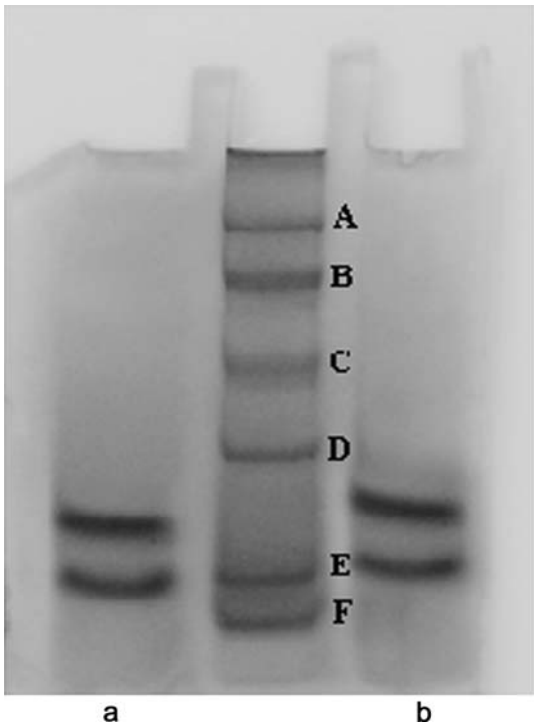

Fig. 3 Molecular weight determination of cystatin by SDS-PAGE electrophoresis on 12.5% polyacrylamide gel at 25°C. *Middle lane* contained the molecular mass standards: *A* phosphorylase b (97.4 kDa), *B* bovine serum albumin (68 kDa), *C* ovalbumin (45 kDa), *D* carbonic anhydrase (29.1 kDa), *E* soyabean trypsin inhibitor (20 kDa), *F* lysozyme (14.3 kDa). *Lane a* contained 40 μg and *lane b* contained 40 μg of 2-mercaptoethanol treated purified inhibitor

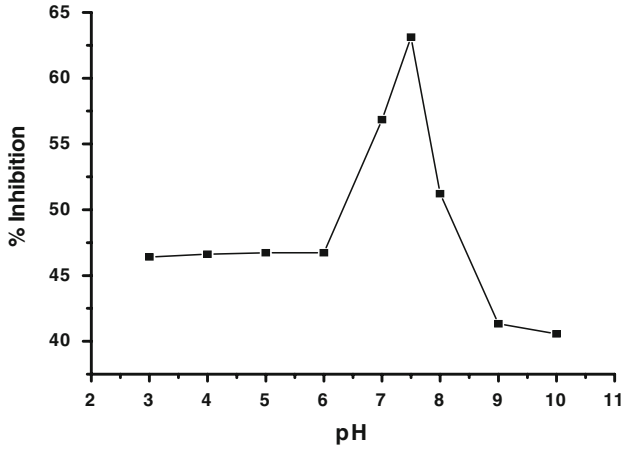

Fig. 4 pH stability profile of PTPI. 50 μg of the inhibitor was incubated in 50 mM sodium acetate buffer, pH 3.0–6.0, sodium phosphate buffer, pH 7.0–8.0, tris-HCl buffer, pH 9.0, for 30 min at 37°C. Remaining % inhibitory activity was analyzed against 50 μg of papain at 37°C

Hydrodynamic parameters for PTPI as ascertained from gel filtration behavior suggested a Stokes' radius of 27.3 Å and diffusion coefficient of 7.87×10^{-7} cm^2 s^{-1}. The sedimentation coefficient of PTPI is 3.83 s. The S_{max}/S ratio of PTPI is 1.2. PTPI possesses 2.32% carbohydrate content and is devoid of any disulphide bonds.

Storage and stability

The purified inhibitor retained 70% of its original anti papain activity after 4 months of storage at 0°C and 2 months of storage at +4°C. Effect of pH on PTPI activity was examined at various pH values. Figure 4 shows that the inhibitor is stable in the pH range 3.0–10.0 and has maximum activity at pH 7.5. Stability of PTPI was also investigated as a function of temperature between 30 and 90°C in 50 mM sodium phosphate buffer, pH 7.5 by means of fluorescence emission spectroscopy and inhibitory activity assay. PTPI remained maximally active within the temperature range of 30–70°C (Fig. 5) and was stable for 120 min at 90°C. An Arrhenius plot showed that activation energy (E_a) of heat denaturation for PTPI is 21 kJ mol^{-1} (Fig. 6). The critical transition temperature T_c, determined as the temperature at which a change in the slope of the plot occurs, was found to be 70°C.

Immunological properties

The PTPI caused good immune response; and the resulting antiserum had a titer of 25,118.86 as determined by direct binding ELISA in rabbit serum. The antibodies raised against purified inhibitor gave a reaction of identity

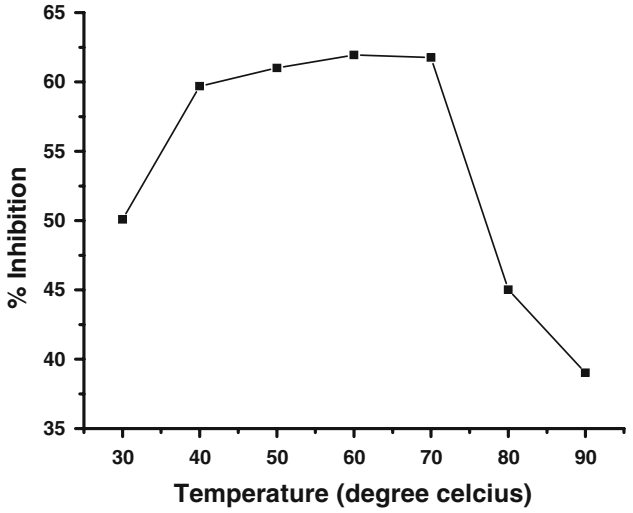

Fig. 5 Effect of temperature on PTPI. 50 μg of the inhibitor was incubated in 50 mM sodium phosphate buffer, pH 7.0, at various temperatures for 30 min. Remaining % inhibitory activity was analyzed against 50 μg of papain at 37°C

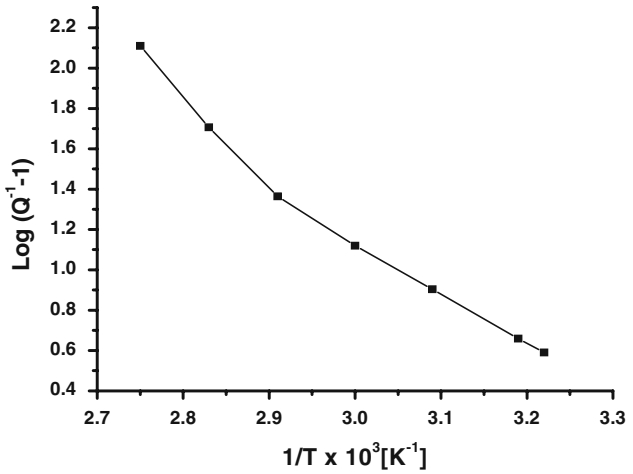

Fig. 6 Arrhenius plot, logarithm of tryptophyl quantum yield versus temperature for PTPI in 50 mM sodium phosphate buffer, pH 7.5

with the inhibitor as indicated by a single precipitin line on immunodiffusion suggesting that the wells contained apparently pure PTPI (Fig. 7). The antiserum exhibited no immunogenic identity with goat lung and brain cystatins.

Antibacterial activity

PTPI does not show any antibacterial activity. The Gram negative strains studied (*E. coli*, *Streptococcus* mutants) were found to be insensitive to PTPI at 50–200 μg ml^{-1} of inhibitor concentrations.

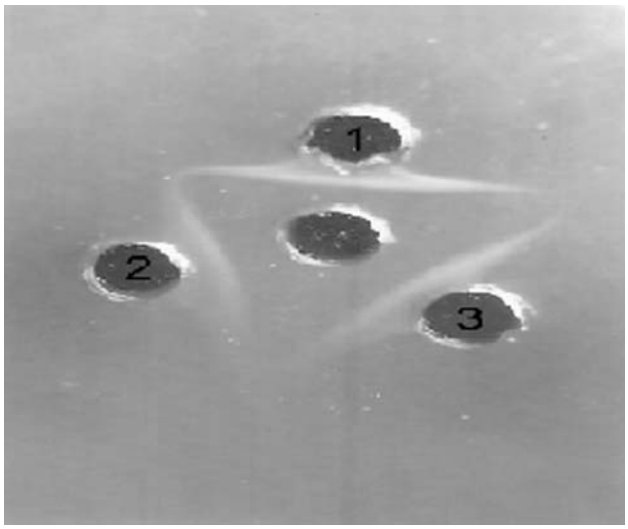

Fig. 7 Ouchterlony immunodiffusion-anti-PTPI antiserum was raised in rabbits. For the immunodiffusion study, the antiserum is allowed to react with inhibitor (60 μg) on agarose plates. The central well contained the antiserum, whereas the surrounding three well contained PTPI

Active site titration

Active papain was titrated with PTPI at protein concentrations much higher than the dissociation equilibrium constants of the complexes formed. The titrations were monitored by the changes in intrinsic fluorescence accompanying the binding. These analyses showed that the inhibitor binds to papain with stoichiometry of 1:1 (Fig. 8).

Kinetic properties of PTPI

Enzyme inhibition spectra

The purified inhibitor, inactivated papain and ficin very efficiently and to a slightly lesser extent, bromelain, but failed to inhibit bovine trypsin, chymotrypsin, and pepsin.

Kinetics and affinity of interaction with proteinases

Dissociation rate constants, as well as dissociation equilibrium constants (measured as K_i), for the binding of PTPI to plant cysteine proteinases were determined by monitoring the loss of enzyme activity (Table 2). Association rate constants were calculated from measured dissociation rate and dissociation equilibrium constants as $K_i = K_{-1}/K_{+1}$. Accurate K_i values were determined by working at lower enzyme concentrations. As other reported cystatins (Zehra et al. 2005; Sumbul and Bano 2006; Shahid et al. 2005; Rashid et al. 2006), PTPI also binds papain tightly and reversibly. The increasing values of K_i (app) with an increase in the substrate concentration suggested a

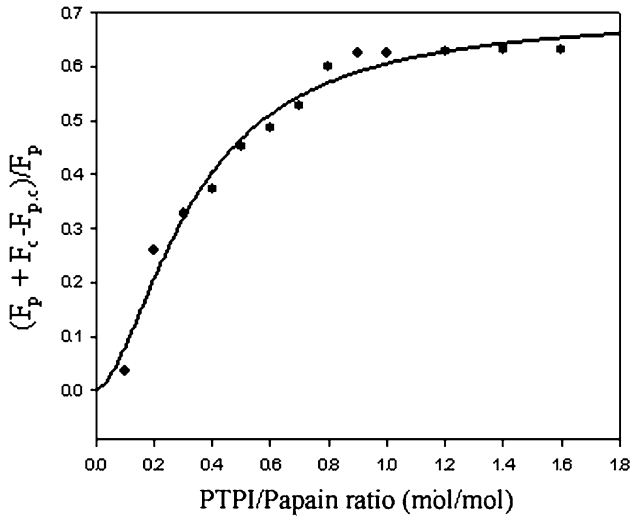

Fig. 8 Titration of papain with PTPI, monitored by measurements of tryptophan fluorescence. Excitation was at 280 nm and fluorescence emission was measured at 350 nm. F_p fluorescence of protienase, F_c fluorescence of added cystatin, $F_{p.c}$ fluorescence of proteinase/cystatin mixture. The continuous *line* represents the computer fit of the data to the theoretical binding equation

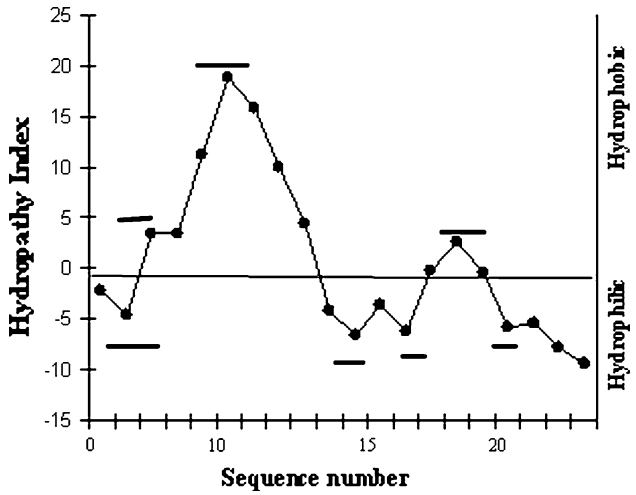

Fig. 9 Hydropathy plot of the N-terminal 24 amino acid stretch of PTPI. A window size of 5 was selected for plot formation

competitive mechanism of inhibition. The true K_i values were obtained from the replot of K_i (app) versus substrate concentration. The K_i values obtained for papain, ficin and bromelain are 5.88, 9.02 nM and 2.22 $\times$ 10^{-8} M, respectively, implying the highest affinity of inhibitor for papain. The K_{-1} values for the enzyme-inhibitor complex were determined by the displacement procedure, in which the inhibitor released from the complex with the increase in time was trapped by excess substrate (casein). The amount of enzyme released from the complex was monitored by continuous measurement of enzyme activity. The K_{-1} values obtained for papain, ficin and bromelain are 8.75 $\times$ 10^{-5}, 3.35 $\times$ 10^{-4} and 1.32 $\times$ 10^{-4} s^{-1}, respectively. The calculated half-life values of enzyme–inhibitor complex using these K_{-1} values for papain, ficin and bromelain are 7.91 $\times$ 10^3, 5.50 $\times$ 10^3, and 5.23 $\times$ 10^3 s. Association rate constant and hence the affinity of the inhibitor for proteinases is in the following order: papain (1.48 $\times$ 10^4 M^{-1} s^{-1}) > ficin (1.39 $\times$ 10^4 M^{-1} s^{-1}) > bromelain (5.94 $\times$ 10^3 M^{-1} s^{-1}). IC50 values, i.e. the concentration of inhibitor at which 50% of the enzyme is inhibited, calculated are 0.08, 0.078 and 0.154 μM, for papain, ficin and bromelain, respectively, again suggesting greater affinity of the inhibitor for papaya proteinase.

Spectroscopic analyses

Absorption spectrum

PTPI gave typical protein absorption with a maximum at 278 nm and a minimum at 250 nm. The ratio of the absorbance at 280/260 nm is 1.3 (Layne 1957). The millimolar absorption coefficient at 280 nm of PTPI is found to be 22.1 mM^{-1} cm^{-1} by taking molecular weight value of 44,000.

Fluorescence emission spectrum

After excitation at 280 nm, where phenol and indole groups absorb, the PTPI exhibits an emission spectrum with a maximum (λ_{max}) at 335 ± 1 nm which points to the non-polar tryptophyl side chains in the protein (Burstein et al. 1973).

CD spectroscopy

The far-UV CD spectra of PTPI revealed α-helical structure of 17.18% (Fig. 10) (calculated by equation as described by Chen et al. 1972).

Amino acid analysis

The N-terminal 24 amino acid residues of the heavier subunit (23.98 kDa) were sequenced, and some interesting results were obtained. As in other cystatins (Table 3), PTPI possesses a conserved glycine residue at 11th position. Maximum sequence homology was observed with bovine skin cystatin C (Cimerman et al. 1996). Sequence homology of PTPI was also observed with bovine parotid cystatin C, bovine colostrum cystatin C, and human cystatin C (Cimerman et al. 1996); sheep plasma LMW K1 (Shahid et al. 2005), human stefin A and B, salivary cystatin S7, chicken cystatin C (Cimerman et al. 1996), placental cystatin (Rashid et al. 2006) and cystatins E (Ni et al. 1997); the details are given in Table 3. Using sequence of

Table 2 Kinetic constants obtained on interaction of PTPI with proteinases—papain, ficin and bromelain

Proteinase	K_i (nM)	K_{+1} (M^{-1} s^{-1})	K_{-1} (s^{-1})	Half life of complex (s)	IC_{50} (μM)
Papain	5.88 ± 0.06	1.49 ± 0.03 × 10^4	8.76 ± 0.01 × 10^{-5}	7.91 × 10^3	0.08
Ficin	9.02 ± 0.08	1.39 ± 0.02 × 10^4	1.26 ± 0.02 × 10^{-4}	5.23 × 10^3	0.078
Bromelain	22.28 ± 0.11	5.94 ± 0.02 × 10^3	1.32 ± 0.02 × 10^{-4}	5.51 × 10^3	0.154

Results represent the mean ± SEM calculated from three independent experiments

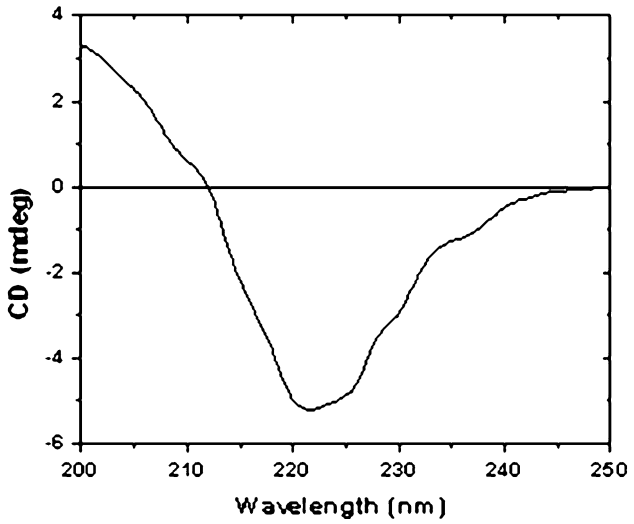

Fig. 10 Far UV-CD spectra of native PTPI. The concentration of the inhibitor used was 0.2 mg ml^{-1} and the pathlength was 0.1 cm

these N-terminal amino acid residues, a hydropathy plot was made using the respective hydropathy indices (Kyte and Doolittle 1982). Among 24 residues sequenced, the stretch of 3–7, 7–11 and 15–19 residues has maximum hydropathy index suggesting that these residues might be present inside the hydrophobic core of the protein (Fig. 9).

Discussion

Cystatins have important regulatory roles to play in normal body processes owing to their cysteine proteinase inhibitory activity. The presence of a thiol protease inhibitor in pancreas has been shown earlier (Ni et al. 1997), however, its isolation and characterization remained unattempted. The present report describes the purification of a thiol proteinase inhibitor from goat pancreas (PTPI, 44 kDa) seen to be specific for cysteine proteinases since no activity against aspartic (pepsin) and serine protienases (chymotrypsin and trypsin) has been detected. The simple purification procedure employed provided 500-fold purification and total recovery of 20.4%. Cystatins from tissues are usually small single subunit proteins with molecular masses in the range of 11–25 kDa (Zehra et al. 2005). However, low molecular mass of tissue cystatins is contrasted with reports on high molecular mass (Zehra et al. 2005; Sumbul and Bano 2006). The values of stokes radius and S_{max}/S ratio can be used to predict the shape of the protein molecule (Schurmann et al. 2001). The values of 27.3 Å and 1.2 for PTPI are in close agreement with those of ovalbumin, suggesting that PTPI is a globular protein. Generally, cystatins type 1 and type 2 isolated from tissues lack carbohydrates (Bode et al. 1988) whereas the presence of carbohydrates is a distinguishing property of type 3 cystatins, the kininogens (Ohkubo et al. 1984). However, presence of carbohydrate has been reported in cystatins E, F, M and those isolated from goat kidney and brain (Ni et al. 1997, 1998; Sotiropoulou et al. 1997). PTPI contains 2.32% carbohydrates and has no thiol group. Considering these data, PTPI may be a variant of cystatin I and cystatin II families. High stability of the purified inhibitor in broad temperature and pH ranges and on storage for long periods is consistently found in other cystatins. For PTPI a straight line Arrhenius plot was obtained from 30 to 70°C with an E_a value of 21 kJ mol^{-1}. This is in accordance with Pace (1975), that the native state of most naturally occurring proteins is only 5–15 kCal/mol more stable than its unfolded conformation. No reports are available on E_a values of other mammalian cystatins. The biphasic Arrhenius plot could be due to temperature induced conformational change in the inhibitor (Allen et al. 1990). The stoichiometry of binding of purified cystatin to papain is 1:1. This value shows that essentially all enzyme molecules were able to bind to the inhibitor, similar results have been demonstrated earlier for the binding of the recombinant human cystatin C (Bjork et al. 1994), recombinant bovine cystatin C (Olsson et al. 1999), recombinant stefin A (Nicklin and Barrett 1984), with papain. Of the enzymes studied, PTPI binds most tightly to papain, i.e. with K_i of 5.88 nM and association rate constant of 1.48 × 10^4 M^{-1} s^{-1} and then to ficin. The affinities measured for the interaction of PTPI with papain, ficin and bromelain close to corresponding affinities of goat kidney cystatin (Zehra et al. 2005), goat brain cystatin (Sumbul and Bano 2006) and HPC (Rashid et al. 2006), for these enzymes. The weaker binding of PTPI to bromelain (K_i = 2.22 × 10^{-8} nM) is largely due to a low dissociation rate constant. The K_i and rate constants for association of the inhibitor with papain and ficin are also comparable with the values measured for chicken cystatin (Björk et al. 1989),

Table 3 N-terminal amino acid sequence of PTPI

PTPI	**V**	**E**	V	**D**	**D**	A	**I**	V	V	**V**	G	Y	Y	**K**	**A**	**A**	Q	**V**	P	**Y**	Q	H	**A**	**H**
Human placental cystatin (8.33%)	Y	**E**	D	**D**	E	P	V	L	L	E	G	L	I	R	L	D	D	G	F	V	I	L	G	L
Human cystatin E (8.33%)	**V**	G	E	L	R	D	L	F	A	R	R	P	A	V	Q	K	A	A	Q	A	A	V	**A**	S
Bovine parotid cystatin C (12.5%)									L	L	G	G	L	M	E	**A**	D	**V**	M	E	E	G	G	Q
Human cystatin C (12.5%)	S	S	P	G	K	P	P	R	L	**V**	G	G	P	H	D	**A**	S	**V**	E	E	E	G	V	R
Chicken cystatin C (4.5%)			S	E	**D**	R	S	R	L	L	G	A	P	V	P	V	D	E	N	D	E	G	L	Q
Bovine skin cystatin– C (20%)										L	G	G	L	H	E	**A**	D	**V**	N					
Bovine colostrum cystatin C (11.8%)								R	L	L	G	G	L	H	E	**A**	D	**V**	N	E	E	G	V	Q
Human salivary cystatin-S7 (5.3%)						S	S	S	K	E	E	N	R	I	I	Q	G	G	I	**Y**	D	A	D	L
Human stefin B (5.8%)							M	M	C	G	A	P	S	A	T	Q	P	A	T	A	E	T	Q	**H**
Human stefin A (5.8%)							M	I	P	G	G	L	S	E	**A**	M	P	A	T	P	E	I	Q	E
Human α_2 kininogen (14.3%)		**E**	–	S	Q	S	E	E	I	D	C	N	D	**K**	D									
Bovine α_2 kininogen (7.1%)		**E**	–	S	S	Q	–	E	I	D	C	N	D	P	Q	Q								
Sheep plasma LMW K1 (10%)	D	Q	H	K	S	E	**I**	A	Q	S														

The highly purified cystatin was transferred to PVDF membrane by Western blotting before doing amino acid analysis. Percent homology with other known cystatin N-terminal sequences has been given in *parenthesis*. Similar amino acids are highlighted in bold

human cystatin C (Bjork et al. 1994) and cystatin D (Balbin et al. 1994) for their interactions with various cysteine proteinases (papain, ficin and cathepsins B, H and L). Studies of the kinetics of binding of chicken cystatin to several cysteine proteinases have shown that these reactions are best described by the simple reversible bimolecular mechanism:

$$P + I \underset{K_{-1}}{\overset{K_{+1}}{\rightleftarrows}} PI$$

where P is the proteinase, I the inhibitor and PI their complex (Björk and Ylinenjärvi 1990). The same conclusion is strongly indicated by studies with PTPI in this work. The linear increase in the observed pseudo-first order rate constant for cysteine proteinases studied; with PTPI concentration is consistent with a simple reversible bimolecular reaction mechanism (Nicklin and Barrett 1984). Moreover, the increasing value of K_i with an increase in substrate concentration suggests the inhibition to be competitive as reported earlier by Li et al. (2000) and by Nicklin and Barrett (1984) for inhibition of human cathepsin B by chicken cystatin.

The CD spectra of PTPI resembles more to that of cystatin A (with low α-helical content ∼15%) (Lindahl et al. 1992; Stubbs et al. 1990).

PTPI is a good partner of other reported goat tissue cystatins in terms of its physical properties. Also, the pH and heat stability, interaction with papain, affinities towards other proteinases and N-terminal sequence analysis of the purified PTPI are quite similar to its other cystatins; but differences in terms of its molecular mass, subunit structure, sulphydryl groups, and carbohydrate content from other tissue cystatins imply different routes of biosynthesis, different in vivo distribution and suggest a variety of physiological functions. The presence of a thiol proteinase inhibitor in goat pancreas should prompt studies on its role in pancreatic (patho) physiology.

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