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Purification, Characterization and Kinetics of Thiol Protease Inhibitor from Goat (*Capra hircus*) Lung

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Abstract—In the present study, two molecular forms of goat lung cystatin (GLC), I and II, were purified to homogeneity by a two-step procedure including ammonium sulfate precipitation (40-60%) and ion exchange chromatography. The inhibitor forms migrated as single bands under native and SDS-PAGE with and without reducing agent giving molecular mass of 66.4 and 76.4 kDa, respectively. GLC-I possesses 0.07% and GLC-II 2.3% carbohydrate content and no -SH groups. GLC-I showed greater affinity for papain than for ficin and bromelain. Immunological studies showed that the inhibitor was pure and there was cross reactivity between anti-GLC-I serum and goat brain cystatin. Both inhibitor forms were stable in the pH range of 3-10 and up to 75°C. GLC-I was found to possess 49% α-helical structure by CD spectroscopy. The inhibitor–papain complexes showed conformational changes as invoked by UV and fluorescence spectroscopic studies.

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Key words: goat lung cystatin, ion-exchange chromatography, kinetics, circular dichroism, ELISA, fluorescence spectroscopy

Proteolytic enzymes are widely distributed in animals, plants, and microorganisms. Cystatins are natural protein inhibitors of cysteine proteases. They are noncovalent, competitive, and reversible binding inhibitors, constituting a single super family of structurally, functionally and evolutionarily related proteins [1]. On the basis of subcellular localization, size, disulfide bonds, and sequence homology, the cystatin superfamily has been classified into three families [2]. Family 1 cystatins (cystatins A and B) are also called stefins; they are intracellular proteins containing about 100 amino acids residues (11-12 kDa) and are devoid of carbohydrate content and disulfide linkages [3]. Family 2 cystatins (cystatins C, D, E, F, S, SA, CD, and SN) are mainly extracellular and transcellular secreted proteins [4] (13-14 kDa) possessing two intra-chain disulfide bonds; they are not glycosylated. Family 3 cystatins, also called as kininogens, include high molecular weight kininogen (120 kDa), low molecular weight kininogen (68 kDa), and T-kininogen (68 kDa, found only in rat plasma), which contain three family 2-like cystatin domains and are only found in serum. They are single chain glycoproteins and serve as precursors for the vasodilator peptides bradykinin and kallidin. Kininogens are also involved in the blood-clotting cascade and participate in the activation of prokallikrein and factor XI and XII [5].

These inhibitors might protect cells from unwanted proteolysis that might otherwise cause a number of pathologies like rheumatoid arthritis [6], osteoporosis [7], renal failure [8], Alzheimer's disease [9], purulent bronchitis [10], cerebral amyloid angiopathy [11], metastasizing cancer [12], atherosclerosis and aneurysm [13], septic shock [14], and microbial invasion [15].

Cystatins have been purified and characterized from various sources including bovine muscles [16], goat kidney [17], human spleen [18], human liver [19], human placenta [20], sheep plasma [21], skin of Atlantic salmon [22], bovine brain [23], and goat brain [24].

Lung diseases like cancer, emphysema, and idiopathic pulmonary fibrosis are known to be caused by an imbalance between the activities of endogenous inhibitors and cysteine proteinases (cathepsins) [25], thus cystatins might play an important role in such diseases. However, isolation and characterization of cystatin from lungs has yet not been undertaken. The present study was designed to purify and characterize cystatin from goat lungs. This will shed light not only on goat lung cystatin properties but will also yield to a better understanding of the pathology of lung diseases.

Abbreviations: GLC, goat lung cystatin.

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MATERIALS AND METHODS

Papain was purchased from Sigma (USA). Medium range molecular mass markers were from Genie (India), and DEAE-cellulose was purchased from SRL Ltd. (India). All other chemicals used were of highest purity grade commercially available.

Purification of cystatin from goat lung. Goat lung cystatin was purified to homogeneity by a two-step procedure including ammonium sulfate precipitation and ion-exchange chromatography. Fresh lung tissue (100 g) was homogenized in extraction buffer (200 ml) of physiological pH containing 1% NaCl, 3 mM EDTA, and 2% *n*-butanol. After centrifugation (5000 rpm for 15 min at 4°C), the supernatant was adjusted to pH 11.0 with 3 M NaOH and then incubated at 4°C for 30 min to remove unwanted proteins. The pH of the solution was brought back to 7.5 with acetic acid. After centrifugation (8000 rpm) for 30 min, the supernatant was fractionated between 40 to 60% ammonium sulfate saturation, and the precipitated proteins were dissolved in 0.1 M sodium phosphate buffer, pH 7.5, and dialyzed against the same buffer containing 1% NaCl. The dialyzed protein was subjected to ion-exchange chromatography on a DEAE-cellulose column (2 × 7 cm) equilibrated with 50 mM sodium phosphate buffer, pH 8.0, and the proteins were eluted by a linear gradient of 0-0.5 M NaCl using 50 mM sodium phosphate buffer, pH 8.0. Fractions obtained were tested for protein and inhibitory activity.

Assay of thiol proteinase inhibitory activity. The inhibitory activity of cystatin was assessed by its ability to inhibit caseinolytic activity of papain by the method of Kunitz [26]. One unit of inhibitor activity is defined as the amount of inhibitor causing 0.001 absorbance change per minute. The protein concentration was determined by the method of Lowry et al. [27].

Electrophoresis. Homogeneity of the purified inhibitors GLC I and II was checked by native and SDS-PAGE in the presence and absence of 2-mercaptoethanol by the method of Laemmli [28]. Native gels (7.5%) were also run for both inhibitor forms. The gels were stained with 0.1% Coomassie Brilliant Blue R-250. The molecular masses of the inhibitors were determined by SDS-PAGE against phosphorylase *b* (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29.1 kDa), soybean trypsin inhibitor (20.1 kDa), and lysozyme (14 kDa).

Mass spectrometry of GLC (MALDI-TOF analysis). GLC-I and GLC-II were freeze dried, desalted, and prepared for analysis on a Voyager Bioworkstation (Perspective Biosystem, USA). The samples were dissolved in 1% trifluoroacetic acid and the matrix sinapinic acid was added. This preparation was then vortexed, and 1 mg/ml of the purified inhibitor (GLC-I and GLC-II) was applied on the sample plate. The spectrometer equipped with delayed extraction system accessory was operated in linear mode. Samples ions were evaporated using a nitrogen laser at 330 nm wavelength and accelerated at a potential of 20 kV with delays of 134 nsec. Around 150 shots of 3 nsec pulse width laser light were required to ionize the sample. Finally, the signal was digitized at a rate of 480 MHz, and averaged data was presented to the data system for correction.

Thiol group estimation. Thiol groups of cystatin were determined by the Ellman procedure [29] using dithionitrobenzoate and molar extinction coefficient of 13,600 $M^{-1}\cdot cm^{-1}$.

Carbohydrate estimation. The carbohydrate content was analyzed by the method of Dubois et al. [30] using glucose as standard.

Thermal stability of the inhibitors. Fifty micrograms of the inhibitors in 50 mM sodium phosphate buffer (pH 7.5) were incubated at various temperatures in the range of 25-100°C for 30 min. These samples were rapidly cooled in an ice-cold water bath and checked for residual activity against 50 μg of papain.

pH stability of the inhibitors. Fifty micrograms of the inhibitors were incubated with 50 mM Tris-glycine buffer (pH 2-6), sodium phosphate buffer (pH 7.0 and 8.0), and Tris-NaOH buffer (pH 9-12) for 30 min at 37°C. Aliquots of this reaction mixture were used for the determination of its remaining inhibitory activity against 50 μg of papain.

Stoichiometry of cystatin–papain complex. The inhibitory activity of cystatin was assessed by its ability to inhibit caseinolytic activity of papain. The concentration of papain was varied from 0.01 to 0.06 μM, while the inhibitor concentration was fixed at 0.06 μM.

Kinetics. IC_{50} was determined as the concentration of the inhibitor at which the enzyme is 50% inhibited. Association rate constant (k_{+1}) values were determined by monitoring the time dependence of the association under second order reaction conditions. The constant k_{+1} was calculated assuming that enzyme (E) and inhibitor (I) react in such a way that the dissociation constant k_{-1} is low enough to neglect the reverse reaction during the initial part of the process. The initial concentrations of the enzyme ($[E]_o$) and inhibitor ($[I]_o$) were identical. The association rate is given by the equation:

$$1/[E] = 1/[E]_o + k_{+1}\cdot t.$$

For the dissociation rate constant, maximal association between proteinase and either inhibitor was achieved before the reaction was shifted towards dissociation by adding excess substrate, which also binds the entire free enzyme. Dissociation of the EI complex obeys first order kinetics. Thus, the integrated form of the dissociation rate equation is given by:

$$\ln([EI]/[EI]_0) = k_{-1}\cdot t.$$

From the above k_{-1}, the half-life of the complex ($\tau_{1/2}$) was calculated as $0.693/k_{-1}$.

Production of antiserum. Antibodies against goat lung cystatins (GLC-I) were raised by injecting 300 μg of the purified inhibitor in Freund's complete adjuvant subcutaneously into a healthy male albino rabbit. The injection was repeated every week in Freund's incomplete adjuvant and the rabbit was bled every second week. The blood collected was allowed to coagulate at 22°C for 3 h. The antisera was decomplemented at 57°C for 30 min and stored at −20°C in small aliquots.

Immunodiffusion. Immunodiffusion was performed essentially by the method of Ouchterlony [31]. One percent agarose in normal saline containing 0.02% sodium azide was poured on a glass plate and allowed to solidify at room temperature. Fifteen microliters of suitably diluted antiserum and the required amount of antigen were added in different wells. The reaction was allowed to proceed for 12-24 h in a moist chamber at room temperature. (The goat brain cystatin used in immunodiffusion studies was purified in our laboratory.)

Direct binding ELISA. The generation of antigen-specific antibody was measured in the sera of GLC-I immunized rabbits by the technique of direct binding ELISA [32]. Ninety-six wells of an Immulon 2 HB microtiter plate (Dynex, USA) were coated overnight with 100 μl of antigen at 4°C. The plate was washed three times with TBST buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20). Nonspecific sorption was blocked during 5-6 h at room temperature with TBS buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl) containing 1% BSA. Plates were washed twice with TBS-T. The test and the control wells were then loaded with 100 μl of serially diluted serum (1 : 10). The plates were incubated for 2 h at room temperature and then overnight at 4°C. One hundred microliters of appropriate conjugate of anti-rabbit alkaline phosphatase (1 : 3000) were coated in each well and kept for 2 h at room temperature. After regular washing with TBS-T and distilled water, the substrate *p*-nitrophenyl phosphate (5 mg/100 ml of 50 mM sodium bicarbonate buffer, pH 9.5, containing 0.02% sodium azide) was added in each well and incubated for 30-45 min. The reaction was stopped by addition of 100 μl of 3 M NaOH in each well. The absorbance of each well was monitored at 405 nm using a Qualigens ELISA reader.

CD spectroscopy. Circular dichroism measurements were carried out on a Jasco spectropolarimeter model J-720 using a SEKONIC XY plotter (model SPL-430 A). The concentration of inhibitor for far-UV CD analysis was 0.2 mg/ml. The path length was 0.1 cm.

Ultraviolet spectroscopy. Ultraviolet (UV) absorption difference spectra were measured for GLC-I and GLC-II (2.66 μM) along with activated papain on a Cintra 10 spectrophotometer.

Fluorescence spectroscopy. Fluorescence measurements were performed on a Shimadzu (Japan) spectrofluorimeter model RF-540 equipped with data recorder DR-3. The excitation wavelength was 280 nm and the slits were set at 5 nm for excitation and emission. The path length of the sample was 1 cm, and the emission wavelength range was 300-400 nm. The protein concentration used in the fluorescent measurements was 2 μM. Each spectrum was the average of at least three scans.

Replicates. The purification procedure was reproduced several times to obtain fresh purified inhibitor forms for each experiment. The results reported were performed at least three times independently.

RESULTS

Purification of cystatins from goat lung. The scheme of purification of cystatins from goat lung is summarized in Table 1. When proteins precipitated after 40-60% ammonium sulfate saturation were dialyzed and loaded on a DEAE-cellulose column, two separate protein peaks with papain inhibitory activity were obtained (Fig. 1). The first inhibitory peak (GLC-I) was procured with purification of 76-fold and yield of 11.3%, while the second inhibitory peak (GLC-II) was obtained with enrichment of 344-fold and yield of 10.5%. (Since GLC-I was

Table 1. Purification of thiol protease inhibitor from goat lungs

Step	Volume, ml	Total protein, mg	Specific activity, units/mg	Yield, %
Lung extract	150	6900	0.154	100
pH 11.0	110	4950	0.206	79.7
$(NH_4)_2SO_4$ fractionation	90	3375	0.215	56.6
Ion exchange chromatography				
peak I	9	8.19	11.7	11.3
peak II	15	1.78	52.9	10.5

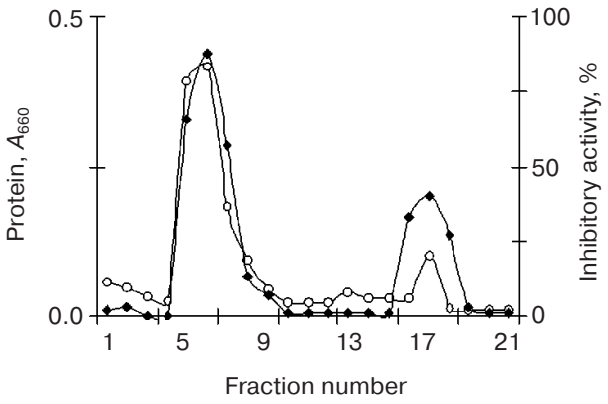

Fig. 1. Ion-exchange chromatography on a DEAE-cellulose column (2 × 7 cm) equilibrated with 50 mM sodium phosphate buffer, pH 8.0. The elution was performed in 3 ml fractions using a linear gradient of 0-0.5 M NaCl at a flow rate of 37 ml/h. Each fraction was assayed for thiol proteinase inhibitory activity (closed symbols) and protein concentration (open symbols).

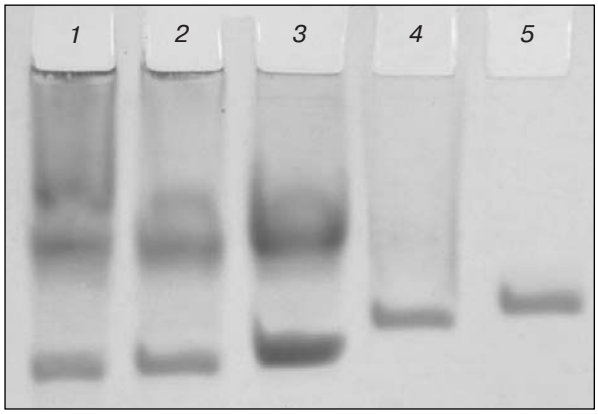

Fig. 2. Gel electrophoresis of goat lung cystatin during various stages of purification. Electrophoresis was performed on 7.5% non-denaturing polyacrylamide gel at 25°C. Lanes: *1*) lung homogenate; *2*) homogenate after alkaline treatment; *3*) dialyzed fraction after ammonium sulfate fractionation; *4*, *5*) purified inhibitors GLC-I and GLC-II after ion-exchange chromatography, respectively.

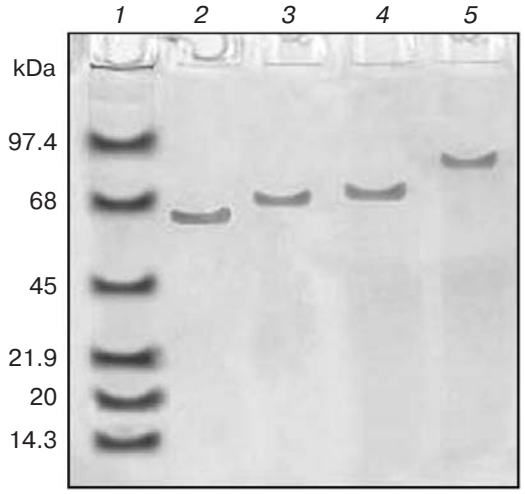

Fig. 3. Molecular mass determination of goat lung cystatin by SDS-PAGE on 10% polyacrylamide gel at 25°C. Lanes: *1*) molecular mass standards; *2*, *3*) 60 μg of purified inhibitor (GLC-I) in the absence and presence of β-mercaptoethanol, respectively; *4*, *5*) 60 μg of purified inhibitor (GLC-II) in the absence and presence of β-mercaptoethanol, respectively.

obtained in higher concentration with significant activity, most of the characterization was done using GLC-I.) GLC-I and -II migrated with different mobilities but as single bands in non-denaturing as well as in denaturing polyacrylamide gel electrophoresis with and without reduction (Fig. 2).

Molecular mass determination. The molecular masses of both inhibitor forms were determined by running the inhibitor and standard proteins molecular mass markers on SDS-PAGE in the presence and absence of β-mercaptoethanol. The mobilities of GLC-I and -II correspond to molecular mass of 63.1 and 73.1 kDa, respectively, in the presence of β-mercaptoethanol (Fig. 3). MALDI-TOF analysis (Fig. 4) showed molecular masses as 66.4 and 76.4 kDa, respectively. Of note, the purified inhibitors showed anomalous migration in SDS-PAGE in the absence of reducing environment (53.1 kDa for GLC-I and 63.1 kDa for GLC-II) (Fig. 3).

Heat and pH stability of the inhibitors. Both inhibitors were stable up to 75°C. However, at temperatures above 90°C GLC-I and GLC-II lost 28 and 22% of its inhibitory activity, respectively. Both the inhibitors were maximally active within the temperature range of 25-75°C. They were found to be stable within the pH range 3-10 but lost most of their inhibitory activity below pH 3.

Sulfhydryl and carbohydrate content. The free thiol content of native cystatins was measured by the method of Ellman. Neither free thiol group nor disulfide linkages were observed in either of the inhibitors under native and denaturing conditions. GLC-I and -II exhibited low glycosylation, 0.07 and 2.3%, respectively. Lack of carbohydrate and sulfhydryl content places GLC-I in cystatin class I.

Kinetics. The kinetics of goat lung cystatins with papain, ficin, and bromelain were analyzed by measurement of the loss of enzyme activity. As seen in Table 2, GLC-I exhibited an IC_{50} of 1.5 nM for papain, 13 nM for ficin, and 26 nM for bromelain, suggesting highest efficiency of the inhibitor for papain. The values hold good comparison with earlier reports on interaction of cystatins with thiol proteases [20, 24].

Tightly binding inhibitors have high association rate constants. Among the three thiol proteinases, as expect-

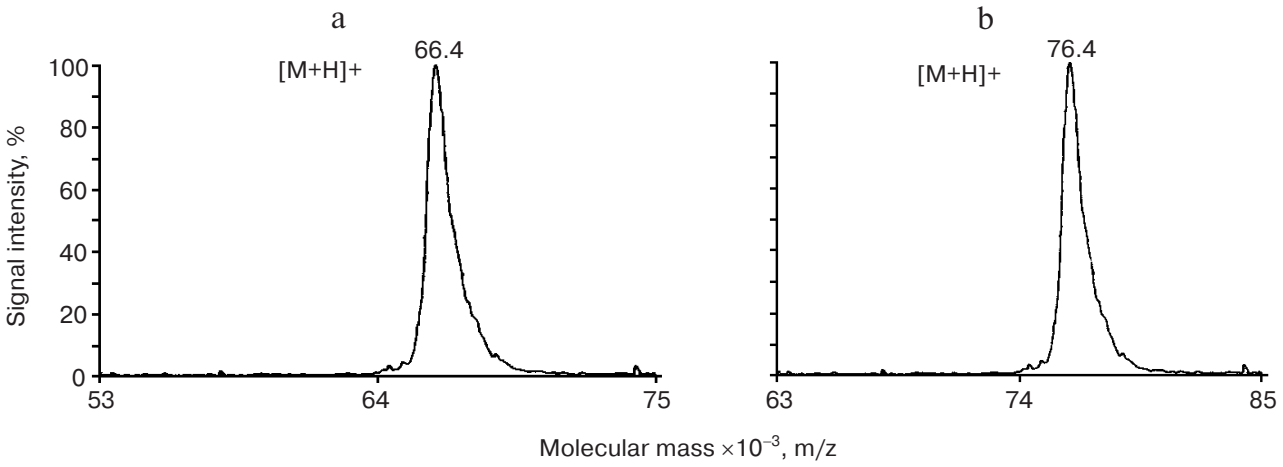

Fig. 4. Molecular mass determination of the purified GLC-I (a) and GLC-II (b) by mass spectrometry (MALDI-TOF).

ed, papain gave the highest k_{+1} of $3.06 \cdot 10^5$ $M^{-1} \cdot sec^{-1}$. High values of k_{+1} and half-life of the complex ($\tau_{1/2}$) suggest the stability of the enzyme inhibitor complex [EI] and that the complex formation is very rapid. These results are in parallel to observations made on cystatin–papain interactions by other groups [17, 20, 24].

Immunological properties. The antibodies raised against purified inhibitor (GLC-I) gave a reaction of identity with GLC-I as indicated by a single precipitin line on immunodiffusion, indicating that the purified inhibitor has immunogenic purity and homogeneity (Fig. 5). The GLC-I antisera also exhibited immunogenic identity with goat brain cystatin and GLC-II, indicating similarity in epitopes. Goat lung cystatin GLC-I showed high immune response and gave a high titer of antibodies owing to its high specificity. The titer of antibodies determined by direct binding ELISA in rabbit serum was 25,600.

CD spectroscopy. Far-UV CD spectra depict the contribution of secondary structure of the protein. The α-helical structure of the protein in the far-UV region is characterized by negative peaks at 208-210 and 222 nm and positive peaks between 190 and 192 nm. The far-UV CD spectra of the purified inhibitor at a concentration of 0.2 mg/ml show a significant peak at 222 nm and a small peak at 210 nm also. The results were expressed as MRE (mean residue ellipticity) in $deg \cdot cm^2 \cdot dmol^{-1}$, which is defined as:

$$MRE = \theta_{obs}/(10nlC_p),$$

where θ_{obs} is the CD in millidegrees, n is the number of amino acid residues, l is the pathlength of the cell, and C_p is the mole fraction. Helical content of GLC-I was calculated from the MRE values at 222 nm using the following equation:

$$\% \ \alpha\text{-helix} = (MRE_{222} - 2340/30300) \times 100.$$

The results show GLC-I to have 49% α-helical content.

Interactions of lung cystatins GLC-I and GLC-II with papain. The UV difference spectrum shows a peak at 260 nm and another peak at 295-300 nm and a minimum at around 280 nm for both inhibitor–papain complexes (Fig. 6).

Fluorescence spectroscopy. Fluorescence emission spectra showed maxima at 325 and 330 nm for GLC-I and GLC-II, respectively. Upon formation of papain–inhibitor complexes, fluorescence intensity

Table 2. Kinetic constants for the interaction of goat lung cystatin (GLC-I) with proteases

Protease	IC_{50}, nM	$k_{+1} \times 10^{-4}$, $M^{-1} \cdot sec^{-1}$	$k_{-1} \times 10^3$, sec^{-1}	$\tau_{1/2}$, sec
Papain	1.5 ± 0.2	30.6 ± 0.1	0.46 ± 0.4	1500 ± 200
Ficin	13 ± 2	8.33 ± 0.2	1.1 ± 0.1	630 ± 10
Bromelain	26 ± 3	8.0 ± 0.4	2.1 ± 0.2	330 ± 30

Note: Measured values are given with standard errors in three independent experiments.

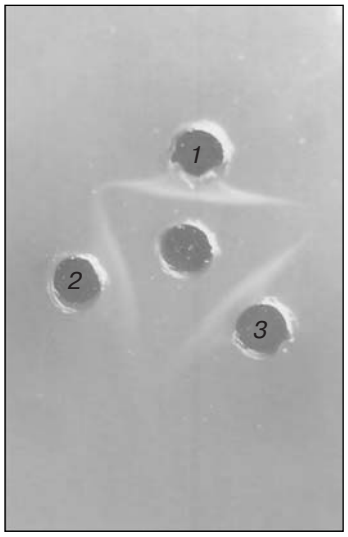

Fig. 5. Ouchterlony immunodiffusion. The central well contains the antiserum toward GLC-I, whereas wells *1-3* contain GLC-I, GLC-II, and goat brain cystatin, respectively.

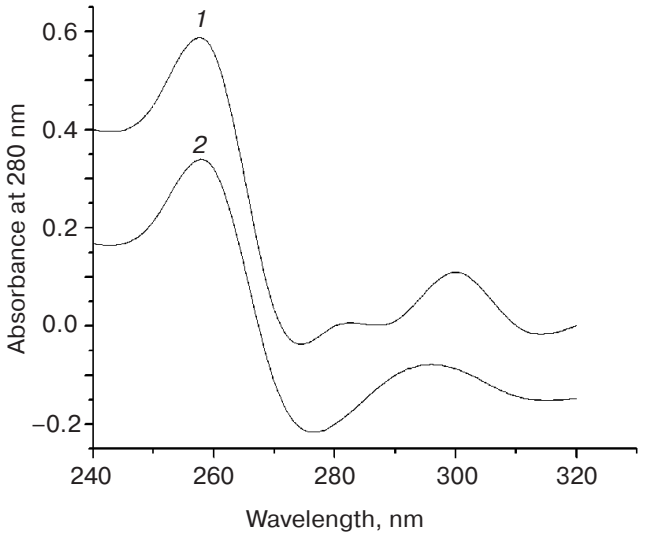

Fig. 6. Ultraviolet absorption difference spectra for GLC-I (*1*) and GLC-II (*2*) bound to papain. For complex formation with papain, both the inhibitors were taken in the molar ratio of 1 : 1. The concentration of proteins used was 2.66 μM.

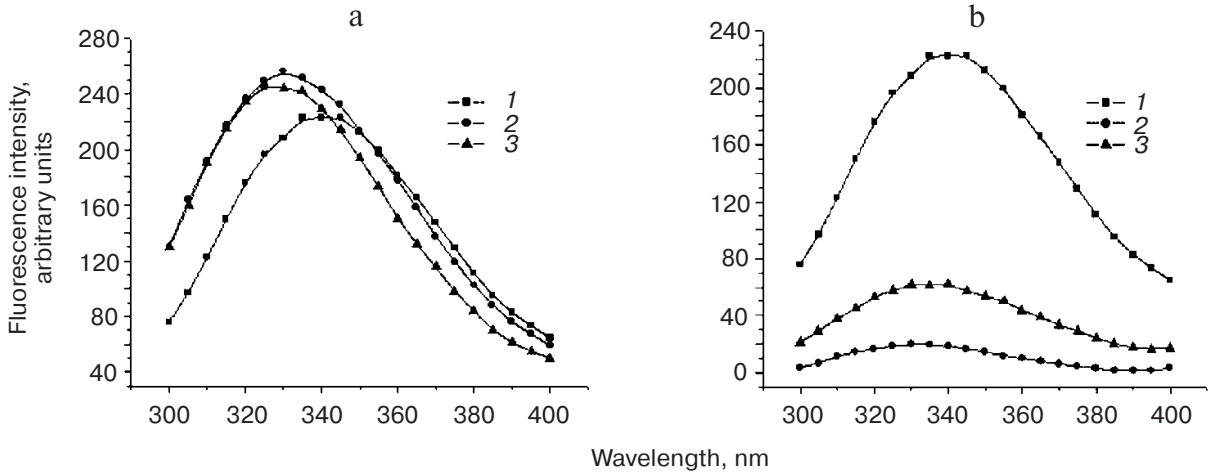

Fig. 7. Corrected fluorescence spectra of GLC-I (a) and GLC-II (b) bound to papain. Excitation wavelength was 280 nm and emission wavelength range was 300 to 400 nm. Papain and GLC-I were taken in a molar ratio of 1 : 1. The protein concentration used was 2 μM. Curves: *1-3*) papain, papain + GLC-I, and GLC-I, respectively (a); *1-3*) papain, GLC-II, and papain + GLC-II, respectively (b).

increased compared to the native inhibitors with red shift of 5 and 10 nm for GLC-I and GLC-II, respectively (Fig. 7, a and b).

DISCUSSION

Numerous inhibitors of cysteine proteases have been described and isolated from a variety of sources [24]. Mammalian cystatins have been isolated from human, rat, and many other sources [20, 33]. Only two cystatin forms have been characterized from goat [17, 24]. There is little existing information on cystatins from this source, and the present work describes for the first time the purification and properties of goat lung cystatins.

Cystatins from mammalian tissues are usually low molecular mass inhibitors having molecular mass in the range of 11-25 kDa [20, 33]. However, high molecular mass inhibitors have also been reported from the skin of Atlantic salmon [22] and goat kidney [17]. High stabilities

of the purified inhibitors (GLC-I and GLC-II) in broad temperature and pH ranges (75°C, pH 3-10) are properties consistent with other cystatins [34, 35].

GLC-I is found to be devoid of carbohydrate content, while the presence of 2.3% carbohydrate content in GLC-II is in accordance with cystatin E and F [36, 37] and those isolated from goat kidney and brain [17, 24]. Sulfhydryl content was found to be absent in both the inhibitors, which is in accordance with result previously reported [38].

The purified inhibitors inhibit papain more readily than ficin and bromelain by a reversible and competitive mechanism [39]. It has been reported that cystatin isolated from other sources generally do not inhibit bromelain, but in the present study bromelain was clearly inhibited. However, bromelain is inhibited by human placental and other purified cystatins [38, 18]. The competitive mechanism of inhibition has been found for other cystatins [40].

Tight binding inhibitors have high association rate constants. The values of k_{+1} and k_{-1} are consistent with rate constants of chicken cystatin with papain [40]. Papain exhibited the highest affinity, followed by ficin and bromelain. Low k_{-1} and high k_{+1} and half-life suggest stability of the enzyme inhibitor complex (EI) and rapid complex formation. Hence, the data obtained gives comprehensive information about the kinetics of the purified goat lung cystatin with papain, ficin, and bromelain.

The nature of interaction of thiol proteinases with the isolated inhibitors was observed through the spectroscopic changes that accompanied the binding of model thiol proteinase papain with the lung cystatins in their specific stoichiometric ratio (experimentally determined, data not shown). These binding interactions were followed by UV difference spectroscopy and fluorescence spectroscopy. Absorption difference spectra between cystatin–papain complexes revealed the pattern of changes in ultraviolet absorption in the aromatic wavelength region. The peaks observed at 260 nm may have the contributions of cysteine or phenylalanine residues or may be due to tryptophan also [41]. Similarly, the peaks around 295 nm for GLC-I and GLC-II–papain complex are suggestive of the involvement of aromatic residues in the binding with papain. Minima at around 280 nm indicate changes around tyrosine residues [41, 42], and the spectra are similar to that for the cystatin purified from goat brain [24]. The negative peaks in difference spectra for GLC-II are indicative of changes around tyrosine residues [42], an attribute similar to the UV spectra of kininogen purified from sheep plasma [21].

The complexes of GLC-I and GLC-II with equimolar papain showed 5 and 10 nm red shift and increase in fluorescence intensity. This suggests that the interaction of the two proteins occurs in a manner leading to the exposure of absorbing groups to the polar environment, or tryptophan has come near charged groups [43].

The CD spectrum of GLC-I in the far-UV region shows a peak at 222 nm and a small peak at 210 nm. The α-helical content is found to be 48.8%, which is higher compared to other previously reported cystatins—stefin A, stefin B, and chicken cystatin—which have 22, 20, and 20% helical content, respectively.

The lung cysteine proteinase inhibitors isolated in this study resemble more other cystatins of type I isolated from various species with respect to carbohydrate content, disulfide linkages, and pH and thermal stability and hence can be placed as a variant of cystatin type I of the cystatin super family.

It is interesting to speculate on the physiological role of these endogenous proteinase inhibitors. It seems likely that these endogenous inhibitors would at least serve a protective function against inappropriate proteolysis both within the cell and outside the cell. The lysosomal proteinases of leukocytes and alveolar macrophages are believed to contribute to the destruction of lung tissue, which occurs in emphysema. It has been shown that α_1-antitrypsin inactivates cathepsin B [44], tending to weaken lung defense against other proteinases. Thus, GLC-I and GLC-II could represent a second line of defense against emphysema. Recently evidence has implicated cystatins in the machinery working against microbes of the trachea [15].

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