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A Cysteine Protease Inhibitor Stored in the Large Granules of Horseshoe Crab Hemocytes: Purification, Characterization, cDNA Cloning and Tissue Localization[1]

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A cysteine protease inhibitor with an apparent $M_r=12,600$, designated limulus (L)-cystatin, was isolated from hemocyte lysates of the Japanese horseshoe crab (*Tachypleus tridentatus*), using two steps of chromatography, including dextran sulfate-agarose, and carboxymethylated papain-agarose. L-cystatin inhibits amidolytic activity of papain by forming a noncovalent 1:1 complex with an equilibrium constant (K_i) of 0.08 nM. It also inhibits cathepsin L ($K_i=0.17$ nM) and ficin ($K_i=0.52$ nM), but not argingipain (a bacterial cysteine protease) and calpains. A cDNA for L-cystatin was isolated and the open reading frame coded for a mature protein of 114 amino acids, of which 99 residues were confirmed by peptide sequencing. L-cystatin shows significant sequence identities to members of the family 2 cystatin, such as bovine colostrum cystatin (33%) and human cystatin S (31%). Northern blotting revealed expression of the mRNA in hemocytes and slightly in heart but expression was negligible in hepatopancreas, intestine, stomach, and muscle. Immunoblotting revealed the localization to be in the large granules of hemocytes. Furthermore, L-cystatin has an antimicrobial activity against Gram-negative bacteria, which is much stronger than that of chicken egg white cystatin. These data suggest that the large granule-derived L-cystatin serves synergistically to accomplish an effective defense against invading microbes, together with other defense molecules that are released in response to external stimuli.

Key words: antimicrobial activity, cystatin, cysteine protease, horseshoe crab, ion-spray mass spectrometry.

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The cystatin superfamily comprises a group of inhibitors for cysteine proteases that are widely distributed in mammalian tissues and body fluids. These inhibitors are assumed to be involved in the regulation of normal or pathological processes in which these proteases participate. Thus, cystatins may influence the intra- and extracellular catabolism of proteins and peptides (*1*), regulate proteolytic processing of prohormones and proenzymes (*2-4*), protect against penetration of normal tissues by malignant cells or microorganisms (*5-7*), and purulent bronchiectasis (*8*).

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Abbreviations: L-, limulus; Cm-papain, carboxymethylated papain; Z, benzyloxycarbonyl; MCA, 4-methylcoumaryl-7-amide; Boc, *N-tert*-butoxycarbonyl; HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; DTT, dithiothreitol; PCR, polymerase chain reaction; kb, kilobase (s); bp, base pair (s).

These data suggest that cystatins may play important roles not only in the protection of cells from unfavorable proteolysis by intracellular and external cysteine proteases but also in biological defense systems against invaders. Based on the molecular structures of various cystatins so far studied, the cystatin superfamily can be subdivided into three families 1, 2, and 3 (*9, 10*). The family 1 cystatins A and B are small proteins consisting of single polypeptide chains of about 100 amino acids residues without disulfide bridges. The family 2 cystatins consist of polypeptide chains of approximately 120 amino acid residues with two intrachain disulfide bonds. Finally, the family 3 cystatins, the kininogens, display a higher degree of structural complexity characterized by the presence of three family 2 cystatin-like domains, each with two disulfide bridges at positions homologous to those in family 2 cystatins. They also differ from the cystatins of family 1 and 2 in that they are glycoproteins.

In invertebrates two cystatins with sequence similarity to family 2 have been isolated and characterized, sarcocystatin A from the hemolymph of *Sarcophaga peregrine* larvae (*11*) and a cystatin-like protein from *Drosophila melanogaster* (*12*). While the physiological roles of these

cystatins are unclear, sarcocystatin A may participate in the morphogenesis of larval and adult structures (*13*). To obtain further information on structures of the invertebrate cystatin family and to make comparisons with those of known vertebrate ones we examined cysteine protease inhibitors in limulus hemolymph. Here, describe isolation, characterization and cDNA cloning of a cystatin from horseshoe crab hemocytes and discuss its physiological roles.

EXPERIMENTAL PROCEDURES

Materials—Hemocytes were prepared from Japanese horseshoe crabs (*Tachypleus tridentatus*) as previously reported (*14*). Large and small granules of hemocytes were prepared according to Shigenaga *et al.* (*15*). Papain, ficin, and porcine pancreatic elastase from Sigma Chemicals, St. Louis, MO, bovine spleen cathepsin C from Boehringer Mannheim GmbH, Germany, actinidin from PROTOGEN AG, Switzerland, dextran sulfate-Sepharose, Superdex™ 200 HR 10/30, pI markers, and electrophoresis calibration kits (cyanogen bromide digested horse-heart globin and the resulting peptides have the following molecular weights: globin, 16,949; globin I+II, 14,404; globin I+III, 10,700; globin I, 8,159; globin II, 6,214; and globin III, 2,512) from Pharmacia LKB Biotechnology, Uppsala, Z-Phe-Arg-MCA, Arg-MCA, Boc-Val-Pro-Arg-MCA, Suc-Leu-Leu-Val-Phe-MCA, Boc-Ala-Pro-Ala-MCA, and *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64) from Peptide Institute, Osaka, trypsin and α-chymotrypsin from Worthington, Freehold, NJ, lysyl endopeptidase from Wako Pure Chemical Industries, Tokyo, pyroglutamyl peptidase from Toyobo, Tokyo, were used. Argingipain purified from *Porphyromonas gingivalis* and the highly purified m and μ calpains were kindly provided by Dr. Kenji Yamamoto (Faculty of Dentistry, Kyushu University) and Dr. Koichi Suzuki (Institute of Molecular and Cellular Biosciences, the University of Tokyo), respectively. Rat cathepsins B, H, and L were kindly provided by Dr. Yukio Nishimura (Faculty of Pharmaceutical Sciences, Kyushu University). Cm-papain-Sepharose was prepared as described Anastasi *et al.* (*16*). A λZipLox cDNA library was constructed from poly(A^+) RNA extracted from hemocytes using a Time Saver™ cDNA synthesis (Pharmacia LKB Biotechnology, Uppsala). λZipLox™, *Eco*RI Arms, and RNA size markers (9.49, 7.46, 4.40, 2.37, 1.35, and 0.24 kb) were obtained from GIBCO BRL. All other reagents were of analytical grade or of the highest quality commercially available.

Enzyme Assays—Enzyme activities were assayed fluorometrically at different pH (*17*), as follows: papain (substrate, Z-Phe-Arg-MCA), cathepsin H (Arg-MCA), and actinidin (Z-Phe-Arg-MCA) at pH 6.8 (*16, 18, 19*), ficin and cathepsin B (Z-Phe-Arg-MCA) at pH 6.0 (*20*), cathepsin L (Z-Phe-Arg-MCA) at pH 5.5, cathepsin C (Z-Phe-Arg-MCA) at pH 6.8 (*21*), trypsin (Boc-Val-Pro-Arg-MCA), α-chymotrypsin (Suc-Leu-Leu-Val-Phe-MCA), and elastase (Boc-Ala-Pro-Ala-MCA) at pH 8.0 (*22*), argingipain (Z-Phe-Arg-MCA) at pH 7.5 (*23*). Calpains were assayed using casein as substrate at pH 7.5 (*24*). Molar concentration of active papain was determined by the active-site titration with E-64 (*20*).

Assay for L-Cystatin—L-cystatin activity was expressed as an inhibitory activity against papain (*16*). For fraction assay, papain (2 nmol) in 0.1% Briji (polyoxyethylene lauryl alcohol ether) was mixed with a sample in 450 μl of 200 mM of potassium phosphate/4 mM EDTA/4 mM DTT/0.01% BSA, pH 6.8, and the mixture was preincubated at 40°C for 5 min. The enzyme activity was measured at 40°C for 10 min in the presence of the substrate at 200 μM and terminated by the addition of 500 μl of 0.1 M sodium acetate buffer, pH 4.3. One unit of the inhibitory activity was defined as the amount of cystatin that inhibited 50% activity of papain (ID50).

Determination of Equilibrium Constants for Dissociation (K_i)—K_m values for papain (96 μM), ficin (132 μM), cathepsin C (190 μM), actinidin (181 μM), and cathepsins B (173 μM), H (158 μM), and L (63 μM) were determined by direct linear plotting (*25*). K_i values were determined by continuous fluorometric assays (*26*). The concentrations of the enzymes during the assay were 0.01 nM (papain), 0.02 nM (ficin), 0.04 nM (cathepsin B), 0.07 nM (cathepsin H), 0.1 nM (cathepsin L), 0.1 nM (cathepsin C), and 5.5 nM (actinidin). Substrates were used at 10 μM, and all measurements were made with less than 2% hydrolysis. To a cuvette containing 980 μl of enzyme solution thermostatted at 40°C was added 20 μl of substrate solution. Once a stable reaction rate had been recorded, the inhibitor was added. The reaction rate was followed for up to 60 min as it relaxed progressively to the new linear rate corresponding to the concentration of enzyme at equilibrium. Values were obtained for five inhibitor concentrations. Apparent K_i values were determined from re-plots of the form $[I]t/(1-V_i/V_o)$ *versus* V_o/V_i (*27*) and, when $[I]t \gg [E]t$, V_o/V_i-1 *versus* $[I]t$. When experiments were made with $[S] \ll K_m$, corrections were made by use of the relationship $K_i = K_{i(app)}/(1+[S]/K_m)$ for simple competition.

Antimicrobial Activity—*Escherichia coli* (clinically isolated), *Salmonella typhimurium* LT2 (S), *Salmonella minnesota* (Re), *Klebsiella pneumoniae*, and *Staphylococcus aureus* were used for determination of the antimicrobial activity by the method of Saito *et al.* (*28*).

Agglutination Activity of L-Cystatin against Bacteria—Bacterial cell agglutinating activity was assayed according to Saito *et al.* (*29*). The following strains were used: *E. coli* K12, *E. coli* B, *Staphylococcus saprophytious*, *S. aureus*, *Micrococcus luteus*, and *Enterococcus hirae*.

Peptide Preparation, Sequencing, and Amino Acid Analysis—The purified L-cystatin was reduced, *S*-alkylated with iodoacetamide, then digested with lysyl endopeptidase, trypsin, or α-chymotrypsin (E/S = 1 : 50, w/w) (*30*). The generated peptides were separated by reversed-phase HPLC using a Chemcosorb 5-ODS-H column (2.1 × 150 mm, Chemco Scientific, Osaka). Peptides were eluted by a linear gradient of 0-80% acetonitrile in 0.06% trifluoroacetic acid at a flow rate of 0.2 ml/min. Amino acid sequence analysis of purified peptides was made using a gas-phase Sequencer model 473A (Applied Biosystems) with the chemicals and program supplied by the manufacturer. For amino acid analysis, samples were hydrolyzed in 6 M HCl in evacuated and sealed tubes at 110°C for 24, 48, and 72 h. The hydrolyzates were analyzed using a Hitachi L-8500 amino acid analyzer with the chemicals and program supplied by the manufacturer. Tryptophan was determined by hydrolysis in 3 M mercaptoethanesulfonic acid (*31*).

L-Cystatin-Specific DNA Probes and Screening of cDNA

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Library—The degenerate nucleotide sequences of primers used for PCR were based on amino acid sequences of peptides -IDANVG- and -NYEVFI-, since the location and spatial relationship of these peptides could be deduced from their sequence similarities to the conserved regions of the cystatin superfamily. Sense and antisense nucleotides were synthesized with an *Eco*RI site at 5′ end, using a DNA Synthesizer model 380B (Applied Biosystems) and the chemicals and program supplied by the manufacturer. Reactions for PCR contained the cDNA template (corresponding to 0.1 μg of poly(A)$^+$ RNA) and 100 pmol of each primer and were carried out in a Perkin-Elmer Cetus thermal cycler. The PCR products were treated with *Eco*RI and purified with agarose gel electrophoresis. Fragments of interest were then ligated into a plasmid pGEM-3Zf(+) (Promega, Madison, WI) for sequence analysis, as described by Sambrook *et al.* (*32*). One clone of 156 bp contained the sequence of L-cystatin-derived peptides. The PCR fragment, labeled with [α-^{32}P]dCTP using a Ready-To-Go™ DNA-labeling Kit (Pharmacia LKB Biotechnology, Uppsala) served as a probe to screen the λZipLox library. After secondary screening, the plasmids containing the cDNA insert were prepared from the positive plaques, following the manual supplied by the manufacturer.

Computer-Assisted Analysis of Sequence Data—L-cystatin sequence was compared with all entries in the database of SWISS-PROT Data-base (release 14.0 October, 1994) with the Gene Works system (IntelliGenetics, Mountain View, CA).

Molecular Mass Determination of the L-Cystatin—The molecular mass of the protein desalted by reverse-phase HPLC was measured with a PE-Sciex API-*III* triple quadrupole mass spectrometer equipped with an ion-spray ion source (PE-Sciex, Thornhill, Ontario, Canada). The desalted and lyophilized protein was dissolved in 0.05% $HCOOH/CH_3CN$ (1 : 1) and introduced into the mass spectrometer through a fused silica tube (100 μm i.d.) at a flow rate of 2 μl/min. The mass spectrometer was scanned from m/z 800–2,000 in the positive ion mode. Step size was 0.1 amu, and dwell time was 1 ms/step. Ion-spray voltage was set at 5.0 kV, and the orifice potential was 70 V.

Determination of Blocked NH_2-Terminal Residue—(a) Ion-spray mass spectrometry: The mass spectrum of the NH_2-terminal peptide dissolved in 0.05% $HCOOH/CH_3CN$ (1 : 1) was obtained on a PE-Sciex API-*III* triple quadrupole mass spectrometer equipped with an ion-spray ion source. The mass spectrum of the peptide was obtained between m/z 400–800 in the positive ion mode. Step size was 0.1 amu, and dwell time was 1 ms/step. Ion-spray voltage was set at 5.0 kV, and the orifice potential was 70 V. (b) Pyroglutamyl peptidase treatment: After the sample (500 pmol) had adsorbed to polyvinylidene difluoride membrane, it was treated with pyroglutamyl peptidase and sequenced according to the method of Hirano *et al.* (*33*).

Northern Blot Analysis—Organs and tissues were obtained from an adult male of *Tachypleus tridentatus* and Northern blot analysis was performed as described by Miura *et al.* (*22*).

Antiserum—An antiserum against L-cystatin was raised in rabbits (male, Japanese White, 3.0 kg). The intact protein (200 μg) was emulsified in a synthetic adjuvant, TiterMax™ (Vaxcel, GA) and given intradermally. After 4 weeks, the rabbits were given 200 μg of the antigen in the same adjuvant, as a booster. Blood was drawn 3 weeks after the second injection and the serum was stored at −80°C.

SDS-PAGE, Immunoblotting, and Isoelectric Focusing—SDS-PAGE was performed in 15% slab gels according to the method of Laemmli (*34*). The gels were stained with Coomassie Brilliant Blue R-250. For immunoblotting, gels were transferred to nitrocellulose membranes, using an electroblot apparatus (Bio-Rad, Italy). The membranes were treated with the anti-serum and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG and visualized with 4-chloro-1-naphthol, as described (*35*). Isoelectric focusing was carried out by Multiphor™ II Electrophoresis with carrier ampholines (pH 3–10) (Pharmacia LKB Biotechnology, Uppsala).

RESULTS

Purification of L-Cystatin—The papain inhibitory activity was mainly present in hemocytes (3,900 units/mg) and was negligible in hemolymph plasma (86 units/mg). The lysate prepared from 38 g of hemocytes (wet weight) was first fractionated on a dextran sulfate–Sepharose CL-6B column (Fig. 1A). There were three peaks with apparent papain inhibitory activities. Peak II showed no dose-dependent inhibitory activity and the protein with inhibitory activity in peak III did not adsorb to Cm-papain–Sepharose (data not shown). Therefore, fractions of peak I were pooled and dialyzed against 50 mM sodium citrate, pH 6.5, then applied to a Cm-papain–Sepharose CL-6B column (2.5 × 4.5 cm), equilibrated with the same buffer. After washing with equilibration buffer containing 1 M NaCl, the inhibitor was eluted with 2 M guanidine hydrochloride in the same buffer (Fig. 1B). The purified inhibitor, named limulus cystatin (L-cystatin), gave a single protein band (M_r = 12,600) on SDS-PAGE, under reducing and nonreducing conditions (Fig. 2A). The pI value of L-cystatin was determined to be 10.0 (Fig. 2B). The molecular weight of L-cystatin was estimated to be 13,000, by gel filtration on a Superdex™ 200 HR 10/30 column (data not shown). The purification scheme is summarized in Table I.

Inhibition of Papain by L-Cystatin—L-cystatin (9.9 nM) was allowed to react with papain (1.8 nM), and the remaining amidolytic activity was assayed at different times. The inhibition was time-dependent and at least 5 min of incubation was required for complete inhibition (inset in Fig. 3). When papain was incubated with increasing amounts of L-cystatin for 10 min, there was a parallel decrease in the amidolytic activity of the enzyme. The intersection of the extrapolated line occurred at an inhibitor:enzyme molar ratio close to 1:1 (Fig. 3).

Inhibition of Cysteine Proteases by L-Cystatin—Inhibitory activities and specificities of L-cystatin against several cysteine proteases were investigated and K_i values were calculated by Henderson plot, as shown in Table II. L-cystatin strongly inhibited papain, cathepsin L, and ficin at similar K_i values. However, the inhibition for cathepsin B was 1/70 lower than that of papain and the lowest reactivity for actinidin. When a 10–20 molar excess of inhibitor was used for argingipain or calpains, there was no inhibition. Henderson plots showed that L-cystatin competitively inhibited papain, cathepsins B, H, and L, ficin and cathepsin C, and the interactions of these proteases with L-cystatin

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were tight-binding. On the other hand, the interaction with actinidin was much weaker than the proteases mentioned above.

Antimicrobial Activity and Bacterial Agglutinating Activity of L-Cystatin—Table III shows concentrations of L-cystatin which inhibit growth of *E. coli*, *S. typhimurium*, *S. minnesota*, *K. pneumoniae*, and *S. aureus*. L-cystatin had significant antimicrobial activity against Gram-negative bacteria, including *E. coli*, *S. typhimurium*, and *K. pneumoniae*, but not the Gram-positive *Staphylococcus* strain. The antimicrobial activity of L-cystatin was much stronger than that of egg cystatin. On the other hand, no bacterial agglutinating activity was observed for L-cystatin against *E. coli* K12, *E. coli* B, *S. saprophytious*, *S. aureus*, *M. luteus*, and *E. hirae*, even at the concentration of 220 μg/ml.

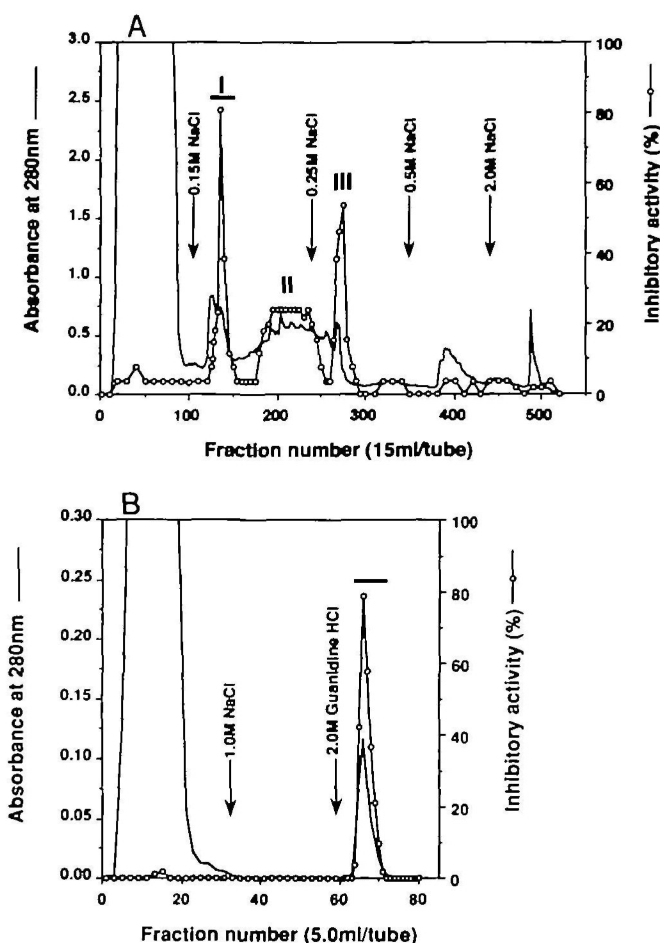

Fig. 1. **Elution profile of L-cystatin on a dextran sulfate-Sepharose CL-6B column (A) and a Cm-papain-Sepharose CL-6B column (B).** A: The hemocyte lysate, 770 ml prepared from 38 g of the *T. tridentatus* hemocytes, was applied to the column equilibrated with 20 mM Tris-HCl buffer, pH 8.0, containing 50 mM NaCl. The column was then washed with the same buffer, and L-cystatin was eluted stepwise with equilibration buffer containing 0.15 M NaCl. Elution was performed at a flow rate of 60 ml/h. Fractions were assayed for inhibitory activity toward papain and indicated by a solid bar were pooled. B: Pooled fraction I was then applied to the column and eluted with 50 mM sodium citrate buffer, pH 6.5, containing 2 M guanidine-HCl at a flow rate of 15 ml/h, after washing extensively with the same buffer and buffer containing 1 M NaCl without guanidine-HCl. Fractions indicated by a solid bar were pooled.

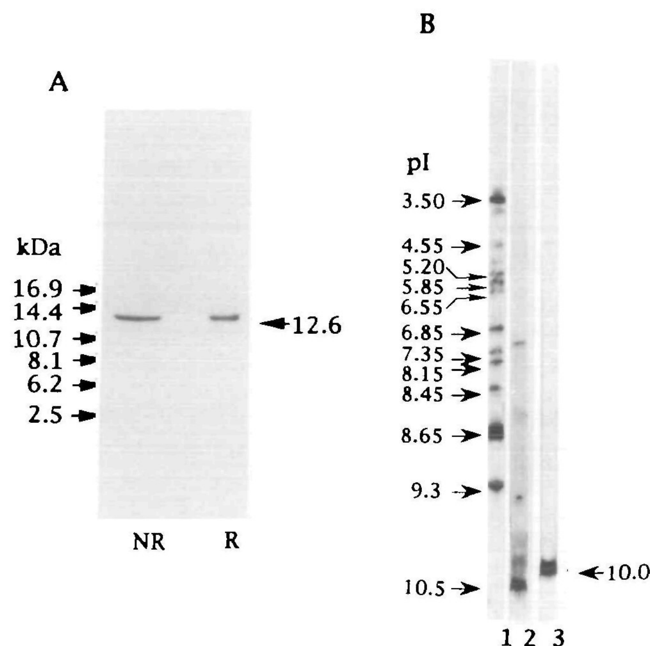

Fig. 2. **SDS-PAGE (A) and isoelectric focusing (B) of L-cystatin.** A: The purified L-cystatin was subjected to SDS-PAGE in the absence (NR) and presence (R) of 2-mercaptoethanol. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250. B: Lane 1, standard; lane 2, cytochrome c; lane 3, L-cystatin. After isoelectric focusing, gel strip was stained with Coomassie Brilliant Blue R-250. Arrows indicate the molecular weight and pI markers.

TABLE II. **K_i values for the inhibition of various thiol-proteases by limulus (L)-cystatin.**

Proteases	K_i values[a] (nM)	Substrates
Papain	0.08	Z-Phe-Arg-MCA
Cathepsin B (Rat kidney)	5.51	Z-Phe-Arg-MCA
Cathepsin H (Rat liver)	1.28	Arg-MCA
Cathepsin L (Rat liver)	0.17	Z-Phe-Arg-MCA
Ficin	0.52	Z-Phe-Arg-MCA
Cathepsin C (Bovine spleen)	2.32	Z-Phe-Arg-MCA
Actinidin	60.9	Z-Phe-Arg-MCA

[a]K_i values were calculated by Henderson plot and expressed as nM.

TABLE I. **Purification of L-cystatin from the limulus hemocyte lysate.**

Step	Volume (ml)	Total protein[a] (mg)	Total activity[b] (units)	Specific activity (units/mg)	Yield (%)	Purification (-fold)
Hemocyte lysate	770	1,850	–	–	–	–
Dextran sulfate-Sepharose CL-6B	450	70	281,000	4,020	100	1
Cm-papain-Sepharose CL-6B	35	1.3	93,300	71,800	33	17.9

[a]The amounts of protein were determined by the method of Lowry *et al.* (*64*). [b]One unit of inhibitory activity was defined as the amount which inhibited 50% activity of papain (ID50).

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Peptide Sequencing—Phenylthiohydantoin-amino acids were not obtained by sequencing the intact protein, thereby suggesting a blocked NH_2-terminus of L-cystatin. The protein was then reduced, *S*-alkylated and digested, respectively, with trypsin, α-chymotrypsin and lysyl endopeptidase. The seven peptides in total were isolated and sequenced, the results of which revealed sequences of 99 amino acids (underlined amino acids in Fig. 4).

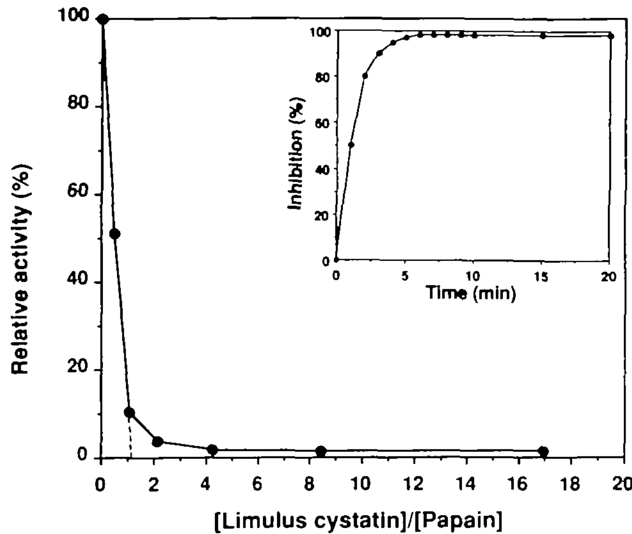

Fig. 3. **Reaction stoichiometry in the inhibition of papain with L-cystatin.** Papain (1.8 nM) was mixed with different concentrations of limulus cystatin (0-30 nM) and incubated at 40°C for 15 min. The remaining amidolytic activity was measured using substrate Z-Phe-Arg-MCA. In the inset, the mixture of papain (1.8 nM) and L-cystatin 9.9 nM was incubated at 40°C, and at intervals, aliquots of the mixture were taken for assay.

Isolation of cDNA Clone and Nucleotide Sequence of L-Cystatin—An L-cystatin-specific probe of 156 bp was identified with oligonucleotides corresponding to peptides derived from L-cystatin, using PCR and DNA sequence analysis. When the probe was used to screen a hemocyte cDNA library (100,000 recombinant phages), six hybridization-positive clones were obtained. Of these, one clone with the longest insert of 0.68 kbp was used to determine the nucleotide sequence of both strands, using designed sequencing primers. The nucleotide and deduced amino acid sequences are shown in Fig. 4. The cDNA included 673 nucleotides. There was one open reading frame beginning at the first Met codon at position 24 with the stop codon at position 424. A consensus sequence for a poly(A) addition signal (AATAAA) starting at position 656 but no poly(A) tail was present in this clone.

The open reading frame codes for a protein of 133 amino acid residues and amino acid sequences of the isolated peptides corresponded exactly to the protein sequence deduced from the cDNA sequence, indicating that the isolated cDNA clone codes for L-cystatin. A hydropathy profile of the deduced protein sequence indicated the presence of a signal sequence with a typical hydrophobic core. From the amino acid composition of L-cystatin (Table

TABLE III. **Antimicrobial activity of limulus (L)-cystatin against various bacteria.**

	IC50[a] (μg/ml)	
	L-cystatin	Egg cystatin
Salmonella typhimurium LT2	82	>131
Klebsiella pneumoniae	100	>131
Escherichia coli	86	ND[b]
Salmonella minnesota (Re)	>100	ND[b]
Staphylococcus aureus	>100	ND[b]

[a]50% inhibitory concentration. [b]Not determined.

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GCATTTTTTGGAAGAATCGACGTTATGGAGGGTTATAACATATTAGCAGTCCTTATTATT  60
                        M  E  G  Y  N  I  L  A  V  L  I  I   12

TTGGTTGGAGTTTCGATGGGACAGATACCTGGAGGATGGATTGACGCTAATGTTGGAGAT 120
L  V  G  V  S  M  G ↑Q  I  P  G  G  W  I  D  A  N  V  G  D   32

ACAGACGTGAAGGAAGCAGCCAGATTCGCAACAGAGGCGCAGAGTTCACGATCTAATTCT 180
T  D  V  K  E  A  A  R  F  A  T  E  A  Q  S  S  R  S  N  S   52

CTTTACCACCACAAATTACTGAAGATTCATAAAGCTCGAACACAGGTGGTTAGTGGTATA 240
L  Y  H  H  K  L  L  K  I  H  K  A  R  T [Q  V  V  S  G] I   72

AACTACGAAGTGTTTATTGAAACTGGTACTACTACCTGCAAGAAAAGTGAAGTGCCCTTG 300
N  Y  E  V  F  I  E  T  G  T  T  T  C  K  K  S  E  V  P  L   92

GAAGATTTGAAAAGGTGTGCAGTTCCTGAAAACGGGGTAAAACATCTTTGCCAAGCGATT 360
E  D  L  K  R  C  A  V  P  E  N  G  V  K  H  L  C  Q  A  I  112

GTCTGGGTTCAAGCATGGATTCCACGCACTAAAGTAACGAAACTTGAGTGTCAAAACAAG 420
V  W  V  Q  A  W  I  P  R  T  K  V  T  K  L  E  C  Q  N  K  132

GGGTAACTTTCTCAGTAAAGTCATCACCAAAATTTGCACTTTTTATTCATCTATGGATTT 480
G                                                            133

ATTGTACCCATGCATTGGACAAAATATTATATTTTATACCTTTTTTAATACCTACATTTA 540

CTATCAAAAATTAAATGAAGATTTTAACTTGTAAAGTTCACTTATGATGCTATATCCAGC 600

TAGAAATTTATTAATTTTAAACTCGGTTGTAAATTGTAAACAAAGCCAATTTAGAAATAA 660

AGTTTCTCACTTT                                                673
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Fig. 4. **Nucleotide and deduced amino acid sequences of L-cystatin.** Number of nucleotides and amino acid residues are given on the far right of each line. Single underlines represent sequences determined by amino acid sequence analysis of isolated peptides. A double underline represents a poly(A) addition signal. A box represent a putative protease binding site. The cleavage site accompanied by maturation is shown by arrowhead.

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IV) and the hydropathy profile, the amino-terminal amino acid seemed likely to be Gln at position 20 that had been converted to pyroglutamate residue. A possible cleavage site by signal peptidase with the highest value, using the weight matrix of von Heijne (*36*), was also Gly 19-Gln 20. Therefore, L-cystatin was treated with pyroglutamyl peptidase and subjected to sequence analysis, and the sequences obtained coincided with those from positions 21 to 30. The NH_2-terminal pyroglutamate residue was also confirmed by ion-spray mass spectrometry, using the NH_2-terminal hexa-peptide, <EIPGGW, which was isolated from α-chymotryptic digest of the *S*-alkylated L-cystatin. The mass spectrum showed a prominent single charged ion signal (protonated form) at m/z 640.2 (Fig. 5) that is consistent with the theoretical mono-isotopic mass

TABLE IV. **Amino acid composition of limulus (L)-cystatin.**

Amino acids	Residues/molecule[a]	
1/2 Cys	3.44[d]	(4)
Asp	9.20	(4)
Asn		(5)
Glu	14.0	(8)
Gln		(6)
Ser	6.38	(6)
Gly	7.25	(7)
His	3.88	(4)
Arg	4.94	(5)
Thr	9.20	(9)
Ala	8.69	(9)
Pro	4.35	(4)
Tyr	2.12	(2)
Val	10.5[b]	(11)
Met	0.19	(0)
Ile	6.69[b]	(7)
Leu	7.15	(7)
Phe	2.0	(2)
Trp	2.82[c]	(3)
Lys	10.80	(11)
$GlcNH_2$	Not detected	
$GalNH_2$	Not detected	
Total	113.6	(114)

[a]Extrapolated or average values estimated from 24-, 48-, and 72-h hydrolysates. Values in parentheses are taken from the sequence deduced from cDNA. [b]Taken from the values of a 72-h hydrolysate. [c]Estimated on a 24-h hydrolysate with 3 M mercaptoethanesulfonic acid. [d]Estimated on a 24-h hydrolysate with performic acid.

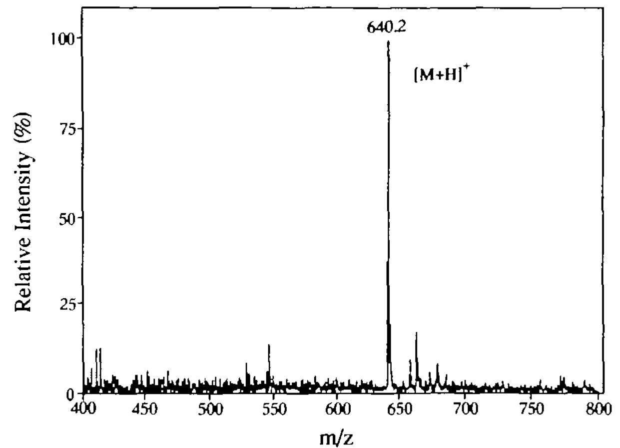

Fig. 5. **Ion-spray mass spectrum of NH_2-terminal peptide of L-cystatin.**

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L-cystatin  :                 Q I P G G W I D A N V G D T D V K E A A R F A T E A Q S S R    30
CYT BOVINE  :                 R L L G G L M E A D V N E E G V Q E A L S F A V S E F N K R    30
CYTS HUMAN  : S S S K E E N R I I P G G I Y D A D L N D E W V Q R A L H F A I S E Y N K A    38
CYTC HUMAN  :   S S P G K P P R L V G G P M D A S V E E E G V R R A L D F A V G E Y N K A    37
CYT BITAR   :                   I P G G L S P R D V T D P D V Q E A A A F A V E K Y N A G    29
CYT CHICKEN :       S E D R S R L L G A P V P V D E N D E G L Q R A L Q F A M A E Y N R A    35
CYTA SARPE  :                 Q C V G C P S E V K - G D K L K Q S E E T L N K S L S K L A    29

L-cystatin  : S N S L Y H H K L L K I H K A R T Q V V S G I N Y E V F I E T - - - - - - G T    63
CYT BOVINE  : S N D A Y Q S R V V R V V R A R K Q V V S G M N Y F L D V E L - - - - - - G R    63
CYTS HUMAN  : T E D E Y Y R R P L Q V L R A R E Q T F G G V N Y F F D V E V - - - - - - G R    71
CYTC HUMAN  : S N D M Y H S R A L Q V V R A R K Q I V A G V N Y F L D V E L - - - - - - G R    70
CYT BITAR   : S K N D Y Y F K E R R V V E A Q S Q V V S G V K Y Y L M M E L - - - - - - L K    62
CYT CHICKEN : S N D K Y S S R V V R V I S A K R Q L V S G I K Y I L Q V E I - - - - - - G R    68
CYTA SARPE  : A G D G P T Y K L V K I N S A T T Q V V S G S K D V I N A D L K D E N D K T    67

L-cystatin  : T T C K K S E V - - - - - - - P L E D L K R C A V P E N G V K H - - - L C Q    91
CYT BOVINE  : T T C T K S Q A - - - - - - - - - - N L D S C P F H N Q P H L K R E K L C S    91
CYTS HUMAN  : T I C T K S Q P - - - - - - - - - - N L D T C A F H E Q P E L Q K K Q L C S    99
CYTC HUMAN  : T T C T K T Q P - - - - - - - - - - N L D N C P F H D Q P H L K R K A F C S    98
CYT BITAR   : T T C K K T V G - - - - R P K G Y Q E I Q N C N L P P E N Q - Q E E I T C R    95
CYT CHICKEN : T T C P K S S G - - - - - - - - - - D L Q S C E F H D E P E M A K Y T T C T    96
CYTA SARPE  : K T C D I T I W S Q P W L E N G I E V T F N C P G E P K V V K K H S A       102

L-cystatin  : A I V W V Q A W I P R T K V T K L E C Q N K G      114
CYT BOVINE  : F Q V Y V V P W M N T I N L V K F S C Q D          112
CYTS HUMAN  : F E I Y E V P W E D R M S L V N S R C Q E A        121
CYTC HUMAN  : F Q I Y A V P W Q G T M T L S K S T C Q D A        120
CYT BITAR   : F E V W S R P W L P S T S L T K                    111
CYT CHICKE  : F V V Y S I P W L N Q I K L L E S K C Q            116
CYTA SARPE  :
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Fig. 6. **Alignment of the amino acid sequence of L-cystatin with those of members of family 2 cystatin.** CYT BOVINE, bovine colostrum cystatin; CYTS HUMAN, human cystatin S; CYTC HUMAN, human cystatin C; CYT BITAR, a cystatin from *Bitis arietans*; CYT CHICKEN, chicken egg white cystatin; CYTA SARPE, sarcocystatin A from flesh fly. Manual alignment and position numbers were based on the sequence of L-cystatin with appropriate gaps. Residues identical to L-cystatin are boxed.

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value (640.3) obtained by calculating the NH_2-terminal residue of the peptide as pyroglutamic acid. The intact L-cystatin was also subjected to ion-spray mass spectrometry to determine whether or not it contained modified amino acid residue(s). The results indicated that the observed mass value of L-cystatin of 12,637.3 agreed well with the theoretical average mass value of 12,638.5 (data not shown), suggesting no modified residues in the material. The amino acid composition of L-cystatin predicted from the nucleotide sequence was consistent with that obtained by amino acid analysis of whole L-cystatin (Table IV).

Comparison of the Amino Acid Sequence of L-Cystatin with Those of Other Cystatins—L-cystatin was closely related to cystatins of the family 2, as shown in Fig. 6, including bovine colostrum cystatin (33% identity) (*37*), human cystatin S (31%) (*38*), human cystatin C (29%) (*39*), snake cystatin from *Bitis arietans* (33%) (*40*), chicken egg white cystatin (27%) (*41*), sarcocystatin A from flesh fly *Sarcophaga peregrina* (16%) (*13*). Based on the sequence homology, disulfide bonds of L-cystatin, Cys^{66}-Cys^{79} and Cys^{90}-Cys^{110}, were postulated. On the other hand, L-cystatin had lower sequence similarity with the family 1 cystatins, such as human cystatin A (22%), rat cystatin α (19%), human cystatin B (20%), and rat cystatin β (21%) and no significant sequence homology with kininogens of family 3. Pro-sequences of neutrophil antibiotics are known to have a sequence homology to a pig leucocyte cysteine protease inhibitor, cathelin (*42*). L-cystatin also had a lower level of sequence identities between the pro-sequences, including precursors of antibacterial peptide Bac5 (23%) (*42*), bactericidal peptide indolicidin (23%) (*43*), and antibiotic dodecapeptide (21%) (*44*) and cathelin (21%) (*45*). The homology is confined to the region upstream of mature neutrophil antibiotics and includes four invariant cysteine residues.

Northern Blot Analysis and Subcellular Localization of L-Cystatin—To determine the size of the mRNA and to investigate tissue-specific expression of L-cystatin, Northern blot analysis was carried out using poly$(A)^+$ RNA (Fig. 7A). An mRNA of 0.8 kb was detected in hemocytes and slightly in heart, but not in hepatopancreas, stomach, intestine, and muscle, indicating the tissue-specific expression of L-cystatin. Furthermore, the length of mRNA indicated that the sequence in Fig. 4 represents almost a full-length cDNA of L-cystatin, taking the length of the poly(A) tail into account.

Antiserum raised against purified L-cystatin was used to identify localization in the hemocyte. The isolated large and small granules from hemocytes (*15*) were first treated with 1% SDS at 100°C for 2 min and subjected to SDS-PAGE, under nonreducing conditions for immunoblotting. The anti-L-cystatin antiserum recognized the 12.6-kDa L-cystatin in the extracts of large granules (Fig. 7B). However, immunoreactive materials were never observed in small granules or in hemolymph plasma, therefore L-cystatin seems to be specifically stored in the large granules.

DISCUSSION

In the present study, a cysteine protease inhibitor (L-cystatin) from horseshoe crab hemocytes was newly purified and characterized, and the entire sequence of a cDNA coding for

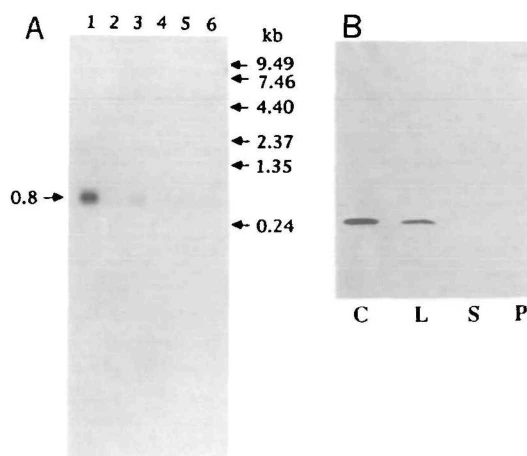

Fig. 7. **Northern blot analysis of L-cystatin (A) and subcellular localization of L-cystatin (B).** A: lane 1, hemocyte; lane 2, hepatopancreas; lane 3, heart; lane 4, stomach; lane 5, intestine; lane 6, muscle. B: 10 μg of proteins from large granules, small granules, and plasma were subjected to SDS-PAGE, under nonreducing conditions. C, L-cystatin; L, large granule; S, small granule; P, plasma. Arrows indicate the RNA size markers.

L-cystatin was determined. L-cystatin is a single-chain protein consisting of 114 amino acids with an apparent $M_r = 12,600$, which has a significant sequence homology with members of family 2 cystatin. Protease inhibitors found in horseshoe crab hemolymph or hemocytes include α_2-macroglobulin (*46*), Kunitz-type protease inhibitor (*47*), trypsin inhibitor (*48*), limulus intracellular coagulation inhibitors type 1 and type 2 (serpins) (*49, 22*), and endotoxin-binding protein-protease inhibitor (*50*). None of these belong to the cystatin superfamily. The present report is the first of a cysteine protease inhibitor stored in the large granules in the hemocytes.

L-cystatin is a tight-binding inhibitor for papain with a $K_i = 0.08$ nM by forming a noncovalent 1:1 complex. L-cystatin is most reactive to cathepsin L of the four cathepsins, B, H, L, and C (Table II). Chicken egg white cystatin of family 2 also has a higher affinity with cathepsin L of $K_i = 0.003$ nM than with other cathepsins (*16*). The affinity of L-cystatin for actinidin from kiwi fruit is 8,000-fold lower than that of papain, which is again similar to the case of egg white cystatin (*19*). Furthermore, argingipain, an asparagine specific cysteine protease isolated from the bacteria *Porphyromonas gingivalis*, was neither inhibited by L-cystatin nor by recombinant chicken egg white cystatin (*23*). Therefore, the inhibitory spectrum of L-cystatin is similar to that of family 2 cystatins. On the other hand, kininogens of family 3 inhibit calpain with $K_i = 1$ nM (*51*), whereas L-cystatin did not. Trypsin and elastase were not inhibited by L-cystatin and for 50% inhibition of α-chymotrypsin (4.8 nM), a 400-times molar excess of cystatin was required.

Mature L-cystatin is composed of 114 amino acid residues, which is consistent with the size of members of family 2. According to structural models (*52-54*) and X-ray crystallographic analyses (*55, 56*) of chicken egg white cystatin, it binds with papain by forming a tripartite wedge slotting into the active site cleft of papain. These three defined binding sites are as follows: an NH_2-terminal Gly 9;

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-Gln-Leu-Val-Ser-Gly- at positions 53 to 57; and -Pro-Trp- at positions 103 and 104. These binding sites are also well conserved in L-cystatin at the corresponding sites, Gly 4, Gln-Val-Val-Ser-Gly- at positions 48 to 52, and -Ala-Trp- at positions 98 and 99 (Fig. 6). Except for *Bitis arietans* cystatin, previously characterized cystatins of family 2 all contain 4 cysteine residues which are involved in the formation of the two disulfide bonds characteristic for the family. L-cystatin contains 4 cysteine residues located at identical positions (Fig. 6). These structural similarities are sufficient to support the proposal that L-cystatin is a member of family 2. Location of one of the disulfide bonds in invertebrate sarcocystatin A differs from those of vertebrate cystatins. Interestingly, L-cystatin is more closely related to mammalian cystatins, for example, bovine colostrum cystatin (33%), than the invertebrate sarcocystatin A (16%) and *Drosophila melanogaster* cystatin-like protein (16%). Pairwise comparisons for sequence identities between family 2 cystatins containing four vertebrate and two invertebrate proteins are 33-16%.

The expression of mRNA for L-cystatin was detected in the hemocyte and slightly in heart but negligibly in all other tissues examined. In the case of sarcocystatin A of flesh fly *Sarcophaga*, it is transiently expressed in the very early embryonic stage and in the pupal stage, probably participating in morphogenesis of larval and adult structures (*13*). Expressions of mammalian cystatins of family 2 are diversified. For example, cystatin D is expressed in the parotid gland but not in the seminal vesicle, prostate, epididymis, testis, ovary, placenta, thyroid, gastric corpus, small intestine, liver, or gallbladder tissue (*57*). Cystatin C, however, is expressed in all these tissues (*58*) and secretory gland cystatins are present in saliva, seminal plasma, and tears (*9*). Like cystatin D, L-cystatin shows restricted expression in the hemocyte and is located in the large granules.

The most characteristic property of L-cystatin is that it has significant antimicrobial activity against Gram-negative bacteria (Table III). Among cystatins of family 2, there are marked differences in their isoelectric point, pI values ranging from acidic for cystatin A (pI=4.5-5.0) and chicken egg white cystatin (pI=5.6-6.5) through neutral cystatin B (pI=6.0-6.6) to basic cystatin C (pI=8.0-9.5) and bovine colostrum cystatin (10.0-10.3) (*9*). Interestingly, the pI value of L-cystatin (pI=10.0) is more basic than that of cystatin C. The basic nature rich in Lys (Table IV) could be an important factor in its antimicrobial activity to interact with surface components of Gram-negative bacteria. The existence of L-cystatin in the large granules also suggests that L-cystatin serves synergistically as a defense molecule against invading microbes, together with the anti-LPS factor (*59, 60*), tachyplesins (*61, 14*), big defensin (*28*), and coagulation factors (*62, 63*), all of which are secreted into the extracellular fluid in response to the external stimulation of bacterial lipopolysaccharides.

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