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Characteristic properties of endoplasmic reticulum membrane m-calpain, calpastatin and lumen m-calpain: a comparative study between membrane and lumen m-calpains

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Previously, we reported that bovine pulmonary smooth muscle endoplasmic reticulum (ER) membrane possesses associated m-calpain and calpastatin and ER lumen contains only m-calpain. Herein, we report characteristic properties of ER membrane m-calpain (MCp), calpastatins and lumen m-calpain (LCp) and a brief comparative study between MCp and LCp. MCp containing 80 kDa large and 28 kDa small subunit is non-phosphorylated, whereas LCp containing only 80 kDa large subunit is phosphorylated. Optimum pH, Ca^{2+} concentration and pI value of both MCp and LCp are 7.5, 5 mM and 4.5, respectively. MCp and LCp have similar kinetic parameters and circular dichroism (CD) spectra. Autolysis of MCp and LCp are different. Coimmunoprecipitation studies revealed that LCp is associated with ERp57 in the ER lumen, which suggests that the regulation of LCp differs from the regulation of MCp. In presence of Ca^{2+}, the activated LCp cleaves inositol 1,4,5-trisphosphate receptor-1 (IP_3R1) in the ER lumen, whereas the activated MCp cleaves Na^+/Ca^{2+} exchanger-1 (NCX1) in the ER membrane. We have determined pI (4.6 and 4.7, respectively) and IC_{50} (0.52 and 0.8 nM, respectively) values of 110 and 70 kDa calpastatins. For first time, we have determined the characteristic properties, regulation and functional activity of LCp in the ER lumen.

Keywords: Calpastatin/endoplasmic reticulum/m-calpain/pulmonary smooth muscle.

Abbreviations: CD, circular dichroism; CHAPS, 3-[3-cholamidopropyl] dimethylammonio-2-hydroxy-1-propanesulfonate; 2-DE, 2-dimensional gel electrophoresis; ER, endoplasmic reticulum; IC_{50}, the concentration of calpastatin that caused 50% inhibition of calpain; IP_3R1, inositol 1,4,5-trisphosphate receptor-1; LCp, lumen m-calpain; MCA, 7--amino-4-methylcoumarin; MCp, membrane m-calpain; MCE, 2-mercaptoethanol; NCX1, Na^+/Ca^{2+} exchanger-1; pI, isoelectric point.

Calpains are calcium-dependent proteinases and they occur as two isozymes that differ in sensitivity to Ca^{2+} (μM and mM Ca^{2+} requiring forms μ-calpain and m-calpain, respectively) (*1*). Each isozyme is a heterodimer consisting of a catalytic subunit and a regulatory subunit of ~80 and 30 kDa, respectively (*2*). Although calpain contains two subunits, yet several reports are also available regarding the presence of calpain consisting of only an 80 kDa catalytic subunit and its proteolytic activity (*3*, *4*).

Calpastatin is an endogenous inhibitory protein that is specific for calpains (*5*). Many of the tissues that contain calpain have also been reported to contain calpastatin (*6*). The calpain-calpastatin system has been implicated in various physiological and pathological calcium-dependent processes such as differentiation of myoblasts and adipocytes, actin reorganization, cell cycle regulation and signal transduction (*5*). Calpain over activation can also contribute to many pathological consequences such as pulmonary hypertension, smooth muscle cell proliferation and apoptosis (*7–9*).

The activity of calpain, as an intracellular protease is strictly regulated by various mechanisms. Among them, the concentration of Ca^{2+} in cells is the most important factor. In addition to intracellular calcium level, the activity of calpain is tightly regulated by a variety of factors including its endogenous inhibitor, calpastatin through the formation of a complex in the presence of Ca^{2+}, binding to phospholipids, autoproteolysis and phosphorylation (*10*, *11*).

Calpain has been purified from a variety of systems such as skeletal muscle, platelets, brain, liver and kidney (*12–15*). Calpastatin has also been isolated from porcine skeletal muscle, chicken skeletal muscle, rabbit skeletal muscle, bovine cardiac muscle and from human erythrocytes (*16–20*). Although the calpain system has been studied extensively in a number of tissues, much less is known about the properties of m-calpain and calpastatin in endoplasmic reticulum (ER). Previously, we have demonstrated the localization of the associated m-calpain and calpastatin in the cytosolic side of the ER membrane and only m-calpain in the ER lumen (*21*). In this communication, we report characteristic properties of membrane m-calpain (MCp), calpastatins and lumen m-calpain (LCp) of the ER of pulmonary artery smooth muscle and a brief comparative study between MCp and LCp. Association between MCp and calpastatin in the ER membrane suggests that MCp is regulated by calpastatin. In our previous report (*21*), we have shown that calpastatin is not present in the lumen of the ER indicating that the regulation of LCp differs from MCp.

Herein, we have demonstrated a novel type of regulation of LCp by ER protein 57 (ERp57) in the lumen of the ER. ERp57 (also known as ER-60, ERp60, ERp61, GRP58) is a member of protein-disulfide isomerase (PDI) family (*22–24*). It is mainly present in the lumen of the ER and can also be found in the nucleus, extracellular space, cytosol and cell surface (*22*). It functions as a molecular chaperone within the lumen of the ER and in correcting the folding and rearranging of the disulfide bonds of misfolded glycoproteins (*22*, *23*). ERp57 mediated regulation of μ-calpain has been reported (*23*). In our recent report (*25*), we have demonstrated that in presence of Ca^{2+}, the activated MCp cleaves NCX1, the Ca^{2+} uptake machinery in the ER membrane. In this communication, we have shown that in presence of Ca^{2+} the activated LCp cleaves the ER calcium release channel, IP_3R1 in the ER lumen. To the best of our knowledge, this is first report regarding the purification of two types of m-calpains (MCp and LCp) and calpastatins, their characterization, comparative study between MCp and LCp in their properties (physicochemical and kinetic), regulations and functional activities in the ER.

Materials and Methods

Materials

Bicinchoninic acid (BCA) protein assay kit was purchased from Pierce (Rockford, IL). Casein was purchased from US Biological Corp (Cleveland, OH). The flurogenic calpain substrate, SLLVY-AMC was purchased from Bachem (King of Prussia, PA). Mouse anti-calnexin was the product of BD Transduction Laboratories (San Jose, CA). Sheep polyclonal anti-Sec16 was kindly donated by Prof. D.J. Stephens, Department of Biochemistry, University of Bristol, Bristol BS8 1TD, UK. Calpeptin was purchased from Calbiochem (San Francisco, CA). Bovine skeletal muscle m-calpain was the product of Fitzgerald Industries International Inc. (Concord, MA). Rabbit polyclonal antibody of m-calpain was kindly donated by Prof. K.K.W. Wang, Brain Institute, University of Florida, Gainesville, Florida; rabbit polyclonal antibody of calpastatin was kindly donated by Prof. J. Takano, Laboratory of Proteolytic Neuroscience, RIKEN Brain Science Institute, Saitama, Japan. Mouse monoclonal calpastatin L-domain-specific antibody was the product of Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-ERp57 was purchased from Stressgen Bioreagents (Michigan, USA). Rabbit polyclonal anti-IP_3R1 was from Chemicon International (Temecula, CA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and anti-rabbit IgG secondary antibodies were purchased from Zymed (San Francisco, CA). DEAE cellulose, phenyl-sepharose and Mono Q HR (5/5) were the products of GE Healthcare Bio-Sciences Corp (Piscataway, NJ). DEAE-TSK (ToyoPearl) 650 S was purchased from Supelco (Bellefonte, PA). ReadyStrip IPG strips (pH 3–10) were the products of Bio-Rad Laboratories (Hercules, CA). Reactive Red agarose, protein A/G agarose beads, A23187, rabbit polyclonal anti-phospho-Ser, anti-phospho-Thr, anti-phospho-Tyr and all other chemicals and reagents used were obtained from Sigma Chemical Co. (St. Louis, MO).

Methods

Isolation of ER from the pulmonary artery smooth muscle tissue. Bovine pulmonary artery smooth muscle tissue was collected according to our previously described method (*21*). RER was isolated from the bovine pulmonary artery smooth muscle tissue by following the method of Phung *et al.* (*26*) with some modifications as described by Samanta *et al.* (*21*). ER was isolated by the removal of ribosomes from RER by following the method of Fujiki *et al.* (*27*) with some modifications as described by Samanta *et al.* (*21*).

Separation of the ER membrane and lumen from the ER by Na_2CO_3 treatment.. The ribosome free ER suspension was diluted with 100 mM sodium carbonate to adjust pH to 11.5, to bring the protein concentration to ~0.5 mg/ml, and then incubated at 0°C for 30 min. The resulting suspension was centrifuged at 150,000 *g* for 1 h in an ultracentrifuge (Beckman, USA). The supernatant (ER lumen fraction) was collected and dialysed in medium B (250 mM sucrose and 100 mM Tris–HCl buffer, pH 7.4) and the pellet (ER membrane) was washed in medium B by resuspension and centrifugation. The pellet was resuspended in medium B and considered as ER membrane suspension (*21*, *27*). To a part of the ER membrane suspension, Triton X-100 (1%) was added, incubated in a rotary shaker for 1 h, and then centrifuged at 150,000*g* for 1 h. The supernatant was considered as the Triton extracted ER membrane lysate. All operations were carried out at 4°C.

Western blot. Western blot was performed according to the procedure described by Towbin *et al.* (*28*).

Purification of MCp and calpastatins from the ER membrane. Purification of MCp and calpastatins from the ER membrane was performed by following the procedure of Thompson and Goll (*29*) with some modifications, briefly the Triton extracted ER membrane lysate was saturated with 50% ammonium sulphate and the precipitated protein was collected by centrifugation at 100,000 *g* in a Beckman Optima TLX ultracentrifuge with TL-100.3 rotor for 1 h. The precipitate was redissolved in the TEM buffer [20 mM Tris–HCl (pH 7.5), 1 mM EDTA, 0.1% 2-mercaptoethanol] and then it was dialysed against several changes of TEM and clarified by centrifugation at 100,000 *g* in a Beckman Optima TLX ultracentrifuge with TL-100.3 rotor for 1 h. The clear dialysed material was applied onto a DEAE-cellulose column (~100 ml packed DEAE cellulose). The DEAE cellulose cake was washed with two volumes of TEM to remove all unbound proteins and then it was suspended in TEM, which was poured into a column (5 × 30 cm). The bound proteins were then eluted with 50–300 mM KCl in TEM. Calpastatin was eluted first at 50–100 mM KCl and then MCp was eluted at 200–300 mM KCl.

The fractions from the DEAE cellulose column containing calpastatins were pooled and adjusted to 1 M ammonium sulphate (23% saturation) by slow addition of solid ammonium sulphate and it was then centrifuged at 100,000 *g* in a Beckman Optima TLX ultracentrifuge with TL-100.3 rotor for 30 min to remove the precipitated proteins. The supernatant was applied onto a phenyl-sepharose column (1.6 × 20 cm), which was previously equilibrated with 1 M ammonium sulphate, TEMA (TEM with 1 mM NaN_3). Then the column was eluted with a descending linear gradient from 1 M to 0 M ammonium sulphate in TEMA, followed by washing with 1 mM EDTA, 0.1% 2-ME, pH 7.5 and calpastatin containing fractions were pooled. The pooled material was applied onto a DEAE-TSK anion exchange column (1.6 × 20 cm). Then the column was eluted with a shallow gradient from 20 to 125 mM KCl in TEM. Then the calpastatin containing fractions were pooled and concentrated by ultra filtration (Amicon YM-10 membrane; mol. wt. cut off 10 kDa). Finally, the pooled and concentrated fraction was applied onto a Mono Q HR (5/5) column for further purification of the calpastatin molecules and eluted fractions were pooled and concentrated by ultrafiltration (Amicon YM-10 membrane; mol. wt. cut off 10 kDa).

The fractions obtained from DEAE cellulose column containing m-calpain (MCp) were pooled and applied onto another phenyl-sepharose column (1.6 × 20 cm) that has been equilibrated with 125 mM KCl, TEMA. The column was then washed with 125 mM KCl, TEMA until OD at 280 nm of the effluent was returned to base line. Then the column was eluted with 1 mM EDTA, 0.1% 2-ME and MCp containing fractions were pooled. Solid NaCl was added to 0.5 M to the pooled material. This solution was loaded onto a Reactive Red column (2.6 × 20 cm) that has been equilibrated in 0.5 M NaCl, TEMA. The column was then washed with 0.5 M NaCl, TEMA until OD at 280 nm of the eluant was returned to base line. Then the column was eluted with 20 mM Tris–HCl (pH 8.5), 1 mM EDTA, 0.1% 2-ME and MCp containing fractions were pooled. The pooled material obtained in the previous step was loaded onto a second DEAE-TSK anion exchange column (1.6 × 20 cm), which was previously equilibrated with 20 mM Tris–MES (pH 6.5), 135 mM KCl, 1 mM EDTA, 0.1% 2-ME and

the column was washed with the same buffer (equilibration buffer) until OD at 280 nm was returned to base line. Finally, the column was eluted with a linear gradient from 135 to 275 mM KCl in 20 mM Tris–MES (pH 6.5), 1 mM EDTA, 0.1% 2-ME and MCp containing fractions were pooled and concentrated by ultra filtration (Amicon YM-10 membrane; mol. wt. cut off 10 kDa).

All the purification steps were performed at 4°C. In each step of purification, the protein contents of the samples were measured. The purities of MCp and calpastatins from the ER membrane fraction were determined by SDS–PAGE and western blot analyses. To determine the extent of purification in each step, the samples were analysed by protease activity measurement (for MCp) and by inhibitory activity measurement (for calpastatins).

Purification of LCp from the ER lumen. For purification of LCp from the ER lumen fraction, it was saturated with 50% ammonium sulphate and the precipitated protein was collected by centrifugation at 45,000 *g* for 1 h. The precipitate was redissolved in the TEM [20 mM Tris–HCl (pH 7.5), 1 mM EDTA, 0.1% 2-mercaptoethanol] buffer and then it was dialysed against several changes of TEM and clarified by centrifugation at 100,000 *g* for 1 h. Then the solution was applied onto a series of chromatographic columns: DEAE-cellulose, phenylsepharose, Reactive Red and DEAE-TSK anion exchange columns as described above. Finally, the DEAE-TSK anion exchange column was eluted with a linear gradient from 135 to 275 mM KCl in 20 mM Tris–MES (pH 6.5), 1 mM EDTA, 0.1% 2-mercaptoethanol, and LCp containing fractions were pooled and concentrated by ultra filtration (Amicon YM-10 membrane; mol. wt. cut off 10 kDa).

All the purification steps were performed at 4°C. In each step of purification, the protein contents of the samples were measured. The purity of LCp from the ER lumen fraction was determined by SDS–PAGE and western blot analyses. To determine the extent of purification in each step, the samples were analysed by protease activity measurement.

Assays for m-calpain and calpastatin activities. The assays for m-calpain and calpastatin were performed according to the procedure described by Murakami *et al*. (*30*) with some modifications. Briefly, the assay mixture contained, in a final volume of 1.0 ml, 0.4% casein as substrate, 100 mM imidazole–HCl buffer (pH 7.5), 5 mM cysteine and 5 mM $CaCl_2$. For determination of m-calpain activity, the protease sample was incubated in the assay mixture at 30°C for 30 min, and then the reaction was stopped by adding 1 ml of 5% trichloroacetic acid. For determination of the acid-soluble products in the filtrate, 0.4 ml of the filtrate was diluted with the reagents to a total volume of 2.8 ml, and the absorbance at 750 nm was read against the blank. For determination of calpastatin activity, the inhibitor sample was preincubated with a fixed amount of calpain at 30°C for 10 min, and then the decrease in proteolytic activity was measured. One unit of calpain was defined as the amount of the enzyme that caused a change of 1.0 in A750 for the trichloroacetic acid-soluble products after incubation for 30 min at 30°C. One unit of calpastatin was defined as the amount of inhibitor that inhibited one unit of calpain activity under the standard assay conditions.

Casein zymogram of the MCp and LCp. Casein zymogram of the purified MCp and LCp were performed according to the procedure described by Raser *et al*. (*31*) with some modifications (*21*). Briefly, casein (0.5% w/v) was copolymerized with 8% (w/v) acrylamide, 0.32% (w/v) bisacrylamide, 375 mM Tris–HCl (pH 8.8) as the separating gel and 4% (w/v) acrylamide, 0.11% (w/v) bis-acrylamide, 330 mM Tris–HCl (pH 6.8) (no casein) as the stacking gel, which were poured into minigel casts. Ammonium persulfate (0.04%, w/v) and TEMED (0.028%, v/v) were used to catalyse the polymerization.

The specificity of calpain activity, assessed by casein zymography, was studied by calcium dependency i.e. in presence and absence of calcium in the purified m-calpains (MCp and LCp). Calcium induced processing was performed before electrophoresis. The effect of calpain inhibitor, calmidazolium was studied by its incubation with each of the purified m-calpains (MCp and LCp) before electrophoresis.

Determination of amino-terminal sequences of the MCp, LCp and calpastatins. The purified MCp (80 kDa large and 28 kDa small subunit), LCp (80 kDa) and calpastatins (110 and 70 kDa) were subjected to SDS–PAGE (10% for m-calpains and 7.5% for calpastatins). Then the gels were electroblotted onto PVDF membranes. The protein bands of interest were cut out and their N-terminal amino-acid sequences were determined according to the procedure described by Matsudaira (*32*).

Determination of phosphorylated status of the MCp, LCp and calpastatins. Immunoblot studies of the purified MCp, LCp and calpastatins with rabbit polyclonal antibodies specific for phosphorylated serine, phosphorylated threonine and phosphorylated tyrosine were performed to determine whether MCp, LCp and calpastatins (110 and 70 kDa) were phosphorylated in the states that they were purified from the ER.

Determination of optimum pH and Ca^{2+} concentration for the maximum activities of the MCp and LCp. Cleavage of casein by the purified MCp and LCp were determined separately at a range of pH values in the following buffers: 50 mM Na citrate (pH 5.0–6.5), 50 mM Tris–HCl (pH 7.0–8.0) and 50 mM glycine–NaOH (pH 8.5–9.5) (*33*).

In order to determine the optimum Ca^{2+} concentration for the activities of the purified MCp and LCp, various concentrations (0.05–7 mM) of Ca^{2+} at pH 7.5 were tested (*34*).

Determination of isoelectric points (pI) and 2D gel electrophoresis (2-DE) of the MCp, LCp and calpastatins. Determination of the pIs and 2-DE of the purified MCp, LCp and calpastatins were performed according to Nakajima *et al*. (*35*) with some modifications. Briefly, the first-dimension isoelectric focusing (IEF) for 2-DE was performed on 11-cm non-linear pH 3–10 immobilized pH gradient strips (ReadyStrip IPG strips). IPG strips were rehydrated in the first-dimension buffer (8 M urea, 2% CHAPS {3-[3-cholamidopropyl]dimethylammonio-2-hydroxy-1-propanesulfonate}, 0.5% IPG buffer, 1% dithiothreitol (DTT), with a trace of bromophenol blue) and 75 µg sample. IEF was performed (in Bio-Rad Protean IEF cell) using a rapid ramp up to 8000 V for 10–12 h to give a total of 35,000 V-hours. The current limit was set to 50 µA per strip. The equilibration of each strip was performed in 5 ml SDS buffer [50 mM Tris–HCl (pH 8.8), 6 M urea, 30% vol/vol glycerol, 2% SDS, and trace bromophenol blue] with 2% DTT, followed by 5 ml SDS buffer with 2.5% iodoacetamide. Then 2-DE was performed on a 10% Bis–Tris gel, with the first-dimension IPG strip embedded at the top in 1% agarose. Proteins were visualized on the gels by silver staining. The gel images were analysed with the help of PDQuest software (Bio-Rad, USA).

Autolysis of the MCp and LCp. The purified MCp and LCp were subjected to autolysis by following the experimental conditions used by Nagainis *et al*. (*36*) with some modifications. Briefly, the purified MCp and LCp (0.2 mg/ml protein) were incubated separately at 0°C in 50 mM Tris–acetate (pH 7.5), 10 mM 2-mercaptoethanol (MCE), 0.5 mM EGTA, with 2 mM final Ca^{2+} concentration in the reaction mixture. Aliquots (0.1 ml) were withdrawn at different times and added to 0.1 ml EGTA (2 mM) to quench autolysis.

Determination of kinetic parameters of the MCp and LCp. The effects of various synthetic fluorogenic substrates on activities of the purified MCp and LCp were determined at room temperature. The fluorescence assays using Suc-Leu-Met-MCA, Suc-Leu-Tyr-MCA, Suc-Leu-Leu-Val-Tyr-MCA and Boc-Val-Leu-Lys-MCA were done as described by Sasaki *et al*. (*37*) with some modifications. Briefly, in a total volume of 0.5 ml, 15–25 µg of each of the purified m-calpains was incubated separately with 0.001-4 mM substrate, 100 mM imidazole-HCl buffer (pH 7.5), 5 mM L-cysteine, 2.5 mM 2-mercaptoethanol, 5 mM $CaCl_2$, and 4% Me_2SO (dimethyl sulfoxide). The reaction was initiated by adding the enzyme and continued for 5–10 min at 30°C. The increase in fluorescence (excitation at 380 nm; emission at 460 nm) of 7-amino-4-methylcoumarin (MCA) produced was continuously monitored using a Hitachi model F-7000 recording spectrofluorometer, and the initial rate of hydrolysis (in Ms^{-1}) was calculated from the straight portion of the graphs. Control runs were performed in the absence of Ca^{2+}. The K_m and k_{cat} values were calculated from double-reciprocal Lineweaver–Burk plots (*37*).

Measurement of circular dichroism (CD) of the MCp and LCp. CD spectra of the purified MCp and LCp were obtained by using a Jasco J 600 spectropolarimeter with a band width of 2 nm and a scan speed of 2 nm/min. Each sample was scanned four times, the scans were automatically averaged. All measurements were done at 22–30°C with protein concentrations ranging from 0.004 to 0.150 mg/ml in 20 mM K-phosphate (pH 7.5), 1.0 mM EDTA by using a CD cell with path length 0.1 dm (*38*). Solvent composition and protein concentration within the range used in our experiment had no effect on the CD spectra of the purified MCp and LCp.

Co-immunoprecipitation of MCp or LCp and ERp57. Five micrograms of the m-calpain antibody (or ERp57 antibody) was incubated with 50 ml of protein A/G agarose beads for 40 min at 4°C as described previously (*21*). The m-calpain antibody (or ERp57 antibody) was substituted with IgG in controls. The protein A/G agarose-anti-m-calpain (or anti-ERp57) complex was washed three times with PBS containing 0.1% Triton X-100; then incubated overnight at 4°C with the ER lumen fraction or with the ER membrane suspension (~1 mg protein). The beads were then washed three times with PBS containing 0.1% Triton X-100. The immunoprecipitate was subsequently subjected to western blotting using ERp57 (or m-calpain) antibody to assess co-immunoprecipatation with m-calpain or ERp57.

Interaction assay of LCp and ERp57. We have determined how much of the protein (LCp) was involved in the interaction according to Ozaki *et al.* (*23*). Briefly, ERp57 antibody (0.2–50 μg) and normal rabbit IgG (0.2–50 μg) were incubated with ER lumen fraction (~500 μg protein). When a precipitate had formed, these samples were centrifuged at 12,000 rpm for 5 min at 4°C. 80 μl of the supernatant obtained was used to assay for LCp activity using SLLVY-AMC as a substrate.

Spectrophotometric assay of LCp. LCp activity was assayed spectrophotometrically using the calpain specific substrate, SLLVY-AMC (Bachem) as described by Atsma *et al.* (*39*) with some modifications. Briefly, before and after the Ca^{2+} treatment, the ER was diluted in the buffer containing 130 mM KCl, 9 mM Tris–PO_4, 4 mM Tris–HCl (pH 7.5) and in the presence of 50 μM SLLVY-AMC. Activity was measured under linear conditions as a function of AMC hydrolysis using excitation and emission wavelengths of 355 nm and 444 nm, respectively. ER, incubated in the absence of substrate, exhibited the same fluorescence as the buffer alone. LCp activity was measured in the ER preincubated with the calpain specific inhibitors, leupeptin (100 μM) or calpeptin (20 μM) for 20 min followed by treatment with 5 mM $CaCl_2$ in presence of A23187 (5 μM).

Determination of IC_{50} values of the calpastatins. Inhibition of proteolytic activity of the purified MCp by the purified 110 and 70 kDa calpastatins were determined according to Takano *et al.* (*40*). Briefly, the incubation mixture (1 ml) contained 0.4% casein, 100 mM imidazole-HCI buffer (pH 7.5), 5 mM cysteine, 5.0 mM $CaCl_2$, 8 nM of the purified MCp and various concentrations of purified 110 or 70 kDa calpastatin. The reaction was terminated after 30 min at 30°C by adding 1 ml of 5% trichloroacetic acid, and the acid soluble products were determined by the method of Murakami *et al.* (*30*).

Statistical analysis. The appropriate ANOVA was performed for each data set by using Sigma Stat statistical software. Individual means were compared with Fisher's protected least-significant difference test, with $P \leq 0.05$ being considered a statistically significant difference between mean values.

Results

Characterizations of the rough ER (RER) vesicles, ribosome free ER vesicles, ER membrane and ER lumen

We ascertained the purities of the RER vesicles, ribosome free ER vesicles and ER membrane by electron microscopic and immunoblot studies as described in our previous report (*21*). Immunoblot studies of the ER membrane and ER lumen fractions with calnexin (ER transmembrane marker) (*21*), Sec16 (ER peripheral membrane marker) (*41*) and calreticulin (ER lumen marker) (*21*) revealed the purities of the isolated ER membrane and lumen fractions (Fig. 1).

Purification of MCp, LCp and calpastatins from the ER

Purification of MCp and calpastatins from the ER membrane.. The final-step elution profile for MCp from DEAE-TSK column was described in Fig. 2A. SDS–PAGE and western blot analyses of all steps of purification of MCp were depicted in Fig. 2B and C, respectively. Both SDS–PAGE (Fig. 2B) and western blot (Fig. 2C) analyses of the purified fraction of MCp exhibited two distinct bands corresponding to molecular masses of 80 and 28 kDa, which revealed that the purified MCp has an 80 kDa large and 28 kDa small subunit. Similarly, the final-step elution profile for calpastatins from Mono Q HR column was described in the Fig. 2D. SDS–PAGE and western blot analyses of all steps of purification of calpastatins were depicted in Fig. 2E and F, respectively. Both SDS–PAGE (Fig. 2E) and western blot (Fig. 2F) of the purified fractions of calpastatins exhibited two distinct protein bands at 110 and 70 kDa, respectively.

The results of purification of MCp and calpastatins as assessed, respectively, by protease activity and inhibitory activity measurements were summarized in Tables I and II.

Purification of LCp from the ER lumen. The final-step elution profile for LCp from DEAE-TSK column was described in Fig. 2G. SDS–PAGE and western blot analyses of all steps of purification of LCp were depicted in Fig. 2H and I, respectively. Both SDS–PAGE (Fig. 2H) and western blot (Fig. 2I) analyses of the purified fraction of LCp exhibited only a single band corresponding to molecular mass of 80 kDa, which revealed that the purified LCp has only 80 kDa large subunit.

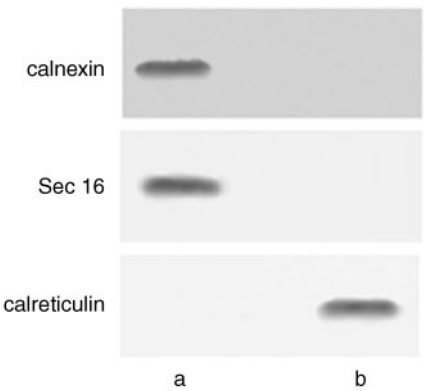

Fig. 1 Immunoblot studies of the ER membrane and lumen fraction with calnexin (transmembrane ER marker) (carried out on 7.5% SDS–PAGE), Sec16 (peripheral ER membrane marker) (carried out on 7.5% SDS–PAGE) and calreticulin (lumen ER marker) (carried out on 10% SDS–PAGE). Lane a, ER membrane fraction; lane b, ER lumen fraction.

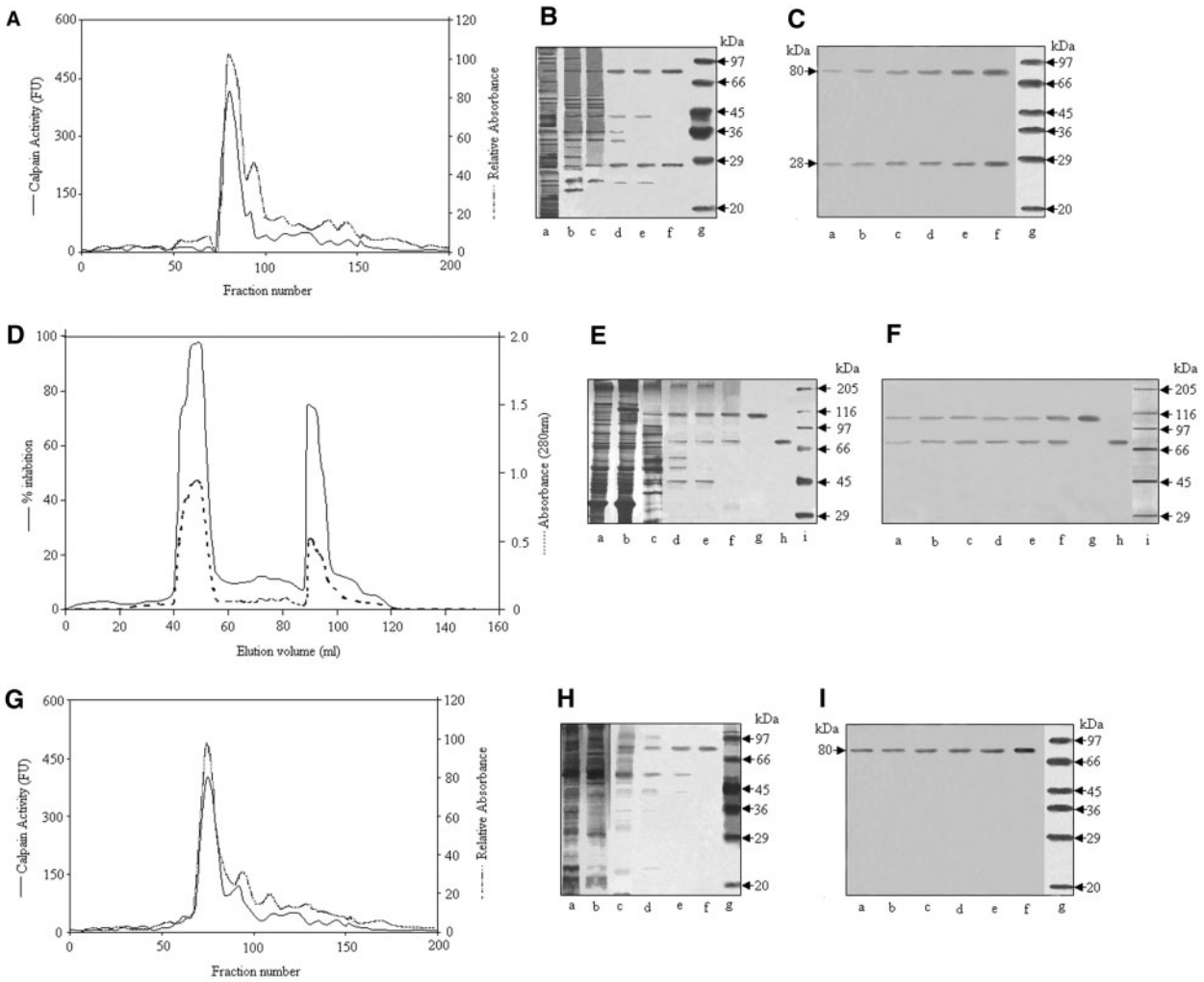

Fig. 2 Purification profiles of MCp and calpastatins from ER membrane and LCp from ER lumen. (A) DEAE-TSK column elution profile of the purified MCp; the column was eluted at a flow rate of 60 ml/h. (B) 10% SDS–PAGE profile of purification of MCp. Lane a, Triton extracted ER membrane lysate; lane b, 0–50% ammonium sulphate precipitate; lane c, DEAE cellulose eluate fraction; lane d, phenyl sepharose eluate fraction; lane e, Reactive Red eluate fraction; lane f, DEAE-TSK eluate fraction; lane g, molecular weight standards. (C) Western blot profile of purification of MCp. Lane a, Triton extracted ER membrane lysate; lane b, 0–50% ammonium sulphate precipitate; lane c, DEAE cellulose eluate fraction; lane d, phenyl sepharose eluate fraction; lane e, Reactive Red eluate fraction; lane f, DEAE-TSK eluate fraction; lane g, molecular weight standards. (D) Mono Q HR (5/5) column elution profile of the purified calpastatins; the column was eluted at a flow rate of 60 ml/h. (E) 7.5% SDS–PAGE profile of purification of the calpastatins. Lane a, Triton extracted ER membrane lysate; lane b, 0–50% ammonium sulphate precipitate; lane c, DEAE cellulose eluate fraction; lane d, 0–23% ammonium sulphate supernatant; lane e, phenyl sepharose eluate fraction; lane f, DEAE-TSK eluate fraction; lane g, Mono Q HR eluate fraction (I); lane h, Mono Q HR eluate fraction (II); lane i, molecular weight standards. (F) Western blot profile of purification of the calpastatins. Lane a, Triton extracted ER membrane lysate; lane b, 0–50% ammonium sulphate precipitate; lane c, DEAE cellulose eluate fraction; lane d, 0–23% ammonium sulphate supernatant; lane e, phenyl sepharose eluate fraction; lane f, DEAE-TSK eluate fraction; lane g, Mono Q HR eluate fraction (I); lane h, Mono Q HR eluate fraction (II); lane i, molecular weight standards. (G) DEAE-TSK column elution profile of the purified LCp; the column was eluted at a flow rate of 60 ml/h. (H) 10% SDS–PAGE profile of purification of LCp. Lane a, ER lumen fraction; lane b, 0–50% ammonium sulphate precipitate; lane c, DEAE cellulose eluate fraction; lane d, phenyl sepharose eluate fraction; lane e, Reactive Red eluate fraction; lane f, DEAE-TSK eluate fraction; lane g, molecular weight standards. (I) Western blot profile of purification of LCp. Lane a, ER lumen fraction; lane b, 0–50% ammonium sulphate precipitate; lane c, DEAE cellulose eluate fraction; lane d, phenyl sepharose eluate fraction; lane e, Reactive Red eluate fraction; lane f, DEAE-TSK eluate fraction; lane g, molecular weight standards.

The result of purification of LCp as assessed by protease activity measurement was summarized in Table III.

Casein zymogram of the MCp and LCp

In the casein zymogram, both the purified MCp and LCp showed zymographic activities (Fig. 3A, lanes b and c) in presence of Ca^{2+} containing proteolysis buffer (pH 7.5) but in absence of Ca^{2+} and in presence of calpain specific inhibitor, calmidazolium (400 μM, determined to be optimum inhibitory concentration, data not shown), no such caseinolytic band has, however, been detected (Fig. 3A, lanes d–g).

Upon treatment of the purified MCp and LCp with 2 mM $CaCl_2$, autolysed m-calpain bands were observed in casein zymogram (Fig. 3B, lanes b and c). The autolysed products of the MCp and LCp have slower migration compared to their native forms in the casein zymogram (Fig. 3B, lanes b and c); this is due to different surface charge of the autolysed m-calpain than the native m-calpain in casein zymogram (*21*, *31*).

Table I. Purification of MCp from the ER membrane[a].

Purification steps	Total protein (mg)	Total activity (units[b])	Specific activity (units/mg)	Fold purification[c]	Recovery (%)
Triton extracted ER membrane lysate	1242	–	–	–	–
0–50% $(NH_4)_2SO_4$ precipitate	441	–	–	3	–
DEAE cellulose eluate fraction	65	650	10	19	100
Phenyl-sepharose eluate fraction	5.6	420	75	143	65
Reactive Red eluate fraction	2.2	264	120	228	41
DEAE-TSK eluate fraction	0.9	153	170	323	24

[a]Initial preparation contained 3,930 mg of ER membrane protein.
[b]One unit of calpain was defined as the amount of the enzyme that caused a change of 1.0 in A750 for the trichloroacetic acid-soluble products after incubation of the enzyme in the assay mixture under the standard assay conditions.
[c]MCp activity can not be detected in the Triton extracted ER membrane lysate or 0-50% $(NH_4)_2SO_4$ precipitate because of the presence of calpastatin. Hence, the fold purification for MCp for the above mention steps are calculated based on protein content and assuming 100% recovery of activity.

Table II. Purification of calpastatins from the ER membrane[a].

Purification steps	Total protein(mg)	Total activity(units[b])	Specific activity (units/mg)	Fold purification[c]	Recovery (%)
Triton extracted ER membrane lysate	1242	–	–	–	–
0-50% $(NH_4)_2SO_4$ precipitate	441	–	–	3	–
DEAE cellulose eluate fraction	66	1915	29	19	100
0-23% $(NH_4)_2SO_4$ supernatant	8.3	1490	180	118	78
Phenyl-sepharose eluate fraction	2.5	810	324	212	42
DEAE-TSK eluate fraction	0.92	472	513	336	25
Mono Q HR eluate fraction (I)	0.16	132	825	541	7
Mono Q HR eluate fraction (II)	0.14	113	807	529	6

[a]Initial preparation contained 3,930 mg of ER membrane protein.
[b]One unit of calpastatin was defined as the amount of inhibitor that inhibited 1 unit of calpain under the standard assay conditions.
[c]Calpastatin activity can not be detected in the Triton extracted ER membrane lysate or 0-50% $(NH_4)_2SO_4$ precipitate because of the presence of m-calpain. Hence, the fold purification for calpastatin for the above mentioned steps are calculated based on protein content and assuming 100% recovery of activity.

Table III. Purification of LCp from the ER lumen.

Purification steps	Total protein (mg)	Total activity (units[a])	Specific activity (units/mg)	Fold purification	Recovery (%)
ER luminal fraction	1153	830	0.7	–	100
0–50% $(NH_4)_2SO_4$ precipitate	413	709	1.7	2	85
DEAE cellulose eluate fraction	61	599	9.8	14	72
Phenyl-sepharose eluate fraction	5.4	405	76	109	49
Reactive Red eluate fraction	1.8	279	155	221	34
DEAE-TSK eluate fraction	0.5	162	324	463	20

[a]One unit of calpain was defined as the amount of the enzyme that caused a change of 1.0 in A750 for the trichloroacetic acid-soluble products after incubation of the enzyme in the assay mixture under the standard assay conditions.

Amino-terminal sequences of the MCp, LCp and calpastatins

Although immunoblot study revealed that the 80 kDa bands from MCp and LCp were the m-calpain large subunit; 28 kDa band from MCp was the m-calpain small subunit; 110 and 70 kDa bands were the calpastatin molecules, but for further confirmation of the purified proteins we have determined the N-terminal amino-acid sequences of the purified 80 kDa large subunits from MCp and LCp and purified 28 kDa small subunit from MCp and also of the purified 110 and 70 kDa calpastatin molecules.

The 80 kDa large subunit of MCp has 88% sequence identity (*25*) with corresponding human m-calpain large subunit (*42*). Herein, we have determined the N-terminal amino-acid (first 10 residues) sequence of the purified 80 kDa LCp, which was homologous to that of the 80 kDa large subunit of MCp (M-A-G-I -A-A-K-L-A-K).

The 28 kDa small subunit of MCp has 84% sequence identity (*25*) with corresponding human m-calpain small subunit sequence (*43*).

Similarly, we have also determined the N-terminal amino-acid (first 10 residues) sequences of the purified 110 and 70 kDa molecules and compared with the

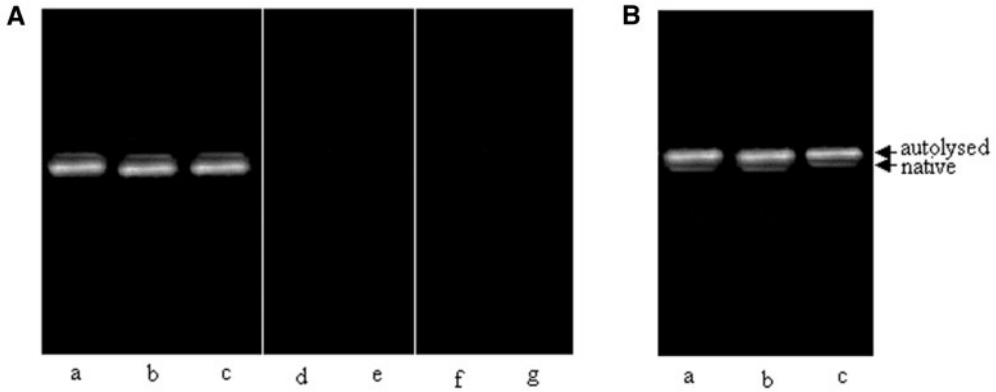

Fig. 3 Casein zymogram of the purified MCp and LCp under different conditions. (A) Casein zymogram of the purified m-calpains (MCp and LCp). Lane a, bovine skeletal muscle m-calpain (as control) (the gel was incubated in Ca^{2+} containing proteolysis buffer); lane b, purified MCp (the gel was incubated in Ca^{2+} containing proteolysis buffer); lane c, purified LCp (the gel was incubated in Ca^{2+} containing proteolysis buffer); lane d, purified MCp (the gel was incubated in proteolysis buffer with 1 mM EGTA instead of Ca^{2+}); lane e, purified LCp (the gel was incubated in proteolysis buffer with 1 mM EGTA instead of Ca^{2+}); lane f, purified MCp treated with calmidazolium (400 μM) followed by addition of 10 mM EGTA (the gel was incubated in Ca^{2+} containing proteolysis buffer); lane g, purified LCp treated with calmidazolium (400 μM) followed by addition of 10 mM EGTA (the gel was incubated in Ca^{2+} containing proteolysis buffer). (B) Casein zymogram of native and autolysed purified m-calpains (MCp and LCp). Lane a, bovine skeletal muscle m-calpain (as control), treated with 2 mM $CaCl_2$ (the gel was incubated in Ca^{2+} containing proteolysis buffer); lane b, purified MCp treated with 2 mM $CaCl_2$ (the gel was incubated in Ca^{2+} containing proteolysis buffer); lane c, purified LCp treated with 2 mM $CaCl_2$ (the gel was incubated in Ca^{2+} containing proteolysis buffer).

amino-acid sequence of bovine cardiac 145 kDa calpastatin molecule. Both the molecules have the similar sequence and the sequence M-N-P-T-E-A-K-A-I-P is homologous to the sequence M_{69}NPTEAKAIP (i.e. the sequence of 1–10 amino-acid residues of L-domain) in bovine cardiac 145 kDa calpastatin molecule (*44*). Taken together, these results suggest that 110 and 70 kDa immunoreactive bands of the purified proteins are of calpastatin molecules present in the ER and both the purified molecules contain L-domain of the calpastatin molecule.

In order to further confirm the presence of the L-domain in the calpastatin molecules, we have performed western blot of the purified 110 and 70 kDa calpastatin molecules with the calpastatin L-domain specific antibody. The results showed that the antibody recognized two immunoreactive bands at 110 and 70 kDa (Fig. 4), which further confirmed the presence of L-domain both in 110 and 70 kDa calpastatin molecules.

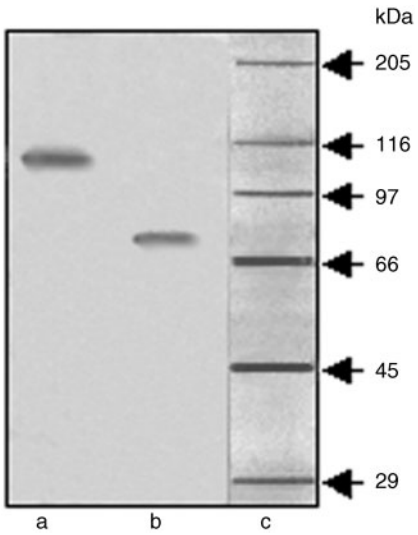

Fig. 4 Western blot of the purified 110 and 70 kDa calpastatins with calpastatin L-domain specific antibody. Lane a, purified 110 kDa calpastatin; lane b, purified 70 kDa calpastatin; lane c, molecular weight standards.

Comparative studies in the characteristic properties of the MCp and LCp

We have compared MCp and LCp by their following physicochemical and kinetic properties and CD spectra.

Phosphorylated status of the MCp and LCp. Immunoblot studies using anti-phospho-Ser, anti-phospho-Thr and anti-phospho-Tyr antibodies indicated that the purified LCp contained phosphorylated Ser, Thr and Tyr residues in the state of its purification, but the purified MCp was not phosphorylated in the state of its purification (Fig. 5A).

Optimum pH and Ca^{2+} concentration for maximum activities of the MCp and LCp. The pH activity profiles of the purified MCp and LCp were examined. As shown in Fig. 5B and C, the activities of both the purified MCp and LCp were maximal at pH 7.5 indicating that both the purified proteins are neutral proteases like other m-calpain (*34*, *45*, *46*).

The optimum concentration of Ca^{2+} for highest activities of both the purified m-calpains (MCp and LCp) were determined to be 5 mM (Fig. 5D and E), which was similar to the result reported by Mkwetshana *et al.* (*34*).

pI and 2-DE of the MCp and LCp. Figure 5F and G depicted the 2-DE of the purified MCp and LCp, respectively. Analyses of the gel images by PDQuest software (Bio-Rad, USA) revealed that the pI of the purified MCp and LCp was same, which was 4.5.

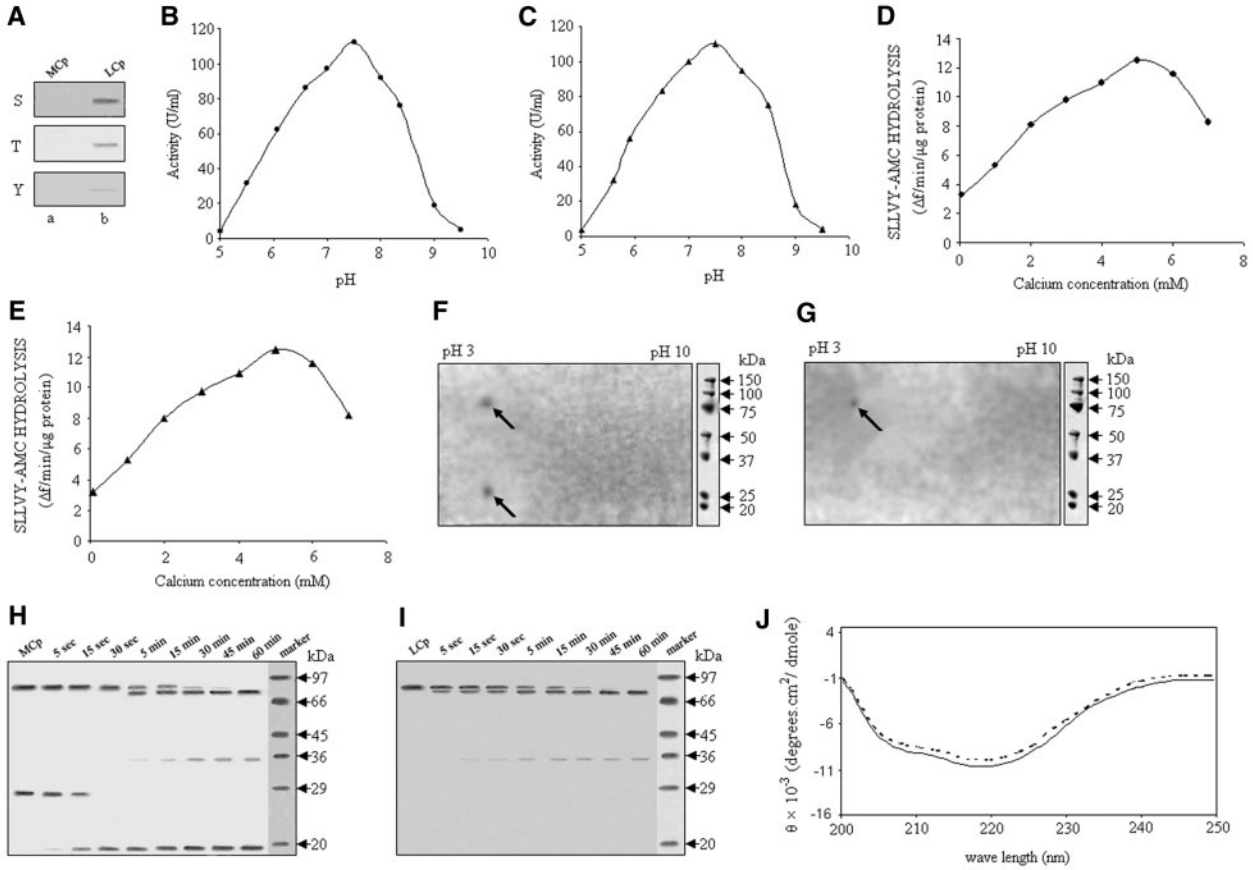

Fig. 5 Characteristic properties of the purified MCp and LCp. (A) Determination of phosphorylated status of the purified MCp and LCp. Western blots of the purified MCp and LCp using rabbit polyclonal anti-phospho-Ser (S), anti-phospho-Thr (T) and anti-phospho-Tyr (Y) antibodies. Lane a, purified MCp; lane b, purified LCp. (B) Effect of pH on the activity of the purified MCp. (C) Effect of pH on the activity of the purified LCp. (D) Effect of Ca^{2+} concentration on the activity of the purified MCp. (E) Effect of Ca^{2+} concentration on the activity of the purified LCp. (F) 2-DE of the purified MCp. Protein spots at pI value (4.5) were indicated by arrows, upper arrow for 80 kDa large subunit of MCp and lower arrow for 28 kDa small subunit of MCp. (G) 2-DE of the purified LCp. Protein spot at pI value (4.5) was indicated by arrow. (H) Immunoblot profile showing time course of the purified MCp (0.2 mg/ml protein) autolysis by 2 mM Ca^{2+}. (I) Immunoblot profile showing time course of the purified LCp (0.2 mg/ml protein) autolysis by 2 mM Ca^{2+}. (J) CD spectra of the purified MCp (—) and LCp (---). Path length 0.1 dm; protein concentration 0.15 mg/ml. The spectra are average of four accumulations.

The value of the pI indicated that like other m-calpain (*47*), the purified MCp and LCp of the ER are acidic in nature.

Autolysis of the MCp and LCp. The autolysed products of the purified MCp exhibited bands at 78, 35 and 18 kDa, whereas LCp exhibited only at 78 and 35 kDa in the immunoblot (Fig. 5H and I). The small subunit of the purified MCp was autolysed rapidly, which took only 30 s for its complete autolysis (Fig. 5H). This result was well co-related with the results obtained by Nagainis *et al.* (*36*) and Crawford *et al.* (*48*). During this time no detectable change in the 80 kDa subunit of MCp was observed (Fig. 5H). But due to the lack of 28 kDa small subunit the autolysis of the 80 kDa LCp started from the beginning (Fig. 5I). The autolysis of the large subunit of the purified MCp was completed after 60 min, which occurred in several sequential steps (Fig. 5H). The time course of autolysis for LCp was similar (60 min) to that of MCp (Fig. 5H and I). The relatively long autolysis time for the large subunit of MCp and LCp was in agreement with that reported by others (*34*, *48*, *49*).

Kinetic parameters of the MCp and LCp. Table IV summarized the K_m, k_{cat} and k_{cat}/K_m values for the purified MCp and LCp determined with various fluorogenic substrates. The K_m values for MCp and LCp with Suc-Leu-Met-MCA, Suc-Leu-Tyr-MCA, and Suc-Leu-Leu-Val-Tyr-MCA were almost similar to that for porcine m-calpain with the exception of Boc-Val-Leu-Lys-MCA. The k_{cat} values for MCp and LCp with Suc-Leu-Met-MCA, Suc-Leu-Tyr-MCA, and Suc-Leu-Leu-Val-Tyr-MCA were almost similar to that for porcine m-calpain with the exception of Boc-Val-Leu-Lys-MCA. The k_{cat}/K_m values for both MCp and LCp decreased in the order Suc-Leu-Leu-Val-Tyr-MCA > Boc-Val-Leu-Lys-MCA > Suc-Leu-Tyr-MCA ≥ Suc-Leu-Met-MCA, which corresponded with results obtained by Sasaki *et al.* (*37*).

CD spectra of the MCp and LCp. CD studies showed that the purified MCp and LCp have almost identical CD spectra (Fig. 5J), which were well co-related with that obtained by Edmunds *et al.* (*38*) and Suzuki and Ishiura (*50*).

Table IV. Comparison of kinetic parameters for ER MCp[a] and LCp[a] and porcine[b] m-calpain at room temperature.

Substrate[c]	Enzyme	K_m (mM)	k_{cat} (s^{-1})	$10^{-2} \times (k_{cat}/K_m)$ ($M^{-1}s^{-1}$)
Suc-Leu-Met-MCA	MCp	4.66	0.186	0.399
	LCp	4.68	0.189	0.404
	Porcine	4.78	0.194	0.405
Suc-Leu-Tyr-MCA	MCp	2.13	0.084	0.394
	LCp	2.10	0.083	0.395
	Porcine	2.21	0.092	0.417
Suc-Leu-Leu-Val-Tyr-MCA	MCp	0.461	0.063	1.37
	LCp	0.459	0.062	1.35
	Porcine	0.466	0.065	1.39
Boc-Val-Leu-Lys-MCA	MCp	8.11	1.06	1.31
	LCp	8.12	1.08	1.33
	Porcine	7.08	0.805	1.14

[a]The amount added per tube: 25 μg of each of ER m-calpain (MCp or LCp).
[b]Taken from (*37*).
[c]Seven to ten different concentrations (0.001–4 mM) for each substrate were used for Lineweaver-Burk plots.

Comparative studies in the regulations of MCp and LCp in the ER

Immunoprecipitation of LCp with ERp57 and *vice versa* were carried out on the ER lumen fraction, using the anti-m-calpain (or anti-ERp57) followed by immunoblotting for the identification of ERp57 (or m-calpain), as described in the method section. As shown in Fig. 6A, 57 kDa immunoreactive ERp57 band was co-precipitated with the immunoprecipitated LCp in the ER lumen fraction, which was detected when immunoblot was performed with ERp57 antibody. On the other hand, 80 kDa band of LCp was co-precipitated with the immunoprecipitated ERp57 in the ER lumen fraction, which was detected when immunoblot was performed with m-calpain antibody (Fig. 6B). Taken together, these results (Fig. 6A and B) demonstrate the association between LCp and ERp57 in the ER lumen, which suggests that the regulation of LCp differs from the regulation of MCp that is regulated by the calpastatin through its association with calpastatin in the ER membrane (*21*).

The interaction assay between LCp and ERp57 showed that around 77% of the LCp activity was precipitated by the ERp57 antibody (Fig. 6C). Interestingly, in case of mitochondrial μ-calpain, Ozaki *et al*. (*23*) reported that ~75% of the calpain activity was precipitated by the ERp57 antibody.

We have also performed the co-immunoprecipitation study of MCp and ERp57. The results revealed that there was no association between MCp and ERp57 (Fig. 6D and E), which indicated that MCp is not regulated by ERp57. Therefore, regulations of MCp and LCp appear to be different.

Comparative studies in the functional activities of MCp and LCp in the ER

In our recent report (*25*) we have shown that in presence of Ca^{2+}, MCp cleaves NCX1 in the ER membrane.

In order to determine the functional activity of LCp in the ER lumen, the isolated ER vesicles were pretreated with membrane impermeable calpain inhibitor, leupeptin (100 μM) for 20 min followed by incubation in a suspending buffer (130 mM KCl, 9 mM Tris–PO_4, 4 mM Tris–HCl, pH 7.5) supplemented with 5 mM $CaCl_2$ in presence of Ca^{2+} ionophore, A23187 (5 μM) at 37°C for 10 min. Stock solution of A23187 (100 mM) was made with DMSO diluted in the suspending buffer prior to treatment.

In another set of experiment ER vesicles were pretreated with membrane permeable calpain inhibitor, calpeptin (20 μM) for 20 min followed by incubation in the suspending buffer supplemented with 5 mM $CaCl_2$ in presence of A23187 (5 μM) at 37°C for 10 min. Dose and time of treatments of the agents used were determined to be optimum in separate experiments (data not shown).

After treatment of the leupeptin (100 μM) pretreated ER with $CaCl_2$ in presence of A23187 (5 μM), we have performed western blots with polyclonal anti-IP_3R1. We found a 40 kDa fragment of IP_3R1 in the immunoblot (Fig. 7A). On the other hand no proteolytic fragment of IP_3R1 was detected in the immunoblot when ER vesicles were pretreated with calpeptin (20 μM) (Fig. 7A).

In order to determine the m-calpain activity under leupeptin (100 μM) or calpeptin (20 μM) pretreatment followed by Ca^{2+}-treated condition, we measured the m-calpain activity with the fluorogenic calpain substrate SLLVY-AMC. Pretreatment of the ER with leupeptin (100 μM) inhibited only MCp but not LCp as it is not membrane permeable. Hence, treatment of Ca^{2+} with leupeptin (100 μM) pretreated ER activated only LCp, which cleaved SLLVY-AMC and we obtained only LCp activity (Fig. 7B). On the other hand pretreatment of the ER with calpeptin (20 μM) inhibited both MCp and LCp as it is membrane permeable and hence no discernible m-calpain (MCp and LCp) activity has, however been detected (Fig. 7B).

Thus, taken together these data suggested that in presence of Ca^{2+}, LCp cleaves IP_3R1 in the ER lumen.

Characteristic properties of the calpastatins

Phosphorylated status of the calpastatins. Immunoblot studies using anti-phospho-Ser, anti-phospho-Thr and anti-phospho-Tyr antibodies indicated that the purified 110 and 70 kDa calpastatins were not phosphorylated in the state of their purification (data not shown).

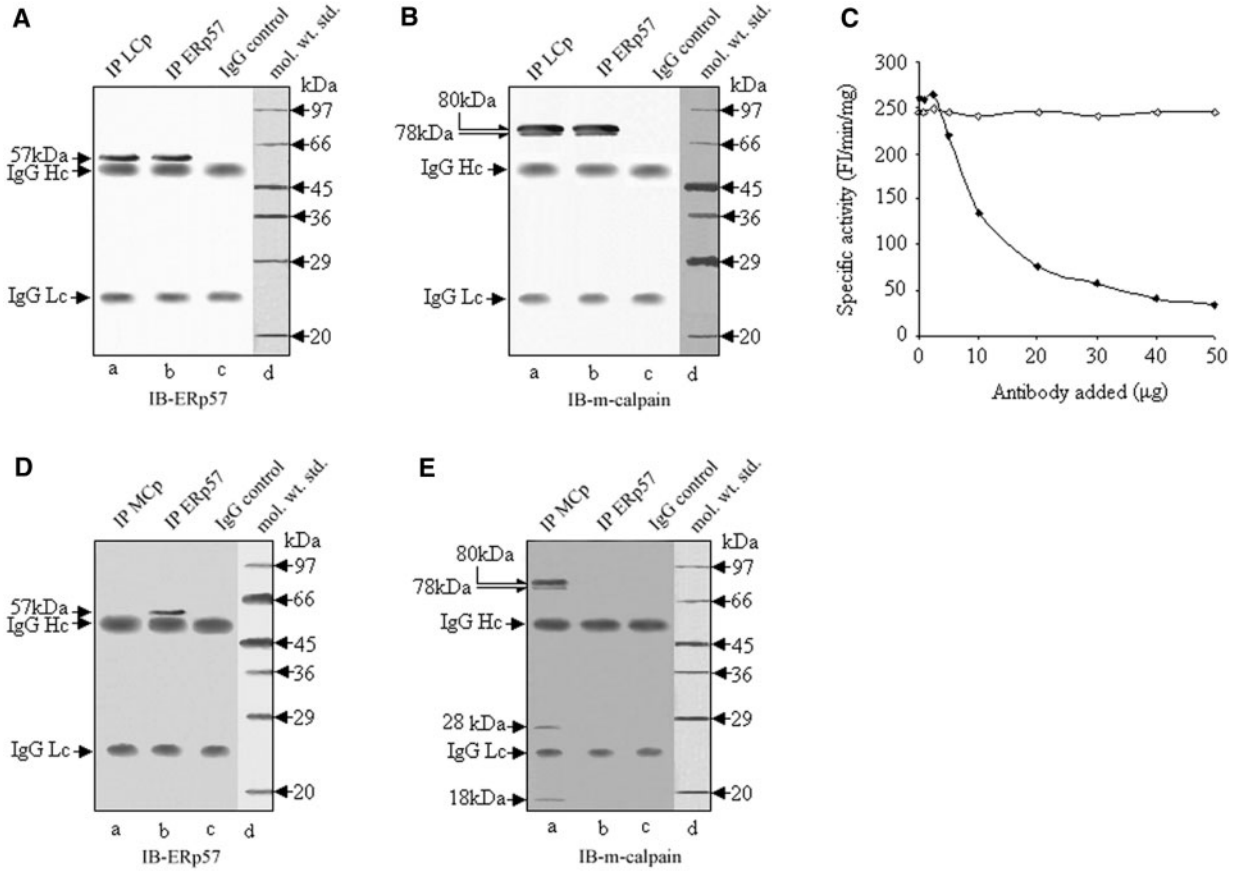

Fig. 6 Association between LCp or MCp and ERp57 in the ER. (A, B) Immunoprecipitation (IP) of the ER lumen fraction with rabbit polyclonal m-calpain and ERp57 antibodies followed by immunoblotting (IB) with the ERp57 and m-calpain antibodies, respectively (carried out on 10% SDS–PAGE). (A) lane a, IP LCp blotted with the ERp57 antibody; lane b, IP ERp57 blotted with the ERp57 antibody; lane c, IgG control blotted with the ERp57 antibody; lane d, molecular weight standards. (B) lane a, IP LCp blotted with the m-calpain antibody; lane b, IP ERp57 blotted with the m-calpain antibody; lane c, IgG control blotted with the m-calpain antibody; lane d, molecular weight standards. (C) Immunoprecipitation (IP) of the ER lumen fraction with ERp57 antibody (0.2–50 μg) followed by LCp activity measurement to determine how much of the protein (LCp) was involved in the interaction. Approximately 77% of the LCp activity was precipitated by ERp57 antibody (filled diamond). Normal rabbit IgG (0.2–50 μg) was used as a control (open diamond). (D, E) Immunoprecipitation (IP) of the ER membrane suspension with polyclonal m-calpain and ERp57 antibodies followed by immunoblotting (IB) with the ERp57 and m-calpain antibodies, respectively (carried out on 10% SDS–PAGE). (D) lane a, IP MCp blotted with the ERp57 antibody; lane b, IP ERp57 blotted with the ERp57 antibody; lane c, IgG control blotted with the ERp57 antibody; lane d, molecular weight standards. (E) lane a, IP MCp blotted with the m-calpain antibody; lane b, IP ERp57 blotted with the m-calpain antibody; lane c, IgG control blotted with the m-calpain antibody; lane d, molecular weight standards.

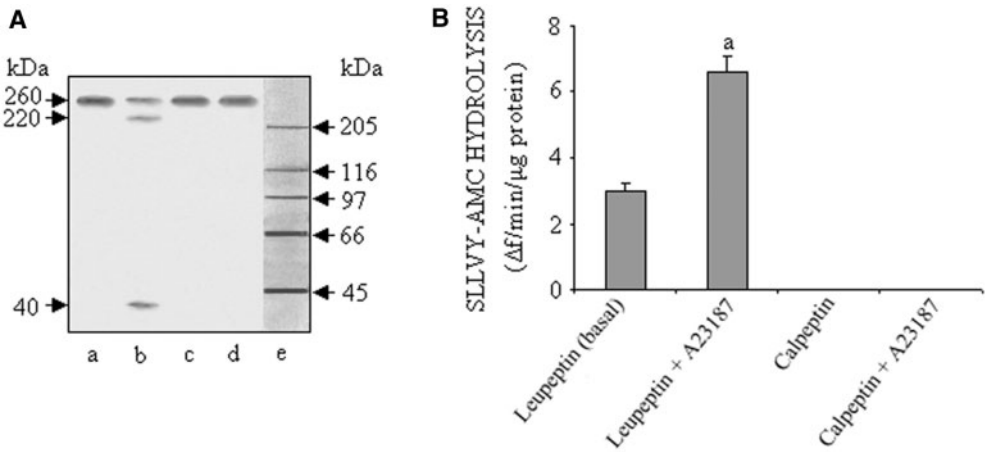

Fig. 7 Effect of treatment of Ca^{2+} (5 mM) in presence of Ca^{2+} ionophore, A23187 (5 μM) on LCp activity and cleavage of IP_3R1 in the ER. (A) Western blot of IP_3R1 (carried out on 7.5% SDS–PAGE). Lane a, leupeptin (membrane impermeable calpain inhibitor) (100 μM) treated ER suspension (basal); lane b, ER was pretreated with leupeptin (100 μM) followed by treatment with Ca^{2+} (5 mM) in presence of A23187 (5 μM); lane c, calpeptin (membrane permeable calpain inhibitor) (20 μM) treated ER suspension; lane d, ER was pretreated with calpeptin (20 μM) followed by treatment with Ca^{2+} (5 mM) in presence of A23187 (5 μM). (B) Effect of different calpain inhibitors, leupeptin (100 μM) and calpeptin (20 μM) on Ca^{2+} induced LCp activity in the ER. Data are mean ± SE ($n = 4$); $^{a}P < 0.001$ compared to the basal condition.

pI and 2-DE of the calpastatins. Figure 8A and B depicted the 2-DE of the purified 110 and 70 kDa calpastatins, respectively. Analyses of the gel images by PDQuest software (Bio-Rad, USA) revealed that the pI of the purified 110 and 70 kDa calpastatins were 4.6 and 4.7, respectively. The values of the pI indicated that like other calpastatin (*40*), the purified calpastatins of the ER are acidic in nature.

IC_{50} values of the calpastatins. The purified 110 and 70 kDa calpastatins were tested to determine their abilities to inhibit the purified MCp. Activity of the purified MCp was inhibited by the purified calpastatins. Figure 8C showed the inhibition of the purified MCp with varying concentrations of the purified calpastatins. The IC_{50} value for 110 kDa calpastatin was 0.52 nM, whereas the IC_{50} value for 70 kDa calpastatin was 0.8 nM. These IC_{50} values were very well comparable with those typically obtained with calpastatins from pig heart muscle and erythrocytes (*40*), indicating that the ER calpastatin molecules are equally efficient with other mammalian calpastatin in inhibiting the calpain.

Discussion

In our previous report, we have demonstrated that in addition to the cytosolic side of the ER membrane, the 80 kDa m-calpain is also present in the lumen of the ER (*21*). Hood *et al*. (*51*) also reported that 80 kDa m-calpain is present within the lumen of the ER. But no report has however been available regarding the characterization of these two types of m-calpains (MCp and LCp) and calpastatins and also the regulation and functional activity of LCp in the ER. Herein, we report the same in the ER of pulmonary artery smooth muscle.

In our present study, a series of chromatographic steps were employed to purify MCp (80 kDa large and 28 kDa small subunit) and calpastatins (110 and 70 kDa) from the ER membrane and LCp (80 kDa) from the ER lumen (Fig. 2B, C, E, F, H and I). Several lines of evidences such as western analyses, zymographic studies and N-terminal amino-acid sequence analyses confirmed the identities of the purified proteins as m-calpains and calpastatins (Figs 2C, 2F, 2I, 3A and B) (*25*). From the N-terminal (first 25 amino-acid residues) sequence analyses, we have found that the large and small subunit of the purified MCp have, respectively, 88 and 84% sequence similarity (*25*) with those of the human m-calpain (*42*, *43*). N-terminal amino-acid (first 10 residues) sequence analyses of the purified 110 and 70 kDa molecules revealed that both have the similar sequence, which is identical with the sequence of L-domain (first 1–10 amino-acid residues of L-domain i.e. 69–78 amino-acid residues) of bovine cardiac 145 kDa calpastatin molecule (*44*).

From the N-terminal amino-acid (first 10 residues) sequence analyses, we have found that both 110 and

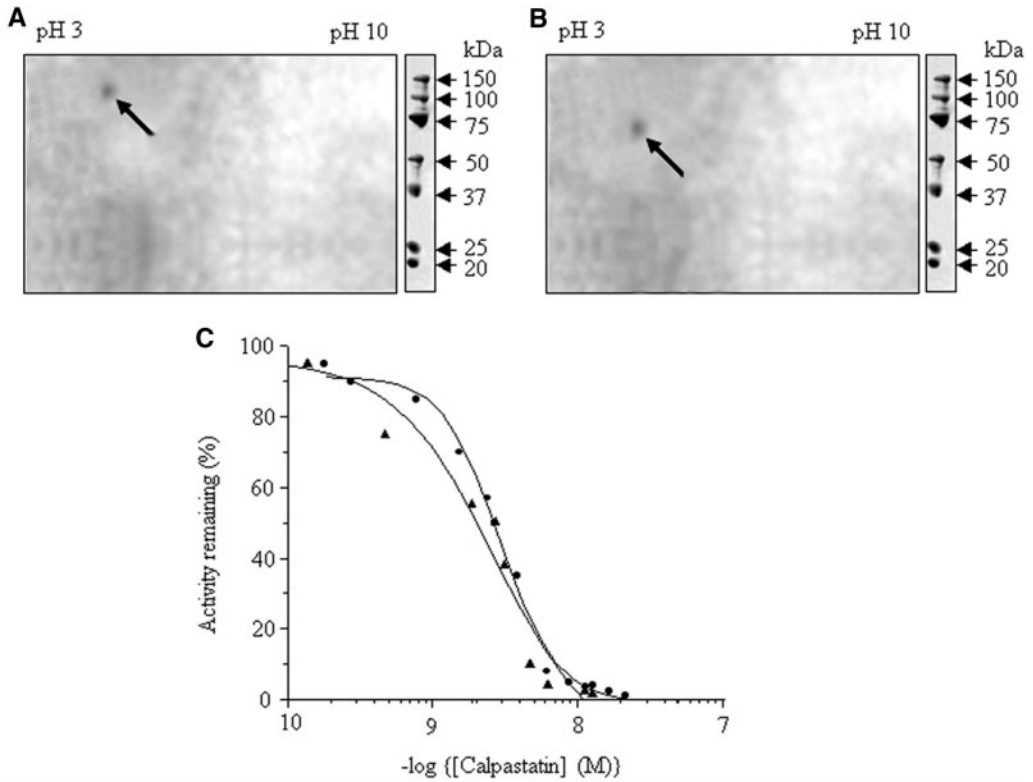

Fig. 8 Characteristic properties of the purified calpastatins. (A) 2-DE of purified 110 kDa calpastatin. Protein spot at pI value (4.6) was indicated by arrow. (B) 2-DE of purified 70 kDa calpastatin. Protein spot at pI value (4.7) was indicated by arrow. (C) Determination of IC_{50} values of the purified calpastatins. Inhibition of proteolytic activity of the purified MCp by the purified 110 kDa (filled circle) and 70 kDa (filled triangle) calpastatins.

70 kDa calpastatin molecules have identical sequence, although their molecular weights are different; which indicates that 70 kDa calpastatin molecule may be the alternative splice variant or proteolysis product of 110 kDa calpastatin molecule. The variability of the molecular weights of calpastatin may be due to protein degradation, while some is likely to result from alternative splicing or different translation initiation sites (*44*, *52*, *53*).

We were unable to detect activity of the MCp by spectrophotometric assay up to $(NH_4)_2SO_4$ precipitation step of purification due to the association of the MCp and calpastatin (Table I), but after 0–50% $(NH_4)_2SO_4$ precipitation step, the MCp was separated from the calpastatin and showed its activity (Table I). Similar type of observation was also reported by Saito *et al*. (*54*), who found that the rainbow trout m-calpain showed its activity after DE-52 column chromatography step of purification. On the other hand, activity of LCp was detected spectrophotometrically from the initial step of purification due to the absence of calpastatin in the ER lumen (Table III).

Association between MCp and calpastatin in the ER membrane indicated that MCp is regulated by calpastatin, which is absent in the ER lumen. This suggested that endogenous regulatory molecule(s) may exist in the lumen of the ER, which indicates that the regulation of LCp differs from MCp. Our results demonstrated that ERp57 is associated with LCp in the ER lumen (Fig. 6A and B). ERp57 mediated stabilization of μ-calpain has been reported (*23*). ERp57 functions mainly in the refolding of the large subunit of calpain, which is involved in the formation of disulfide bonds to form functional conformations and also in prevention of proteolytic degradation of calpain (*23*). Considering these findings and the results of our study, we suggest that ERp57 is the regulatory molecule of LCp in the ER lumen, which stabilizes the LCp. Unfolded proteins have surface-exposed hydrophobic parts, which are recognized by the molecular chaperons (*23*) suggesting that ERp57 is associated with the hydrophobic amino-acid sequence of LCp in the ER lumen. Kozlov *et al*. (*55*) reported that ERp57 has 4 thioredoxin-like domains, abb′a′, where the a and a′ domains are the catalytic domains and the b and b′ are the non-catalytic domains. They also demonstrated that the bb′ fragment of human ERp57 interacts with the lectin-like chaperones, calnexin and calreticulin (*55*). Ozaki *et al*. (*23*) demonstrated that calpain also interacts with ERp57 via b and b′ domains. We suggest that in a similar fashion LCp may also interact with ERp57 through b and b′ domains in the ER lumen.

Our results reveal that although LCp is associated with ERp57 (Fig. 6A and B), but there appears to be no association between MCp and ERp57 (Fig. 6D and E). This could possibly be due to the association of MCp with its endogenous inhibitor, calpastatin (*21*), although exact reason remains to be resolved. Ozaki *et al*. (*23*) also demonstrated that cytosolic μ- and m-calpains do not associate with ERp57. Therefore, the regulations of MCp and LCp appear to be different.

In the casein zymogram, both the purified MCp and LCp showed zymographic activities (Fig. 3A, lanes b and c). Prominent autolysed bands of both the purified MCp and LCp were observed in the zymogram after addition of 2 mM $CaCl_2$ (Fig. 3B, lanes b and c). The autolysed band has slower mobility than native m-calpain, which appears to be due to different surface charge of the autolysed m-calpain than that of the native m-calpain in casein zymogram (Fig. 3B, lanes b and c). Similar observation has also been reported by Raser *et al*. (*31*). No other caseinolytic band has, however, been detected in the zymogram (Fig. 3B, lanes b and c), which could possibly be due to the fact that the 35 kDa autolytic fragments of both MCp and LCp and 18 kDa autolytic fragment of MCp (Fig. 5H and I) represent the inactive form of m-calpain (*21*).

In the phosphorylation study we have found that only 80 kDa LCp was phosphorylated in its Ser, Thr, Tyr residues in the state of its purification. On the other hand no phosphorylated residues were detected in either MCp or calpastatins in the states of their purification, it may be due to the association of MCp and calpastatins in the ER membrane. Melloni *et al*. (*56*) demonstrated that phosphorylation of calpastatin in L-domain reduces its association with calpain. Thus, our present study is in agreement with the observation of Melloni *et al*. (*56*).

The specificity of calpain can be understood by observing the effects of subsite residues (*37*). The general rule is that calpain cleaves peptides preferentially whose P_1 positions are occupied by either Met, Tyr, or Arg preceded by a hydrophobic amino-acid residue at P_2 position (*37*). In our present study, hydrolysis of various fluorogenic substrates by the purified m-calpains (MCp and LCp) follows this general rule (Table IV).

In our recent report (*25*) we have shown that in presence of Ca^{2+}, MCp cleaves NCX1 in the ER. Herein, we have reported that in presence of Ca^{2+}, LCp cleaves IP_3R1 in the ER (Fig. 7A). ER sequesters a considerable amount of mobilizable Ca^{2+} and plays a pivotal role in regulating Ca^{2+} dynamics under stimulated condition (*57*). In the present study, we have used Ca^{2+} ionophore, A23187 in order to increase $[Ca^{2+}]$ in the lumen of the ER. This increase in $[Ca^{2+}]$ in the ER lumen activates LCp, which subsequently cleaves IP_3R1 (Fig. 7A).

Inositol 1,4,5-trisphosphate receptors (IP_3Rs) are Ca^{2+} release channels on the ER that play a critical role in the generation of complex $[Ca^{2+}]_c$ patterning, e.g. Ca^{2+} waves and oscillations (*58*). IP_3Rs also supplied Ca^{2+} to the cytosol from the ER store (*58*). Three different subtypes of IP_3Rs are reported in the mammals, which are IP_3R1, IP_3R2 and IP_3R3 (*58*). Almost all tissues express differentially all the three IP_3R subtypes, while IP_3R1 predominates in smooth muscle cells (*59*).

IP_3R1 contains six transmembrane domains and as a result, there are three loops that reside in the ER lumen among them third luminal loop (L3) is the largest (*58*). Such orientation of IP_3R1 in the ER provides opportunity of activated LCp to act on IP3R1 as substrate. We suggest that this cleavage could lay a key role in

Ca^{2+} dysregulation of cell due to inability to release Ca^{2+} from the ER.

In summary, (i) both MCp and LCp exhibit optimum activities at pH 7.5; (ii) the optimum Ca^{2+} concentration for the activation of both MCp and LCp is 5 mM; (iii) both MCp and LCp are acidic in nature and their pI value is same, which is 4.5; (iv) MCp and LCp cleave several synthetic fluorogenic substrates and their kinetic properties (K_m, k_{cat} and k_{cat}/K_m values) are almost similar to that of m-calpain from other mammalian systems; (v) MCp and LCp have almost identical CD spectra in the far ultraviolet with that of m-calpain studied from other mammalian systems; (vi) autolysis of the MCp gives a final stable mixture of peptides of 78, 35 and 18 kDa, whereas LCp gives only 78 and 35 kDa. The small subunit of MCp takes only 30 s for its complete autolysis. During this time there is no detectable change in the 80 kDa subunit of MCp. But due to the lack of 28 kDa small subunit the autolysis of the 80 kDa LCp started from the beginning, although a long autolysis time (60 min) is required for completion of autolysis of the large subunit of MCp and LCp; (vii) MCp is not phosphorylated in the state of its purification, whereas LCp contains phosphorylated Ser, Thr and Tyr residues in its state of purification; (viii) MCp is regulated by calpastatin in the ER membrane, whereas LCp is regulated by ERp57 in the ER lumen; (ix) in presence of Ca^{2+} the activated MCp cleaves NCX1 in the ER membrane, whereas the activated LCp cleaves IP_3R1 in the ER lumen; (x) both 110 and 70 kDa inhibitor proteins contain L-domain of calpastatin molecule; (xi) pI values of the 110 and 70 kDa inhibitor proteins are 4.6 and 4.7, respectively; (xii) 110 and 70 kDa inhibitor proteins inhibit the MCp with IC_{50} values of 0.52 and 0.8 nM, respectively.

ER MCp is almost similar with cytosolic m-calpain in molecular mass, optimum pH and Ca^{2+} concentration, isoelectric point, kinetic parameters (K_m, k_{cat} and k_{cat}/K_m values), autolysis and CD spectra; but differs in its state of phosphorylation (*34*, *36–38*, *45–50*, *54*). Additionally, our results (Fig. 6D and E) reveal that there is no association between MCp and ERp57. Ozaki *et al.* (*23*) also demonstrated that cytosolic m-calpain does not associate with ERp57. Considering these findings and the results of our study, it may be suggested that MCp is closely related to the cytosolic m-calpain.

On the other hand, although LCp is similar with cytosolic m-calpain in optimum pH and Ca^{2+} concentration, isoelectric point, kinetic parameters (K_m, k_{cat} and k_{cat}/K_m values) and CD spectra, state of phosphorylation; but differs in molecular mass, autolysis and regulation by interacting with ERp57 (*23*, *34*, *36–38*, *45–50*, *54*).

In conclusion, pulmonary smooth muscle ER MCp and LCp are similar in optimum pH and Ca^{2+} concentration for their maximal activities, isoelectric point, kinetic parameters (K_m, k_{cat} and k_{cat}/K_m values) and CD spectra; but differ in molecular mass, state of phosphorylation, autolysis and their regulations and functional activities. MCp, LCp and calpastatins of ER are closely related to the corresponding enzyme (m-calpain) and its endogenous inhibitor calpastatin molecule from other species as evidenced by physico-chemical, kinetic and functional properties and also by N-terminal amino-acid sequence analyses.

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Conflict of interest

None declared.

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