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Identification, purification and characterization of matrix metalloproteinase-2 in bovine pulmonary artery smooth muscle plasma membrane

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Abstract

Bovine pulmonary artery smooth muscle tissue possesses matrix metalloproteinase-2 (72 kDa gelatinase: MMP-2; E.C. 3.4.24.24) as revealed by immunoblot studies of its plasma membrane suspension with polyclonal MMP-2 antibody. In this report, we described the purification and partial characterization of MMP-2 in the plasma membrane fraction of the smooth muscle. MMP-2 has been purified from plasma membrane fraction of bovine pulmonary artery smooth muscle to homogeneity using a combination of purification steps. Heparin sepharose purified preparation of 72 kDa progelatinase is composed of two distinct population of zymogens: a 72 kDa progelatinase tightly complexed with TIMP-2 (an ambient tissue inhibitor of metalloprotease in the smooth muscle plasma membrane), and a native 72 kDa progelatinase free of any detectable TIMP-2. The homogeneity of the native 72 kDa progelatinase form is demonstrated by SDS-PAGE under non-reducing condition, non-denaturing native gel electrophoresis. The purified TIMP-2 free proenzyme electrophoresed as a single band of 72 kDa which could be activated by APMA with the formation of 62 and 45 kDa active species. The proenzyme is activated poorly by trypsin but not by plasmin. The purified 72 kDa progelatinase is stable at aqueous solution and does not spontaneously autoactivate. The purified 72 kDa gelatinase exhibited properties that are typical of MMP-2 obtained from other sources. These are: (i) its activity is dependent on the divalent cation, Ca^{2+}, and is inhibited by EDTA, EGTA and 1:10-phenanthroline; (ii) it was inhibited by α_2 macroglobulin but not by the inhibitors of serine, cysteine, thiol, aspartic proteinases and calpains; (iii) it was found to be inhibited by TIMP-2, the specific inhibitor of MMP-2; (iv) like MMP-2, obtained from other sources, its major substrates were found to be collagens (type IV and V) and gelatins (type I, IV and V). Additionally, the purified MMP-2 degrades Dnp-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg-OH (dinitrophenyl labelled peptide), a well known synthetic substrate for the MMP-2. (Mol Cell Biochem **258**: 73–89, 2004)

Key words: matrix metalloproteinase-2, tissue inhibitor of metalloproteinase-2, pulmonary smooth muscle plasma membrane

Abbreviations: ECM – extracellular matrix; HBPS – Hank's buffered physiological saline; MMP-2 – matrix metalloproteinase-2; TIMP-2 – tissue inhibitor of metalloproteinase-2; TIMP-1 – tissue inhibitor of metalloproteinase-1; APMA – aminophenylmercuric acetate; H_2O_2 – hydrogen peroxide; t-buOOH – tert-butylhydroperoxide, $ONOO^-$ – peroxynitrite; O_2^- – superoxide radical; XO – xanthine oxidase; HPX – hypoxanthine; $C_{12}E_8$ – octaethylene glycol mono-n-dodecyl ether; Brij-35 – polyoxyethlene lauryl ether

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Introduction

The extracellular matrix (ECM) plays a central role in maintaining the structural integrity of primitive multicellular organisms as well as highly complex mammals. In addition, the matrix metalloproteinases (MMPs) influence basic cellular processes such as proliferation, differentiation, migration and adhesion. The early notion that the extracellular matrix is an inert and stable structure has been dispelled and it is clear that a dynamic equilibrium between synthesis and degradation of matrix components is required for matrix maintenance. Although many proteinases can cleave ECM molecules, the MMPs are believed to be the normal, physiologically relevant mediators of matrix degradation. There are multiple levels at which the expression and activity of MMPs are regulated, suggesting tight control is required for the continuation of normal processes given the potency of the enzymes. The apparent consequences of abnormally high levels of expression of some metalloproteinases in pathological conditions such as wound healing, angiogenesis, tumor invasion, metastasis [1–5], arthritis, emphysema and apoptosis [6] seem to support this contention.

So far 25 matrix metalloproteinases (MMPs) have been described that collectively degrade all extracellular matrices and a number of non-matrix proteins involved in inflammation, cell growth and differentiation [7]. The MMPs and their specific inhibitors, the respective tissue inhibitors of metalloproteinases (TIMPs) have been associated directly and indirectly with these events.

Matrix metalloproteinases (MMPs) are a family of Zn^{2+} and Ca^{2+} dependent endopeptidases secreted by both normal and transformed cells and are capable of degrading collagenous and non-collagenous components of extracellular matrix (ECM) [8, 9]. Several members of MMP family have been identified e.g. stromelysins, collagenases and gelatinases [6, 7]. All of these enzymes have several features: they are produced as zymogens and are activated by a variety of seemingly disparate means [10, 11]. Examples of these activation methods include treatment with proteases; conformational perturbants such as sodium dodecylsulfate and NaSCN; heavy metals such as Au(I) compounds, Hg(II), and organomercurials; oxidants such as NaOCl; sulfhydryl-alkylating agents such as N-ethylmaleimide (NEM); [12] and disulfide compounds, for example, oxidized glutathione [6]. There have also been numerous reports of spontaneous autoactivation [12]. These activation processes resulted in the autocatalytic cleavage of an 8–10 kDa amino terminal fragment [13]. A highly ordered and conserved domain structure found in the MMP's, including a propeptide domain, a zinc-binding active site and a carboxy terminal domain [1, 9].

Two members of the MMP family, the 92 kDa and 72 kDa progelatinase, possess a gelatin-binding domain [6], that may influence their substrate specificity and allows them to be purified by gelatin-sepharose affinity chromatography [14].

A well-characterized member of the MMP family is the 72 kDa progelatinase (MMP-2). The 72 kDa progelatinase is a zymogen and can undergo proteolytic processing to a 62 kDa active enzyme mediated by intact cells or cell membranes. The activation involves the removal of 80 residues from the amino terminus, which contains an unpaired cysteine. This cysteine is thought to coordinate with the active site zinc in the zymogen, maintaining it in non-catalytic form [11–13, 15].

The activity of 72 kDa progelatinase is regulated by TIMP-2, a 21 kDa-non-glycosylated protein [13, 15]. TIMP-2 forms a high affinity complex with the latent 72 kDa progelatinase and copurifies with the zymogen under non-denaturing conditions [6]. It appears that TIMP-2 may have a dual role in the regulation of 72 kDa progelatinase. This was evidenced by the observations that TIMP-2 inhibits the enzymatic activity of the activated 62 kDa gelatinase [16–19] and also retards the membrane mediated activation of the latent 72 kDa progelatinase [20–23].

We found that bovine pulmonary vascular smooth muscle plasma membrane possesses not only the 72 kDa progelatinase/TIMP-2 complex, and TIMP-2, but also free 72 kDa progelatinase. In this communication, we described the identification, purification, and characterization of 72 kDa gelatinase from bovine pulmonary artery smooth muscle plasma membrane.

Materials and methods

Materials

MMP-2, TIMP-2 and their polyclonal antibodies were obtained from Chemicon International, Temecula, CA, USA. [^{14}C]-gelatin was obtained from New England Nuclear, Wilmington, DE, USA. BCA protein assay kit, Pierce Biotechnology, Rockford, IL, USA. Trypsin, plasmin, Soyabean trypsin inhibitor, EGTA, 1:10 phenanthroline, α_2-macroglobulin, EDTA, PMSF, pepstatin, N-ethylmaleimide, leupeptin, antipain, chymostatin, Bowman–Birk inhibitor (BBI), acetyl-calpastatin, collagen I, IV and V, fibronectin, laminin, $C_{12}E_8$, Brij-35, DEAE cellulose, SDS, aminophenylmercuric acetate (APMA), dimethylsulphoxide, NaCl, $CaCl_2$ were obtained from Sigma Chemicals, St. Louis, MO, USA. Gelatin-sepharose, lentil lectin-sepharose and heparin-sepharose were obtained from Pharmacia, Upsala, Sweden. The synthetic substrate Dnp-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg-OH (dinitrophenyl labelled peptide) was obtained from Bachem, USA. All other chemicals used were of analytical grade and obtained from Sigma Chemicals.

Methods

Isolation of smooth muscle membrane

Bovine pulmonary artery collected from slaughterhouse was washed several times with Hank's buffered physiological saline (HBPS) (pH 7.4) and kept at 4°C. The washed pulmonary artery was used for further processing within 4 h after collection. The intimal and serosal (external) layers were removed and the tunica media, i.e. the smooth muscle tissue was collected and used for the present studies [24].

The smooth muscle membrane fraction was isolated by following the procedure as described previously [25]. Briefly, the smooth muscle tissue was homogenized with a cyclomixer in ice-cold medium containing 0.25 M sucrose and 10 mM Tris-HCl buffer, pH 7.4. The homogenate was centrifuged at 600 × g for 15 min at 4°C. The resulting supernatant was centrifuged at 15,000 × g for 20 min to sediment mitochondria and lysosomes. The supernatant was centrifuged at 100,000 × g for 1 h. The pellet was suspended in 10% (w/v) sucrose containing 10 mM Tris-HCl buffer, pH 7.4, and was layered on a discontinuous gradient consisting of 40% (w/v) and 20% (w/v) sucrose both containing 10 mM Tris-HCl buffer, pH 7.4. The gradient was centrifuged at 105,000 × g for 2 h. The fraction collected at the 20–40% sucrose interface was diluted with 10 mM Tris-HCl (pH 7.4) and was used as the plasma membrane fraction. The pellet was considered as the microsomal fraction. All operations were carried out at 4°C. Plasma membrane fraction was stored under liquid nitrogen and thawed before use.

Assay of marker enzymes

Assay of rotenone-insensitive NADPH-cytochrome C reductase

Rotenone-insensitive NADPH-cytochrome C reductase activity was assayed in bovine pulmonary artery smooth muscle membrane fraction by following the procedure previously described [26].

Assay of cytochrome C oxidase activity

Cytochrome C oxidase activity was determined by measuring oxidation of reduced cytochrome C by following the method previously described [27].

Assay of acid phosphatase activity

Acid phosphatase activity was determined at pH 5.5 using p-nitrophenylphosphate as the substrate by following the procedure previously described [28].

Assay of 5′ nucleotidase activity

5′ Nucleotidase activity was assayed by following the procedure as described previously [29].

Determination of protein

Protein concentration was estimated by the Pierce Micro BCA protein assay kit (Pierce) using bovine serum albumin (BSA) as the standard.

Enzyme purification

All purification steps were performed at 4°C.

(I) Ammonium sulfate precipitation

250 ml of plasma membranes (4–4.5 mg/ml) were rapidly thawed and solubilized with 0.5% $C_{12}E_8$ in 50 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl [30]. The resulting solubilized material was centrifuged at 1,00,000 × g. This gave a clear supernatant containing the solubilized enzyme. The supernatant containing the solubilized enzyme was precipitated overnight (12 h) at 4°C with 70% saturation with solid ammonium sulfate [31]. The precipitate was collected by centrifugation at 15,000 × g for 1 h at 4°C, reconstituted in the DEAE cellulose column buffer (50 mM Tris-HCl, 150 mM NaCl, 0.05% $C_{12}E_8$, pH 8.0) as 3 ml of buffer per g of precipitate and dialyzed extensively against the same buffer. During dialysis overnight, the buffer was changed with a fresh one 3 times to wash out of ammonium sulfate and to bring this in a super equilibrium with this buffer. After equilibration the material was filtered using Millipore membrane (0.45 μm pore size).

(II) DEAE cellulose chromatography

The clear dialyzed and filtered material was applied to a DEAE cellulose column (5 × 30 cm) equilibrated with the same buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% $C_{12}E_8$) at a flow rate of 30 ml/h [22]. The unbound fractions containing the 72 kDa gelatinase activity were pooled and dialyzed in the gelatin-sepharose column buffer A (50 mM Tris-HCl pH 7.0, 5 mM $CaCl_2$, 0.05% Brij 35, 0.05% NaN_3,) containing 200 mM NaCl. During dialysis overnight, the buffer was changed with a fresh one 3 times.

(III) Gelatin-sepharose chromatography

The clear dialyzed material was applied to a column of gelatin-sepharose (2 × 10 cm) equilibrated in buffer A containing 200 mM NaCl at a flow rate of 12 ml/h. The flow through material from the gelatin-sepharose column was collected and recirculated 3 times and the unretained protein was collected. Then the column was washed with 5 column vol. of buffer A, followed by buffer A containing 1 M NaCl at a flow rate of 40 ml/h until the absorbance of the eluent returned to back ground. Then the column was eluted in two-step procedure. 92 kDa gelatinase, free of 72 kDa gelatinase, was eluted from the column with buffer A containing 1 M NaCl and 1% DMSO

[32]. 72 kDa progelatinase and 72 kDa progelatinase/TIMP-2 complex were eluted with buffer A containing 1 M NaCl and 5% DMSO at a flow rate of 30 ml/h and the fractions were collected as 3 ml per tube [23]. The 72 kDa gelatinase containing fractions were pooled and concentrated by ultrafiltration (Amicon, YM-30 membrane; mol. wt. cut off 30 kDa), dialyzed overnight against 20 mM HEPES buffer pH 7.5 (20 mM HEPES, 500 mM NaCl, 1 mM $CaCl_2$, 0.05% Brij 35 and 0.02% NaN_3) to remove DMSO and to equilibrate in this buffer with several changes.

(IV) Lentil lectin-sepharose chromatography

The dialyzed eluate obtained in the previous step was recirculated 4 times over a column of lentil-lectin sepharose (2 × 10 cm) equilibrated with HEPES buffer pH 7.5 at a flow rate of 12 ml/h. 92 kDa gelatinase but not the 72 kDa progelatinase and 72 kDa progelatinase-TIMP-2 complex binds to the column. The flow through from the column contained the 72 kDa progelatinase and 72 kDa progelatinase-TIMP-2 complex. The enzyme as well as the complex were concentrated by ultrafiltration (Amicon, YM-30 membrane; mol. wt. cut off 30kDa), and dialyzed extensively overnight in buffer B (20 mM Tris-HCl, pH 8.0, 5 mM $CaCl_2$, 0.05% Brij 35 and 0.02% NaN_3).

(V) Heparin sepharose chromatography

The dialyzed material was applied to a column of heparin-sepharose CL-6B (2 × 8 cm) equilibrated in buffer B at a flow rate of 18 ml/h. The flow through material was collected each time and recirculated 3 times and the unretained protein from the column was collected. Then the column was washed with buffer B extensively at a flow rate of 40 ml/h until the absorbance returned back to ground. After that two-step gradient by NaCl in buffer A were performed at a flow rate of 30 ml/h and the fractions were collected as 3 ml per tube: (1) 72 kDa progelatinase-TIMP-2 complex was eluted with buffer A containing 100 mM NaCl; (2) free 72 kDa progelatinase was eluted with 200 mM NaCl in buffer A. The eluted fractions, which contained progelatinase-TIMP-2 complex and free progelatinase, were pooled separately and concentrated by ultrafiltration (Amicon, YM-30 membrane) and aliquoted. The aliquots were kept under liquid nitrogen and thawed for further study.

Polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was performed according to the procedure of Laemmli [33] using a minigel system apparatus (Bio-Rad). Samples (0.1 µg of protein) were diluted in SDS-containing sample buffer without β-mercaptoethanol (under non-reducing condition) prior to being loaded. Electrophoresis was performed at room temperature at 16 mA during stacking and 18 mA per plate during resolving. Protein containing bands were visualised by silver staining method [34].

Non-denaturing discontinuous polyacrylamide gels were prepared in a Laemmli buffer system lacking SDS [15]. Samples (0.1 µg of protein) were applied to the gel and the electrophoresis was carried out at 4°C at 16 mA per gel during stacking and 18 mA per gel during resolving. The protein containing bands were stained with silver [34].

Assay of Protease Activity by [^{14}C]-Gelatin Degradation

Ca^{2+}dependent matrix metalloprotease activity was determined as follows: the radiolabelled gelatin substrate was prepared by diluting 20 µl (1.2 µCi) of [^{14}C]-labelled gelatin with 20 µl of 1 mg/ml cold gelatin. The substrate mixture was then heated at 55°C for 25 min and allowed to cool slowly to room temperature. The final assay mixture contained 40 µl of substrate, 10 mM $CaCl_2$, 0.05% Brij 35 and APMA (2 mM) activated (for 15 min) sample. The resulting assay mixtures were incubated for 1 h at 37°C and then the reaction was stopped by the addition of 20 µl of 0.25M EGTA. Undigested gelatin was precipitated by the addition of 60 µl of 10% TCA. After chilling on ice for 10 min, samples were centrifuged at 10,000 × g for 10 min and radioactivity in the supernatant was counted by liquid scintillation counter (Beckman) [35].

Zymogram of protease activity

Polyacrylamide minigels (Bio-Rad) (12 or 10%) were cast containing 0.1% gelatin. Gelatin solution was made up as 2% stock in distilled water and dissolved by heating. Samples (0.1 µg of protein) was applied to the gel in standard SDS loading buffer containing 0.1% SDS but lacking β-mercaptoethanol, it was not boiled before loading [36]. The gels were run in 4°C at 16 mA per gel during stacking and 18 mA per gel during resolving until the dye front reaches the end of the gels. Then soaked the gels in 200 ml of 2.5% (v/v) triton X-100 in distilled water in shaker for 1 h with one change after 30 min at 20°C to remove SDS. Next the gels were soaked in the assay buffer (50 mM Tris, 200 mM NaCl, 10 mM $CaCl_2$, 0.05% Brij 35, pH 7.5) for 12 h at 37°C and then stained with Coomassie brilliant blue R-250 in 50% methanol and 10% acetic acid and this was followed by washing with distilled water for 1 min. The clear zone of lysis against a blue background indicates enzyme activity. The zones of gelatin lysis increased with increasing dose of enzyme-containing samples and time of gel incubation. The marker lane was separated from the gel and then destained with destaining solution containing methanol:acetic acid:water (4:1:5 by vol.) [36].

Western immunoblot

MMP-2 and TIMP-2 were identified in bovine pulmonary artery smooth muscle plasma membrane fraction by Western immunoblot method by using their polyclonal antibodies. Western immunoblot was performed according to Towbin [37] with some modifications. Purified MMP-2 (~1 µg pro-

tein) or complex of MMP-2/TIMP-2 (~2 μg protein) obtained from bovine pulmonary artery smooth muscle plasma membrane fraction were also identified in the same manner by Western immunoblot method by using their polyclonal antibodies whenever it was required.

Determination of pI by chromatofocusing

A column (0.5 × 10 cm) of polybuffer exchanger 94 (PBE 94) was prepared at a flow rate of 12 ml/h. Column was equilibrated (until the pH and conductivity of the eluent match that of the start buffer) with the start buffer which contains 25 mM imidazole-HCl (pH 7.4), 0.05% Brij 35 at the same flow rate. Purified enzyme was equilibrated in start buffer by dialysing with the same buffer with several changes. The eluent buffer, polybuffer 74-HCl (pH 4) containing 0.05% Brij 35 was prepared at a ratio of 1:8 (v/v) in water. Before loading the sample on the column, a 10 ml (5 column vol.) of eluent buffer was passed at a flow rate 5 ml/h. Enzyme (0.5 ml having 0.2 mg of protein) was loaded on the column. Enzyme was eluted with polybuffer 74-HCl (pH 7.4) at a flow rate of 5 ml/h. The pH gradient of the column was established between 7.4–4.0. The protein is eluted according to its isoelectric pH. Fractions of 0.5 ml were collected in different tubes. Their pH was checked, and immediately adjusted to pH 7.5 by the addition of tris base. The absorbances of different eluates were taken at 280 nm [15]. The pH of the tube containing the highest gelatinase activity is determined to be the pI of the 72 kDa gelatinase.

Determination of pH optima

pH optima of the protease was also determined at various pH of the suitable buffers from pH values 4–11 in zymogram. The assay systems with samples were incubated in the appropriate buffer 0.1M sodium acetate buffer (pH 4 and pH 5) containing 0.05% Brij 35; 0.1M Tris-HCl buffer (pH 6, pH 7, pH 8 and pH 9) containing 0.05% Brij 35; 0.1M Glycine-NaOH buffer (pH 10 and pH 11) containing 0.05% Brij 35 for 12 h at 37°C [36, 38].

Determination of optimum concentration of Ca^{2+}

Zymogram assay was performed with various concentrations of Ca^{2+} to identify the optimum amount of calcium requirement for the maximum activity of protease.

Activation of progelatinase studies

These were carried out as described by Murphy *et al.* [39]. Progelatinase incubated with either APMA (2 mM), trypsin (10–100 μg/ml) or plasmin (10–100 μg/ml) was analyzed for activity and molecular mass change. The enzyme was activated at 37°C with a final concentration of 2 mM APMA from a 10X stock solution in 50 mM NaOH in calcium assay buffer (CAB) composed of 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 10 mM $CaCl_2$, and 0.05% Brij 35 at different time course. Control enzyme received an equal amount of NaOH without APMA. In some experiments the 72 kDa progelatinase was incubated with TIMP-2 for 10 min at 22°C before or after activation by APMA. Those studies were done in zymogram and by [^{14}C]-gelatin degradation.

The 72 kDa progelatinase (~0.1 μg protein) was treated with $ONOO^-$(100 μM), H_2O_2 (1 mM), t-butyl hydroperoxide (300 μM) and O_2^- generating system (2 mM HPX + 0.03 unit/ml of XO) for 1 h at 37°C, and the matrix metalloproteinase activity was determined.

Inhibition studies

The inhibitors EGTA (10 mM), 1:10 phenanthroline (10 mM), α_2-macroglobulin (100 μg/ml), TIMP-2 (100 μg/ml), EDTA (20 mM), PMSF (1 mM), Bowman–Birk inhibitor (BBI) (100 μg/ml), chymostatin (100 μg/ml), antipain (100 μg/ml), leupeptin (100 μg/ml), N-ethylmaleimide (5 mM), calpastatin (100 μg/ml) and pepstatin (100 μg/ml) were added to the 2.5% (v/v) triton X-100 soaked gels for 1 h, then incubated with reaction buffer CAB (50 mM Tris, 200 mM NaCl, 10 mM $CaCl_2$ and 0.05% Brij 35 pH 7.5) containing these inhibitors for 12 h at 37°C. These were then stained with Coomassie brilliant blue R-250 followed by washing with distilled water for 1 min as above. The clear zone of lysis against the dark Coomassie background indicates protease activity. Purified 72 kDa gelatinase preparation (~0.1 μg protein) was treated with these inhibitors for 1 h at 37°C, and the matrix metalloprotease activity was determined.

Degradation of synthetic substrates

Gelatinase activity was assayed with the synthetic substrate Dnp-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg-OH (dinitrophenyl labelled peptide) [40]. DNP peptide was dissolved in Tris-NaCl-$CaCl_2$ (50 mM tris-HCl buffer, pH 7.5, containing 150 mM NaCl, 10 mM $CaCl_2$ and 0.05% Brij 35) buffer containing 0.02% bovine serum albumin to make a concentration of 500 μM. The purified protease was activated with APMA (2 mM) at 37°C for various time of incubation. After that the peptide (0.1 ml) was mixed with an equal vol. of purified 72 kDa gelatinase (0.1 μg) and incubated for a period of 2 h at 37°C. After stopping the enzymatic reaction by adding 0.5 ml of 1 M HCl, the DNP peptide fragments released were extracted by vigorous shaking with 1 ml of ethyl acetate followed by centrifugation at 5000 × g at room temperature for 10 min to separate two layers completely. The degree of hydrolysis was determined by measuring the absorbance of the organic layer at 365 nm.

Digestion of extracellular matrix macromolecules

The purified 72 kDa gelatinase (0.1 µg) was activated by 2 mM APMA at 37°C for 15 min and then incubated with the protein substrates (10 µg): collagens (type I, IV and V), gelatins (type I, IV and V) at 33°C (for collagens) or 37°C (for gelatins) for 16 h in Ca^{2+} assay buffer (50 mM Tris-HCl, 200 mM NaCl, 10 mM $CaCl_2$ and 0.05% Brij 35 pH 7.5). Additional experiments included the matrix glycoprotein fibronectin (10 µg) or laminin (10 µg) as the substrate. The reactions were stopped by 20 mM EDTA and products were analyzed by 7.5% SDS/PAGE under reducing conditions [20]. Substrate and cleavage products were visualised by staining with Coomassie blue.

Statistical analysis

Data were analyzed by unpaired *t*-test and analysis of variance followed by the test of least significant difference [41] for comparisons within the groups.

Results

Histological study of bovine pulmonary artery smooth muscle indicates that the tissue has spindle shaped cells which confirms that our studied tissue is enriched with typical smooth muscle cells (Fig. 1).

Fig. 1. Transverse section through the tunica media of bovine pulmonary artery showing the characteristic smooth muscle cells (magnification × 200) (Eosin–hematoxylin preparation).

Characterization of plasma membrane fraction

We characterized the membrane fraction at different steps in the preparation process by measuring the activities of cytochrome C oxidase (a mitochondrial marker [42]), acid phosphatase (a lysosomal marker [43]), rotenone-insensitive NADPH-cytochrome C reductase (a microsomal marker [44]) and 5′ nucleotidase (a plasma membrane marker [43]). Compared with the 600–15000 g pellet and the microsomal fractions, the plasma membrane fraction showed, respectively, 17- and 19-fold increase in the specific activity of 5′ nucleotidase (Table 1). The plasma membrane fraction showed a 37-fold decrease in the specific activity of cytochrome C oxidase compared with 600–15000 g pellet, a 24-fold decrease in the specific activity of acid phosphatase compared with the 600–15000 g pellet and a 29-fold decrease in the specific activity of NADPH-cytochrome C reductase compared with the microsomal fraction (Table 1).

Identification of MMP-2 and TIMP-2 in the plasma membrane fraction

Western immunoblot study of bovine pulmonary artery smooth muscle membrane suspension with polyclonal MMP-2 and TIMP-2 antibodies revealed, respectively, that the fraction possesses MMP-2 and TIMP-2 (Figs 2A and 2B).

Table 1. Specific activities of cytochrome C oxidase, acid phosphatase, rotenone-insensitive NADPH-cytochrome C reductase and 5′-nucleotidase at different steps in the preparation of bovine pulmonary artery smooth muscle plasma membrane

Fraction	Cytochrome C oxidase	Acid phosphatase	Rotenone-insensitive NADPH-cytochrome C reductase	5′-nucleotidase
600–15,000 g pellet	2.98 ± 0.14	3.42 ± 0.21	0.18 ± 0.01	0.12 ± 0.01
15,000–100,000 g pellet	0.26 ± 0.02 (9)	0.34 ± 0.02 (10)	2.75 ± 0.15 (1528)	1.86 ± 0.11 (1550)
Microsomes	0.10 ± 0.01 (3)	0.17 ± 0.01 (5)	3.44 ± 0.19 (1911)	0.11 ± 0.01 (92)
Plasma membrane	0.08 ± 0.01 (3)	0.14 ± 0.01 (4)	0.12 ± 0.01 (67)	2.05 ± 0.11 (1708)

Cytochrome C oxidase activity is expressed as µmole of cytochrome C utilized/30 min/mg protein. Acid phosphatase activity is expressed as µmole of p-nitrophenol/30 min/mg of protein. NADPH cytochrome C reductase (rotenone-insensitive) activity is expressed as reduction of cytochrome C at 550 nm/30 min/mg of protein. 5′-nucleotidase activity is expressed as µmole of P_i/30 min/mg of protein. Results are mean ± S.E. (n = 4). The values in parentheses indicate the activity as percentage of that of the 600–15,000 g pellet (values of 600–15,000 g pellet are set at 100%).

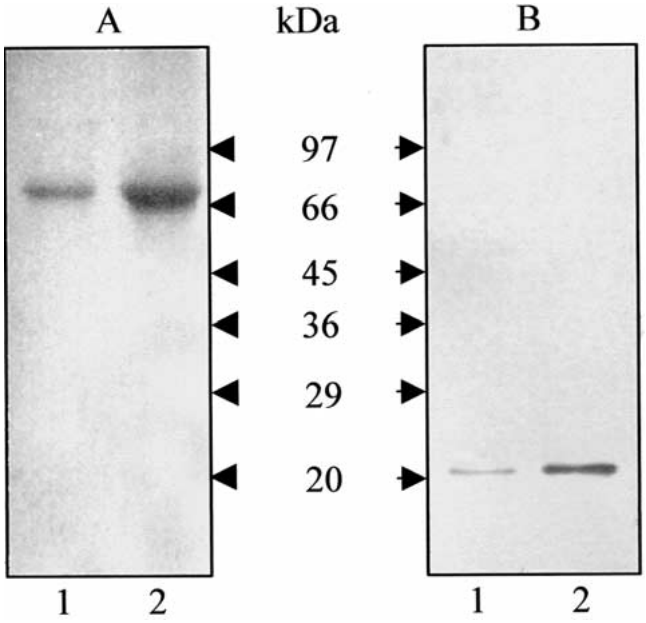

Fig. 2. (A) Identification of matrix metalloproteinase-2 (MMP-2) in bovine pulmonary vascular smooth muscle plasma membrane with polyclonal rabbit anti (bovine MMP-2) IgG by Western immunoblot. Lane 1, bovine pulmonary artery smooth muscle plasma membrane suspension; lane 2, pure bovine MMP-2 (obtained from Chemicon International, CA, USA). (B) Identification of tissue inhibitor of metalloproteinase-2 (TIMP-2) in bovine pulmonary vascular smooth muscle plasma membrane with polyclonal rabbit anti (bovine TIMP-2) IgG by Western immunoblot. Lane 1, bovine pulmonary artery smooth muscle plasma membrane suspension; lane 2, pure bovine TIMP-2 (obtained from Chemicon International, CA, USA).

Purification of the 72 kDa gelatinase

In each step of purification the protein content of the samples was measured. To determine the extent of purification in each step, the samples were electrophoresed on SDS-PAGE in non-reducing conditions and assayed by gelatin zymography and also by [^{14}C]-gelatin degradation.

The results of purification of proMMP-2 as assessed by [^{14}C]-gelatin degradation are summarized in Table 2. Figure 3 (A, B, C) illustrates the results of these analyses. The purity of fractions was ascertained by silver stained SDS polyacrylamide gels and gelatin substrate gels carried out under non-reducing conditions.

DEAE cellulose chromatography was employed before the gelatin-sepharose affinity chromatography in order to remove a significant amount of contaminants. The unretained proteins of the DEAE cellulose (Figs 3A and 3B, lane 3) column were collected and loaded to a gelatin-sepharose column. The column was washed extensively and then eluted with buffer containing 1% and 5% step gradient of DMSO. The 5% DMSO eluate was analyzed for protein (silver stained SDS gels) (Fig. 3A, lane 6) and gelatinase activity (gelatin zymogram) (Fig. 3B, lane 6). The bulk of proteins in the DEAE cellulose eluate does not bind to the gelatin-sepharose and are found in the unretained fraction (Fig. 3A, lane 4; Fig. 3B, lane 4). The 5% DMSO elutes a 72 kDa protein as the major component along with other contaminants (Fig. 3A, lane 6; Fig. 3B, lane 6). Gelatin substrate gel analysis indicates that the gelatin-sepharose column specifically binds gelatinases since little or no enzyme activity was detected in the unretained protein and the washed fractions (Fig. 3B, lane 4). The 92 kDa and 72 kDa gelatinase activities bound to the gelatin sepharose column (Fig. 3B, lane 4) are selectively eluted from the column by 1% and 5% of DMSO (Figs 3A and 3B, lanes 5, 6). The 5% DMSO eluates from the gelatin-sepharose column which preferentially enriched with 72 kDa gelatinase (Figs 3A and 3B, lane 6) were pooled, concentrated and passed over a lentil lectin-sepharose column to remove traces of 92 kDa progelatinase completely. The unretained protein from the lentil lectin-sepharose column which contained 72 kDa gelatinase and also 72 kDa gelatinase-TIMP-2 complex was collected, concentrated and analyzed by SDS-PAGE (Fig. 3A, lane 7). It was evident from Figs 3A and 3B (lane 7) that the higher component molecular weight protein i.e. the 92 kDa gelatinase was disappeared completely. The unretained protein fraction from lentil lectin-sepharose column was concentrated and passed over a heparin-sepharose column. This column elutes two fractions at two different concentrations of NaCl. The 72 kDa progelatinase-TIMP-2 complex and free 72 kDa progelatinase were separated by 100 mM NaCl (peak 1) and 200 mM NaCl (peak 2), from the heparin-sepharose column (Fig. 3C) respectively. The resulting two fractions obtained by NaCl step gradient from the heparin-sepharose

Table 2. Purification of 72 kDa gelatinase from bovine pulmonary artery smooth muscle plasma membrane

Step	Total protein (mg)	Total activity (units*)	Specific activity (units/mg)	Fold purification	Recovery %
Plasma membrane suspension	1142	2754	2.4	–	100
0–70% $(NH_4)_2SO_4$ precipitate	441	2421	5.5	2	88
DEAE cellulose flow through	64	2118	33.1	14	77
Gelatin sepharose eluate (5% DMSO)	2.1	1101	524.3	218	40
Lentil-lectin sepharose flow through	1.3	794	610.8	255	29
Heparin sepharose eluate (200 mM NaCl)	0.4	418	1045.0	435	15

*Unit = 1 μg gelatin degraded per min at 37°C.

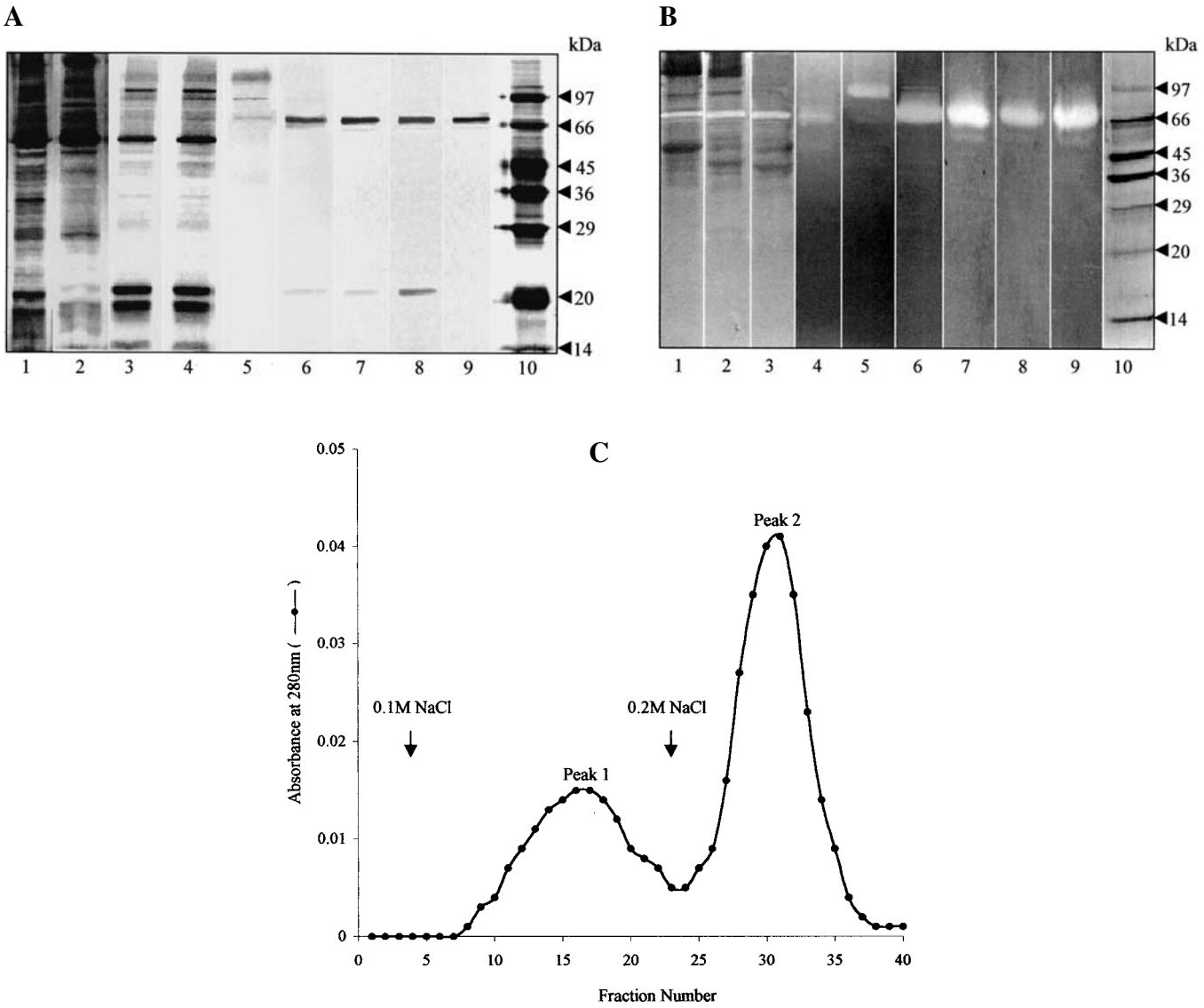

Fig. 3. (A) 10% SDS-PAGE of purification profile of the 72 kDa matrix metalloproteinase-2 from bovine pulmonary artery smooth muscle plasma membrane fraction. Lane 1, solubilized plasma membrane fraction; lane 2, 0–70% ammonium sulfate precipitation; lane 3, DEAE cellulose chromatography unretained fraction; lane 4, non-stick gelatin sepharose eluate; lane 5, 1% DMSO eluate from the gelatin-sepharose column; lane 6, 5% DMSO eluate from the gelatin-sepharose column; lane 7, unretained protein from the lentil lectin-sepharose column; lane 8, 100 mM NaCl heparin-sepharose eluate; lane 9, 200 mM NaCl heparin-sepharose eluate; lane 10, molecular weight standards. (B) 12% zymogram of purification profile of the 72 kDa matrix metalloproteinase-2 from bovine pulmonary artery smooth muscle plasma membrane fraction. Lane 1, solubilized plasma membrane fraction; lane 2, 0–70% ammonium sulfate precipitation; lane 3, DEAE cellulose chromatography unretained fraction; lane 4, non-stick gelatin sepharose eluate; lane 5, 1% DMSO eluate from the gelatin-sepharose column; lane 6, 5% DMSO eluate from the gelatin-sepharose column; lane 7, unretained protein from the lentil lectin-sepharose column; lane 8, 100 mM NaCl heparin-sepharose eluate; lane 9, 200 mM NaCl heparin-sepharose eluate; lane 10, molecular weight standards. (C) Elution profile and the separation of 72 kDa progelatinase and the 72 kDa progelatinase-TIMP-2 complex on heparin-sepharose chromatography column. The complex was eluted (3 ml each fraction) with 100 mM NaCl in buffer A (peak 1). Free latent gelatinase was eluted (3 ml each fraction) with 200 mM NaCl in buffer A (peak 2).

column were concentrated, analysed for protein contents and assayed for gelatinolytic activity on gelatin substrate gels (Fig. 3B, lanes 8, 9). The 200 mM NaCl eluate yields a distinct zone of lysis at 72 kDa in gelatin substrate gels (Fig. 3B, lane 9). Silver stained SDS-PAGE (Fig. 3A, lane 9) shows a corresponding distinct protein band migrating at 72 kDa. The overall yield of proMMP-2 was 15% with 435 fold of purification after heparin-sepharose chromatography (Table 2).

Confirmation of free MMP-2 and MMP-2/TIMP-2 complex in the heparin sepharose purified preparations

Western immunoblot study of peak 1, 100 mM NaCl eluate of heparin sepharose purified preparation with the mixture of polyclonal antibodies of MMP-2 and TIMP-2 revealed that the peak 1 eluate contains the MMP-2/TIMP-2 complex (Fig. 4A). And also Western immunoblot study of peak 2, 200 mM NaCl eluate of heparin sepharose purified preparation with the polyclonal antibody of MMP-2 revealed that the peak 2 eluate contains free MMP-2 (Fig. 4B).

Analysis of 72 kDa gelatinase and the 72 kDa gelatinase-TIMP-2 complex by native PAGE

To further ascertain clearly the purity of the 72 kDa gelatinase and the 72 kDa gelatinase-TIMP2 complex in the eluate from heparin-sepharose chromatography, electrophoresis by native-PAGE was carried out (Fig. 5). The unretained lentil lectin-sepharose pool exhibits two distinct protein bands corresponding to two species having approximate molecular mass of 90–95 kDa and 72 kDa that were eluted in peaks 1 and 2 (Fig 5, lane 1). Figure 5 illustrates that peak 1 from the heparin-sepharose column that migrates as a single band (lane 2) while peak 2 also migrates as single band but at a faster electrophoretic rate (lane 3). The native gel profile, therefore, indicates that the plasma membrane fraction contains a mixed population of free 72 kDa progelatinase and 72 kDa progelatinase/TIMP-2 complex.

pI of 72 kDa progelatinase

Chromatofocusing study of the purified 72 kDa progelatinase revealed that the pI of the enzyme is 6.2–6.4 (Fig. 6, lanes 3 and 4). This indicates that like other MMPs [15, 19–23], the 72 kDa progelatinase is a neutral protease.

Optimum pH for maximum activity

The enzyme shows significant activity from pH 6 to 9 with maximum activity at pH 8.0 (Fig. 7, lane 5).

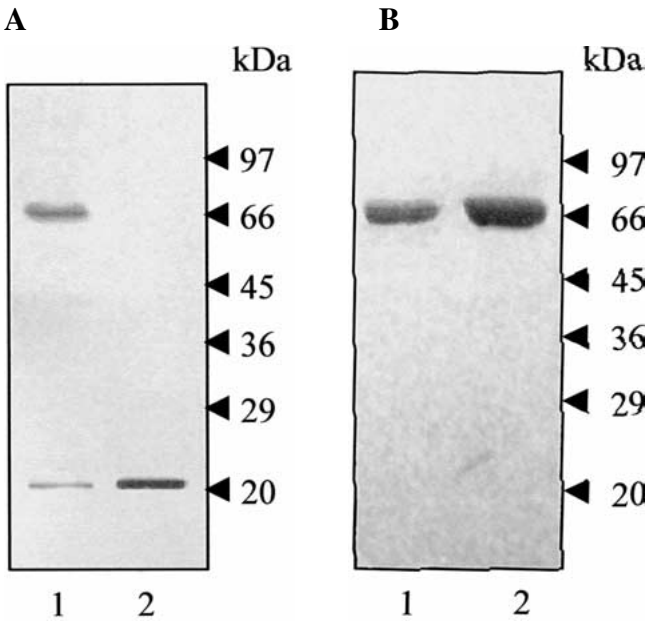

Fig. 4. (A) Identification of heparin sepharose purified complex of matrix metalloproteinase-2 (MMP-2) and tissue inhibitor of metalloprotease-2 (TIMP-2) (100 mM NaCl, peak 1) with a mixture of two different polyclonal antibodies to the MMP-2 [rabbit anti (bovine MMP-2) IgG] and the TIMP-2 [rabbit anti (bovine TIMP-2) IgG] by Western immunoblot. Lane 1, heparin sepharose purified complex of matrix metalloprotease-2 (MMP-2) and tissue inhibitor of metalloprotease-2 (TIMP-2) from bovine pulmonary artery smooth muscle plasma membrane suspension; lane 2, pure bovine TIMP-2 (obtained from Chemicon International, CA, USA). (B) Identification of heparin sepharose purified matrix metalloprotease-2 (MMP-2) (200 mM NaCl, peak 2) with polyclonal rabbit anti (bovine MMP-2) IgG by Western immunoblot. Lane 1, heparin sepharose purified (200 mM NaCl eluate) matrix metalloprotease-2 (MMP-2); lane 2, pure bovine MMP-2 (obtained from Chemicon International, CA, USA).

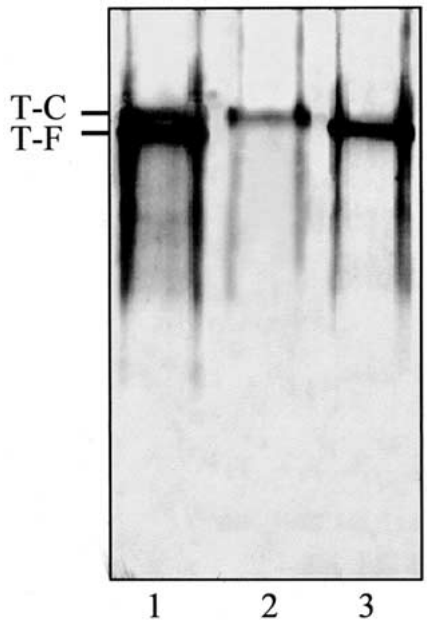

Fig. 5. Analysis of the TIMP-2-free 72 kDa progelatinase (T-F) and the TIMP-2-complex 72 kDa progelatinase (T-C) by native PAGE. Lane 1, lentil lectin-sepharose pool; lane 2, 100 mM NaCl eluate of heparin-sepharose (peak 1); lane 3, 200 mM NaCl eluate of heparin sepharose (peak 2).

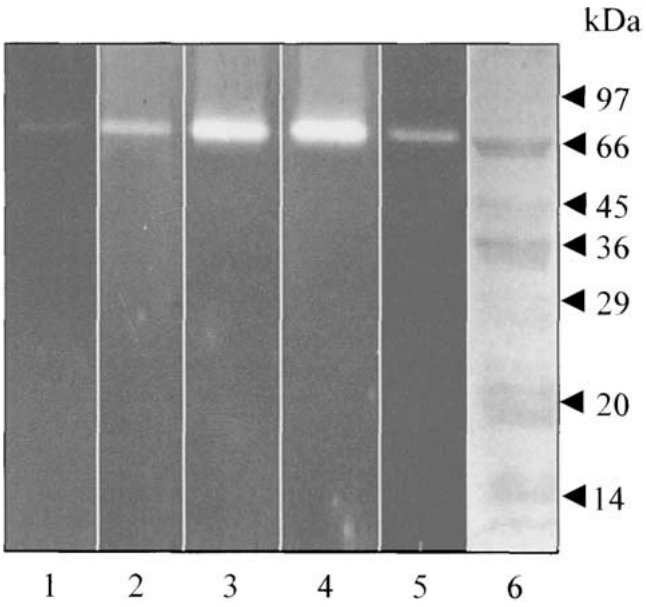

Fig. 6. 12% zymogram of the elution profile of 72 kDa progelatinase, eluted according to isoelectric pH from chromatofocusing column by polybuffer 74-HCl (pH 4). Highest activity obtained in lane 3, pH 6.2 and in lane 4, pH 6.4. The pI of the enzyme is 6.2–6.4. Lane 6, molecular weights standards.

Optimum concentration of Ca^{2+} for maximum activity of progelatinase

Since the MMP-2 requires Ca^{2+} for its activity [8, 9, 20], a dose-response profile for Ca^{2+} in this regard was determined. This was tested using gelatin zymography. The optimum concentration of Ca^{2+} for highest activity of MMP-2 was determined to be 10 mM (Fig. 8, lane 5).

Activation

The purified 72 kDa progelatinase exists mainly as an inactive zymogen. It exhibits little or no gelatinolytic activity in solution unless it is activated by organomercurial (APMA) treatment or is electrophoresed in gelatin substrate gels. Figures 9A and 9B demonstrates that the isolated 72 kDa progelatinase is unable to degrade [^{14}C]-gelatin and the synthetic substrate in solution unless it is activated by APMA. The time course of activation of the 72 kDa progelatinase was tested with both the [^{14}C]-gelatin degradation and the degradation of synthetic substrate. The 72 kDa proenzyme activation was found to be optimum at 15 min, after that the activity decreases with time (Figs 9A and 9B). The APMA activated enzyme is inhibited by 1,10-phenanthroline (10 mM), EDTA (20 mM), EGTA (10 mM), TIMP-1 and TIMP-2 (100 µg/ml) (Table 3).

Incubation of the purified 72 kDa gelatinase preparation with 2 mM APMA at 37°C for different time results partial conversion of the enzyme to a major species of 62 kDa and a minor species of 45 kDa in SDS-PAGE (Fig. 10A). Corresponding substrate gel indicates that the 62 kDa and 45 kDa species are enzymatically active yielding enhanced zones of gelatinolysis in the 62 kDa and 45 kDa position (Fig. 10B). Western blot of the fully activated purified gelatinase after 15 min of exposure to APMA was developed with polyclo-

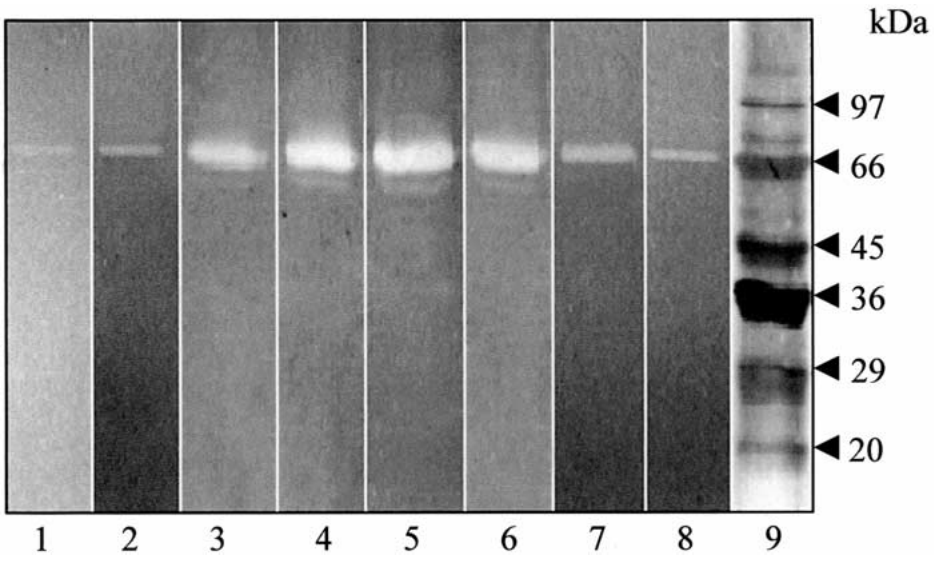

Fig. 7. 10% zymogram profile of the purified 72 kDa progelatinase as a function of pH for the determination of optimum pH for maximum activity. The buffers were formulated and zymogram was incubated as described by materials methods. Lane 1, pH 4 (0.1 M sodium acetate buffer); lane 2, pH 5 (0.1 M sodium acetate buffer); lane 3, pH 6 (0.1 M Tris-HCl buffer); lane 4, pH 7 (0.1 M Tris-HCl buffer); lane 5, pH 8 (0.1 M Tris-HCl buffer); lane 6, pH 9 (0.1 M Tris-HCl buffer); lane 7, pH 10 (0.1 M glycine-NaOH buffer); lane 8, pH 11 (0.1 M glycine-NaOH buffer); lane 9, molecular weight standards.

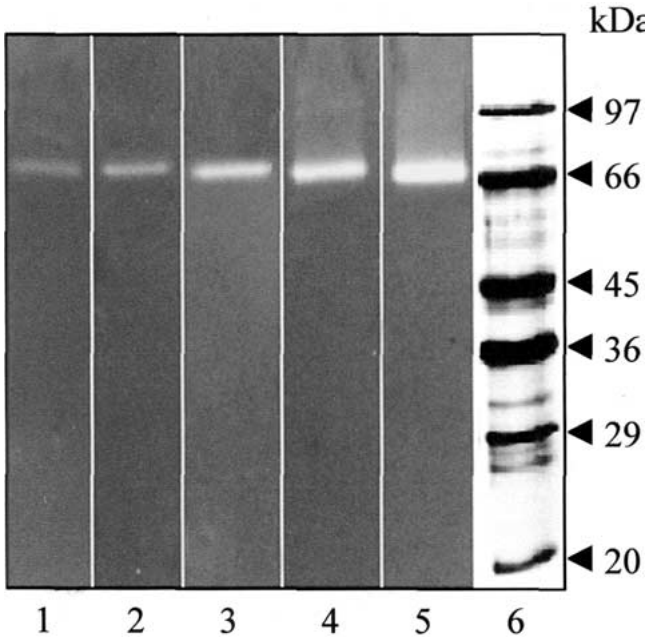

Fig. 8. 10% zymogram profile of the purified 72 kDa progelatinase as a function of Ca^{2+} concentration for the determination of optimum Ca^{2+} ion concentration for maximum gelatinase activity. After triton X-100 wash, the gel slices were soaked in the assay buffer (50 mM Tris, 200 mM NaCl, 0.05% Brij 35, pH 7.5) containing different concentration of $CaCl_2$ ranging from 6 mM to 10 mM for 12 h at 37°C and then stained with Coomassie Brilliant blue. The clear zone of lysis against a blue background indicates enzyme activity. Lanes 1–5 incubated in the assay buffer with $CaCl_2$ concentration as 6, 7, 8, 9 and 10 mM respectively. Lane 6, molecular weight standards.

nal MMP-2 antibody (Fig. 11) showed a complete conversion of the free 72 kDa progelatinase, yielded two different immunoreactive distinct species at 62 and 45 kDa respectively providing immunological evidence that the two species (62 and 45 kDa species) are derived from the 72 kDa species (Fig. 11) as described by SDS-PAGE (Fig. 10A). Activation studies showed that maximal activity of the purified 72 kDa gelatinase against [^{14}C]-gelatin and synthetic substrate was achieved after 15 min of treatment to APMA, after which time the enzyme activity decreased (Figs 9A and 9B). Zymogram showed that the 45 kDa species was observed after 5 min of exposure to APMA (Fig. 10B). EDTA and TIMP-2 (Fig. 12) blocked the APMA mediated conversion. This indicates that the conversions may be due to an autolytic cleavage of the purified 72 kDa gelatinase following APMA treatment [12, 20].

Trypsin treatment of the purified 72 kDa gelatinase caused a very minor conversion to 62 kDa active species (Fig. 12). In a series of experiments employing different trypsin concentrations and a variety of incubation conditions, only 5–20% of 72 kDa zymogen was activated. Purified plasmin under a variety of conditions was unable to convert or activate the 72 kDa zymogen (results not shown).

$ONOO^-$(100 µM), H_2O_2 (1 mM), t-butyl hydroperoxide (300 µM) and $O_2^{\cdot-}$generating system (2 mM HPX + 0.03 unit/ml of XO) treatment of the purified 72 kDa gelatinase caused appreciable activation and stimulated gelatin degradation (Table 4).

Inhibition studies

Inhibition of the purified 72 kDa zymogen by TIMP-1 and TIMP-2

To test the efficacy of TIMP-1 and TIMP-2 to inhibit the purified 72 kDa gelatinase activities, equal amounts of the inhibitors were tested separately with the purified enzyme for [^{14}C]-gelatin degradation study (Tables 3 and 5). As shown in Table 5, 100% of inhibition of the enzyme activity was observed when 100 µg/ml of TIMP-2 was used, while the same concentration of TIMP-1 elicited very less inhibition of the enzyme activity of the 72 kDa zymogen.

Inhibition by other protease inhibitors

100% inhibition of gelatinase activity on gelatin has been observed using α_2-macroglobulin (100 µg/ml), 1,10-phen-

Table 3. Activation of 72 kDa gelatinase by APMA and inhibition by TIMP-1, TIMP-2 and the metal ion chelators

Fraction	Pre-treatment	Inhibitor	[^{14}C]-Gelatin degradation	% inhibition
72 kDa preparation	none	none	43 ± 4	–
72 kDa preparation	APMA	none	1548 ± 51[a]	–
72 kDa preparation	APMA	1,10-phenanthroline	97 ± 7[a,b]	94
72 kDa preparation	APMA	EDTA	81 ± 5[a,b]	95
72 kDa preparation	APMA	EGTA	83 ± 6[a,b]	95
72 kDa preparation	APMA	TIMP-2	67 ± 3[a,b]	96
72 kDa preparation	APMA	TIMP-1	156 ± 10[a,b]	90

0.1 µg of samples of purified 72 kDa gelatinase were pretreated as indicated with 2 mM APMA for 15 min at 37°C. The pretreated or control samples were then added into a [^{14}C]-gelatin degradation assay as described in Materials and methods in the absence or presence of 10 mM 1,10-phenanthroline, 20 mM EDTA, 10 mM EGTA, 100 µg/ml of TIMP-1 and TIMP-2 each as indicated. Incubation was carried out at 37°C for 1 h and the radioactivity was measured. The gelatinase activity is expressed as [^{14}C]-gelatin degradation by the protease (cpm/mg protein/30 min). Results are mean ± S.E. (n = 4). [a]$p < 0.001$ compared with basal condition; [b]$p < 0.001$ compared with the APMA treated condition.

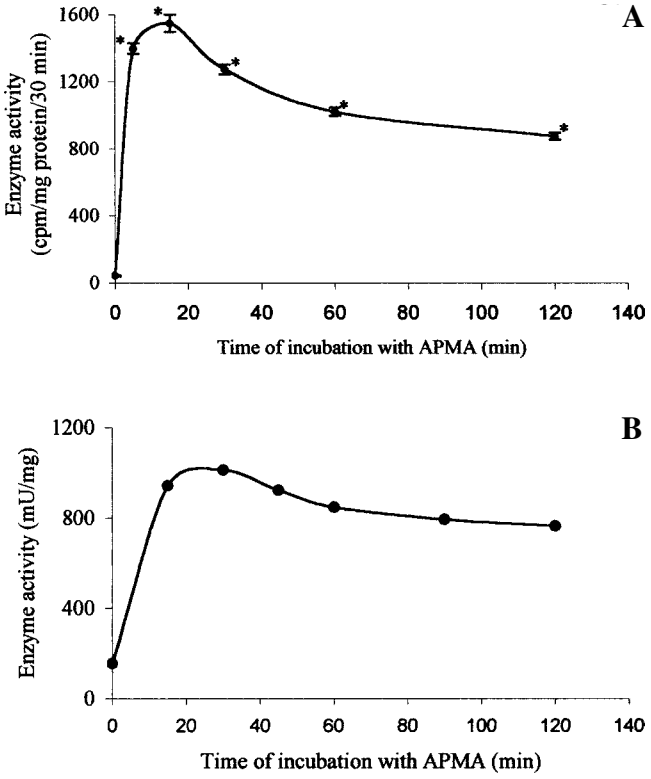

Fig. 9. (A) Time course of activation of the purified 72 kDa progelatinase (MMP-2) (0.1 µg) with 2 mM APMA. Samples were activated with 2 mM APMA at 37°C for the indicated times and incubated with [^{14}C]-gelatin for 1 h at 37°C and the count was taken. MMP-2 activity is expressed as [^{14}C]-gelatin degradation by the protease (cpm/mg protein/30 min). Results are mean ± S.E. (n = 4). *$p < 0.001$ compared with basal condition. (B) Degradation of a dinitrophenyl labelled synthetic substrate by purified 72 kDa progelatinase (0.1 µg) and time course of activation of 72 kDa progelatinase with APMA by using the synthetic substrate. Samples were activated with 2 mM APMA at 37°C for the indicated times and incubated with the dinitrophenyl labelled peptide for 2 h at 37°C. The degree of hydrolysis was determined by measuring the absorbance of the organic layer at 365 nm as (details are in the text). 1U gelatinase catalyses the hydrolysis of 1 µmol dinitrophenyl-labelled peptide/min at 37°C.

anthroline (10 mM), EGTA (10 mM) and EDTA (20 mM) as the inhibitors in [^{14}C]-gelatin degradation assay systems (Table 6). Serine, chymotrypsin, cysteine, thiol, calpain and aspartic protease inhibitors such as PMSF (1 mM), Bowman–Birk inhibitor (BBI) (100 µg/ml), chymostatin (100 µg/ml), antipain (100 µg/ml), leupeptin (100 µg/ml), N-ethylmaleimide (5 mM), calpastatin (100 µg/ml) and pepstatin (100 µg/ml) had no discernible inhibitory effect towards the purified 72 kDa gelatinase (data not shown).

Digestion of extracellular matrix macromolecules

The substrate specificity of the active 72 kDa gelatinase was examined by employing a number of purified extracellular matrix proteins as potential substrates for the enzyme (Fig. 13). Native type I collagen is resistant to digestion by active gelatinase (lanes 1 and 2), whereas thermally denatured type I collagen (or gelatin I) is rapidly and completely digested by the purified enzyme (lanes 3 and 4). Native type IV collagen is digested by the bovine gelatinase (lanes 5 and 6), as type IV gelatin was hydrolyzed by the enzyme (lanes 7 and 8). Native type V collagen was digested to lesser extent (lanes 9 and 10), whereas type V gelatin was hydrolyzed completely by the enzyme (lanes 11, 12). The purified gelatinase was unable to degrade laminin and fibronectin (data not shown). Our results are in accordance with the observation of previous researchers [20]. Based on these studies, the 72 kDa MMP appears to be identical to previously characterized MMP-2.

Degradation of synthetic substrates

Degradation of the synthetic peptide substrate Dnp-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg-OH by the APMA activated purified 72 kDa gelatinase is presented in Fig. 9B as a function of time. The degradation of the synthetic substrate was found to be optimum at 15 min, and then decreases with increase in incubation time.

Discussion

In this present study a series of chromatographic procedures have been employed to purify the 72 kDa matrix metalloproteinase (gelatinase A; MMP-2) from the plasma membrane of bovine pulmonary vascular smooth muscle tissue. In this regard, we have undertaken ammonium sulfate precipitation of the solubilized membrane fraction followed by (i) DEAE-cellulose chromatography; (ii) gelatin-Sepharose chromatography; (iii) lentil lectin-Sepharose chromatography; and (iv) heparin-Sepharose chromatography. At the outset, we obtained free MMP-2 and MMP-2/TIMP-2 complex. The free enzyme activity was completely inhibited by EDTA, EGTA and 1:10-phenanthroline (Tables 3 and 6). α_2-macroglobulin, TIMP-1 and TIMP-2 also showed inhibition towards the free enzyme activity (Table 3, 5 and 6). TIMP-2 was found to be more effective than TIMP-1 for inhibiting the purified free MMP-2 activity (Table 5).

In the present study, two lines of evidence suggest that there exist a complex of MMP-2 and TIMP-2. These are (i) native PAGE of the peak 1 (100 mM NaCl eluate from heparin-sepharose column) showed a band in the 92–95 kDa region which indicates that the peak contains a complex consisting of MMP-2 and TIMP-2 (Fig. 5); (ii) separation of this complex in SDS-PAGE yields MMP-2 and TIMP-2 as revealed by Western immunoblot assay of the complex with

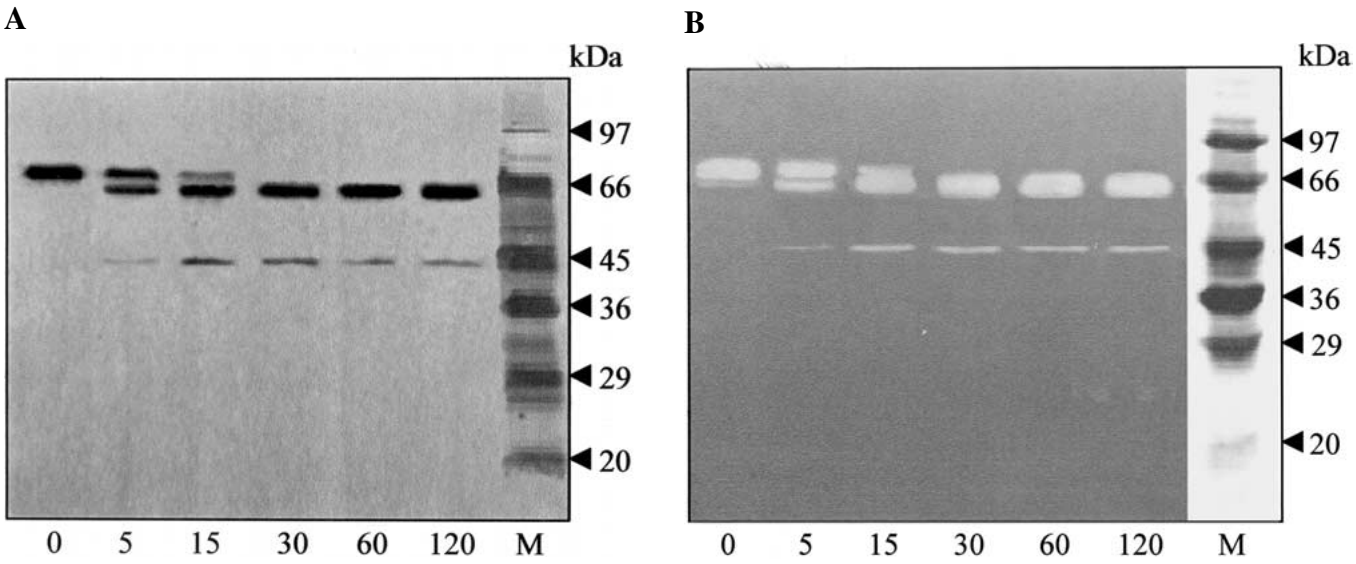

Fig. 10. (A) Time course of activation of the purified 72 kDa progelatinase (0.1 μg protein) with APMA in 10% SDS-PAGE. The different time courses (0–120 min) of incubation with APMA were indicated below the lanes in min. Lane M, molecular weights standards. (B) Time course of activation of the purified 72 kDa progelatinase (0.1 μg protein) with APMA in 10% gelatin containing zymogram. The different time courses (0–120 min) of incubation with APMA were indicated below the lanes in min. Lane M, Molecular weights standards.

a mixture of polyclonal MMP-2 and TIMP-2 antibodies (Fig. 4A). Previous research have also indicated that the 72 kDa MMP-2 progelatinase was secreted as a complex with TIMP-2 in simian virus 40 transformed human lung fibroblasts, harvey murine sarcoma virus transformed human bronchial epithelial cells, human A2058 melanoma cells, human rheumatoid synovial cells and skin fibroblasts [6, 19, 22, 23, 45]. One of the major functions of TIMP-2 was suggested to stabilize the otherwise quiescent form of proMMP-2 [6, 19, 22, 23, 45].

Although the smooth muscle plasma membrane contains TIMP-1 [23, 46], this inhibitor is unable to form a complex with the free 72 kDa progelatinase (MMP-2) (since no MMP-2/TIMP-1 complex has been detected after gelatin-sepharose chromatography) (Fig 3A and 3B, lanes 6, 7 and 8), supporting the observation that TIMP-2 specifically binds to the latent 72 kDa gelatinase.

The activity of the 72 kDa progelatinase is regulated by TIMP-2, a 21 kDa molecule. Like TIMP-1, TIMP-2 inhibits the activity of the activated MMPs [47]. However, in comparison to TIMP-1, TIMP-2 is more effective and specific to inhibit the purified MMP-2 (Fig. 12, lane 4 and Tables 3, 5 and 6).

One of the major characteristics of the 72 kDa gelatinase is that it produces inactive zymogens which can be partially or fully activated *in vitro* with organomercurial compounds

Table 4. Activation of the purified 72 kDa gelatinase by oxidants and $O_2^{\cdot-}$ generating system (2 mM HPX + 0.03 unit/ml of XO)

Treatment	Matrix metalloproteinase activity	% change vs. control
Basal	43 ± 4	–
$ONOO^-$ (100 μM)	301 ± 11[a]	+600
H_2O_2 (1 mM)	306 ± 12[a]	+612
t-buOOH (300 μM)	344 ± 14[a]	+700
$O_2^{\cdot-}$ generating system (2 mM HPX + 0.03 unit/ml of XO)	546 ± 16[a]	+1170

0.1 μg samples of purified 72 kDa progelatinase was treated with $ONOO^-$ (100 μM), H_2O_2 (1 mM), t-buOOH (300 μM) and $O_2^{\cdot-}$ generating system (2 mM HPX + 0.03 unit/ml of XO) for 1 h at 37°C, and the matrix metalloproteinase activity was determined. The matrix metalloproteinase (gelatinase) activity is expressed as [^{14}C]-gelatin degradation by the protease (cpm/mg protein/30 min). Results are mean ± S.E. (n = 4). [a]$p < 0.001$ compared with basal value.

Table 5. Inhibition of 72 kDa progelatinase by normal physiological inhibitor TIMP-1 and TIMP-2

Fraction	Inhibitor	Gelatin degradation	% inhibition
72 kDa Zymogen	none	43 ± 4	–
72 kDa Zymogen	TIMP-2	N.D.[a]	100
72 kDa Zymogen	TIMP-1	38 ± 3	12

0.1 μg samples of purified 72 kDa progelatinase were pretreated as indicated with 100 μg/ml of TIMP-1 and TIMP-2 for 1 h. The pretreated or control samples were then added into a [^{14}C]-gelatin degradation assay as described in Materials and methods. Incubation was carried out at 37°C for 1 h and the radioactivity was measured. The gelatinase activity is expressed as [^{14}C]-gelatin degradation by the protease (cpm/mg protein/30 min). Results are mean ± S.E. (n = 4). [a]$p < 0.001$ compared with basal value. (N.D., not detected)

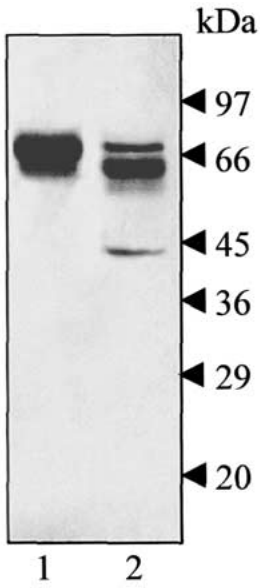

Fig. 11. Identification of 62 and 45 kDa smaller species of APMA activated 72 kDa purified MMP-2 with polyclonal rabbit anti (bovine MMP-2) IgG by Western immunoblot. Lane 1, control [bovine matrix metalloprotease-2 (MMP-2) purified by heparin sepharose chromatography (200 mM NaCl)]; lane 2, APMA activated heparin sepharose purified (200 mM NaCl) matrix metalloprotease-2 (MMP-2).

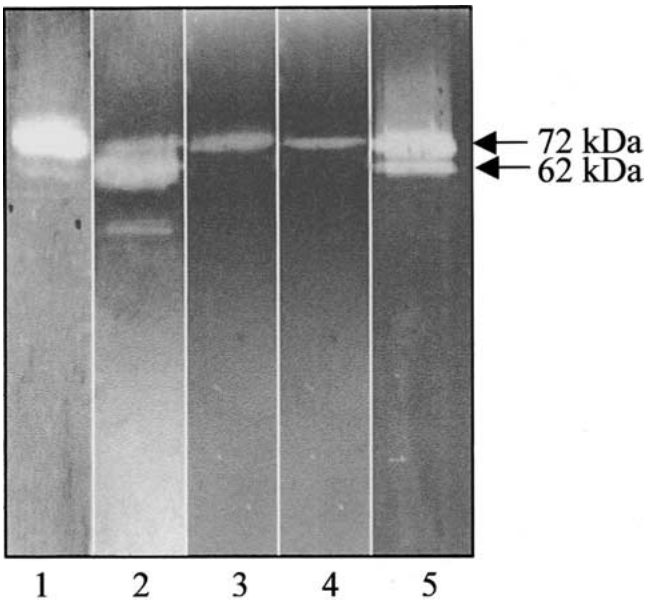

Fig. 12. 10% zymogram profile of the blocking of APMA mediated activation of 72 kDa gelatinase by EDTA and TIMP-2, and activation of 72 kDa gelatinase by trypsin treatment. Purified preparation of 72 kDa gelatinase (2.5 μg protein) was incubated alone at 37°C for 15 min in 0.1 ml of 50 mM Tris, 200 mM NaCl, 10 mM $CaCl_2$, 0.05% Brij 35, pH 7.5 buffer (lane 1); in buffer containing 2 mM APMA (lane 2); in buffer containing 2 mM APMA plus 20 mM EDTA (lane 3); in buffer containing 2 mM APMA plus 100 μg/ml TIMP-2 (lane 4); in buffer containing purified 50 μg purified trypsin for 30 min at 37°C followed by addition of 200 μg soybean trypsin inhibitor (lane 5); The incubations were terminated by the addition of SDS sample buffer and the sample analysed by gelatin substrate zymography.

such as APMA by removing the NH_2 terminal propeptides [14, 20, 22]. The purified free 72 kDa progelatinase is quickly activated by treatment with APMA (Figs 9A, 9B, 10A, 10B, 11 and 12) and partially degraded by trypsin (Fig. 12). During APMA activation, the purified MMP-2 autocatalytically produces active species having molecular mass of 62 kDa and 45 kDa (Figs 11 and 12), which are found to be inhibited by EDTA and TIMP-2 (Fig. 12, lanes 3 and 4).

Table 6. Effect of general protease inhibitors on APMA activated 72 kDa progelatinase

Treatment	[^{14}C]-gelatin degradation	% inhibition
APMA treated 72 kDa gelatinase	1548 ± 51	–
+ EGTA (10 mM)	83 ± 6[a]	95
+ 1,10-phenanthroline (10 mM)	97 ± 7[a]	94
+ EDTA (20 mM)	81 ± 5[a]	95
+ α_2-macroglobulin (100 μg/ml)	75 ± 3[a]	95
+ TIMP-2 (100 μg/ml)	67 ± 3[a]	96

0.1 μg of samples of purified 72 kDa gelatinase were pretreated as indicated with 2 mM APMA for 15 min at 37°C. The pretreated samples were then added into a [^{14}C]-gelatin degradation assay as described in the text (Materials and methods) in the absence or presence of different protease inhibitors. Incubation was carried out at 37°C for 1 h and the radioactivity was measured. The gelatinase activity is expressed as [^{14}C]-gelatin degradation by the protease (cpm/mg protein/30 min). Results are mean ± S.E. (n = 4).
[a]$p < 0.001$ compared with basal value.

It appears conceivable that in *in vivo* conditions, tissue degradation could occur by the 72 kDa gelatinase. The ability of the purified MMP-2 to degrade native and denatured type IV collagen (gelatin) (Fig. 13, lanes 5, 6, 7 and 8) implies its role in basement membrane degradation that could occur in many normal and pathological processes such as aneurysm, angiogenesis, macrophage infiltration and tumor cell invasion [1, 4, 5, 7, 8, 20].

Several lines of evidence suggest that the purified 72 kDa gelatinase is the MMP-2. First, the enzyme is inhibited by the general matrix metalloproteinase inhibitor α_2-macroglobulin [45] and also by 1:10-phenanthroline, EGTA and EDTA, chelators for Zn^{2+} and Ca^{2+}, respectively (Tables 3 and 6). Second, the purified MMP-2 has the ability to bind to gelatin-sepharose (Figs 3A and 3B). Third, serine, chymotrypsin, cysteine, thiol, calpain and aspartic protease inhibitors such as PMSF, Bowman–Birk inhibitor (BBI), chymostatin, antipain, leupeptin, N-ethylmaleimide, calpastatin and pepstatin were ineffective in preventing the enzyme activity in the assay systems. Fourth, the enzyme is activated by oxidants such as hydrogen peroxide, peroxynitrite and t-butyl hydroperoxide, and O_2^-generating system (2 mM HPX + 0.03 unit/ml of XO)

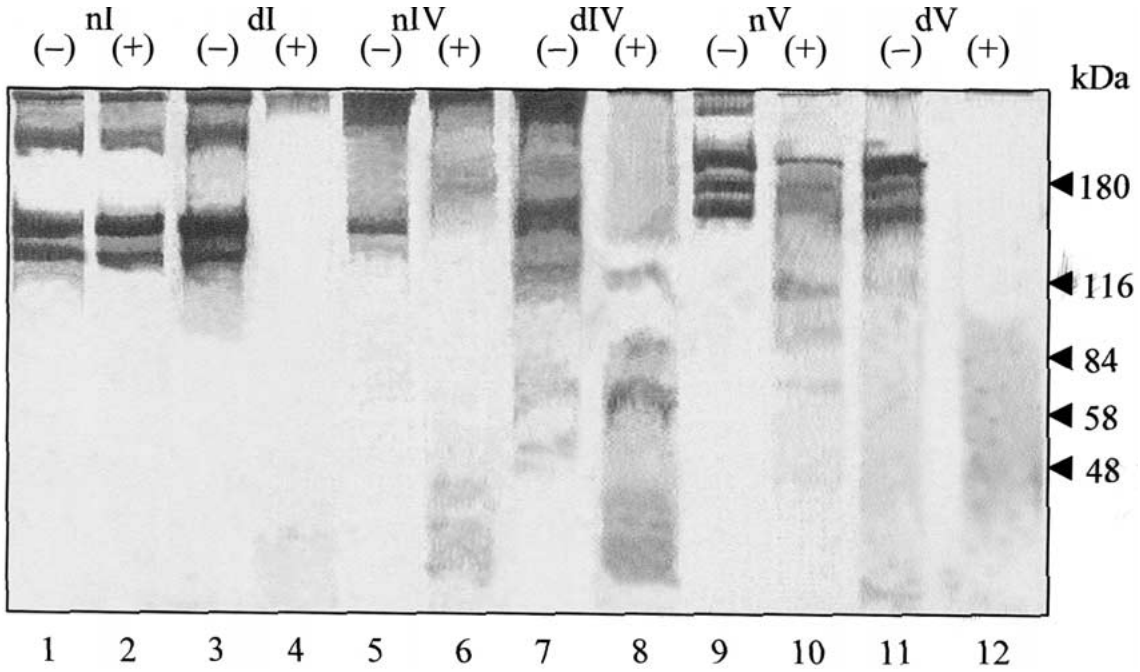

Fig. 13. 7.5% SDS-PAGE for the determination of substrate cleavage specificity of the activated purified bovine 72 kDa gelatinase. Substrate degradation assays were carried out by incubating various extracellular proteins (10 μg): collagens (type I, IV and V), gelatins (type I, IV and V) at 33°C (collagens) or 37°C (gelatins) for 16 h in Ca^{2+} assay buffer (50 mM Tris-HCl, 200 mM NaCl, 10 mM $CaCl_2$ and 0.05% Brij 35 pH 7.5, in the absence (–) or presence (+) of 0.1 μg of preactivated 72 kDa purified gelatinase (enzymes to substrate ratio 1:100, w/w). Preactivation was carried out by 2 mM APMA at 37°C for 15 min. The collagen substrates employed were native (n) and thermally denatured (d) (100°C for 5 min) or gelatin. Following incubation the reactions were stopped by 20 mM EDTA and products analyzed by 7.5% SDS/PAGE under reducing conditions. Substrate and cleavage products were visualised by staining with Coomassie blue. Lanes 1 and 2, native type I collagen in the absence (–) and presence (+) of purified MMP-2 respectively; lanes 3 and 4, denatured type I collagen in the absence (–) and presence (+) of purified MMP-2 respectively; lanes 5 and 6, native type IV collagen in the absence (–) and presence (+) of purified MMP-2 respectively; lanes 7 and 8, denatured type IV collagen in the absence (–) and presence (+) of purified MMP-2 respectively; lanes 9 and 10, native type V collagen in the absence (–) and presence (+) of purified MMP-2 respectively; lanes 11 and 12, denatured type V collagen in the absence (–) and presence (+) of purified MMP-2 respectively.

(Table 4) [25, 48–51]. Fifth, immunoblot study of the purified 72 kDa gelatinase with polyclonal MMP-2 antibody revealed that the 72 kDa protein cross-react with the antibody (Fig. 4B). Sixth, the 72 kDa gelatinase degrades the specific MMP-2 substrates: native type IV, V collagens and denatured collagens/gelatins (type I, IV and V) but not fibronectin and laminin (Fig. 13). Seventh, the purified 72 kDa gelatinase is able to degrade the synthetic substrate, Dnp-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg-OH (dinitrophenyl labelled peptide), a well known substrate for MMP-2 (Fig. 9B) [23, 32, 40]. Eighth, the 72 kDa gelatinase is inhibited more effectively by TIMP-2 (a specific inhibitor of 72 kDa gelatinase) [19, 47] than TIMP-1 (a specific inhibitor of 92 kDa gelatinase) (Fig. 12, lane 4 and Tables 3, 5 and 6). Ninth, the 62 kDa and the 45 kDa active forms of the purified 72 kDa gelatinase, obtained upon APMA activation, were found to be inhibited by EDTA and TIMP-2 (Fig. 12). Tenth, the 62 kDa and the 45 kDa active fragments of the 72 kDa gelatinase cross-react with polyclonal MMP-2 antibody in Western immunoblot assay (Fig. 11). Eleventh, the activity of the purified 72 kDa gelatinase is stimulated by Ca^{2+} in a dose-dependent manner (Fig. 8). Twelfth, the activity of the 72 kDa gelatinase is poorly activated by trypsin but not activated by plasmin (Fig. 12, lane 5) [20]. Thirteen, like MMP-2 characterized from other sources the pI of the 72 kDa gelatinase is determined to be 6.2–6.4 (Fig. 6). Fourteen, the pH optima for maximum activation is found to be 8 (Fig. 7). This is in accordance with the previous findings that purified MMP-2 obtained from other sources [6, 19, 22, 23, 45]. These 14 pieces of evidence strongly support that the purified 72 kDa gelatinase is the MMP-2.

Reactive oxygen species (ROS) can modulate MMP-2 activity either directly or via a derivative radical(s), for example, peroxynitrate $ONOO^-$ [48–50]. Similar radicals may also be responsible for MMP-2 activation in ischemia-reperfusion, inflammation, or other conditions associated with oxidative stress [50]. Reactive oxygen species, conceivably, undergo fascile reaction with thiol groups of proMMP-2 and thereby could serve as a mechanism of activation of MMP-2 [48–51]. Additionally, the reactive radicals may inactivate TIMP-2, the ambient inhibitor of MMP-2 [25, 48, 51, 52]

thereby causing an imbalance of protease-antiprotease (MMP-2-TIMP-2) leading to an increase in MMP-2 activity in the system.

Circulating levels of pro-MMP-2 are increased in some patients with pulmonary vascular aneurysm [53]. Increased serum and tissue bound MMP-2 has been suggested to play a crucial role for the occurrence of pulmonary diseases, for example, pulmonary hypertension [54]. Under normal circumstances the transcription, secretion and activation of MMP-2 is tightly regulated [54], but under pathophysiological conditions, for instance, oxidant burden in the lung, MMP-2 level increases [55]. It thus appears conceivable that MMP-2 could play an important role in pulmonary vascular pathophysiology.

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