J. Biochem. 2012;151(5):493–499 doi:10.1093/jb/mvs014

JB THE JOURNAL OF BIOCHEMISTRY

Matrix metalloproteinase-9 activity detected in body fluids is the result of two different enzyme forms

Received July 15, 2011; accepted January 2, 2012; published online February 17, 2012

Tiziana Bellini[1], Alessandro Trentini[1], Maria C. Manfrinato[1], Carmine Tamborino[2], Carlo Alberto Volta[3], Valentina Di Foggia[1,*], Enrico Fainardi[4], Franco Dallocchio[1] and Massimiliano Castellazzi[1,†]

[1]Section of Biochemistry and Clinical Biochemistry, Department of Biochemistry and Molecular Biology, University of Ferrara, Via Luigi Borsari 46, [2]Section of Neurology, Department of Medical and Surgical Sciences of the Communication and Behaviour, [3]Section of Anesthesia and Intensive Care, Department of Surgical, Anesthesiological and Radiological Science, University of Ferrara, and [4]Neuroradiology Unit, Department of Neurosciences and Rehabilitation, Azienda Ospedaliera-Universitaria, Arcispedale S. Anna, Corso della Giovecca 203, Ferrara I-44100, Italy

*Present address: Blizard Institute, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, 4 Newark Street, E1 2AT London, UK

†Massimiliano Castellazzi, Department of Medical and Surgical Sciences of the Communication and Behaviour, Section of Neurology, University of Ferrara, Corso della Giovecca, 203, Ferrara I-44121, Italy. Tel: +39-(0)532-236388, Fax: +39-(0)532-205525, email: massimiliano.castellazzi@unife.it

***In vitro* activation of matrix metalloproteinase-9 (MMP-9) (Gelatinase B) with MMP-3 shows the presence of two different forms: an 82 kDa, N-terminal truncated form, and a 65 kDa, N- and C-terminal truncated form. So far the presence of the 65 kDa form has not been reported *in vivo*. Affinity chromatography was performed to separate MMP-9 from MMP-2 and immunoprecipitation to isolate ~65 kDa MMP-9 from 82 kDa MMP-9 in sera of healthy donors. The presence of ~65 kDa active MMP-9 was demonstrated both with gelatin zymography and western blot analysis. The ~65 kDa MMP-9 lacks the haemopexin domain required for the high-affinity binding of the tissue inhibitor TIMP-1, and can be evaluated by activity assay in the presence of TIMP-1. This opens the possibility to investigate the role of this form of MMP-9 that escapes physiological regulation.**

Keywords: activity assay/biological markers/metalloproteinase/MMP-9/zymography.

Abbreviations: APMA, 4-aminophenylmercuric acetate; ConA, concanavalin-A; CV, coefficient of variation; ECM, extracellular matrix; MMP, matrix metalloproteinase; OD, optical density; PBS, phosphate buffered saline; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TIMP, tissue inhibitor of matrix metalloproteinase.

Extracellular matrix (ECM) composition and turnover are regulated by a variety of enzymes among which matrix metalloproteinases (MMPs) seem to be the most important (*1*–*3*). The MMPs are zinc-containing and calcium-requiring endopeptidases known for their ability to cleave one or several ECM constituents, as well as non-matrix proteins (*1*). MMPs are classified into different families (*4*), one of which is the family of gelatinases that includes MMP-9 (Gelatinase B) and MMP-2 (Gelatinase A).

Gelatinase B has been associated with a large number of pathological conditions, such as acute respiratory distress syndrome, destructive lung disease, Sjögren's syndrome, peripheral nerve injury, Guillain–Barré syndrome, blood–brain-barrier damage, Multiple sclerosis, Alzheimer's diseases, cancers and brain ischaemia (*5*). MMP-9 is synthesized as a pre-proenzyme, and the signal peptide is removed during translation producing a 92 kDa proenzyme (*5*). Its structure is organized into three principal domains: a propeptide domain that maintains the enzyme in an inactive form, a catalytic domain that interacts with substrate and a haemopexin-like domain, which is important for interaction with substrate and its inhibitor, the tissue inhibitor of metalloproteinase (TIMP)-1 (*5*).

MMP-9 is released in the inactive form as proenzyme and can be secreted in physical association with its specific tissue inhibitor (TIMP-1) by monocyte/macrophage cells as proMMP-9/TIMP-1 complex (*6*) or as TIMP-1 free protein by tertiary granules of neutrophil cells (*5*, *7*). Activation of MMP-9 requires a complex network of mechanisms involving other MMPs (*8*). The most commonly used activator in *in vitro* experiments is 4-aminophenylmercuric acetate (APMA). APMA activation induces an autoproteolytic activation causing the removal of the propeptide followed by a further breakage and subsequent formation of a N- and C-truncated form of 67 kDa (*9*, *10*). The most likely physiological activator of MMP-9 is thought to be MMP-3 since a catalytic amount of MMP-3 is sufficient to activate proMMP-9 (*9*, *11*). The activation promoted by MMP-3 is initially characterized by the removal of the amino-terminal propeptide with the hydrolysis of the Glu_{40}–Met_{41} and Arg_{87}–Phe_{88} bonds generating an N-truncated active enzyme of 82 kDa (*11*) that can be regulated by TIMP-1 (*12*) and by non-specific inhibitors, such as α2-macroglobulin (*13*). A subsequent processing by MMP-3 produces a 65 kDa active MMP-9 that lacks both the N- and C-terminal domains (*9*). Densitometric analysis showed that this MMP-9

species was associated with the gelatinolytic activity and this active enzyme was not recognized by monoclonal antibodies specific for both N- and C-terminal domains by immunoblot analysis (*9*). Removal of the MMP-9 C-terminal domain causes an increase of TIMP-1 dissociation constant from picomolar to nanomolar (*10*, *14*) demonstrating the important role of the C-terminus domain in the high-affinity interaction between TIMP-1 and MMP-9. For these reasons, the 82 kDa form is still able to bind, and thus could be inhibited by TIMP-1, while the 65 and the 67 kDa form, N- and C-truncated, should escape the control of the specific inhibitor at physiological conditions (*10*).

At present, the N- and C-truncated active enzyme have never been described in body fluids where MMP-9 activity is measured as a whole, in absence of discrimination between the two active forms (*15–17*).

The aim of this study is to demonstrate the presence of the N- and C-truncated active MMP-9 in serum samples.

Materials and Methods

Blood specimen collection and storage

Serum samples from 25 healthy volunteers (12 females and 13 males; mean age ± SD: 32.4 ± 8.2 years) were collected in Vacutainer™ tubes and centrifuged within 30–60 min after venipuncture at 3,000 rpm for 12 min at room temperature. After removal of the supernatants, serum samples were aliquoted and stored at −70°C until assay. Each sample was analysed in duplicate in the same session. Serum specimens were chosen since active MMP-9 concentrations are not altered by clotting phenomena that take place in collector tubes (*16*, *17*). Informed consent was given by all volunteers before inclusion and the study design was approved by the Local Committee for Medical Ethics in Research.

Enzymes, antibodies and reagents

In all experiments, recombinant human MMP-2 and MMP-9 proenzymes used as standards as well as APMA and buffers, were included in commercially available Activity Assay System kits (Activity Assay System, Biotrak, Amersham Biosciences, UK, Cat. No. RPN 2631 and Cat. No. RPN 2634). In preliminary experiments, MMP-9 and MMP-2 proenzymes were tested for the presence of MMP-2 and MMP-9, respectively, with MMP-2 and MMP-9 activity assays. No contamination was found in either experiment. TIMP-1 (Cat. No. T8947), Concanavalin A (ConA) Sepharose (Cat. No. C9017), Protein G Sepharose (Cat. No. P3296), sodium chloride, calcium chloride, zinc chloride, BRIJ™ 35 and Comassie Blue-R were purchased from Sigma-Aldrich (Sigma-Aldrich, Milan, Italy). Tris base was purchased from Merck (VWR International, Milan, Italy). The assay buffer used for immunoprecipitation and activation of MMP-9 was composed as follows: 50 mM Tris–HCl buffer pH 7.6 containing 1.5 mM sodium chloride, 0.5 mM calcium chloride, 1 μM zinc chloride and 0.01% (v/v) BRIJ™ 35.

Gelatin zymography

Zymographic patterns of gelatinases were evaluated by using the method described by Heussen and Dowdle (*18*) on gelatin-copolymerized gel. Briefly, samples were applied without reduction and boiling to a 10% polyacrylamide gel containing 0.1% gelatin. Gel electrophoresis was performed with these settings: 30 mA, 35 W and 450 V/h for about 45 min. Then electrophoresis gels were incubated with 2.5% (v/v) Triton for 20 min three times and then in 50 mM Tris, pH 7.6, containing 150 mM NaCl, 5 mM $CaCl_2$ and 0.02% (v/v) Brij 35 for 22 h. The gels were then stained with 0.3% Comassie Blue-R.

With this zymographic method, we were unable to discriminate between 67 and 65 kDa active MMP-9 forms; for this reason, we chose to define the average weight of the N- and C-truncated active MMP-9 at about 65 kDa.

Densitometric analysis of the zymographic patterns

Gel was scanned using a Canon scanner (CanoScan D660U) interfaced with a Windows PC and acquired with Adobe Photoshop (Adobe System Inc., Mountain View, CA, USA) in grey scale mode at 600 dpi. The corresponding images were saved to disk in TIFF format and then analysed using Quantity One® software (Bio-Rad Laboratories). The bands were manually selected using the volume tool within the program and their densitometric values were adjusted for the background, giving an integrated density reported in volume units of pixel intensity × square millimeter.

MMP-9 activity assays

As reported in our previous studies (*15–17*), active forms of MMP-9 were measured using a commercially available activity assay system (Activity Assay System, Biotrak, Amersham Biosciences, UK, Cat. No. RPN 2634) following the manufacturer's instructions. Briefly, each sample was analysed in duplicate into 96-microwell microtiter plates precoated with anti-MMP-9 antibodies. Six serial dilutions of standard were dispensed on each plate in duplicate in the range of 0.125–4 ng/ml. After an overnight incubation at 2–8°C and four washing cycles, only the enzymes had bound to the wells. The standards, human proMMP-9, were activated by adding 50 μl/well of APMA. In this way, total amounts of MMP-9 were measured. Conversely, to detect only endogenous levels of active MMP-9, 50 μl/well of dilution buffer, instead of APMA, was dispensed into each well. Fifty microlitres of detection reagent, a modified pro-urokinase and urokinase substrate, were pipetted into each well. The amount of active MMP-9 in all samples was determined by interpolation from the standard curve. As reported in the manufacturer's instructions, the lower limit of quantification was assumed at 0.125 ng/ml, the range of intra-assay coefficient of variations (CV) was 3.4–4.3%, whereas the range of inter-assay CV was 20.2–21.7%. The MMP-9 activity assay was not able to distinguish between the two active MMP-9 forms, but only detected both MMP-9 activities.

Inhibition of MMP-9 by TIMP-1 was measured by slight modification of the above mentioned activity assay. Briefly, after an overnight incubation of the samples in microtiter plates pre-coated with anti-MMP-9 antibodies, 100 μl/well of purified TIMP-1 (100 ng/ml) were added to all samples in order to inhibit the 82 kDa MMP-9 active form. After 90 min of incubation at 37°C and three cycles of washing the detection reagent was applied, and the residual MMP-9 activity was assigned to ~65 kDa active MMP-9. The optimal concentration of TIMP-1 used for these determinations was tested in preliminary experiments which showed that increasing TIMP-1 concentration up to 200 ng/ml did not cause a further decrease in MMP-9 activity (data not shown). To verify the reliability of both the activity assays, we tested three serum samples at the dilution of 1:15, 1:20, 1:25, 1:30, 1:40 and we checked the linearity between the concentrations of total active MMP-9 and of TIMP-1 resistant MMP-9 and the respective optical density (OD) values. In all these determinations, a strong correlation was found with an R^2 never <0.96. Moreover, the differences between expected values and measured values were <12% in all the determinations (data not shown).

MMP-2 activity assay

Active forms of MMP-2 were measured using a commercially available activity assay system (Activity Assay System, Biotrak, Amersham Biosciences, UK, Cat. No. RPN 2631) as previously reported (*16*, *17*). The principle is the same as the MMP-9 activity assay. According to the manufacturer's instructions, the lower limit of quantification of the assay was 0.19 ng/ml, the range of intra-assay CV was 4.4–7.0%, whereas the range of inter-assay CV was 16.9–18.5%.

Activation of proMMP-9

A sample of recombinant MMP-9 (8 ng/ml) was incubated with 1 mM APMA (diluted in assay buffer) at 37°C up to 6 hr in order to activate the enzyme. Samples were withdrawn from the incubation mixture at baseline, 2, 4 and 6 hr. Then, the samples were incubated overnight at 4°C in microtiter plates precoated with anti-MMP-9 antibodies to test by activity assay with and without TIMP-1, or kept at 4°C overnight and assayed by gelatin zymography.

Immunoprecipitation

To isolate the 65 kDa MMP-9 from a serum sample, a two-step immunoprecipitation procedure was conducted by using monoclonal antibody against MMP-9 (R&D Systems, Minneapolis, MN, USA, Cat. No. MAB936). As indicated by the manufacturer's instructions, this antibody is able to bind pro (92 kDa) and active (82 kDa) forms of recombinant human MMP-9, does not react with the C-terminal truncated form (~65 kDa) of MMP-9 and does not show cross-reactivity with recombinant human MMP-2. Diluted serum sample (1:20 in assay buffer) was incubated overnight at 4°C on a tube rotator with 4 µg/ml of anti-MMP-9 antibody and 1 µl of 2.5% Triton. After this first step, Protein G Sepharose fast flow was added to the sample in a ratio of 1:3 and incubated for 3 h at 4°C on a tube rotator. After centrifugation, supernatant was removed and immunoprecipitation was repeated with the same antibody. The sample was then treated with Protein G Sepharose fast flow, as previously indicated, and finally centrifuged to obtain supernatant. The untreated serum and supernatant obtained after the two-step immunoprecipitation were then tested for MMP-9 activity in presence and in absence of TIMP-1.

Gelatinases separation by concanavalin-A chromatography

To identify the 65 kDa MMP-9 form, serum MMP-2 and MMP-9 were separated by using a concanavalin-A-Sepharose affinity chromatography following the method described earlier by Rantala-Ryhänen (*19*) and adapted by us. Briefly, a microcolumn with 500 µl of concanavalin-A-Sepharose was equilibrated with phosphate buffered saline (PBS). After serum sample application the column was first washed with a 0.3 M NaCl buffer and then with a 0.3 M NaCl and 50 mM methyl α-D-mannopyranoside buffer. Fractions of the flow through and wash were kept. Finally, the column was eluted with a buffer containing 0.5 M methyl α-D-mannopyranoside. The fractions were analysed with gelatin zymography, MMP-2 and MMP-9 activity assays and MMP-9 inhibition by TIMP-1.

Gelatinases separation by gelatin agarose chromatography

To enrich a sample in gelatinases, 6 ml of serum from healthy donors was subjected to gelatin-agarose affinity chromatography. Briefly, a column with 4 ml of gelatin-agarose (Sigma-Aldrich, Cat. No. G5384) was equilibrated with 50 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, Brij35 0.02% pH 7.5 (equilibration buffer). After serum sample application, the column was washed with equilibration buffer and eluted with 7% dimethylsulfoxide in equilibration buffer. The fractions with higher gelatinolytic activity evidenced by gelatin zymography were pooled and concentrated by ultrafiltering in centriplus filter devices (YM-30, Amicon-Millipore).

Western blot and analysis of MMP-9

For western blot analysis, samples electrophoresed by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing condition were transferred to polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Hybond-LFP) in tris–glycine–SDS blotting buffer with 20% methanol. Non-specific sites were then blocked and blots were incubated overnight with rabbit polyclonal antibodies anti-human MMP-9 at 1.25 µg/ml, which recognize a sequence in the catalytic domain of the enzyme (Novus Biologicals, NBP1-57940). Blots were washed and then incubated with Cy5 conjugated secondary anti-rabbit antibodies (Amersham ECL Plex Western Blotting System) diluted 1:2,500 in PBS. Finally, fluorescence band detection was performed using a molecular imager scanner (Molecular Imager Pharos FX imaging system, Bio-Rad). Band molecular weights were determined by Quantity One® software (Bio-Rad Laboratories).

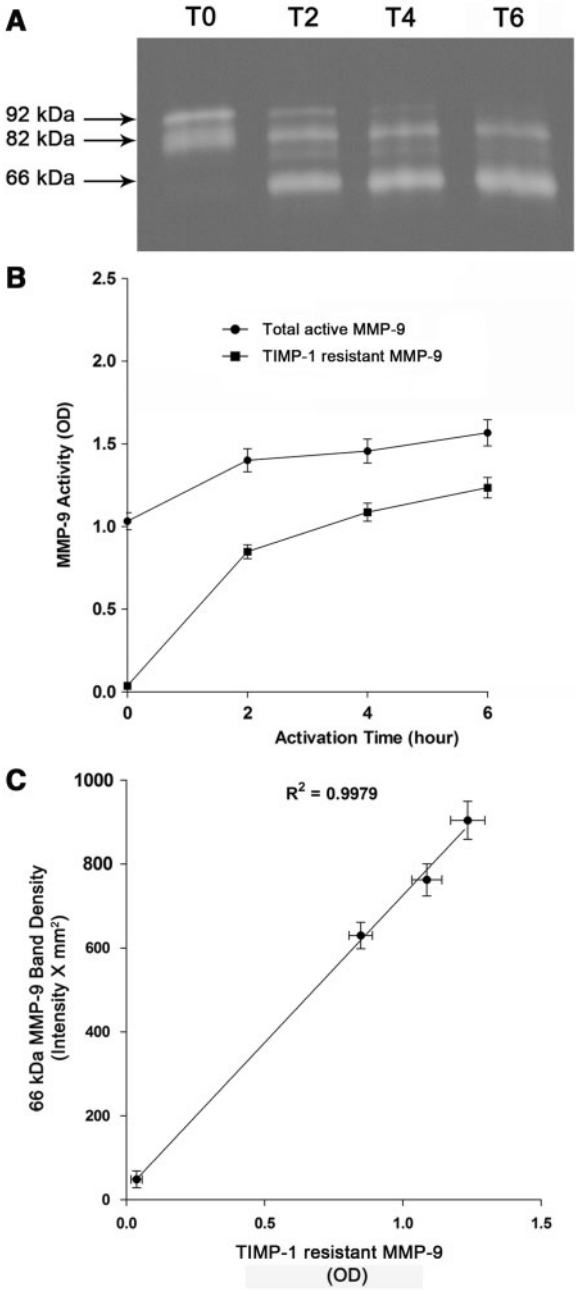


Fig. 1 Time course activation of proMMP-9. (A) Gelatin zymography time course activation of proMMP-9 with APMA. An 8 ng/ml of MMP-9 was incubated with 1 mM APMA for a few minutes at baseline (T0) and at 2 (T2), 4 (T4) and 6 h (T6) at 37°C before the assay; (B) total active MMP-9 (circles) and TIMP-1-resistant active MMP-9 (squares) values were measured at the same time points with the different activity assays. Experiments were repeated three times; values are indicated as mean ± standard deviation; (C) Correlation between temporal changes of TIMP-1-resistant active MMP-9 and ~65 kDa MMP-9 band density expressed as mean value ± standard of optical density and intensity × square millimetre, respectively.

Results

Inhibition of MMP-9 by TIMP-1

To verify whether inhibition by TIMP-1 is useful to distinguish between the two active forms of MMP-9, we performed a time-dependent activation of MMP-9 by APMA.

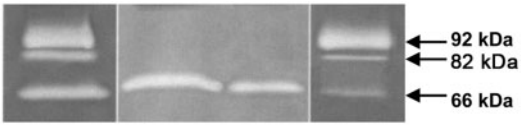
As shown in Fig. 1A, an increasing incubation time with APMA corresponded to a decreasing intensity of the gelatinolytic bands at high-molecular weights (proMMP-9 and 82 kDa MMP-9, respectively) and an associated increasing intensity of the lower molecular weight band (~65 kDa MMP-9). It must be noted that there was a band between 82 and ~65 kDa forms, most likely representing an intermediate form in the process of activation (*9*). These findings confirmed

that in the presence of APMA, proMMP-9 was converted into the 82 kDa form and then into the ~65 kDa form.

The same samples were assayed also for MMP-9 activities. As indicated in Fig. 1B, the total MMP-9 activity (circles) increased with the incubation time, due to the progressive conversion of the inactive 92 kDa form to the active 82 kDa and ~65 kDa forms. When TIMP-1 was added to the assay mixture, the uninhibited activity (Fig. 1B, filled squares), which was very low at time zero, showed a remarkable time-dependent increase indicating that under the assay conditions only a part of the active enzyme was inhibited. Furthermore, the uninhibited activity increased in parallel with the ~65 kDa band intensity, showing a strong positive correlation with the zymographic data (Fig. 1C) ($P < 0.01$; Pearson correlation $r = 0.9987$). This suggested that the assay performed in presence of TIMP-1 is a useful indicator of the presence of the ~65 kDa form.

Identification of ~65 kDa MMP-9 form in serum

A fresh serum sample was subjected to an affinity chromatography on ConA-Sepharose in order to verify whether the ~65 kDa MMP-9 form was present in biological fluids or whether it was an '*in vitro*' artefact. The obtained chromatographic fractions were analysed by gelatin zymography, MMP-2 activity assay and MMP-9 activity assays. Gelatin zymography (Fig. 2) showed the presence of a single ~65 kDa band in the flow through (lane B) and wash (lane C) samples corresponding to the unbound MMP-2. This was supported by the MMP-2 activity assay data, which showed the presence of the enzyme only in the flow through and wash fractions (Fig. 2 and Table I). In the eluate, three bands were present (lane D), which migrated at 92, 82 and ~65 kDa in accordance with the three forms of MMP-9. In fact, MMP-2 activity assay did not show the presence of the enzyme in the eluate, ruling out the possibility that the ~65 kDa band in the eluate was residual MMP-2. Additionally, when MMP-9 activity was assayed in the eluate, the addition of TIMP-1 only partially inhibited its activity (Fig. 2 and Table I). This indicated that both MMP-9 active forms, 82 and ~65 kDa, were present in the serum sample. To further demonstrate the presence of the ~65 kDa MMP-9 form in biological fluids, we performed a two-step immunoprecipitation of a serum sample with an anti-MMP-9 antibody, which recognizes only the 92 and 82 kDa forms of the enzyme; thus, only the ~65 kDa form was expected to be present in the supernatant. However, gelatin zymography (data not shown) demonstrated that even a second treatment with the antibody did not remove all 92 and 82 kDa forms of MMP-9. Nevertheless, as shown in Fig. 3 when TIMP-1 was added to the MMP-9 assay of a native serum sample, an inhibition in MMP-9 activity of ~54% was observed. Conversely, only a slight inhibition was found in the supernatant obtained with the two-step immunoprecipitation after the addition of TIMP-1. These data indicate that an active form of MMP-9, not inhibited by TIMP-1 and not recognized by antibodies specific for the 92 kDa and 82 kDa forms of the protease, occurs in serum. On the basis of the results obtained also by ConA chromatography, this MMP-9 should be the ~65 kDa form.



	Total active MMP-9	TIMP-1 resistant active MP-9	Active MMP-2
	(OD)	(OD)	(OD)
Flow through (**B**)	0	0	1.324
Wash (**C**)	0	0	1.019
Elution (**D**)	0.439	0.288	0

Fig. 2 MMP-9 and MMP-2 activity before and after concanavalin-A-Sepharose chromatography. Gelatin zymography profiles of native serum (A), flow through (B), wash (C) and elution (D) obtained after concanavalin-A-Sepharose chromatography. Table I describes absorbance values in flow through, wash and elution samples of MMP-2 and MMP-9 activity assay in the presence or absence of TIMP-1.

Table I. Serum concentrations of total active MMP-9 and of TIMP-1-resistant active MMP-9 in 25 healthy donors (mean ± standard deviation; range).

Samples	Total active MMP-9 (ng/ml)	TIMP-1-resistant active MMP-9 (ng/ml)
Healthy donors ($n = 25$)	0.57 ± 0.25 (0.15–1.18)	0.36 ± 0.21 (0.06–0.77)

Western blot analysis

Gelatinases-enriched serum sample was analysed by western blot in non-denaturing (Fig. 4, lane A) and denaturing (Fig. 4, lane B) conditions. In agreement with the manufacturer's instructions, antibody used in this experiment recognized the catalytic domain only in denatured enzyme. In fact, no bands were present in lane A, whereas two bands were clearly identified in lane B, corresponding to proMMP-9 (92 kDa) and to N- and C-truncated active MMP-9 (~65 kDa). In lane C, fully activated purified MMP-9 was applied after 6 h of incubation with APMA. Only one band was present at ~64 kDa. This experiment confirmed that a ~65 kDa active MMP-9 is present in serum sample without chemical or enzyme activations of circulating proMMP-9.

Evidences of ~65 kDa MMP-9 form in serum of healthy donors

In order to measure the levels of ~65 kDa form in biological samples, we assayed the MMP-9 activity and TIMP-1 inhibition on the sera of 25 healthy donors.

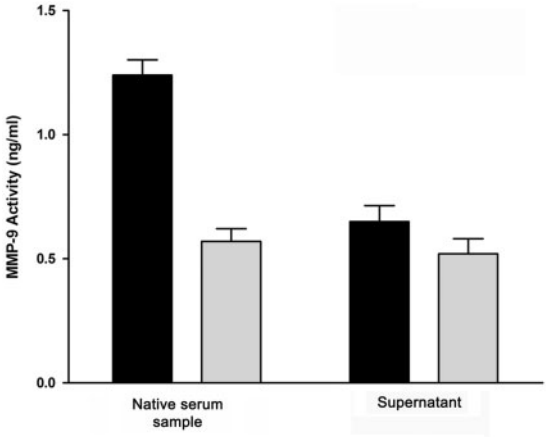


Fig. 3 MMP-9 activity before and after two-step immunoprecipitation. Total MMP-9 activity (black) and TIMP-1 resistant MMP-9 activity (grey) in native serum sample and supernatant obtained after two-step immunoprecipitation.

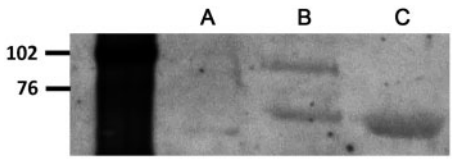


Fig. 4 Western blot analysis. Gelatinases enriched serum sample and APMA-activated purified MMP-9 were analysed. Lane A: non-denatured serum sample; Lane B: denatured serum sample; Lane C: fully activated purified MMP-9. Molecular weights are indicated on the left (Rainbow fluorescent marker, GE Healthcare).

Table I depicts serum levels of total active (82 and ~65 kDa) and ~65 kDa active MMP-9. Detectable levels of active MMP-9 were measured in all the samples with both methods. Comparison between the results obtained by MMP-9 activity assay and MMP-9 inhibition by TIMP-1 in each serum sample showed that the percentage of ~65 kDa MMP-9 had a great individual variability fluctuating from 10.6% to 92.8% with respect to the total activity.

Discussion and Conclusions

In this study, we demonstrated for the first time the presence of ~65 kDa active MMP-9 in serum samples.

It is known that activation of MMP-9 with organomercurial compounds, as well as with other proteases like MMP-3, can produce two different active enzyme forms: a 82 and a 65 kDa form (*9*, *11*, *20*). The difference between these two active MMP-9 is that the latter lacks the haemopexin-like domain, giving a lower affinity for TIMP-1, which can no longer inhibit the 65 kDa MMP-9 at physiological concentration (*10*).

In fact, we have shown that there is a direct correlation between the appearance of the ~65 kDa form and the loss of TIMP-1 inhibition, and it has been reported that activation of human plasma MMP-9 by APMA strongly reduces the inhibition by TIMP-1 (*21*). Nevertheless, also untreated MMP-9 from serum samples showed a residual activity resistant to TIMP-1, suggesting that a MMP-9 form, similar to that obtained by APMA activation, is physiologically present in human blood.

So far, most attention has focused on the 82 kDa active form which can easily be detected in biological samples by gelatin zymography and commercially available activity assay systems, but little attention has been devoted to the minor form. This lack of interest is mainly due to the fact that the most common method used to reveal gelatinases is zymography, in which the ~65 kDa MMP-9 form is shielded by the more abundant 66 kDa form of MMP-2. Furthermore, to the best of our knowledge, commercial ELISA kits cannot distinguish between inactive and active forms. Also, activity assay systems can evidence only 82 and ~65 kDa activities together (*22*). Thus, until now the presence of ~65 kDa MMP-9 has never been reported *in vivo*.

Using ConA-Sepharose affinity chromatography, we separated serum MMP-9 from MMP-2: since glycosylation sites of MMP-9 are found in the catalytic domain all forms were expected to bind to ConA, while MMP-2, which is not glycosylated, should not bind to the resin (*19*). The sample eluted from the column, despite the total absence of MMP-2, showed a ~65 kDa band in zymography that can only be assigned to the ~65 kDa form of MMP-9. In fact, exploiting the differential TIMP-1 binding ability of the two active forms, we saw that the addition of TIMP-1 to the sample caused only a moderate inhibition in MMP-9 activity. The immunoprecipitation were consistent with our hypothesis. In fact, when a serum sample was depleted from 92 kDa proMMP-9 and 82 kDa active MMP-9 by immunoprecipitation with specific monoclonal antibodies, the whole MMP-9 activity strongly decreased, whereas the MMP-9 activity insensitive to TIMP-1 did not change. In addition, a ~65 kDa MMP-9 was also detected in serum sample by western blot analysis using a specific antibody that recognizes denatured enzyme forms. With this method, we clearly identified two major circulating MMP-9 components: 92 kDa proMMP-9 and ~65 kDa active MMP-9. The absence of the 82 kDa active MMP-9 could be attributable to a difference in sensitivity between zymography and western blot. Collectively taken, our data confirm the presence of circulating ~65 kDa MMP-9 in serum samples.

Taking advantage of the different affinity that 82 and ~65 kDa MMP-9 have for TIMP-1, we were also able to measure the levels of ~65 kDa active form in sera of a small group of healthy donors. Our analysis showed a high individual variability in its levels ranging from 10.6% to 92.8% in relation to total endogenous active MMP-9 concentrations. This was likely due to the different health conditions of the controls, since several factors can alter MMP-9 levels (*6*, *23*), but nothing is known about the ~65 kDa form. However, the fact that this form was detectable in all samples indicated that it is normally present in serum. The mean extent of inhibition observed is lower than the inhibition reported by Grierson *et al.* (*21*), who found an 80% inhibition. However, these data are obtained in lithium-heparin plasma, while our data are

from sera. We have already shown that heparin interferes with MMP-9 assay (*17*), but we cannot exclude that in serum higher levels of ~65 kDa MMP-9 could be formed.

The low extent of TIMP-1 inhibition (37%) might suggest that ~65 kDa MMP-9 is the main active form present in serum. However, it must be taken into account that TIMP-1 is present in normal sera at concentrations similar to that used in the inhibition experiments, and at least part of the active MMP-9 could have already bound TIMP-1. Thus, despite the activity assay's usefulness for estimating the total enzymatic activity, it could underestimate the 82 kDa form. The presence of tightly bound TIMP-1 could also explain the discrepancy between activity assay, where the ~65 kDa form appears higher than the 82 kDa form, and zymography, where the 82 kDa form is more abundant than the ~65 kDa.

In fact while in the activity assay TIMP-1 could be still bound to the 82 kDa MMP-9, during electrophoresis TIMP-1 is displaced and the gelatinolytic activity of the 82 kDa MMP-9 is fully displayed.

Moreover, a possible limitation of our study could be due to the presence in serum of MMP-9 dimers (*24*) or circulating 82 kDa complexed to other molecules via its haemopexin domain, which would escape pM affinity TIMP-1 inhibition leading to an overestimation of the TIMP-1-resistant activity.

At present, it is still not clear whether the balance between 82 and ~65 kDa MMP-9 has a physiological significance and whether these two enzymatic forms should have a different activity. Although it has been demonstrated that both N-truncated and N- and C-truncated active MMP-9 share the same catalytical activity (*14*) a limitation of our study is the lack of a kinetic study of the two enzymatic forms purified from human sera to confirm previously reported data.

In conclusion, the lack of regulation of ~65 kDa MMP-9 by its specific inhibitor suggests that the role of this form deserves more attention. This could be relevant in order to define the degradative potential of active MMP-9 in some body compartments, such as cerebrospinal or synovial fluids (*15*, *22*) or, more generally, in other inflammation sites. In addition, in an *in vitro* 3D co-culture system for the study of cancer invasion, it has been demonstrated that the ~65 kDa active MMP-9 may play a crucial role in the mechanisms controlling cell invasion (*25*).

Therefore, further studies, including a large number of healthy volunteers and patients with different inflammatory diseases, are required to clarify the significance of this ~65 kDa MMP-9 form in both physiological and pathological conditions.

Acknowledgements

The authors thank Dr Elizabeth Jenkins for her helpful corrections to the manuscript. Study sponsors did not have a role in study design; in the collection, analysis, and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

Funding
Fondazione Cassa di Risparmio di Ferrara, Italy; Research Program Regione Emilia Romagna-University 2007-2009 (PRUa1a-2007-008).

Conflict of interest
None declared.

References

1. Massova, I., Kotra, L.P., Fridman, R., and Mobashery, S. (1998) Matrix metalloproteinases: structures, evolution, and diversification. *FASEB J.* **12**, 1075–1095
2. Nagase, H., Visse, R., and Murphy, G. (2006) Structure and function of matrix metalloproteinases and TIMPs. *Cardiovasc. Res.* **69**, 562–573
3. Lemaître, V. and D'armiento, J. (2006) Matrix metalloproteinases in development and disease. *Birth Defects Res.* **78**, 1–10
4. Sang, Q.X., Birkedal-Hansen, H., and Van Wart, H.E. (1995) Proteolytic and non-proteolytic activation of human neutrophil progelatinase B. *Biochim. Biophys. Acta* **1251**, 99–108
5. Lacraz, S., Nicod, L.P., Chicheportiche, R., Welgus, H.G., and Dayer, J.M. (1995) IL-10 inhibits metalloproteinase and stimulates TIMP-1 production in human mononuclear phagocytes. *J. Clin. Invest.* **96**, 2304–2310
6. Van den Steen, P.E., Dubois, B., Nelissen, I., Rudd, P.M., Dwek, R.A., and Opdenakker, G. (2002) Biochemistry and molecular biology of Gelatinase B or matrix metalloproteinase-9 (MMP-9). *Crit. Rev. Biochem. Mol. Biol.* **37**, 375–536
7. Ardi, V.C., Kupriyanova, T.A., Deryugina, E.I., and Quigley, J.P. (2007) Human neutrophils uniquely release TIMP-free MMP-9 to provide a potent catalytic stimulator of angiogenesis. *Proc. Natl Acad. Sci. USA* **104**, 20262–20267
8. Cuzner, M.L. and Opdenakker, G. (1999) Plasminogen activators and matrix metalloproteases, mediators of extracellular proteolysis in inflammatory demyelination of the central nervous system. *J. Neuroimmunol.* **94**, 1–14
9. Okada, Y., Gonoji, Y., Naka, K., Tomita, K., Nakanishi, I., Iwata, K., Yamashita, K., and Hayakawa, T. (1992) Matrix metalloproteinase 9 (92-kDa gelatinase/type IV collagenase) from HT 1080 human fibrosarcoma cells. Purification and activation of the precursor and enzymic properties. *J. Biol. Chem.* **267**, 21712–21719
10. Vempati, P., Karagiannis, E.D., and Popel, A.S. (2007) A biochemical model of Matrix Metalloproteinase 9 activation and inhibition. *J. Biol. Chem.* **282**, 37585–37596
11. Ogata, Y., Enghild, J.J., and Nagase, H. (1992) Matrix metalloproteinase 3 (stromelysin) activates the precursor for the human matrix metalloproteinase 9. *J. Biol. Chem.* **267**, 3581–3584
12. Murphy, G. and Docherty, A.J. (1992) The matrix metalloproteinases and their inhibitors. *Am. J. Respir. Cell. Mol. Biol.* **7**, 120–125
13. Birkedal-Hansen, H., Moore, W.G., Bodden, M.K., Windsor, L.J., Birkedal-Hansen, B., DeCarlo, A., and Engler, A. (1993) Matrix metalloproteinases: a review. *Crit. Rev. Oral. Biol. Med.* **4**, 197–250
14. O'Connell, J.P., Willenbrock, H.F., Docherty, A.J.P., Eaton, D., and Murphy, G. (1994) Analysis of the role of the COOH-terminal domain in the activation, proteolytic activity and tissue inhibitor of Metalloproteinase

interactions of Gelatinase B. *J. Biol. Chem.* **269**, 14967–14973

15. Fainardi, E., Castellazzi, M., Bellini, T., Manfrinato, M.C., Baldi, E., Casetta, I., Paolino, E., Granieri, E., and Dallocchio, F. (2006) Cerebrospinal fluid and serum levels and intrathecal production of active matrix metalloproteinase-9 (MMP-9) as markers of disease activity in patients with multiple sclerosis. *Mult. Scler.* **12**, 294–301
16. Fainardi, E., Castellazzi, M., Bellini, T., and Dallocchio, F. (2007) Matrix metalloproteinases (MMP): determination of different forms by different techniques may require different preanalytical strategies. *Mult. Scler.* **13**, 691–692
17. Castellazzi, M., Tamborino, C., Fainardi, E., Manfrinato, M.C., Granieri, E., Dallocchio, F., and Bellini, T. (2007) Effects of anticoagulants on the activity of gelatinase. *Clin. Biochem.* **40**, 1272–1276
18. Heussen, C. and Dowdle, E.B. (1980) Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. *Anal. Biochem.* **102**, 196–202
19. Rantala-Ryhänen, S., Ryhänen, L., Nowak, F.V., and Uitto, J. (1983) Proteinases in human polymorphonuclear leukocytes. Purification and characterization of an enzyme which cleaves denatured collagen and a synthetic peptide with a Gly-Ile sequence. *J. Biochem.* **134**, 129–237
20. Shapiro, S.D., Fliszar, C.J., Broekelmann, T.J., Mecham, R.P., Senior, R.M., and Welgus, H.G. (1995) Activation of the 92-kDa gelatinase by stromelysin and 4-aminophenylmercuric acetate. Differential processing and stabilization of the carboxyl-terminal domain by tissue inhibitor of metalloproteinases (TIMP). *J. Biol. Chem.* **270**, 6351–6356
21. Grierson, C., Miller, D., LaPan, P., and Brady, J. (2010) Utility of combining MMP-9 enzyme-linked immunosorbent assay and MMP-9 activity assay data to monitor plasma enzyme specific activity. *Anal Biochem.* **404**, 232–234
22. Hanemaaijer, R., Visser, H., Konttinen, Y.T., Koolwijk, P., and Verheijen, J.H. (1998) A novel and simple immunocapture assay for determination of Gelatinase-B (MMP-9) activities in biological fluids: saliva from patients with Sjogren's syndrome contain increased latent and active Gelatinase-B levels. *Matrix Biol.* **17**, 657–665
23. Raitio, A., Tuomas, H., Kokkonen, N., Salo, T., Sorsa, T., Hanemaaijer, R., and Oikarinen, A. (2005) Levels of matrix metalloproteinase-2, -9 and -8 in the skin, serum and saliva of smokers and non-smokers. *Arch. Dermatol. Res.* **297**, 242–248
24. Cha, H., Kopetzki, E., Huber, R., Lanzendörfer, M., and Brandstetter, H. (2002) Structural basis of the adaptive molecular recognition by MMP9. *J. Mol. Biol.* **320**, 1065–1079
25. Shimizu, S., Ozawa, S., and Kubota, E. (2005) Activation of cancer cell-derived MMP-9 correlates with cancer invasion—an *in vitro* study with 3-dimensional co-culture system. *Oral Med. Pathol.* **10**, 63–72