**Review**

Serena Ricci, Vittoria D'Esposito, Francesco Oriente, Pietro Formisano and Angelina Di Carlo*

# Substrate-zymography: a still worthwhile method for gelatinases analysis in biological samples

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**Abstract:** Matrix metallo-proteinases (MMPs) are a family of zinc-dependent endopeptidases, capable of degrading all the molecular components of extracellular matrix. A class of MMPs is gelatinases which includes gelatinase A or MMP-2 (72 kDa) and gelatinase B or MMP-9 (92 kDa), which have been shown to play critical roles in pathophysiology of many human disease and, in particular, cancer progression. For these reasons they obtained a great interest as potential non-invasive biomarker in providing useful clinical information in cancer diagnosis and therapy. A sensitive and unexpensive method for analysis of gelatinases is the gelatine zymography, which allows to measure the relative amounts of active and inactive enzymes in body fluids and tissue extracts. The procedure involves the electrophoretic separation of proteins under denaturing but non reducing conditions through a polyacrylamide gel containing a synthetic substrate (gelatin). The aim of this mini-review has been to describe the general principles of gelatine zymography technique, underling the main advantages and disadvantages. Even though an improvement of this method is necessary for a better applicability in laboratory medicine, gelatine zymography represents the most convenient method to detect the activity of the different gelatinases from a wide range of biological samples.

**Keywords:** gelatinases; matrix metalloproteinase-2; matrix metalloproteinase-9; substrate zymography.

***Corresponding author: Angelina Di Carlo,** Full Professor of Clinical Pathology, Department of Medico-Surgical Sciences and Biotechnologies, "La Sapienza" University of Rome, Corso della Repubblica 79, Latina 04100, Italy, Phone: +39 0773 1757210, Fax: +39 0773 1757219, E-mail: angelina.dicarlo@uniroma1.it
**Serena Ricci:** Department of Translational Medical Science, University of Naples "Federico II", Naples, Italy
**Vittoria D'Esposito, Francesco Oriente and Pietro Formisano:** Department of Translational Medical Science, University of Naples "Federico II", Naples, Italy; and URT Genomica del Diabete, IEOS-CNR, Naples, Italy

## Introduction

The term "metalloprotease" encompasses esopeptidases and endopeptidases involved in many biological processes, and are grouped in 14 different clans. Some clans require only one catalytic metal ion and others require two metal ions acting co-catalytically. The divalent metal ion contained in the active site is, in the vast majority of cases, a zinc ion, but cobalt, manganese or nickel are also represented. In human, the majority of metallo-proteases are zinc metallo-endopeptidases distributed in five clans (i.e. MA, ME, MJ, MK, MM) [1]. Among these, the clan MA is the main clan of metallo-proteinases and it is characterized by the "HEXXH" zinc-binding motif with two histidines acting as a ligands of the catalytic Zn++ and the glutamate as the general basis. This clan include the following families: M3, M10, M12, M13, M41, M43, and M48. In particular, M10 family includes human zinc-endopeptidases known as "matrix metalloproteinases" (MMPs). It is becoming increasingly recognized that MMPs are a multifunctional group of biologically important molecules with diverse roles in normal cell growth and differentiation. This family consists in 25 members and numerous homologues from other species which are able to degrade basement membranes and extracellular matrix (ECM) components. Although MMPs have overlapping substrate specificities, they are divided into five groups with respect to their preferential degradation of different matrix substrates [matrilysin, collagenases, gelatinases, stromelysin and membrane MMPs (MT-MMPs)] and in eight structural classes (Table 1), three of which are membrane bound [2, 3].

The basic structure of these enzymes is characterized by (i) a pre-peptide domain involved in the pro-enzyme secretion process, (ii) the auto-inhibitory pro-domain, (iii) the catalytic domain and (iv) the C-terminal hemopexin-like domain often involved in the recognition/positioning of substrates (Figure 1). The pro-domain, composed of about 80 residues, extends from the N-terminus to the catalytic domain and it is responsible for the enzyme latency. A cysteine sulphydryl group present in the N-terminal pro-domain ("Cys-switch") interacts with the $Zn^{2+}$ ion and

**Table 1:** Members of the MMP family.

| Structure | Name | Pseudonym | Human chromosome | Molecular weight, Da (latent/active) | Matrix substrates | Bioactive substrates |
|---|---|---|---|---|---|---|
| Minimal-domain | MMP-7<br>MMP-26 | Matrilysin-1<br>Matrilysin-2 | 11q21-q22<br>11p15 | 28.000/19.000 | Collagen IV, X IV, aggrecan, elastin, fibronectin, gelatin, laminin, fibrinogen, fibronectin, gelatin | TNF-α, α-PI, IGFBP-3, TNF-α, RANK ligand, IGFBP-3, fas ligand, plasminogen, E-caderin, proα-defensin, HB-EGF, MMP-9 |
| Simple hemopexin-domain | MMP-1 | Collagenase-1 | 11q22-q23 | 55.000/45.000 | Collagen I, II, III,VII,VIII, X, aggrecan, gelatin | IGFBP-3, IGFBP-2, IL-1β, MCP-3, CTGF, $\alpha_2$-M, α-PI, MMP-2, -9 |
| (Archetypal) | MMP-8 | Collagenase-2 | 11q21-q22 | 75.000/58.000 | Collagen I, II, III,V, VII,VIII, X, aggrecan, elastin, fibronectin, gelatin, laminin | α-PI |
| | MMP-13 | Collagenase-3 | 11q22.3 | 60.000/48.000 | Collagen I, II, III, IV, IX, X, XIV, aggregan, gelatin | MCP-3, $\alpha_1$-antichymotrypsin, plasminogen, TGFβ |
| | MMP-18 | Collagenase-4 (*Xenopus*)[a] | – | 70.000/53.000 | Unknown | Unknown |
| | MMP-3 | Stromelysin-1 | 11q23 | 57.000/45.000 | Collagen II, III, IV, IX, X, XI, aggrecan, elastin, fibronectin, gelatin, laminin | IGFBP-3, IL-1β, MCP-3, HB-EGF, E-caderin, plasminogen, uPA, $\alpha_1$-antichymotrypsin, $\alpha_2$-M, α-PI, MMP-7, -8, -13 |
| | MMP-10 | Stromelysin-2 | 11q22.3-q23 | 57.000/44.000 | Collagen III, IV, V, aggrecan, elastin, fibronectin, gelatin, laminin | MMP-1, -8 |
| | MMP-12<br>MMP-19 | Metallo-elastase | 11q22.2-q22.3<br>12q14 | 54.000/45.000 | Collagen IV, elastin, fibronectin, gelatin, laminin, aggregan, fibronectin, amelogenin | IGFBP-3, plasminogen, apolipoprotein (a), uPA, $\alpha_1$-antichymotrypsin, $\alpha_2$-M, α-PI, E-caderin, MMP-1, -7, 8, -9, -13 |
| | MMP-20<br>MMP-27 | Enamelysin | 11q22.3<br>11q24 | 54.000/22.000 | Aggregan, amelogenin, gelatin | Unknown |
| Gelatin-binding | MMP-2<br>MMP-9 | Gelatinase-A<br>Gelatinase-B | 16q13<br>20q11.2-q13.1 | 72.000/66.000<br>92.000/86.000 | Collagen I, II, III, IV, V, VII, X, XI, aggrecan, elastin, fibronectin, gelatin, laminin | IGFBP-3, -1, -5, IL-1β, ICAM-1, MCP-3, IL-2Rα, TGFβ, SDF-1, IL-8, IFNγ, FGFR-1, Kit-L, plasminogen, galectin-3 |
| Furin-activatedsecreted | MMP-11<br>MMP-28 | Stromelysin-3<br>Epilysin | 22q11.2<br>17q21.1 | 51.000/44.000 | Collagen IV, aggrecan, fibronectin, laminin | $\alpha_2$-M, α-PI, IGFBP-1, casein |
| Vitronectin-likeinsert | MMP-21 | – | 10q26.13 | 65.000/62.000 | Unknown | Unknown |

**Table 1** (continued)

| Structure | Name | Pseudonym | Human chromosome | Molecular weight, Da (latent/active) | Matrix substrates | Bioactive substrates |
|---|---|---|---|---|---|---|
| Trans-membrane Type I (MT-MMPs Type I) | MMP-14 | MT1-MMP | 14q11-q12 | 66.000/56.000 | Collagen I, II, III, aggrecan, elastin, fibronectin, gelatin, laminin, vitronectin, fibrin | MCP-3, MUC-1, CD-44, transglutamatase, MMP-2, -13 |
| | MMP-15 | MT2-MMP | 15q13-q21 | 72.000/60.000 | | |
| | MMP-16 | MT3-MMP | 8q21 | 64.000/52.000 | | |
| | MMP-24 | MT5-MMP | 20q11.2 | –/62.000 | | |
| Glycosyl-phosphatidyl-inositol MT-MMPs (GPI-anchored MT-MMPs) | MMP-17 | MT4-MMP | 12q24.3 | 57.000/53.000 | Collagen IV, fibronectin, fibrin, gelatin, laminin | Unknown |
| | MMP-25 | MT6-MMP | 16p13.3 | | | |
| Trans-membrane type II (MT-MMPs Type II) | MMP-23A | CA-MMP/ Femalysin | 1p36.3 | 44.000/36.000 | Gelatin | Unknown |
| | MMP23B | | | | | |

[a]No human homologue known.




**Figure 1:** Structure of secreted MMPs and trans-membrane-type MMPs (TM-MMPs).
(A) Minimal domain MMPs: MMP-7, MMP-26; (B) archetypal MMPs (simple hemopexin-domain): MMP-1, MMP-8, MMP-13, MMP-18, MMP-3, MMP-10, MMP-12, MMP-19, MMP-20, MMP-27; (C) gelatin binding MMPs: MMP-2, MMP-9; (D) furin activated MMPs: MMP-11, MMP-28; (E) vitronectin insert MMPs: MMP-21; (F) trans-membrane-type I MMPs: MMP-14, MMP-15, MMP-16, MMP-24; (G) glycosyl-phospatidyl-Inositol (GPI-anchored) MMPs: MMP-17, MMP-25; (H) trans-membrane-type II MMPs: CA-MMP (MMP-23A,MMP-23B). Key: Pre, amino-terminal-signal sequence; Pro, pro-peptide; SH, thiol group; catalytic, catalytic domain; Zn, zinc-binding site; Fi, fibronectin; Fu, furin; Vn, vitronectin; TM, trans-menbrane domain; CY, cytoplasmic domain; GPI, glycosyl-phospatidyl-inositol; SA, N-terminal signal anchor; CA, cysteine array; Ig-like, immunoglobulin like domain.

blocks the active site. The pro-domain of some MMPs shows a recognition sequence for furin-like serine proteinases, which is needed for the pro-domain cleavage and, consequently, for the MMPs activation [4].

Concerning biological function, MMPs have been shown to be critically involved in many normal biological processes, including organ development, wound healing, tissue remodeling, morphogenetic changes, etc., as well as in several pathological conditions, such as cancer, arthritis, cardiovascular diseases, nephritis, neurological diseases, breakdown of blood-brain barrier, skin ulceration, inflammation, diabetes, etc. [5]. They are important in creating an environment that support the initiation and maintenance of growth of primary and metastatic tumors, and have an important regulatory role, as they can modulate cytokines and chemokines activity by proteolytic processing [6]. Primary function of MMPs is tissue growth and remodeling by selective proteolytic degradation. Therefore, in order to avoid uncontrolled

ECM turnover, inflammation, cell growth and migration, under physiological conditions, the activity of matrix metallo-proteinases is accurately regulated at the levels of transcription, zymogen activation and inhibition by endogenous inhibitors. Normally, MMPs are specifically inhibited by tissue inhibitors of metallo-proteinases (TIMPs), and the MMP/TIMP balance is considered to be a major factor in the regulation of the net proteolytic activity of the individual MMPs. In humans, four individual species of TIMPs are known: TIMP-1, -2, -3, and -4. TIMP expression is regulated during development and tissue remodeling and under pathological conditions associated with unbalanced MMPs activity [7]. All MMPs are secreted in a latent form as inactive pro-enzyme owing to the presence of a pro-domain, which binds the zinc atom in the catalytic site and inhibits enzymatic activity (Figure 1). MMPs become active after the disruption of this bond by chemical modification or by enzymatic removal of the pro-domain. This activation step may occur either intracellular (catalyzed by furin) or extracellular (by other MMPs, TIMPs or serine proteases) [3–7]. The basic action of MMPs (e.g. cleavage of proteins) has proven sufficiently sophisticated to orchestrate various functions, specific modulators of angiogenesis as well as fine-tuners of cell signaling pathways and the inflammatory response. Moreover, recent evidences indicate that MMPs may even work in a non-proteolytic manner [6].

## Gelatinases

One of the most studied classes of matrix metallo-proteinases are the gelatinases or collagenases type IV, historically defined as their affinity for denatured collagen (e.g. gelatin), which are widely recognized to participate to the etiology of a plethora of neoplastic and non-neoplastic pathologies.

This class includes two members, namely (i) gelatinase A or MMP-2 (72 kDa and 62 kDa for the pro-enzyme and the active enzyme, respectively); (ii) gelatinase B or MMP-9 (92-85-82 kDa for the pro-enzyme, the intermediate form and active enzyme, respectively). Gelatinase A (MMP-2) is a no-glycosylated protein, which is strictly present as a monomer in plasma. MMP-2 is expressed in normal fibroblasts, endothelial and epithelial cells as well as in many transformed cells and in tumor-associated fibroblasts. MMP-2 digests native collagen types I, II and III in a similar manner to the collagenases. However, the collagenolytic activity of MMP-2 is much weaker than MMP-1 or other collagenases. Nevertheless, because pro-MMP-2 is recruited to the cell surface and activated by the membrane-bound MT-MMPs, it may accumulate pericellularly and express reasonable local collagenolytic activity. It may also act as a 'collaborator' activity, digesting collagenase-clipped collagen to smaller fragments because those fragments denature at body temperature (37 °C). Moreover, MMP-2 cleaves several different substrates, including cytokines, growth factor, receptors or binding factors. The MMP-2 activity is tightly regulated by TIMPs. In particular, TIMP-2 display relevant affinity for MMP-2 as well as TIMP-3 and -4. Therefore, their adequate secretion is required for a balanced MMP-2/TIMP ratio. The regulation of MMP-2 activity occurs at many levels, among which regulation through TIMP-2 and its cell surface receptor, MT1-MMP (MMP-14) are critically decisive. In fact, TIMP-2 forms a binary complex with MT1-MMP by binding its catalytic site, leaving no free MT1-MMP receptors. Therefore, the complex is able to recruit proMMP-2, by linking the hemopexin domain, and to inhibit its activation [8]. Moreover, the expression of TIMP-2, MMP-2 and MT1-MMP is co-regulated transcriptionally, indicating an intricate network of regulation. Pro-MMP-2 activation is also seen by complex signaling induced by ECM proteins like osteopontin or IL-8 produced by endothelial cells [9–11]. Gelatinase B (MMP-9) contains two N-glycosylated sites in the pro-domain and the catalytic domain, and a number of O-linked glycans. In plasma, MMP-9 exists as a monomer or a dimer, complexed with neutrophil gelatin-associated lipocalin (NGAL). It is expressed in normal leukocytes, macrophages, pericytes, endothelial cells as well as in transformed cells. MMP-9, as well as MMP-2, is involved in the mechanical removal of structural proteins in the extracellular matrix, but also in regulation of multiple cellular functions including cell growth, apoptosis, angiogenesis, invasion, metastasis and immune response by cleaving growth factor-precursors, cell adhesion molecules, cell surface receptors and other bioactive proteins. The structure is almost similar to MMP-2. The nascent form of MMP-9 shows an N-terminal signal sequence (pre-domain) which directs the protein to the endoplasmic reticulum. The pre-domain is followed by a pro-peptide (pre-pro-domain) that maintains enzyme-latency until it is cleaved or disrupted, and a catalytic domain that contains the conserved zinc-binding region. A hemopexin domain is connected to the catalytic domain by a hinge or linker region. The hemopexin domain is involved in NGAL, extracellular matrix components, β-hematin and TIMPs (TIMP-1 and TIMP-3) binding. Moreover, the structure also shows a series of three head-to-tail cysteine-rich repeats within its catalytic domain. These inserts resemble the collagen-binding type II repeats of fibronectin

and are required to bind and cleave collagen and elastin. Like other proteolytic enzymes, MMP-9 is first synthesized as inactive proenzyme or zymogens. Activation of proMMP-9 is mediated by plasminogen activator/plasmin (PA/plasmin) system [12]. MMP-9 expression is regulated by several cytokines and growth factors, including interleukins, interferon, epidermal growth factor (EGF), nerve growth factor (NGF), basic-fibroblast growth factor (b-FGF), vascular endothelial growth factor (VEGF), platelet derived growth (PDGF), tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), the extracellular matrix metalloproteinase inducer EMMPRIN and also osteopontin. Many of these stimuli induce the expression and/or activation of c-fos and c-jun proto-oncogene products, which promote the binding of activator protein-1 complex (AP-1) sites within of MMP-9 gene promoters [13]. Primary function of MMP-9 is degradation of proteins in the extracellular matrix. It proteolytically digests decorin, elastin, fibrillin, laminin, gelatin, and types IV, V, XI and XVI collagen and also activates growth factors like pro-TGF-β and pro-TNF-α. Physiologically, MMP-9 in coordination with other MMPs, play a role in normal tissue remodeling events such as neuronal growth, embryonic development, angiogenesis, ovulation, mammary gland involution and wound healing, osteoblastic bone formation and/or inhibition of osteoclastic bone reabsorption [10, 14, 15]. In chronic lymphocytic leukemia, MMP-9 promotes B cell survival in a non-proteolytic fashion via its hemopexin domain by docking to the surface receptors α4β1 and CD44, which induce intracellular signaling, involving Lyn activation and STAT3 phosphorylation, which prevents B cell apoptosis [16].

The role of MMP-2 and MMP-9 in human diseases is supported by several in vivo and in vitro evidences, even though they follow distinct and even opposite patterns, consistently with the notion that MMP-9 has a pro-inflammatory property, whereas MMP-2 has a pro-homeostatic one [17].

Nowadays, it is widely recognized that gelatinases participate to the etiology of a plethora of pathologies such as cardiovascular and auto-immune diseases which, taken altogether, represent a leading cause of mortality and morbidity in Western countries. In particular, in the case of cardiovascular diseases, gelatinases participate both to the genesis of the atherosclerotic lesions and to the acute event (i.e. stroke or myocardial infarction) [18]. In fact, high levels of MMPs have been detected in the atherosclerotic plaque, and activation of MMPs appears to facilitate atherogenesis, platelet aggregation and plaque destabilization which follow the fibrous cup rupture [19, 20].

In the auto-immune diseases, gelatinases are involved both in the generation of remnant epitopes and in the modulation of cross-talking between immune system compartments [21]. Moreover, gelatinases have been found to have an important role also in metabolic disorders, such as type 2 diabetes and obesity [22], and in neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) and Alzheimer's dementia (AD) [23].

Among the wide variety of physiological and pathological conditions in which gelatinases are involved, cancer progression is the most extensively studied. Initially gelatinases were thought to have a role exclusively in invasion and metastasis processes, but recent studies demonstrated that they are also critically involved in several steps in cancer formation and during the growth of tumor cells by releasing of cell membrane bound precursors of some growth factors, or the inhibition of apoptosis pathways, for example, by cleaving the Fas ligand [24]. Several studies, in fact, identified that the expression of gelatinases is enhanced in many primary tumors, such as colon-rectal cancer, breast cancer or gastric cancer [25–27]. The overexpression of gelatinases, both in neoplastic tissue and in body fluids, has been shown to correlate with grade or stage and they may also be predictor of disease-free survival after treatment, or may correlate to overall cancer-specific survival [28]. Our previous studies, in fact, identified a higher MMP-9 gelatinolytic activity in urine from bladder cancer patients [29] compared to healthy controls, as well as in sera from clear cell renal cell carcinoma (ccRCC) subjects compared to oncocytoma patients [30].

Therefore, thanks to their value as potential prognostic indicators, MMP-9 and MMP-2 represent the most promising cancer biomarkers which might offer useful clinicians information for improving cancer patients managements.

## Detection of gelatinases

Several methods have been developed for analysis of active and latent forms of gelatinases A and B in biological samples. Here, we briefly describe the main techniques used for detection of MMP-2 and MMP-9, focusing on substrate-zymography and discussing the major advantages and disadvantages.

### Enzyme-linked immunosorbent assay (ELISA)

In vitro ELISA kits for human, as well as other species, MMP-2 and MMP-9 are commercially available (e.g.

Biotrack®, Quantikine HS® R&D Systems, etc.). However, these assays are not able to distinguish between the active and latent forms of the enzymes or between specific MMPs and their TIMP complexes. The major disadvantage of this technique is that, even if the high sensitivity, it requires two different antibodies for each individual MMP, and a separate assay plate must be used for the measurement of each MMP, making for a very time consuming assay. Also multiplexed ELISAs (e.g. Biorad Bioplex®), which allow the simultaneous determination of each of gelatinases, have been reported to exhibit high cross-reactivity among various MMPs because these proteins share common domains [31, 32].

## Western blotting

For Western blot analysis, samples electrophoresed by 10% SDS–PAGE are transferred onto PVDF or nitrocellulose membranes. After incubation with a blocking buffer, saturated membrane is blotted overnight with a primary antibody anti-MMP-2 or anti-MMP-9, respectively. The following incubation of membrane with a secondary antibody and a luminescent substrate allows signal revelation which is directly related to protein amount. Compared to ELISA, Western blotting allows separation of the protein mix by size, charge, and/or conformation and allows the detection of several targets, contrary to ELISA where only one protein can be detected. However, the technique is time-consuming, requires optimizing the experimental conditions (i.e. protein isolation, buffers, type of separation, gel concentration, etc.), and most of all the detection limits are much lower than those of other methods heals, potentially leading to false negative results. Moreover immunoblotting analysis requires the availability of antibodies and the inhibition of proteolytic activity of enzymes during protein blocking and immunochemical reactions [31, 32].

## High performance liquid chromatography-mass spectrometry (HPLC/MS)

Mass spectrometry-based proteomics has become a very powerful tool in MMP research. Identification of gelatinases by mass spectrometry involves enzymatic digestion after capture of the MMP using biotin-azide tags and avidin chromatography followed by analysis of the MMP-digests by HPLC/MS. This technique is able to reveals all the proteins present in a sample, and unlike classical Western blotting it is quantitative. However, despite the high sensitivity, it requires high costs and also more technically challenging to use compared to Western blotting. In addition, the method is very lengthy and still not fully reliable when it comes to identifying proteins in the digested form, because the identification often relies on the presence of only a few peptides from the digested protein [31, 32].

## In situ zymography (ISZ)

Zymography in situ (ISZ) allows the localization of MMPs in tissue sections. The process provides the incubation of frozen sections of unfixed tissue sample with a specific substrate (e.g. gelatin, in case of MMP-2 and MMP-9) which will be digested by the activated MMPs in time- and dose-dependent manner. The degradation of substrate, detected by light or fluorescent microscopy as white spots on black background, directly correlates with the enzymatic activity. A big limitation of this technique is that it is not able to discriminate between different classes of MMPs and digestion process may be influenced by other proteases (e.g. serine or cysteine proteases). This limitation may be reduced by combining ISZ with immunohistochemistry (IHC). The immunolocalization of the specific MMPs, in fact, can be compared with the localization pattern of the ISZ. Moreover, the combination ISZ-HIC may be useful for co-location of proteins such as cell type markers or other proteins of interest, and can be also adapted for time-lapse analysis of MMPs activity and analysis of MMPs activity in migrating cells. However, while IHC is not able to discriminate between active and inactive forms of the enzymes, ISZ can evaluate net functional activity. A sensitive advance in this technique is the use of quenched fluorogenic substrates which allow evaluation of enzymatic activity by using confocal microscopy [33, 34].

## Substrate-zymography

Substrate-zymography still represents the most simple, sensitive and quantifiable, unexpensive and functional assay for MMPs analysis, which is able to identify, simultaneously in the same sample, the entire panel of enzymes that are capable of degrading a specific substrate. In particular, the identification of gelatinases A and B activities is possible by using of gelatin as substrate (gelatin zymography). Gelatin-zymography allows determining simultaneously both active and latent forms of gelatinases A and B, in biological fluids (e.g. serum, urine, pleural effusion,

cell culture medium, etc.) as well as in tissues. The proteins are separated by electrophoresis under denaturing [sodium dodecyl sulfate (SDS)] but non reducing conditions, without prior boiling. In fact, boiling cause protein precipitation of the enzymes, and reducing agents (i.e. mercaptoethanol, 1,4-dithiothreitol, urea) break the disulphide bond and inhibit the MMP refolding after electrophoresis. The separation occurs in a polyacrylamide gel containing the gelatin substrate which is co-polymerized with the acrylamide. During electrophoresis, the SDS causes the gelatinases denaturation, so they become inactive. The inactivation of gelatinases during zymography is believed to involve the dissociation of $Cys^{73}$ from the zinc molecule, caused by SDS. After the gel has been run, it is incubated overnight at 37 °C in a nonionic detergent (such as Triton® X-100) which causes the exchange of the SDS, and then in an appropriate "refolding" buffer which contains $Ca^{2+}$, because gelatinases are Ca-dependent enzymes. During this incubation, the enzymes partially recover their structure and activity and the latent gelatinases are auto-activated without cleavage. If the proteinases are active, the gelatin is digested and converted to low-molecular weight peptides that are washed out of the gel. Subsequently staining with Coomassie® Blue indicates the zone of lysis as a clear region on a uniform blue background of undegraded substrate (e.g. Figure 2, panels A, B and C). The clear bands in the gel can be quantified by densitometry. By using an image analysis software, it is possible to determine the molecular weight, volume and background of each band. The pixel density may be determined after background subtraction and used to calculate the integrated density of a selected band. Values of integrated density are reported in volume units of pixel intensity per $mm^2$. The relative amounts of the different forms of both fluid and tissue gelatinases are expressed as the integrated density $\times 10^{-3}$ (volume) of all the pixels

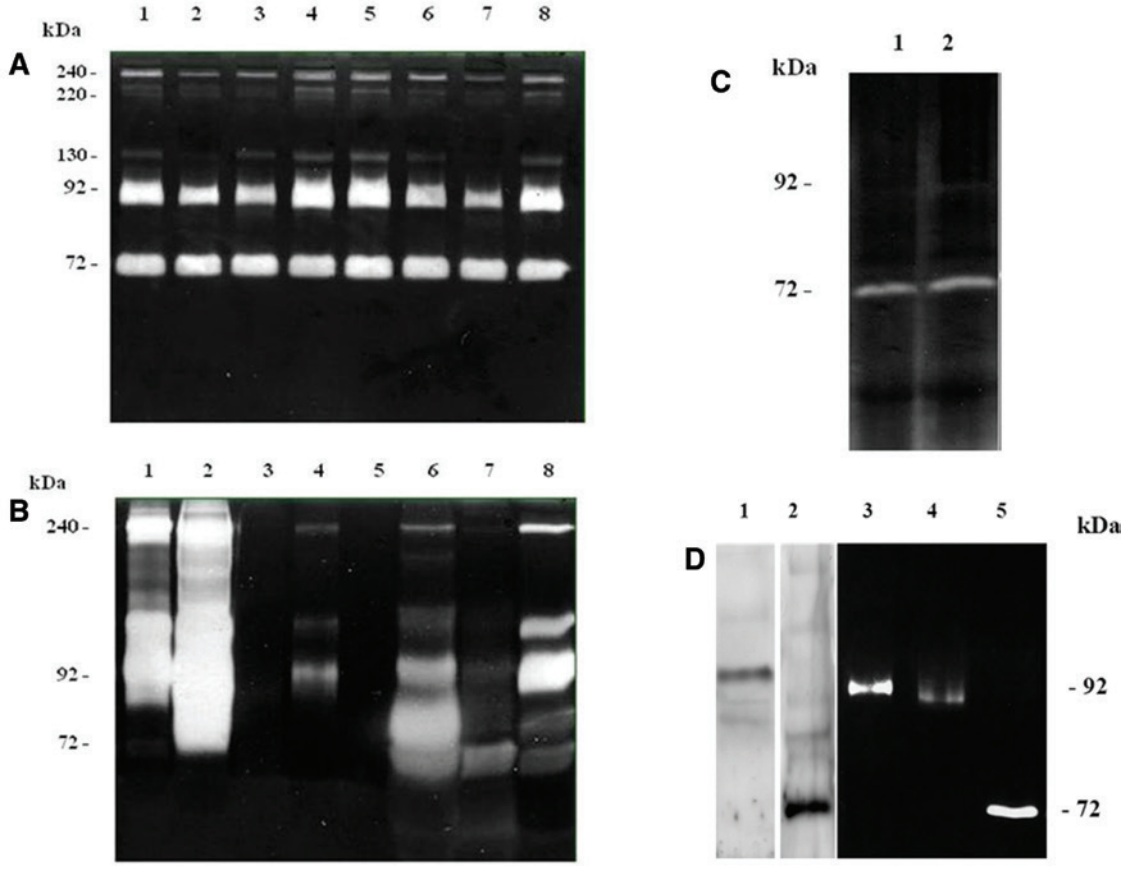


**Figure 2:** Panels of representative gelatin zymograms.
(A) Zymogram (0.6% gelatin) of sera from kidney cancer patients. In all samples 25 μg of protein was loaded onto the gel. (B) Zymogram (0.1% gelatin) of urine from kidney cancer patients. In all samples 12 μL of concentrated urine was loaded onto the gel. (C) Zymogram (0.1% gelatin) of brain tissue samples. In all samples 50 μg of protein was loaded onto the gel. (D) 1: Western blotting of pro-MMP-9 (92 kDa); 2: Western blotting of pro-MMP-2 (72 kDa); 3: Zymograms (0.6% gelatin) of purified gelatinase B (20 μU); 4: Zymograms (0.6% gelatin) of purified gelatinase B (10 μU); 5: Zymograms (0.6% gelatin) of purified gelatinase A (120 mU).

above the background of each band. The molecular size of bands displaying enzymatic activity is identified by comparison with pre-stained standard protein, as well as with purified gelatinase A or gelatinase B. The nature of lytic bands observed in zymograms is further confirmed by an inhibition assay, which is performed by using control gels containing either of the MMP selective inhibitors, EDTA or phenanthroline, in the sample loading buffer. Furthermore, the character of proteolytic bands is analysed by incubating the identical zymograms in PMSF, a serine protease inhibitor; or Pefabloc, an irreversible serine protease inhibitor. The immunological detection of lytic bands can be also performed by Western blotting with antibodies against MMP-2, MMP-9 which recognize the pro-forms of both enzymes (Figure 2, panel D) [33–36].

Critical step in performing substrate zymography is sample preparation. Body fluids samples, such as peripheral venous blood or first morning urine, should be used fresh and only once in order to prevent enzyme activation due to freeze-thawing processes. The discrepancy in MMP zymographic profiles between serum and plasma has been widely reported. In particular, referring to gelatinases, MMP-2 activity and concentration show no significant difference between plasma and serum, while the levels and zymographic separation of the MMP-9 is strongly affected by anticoagulants, which suggests a releasing mechanism during coagulation and fibrinolysis [37].

Regarding urine samples, hematuric samples or urine positive for leukocytes should be eliminated in order not to confound leukocytic gelatinases. Moreover, prior to analysis, urine should be concentrate by using membrane with a molecular weight cut-off of about 30,000 Da [29, 30].

Also tissue extracts should be used fresh or after rapid freezing using liquid nitrogen and stored at −80 °C. The same tissue protein extraction procedure represents a critical step in substrate-zymography, because it may cause interactions of enzymes with their respective inhibitors or may inactive them. For this reason, the extraction buffer does not have to contain protease inhibitors, EDTA or any other Zn-chelator. For many tissue types it would be preferable to purify homogenate by using gelatin-sepharose. Zang et al., in fact, demonstrated that use of gelatin-Sepharose 4B allows both the effectively separation of gelatinases from the dominance of other tissue proteins and the concentration of extracted activity from the entire tissue [38].

Substrate-zymography presents several advantages compared to other main analytical technique for studying of proteases (Table 2). The main advantage of using gelatin-zymography is that this technique does not require expensive materials (e.g. antibodies) and it is extremely sensitive because levels of 10 pg of MMP-2 and 32 pg of MMP-9 can be already detected. For this reason, zymography has been proven to be much more sensitive than Western blot analysis, because it is very difficult to identify antibodies sensitive enough to detect small amounts of MMPs.

**Table 2:** Advantages and disadvantages of substrate zymography.

| Advantages | Disadvantages |
|---|---|
| Low cost | No discrimination between free MMPs and MMPs complexed with TIMPs and/or NGAL |
| High sensitivity, pg | No information about localization of proteolitical activity |
| Distinguish different species of enzymes due to mobility difference in same gel | Interference by other MMPs |
| Detection of active and latent form of the same protease | Long protocol (2 days) |
| No interference by gelatinases inhibitors (TIMPs) | |

Quantification of proteolytic activity of samples subjected to zymography may be difficult at times because (i) the limited number of wells per gel does not allow a full standard curve and several samples to be run on the same gel and (ii) the two-step staining/destaining method is not reliable and is difficult to reproduce. Leber et al., developed an enhanced method which use a single-step staining-destaining procedure which leads to faster and more reproducible results during quantification [39].

A particular advantage of this system is also the opportunity to detect and to quantify on a single gel proteases with different molecular weights, which show activity towards the same substrate. Indeed, the pro-forms of MMPs become activated during the process of denaturation and renaturation; after gel electrophoresis, the active forms and the originally inactive forms degrade gelatin, and both forms can be detected on zymogram. Another positive aspect which needs to be considered is that during electrophoresis, SDS promotes the dissociation between MMPs and their endogenous inhibitors (TIMPs) so they do not interfere with detection of the enzymatic activity.

On the other hand, substrate zymography is not able to discriminate between free MMPs and complexed to TIMPs or other protein. In fact, MMP-9 can be associated with lipocalin-2 (NGAL) giving a band at ~130 kDa, and can also form dimer and multimer giving bands at ~220 kDa and ~240 kDa, respectively. These complexes are not dissociated in zymogram and might represent a complication during the analysis of data. Moreover, substrate

zymography cannot give information about localization, in cells or tissues, of the proteolytic activity. In addition, it should be considered that other MMPs, such as MMP-1, MMP-8, and MMP-13 can also lyse the gelatin substrate.

The new advances of this method are basically focused towards two dimensional zymography which combines isoelectric focusing (IEF) with zymographic electrophoresis to achieve significant improvement in separation of the enzymatic isoforms and, most of all, to identify possible post-translational modification which can modulate enzymatic activity. For example, Chen et al., by using 2D gelatin zymography were able to detect organomercuric chemical 4-aminophenylmercuricacetate-induced activation of MMP-2 isoforms with variant pI values in the conditioned medium of human fibrosarcoma cells and also several MMP-9 pro-forms, with different pI values, in murine LPS-stimulated BV-2 cells [40].

Finally, as reported by Vandooren et al., zymography technique has been proposed as the most suitable and convenient methods in "reverse degradomics" studies for measurement of the total impact of a complex biological sample on a particular substrate, which, for example, may be linked to a particular human disease. To reach this aim, zymography has been demonstrated to be worthwhile technique in providing information on the spatial distribution of activity, an important parameter which cannot be obtained with the currently available high-throughput (reverse) degradomics platforms [36].

## Concluding remarks

Matrix-metalloproteinase 2 (gelatinase A) and matrix-metalloproteinase 9 (gelatinase B) are known to have clear roles in physiological tissue turnover and remodeling, but contribute also to a number of pathological events. Therefore, they obtained a deep interest as potential non-invasive biomarkers. The study of MMP-2 and MMP-9 in physio-pathological processes requires a reliable method for the assessment of the enzymatic activity, and substrate zymography still represents a fascinating and advantageous technique in molecular biology for the study of MMPs proteolytic activity. Further improvements of zymography-based techniques will expedite the development of new diagnostic and/or prognostic tools for a wide variety of human disease.

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