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Assessment of Gelatinases (MMP-2 and MMP-9) by Gelatin Zymography

Marta Toth and **Rafael Fridman**

1. Introduction

The invasion and metastasis of tumor cells has been shown to require proteolytic activity in order to degrade components of the extracellular matrix (ECM). The hydrolysis of the ECM appears to facilitate tumor cell migration contributing to the metastatic dissemination of malignant cells (1). A major group of proteases that has been directly associated with tumor metastasis is the matrix metalloproteinases (MMPs), a family of endopeptidases known to cleave many ECM proteins (1). The MMPs are multidomain proteases that contain a zinc atom in the active site and are produced in a latent inactive form (zymogen) (2). Acquisition of enzymatic activity requires cleavage of the inhibitory N-terminal domain (3). Thus, generation of the active form usually occurs concomitantly with a decrease in molecular mass and exposure of the active site. Once activated, all the MMPs are specifically inhibited by a group of endogenous protease inhibitors known as the tissue inhibitors of metalloproteinases (TIMPs), which bind to the active site inhibiting catalytic activity (4).

The gelatinases MMP-2 (gelatinase A, EC 3.4.24.24) (5) and MMP-9 (gelatinase B, EC 3.4.24.35) (6) are two members of the MMP family that have been extensively studied owing to their consistent association with tumor invasion and metastasis. It has been reported that the expression and activity of gelatinases are elevated in many malignant human tumors and correlate with tumor progression (7,8). MMP-2 and MMP-9 were originally described as type IV collagenases because of their ability to promote the hydrolysis of collagen IV (5,6), a major component of basement membranes and a major structural barrier for tumor cell invasion. Both enzymes can also cleave a variety of ECM proteins but they are extremely efficient in hydrolyzing denatured collagen I (gelatin) and consequently they are referred to as gelatinases (9). The ability of MMP-2 and MMP-9 to degrade denatured collagen I was developed into a relatively easy yet powerful technique to detect their presence in biological samples. This technique, known as gelatin zymography, identifies gelatinolytic activity in biological samples using sodium dodecyl sulfate (SDS)-polyacrylamide gels impregnated (copolymerized) with gelatin (10,11). The methodology for SDS-polyacrylamide gel electrophoresis (PAGE) has already been described in the chapter by Blancher and Jones in this volume. To maintain enzymatic activity, the samples are electrophoresed under nonreducing conditions. After removal of the SDS from the gel by Triton X-100 and incubation in a calcium-containing buffer, the partially renatured enzymes can degrade the gelatin leaving a cleared zone that can be detected after staining of the gel. In the presence of SDS the enzymes are denatured exposing their active site, which permits both the latent and active forms of the gelatinases to exhibit gelatinolytic activity after the partial renaturation. Furthermore, SDS disrupts the noncovalent interactions between gelatinases and TIMPs, allowing for the detection of gelatinase activity independently of the presence of TIMPs. Detection of gelatinase activity in latent enzymes and/or in enzymes derived from samples rich in TIMPs is a major paradox of gelatin zymography. Under

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physiological conditions, latent gelatinases do not possess enzymatic activity (3,9). Likewise, in the presence of TIMPs, gelatinase activity is usually inhibited (4). Thus, gelatin zymography cannot provide definite information on the net MMP-2- and MMP-9-dependent proteolytic activity present in a given sample because it does not take into account endogenous inhibitors. Gelatin zymography, however, is a useful qualitative tool for the detection and analysis of the level and type of the gelatinases expressed in different cell types/tissues at any given time and/or after different treatments. For example, it is possible to determine which gelatinases are expressed in tumor cells with various degrees of invasive potential whether they are derived from established cancer cell lines or from tumor biopsies. Furthermore, the regulation of gelatinase expression in vitro in response to a variety of factors can be studied. It should be pointed out, however, that owing to the enzymatic nature of the method and the many variables involved, zymography is too crude to be used as a quantitative technique (discussed in Note 1).

Another important feature of the gelatin zymography technique is the ability to assess the activation status of the enzymes. Because the active forms of the gelatinases do not normally possess the inhibitory N-terminal domain (~10 kDa), the molecular mass of the species detected can be used, in some cases, as an indicator of the degree of zymogen activation in the sample. Therefore, it is possible to correlate the presence of active forms with the invasive characteristics of a given cell/tumor type. It is also possible to study the factors that play a role in gelatinase activation. However, as will be discussed in **Subheading 2** (*see* Note 2), discrimination between active and latent gelatinase species using gelatin zymography is prone to serious pitfalls. Nevertheless, owing to the simplicity and sensitivity of the gelatin zymography it is the method of choice to assess expression of gelatinases in tumor cells. Here we provide a detailed protocol on how to set up the gelatin zymography technique and how to use it in samples relevant for the study of tumor cell invasion and metastasis. We have also provided hints for the interpretation of results.

2. Materials

1. Disposable plastic gel casting cassettes, 1.0 mm thick, 10 × 10 cm.
2. Acrylamide–*bis*-acrylamide stock: Prepare 200 mL of 30% w/v acrylamide, 0.8% w/v *bis*-acrylamide solution in distilled water (dH_2O) (*see* Note 3). Use a surgical mask when weighing acrylamide powder. Store the acrylamide solution in a dark

[1]Regarding the use of gelatin zymography for quantitative assessments: Although gelatin zymography has been claimed to be a quantitative technique, major problems arise during the process of method standardization. As with any quantitative assay, a reproducible and accurate standard is essential. However, owing to the many variables involved in the assay including (a) variations in the amount of copolymerized gelatin; (b) nature, source, and loading of the sample; (c) incubation time and temperature; (d) washing conditions; (e) staining and destaining conditions; and (f) source and condition (latent, active) of the standards, it is unreliable to establish reproducible assay conditions when analyzing multiple samples.

[2]Latent vs active species: Because gelatin zymography is widely used to study gelatinase activation, it is important to be aware of the pitfalls. Indeed, the complexity of the activation process and the inherent limitations of SDS-PAGE make identification of active species by gelatin zymography not straightforward. Gelatinases are usually activated in a sequential process involving generation of inactive intermediate species. Therefore, if good band separation and mass determination are not achieved, the intermediate species can be easily confused with the active forms. In addition, owing to differences in glycosylation between the precursor (intracellular) and the mature (secreted) form of latent MMP-9 (16), the intracellular precursor form of MMP-9 (~85 kDa) can be easily mistaken for the active species. This is prone to occur when the samples examined are derived from cell lysates or tissue extracts, all of which may contain the intracellular precursor enzyme (Fig. 1). Inclusion, in the same gel, of active enzymes (**Subheading 3.2.5.**) as standards and/or reliable unreduced molecular weight markers (Mark 12 from Novex, cat. no. LC5677) will aid in discriminating between inactive (intermediate, precursor) and active species. When in doubt, additional methods including immunoblot analysis with antibodies recognizing latent or active species, inhibitor trapping (α2-macroglobulin) (3) and/or enzymatic assays are required to determined the activation status of the gelatinases.

[3]For reliable electrophoresis results, it is crucial to prepare a good acrylamide–*bis*-acrylamide stock solution from quality reagents. Ready-to-use 30% acrylamide–*bis*-acrylamide 37.5:1 (2.6% C) solution can be purchased from Bio-Rad Laboratories (Hercules, CA; cat. no. 161-0158), allowing reproducible results and preventing the handling of acrylamide powder.

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bottle at 4°C where it is stable for at least 6 mo. Unpolymerized acrylamide is a neurotoxin; **always handle with gloves**!

3. Separating gel buffer stock: Prepare 200 mL of 1.88 *M* Tris-HCl buffer, pH 8.8. Autoclave and store it at room temperature. It is stable for at least 6 mo.
4. Stacking gel buffer stock: Prepare 200 mL of 1.25 *M* Tris-HCl buffer, pH 6.8. Autoclave and store it at room temperature. It is stable for at least 6 mo.
5. 1% w/v Gelatin (Sigma, St. Louis, MO, cat. no. G-8150): Dissolve the gelatin in 5 mL of dH_2O and heat the solution at 60°C in a water bath for at least 20 min; mix well. Make sure that the gelatin is completely dissolved. Cool down the gelatin solution to room temperature before use. Prepare it fresh.
6. 20% w/v SDS: Prepare 200 mL of 20% SDS in dH_2O. Use a surgical mask when weighing SDS. Store it at room temperature. It is stable for 1 yr.
7. 10% w/v Ammonium persulfate (APS): Prepare 1–5 mL of 10% APS in dH_2O, depending on the number of gels to be prepared. Store it at 4°C for no longer than 2 wk.
8. *N*, *N*, *N*′, *N*′-tetramethylethylenediamine (TEMED): Store it in a dark bottle at 4°C.
9. Running buffer stock (10X): Prepare 1 L of 0.25 *M* Tris base and 1.92 *M* glycine, pH 8.3. The pH should be correct without adjusting. Store it at room temperature. It is stable for months.
10. Running buffer: Dilute the running buffer stock 10× with dH_2O to make 1 L and supplement with 5 mL of 20% SDS to a final concentration of 0.1%. Store it at room temperature. It is stable for months.
11. Sample buffer (4X): Prepare 10 mL of 250 m*M* Tris-HCl, pH 6.8; 40% glycerol; 8% SDS; and 0.01% bromophenol blue. Store it at −20°C in 0.5-mL aliquots. Before use, warm it up to dissolve the SDS.
12. Renaturing solution stock (10X): Prepare 200 mL of 25% v/v Triton X-100 in dH_2O. Store it at room temperature. It is stable for months.
13. Developing buffer stock (10X): Prepare 1 L of 500 m*M* Tris-HCl, pH 7.8; 2 *M* NaCl, 50 m*M* $CaCl_2$; and 0.2% Brij 35. Store it at 4°C for 6 mo.
14. Staining solution: Prepare 1 L of 0.5% Coomassie blue R-250, 5% methanol, and 10% acetic acid in dH_2O. Filter. Store it at room temperature. This solution is reusable.
15. Destaining solution: Prepare 1 L of 10% methanol, 5% acetic acid in dH_2O. Store it at room temperature for months.
16. Phosphate-buffered saline (PBS): Prepare 1 L of 10 m*M* phosphate buffer, pH 7.1; 137 m*M* NaCl; and 2.7 m*M* KCl. Autoclave and store it at room temperature. It is stable for months.
17. Lysis buffer: Prepare 100 mL of 25 m*M* Tris-HCl, pH 7.5; 100 m*M* NaCl; and 1% Nonidet P-40 (NP-40). Store it at 4°C for 6 mo. Right before use add protease inhibitors: 10 μg/mL aprotinin, 2 μg/mL leupeptin, and 4 m*M* benzamidine.
18. Tris-buffered saline (TBS): Prepare 1 L of 50 m*M* Tris-HCl, pH 7.5; 150 m*M* NaCl. Store it at 4°C for months.
19. TBS-CM: Prepare 200 mL of TBS supplemented with 1 m*M* $CaCl_2$ and 1 m*M* $MgCl_2$. Store it at 4°C and use within a week.

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20. TBS-B: Prepare 50 mL of TBS containing 5 m*M* $CaCl_2$ and 0.02% Brij-35. Store it at 4°C for 1 mo or until a visible precipitate appears.

21. TBS-CM-Triton X-114: Prepare 50 mL of 1.5% v/v Triton X-114 in TBS-CM. Store it at 4°C. It is stable for months. Right before use add protease inhibitors: 10 μg/mL aprotinin, 2 μg/mL leupeptin, and 4 m*M* benzamidine.

22. Gelatin-agarose beads (Sigma cat. no. G-5384): Wash the beads (the amount needed for the experiment) twice with TBS-B before use to remove preservative solution. Make a 50% suspension of gelatin–agarose beads in TBS-B.

3. Methods

3.1. Gel Preparation

The following protocol is for the preparation of eight gels (10% polyacrylamide–0.1% gelatin) (*see* Notes 4 and [5]).

1. Separating gel: Mix 17.8 mL of dH_2O, 5 mL of 1% gelatin, 16.6 mL of acrylamide–*bis*-acrylamide stock, 10 mL of separating gel buffer stock, 0.25 mL of 20% SDS; and 150 μL of 10% APS in a 125-mL filtration flask at room temperature. Degas the solution for approx 5 min.

2. Stacking gel: Mix 11 mL of dH_2O, 2 mL of acrylamide–*bis*-acrylamide stock, 2 mL of stacking gel buffer stock, 0.1 mL of 20% SDS, and 75 μL of 10% APS in a 125-mL filtration flask at room temperature. Degas the solution for approx 5 min.

3. Add 30 μL of TEMED to the separating gel solution to initiate polymerization. Swirl the solution rapidly without causing bubble formation or aeration.

4. Immediately, pipet 6.2 mL of separating gel solution into each cassette avoiding the formation of bubbles.

5. Carefully overlay the separating gel solution with dH_2O up to the top of the cassette using a syringe. Do not disturb the surface of the separating gel solution.

6. Let the gel polymerize for at least 1 h at room temperature. Polymerization is complete when a discrete line of separation can be noted between the gel and the water overlay.

7. Decant the overlay water from the separating gel.

8. Immediately, add 10 μL of TEMED to the stacking gel solution, swirl rapidly, and pipet the solution on top of the polymerized separating gels until it reaches the top of the front plate.

9. Rapidly, insert the appropriate combs (usually 10 wells) into the liquid stacking gel, making sure that no bubbles remain trapped under the comb. Let the stacking gel polymerize at room temperature (about 30–60 min).

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[4]We commonly use 10% polyacrylamide gels for separating gelatinases. However, the percentage and the thickness of the separating gel can be varied depending on the aim of the separation. For instance, to better visualize the dimeric form of MMP-9 (~ 200 kDa) (Fig. 1) and/or to obtain a better resolution of closely opposed bands (latent and active forms), a lower percentage (7–8%) of polyacrylamide solution can be used. However, the gelatinolytic bands will be less sharp. Alternatively, the gel can be run for an additional 30 min after the tracking dye have reached the bottom of the gel.

[5]It is very convenient to prepare the gels in advance since the polymerized gels can be stored at 4°C for 2–3 wk without any effects on resolution. Avoid bacterial contamination of buffers and solutions as bacterial proteases may result in the appearance of nonspecific gelatinolytic bands. This can be minimized by sterile filtration of buffers and stock solutions and storage at 4°C, as indicated.

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10. Store the gels (comb on top) in a sealed plastic bag or container containing 1X running buffer to keep them moist. Store them at 4°C for up to 2–3 wk (*see* Note 5).

3.2. Sample Preparation

3.2.1. Serum-Free Conditioned Media—The gelatinases are secreted enzymes (9). Therefore, in cultured cells, a significant part of the gelatinase pool is found in the media. Because serum contains gelatinases, it is necessary to prepare serum-free conditioned media for gelatin zymography (Fig. 1).

1. Grow the cells to be tested to approx 80% confluence in complete growth media.
2. Wash the cell monolayer with sterile PBS or serum-free media to remove the serum completely.
3. Incubate the cells with serum-free media at 37°C for at least 12–16 h (*see* Note 6).
4. Collect the media and centrifuge (400*g*, 5 min at 4°C) to remove cells and debris. Keep the supernatant.
5. Mix 75 μL of the clarified supernatant with 25 μL of 4X sample buffer; vortex-mix. Let the sample stand at room temperature for 10–15 min. Do not heat the sample! Load 30 μL per lane. If the intensity of the gelatinolytic bands is very low, the conditioned media can be concentrated using commercially available concentrators (10 kDa cutoff) or subjected to gelatin-agarose purification as follows:
6. In a microcentrifuge tube, mix 1 mL of clarified conditioned media with 30 μL of the gelatin–agarose bead suspension and rotate at 4°C for at least 1 h.
7. Centrifuge (16,000*g*) the tubes in a microcentrifuge (approx 1–2 min) and carefully aspirate the supernatant.
8. Wash the beads (containing the bound gelatinases), at least twice, with 1 mL of cold TBS-B.
9. After the last wash, carefully aspirate the supernatant. Add 20 μL of 1X sample buffer to the beads to elute the bound enzymes. Do not heat the samples! Spin the tubes and load the supernatants on the gel.

3.2.2. Cell Lysates—In addition to being secreted into the media, the gelatinases are also cell associated, intracellularly and extracellularly. Intracellular enzymes represent gelatinases undergoing different stages of posttranslational processing whereas the extracellular gelatinases represent enzymes that are associated with the cell surface/matrix. Analysis of cell lysates therefore can provide valuable information on the rate of synthesis, cell surface–matrix association and activation status of surface-associated gelatinases. Cell lysates are prepared as follows:

1. Wash the cells twice with cold PBS.
2. Add cold lysis buffer. Use 2 mL of lysis buffer per 150-mm dish to obtain a final total protein concentration of approx 2–3 mg/mL.
3. Scrape the cells into the lysis buffer with a rubber policeman, collect the lysate, and incubate it on ice for at least 15 min.

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[6]Preparation of conditioned media: Be aware that long incubation times (48–72 h) of cells in serum-free media can affect viability. Incubate the cells in a minimum amount of serum-free media (~50–60% of the amount of growth media) to obtain more concentrated samples.

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4. Vortex-mix and centrifuge (16,000*g*) for 20 min at 4°C in a microcentrifuge. Collect the supernatant and measure protein concentration.

5. Mix 75 μL of the supernatant with 25 μL of 4X sample buffer. Do not heat the sample. Load up to 30 μL per lane. In the minigel system described here, do not load more than 40 μg of total protein per lane, as excessive amount of protein will disrupt band separation and resolution (*see* Note 7). If the level of gelatinases is low, phase partition with Triton X-114 can be used to concentrate the cell-associated gelatinases.

3.2.3. Phase Extraction with Triton X-114—Triton X-114 is a biphasic detergent that is soluble in aqueous buffers at 4°C but above 20°C samples containing Triton X-114 separate into two distinct phases (aqueous and detergent). In general, hydrophilic proteins allocate to the aqueous phase and hydrophobic proteins to the detergent phase. However, anomalous distributions of proteins have been reported. Being hydrophilic in nature, gelatinases are found mostly in the aqueous phase. However, gelatinases (most evident with MMP-2) have also been found in the detergent phase (12,13), suggesting a strong association with the plasma membranes. Phase partition is carried out as follows:

1. Wash the cells twice with cold PBS. During all the following steps, keep samples on ice unless otherwise stated.

2. Add 2 mL of cold TBS-CM-Triton X-114 solution containing protease inhibitors per 150-mm dish. This should yield approx 2–3 mg of total protein per milliliter.

3. Scrape the cells into the solution and transfer to a tube.

4. Incubate the extract on ice for at least 15 min and then centrifuge (16,000*g*, 20 min) at 4°C.

5. Collect the supernatant, transfer to a new tube and incubate it for 2 min in a 37°C water bath.

6. Centrifuge (16,000*g*) the sample at room temperature for approx 5 min to obtain the lower detergent and upper aqueous phases.

7. Carefully collect and transfer the aqueous phase into a new tube without disturbing the detergent phase. Cool down the tubes briefly. Keep the tube with the detergent phase on ice.

8. Add 30 μL of the gelatin-agarose beads to 500 μL of the aqueous phase and proceed as described in **Subheading 3.2.1., steps 6–9**. Load the samples onto the gel.

9. Add 2 vol of TBS-B and 1 vol of 4X sample buffer to the detergent phase (4X dilution) (*see* Note 8). Do not heat the sample. Load 30 μL per lane.

3.2.4. Preparation of Tissue Extracts—Fresh tissue biopsies derived from tumor samples are an important source for examining gelatinase expression during tumor progression (Fig. 1).

1. Cut the tissue of interest (~50 mg) into small pieces. Remove any visible fat.

[7]Triton X-114 extraction and detergent phase: Owing to the high sensitivity of gelatin zymography, presence of gelatinases in the detergent phase may be the result of contamination with enzymes coming from the aqueous phase. To minimize contamination, repeat again the extraction by adding ice-cold TBS-CM to the detergent phase. Vortex-mix and repeat phase partition as described in **Subheading 3.2.3., steps 5–7**. Discard the upper phase and continue as described in **Subheading 3.2.3., step 9**.

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2. Add approx 500 μL of cold lysis buffer with protease inhibitors (**Subheading 2; item 17**).
3. Homogenize the tissue on ice with a pestle (Kontes, Vineland, NJ, cat. no. 749520-0000) in a microcentrifuge tube (*see* Note 9).
4. Centrifuge (16,000*g*) the homogenate for 10 min at 4°C. Collect the supernatant and measure the protein concentration.
5. Adjust the protein concentration to 1 μg per μL of 1X sample buffer. Load onto the gel equal amounts of protein per lane (*see* Note 7).

3.2.5. Gelatinase Standards—It is important to include gelatinase standards in each zymogram to accurately determine the type and activation status of the enzyme(s) expressed in a given sample. Conditioned medium from HT1080 human fibrosarcoma cells (American Type Culture Collection, CCL-121) is optimal because it contains both MMP-2 (72 kDa) and MMP-9 (92 kDa) (Fig. 1). Conditioned media obtained from HT1080 cells treated for 16 h with phorbol ester (100 n*M*) or concanavalin A (10 μg/mL) contain active MMP-2 (62 kDa) and can be used as reference for active MMP-2 (7). If available, purified natural or recombinant enzymes can be used (Fig. 1) (*see* Note 10).

1. Dilute the purified gelatinases to a final concentration of 1 ng per μL of 1X sample buffer. Do not heat the sample.
2. Load 1–5 ng of the enzymes per lane.

3.3. Running and Developing of Gel

1. Gently pull the comb out from the stacking gel and peel off the tape from the bottom of the cassette. Place the cassette into the gel apparatus and fill the buffer chambers with 1X running buffer.
2. Load the samples and run the gel at constant voltage (125 V, starting current should be approx 30–40 mA/gel) until the bromophenol blue tracking dye reaches the bottom of the gel (approx 90 min). These running conditions will prevent overheating of the gel (*see* Note 4).

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[8]Gelatinolytic bands: Under optimal conditions, gelatinolytic bands should be sharp and well defined (Fig. 1). This is generally true with samples derived from conditioned media or with purified enzymes. Cell lysates and tissue extracts, in contrast, may produce distorted bands owing to the high protein content. This is resolved by minimizing the amount of protein loaded per lane or concentrating the sample using phase partition and agarose-beads purification as described in **Subheading 3.2.3.** The sharpness and resolution of the bands also depend on the time of staining and destaining of the gels. Because other proteases besides MMPs can cleave gelatin, it is important to ascertain the nature of the enzymes detected. Incubating the gel in developing buffer containing 20 m*M* EDTA will cause the disappearance of bands produced by metalloproteases. By comparing the molecular weight of the bands in the samples with known gelatinase standards and the use of EDTA, a reliable assessment of the nature and molecular mass of the enzymes can be made. Other major MMPs showing gelatinolytic activity are stromelysin 1 (MMP-3) and interstitial collagenase (MMP-1) (3). However, the intensity of the bands produced by these enzymes is significantly lower than that elicited by gelatinases.

[9]Preparation of tissue extracts: The amount of lysis buffer can be varied depending on the source of the tissues. Be aware that owing to inherent differences in tissue structure, between and within specimens, protein extractability may vary (11). Therefore, the results should be carefully interpreted! After centrifugation, the tissue homogenates may contain floating lipids. Repeat the centrifugation to obtain a clear homogenate.

[10]Gelatinase standards: Purified gelatinases can now be obtained from commercial sources. To activate purified gelatinases, incubate the enzymes in 1 m*M* *p*-aminophenylmercuric acetate (APMA) (**Toxic!**). This should be prepared from a fresh stock of 10 m*M* APMA in 50 m*M* NaOH and then diluted 10-fold in TBS-B. Incubate the purified latent enzymes (2 ng/μL) with APMA in a 37°C water bath. MMP-2 is readily activated to the 62-kDa form after approx 30 min while MMP-9 is partially activated to the 82-kDa form after 2 h. Be aware that presence of TIMPs in the enzyme preparation will inhibit or slow the activation process causing generation of intermediate inactive forms (64 kDa for MMP-2 and 85 kDa for MMP-9) (4). On the other hand, long incubation times with APMA- or TIMP-free gelatinases will cause the appearance of low molecular mass active forms—a 45-kDa form for MMP-2 (14) and a 67-kDa form for MMP-9 (15).

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3. Carefully remove the gel from the cassette and place it in plastic tray containing 100 mL of renaturing solution. Incubate the gel for 30 min at room temperature with gentle agitation.
4. Decant the solution and rinse the gel at least once with 300 mL of dH_2O.
5. Incubate the gel at room temperature for an additional 30 min in 100 mL of developing buffer with gentle agitation.
6. Decant the developing buffer and replace it with 100 mL of fresh developing buffer. Incubate the gel at 37°C for approx 16 h in a closed tray (*see* Note 11).
7. Decant the developing buffer and stain the gel in staining solution for at least 1 h or until the gel is uniformly dark blue. The staining solution can be collected and used again. However, it will require a longer staining time.
8. Destain the gel with destaining solution until areas of gelatinolytic activity appear as clear sharp bands over the blue background (*see* Note 7).

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[11]The time of incubation of the gel in the developing buffer is critical. Because the presence of the gelatinolytic bands is the result of enzymatic activity, varying the incubation time will affect the size of the bands. For most conditions, overnight incubation will provide optimal resolution and reproducible results. Therefore, for better resolution, it is preferable to accordingly increase or decrease the amount of sample loaded into the gel rather than changing the incubation time.

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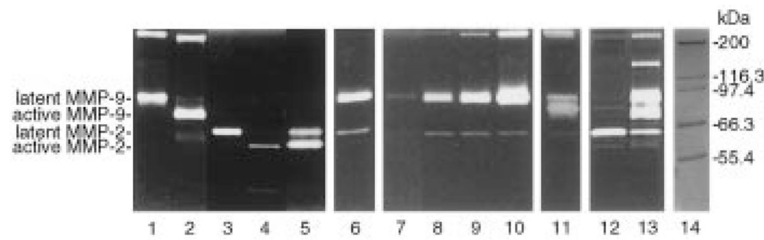

Fig. 1.

Gelatin zymogram of biological samples from different sources. *Lanes 1–5*, purified recombinant MMP-9 (*lane 1*, latent form; *lane 2*, activated form) and MMP-2 (*lane 3*, latent form; *lane 4*, active form; *lane 5*, mixture of active and latent forms). Note the presence of the MMP-9 dimer form (~200 kDa). *Lane 6*, serum-free conditioned media of HT1080 fibrosarcoma cells. *Lanes 7–10*, serum-free conditioned media of nonmalignant breast epithelial MCF10A cells untreated (*lane 7*) or treated with increasing concentrations (10, 25, and 50 ng/mL) of tumor necrosis factor-α (TNF-α) (*lanes 8–10*). Note the obvious induction of MMP-9 in response to TNF-α compared to untreated cells. *Lane 11*, cell lysate of TNF-α treated MCF10A cells. Note the presence of the intracellular, lower molecular mass (~83–85 kDa), precursor (partially glycosylated) form of latent MMP-9. Lanes 12 and 13, tissue extracts (30 μg of protein) from the benign (*lane 12*) and carcinoma (*lane 13*) section of a breast biopsy. Note the induction of MMP-9 and a 130–140-kDa band of unknown origin in the carcinoma. Also note the appearance of a approx 80–85 kDa form in the carcinoma. Note that it is difficult to determine whether this is active MMP-9 or the intracellular precursor form. *Lane 14*, unreduced molecular weight standard (Novex).