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Quantitative Zymography: Detection of Picogram Quantities of Gelatinases

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Zymography is an electrophoretic technique used to identify proteolytic activity in enzymes separated in polyacrylamide gels under nonreducing conditions. It has been used extensively in the qualitative evaluation of proteases present in tumors and cell culture conditioned media. Using commercially available precast gels and a modern image analysis system, we have evaluated zymography as a quantitative technique. The degree of digestion of gelatin within the zymogram by purified gelatinase A, a matrix metalloprotease, is directly proportional to the amount of enzyme loaded over a 10- to 20-fold range. With an overnight (18 h) digestion period, the linear range of this assay extended from 10 to 120 pg of enzyme. The initial rate of digestion is proportional to the enzyme loading and varying the incubation time results in a shift in the linear range of the assay. Active and latent forms of gelatinase A show the same degree of digestion in this assay system. These results justify the use of zymography in the quantitative assessment of gelatinase activity as well as demonstrate its usefulness as a qualitative technique for the analysis of gelatinase species present.

Proteolytic degradation of extracellular matrix components is an important aspect of many physiological and pathological processes, from wound repair and uterine involution to inflammatory diseases and invasion of tumors (1,2). Numerous methods have been developed to assess the levels of active and latent forms of proteolytic enzymes in tissues, biological fluids, and purified preparations. One of the more common methods is the use of zymography, which is an electrophoretic technique in which a large protein substrate, often gelatin, is copolymerized with the acrylamide during casting of the running gel (3,4). The enzymes are separated under denaturing (sodium dodecyl sulfate) but nonreducing conditions, refolded in detergent that will remove the SDS[1] (usually Triton X-100) and then incubated in a suitable buffer system for the enzymes under study. Zymogen forms of the matrix metalloproteases, a family of extracellular matrix degrading enzymes, are activated by this process of denaturation and renaturation (5), and so may be visualized in zymograms along with active forms. The gels are stained with Coomassie blue and evidence of enzymatic activity is demonstrated by the absence of staining in areas where the large protein substrate has been degraded. Zymography provides useful qualitative information on the species of enzymes present as well as an estimation of molecular weight.

In the case of the matrix metalloproteases, the ability to detect proteolytic activity based on molecular weight separation becomes a powerful tool, since the active and latent forms of the enzymes have different molecular weights and may both be visualized in zymograms (6,7). Furthermore, the electrophoretic process efficiently separates these enzymes from endogenous inhibitors—the tissue inhibitors of metalloproteinases-1 and -2 (TIMP-1 and -2). The inhibitors may be found in a tight-binding 1:1 complex with the active forms of any of the members of this family or with the latent forms of gelatinase A (TIMP-2) or gelatinase B (TIMP-1) (8,9). Since gelatinase A is most often isolated in complex with the inhibitor, we have used the enzyme–inhibitor complex as the basis of our evaluation.

The use of zymography to quantitate total activity has been used previously (10–12), but in the cases where the linear range of the assay was established, the gelatinase standard was an unpurified cell culture conditioned media preparation. We have characterized the use of zymography as a quantitative technique using purified gelatinase A–TIMP-2 complex (both active and latent

[1] Abbreviations used: SDS, sodium dodecyl sulfate; TIMP-1 and -2, tissue inhibitors of metalloproteinases-1 and -2; BCA, bicinchonic acid; APMA, *para*-aminophenylmercuric acetate; ELISA, enzyme-linked immunosorbent assay.

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forms) and a modern optical scanning and image analysis system. Under the conditions we present, zymography can be a extremely sensitive technique for the detection of picogram quantities of gelatinase.

MATERIALS AND METHODS

Materials

Precast 10% polyacrylamide minigels (80 × 60 × 1 mm) containing 0.1% gelatin and concentrated Tris–glycine electrophoresis running buffer were from Novex. A single lot of gels was used to perform the majority of experiments.

Methods

Enzymes. The gelatinase A-TIMP-2 complex was purified from the conditioned media of A2058 melanoma cells as previously described (13). TIMP-2 was separated from the enzyme under acid conditions on an HPLC also as previously described (13). Protein concentrations were determined using a microtiter plate BCA assay (Pierce) with bovine serum albumin as a control. The enzyme–inhibitor complex was activated with *para*-aminophenylmercuric acetate (APMA) (Sigma) as previously described (13) and dialyzed to remove residual APMA. The protein concentration of the activated enzyme–inhibitor complex was remeasured prior to evaluation by zymography.

Zymography. Samples for analysis were prepared by dilution into zymogram sample buffer (5×) consisting of 0.4 M Tris, pH 6.8, 5% SDS, 20% glycerol, and 0.03% bromphenol blue. The samples were loaded into the wells of a precast gel and the electrophoresis was carried out at 20 mA constant current for 1.5 to 2 h, at which time the bromphenol blue dye front had reached the bottom of the gel. The gel was removed and incubated for 1 h at room temperature in 100 ml of 2.5% Triton X-100 on a rotary shaker. The Triton X-100 solution was decanted and replaced with 100 ml of enzyme buffer (50 mM Tris, pH 7.5, 200 mM NaCl, 5 mM $CaCl_2$, 0.02% Brij-35). The gel was then incubated at 37°C for various lengths of time depending on the experiment, but for most experiments, 18 h was used. Staining and destaining were carried out at room temperature on a rotary shaker. Each gel was stained with 100 ml of 0.5% Coomassie blue G-250 in 30% methanol, 10% acetic acid for 3 h and then destained with three changes of 30% methanol, 10% acetic acid (for 15, 30, and 60 min of destain time, respectively, for each change). Finally the gel was incubated for 15 min in 30% methanol, 5% glycerol prior to drying between sheets of cellophane (Ann Arbor Plastics, Ann Arbor, MI). When gels were cut for the purpose of running parallel experiments, the volumes of incubation buffer, stain, and destain were reduced proportionately. Areas of digestion are visualized as nonstaining regions of the gel.

For the rezymography experiment, two gels were prepared. In the first gel, two lanes were loaded with identical amounts of enzyme. Following electrophoresis, the gel was washed in Triton X-100 as described above and then incubated for 1 h in enzyme buffer. At this time, one lane was excised and the region between R_f 0.41 and 0.64 was excised and sliced into nine pieces. These pieces were pushed into wells 2 through 10 (in order from low R_f to high R_f) of a second gel and re-electrophoresed, washed, incubated, stained, and destained as described above.

Quantification. The degree of digestion was quantified using an Arcus scanner (Agfa-Gevaert) equipped with a transparency option interfaced to a Macintosh personal computer. Gels were scanned using Adobe Photoshop (Adobe Systems Inc., Mountain View, CA) in gray-scale mode at 600 dpi using the autodensity feature. The scanner records the density for each pixel on a scale from 0 (clear) to 255 (opaque). The image was digitally inverted (new pixel value = 255 − old pixel value), so that the integrations of bands would be reported as positive values. Images saved to disk in TIFF format were analyzed using Image 1.41, a public domain program available through the NIH. Within this program, the integrated density of a particular band is calculated by summing the pixel values within a selected area and subtracting from this total the modal density. This assumes that the background pixel intensity is relatively even and can be approximated by selecting the most common pixel (this method restricts the background to integral values). Alternatively, the average pixel density of the background could be separately determined and used to calculate the integrated density of the selected band. Values of integrated density are reported in volume units of pixel intensity × mm^2. Each gel was scanned three times and the average value of the integrated density for a particular band was used in further calculations. The standard deviation of the mean value was generally only 1 to 2% of the mean.

RESULTS AND DISCUSSION

One of the properties of zymography that makes it such a useful technique is that both active and proenzyme forms of gelatinases may be visualized. It is hypothesized that these proenzymes may be activated by gentle denaturation and refolding (5). If so, then once refolded into an active conformation, the proenzymes could undergo autolytic self-cleavage in a manner analogous to organomercurial activation. Figure 1 shows the results of an experiment designed to demonstrate a shift toward lower molecular weight activated form after a zymography experiment. After re-electrophoresis, bands of digestion were seen in three lanes. The bands were integrated, demonstrating a shift in the amount of lower molecular weight activated forms from 18% of the total integrated density in the control to 61%

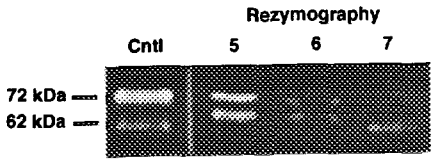

FIG. 1. Rezymography to demonstrate activation of gelatinases within the gel. Cntl indicates the control lane run in parallel with the excised lane that was reelectrophoresed. Lanes 5 through 7 show digestion following re-electrophoresis. Pieces of gel from the first electrophoresis were loaded onto the second in order from low R_f to high R_f.

following rezymography. This experiment demonstrates that there is activation and autolytic self-cleavage of progelatinases within the gelatin gel during the course of the zymogram experiment.

We evaluated the limits of sensitivity of the technique at three basic time points—4.25 h (same day), 18 h (overnight), and 43 h (two overnights) (Figs. 2 and 3). In each case there was a linear range at the low end of the curve with gradual flattening of the curve at high concentrations. Increasing the incubation time allows the detection of smaller quantities of enzyme. With a double overnight incubation we were able to detect 2 pg (0.022 fmol) of enzyme–inhibitor complex. Since the sample could be diluted in up to 20 μl of buffer, this method allows the detection of enzyme concentrations as low as 1 nM. This detection limit compares favorably with other methods, including ELISAs and Western blots, without the need for specialized reagents such as antibodies. With a more reasonable single overnight incubation, we were able to reproducibly detect 10 to 20 pg of enzyme. The linear range of the assay covered a 10- to 20-fold range, beginning at the detection limit and extending up to an integrated density of approximately 1000 units. In the case of an overnight incubation the linear range extended from 10 to 120 pg of enzyme. At the lower end of the linear range, the bands of digestion are barely perceptible by eye and though the scanner is able to detect these bands (Fig. 2B), they do not photograph well. This is unfortunate since it suggests that gels showing sufficient digestion to reproduce well after standard photography would be overloaded for the purposes of quantitation in the linear range.

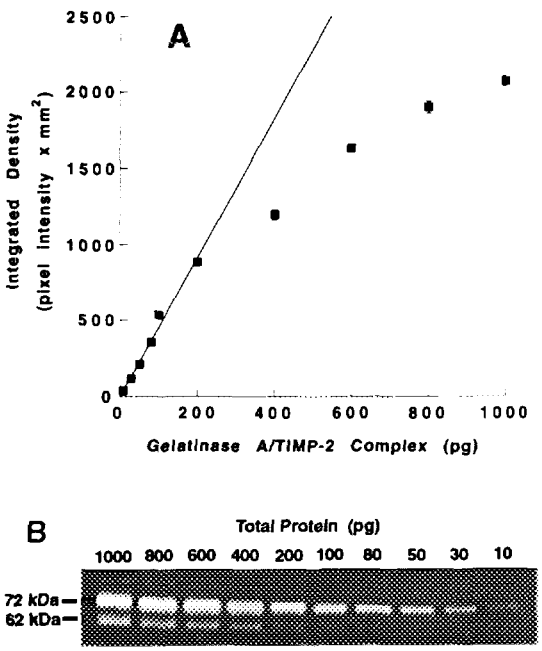

FIG. 2. *Sensitivity of a typical zymogram experiment.* (A) The integrated density for each band was calculated from three scans of the zymogram gel and then plotted against the amount of enzyme loaded in each well. The curve is approximately linear over the low concentration range. (B) Gel scan of zymogram used to produce data for Fig. 2A. At the higher enzyme concentration, the enzyme is seen to be a mix of latent and active forms of the enzyme. These are present in approximately a 7:1 ratio. Only the 72-kDa band was quantitated to produce the integrated density. Integrating both bands together does not alter the slope in the linear range as at the low concentrations of total enzyme, the band of active gelatinase is below the detection limit.

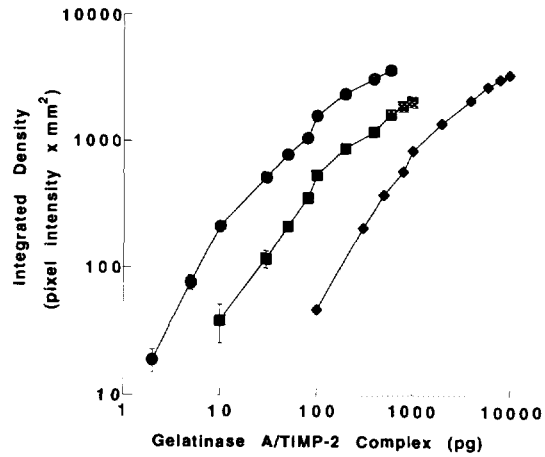

FIG. 3. Variation of sensitivity with time. The absolute sensitivity of the zymogram technique was evaluated as a function of time. A log–log scale was used to display the data from three incubation times: (●) 43 h, (■) 18 h, and (♦) 4.25 h. Replot of the data for any individual experiment on a linear–linear plot reveals a linear relationship between concentration and integrated density similar to Fig. 2A.

The rate of digestion with time was examined by incubating gels loaded with four different concentrations of enzyme for varying lengths of time in the enzyme buffer (Fig. 4). For the first half-hour of incubation there is no apparent digestion at any of the tested sample loadings and at 1 h only the 4-ng sample showed digestion. This lag phase was followed by an increase in the relative intensity for the remainder of the experiment. The rise in relative intensity of digestion was approximately linear for the three lower concentration samples over the next 27 h. When the initial rate of digestion was plotted against enzyme loading, the rate is seen to be approximately directly proportional to total

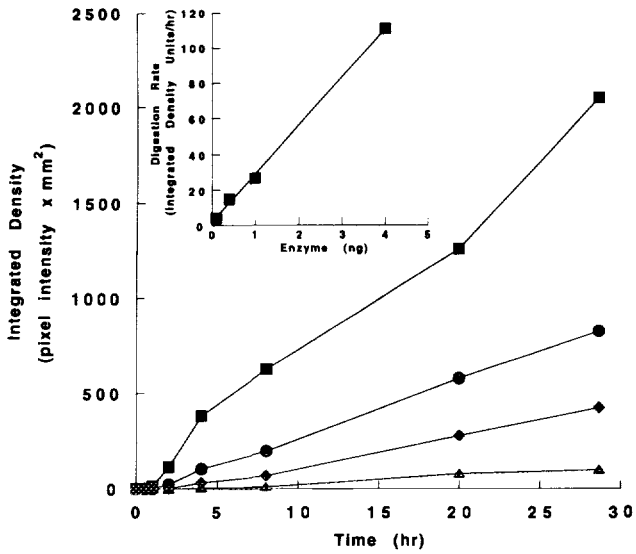

FIG. 4. Development of gelatin digestion with time. The change in integrated density was evaluated at four concentrations of gelatinase A: (■) 4 ng, (●) 1 ng, (♦) 0.4 ng, and (▲) 0.1 ng of total enzyme. Gels were removed at various time points between 0 and 28.5 h, stained, and destained identically. After an initial lag phase of 30 min, the rise in integrated density with time was linear up through 500 to 1000 integrated density units. Replot of this rate against enzyme concentration (inset) demonstrates a direct proportion between rate of gelatin digestion as measured in the zymogram and enzyme loading.

enzyme (Fig. 4, inset), with a "specific activity" for zymogram development of 27 relative intensity units/h/ng. This suggests that it is not biochemically unreasonable to use zymography to determine total enzyme activity and hence total enzyme protein. As an aside, the digestion rate experiments were performed using a different lot of gels from the rest of the data presented here. Although the intralot variation in digestion and sensitivity was low (data not shown), we have noticed that there is sufficient interlot variation between different lots of precast gels to make direct comparisons difficult. However, inclusion of internal standards on each gel allows correction for this variation.

Activation of the matrix metalloproteinases with organomercurial agents results in the loss of the propeptide domain of the enzyme through an autolytic cleavage reaction known as the "cysteine switch" (5). When gelatinase A is activated, it is converted from a 72-kDa proenzyme to a 62-kDa active form (6). On a 10% polyacrylamide gel the bands are sufficiently separated to allow easy distinction between the two forms using the image analysis software. Since both forms are visualized in the zymogram it is pertinent to ask whether they show differences in apparent specific activity. A sample of the enzyme–inhibitor complex was activated with APMA so that the 72-kDa form was quantitatively converted to the 62-kDa form (13). Equivalent protein concentrations of active and latent enzyme–inhibitor complex were evaluated by zymography in the same gel (Fig. 5). Over the range of concentrations previously determined to be in the linear range, there is essentially no difference in the level of digestion between progelatinase and activated enzyme as measured by the integrated density. Thus the assay can be used to quantitate not only total enzyme, but also the proportion of enzyme present in both the latent and active forms. This aspect distinguishes zymography from whole solution methods such as ELISAs, which quantitate total enzyme but do not distinguish between different molecular weight forms, and from solution activity assays, which can only quantify the active forms of the enzyme.

Several protocols are present in the literature for zymography of the gelatinases. In fact, each method is a little different from the rest. Most of these differences are minor and consist of slight variation in buffer concentration or pH. Some modifications incorporate the use of metalloproteinase inhibitors to check specificity (13); others have used zymography as a qualitative enzyme assay to evaluate the pH profile of newly characterized enzymes (10). Two common variations of Heussen and Dowdle's original protocol (4) were investigated for their effect on zymogram sensitivity, including APMA in the enzyme incubation buffer (14) and buffering the Triton X-100 refolding solution (10,14,15). Adding APMA to the enzyme incubation buffer might have the effect of activating any latent gelatinase that had refolded from the SDS in its latent conformation. One might expect to see either no effect or augmentation of digestion. We observed little change in the degree of digestion seen in the linear range of the assay (Fig. 6A); however, at higher enzyme concentrations there appeared to be a decreased amount of digestion. When the Triton X-100 solution was buffered with the same buffer used in the zymogram incubation step, the sensi-

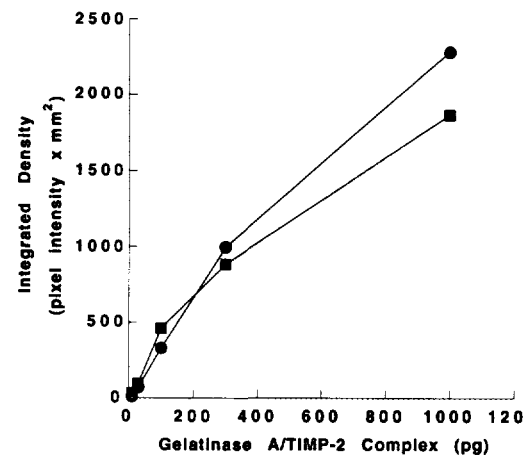

FIG. 5. Comparison of active versus latent enzyme digestion. Gelatinase A was activated using the organomercurial APMA and equal amounts of active (●) and latent (■) enzyme were run on the same gel. Scanned values for integrated density were very similar over the lower concentration range and only appeared to deviate significantly at the highest concentration.

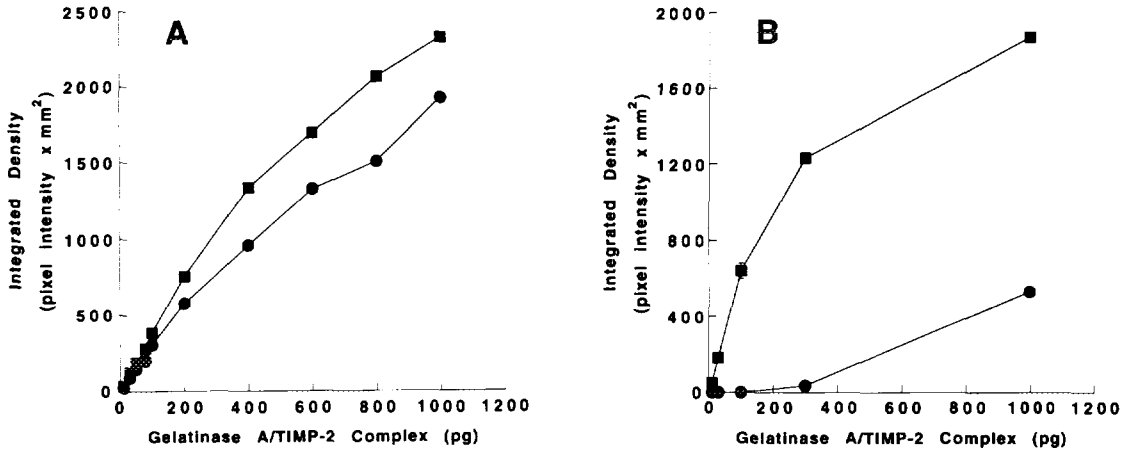

FIG. 6. Comparison of alternate protocols. (A) Comparison of digestion in the presence (●) or absence (■) of 1 mM APMA in the incubation buffer. The figure demonstrates that there is no beneficial effect (and possibly some loss of sensitivity) of the inclusion of APMA in the incubation buffer. (B) Comparison of digestion when the Triton X-100 wash solution is buffered (●) or unbuffered (■). Washing the gel in buffered Triton X-100 results in a significant loss of sensitivity.

tivity was markedly reduced (Fig. 6B). The precise mechanism responsible for the loss of sensitivity was not further characterized, but it would seem prudent to leave the refolding solution unbuffered to achieve the higher level of sensitivity.

Work in the field of tumor cell invasion and metastasis has demonstrated a role for a number of extracellular proteinases involved in matrix degradation (16). Although most studies have quantitated total enzyme levels to correlate with tumor invasiveness, recent publications have emphasized that the amount of activated enzyme present in a tumor sample may be more important than evaluating total enzyme (11,17). Since the zymography process efficiently separates the metalloproteinases from their low-molecular-weight inhibitors, a value for total potential enzymatic activity is obtained. This information is complementary to the results of direct enzyme assay by solution methods, which would determine the level of net activity present. This value is likely to be less than the total potential activity due to the presence of inhibitors and latent enzyme forms. The technique of zymography permits the rapid qualitative evaluation of samples without the requirement of specialized reagents. We have demonstrated that this technique can be used reliably as a quantitative method as well, with a sensitivity level that compares favorably with other techniques (Western blotting and ELISAs).

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