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BRIEF METHOD

Analysis of Gelatinases in Complex Biological Fluids and Tissue Extracts

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Gelatinase B (matrix metalloproteinase-9 [MMP-9]) and gelatinase A (MMP-2) are two closely related members of the MMP family that efficiently degrade denatured collagens or gelatins (Van den Steen et al, 2002). Specific MMPs play a major role in physiological processes, including angiogenesis, wound healing, bone remodeling, and cell migration. Moreover, MMP-9 and MMP-2 are key effector molecules in inflammation, autoimmunity (Opdenakker and Van Damme, 1994), and cancer (Sehgal et al, 1998). Analysis of both enzymes in complex biological samples, especially those with low gelatinase content, therefore is essential. We combined a miniaturized gelatin affinity chromatography with gelatin zymography and Western blot analysis. This strategy allows extremely sensitive and unambiguous detection of gelatinases.

Gelatinases are often detected with specific antibodies or by substrate conversion assays. Gelatinase activity assays measure overall gelatinase activity often with the use of labeled gelatins (Paemen et al, 1996). Because these do not discriminate between gelatinase A and B and even other gelatin-degrading enzymes, specificity is low, especially in the analysis of complex biological samples. Introducing affinity prepurification with the use of monoclonal antibodies enhances the specificity (Hanemaaijer et al, 1998). Moreover, only activated enzymes are recognized. ELISA detects specific forms of MMP-9 or MMP-2. Unfortunately, this method does not necessarily differentiate between pro-enzyme and activated forms. Discrimination between different gelatin-degrading enzymes and their respective activation status may be achieved by zymography or Western blot analysis.

Gelatin zymography detects picogram levels of MMP-9 (Masure et al, 1991). The sensitivity of Western blot analysis is usually lower and depends on the antibody affinity for MMP-9. In our hands, a combination of two monoclonal antibodies against mouse MMP-9 resulted in a detection limit of 100 pg of MMP-9. Nevertheless, such low detection limits are frequently not attained because the ratio of MMP-9 versus total protein is generally extremely low in crude samples, whereas the total protein load per lane is limited to 25 μg so as not to distort the electrophoresis. After protein overloading, gelatinolysis may be eclipsed in zymography. Interference by gelatinolytic activity from other (abundant) enzymes constitutes an additional problem for zymography. For instance, in stomach extracts, pepsin is abundantly present and because this and other proteases also cleave gelatin, it will mask the gelatinases.

To improve the ratio of MMP-9 versus total protein and to exclude interfering gelatinolytic activity, we developed a simple strategy for optimal preparation of complex samples, including tissue extracts. We used a miniaturized affinity chromatography purification step, taking advantage of gelatinases' strong affinity for gelatin (Masure et al, 1991). For rapid and reproducible purification of the samples, we made use of mini-spin columns (Bio-Rad Laboratories, Hercules, California) and gelatin-Sepharose beads (Amersham Pharmacia Biotech, Uppsala, Sweden). Equilibration buffer was composed of 50 mM of Tris (pH 7.5), 0.5 M of NaCl, 10 mM of $CaCl_2$, 0.01% Tween 20, and 5 mM of o-phenanthroline; washing buffer 1 had a similar composition except that the concentration of Tween 20 was increased to 0.05%. Washing buffer 2 was with omission of NaCl because high salt concentrations hinder the electrophoresis. The o-phenanthroline was added to the samples as a gelatinase inhibitor to prevent the gelatinolytic activity from breaking down the gelatin from the Sepharose beads, which are used for the affinity purification of the enzymes. The binding of gelatinases to gelatin-Sepharose is not disturbed by the presence of o-phenanthroline because this inhibitor acts by the chelation of the catalytic Zn^{2+}, whereas binding to gelatin is mediated by the three fibronectin type II–like repeats. Elution buffer was at once the electrophoresis loading buffer and was composed of 100 mM of Tris/HCl (pH 6.8), 4% sodium dodecyl sulfate, 20% glycerol, and 200 μg/ml of bromophenol blue as tracking dye.

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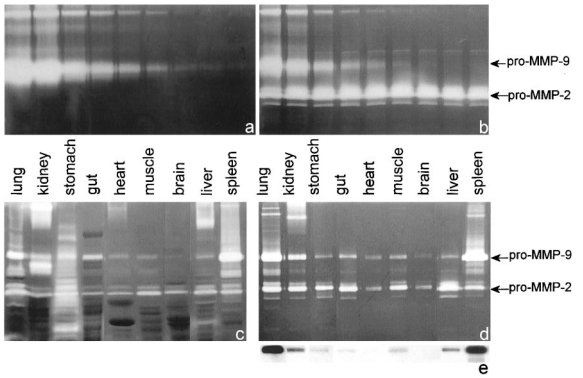

Figure 1.

Quantitative and qualitative aspects of gelatinase B prepurification. a, Standard human neutrophil gelatinase B dilution series. b, Equivalent amounts were added to a complex mixture of mouse serum from a gelatinase B knockout mouse, and a 1:3 dilution series was prepurified and processed for zymography. The reproducible recovery yield over a broad concentration range is obvious. c, Crude tissue extracts induce limitations for zymography analysis. Gelatinases A and B forms may be hidden among other proteases and eclipsed by the presence of extra protein. d, The proposed technique allows the specific recovery of gelatinases. e, Compatibility with immunoblot analysis. The detection of mouse gelatinase B in Western blot was with the combination of two mouse anti-mouse IgG monoclonal antibodies, CDEM-ABA and CDEM-CIA, generated in a gelatinase B knockout mouse.

The mini-spin columns were loaded with 45 μl of gelatin-Sepharose beads (one third beads in two thirds equilibration buffer), and crude samples were added. The mixture was then equilibrated on a shaker at room temperature for 20 minutes. When the sample volume exceeded 100 μl, the columns were equilibrated end-over-end. Subsequently, the cap was removed so that unbound sample was allowed to leave the column in vertical position by gravity flow. The beads were washed three times with washing buffer 1 and once with washing buffer 2. Excess fluid was removed by a short centrifugation step at 4000 $\times g$ before elution with 15 μl of elution buffer (loading buffer). The eluate was totally recovered by centrifugation at maximum speed (20,000 $\times g$).

In Figure 1, we demonstrate the gelatinase recovery. A control dilution series of pure human pro-MMP-9 (10 ng as the highest amount, diluted 1:3) was prepared in water (Fig. 1a). Next, a parallel dilution series was prepared in 10 μl of MMP-9–free serum ($\sim$1 mg) collected from gelatinase B knockout mice to produce representative crude samples with known gelatinase B content. We purified these samples according to the method described above and analyzed the purified samples by zymography. Comparison of both zymograms shows that gelatinase recovery is 30% over a broad concentration range. The aforementioned detection limit for zymography (5 pg) is also obvious. Analysis of unpurified serum samples led to distorted electrophoresis (not shown).

To illustrate the efficacy of the gelatin chromatography purification step before zymography and Western blot analysis, we prepared tissue extracts from various mouse organs and performed both assays before and after purification. Figure 1c shows a zymogram from crude organ extracts (25 μg). Pro-MMP-9 and pro-MMP-2 are detectable in most organs, but other gelatinolytic activities interfere with the detection of the activated and other forms of MMP-9 and MMP-2. This interference was totally excluded by gelatin affinity prepurification as shown in Figure 1d for zymography. Figure 1e demonstrates that the prepurification is also compatible with semiquantitative Western blot analysis. Because most irrelevant proteins are excluded from the samples by the prepurification, total protein content of the samples is no longer limiting. Because recovery of gelatinases is 30%, we started from 75 μg of protein instead of 25 μg for zymography, and because Western blot analysis is at least 10 times less sensitive in comparison with zymography, we used 750 μg of total protein for this assay. In both analytical techniques, the excess starting protein did not distort the electrophoresis. It is obvious that this improvement enables the study of gelatinases in complex samples, including tissue extracts. It also allows the concentration of enzymes from sources low in gelatinases A and B. Because of reproducible recovery yields, the technique is useful for semiquantitative comparison of samples. By the inclusion of dilutions of standard enzyme preparations, quantitative analysis of gelatinases also may be performed.

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