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Assays of matrix metalloproteinases (MMPs) activities: a review

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Abstract

Measurement of matrix metalloproteinase (MMP) activity often remains a challenge, mainly in complex media. Two sets of methods are currently used. The first one measures the hydrolysis of natural protein substrates (labeled or not) and includes the popular zymography. These techniques which are quite sensitive, cannot generally be carried out on a continuous basis. The second one takes mainly advantage of the increase of fluorescence, which is associated to the hydrolysis of initially quenched fluorogenic peptide substrates. Quite recently, another group, which is a compromise between the other two, has been developed. It measures the hydrolysis of synthetic triple-helical peptide substrates. These different methods are described and discussed.
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1. Introduction

The choice of an assay for measuring the activity of an enzyme is critically depending on the qualitative and quantitative objectives of the study. This is particularly important in the case of the matrix metalloproteinases (MMPs), as their natural substrates are usually insoluble (or poorly soluble) proteins, complex mixtures of proteins and associate macromolecules, structural components of extracellular matrices. They are synthesized as inactive zymogens and must be enzymatically activated for being active. Furthermore, their activities are in vivo regulated by endogenous inhibitors (TIMPs). For measuring their enzymatic activity, the choice is between easy-to-use techniques, generally with synthetic substrates, not always figuring the biological activity of the enzymes, and more cumbersome and complex methods using native or modified proteins which are more difficult to use for screening. In the case of studies aimed at determining the role of MMPs, it is obviously better to use native proteins or derivatives as substrates whereas the screening of inhibitors, in presence of purified enzymes, will be easier with synthetic substrates, which allow to perform rapid and numerous assays within quite a short time and high-throughput screening. The situation becomes more complicated when assays are carried out in biological fluids, due to the presence of interfering molecules, mainly inhibitors and also due to the fact that MMP specificities are similar.

In many circumstances, immunochemical procedures can replace enzymatic assays, but they cannot discriminate between zymogens and active enzymes.

The different assays of matrix metalloproteinase activities may be roughly classified in:

- Assays using proteins (modified or not);
- Assays using synthetic linear peptides and their derivatives;
- Assays using synthetic "mini-collagens".

In this brief review, we will describe, illustrate, and compare these assays, referring to recent published results from various laboratories.

Abbreviations: A_2pr, 2,3-diaminopropionic acid; Adp, 2-amino-3-(6,7-dimethoxy-4-coumaryl)propionic acid; APMA, *p*-aminophenylmercuric acetate; Ctmr, 5-carboxytetramethylrhodamine; DNFB, dinitrofluorobenzene; Dnp, dinitrophenyl; Dpa, diphenylalanine; DTNB, dithiobis-(2-nitrobenzoic acid); FIA, flow-injection analysis; FITC, fluorescein isothiocyanate; HTS, high-throughput screening; HRP, horseradish peroxidase; Mca, 7-methoxycoumarin-2-acetic acid; MOCAc, (7-methoxycoumarin-4-yl)acetyl; Nma, *N*-methylanthranilic acid; TNBSA, 2,4,6-trinitrobenzene sulfuric acid; TNF, tumor necrosis factor; uPA, urokinase-type plasminogen activator.

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2. Assays using proteins

Ideally, it would be better to measure the MMP activities with their most specific native proteins as substrates. But the hydrolysis rates are low and the classical methods are not sensitive enough. This is the reason why current methods preferably use labeled proteins, whereas some other original procedures have also been described.

2.1. Non-modified proteins

While collagenolytic activity may be evaluated by the spectrophotometric assay of hydroxyproline in the hydrolysates, this technique is more valuable for bacterial metalloproteinases than for matrix ones [1]. Contrastingly, cleavage rates of insoluble and soluble collagens by tissue collagenases could be measured from dansylation at the N-terminal residues of the cleaved peptides, after hydrolysis. Extensive hydrolysis and HPLC separation were monitored by fluorescence measurements. Using such a technique, kinetic parameters could be determined (K_M values were calculated to be in the micromolar range) [2]. A similar approach has been conducted by Mecham et al., in order to characterize the cleavage sites in insoluble elastin. Prior to Edman sequencing of hydrolyzed peptides, they used DNFB, which reacts covalently with primary and secondary amino groups. They could then identify the P_1' residues of proteolytic hydrolysis. Theoretically, it would be possible to quantify the cleavage rates, even if it would obviously be a tedious method [3].

Very recently, three collagenase assays (MMP-1, MMP-8, and MMP-13) with triple-helical collagens as substrates, have been developed using capillary gel electrophoresis with laser-induced dynamic fluorescence detection, with a non-covalent fluorescent dye (NanoOrange). A good linearity of peak areas was obtained over each assay range (15–150 ng/tube for MMP-1, 3-30 ng/tube for MMP-8, and 1.5–30 ng/tube for MMP-13). The method has been applied to the estimation of inhibitors and the results well agree with those obtained with fluorescent peptides [4].

A great number of data concerning MMP activities has been obtained by zymography, which allows the visualization of protease activities. This technique involves the electrophoresis of enzymes through polyacrylamide gels containing copolymerized protease substrate. It has been used for many years mainly with gelatin as a substrate. Electrophoresis is generally performed in sodium dodecylsulfate (SDS) and proteases are then renatured, enabling the hydrolysis of the substrate. After staining, the zones of digestion may be evidenced and/or quantified. In the early 90s, zymography was used to analyze the gelatinolytic activities of secreted proteinases of metastatic cell lines [5–7], but it was only in 1994 that the technique became quantitative. With the help of a purified MMP-2/TIMP-2 complex, Kleiner and Stetler-Stevenson could detect picogram quantities of the enzyme. A linear relationship could be evidenced between 10 and 120 pg after a 18 h-digestion period. After a 43 h-incubation, the detection limit was lowered to 2 pg. Initial rates of hydrolysis could be determined from density measurements within 30 h of digestion for enzyme concentrations ranging from 0.1 to 4 ng MMP-2 [8]. In a similar way, but using a single-step staining method, which led to fast and reproducible results, Leber and Balkwill [9] could demonstrate a detection limit of 32 pg for pro-MMP-9 and a linear range below 1 ng. More recently, Gogly et al. reported a detection limit as low as 0.1 pg in the case of an MMP-1 assay on collagen zymograms. Such a sensitivity is comparable to an immunodot blot assay using chemiluminescence but it may be noticed that immunoassays do not discriminate between pro- and active forms of enzyme which is not the case for zymography, in the experimental conditions described by the authors. The specificity of this method for collagenase was demonstrated, as no lysis could be observed with 300 pg of either MMP-2 or MMP-3 [10].

Unlike gelatinases, matrilysin (MMP-7), and collagenases (MMP-1 and MMP-13) are difficult to detect. In a recent report, it was demonstrated that heparin may enhance the assays, lowering the detection limit to 30 pg for MMP-7 and to 0.2 ng for MMP-1 and MMP-13. The mechanism of activity enhancement is not fully explained, but it has been suggested that heparin may act by inducing a conformation change, facilitating refolding, reducing autolysis or helping anchoring of the enzyme to the gel [11].

Zymography has also been applied to in situ studies. Initially, limited to soft and homogenous tissues, it has recently been used to demonstrate the sub-lamellar location of gelatinases in hard epidermal layers and a comparison has been made with an adjacent soft connective tissue matrix. The authors could then evaluate the influence of tissue topography on the technique [12].

Zymography may also be performed in a reverse mode, designed to detect the presence of metalloproteinase inhibitors. In this case, a proteinase is directly incorporated into the substrate-containing gel. The method was applied for the quantitative assessment of metalloproteinase inhibitor activity either with gelatin [13] or FITC-labeled collagen and casein [14] as substrates.

Today, zymography is probably one of the most popular techniques for measuring MMP activities. Some experimental procedures have recently been detailed and discussed in the case of enzymatic activities in a crude medium of cultured synoviocytes [15].

2.2. Labeled proteins

Various methods have been developed, using chemically modified or engineered proteins. In the first category, the most popular are proteins labeled either by radioelements or by fluorescent groups, but recent results have emphasized the usefulness of other modifications.

2.2.1. Radiolabeled proteins

For sensitivity and ease of detection, labeling by means of [^{3}H] acetic anhydride is frequently performed. Assays have

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