

REVIEW

Advances in assays of matrix metalloproteinases (MMPs) and their inhibitors

XIAN-CHAO CHENG, HAO FANG & WEN-FANG XU*

Institute of Medicinal Chemistry, School of Pharmaceutical Sciences, Shandong University, Jinan 250012, China

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Abstract

Matrix metalloproteinases (MMPs) play an important role in many physiological and pathological processes. To assay the activities of MMPs is important in diagnosis and therapy of the MMPs associated diseases, such as neoplastic, rheumatic and cardiovascular diseases. Several assay systems have been developed, which include bioassay, zymography assay, immunoassay, fluorimetric assay, radio isotopic assay, phage-displayed assay, multiple-enzyme/multiple-reagent assay and activity-based profiling assay. The principle, application, advantage and disadvantage of these assays have been reviewed in this article.

Keywords: *Matrix metalloproteinases, inhibitors, assays*

Introduction

The matrix metalloproteinases (MMPs) are zinc-binding endopeptidases that collectively degrade all components of the extracellular matrix. Overall, the MMP family consists of more than 25 enzymes, which include collagenases (MMP-1, 8, 13, 18), gelatinases (MMP-2, 9), stromelysins (MMP-3, 10, 11), matrilysins (MMP-7, 26), membrane type MMPs (MMP-14, 15, 16, 17, 24, 25) and others. They are important in normal physiological functions such as angiogenesis, wound healing, mammary gland and postpartum uterus involution, and cervical dilatation, but excess MMP activities are correlated with various diseases, such as tumor, arthritis, periodontal diseases, liver cirrhosis, atherosclerosis, and multiple sclerosis [1–4].

The activities of these enzymes are well regulated by endogenous tissue inhibitors of metalloproteinases (TIMPs). The synthetic inhibitors of MMPs, such as peptidomimetic compounds, nonpeptidic compounds, tetracycline derivative, biphosphonate are also of interest for the treatment of cancer and arthritis. Many MMP synthetic inhibitors have been studied using preclinical studies *in vivo* models.

Although preclinical studies were so compelling to encourage several clinical trials, the past years have seen a consistent number of disappointments and limited success [5]. Most preclinical studies have focused on the role of MMPs in the early stages (progression and metastases) of cancer, in which MMP inhibition seems to have its greatest effect. Unfortunately, clinical trials of MMP inhibitors were conducted almost uniformly in patients with advanced, metastatic disease, and all have failed to show any beneficial effect on patients.

The failure of these inhibitors in clinical trials has stimulated the methodology research in assay of MMPs and their inhibitors in tissue samples and body fluids. In 2005, Lombard et al [6] published a very important review about MMPs activities assay, in which the assay methods were classified mainly by substrates, such as proteins, synthetic linear peptides and synthetic “mini-collagens”. In order to summarize the further development of MMPs assay methods since 2005, such as *in vivo* zymography and activity-based profiling assay, we delivered this review. Moreover, the assay methods of MMPs and their inhibitors were classified by the assay techniques,

*Correspondence: W.-F. Xu, Institute of Medicinal Chemistry, School of Pharmaceutical Sciences, Shandong University, Jinan 250012, China. E-mail: xuwenf@sdu.edu.cn

which were outlined below: bioassay, zymography assay, immunoassay, fluorimetric assay, radio isotopic assay, phage-displayed assay, multiple-enzyme/multiple-reagent assay, and activity-based profiling assay. The principle, application, advantage and disadvantage of these assay methods were described in this review.

Bioassay

Most of previous assays for MMPs rely on the biological activity of the enzyme to degrade natural substrates, and are so-called "bioassay".

Assays using biotinylated gelatin (BG)

Gelatin is a substrate for MMPs such as MMP-2 and MMP-9. Gelatin was biotinylated to increase the sensitivity of the detection system. Biotinylated gelatin (BG) coated on microtiter plates provides a useful substrate for detecting gelatinase activity. Individual wells (illustration) of a microtiter plate were coated with avidin to which biotinylated gelatin substrate was bound. Enzymatic activity of gelatinases liberated gelatin fragments and decreased the number of residual biotin proportionally. The latter was spectrophotometrically quantified by binding of streptavidin-peroxidase and development of peroxidase activity (PO), using peroxide and tetra methyl benzidine as a color reagent (Figure 1) [7,8].

This 2 h assay permits detection of MMP-2 activity in concentrations as low as 0.16 ng/mL and can be used for monitoring MMP activity in tumor-derived samples and for evaluation of efficiencies of synthetic MMP inhibitors [9].

Assays using succinylated gelatin

The assay is based on the use of succinylated gelatin as substrate and measurement of primary amines exposed by hydrolysis of the substrate by gelatinases. The exposed primary amines are detected by reacting with 2,4,6-trinitrobenzene sulfonic acid (TNBSA) that produces a quantifiable color reaction with a λ_{max} at 450 nm, which is directly proportional to the gelatinase activity (Figure 2). Succinylated gelatin was primarily digested by MMP-2 and -9. The detection limit for catalytically active MMP-9 was 12.5 ng (1.0 nM) and that for MMP-2 was 20 ng (2 nM), using a 30 min incubation [10].

The assay can be used to detect gelatinolytic activity in purified or crude samples, and as a high throughput screen for enzyme inhibitors of MMP-2 and -9, in particular.

Zymography assay

Zymography is an accurate and sensitive quantitative technique for the evaluation of matrix metalloproteinases

or their inhibitors. This technique is a variation on acrylamide gel electrophoresis. The gel used for zymography, commonly referred to as a zymogram, contains a protease substrate (usually gelatin) incorporated directly into the gel during polymerization. Following electrophoresis of the sample containing a protease, SDS is removed from the gel by exchange in Triton X-100. This allows the gelatinases in the sample to renature and autoactivate. Its activity is revealed by an absence of protein staining in the region where the substrate has been digested.

Gelatin zymography

Gelatin zymography is the common method for examining gelatinases in cells and media samples. This is an SDS-PAGE-based method performed with copolymerized gelatin. This method allows the relative amount of each gelatinase to be determined in small samples of tissue (<10 mg) [11] and can be used qualitatively for the analysis of gelatinase species present [12,13]. For collagenases 1 and 3, adding heparin could enhance the detection on gelatin zymograms at least 5-fold [14].

There are controversial data on the effects of different anticoagulants as pre-analytical determinants of plasma MMP activities by gelatin zymography. In order to solve this problem, Gerlach et al [15–17] examined whether different anticoagulants affect plasma MMP-2 and MMP-9 activities using gelatin zymography. Their results suggest that similar MMPs activities are found in plasma samples separated from blood collected into EDTA, citrate, or heparin tubes. Further studies found that the circulating levels of MMP-9 should be assessed in citrate or heparin plasma samples. Serum samples, however, should not be used to assess MMPs activities for two main reasons: because artificially higher MMP-9 levels are found, independently of how rapidly the serum is separated from blood, and because they do not correlate with the MMP-9 levels found in plasma samples.

Despite its numerous applications [18], the utility of gelatin zymography is limited by poor inter-laboratory comparison due to lack of a readily available MMP Mr referenced standard. Toward the goal of defining a readily available MMP Mr standard, Makowski et al [19] investigated the feasibility of using gelatinase standards from human capillary blood for calibrating gelatin zymograms. The standards are easily obtained and well characterized and should thus facilitate inter-laboratory comparison and standardization.

Casein zymography

For stromelysins 1 and 2 (MMP-3 and MMP-10, respectively) and matrilysin (MMP-7), casein zymography is more suitable. However, casein zymography has a detection limit at least two orders of magnitude

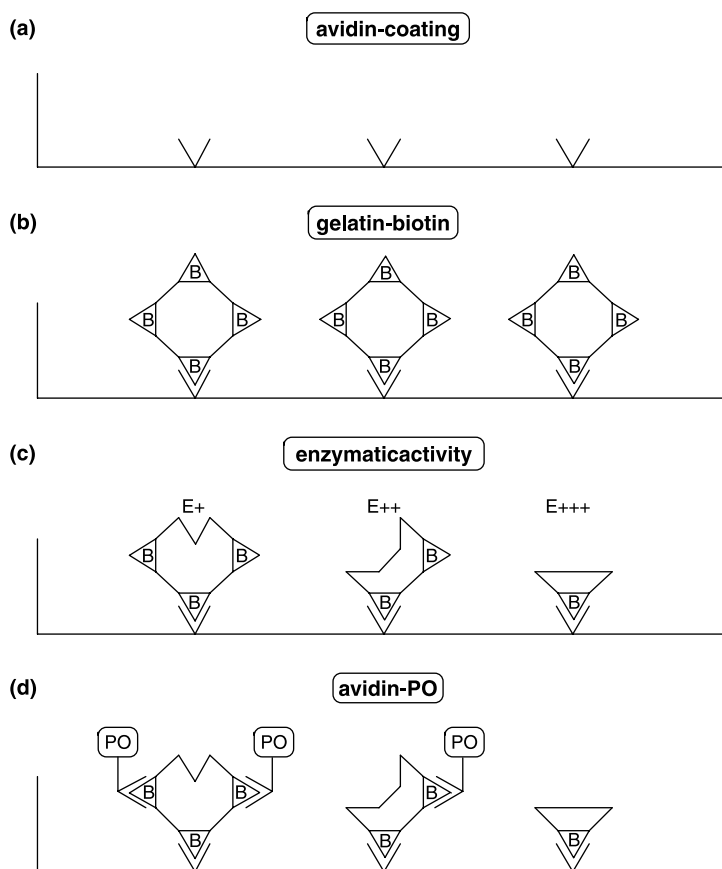


Figure 1. Schematic representation of the gelatinase assay using biotinylated gelatin as substrate.

less sensitive than gelatin zymography; moreover, due to its relatively low molecular weight (23 kDa), casein migrates during the electrophoresis, resulting after

staining in the existence of two clearly defined zones in the gel [20]. The anodic migration of the substrate turns out to be especially problematic in the case of

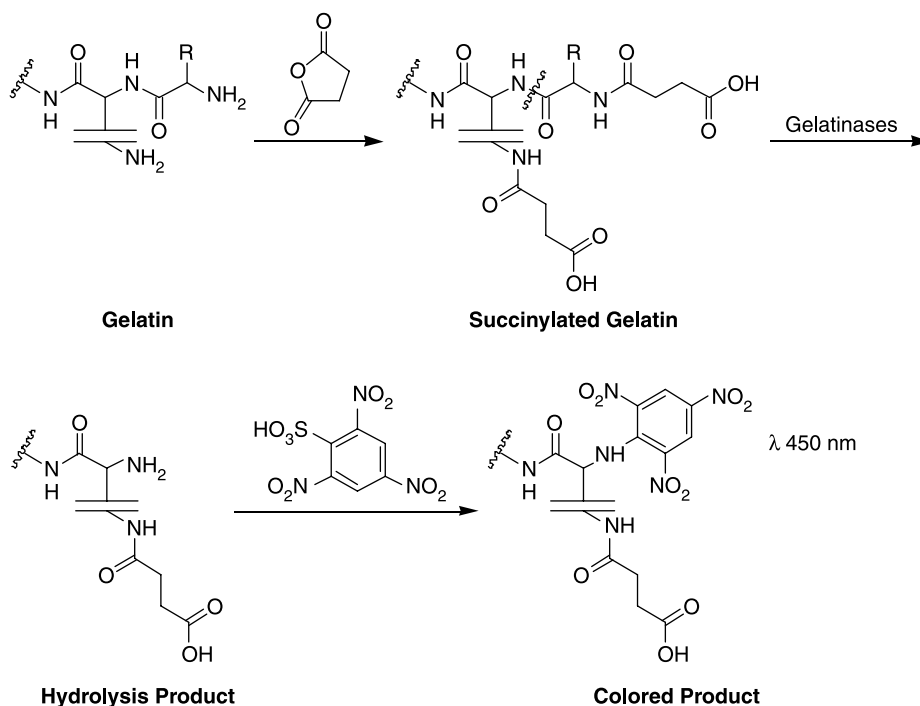


Figure 2. Schematic representation of the gelatinase assay using succinylated gelatin as substrate.

proteinases which are located near the casein migration boundary. This is the case of the latent and activated forms of matrilysin (29 and 20 kDa, respectively). Pre-running casein-embedded gels before the loading and electrophoresis of the samples can improve the definition of the caseinolytic band, increase the method sensitivity, and avoid the problem of substrate migration observed in the classical casein zymography [21].

Collagen zymography

Unlike the gelatinases (MMP-2 and -9) and matrilysin (MMP-7), collagenases (MMP-1 and -13) are difficult to detect at low levels in conventional casein or gelatin zymography. Gogly et al [22] describe the use of casting native collagen type I in SDS-polyacrylamide gel (collagen zymography) for the determination of interstitial collagenase.

This method proved to be very sensitive: 0.1 pg of APMA (p-aminophenylmercuric acetate)-activated procollagenase could be detected, and specific levels of active gelatinase or stromelysin lower than 5 ng were not detected under their experimental conditions [23].

In situ zymography

In situ zymography is based on the same principles as gel-based zymography. Either a photographic emulsion containing gelatin or a fluorescence-labeled proteinaceous macromolecular substrate is brought into contact with a tissue section or cell preparation. After incubation, enzymatic activity is revealed as white spots in a dark background or as black spots in a fluorescent background [24].

This technique is extremely useful for the detection and localization of MMP-2, 7, 9 *in situ* as has been demonstrated in several unrelated tissues [25–27].

In situ zymography preserves the fine morphological details of the tissue and can complement the study of enzyme expression by other microscopic techniques, such as immunocytochemistry [28]. However, researchers must be aware of potential variations in zymographic analysis may be influenced by physical tissue parameters in addition to suspected gelatinase activity [29].

Reverse zymography

Reverse zymography is an electrophoretic technique used to detect the presence of MMP inhibitors rather than MMPs. This technique allows concurrent semi-quantitative analysis of inhibitor levels and molecular weight. The technique has its origin in a fibrinogen-agarose plate assay for protease inhibitory activity in which a digesting agent was added to grooves after electrophoresis and allowed to diffuse into the

gel [30]. The original method had very crude sensitivity and resolving power by current standards, but was modified and improved by directly incorporating a proteinase source into the acrylamide-substrate gels. Recent methods have focused on the detection of MMP inhibitory activity using conditioned media as a good source of MMPs [31].

To minimize the number of variables and make reverse zymography more consistent, sensitive, and quantitative, Oliver et al [30] describe a novel system for reverse zymography using purified recombinant human gelatinase A or gelatinase B in place of conditioned media. This system can detect TIMPs 1, 2, and 3 simultaneously, but with differential sensitivities for TIMPs 1 and 2.

However, this technique is still problematic. For example, it is very hard to predict the proper incubation time for a sample containing an unknown concentration of TIMPs because the gel staining procedure terminates the reaction. As a result, multiple gel samples are typically required to obtain the best reaction time.

Real-time zymography

Hattori et al [32] utilized a fluorescein-isothiocyanate (FITC, a more popular and readily available fluorescent dye)-labeled denatured collagen as the substrate to develop novel zymographic and reverse zymographic methods for detecting MMPs and TIMPs, respectively. Using a trans illuminator, the results can be observed visually without stopping the enzymatic reaction. For this reason, they have named these methods real-time zymography and real-time reverse zymography.

They further expanded this technique by incorporating two substrates with distinct fluorophores (FITC-labeled gelatin and Texas-red-labeled casein) into a single polyacrylamide gel as substrates. The separate detection of the gelatin and casein degradation could be assessed on the same gel using two types of optical filters. With this fluorescent detection system, MMP-3 could be distinguished from MMP-2 and MMP-9 and activities could be measured concurrently and in real time [33].

Using this dual detection system, the time and reagent can be saved, and quantitative analysis of MMPs is possible. Higher sensitivity is achieved with a lower amount of substrate as compared with conventional methods.

In vivo zymography

Bremer and Crawford [34,35] developed a novel approach to assay MMP activity *in vivo*, which was named *in vivo* zymography. This technique is based on the injection and *in situ* degradation of heavily fluoresceinated native collagens. These substrates are

only weakly fluorescent in their native state due to intra-molecular quenching. However, when proteolytically degraded, the quenching is relieved, resulting in an increase in fluorescent signal, which is proportional to the degree of collagen degradation.

Using this technique, Crawford et al [34] characterized patterns of MMP activity in the zebrafish embryo, and Bremer et al [35] sensed or imaged MMP-2 activity directly in intact tumors in nude mice. This technique could also be used to image the effect of MMP inhibition directly within hours after initiation of treatment using the potent MMP inhibitor, prinomastat (AG3340). This technology will enable the detailed analysis of MMPs and their inhibitors directly *in vivo* in intact tumor environments.

Immunoassay

Immunoassays have been developed for MMPs, TIMP and MMP-TIMP complex. Selection of antibodies of defined specificity enabled measurement of both the pro and active forms of the metalloproteinase. Free TIMP was quantified by the selection of a monoclonal antibody which did not recognize TIMP when complexed with metalloproteinases. Detection of enzyme-inhibitor complexes was achieved by capturing the TIMP component of the complex and revealing the metalloenzyme using specific antibodies.

Immuno capture assay

The immuno capture assay, also named biotrak assay [36], was first described by Verheijen et al [37]. MMPs was captured and immobilized by specific antibody, and Prior activation of the latent MMPs by APMA produced the active MMPs, which was necessary to perform this assay. Using protein engineering, a modified pro-urokinase (proUKCOL), as a substrate, was made in which the activation sequence, normally recognized by plasmin (ProArg-PheLysIleIleGlyGly), was replaced by a sequence that is specifically recognized by MMPs (ArgProLeuGlyIleIleGlyGly). The active urokinase (UKCOL) resulting from the activation of proUKCOL by active MMPs can be measured directly using a chromogenic peptide substrate for UKCOL. The assay has been made specific for MMP-9 using an MMP-9 specific monoclonal antibody S22.2 (Figure 3) [38,39]. The detection limit for MMP-9 was below 15 pM, corresponding to 3.75×10^{-15} mol per assay.

By using other antibodies, they then adapted the assay to specific assays for the MMP-2 [40]. Using this technique, Yoshida et al [41] determined the molecular mechanism of SI-27, an anti-MMP agent.

This method was not time-consuming, and yet was quantitative for each subtype of MMP, so it may become a new standard that avoids the deficiencies of zymography and collagenolysis. This assay can easily

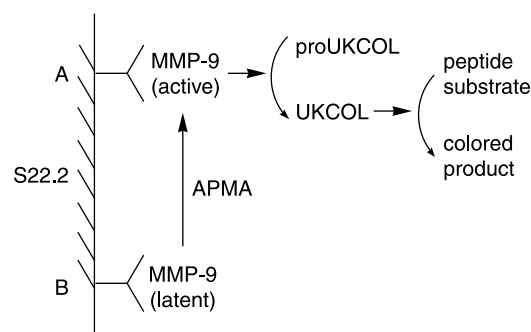


Figure 3. Schematic representation of the setup of the MMP-9 immunocapture activity assay.

be used for high throughput screening of compounds or samples.

Western blotting

Western blotting (protein blotting or immunoblotting) is a powerful and important procedure for the immunodetection of MMPs post-electrophoresis [42]. Western blotting follows five procedures (Figure 4): (A) Unstained SDS-PAGE gel prior to western blot. (B) Exact replica of SDS-PAGE gel obtained as a blot following western transfer. (C) Primary anti-MMPs antibody binding to a specific band on the blot. (D) Secondary antibody conjugated to alkaline phosphatase or horse radish peroxidase (along with soluble substrates) binding to primary antibody. (E) Color development of specific band [43].

Western Blotting offers the following specific advantages: (a) wet membranes are pliable and easy to handle, (b) the proteins immobilized on the membrane are readily and equally accessible to

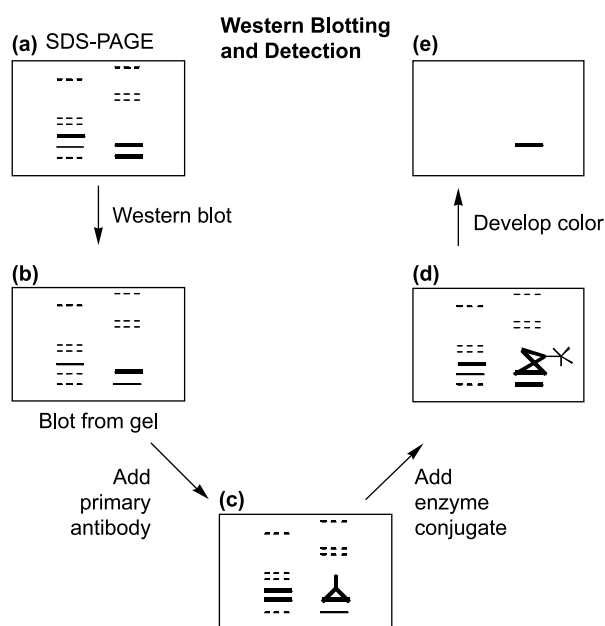


Figure 4. Schematic representation of western blotting and detection procedures.

different ligands, (c) multiple replicas of a gel is possible, (d) prolonged storage of transferred patterns, prior to use, becomes possible and (e) the same protein transfer can be used for multiple successive analyses [44].

However, western Blotting requires knowledge of the MMPs native substrate and the availability of anti-MMPs antibodies. And what is more, it is expensive and time-consuming.

Sandwich enzyme immunoassay

Obata et al [45] developed a one-step sandwich enzyme immunoassay (EIA) for MMP-3 (stromelysin-1). The assay system used two simultaneous immunoreactions using a solid phase monoclonal antibody and a horseradish peroxidase-labeled monoclonal antibody (Fab'). The sensitivity of the assay system was 20 µg/mL and linearity was obtained between 31 and 500 µg/mL. The EIA system was capable of measuring both precursor and active forms of MMP-3 as well as the forms of MMP-3 complexed with tissue inhibitors of metalloproteinases. They further established the one-step sandwich enzyme immunoassay (EIA) for MMP-1 [46], MMP-2 [47], TIMP-2 [48], MMP-7 [49], MMP-8 [50], MMP-20 [51]. (Table I) Compared to the two-step sandwich enzyme immunoassay system [52], this system is more sensitive and has advantages of simplicity and rapidity. This system can be applied to determine the levels of MMPs and proMMPs in human plasma from normal subjects and patients with cancer.

Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assays (ELISA) have recently been developed for the quantification of MMPs [53,54]. Clark et al [55] has raised and characterized monoclonal antibodies against purified human fibroblast collagenase. One of these antibodies has been used in combination with a polyclonal anticollagenase antibody in a double antibody sandwich ELISA to measure collagenase. Two combinations were applicable to the immunoassay: (i) a monoclonal capture antibody with a polyclonal detecting antibody; (ii) two monoclonal antibodies. The assay measures total collagenase, i.e. pro- and active enzyme as well as collagenase in complex with TIMP [56–58]. This assay has also been used to quantify stromelysin [59,60], and type II collagen [61]. These commercially available ELISAs

have good sensitivity.

Maliszewska et al [62] further developed an ultra-sensitive two-side ELISA which allows for the first time to measure reliably MMP-9 concentrations in human cerebrospinal fluid.

Using ELISA, Jung et al [63,64] studied the pre-analytical pitfalls of blood sampling to measure true circulating MMP-9, TIMP-1 and TIMP-2. The results showed that serum does not seem to be an appropriate sample for determining circulating MMP-9 and TIMP-1 whereas the TIMP-2 determination is interfered by heparin. In order to avoid pre-analytical misinterpretations, citrate plasma has been suggested to be the sample of choice for measuring circulating MMP-2 and MMP-9. Their results were consistent with the above mentioned report from Gerlach [15–17] using gelatin zymography.

Urinary type II collagen neoepitope assay (uTIINE assay)

uTIINE assay is a technique that utilizes urinary type II collagen neoepitope to measure MMP activity. When an antibody is made that specifically recognizes a cleavage site that includes either the free amine or the carboxyl terminus in a peptide sequence, it is referred to as a neoepitope antibody. Poole et al [65] applied neoepitope antibodies to the sequence GPQG-COOH, a principal cleavage site in type II collagen, detecting type II collagen fragments in synovial fluid from rheumatoid and osteoarthritic patients. Another neoepitope antibody to a new terminus GPP(OH)GPQG-COOH was generated for collagenase 1, 2, and/or 3 cleavage of type II collagen. Collagen fragments were detected in urine when an upstream anti-collagen II-specific antibody appeared. The urinary TIINE activity correlated well with osteoarthritis (OA) disease activity, reflecting the importance of collagenase activity in the OA disease process. In addition, quantitative assays of type II collagen neoepitopes may be useful markers of cartilage degeneration and joint disease in humans [66]. However, these assays are cumbersome and do not discern a particular collagenase or the relative contribution of the various collagenases.

Fluorimetric assay

Fluorogenic substrates provide a particularly convenient enzyme assay method, as they can be monitored continuously and utilized at reasonably low concentration ranges. There are three types

Table I. The sensitivity and linearity of sandwich enzyme immunoassay for various MMP subtypes.

MMP subtype	MMP-1	MMP-2	TIMP-2	MMP-3	MMP-7	MMP-8	MMP-20
Sensitivity (µg/mL)	0.12	0.24	1.6	20	0.00005	0.00034	0.001
Linearity (µg/mL)	0.12-10	1.0-500	6.3-50	31-500	0.000156-0.01	0.0005-0.5	0.0025-0.16

of fluorogenic substrates: (i) fluorescein isothiocyanate (FITC)-labeled proteins; (ii) intra-molecular fluorescence energy transfer substrates (IFETS); and (iii) fluorogenic triple-helical peptide (fTHP).

Fluorescein isothiocyanate (FITC)-labeled proteins

Intact fluorescein isothiocyanate (FITC)-labeled proteins are internally quenched due to the close proximity of the labels, so they have relatively low background fluorescence at excitation and emission wavelengths of 495 and 525 nm, respectively. Degradation of these substrates leads to exposure of covalently linked fluorescein isothiocyanate molecules and to a concomitant increase in relative fluorescence at these wavelengths. The increase in relative fluorescence is proportional to the degree of protein degradation. This phenomenon provides the basis for a sensitive assay for MMP activity [67]. There is no requirement for the removal of undegraded substrate from the assay mixture prior to the measurement of fluorescence. Assays can be performed in 96-well micro-titer trays, enabling a large number of samples and their respective controls to be processed simultaneously and repeated determinations of fluorescence values may be made on the same assay [68–70].

FITC-proteins exhibit several advantages such as their low cost, stability, easy preparation and waste disposal. However, they are time-consuming due to the procedures required to separate bound from unbound marker.

Intra-molecular fluorescence energy transfer substrates (IFETS)

The substrates consist of peptide chains constructed to match the specificity of the particular enzyme and to bear a suitable chromophore at each side of the cleavable bond [71]. One of the chromophores is a fluorophore (the fluorescence donor) and the other is a light-absorbing group (quencher, the fluorescence acceptor). The intact peptide has low intrinsic fluorescence because of the close proximity of the donor and quencher. When a proteinase cleaves the substrate, the liberated fluorophore is no longer quenched efficiently. The rate of fluorescence increase is a measure of enzyme activity. Quenching is almost unaffected by the nature of the intervening amino acids, so that with appropriate sequences some degree of specificity for the individual MMPs can be achieved [72–74].

Standard methods for performing such assays employ commercial 96-well micro-titer plates and compatible detection devices, with assay volumes of 0.05 to 0.2 mL and times of 30 to 60 minutes, which make this assay suitable for use in high-throughput screening of MMP inhibitors [75].

In contrast to the known fluorogenic substrates that are used widely in the scientific and medical research, IFETS allow control of the enzymatic cleavage at any point of the peptide chain and thus permit simultaneous studies of enzymes of different specificity (Table II) [76–84]. However, IFETS is limited by poor solubility, quenching and high background readings [85].

Fluorogenic triple-helical peptide (fTHP)

Fluorogenic triple-helical peptide (fTHP) substrates mimic the behavior of the native collagen substrate and may be useful for the investigation of collagenase triple-helical activity. Based on this idea, chemically synthesized triple-helical substrates have been used to study the mechanisms of MMP action on collagenous substrates [86,87].

Fluorogenic triple-helical peptide (fTHP) substrates could be assembled by either (a) covalent branching or (b) the “peptide-amphiphile” method (non-covalent self-assembly of lipophilic molecules) (Figure 5) [88].

Radio isotopic assay

Method based on the degradation of radio-labeled (such as ^{14}C or ^3H) natural substrates has been used extensively for the detection of MMP activity, especially MMP-2 and -9 [89–92]. The method is performed in plastic plates of 96 flat-bottom micro-wells in which labeled substrates are dried to a film from thermally reconstituted fibrils. The enzyme samples were added to the wells and were incubated, and the fluid was removed for scintillation counting of the release of labeled degradation products [93,94].

However, the method requires expensive devices for registering the released marker, as well as isotope handling, and generates radioactive waste disposal problems [95,96].

Phage-displayed assay

Recently two biological systems using phage display have been developed to quickly search and decode a large peptide library for substrate optimization. The first system uses monovalent substrate phage display to study substrate specificity of membrane type-1 MMPs [97,98]. The second system uses polyvalent substrate phage display to more quickly optimize substrates of stromelysin, matrilysin and human collagenase 3 [99–102].

In Figure 6 is shown a diagram of the fTC phage (for clarity, not drawn to scale). The gene III protein extends from phage body from the C to the N terminus. At the N termini are the peptide epitopes and the random hexamer domains that may or may not act as substrates, depending on the encoded

Table II. The fluorophore, quencher, cleavage site and enzyme of various substrates*.

Substrate	Fluorophore	Quencher	Cleavage site	Enzyme
(Mca)Acetyl-Pro-Leu-Gly-Leu-(3-Dnp-L-2,3-diaminopropionyl)-Ala-Arg-NH ₂ (FS-1)	Mca	Dnp	Gly-Leu	MMP-2, 7, 9, 13
Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH ₂ (FS-6)	Mca	Dnp	Gly-Leu	MMP-1, 8
Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(Dnp)-NH ₂	Mca	Dnp	Glu-Nva	MMP-3
Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly-Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Ile/Val-Arg	Mca	Dnp	Gly-Leu	MMP-1, 2, 13
DNP-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH ₂	Trp	Dnp	Gly-Leu	vertebrate collagenase, gelatinase
DNP-Pro-Leu-Gly-Met-Trp-Ser-Arg	Trp	Dnp	Gly-Met	fibroblast MMP-2, neutrophil MMP-9
DNP-Pro-Leu-Ala-Leu-Trp-Ala-Arg	Trp	Dnp	Ala-Leu	fibroblast collagenase
DNP-Pro-Leu-Ala-Tyr-Trp-Ala-Arg	Trp	Dnp	Ala-Tyr	neutrophil collagenase
DNP-Pro-Tyr-Ala-Tyr-Trp-Met-Arg	Trp	Dnp	Ala-Tyr	stromelysin
Dabcyl-Gaba-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Glu-(EDANS)-Ala-Lys-NH ₂ (TNO003)	EDANS	Dabcyl	Glu-Nva	MMP-3
Dabcyl-Gaba-Pro-Gin-Gly-Leu-Glu(EDANS)-Ala-Lys-NH ₂ (TNO211)	EDANS	Dabcyl	Gly-Leu	MMP-1, 2, 3, 9
C ₆ -(Gly-Pro-Hyp) ₅ -Gly-Pro-[Amp/Adp]-Gly-Pro-Gln-Gly-Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-(Gly-Pro-Hyp) ₅ -NH ₂	Amp or Adp	Dnp	Gly-Leu	MMP-1, 2
DNP-Pro-β-cyclohexyl-Ala-Gly-Cys(Me)-His-Ala-Lys-NMA-NH ₂	NMA	Dnp	Gly-Cys(Me)	MMP-1, 8, 9

*Abbreviations used: Mca, 7-methoxycoumarin; Dnp, 2,4-dinitrophenyl; Trp, tryptophan; EDANS, 5-((2-aminoethyl)amino)naphthalene-1-sulphonic acid; Dabcyl, 4-(4-methylaminophenylazo)benzoyl; Amp, L- or D-2-amino-3-(7-methoxy-4-coumaryl)propionic acid; Adp, L- or D-2-amino-3-(6,7-dimethoxy-4-coumaryl)propionic acid; NMA, N-methylanthranilic acid.

peptide sequence. The phage were treated in solution with MMPs, and those carrying substrate sequences (Left) were cleaved in the hexamer site, whereas non-substrates (Right) were not. The entire digest was treated with antibodies to the tethers and captured by using a resin that carries protein A. The protein A-antibody-phage complexes were precipitated by centrifugation, and the phage that was not bound by the antibodies remained in solution and could be recovered for amplification. After amplification, the

phage could be analyzed or subjected to further rounds of selection.

The differences in the approaches of monovalent and polyvalent phage as protease substrate discovery tools invite comparison of the two systems. While the monovalent systems have been shown to be quite useful, the polyvalent system possesses certain advantage: (a) all phage can act as substrate phage in the polyvalent system. In monovalent systems, it is estimated that only 10% of the phage particles carry



Figure 5. Structures of fluorogenic triple-helical peptide (fTHP) The top one using a covalent branching protocol and the bottom one using a peptide self-assembly approach.

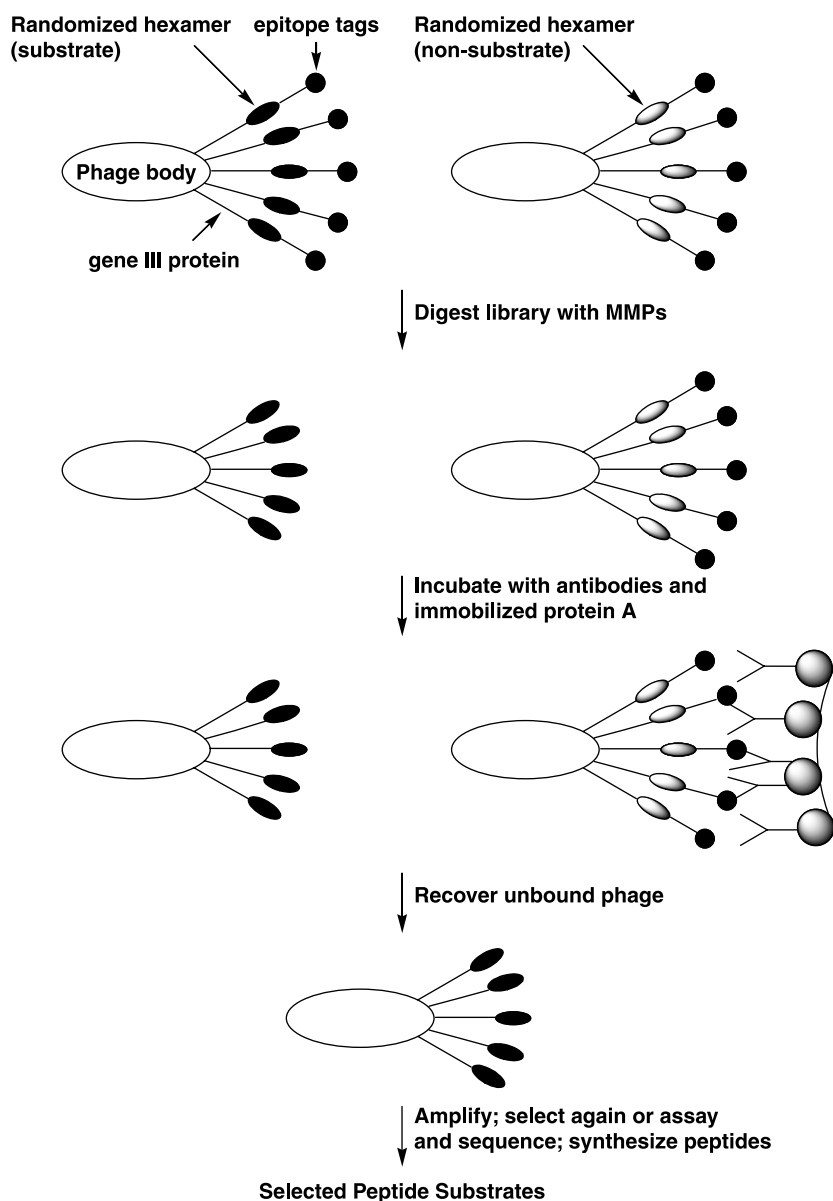


Figure 6. Outline of the tether phage selection.

one copy of the recombinant pIII protein. This increases the effective substrate concentration in the polyvalent system, thus increasing the sensitivity of the system (due to higher concentration of substrate at a given level of phage). (b) Since 90% of the monovalent phage do not carry pIII fusions, the non-recombinant phage lacking the tether must be removed prior to selection. This is accomplished by immobilizing the recombinant phage in microtiter plates coated with tether binding protein and treating the phage with protease while immobilized. Polyvalent phage, being 100% recombinant, is digested in solution rather than immobilized on a solid surface. The advantages of this are: (a) there is little restriction on number of phage that can be screened in solution, but the surface system limits the number of phage that can be routinely immobilized on micro-titer plates. Scale

up of the solution phage system is thus very convenient when significantly larger libraries are prepared; (b) protease resistance of tether binding protein is not an issue; (c) solution proteolysis offers more precise control of cleavage conditions. This has proven especially useful in the quantitative dot-blot assay [103].

The major disadvantage of the polyvalent system is the appearance of non-reactive phage clones, which does not occur in the monovalent system because of the pre-binding step which essentially eliminates clones with defective epitopes. Nothing about the polyvalent method, however, precludes the use of a binding step in later rounds to eliminate non-reactive clones. Thus, a strategy using a combination of conditions favoring multivalent and monovalent interactions may be used to advantage [104].

Future development and screening of phage-displayed peptide library should lead to the discovery of novel ligands for many purposes, including the identification of new drug candidates [105].

Multiple-enzyme/multiple-reagent assay

Based on the fluorogenic triple-helical peptide (fTHP) assay, Rasmussen et al [106] reported on a novel technique that can be used to simultaneously measure activity levels for MMPs. The technique, termed the multiple-enzyme/multiple-reagent assay system (MEMRAS), relies on the use of reagents such as substrates with varying selectivity profiles against a group of enzymes. When reaction rates are measured by following a change in fluorescence with time, for mixtures of enzymes, an equation with unknown concentrations for each activity is generated for each reagent used. Simultaneously solving the set of equations leads to a solution for the unknown concentrations.

They have successfully applied a two-by-two MEMRAS to measure activity levels for mixtures of MMPs such as collagenase 3 and gelatinase A.

The five steps in the experiment and analysis are described below. First, in step 1, the enzyme standard dilutions, mixed-enzyme samples, and reagents were added to a 96-well plate. More specifically, four combinations of concentrations of collagenase 3 and gelatinase A were placed in twice-duplicated wells. To sets of duplicated wells were added reagent A and reagent B. Other wells were used for standard curves of collagenase 3 and gelatinase A *versus* reagents A and B. In step 2, the plate reader recorded the fluorescence values every 5 min for 5 h. In step 3, the values of the slope of the net fluorescence *versus* time curves were plotted *versus* enzyme concentration for the standard enzyme dilutions, for each enzyme/reagent pair. In step 4, the net fluorescence *versus* time was plotted for the mixed-enzyme samples when reacted with each reagent. Finally, in steps 5 and 6, the slopes from steps 3 and 4 were combined into a set of two equations in two unknowns and solved for the “unknown” enzyme concentrations in the mixed-enzyme samples.

The two-by-two MEMRAS system can quantify how much of the total activity is due to collagenase 3 and how much is due to gelatinase A. The measurement error of the MEMRAS system is significantly less (10-fold) than the error arising from a single-reagent system.

The substrates developed, even if not very selective, also became useful when the MEMRAS technique was developed. As opposed to a single reagent that is highly selective for a given MMP, MEMRAS relies on using as many reagents as there are enzymes, and the reagents do not necessarily have to be extremely selective. This indicates that the MEMRAS system is a unique solution to the problem that substrates are

never quite selective enough to directly assay activities in biological fluids.

Activity-based profiling assay

Activity-based proteomic probes (ABPP) is a chemical strategy that utilizes active site-directed probes to record variations in the activity of enzymes in whole proteomes. ABPP typically possess three general elements: (a) a binding group that promotes interactions with the active sites of specific classes of enzymes, (b) a reactive group for covalent label with the active-site of enzymes, and (c) a reporter group (e.g., fluorophore or biotin) for the visualization and affinity purification of probe-labeled enzymes. Using this technique, Saghatelian et al [107] designed a rhodamine-tagged hydroxamate benzophenone probe (HxBP-Rh) for MMPs by incorporating a zinc-chelating hydroxamate and a benzophenone photocrosslinker group, which promoted selective binding and modification of MMP active sites, respectively. HxBP-Rh labeled active MMPs but not their zymogen or inhibitor-bound forms. HxBP-Rh were applied to identify MMPs up-regulated in invasive cancer cells and to evaluate the inhibitor sensitivity of MMPs in whole proteomes. With great sensitivity, HxBP-Rh was able to detect active MMP-2 at concentrations as low as 3 nM in tissue proteomes. Despite the potential of this technique, no endogenous active MMPs have been identified yet.

Another activity based profiling assay of MMPs relies on the use of group-specific inhibitor affinity sorbents for the selective enrichment prior to standard proteomic approaches. The use of MMP inhibitor affinity sorbents with immobilized affinity ligands allows selective enrichment of proteins based on common structural or functional properties, prior to separation and analysis, thereby serving as an effective sample cleanup step resulting in enhanced sensitivity. Freije et al [108] applied this technique to synovial fluid from a rheumatoid arthritis patient followed by gelatin zymography. Using a broad-spectrum MMP inhibitor with nM affinity (TAPI-2), they revealed a strong enrichment of distinct MMPs from this biological sample that were not clearly visible in the original sample. This resulted in an increased extraction yield for all tested MMPs. For MMP-1, -7, -8, -10, -12, and -13 extraction yields of at least 98.8% were obtained, while for MMP-9 an extraction yield of at least 96.1% was reached. This technique is a powerful tool to study low abundance, active MMPs in complex biological samples.

Conclusions and future perspectives

A wide range of methods are available for detecting MMPs activity, however, most of the current approaches are limited by some disadvantages.

A fundamental problem with bioassays is that they lack specificity, i.e. they are unable to distinguish between two enzymes that degrade the same substrate, thus leading to the inaccurate measurements with elevated enzyme levels [109]. Radio isotopic assay is time-consuming, difficult to automate, and generates waste disposal problems. Fluorogenic substrates have the advantage of allowing continuous monitoring of activity and are thus suitable for mechanistic studies, but their use in high throughput screening is often limited by their high cost, low stability and limited solubility in aqueous solvents [110]. Western blotting requires knowledge of the MMPs native substrate and the availability of anti-MMPs antibodies. And what is more, it is expensive and time-consuming [111]. Zymography is more suitable for screening of MMP inhibitors; however, zymography gives a measure of total potential enzymatic activity but does not allow determination of the net level of activity present in a sample. As compared with the above mentioned methods, the immuno capture assay seems better that can meet the sensitive, selective and rapid requirements.

The recently developed phage-displayed technique enables the high-throughput screening of large combinatorial chemical libraries in a short period feasible. Activity-based profiling assay offers a powerful tool to study low abundance, active MMPs directly in complex biological samples. Multiple-enzyme/multiple-reagent assay provides us the unique solution to the problem that substrates are never quite selective enough to directly assay activities in biological fluids. These techniques, still developing, need further validation in the future. In addition, a combinational use of the above mentioned assay methods, such as fluorescence quenching immunoassay [112], zymography incorporating fluorescent substrates [32,34,35,113,114], will combine the advantages and avoid the disadvantages of the single method. This is also a promising way to assay MMPs and their inhibitors in the future.

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