

Determination of species cross-reactivity using MMP-2 Activity Assay and correlation with ELISA and gelatin zymography

L. Smith, A. S. Marengi, and S. J. Capper

Amersham Biosciences UK Ltd., Cardiff Laboratories, Forest Farm, Whitchurch, Cardiff, UK

Three methods for the detection of MMP-2, zymography, ELISA, and activity assay, were compared for their quantitative performance and for their range of testable species. Unlike the ELISA, Biotrak™ MMP-2 Activity Assay readily detected low levels of MMP-2 in cell culture supernatants from a variety of animal species. In addition, MMP-2 concentrations were easily determined for these samples, which is not the case with zymography.

Introduction

Matrix metalloproteinases (MMPs) are a family of Zn²⁺ endopeptidases that possess the ability to break down extracellular matrix molecules associated with tissue destruction (1). They can be grouped by their domain structure into collagenases, gelatinases, stromelysins, matrilysin, and membrane type MMPs (MT-MMPs). MMP-2, a member of this family, is a gelatinase (gelatinase A/type IV collagenase) known to cleave a number of substrates including gelatins; collagen types IV, V, VII, X, XI; fibronectin; proteoglycan; and elastin. It is secreted as a 72 kDa precursor form, proMMP-2, that can be cleaved *in vitro* by organomercurial compounds such as p-aminophenylmercuric acetate (APMA) to an active enzyme of 66 kDa.

Various techniques, such as zymography, ELISA, and activity assays, are used to detect MMP-2. Zymography is an electrophoretic technique that relies on the gelatinolytic activity of MMP-2. The degree of gelatin degradation is proportional to the amount of MMP-2 contained in the sample. Although sensitive, this method is time consuming, lacks specificity, and is only semi-quantitative. ELISAs and activity assays allow precise and accurate measurement of MMPs. Both of these types of assays, however, often use highly specific antibodies that do not cross react with sample types from other animal species, limiting the range of their usefulness.

Here we present results that demonstrate that the new Biotrak MMP-2 Activity Assay quantitatively measures MMP-2 in samples from a range of animal species, overcoming the limitations of these other methods.

Preparation of cell culture supernatants

Mouse Swiss 3T3 fibroblasts, rat aorta smooth muscle fibroblasts, porcine aorta smooth muscle fibroblasts, rabbit synoviocytes (HIG-82), human monocytes (U937), and human lung fibroblasts (MRC-5) were grown for 5 days to confluency in 75 cm³ flasks. The media was recovered, concentrated (apart from U937, MRC-5), and stored at -80 °C prior to use in the various assays.

Gelatin zymography of cell culture supernatants

Zymography was performed using Novex™ gel electrophoresis apparatus, zymogram gels and reagents (2). The cell culture supernatants were diluted 1:10, and samples were mixed with an equal volume of sample buffer. Ten microlitres was loaded into each well of a 10% Tris-Glycine gel containing 0.1% (w/v) gelatin as a substrate. The gel was run for 1.5 h, treated with renaturing buffer, and then incubated overnight at 37 °C in zymogram developing buffer. Areas of MMP digestion were visualized by negative staining with Coomassie™ Blue.

Biotrak MMP-2 Activity Assay

MMP-2 activity was determined using Biotrak MMP-2 Activity Assay System. This assay can measure both total MMP-2 (following activation by APMA) and endogenously active MMP-2 in tissue culture supernatants, serum, plasma, urine, synovial fluid, and tissue homogenates. The assay uses a pre-cursor (pro) form of the detection enzyme that is proteolytically cleaved by captured active MMP-2 into an active detection enzyme. Using protein engineering, the natural activation sequence in the pro detection enzyme has been replaced with an artificial sequence

recognized by specific MMPs (3). MMP-activated detection enzyme can then be measured using a specific chromogenic peptide substrate (Fig 1).

The activity assay was performed as described in the protocols supplied (see also Fig 1). Standards and samples were incubated in microplate wells pre-coated with anti-MMP-2 antibody. Any MMP-2 present will be bound to the wells, other components of the sample being removed by washing and aspiration. In order to measure total (pro and active) levels of MMP-2, APMA was added to the assay along with the detection reagent. Endogenous levels of active MMP-2 may be detected without APMA treatment. The standard used was proMMP-2 that is activated in parallel for either sample type. The resultant colour was read at 405 nm using a microplate spectrophotometer, and the concentration of MMP-2 in the samples determined by interpolation from a standard curve.

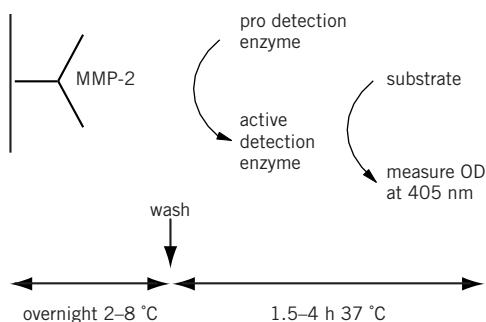


Fig 1. Schematic diagram of Biotrak MMP-2 Activity Assay.

MMP-2 ELISA

ELISAs were performed using Biotrak Human MMP-2 ELISA (RPN 2617) from Amersham Biosciences, which is specific for proMMP-2. Standards and samples were incubated in microplate wells pre-coated with an anti-MMP-2 antibody. The plates were washed and aspirated to remove any excess material. Bound MMP-2 was detected by a peroxidase-labelled Fab' antibody to MMP-2. The amount of peroxidase bound to each well was determined by addition of TMB substrate. The reaction was stopped and the resultant colour read at 450 nm (Fig 2).

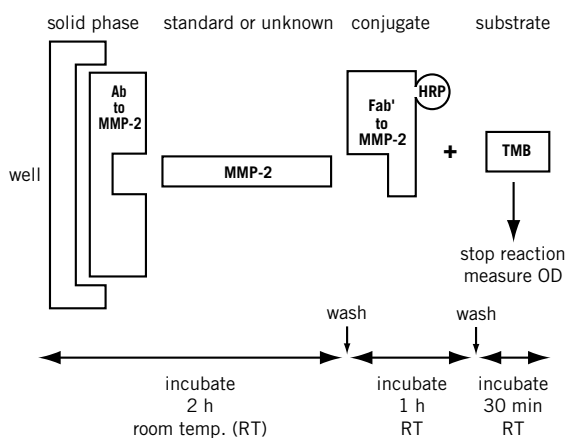


Fig 2. Schematic diagram of Biotrak MMP-2 Human ELISA.

Qualitative MMP-2 detection using zymography

Zymography demonstrated that all species produced bands that compare favourably with the 72 kDa band detected for human proMMP-2 (Fig 3). Areas of gelatinolytic activity were visualized as clear, non-staining regions of the gel. The rabbit HIG-82 cell culture supernatant, however, showed no significant

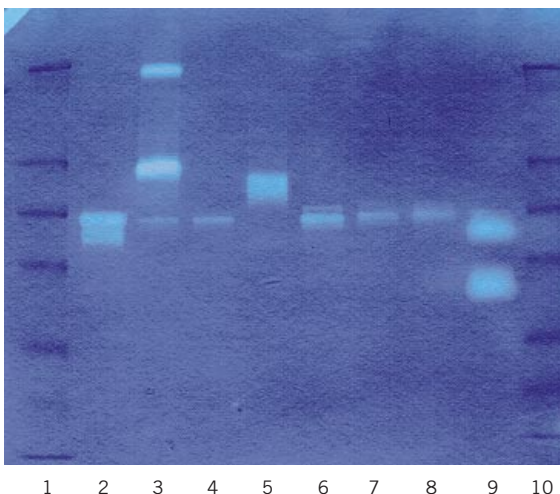


Fig 3. Typical zymogram of cell culture supernatant samples from different animal species. All samples were diluted 1:10 prior to loading. Lane 1, molecular weight marker; lane 2, proMMP-2 standard (human); lane 3, U937 (human); lane 4, MRC-5 (human); lane 5, HIG-82 (rabbit); lane 6, porcine smooth muscle; lane 7, rat smooth muscle; lane 8, 3T3 (mouse); lane 9, active MMP-2 (human); lane 10, molecular weight marker.

gelatinolytic activity, although a 72 kDa band had been observed in previous zymograms. The absence of activity may be due to the sensitivity of this particular zymogram. The samples containing the most MMP-2 activity based on band intensity are porcine, mouse, and rat cells, followed by human MRC-5 and U937. High molecular weight bands were visible for U937 cells, which may represent MMP-2/TIMP-2 complexes or MMP-2 dimers that can dissociate following activation (4). Bands corresponding to MMP-9 (92 kDa) were also seen for U937 cells.

Quantitative MMP-2 Activity Assay for a variety of species

Detectable levels of MMP-2 were found in all cell culture samples tested by Biotrak MMP-2 Activity Assay (Table 1). A correlation existed between the final corrected dilution activity levels and the size/intensity of the bands from zymography. For example, porcine cell culture supernatant produced the most intense bands in zymography and therefore required a greater dilution for accurate measurement by the activity assay. Similarly for rabbit HIG-82 cell culture supernatants, the low levels detected by the MMP-2 Activity Assay were not visible by zymography.

Table 1. Summary of MMP-2 activity in a variety of cell culture supernatants as determined using Biotrak MMP-2 Activity Assay.

species	dilution	observed (ng/ml)	actual concentration (dilution corrected) (ng/ml)
U937 (human)	1:20	0.84	16.8
	1:40	0.55	22.0
MRC-5 (human)	1:20	1.10	22.0
	1:40	0.73	29.2
porcine	1:200	0.64	128
	1:400	0.41	164
HIG-82 (rabbit)	1:20	0.47	9.4
	1:40	0.18	7.2
mouse	1:200	0.85	170
	1:400	0.47	188
rat	1:20	0.87	17.4
	1:40	0.59	23.6

Limited cross-reactivity of Human MMP-2 ELISA

Only the porcine cell culture supernatant demonstrated cross-reactivity to the Human MMP-2 ELISA, limiting its usefulness for screens involving a broad range of animal species. Results by zymography and the MMP-2 Activity Assay illustrated the presence of MMP-2 in all cell culture supernatants tested, but due to antibody specificity of this ELISA, all remaining samples failed to produce any results by ELISA.

Conclusions

Table 2. Summary of MMP-2 measurement by zymography, MMP-2 Activity Assay, and Human MMP-2 ELISA in cell culture supernatants from a variety of animal species.

Species	MMP-2 species cross-reactivity		
	zymography	ELISA	activity assay
Human	✓	✓	✓
Mouse	✓		✓
Rat	✓		✓
Rabbit	✓		✓
Porcine	✓	✓	✓

The MMP-2 Activity Assay provides a simple, specific, and quantitative determination of active and proMMP-2 using samples from a range of animal species (Table 2). The MMP-2 Activity Assay has also been successfully applied to human serum and plasma samples (data not shown). Using this assay system, users are able to test a variety of cell lines without the limitations of species non-cross-reactivity associated with most ELISAs. While zymography was shown to be a useful qualitative technique for a variety of animal species, it lacks the specificity and true quantitative nature of an antibody-based activity assay such as Biotrak MMP-2 Activity Assay.

References

1. Corcoran, M. L. *et al.*, *Enzyme Protein* 49, 7–19 (1996).
2. Kleiner, D. E. and Stetler-Stevenson, W. G., *Anal. Biochem.* 218, 325–329 (1994).
3. Verheijen, J. H. *et al.*, *Biochem. J.* 323, 603–609 (1997).
4. Zucker, S. *et al.*, *J. Immunol. Methods* 148, 189–198 (1992).

ORDERING INFORMATION

Biotrak MMP-2 Activity Assay

96 wells

RPN2631