Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Assays for matrix metalloprotein activity

Kazushi Imai
Department of Biochemistry, Nippon Dental University, 1-9-20 Fujimi, Chiyoda-ku, Tokyo 102-8159, Japan
Yasunori Okada
Department of Pathology, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-0016, Japan

Method Article

Keywords: MMP, matrix metalloproteins, collagenase, gelatinase, transferrin, carboxymethylated transferrin, zymography

Posted Date: April 3rd, 2008

DOI: https://doi.org/10.1038/nprot.2008.85

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Abstract

Introduction

To detect activities of MMP-1, MMP-2, MMP-3, MMP-7 and MMP-9, use radio-labeled fibrillar collagen \(for MMP-1), gelatin \(for MMP-2 and MMP-9) and carboxymethylated transferrin \(for MMP-3 and MMP-7) substrates for the assays [1], although these can be substituted with commercially available FITC-labeled substrates and fluorogenic synthetic peptides for the MMPs. Zymography using gelatin or casein substrate gels is also useful to monitor gelatinolytic activity of MMP-2 and MMP-9 or caseinolytic activity of MMP-3 and MMP-7 in each column fraction, although zymography is not suitable for calculation of purification fold and recovery of proteinases.

Procedure

Collagenase assay for MMP-1 1. Mix a small amount of \[^{14}C]-labeled collagen \(2$_{3\ \text{ml}}$) with unlabeled collagen in 3% \(vol/vol) acetic acid \(45 ml; 3 mg/ml) to make the count 3000 c.p.m. in 50 µl. 2. Dialyze the mixture against 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.02% \(wt/vol) NaN_3 and then 0.01 M Tris-HCl, pH 7.5, 0.2 M NaCl, 0.02% \(wt/vol) NaN_3 at 4˚C, and store the collagen at 4˚C. 3. For collagen fibril assay, take 50 µl of the labeled collagen solution into 400 µl long centrifuge tubes \(150 µg/tube), and incubate with 10 µl of each or combined fraction in TNC or TNC+B buffer, pH 7.5 \(225 µl) in the presence or absence of 1 mM APMA \(15 µl of 20 mM APMA) at 37°C \(total volume of 300 µl). 4. After incubation, spin the tubes at 10,000 g for 15 min using Beckman microcentrifuge and take 200 µl of the supernatant for counting using a scintillation counter. ▲CRITICAL For the controls, add 250 µl of TNC or TNC+B buffer, pH 7.5 \(blank), 10 µl of 100 µg/ml trypsin and 240 µl of the buffer \(negative control) or 10 µl of 4 mg/ml bacterial collagenase and 240 µl of the buffer \(positive control) in the assay. Activity is calculated as follows: \(count of enzyme solution digestion – count of trypsin digestion) / \(count of bacterial collagenase digestion – count of trypsin digestion) x 150 µg/min. Although we have used \[^{14}C]-labeled collagen as a substrate, collagenase assays using FITC-labeled type I collagen \(collagenase assay kit for collagenase, Chondrex, "http://www.chondrex.com":http://www.chondrex.com), fluorogenic peptide substrate \(MMP-1 Substrate I, Fluorogenic, MERC, "http://www.merckbioscience.com/":http://www.merckbioscience.com/) or FRET substrate \(SensoLyte Plus™ 520 MMP-1 assay kit, AnaSpec, "http://www.anaspec.com":http://www.anaspec.com) are commercially available and can be used to detect MMP-1 activity. **Gelatinase assay for MMP-2 and MMP-9** 1. Prepare heat-denatured gelatin by incubation of the \[^{14}C]-labeled collagen at 60˚C for ~30 min. 2. Take 50 µg of the gelatin solution into 400 µl long centrifuge tubes, and incubate with enzyme solution in TNC or TNC+B buffer, pH 7.5 \(135 µl) in the presence or absence of 1 mM APMA \(15 µl of 20 mM APMA) at 37˚C \(total volume of 200 µl). 3. After incubation, add 100 µl of cold 45% \(vol/vol) trichloroacetic acid. Keep samples in ice for at least 10 min and spin at 10,000 g for 15 min. 4. Take 100 µl of the supernatant for counting using a scintillation counter. ▲CRITICAL As for controls, add 150 µl of TNC or TNC+B buffer, pH 7.5 \(blank) or 10 µl of 100 µg/ml trypsin and 140 µl of the buffer \(positive

control) in the assay. Activity is calculated as follows: \(count of enzyme solution digestion – count of blank) / \(count of trypsin digestion – count of blank) x 150 µg/min. Although we have used \[14^C]-labeled gelatin as a substrate, quenched fluorescent peptide substrate \(EnzChek for gelatinases, Invitrogen, "http://www.invitrogen.com/":http://www.invitrogen.com/) is commercially available and can be used to detect MMP-2 and MMP-9 activities. **Carboxymethylated transferrin \(Cm-Tf) assay for MMP-3 and MMP-7** 1. Reduce human transferrin \(Tf) \(20 mg) by incubation with 20 mM dithiothreitol in 20 ml of 0.2 M Tris-HCl, pH 8.6 containing 8 M urea for 4 h at 37˚C. 2. Apply the reduced Tf to a column of Sephadex G-50 \(Φ 2.5 cm x 20 cm) equilibrated in 0.2 M Tris-HCl, pH 8.6 containing 8 M urea at room temperature to remove dithiothreitol, and combine the void volume. 3. Incubate the pooled sample with 1 mCi of \[^{3}H]iodoacetic acid for 30 min at 23˚C, and then with 30 mM iodoacetic acid for 30 min at 23˚C to complete carboxymethylation. 4. After the reactions, add the same volume of cold 10% \(vol/vol) trichloroacetic acid to the radiolabeled Tf and obtain the precipitate by centrifugation at 21,000 g for 5 min at 4˚C using a benchtop centrifuge. Repeat this precipitation step three times. 5. Dissolve the precipitate in 50 mM Tris-HCl, pH 7.5, 0.15 M NaCl at a concentration of 3 mg/ml. Store the substrate at -20˚C in 1 ml portions. 6. For the assay, take 10 µl of \[^{3}H]Cm-Tf into 1.5 ml Eppendorf tubes, and incubate with 10 µl of each or combined fraction in TNC or TNC+B buffer, pH 7.5 in the presence or absence of 1 mM APMA \(10 µl of 3 mM APMA) at 37˚C \(total volume of 30 µl). After incubation, add 200 µl of cold 3.3% \(vol/vol) trichloroacetic acid. Keep samples at room temperature for 15 min and spin at 10,000 g for 5 min. Take 100 µl of the supernatant for counting by a scintillation counter. ▲CRITICAL As for controls, add 10 µl of TNC or TNC+B buffer, pH 7.5 \(negative control) or 10 µl of 100 µg/ml trypsin in TNC buffer, pH 7.5 \(positive control) in the assay. Activity is calculated as follows: \(count of enzyme solution digestion – count of blank) / \(count of trypsin digestion – count of blank) x 30 µg/min. Although we have used \[^{3}H]-labeled Cm-Tf as a substrate, fluorogenic peptide substrates \(Oncogene, "http://www.apoptosis.com":http://www.apoptosis.com or MERCK, "http://www.merckbioscience.com":http://www.merckbioscience.com) are commercially available and can be used to detect MMP-3 and MMP-7 activities. **Zymography** 1. Make SDS-PAGE gels \(1 or 2 mm in thickness) containing 0.2% \(wt/vol) gelatin or 0.1% \(wt/vol) casein. 2. Take $20_{40\ \mu l\ of\ samples\ from\ column\ fractions,\ and\ incubate\ them\ with\ equal\ volume\ of\ SDS\text{-}PAGE\ sample\ buffer\ without\ 2\text{-}mercaptoethanol\ for}$ 30 min at 37˚C. Subject the samples to SDS-PAGE at 4˚C. 3. After electrophoresis, wash the gels in 2.5% \(vol/vol) Triton X-100 in TNC buffer, pH 7.5 for 15 min twice and then incubate in the incubation buffer at 37˚C for 12 - 24 h under shaking. 4. After incubation, stain with Coomassie Brilliant Blue R and destain the gels. ▲CRITICAL Rinsing the gels with 2.5% \(vol/vol) Triton X in TNC buffer, pH 7.5 is necessary to remove SDS from the gels. ▲CRITICAL Activation of proMMPs with APMA is not necessary for zymography, since proMMPs are spontaneously activated during SDS-PAGE probably because of their conformational changes. Proteolytic bands corresponding to proMMPs and active MMPs are seen as transparent bands within dark blue background. ▲CRITICAL Inhibitor studies can be done by adding appropriate inhibitors \(e.g., 15 mM EDTA for MMPs, 2 mM phenylmethane sulfonyl fluoride for serine proteinases, 5 mM N-ethylamaleimide for cysteine proteinases and 0.5 mM pepstatin A for aspartic acid proteinases) into the incubation buffer during incubation of the gels.

References

1. Okada, Y., Nagase, H. & Harris, E.D., Jr. A metalloproteinase from human rheumatoid synovial fibroblasts that digests connective tissue matrix components. Purification and characterization. _J Biol Chem_ **261**, 14245-55 \(1986).