A Specific Immunocapture Activity Assay for Matrix Metalloproteinases Using 5-FAM/QXL 520™ FRET Peptide

Xudong Zhu, Yi Tang, Xiuqing Li,, Xing Han, Zhenjun Diwu and Vera Rakhmanova

AnaSpec Inc., 2149 O'Toole Ave, San Jose, CA 95131, USA

Introduction

Matrix metalloproteinases (MMPs) belong to a family of secreted or membrane-associated zinc endopeptidases capable of digesting extracellular matrix components. MMPs play roles in the multiple stages of tumor development and metastasis; rheumatoid arthritis; wound healing; angiogenesis and other pathological and physiological events. Some assays, such as zymography, ELISA, and peptide-based activity assays, have been developed to facilitate the research and drug discovery for MMPs. Zymography is a relatively low-throughput method, and ELISA does not provide MMP activity information. FRET-peptide based activity assay offers significant advantages because of their simple and fast format - a feature ideal for large scale drug compound screening. When a FRET-peptide based assay for MMPs is used in cell or tissue lysates, non-specific cleavages by MMPs and other proteases are also measured, which may simultaneously be present in biological fluids. To solve this problem of non-specific cleavages and allow the assay of one particular MMP, an anti-MMP is first used to capture the specific MMP from the mixture, and then the activity of this particular MMP is measured by a FRET-peptide substrate.

In previous studies, we developed highly sensitive FRET-peptide substrates for MMPs using the strongly-fluorescent 5-FAM as the FRET donor and QXL™520 as the quencher. In this study, we incorporate the 5-FAM/QXL™520 FRET-peptide substrate to the above specific immunocapture assay. This results in a high sensitive, specific detection of MMP-1, MMP-9 and MMP-13 activity in mixed biological samples.

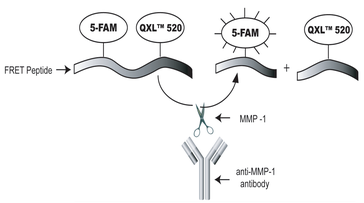
Methods

Materials: 5-FAM/QXL™520 MMP FRET substrate, anti-MMP antibodies, and recombinant MMPs are from AnaSpec. MMPs are full-length MMPs, containing pro-domain, catalytic, hinge, and hemopexin domain. Plasmids encoding MMPs are from Origene.

Preparation of MMP-containing cell supernatant: Plasmids encoding MMP-1, 2, 3, 9, and 13 were individually and transiently transfected to CHO cells using Lipofectamine 2000 (Invitrogen), following the manufacturer's protocol. Supernatant was collected at 24-48 h after transfection and concentrated with an Amicon centrifugal filter device (Millipore) 20-30 fold. The concentrate was stored at –80°C.

Immunocapture MMP activity assay: Antibodies for MMP-1, MMP-9, and MMP-13 were separately coated onto 96-well black microplates. Samples containing MMPs were added to the microplate and incubated for 1 h at RT. Plates were washed and incubated with APMA at a final concentration of 1 mM. Alternatively, MMPs can be first activated by APMA and then applied to microplate for immunocapture. The plates were washed again and a 5-FAM/QXL™520 FRET substrate solution was added. Fluorescence signal was recorded continuously using either FlexStation 384II fluorescence microplate reader (Molecular Devices) at Ex/Em=490/520 nm, cut off 515 nm, or Flx800 fluorescence microplate reader (BioTek Instruments) at Ex/Em=485±20/528±20 nm.

Results



The principle of immunocapture MMP activity assay.

MMP-1 in biological samples is first captured by the immobilized anti-MMP-1, and its proteolytic activity measured by the 5-FAM/QXL™520 FRET peptide. The fluorescence signal, upon cleavage, is monitored at Ex/Em=490±20 nm/520± 20 nm.

By changing the anti-MMP antibody, the assay can be used to measure MMP-9 or 13 activity.

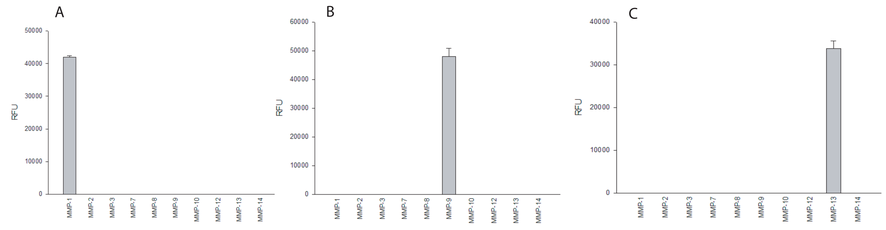


Figure 1. Specificity of immunocapture activity assay as tested by purified recombinant MMPs.

APMA-activated recombinant MMP-1, 2, 3, 7, 8, 9, 10, 12, 13, and 14, at 30 ng each, were added to the anti-MMP antibody pre-coated microplates. (A). Anti-MMP-1; (B). Anti-MMP-9; and (C) anti-MMP-13. After incubation, plates were washed and the activity of MMPs detected by a 5-FAM/QXL™520 FRET peptide substrate. Fluorescence was monitored 1 hour after addition of the substrate. The reading from the substrate control well was subtracted from the readings of the other wells. (n=3, mean±S.D.)

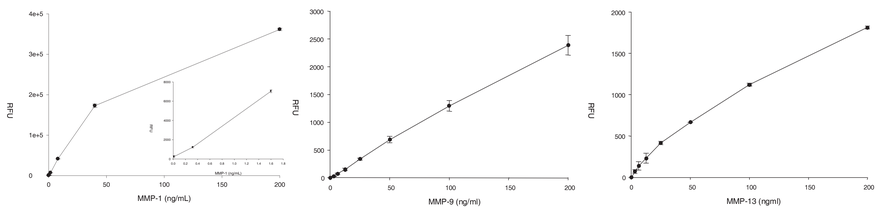


Figure 2. The sensitivity of immunocapture MMP activity assay.

Purified recombinant pro-MMP-1, 9, and 13 were serially diluted and added to the anti-MMP-1, anti-MMP-9, and anti-MMP-13 coated plates, respectively. APMA solution was used to activate MMPs bound on the microplate. MMP activity was measured by the cleavage of the 5-FAM/QXL™520 FRET peptide. Fluorescence reading (RFU) after 2 h was plotted versus the amount of MMPs. The assay can detect low picogram range of MMP-1 and sub-nanogram range of MMP-9 and 13. (n=3, mean±S.D.)

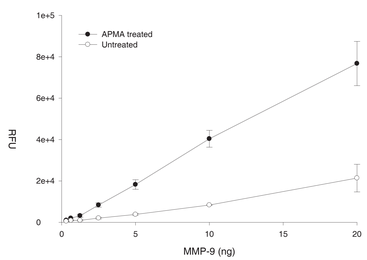


Figure 3. The activity of total MMP-9 (pro-form plus active form) in the immunocapture assay.

Purified recombinant MMP-9 was serially diluted and added to anti-MMP-9 coated plate. 1 mM APMA solution was added to half of the sample wells and assay buffer was added to the other half of the sample wells. After a 2 h incubation at 37°C, the plate was washed. The activity of MMP-9 was measured by the cleavage of the 5-FAM/QXL™520 FRET peptide. (n=3, mean±S.D.)

Pro-MMPs tend to be partially activated during storage. Therefore, the recombinant MMP-9 solution may contain both active and latent enzymes. The MMP-9 antibody captures both active and pro-enzyme. Even without the addition of APMA, MMP-9 showed some activity in the assay. After APMA incubation, the latent form converts to the active form and the activity of total MMP-9 is measured. Therefore, by using APMA, the activity of both pre-existing active form and total enzyme can be differentiated and measured.

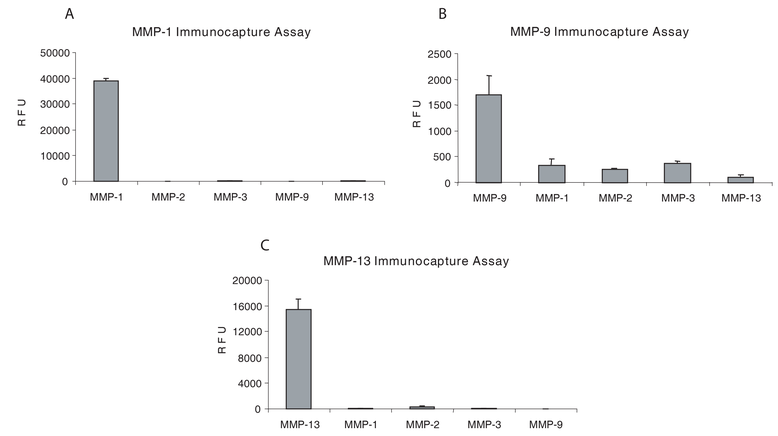


Figure 4. Specificity of MMP immunocapture assay with biological samples.

CHO cells were transfected with MMP-1, 2, 3, 9, or 13 plasmid individually. Supernatant of cell culture was collected 24 h and 48 h after transfection. The enzyme activity in the supernatants containing MMP-1, 2, 3, 9, or 13 was tested by (A). MMP-1; (b). MMP-9 and (C). MMP-13 immunocapture assays.

Discussion and Conclusion

FRET-based peptide substrates are increasingly being used for measuring the activity of MMPs. We previously developed several peptide substrates for MMPs, which contain a novel 5-FAM/QXL™520 FRET pair. Although these FRET peptides are highly sensitive for measuring the activity of MMPs, we found that none of these FRET peptides are exclusively cleaved by any one particular MMP. Since multiple MMPs generally co-exist in biological samples, the assay employing the FRET peptide alone makes differentiation between the activities of different MMPs difficult, if not impossible. To solve this issue, we utilize MMP antibodies to first immobilize a specific MMP from biological samples, then, after washing, apply the FRET peptide to measure the activity.

These immunocapture-based FRET assays are highly sensitive and specifically detect the activity of a particular MMP in a mixed biological sample. The assay has a large and linear assay range and can detect low picogram range for MMP-1 and sub-nanogram range for MMP-9 and 13. The assay can also be used to study the interaction between different MMPs.