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Monitoring of proteolytic enzyme activity using phase transition-based peptide arrays

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ABSTRACT

We have developed an assay using peptide arrays based on phase transition from the glass substrate to the liquid for monitoring quantitative protease activity in real-time. Peptide arrays were fabricated using a bifunctional cross-linker, N-[γ-maleimidobutyryloxy] sulfosuccinimide ester, and a substrate peptide containing two functional groups, cysteine and tetramethyl-6-carboxyrhodamine (TAMRA) on the C- and N-terminus, respectively. The phase transition-based peptide arrays were characterized by analyzing the substrate peptide cleaved from the solid substrate by matrix metalloproteinase-3 (MMP-3). We successfully used this assay to determine the quantitative proteolytic activity of MMP-3 in a dose-dependent manner. In addition, parameters including Michaelis constant (K_m), maximum rate of enzymatic reaction (V_{max}), and half maximal inhibitory concentration (IC_{50}) were determined by analyzing the concentrations of substrate peptide cleaved by MMP-3. Therefore, this new assay has potential for the quantitative analysis of enzyme kinetics of protease and informs research developments in drug discovery utilizing kinetic studies.

1. Introduction

Microarray technology is a promising approach for rapid analyses of a wide variety of biomolecular interactions and enzymatic activities using only small amounts of sample (Jung et al., 2010; Kaushansky et al., 2010; MacBeath, 2002; Templin et al., 2002). These microarrays are combined with non-labeling and labeling detection methods to characterize biomolecular interactions and enzymatic activities of target biomolecules of interest (Chandra et al., 2011; Jung et al., 2010; Kong et al., 2010). Non-labeling methods include surface plasmon resonance, mass spectrometry, and microcantilevers, while fluorescence detection is the most widely used labeling approach (Chandra et al., 2011; Gosalia et al., 2005a; Jung et al., 2010; Yuk and Ha, 2005). Activity-based proteomics based upon fluorogenic microarrays has been used for the detection of enzymatic activities (Sun et al., 2006). Enzymes are involved in a wide variety of metabolic pathways and serve various functions inside living organisms through enzyme-catalyzed chemical modifications (Chen et al., 2009; Sun et al., 2006). Thus, accurate analysis of enzyme activity and identification of kinetic parameters of enzymes, including Michaelis constant (K_m), maximum rate of enzymatic reaction (V_{max}), and half maximal inhibitory concentration (IC_{50}), are very important for characterizing enzymes as well as for developing specific substrates and inhibitors (Johnson and Goody, 2011).

In studies using microarrays, enzymatic reactions occur between enzymes in a liquid phase and substrate molecules in a solid phase (Deere et al., 2008; Lee et al., 2005, 2006; Mugherli et al., 2009). However, this heterophase approach has limitations in the quantitative analyses of enzyme activities and in identifying kinetic parameters. It is difficult to determine the exact concentrations of substrates or products bound to the array surface. The surface concentration of substrates is not the same as the applied concentrations, which may be caused by variations in binding affinity and steric configuration (Nygren et al., 1987). In addition, microarrays do not facilitate continuous measurement of enzymatic reactions (Mugherli et al., 2009). To overcome these limitations, the 'wash-off measurement' was proposed as an alternative approach. It determines exact concentrations of fluorescently conjugated carbohydrates immobilized on a solid surface by calculating the ratio of the fluorescence intensities before and after quenching (Liang et al., 2007; Park et al., 2009). However, there are variations in fluorescence intensity resulting from phase differences and variations in volumes arrayed on each spot. Another approach uses liquid monophase microarrays, including surface tension microarrays and sol–gel-derived microarrays. Enzyme activities and presence of possible inhibitors are

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determined by conducting the enzymatic reaction in liquid droplets printed on the surface of a glass slide (Gosalia and Diamond, 2003; Gosalia et al., 2005b; Monton et al., 2010; Mugherli et al., 2009; Song and Zhao, 2009). However, to prevent evaporation of the liquid droplet, these approaches must be performed in solvents with low volatility such as glycerol and dimethyl sulfoxide, which are often not suitable for enzymatic reactions. Furthermore, these approaches are not suitable for multi-step analyses because products are not immobilized on the array surface (Mugherli et al., 2009). Thus, accurate quantitation of enzymatic products on the surface of the array remains a major challenge.

In this study, we developed an assay using peptide arrays based on phase transition from the solid state to the liquid state for quantitative analysis of enzyme kinetics. Our strategy was to determine the protease activity by quantitatively monitoring the amount of cleaved substrate peptide that is transferred from the solid state on a glass surface to the liquid state subsequent to cleavage by the protease. We used matrix metalloproteinase-3 (MMP-3), one of the MMP family members, as a model protease, because MMP plays essential roles in various physiological and pathological processes such as angiogenesis, morphogenesis, tissue repair, tumor metastasis, tumor invasion, and inflammatory diseases (Hiller et al., 2000; Vihinen and Kahari, 2002; Visse and Nagase, 2003). The MMP-3 activity assay uses peptide arrays fabricated by covalent modification of a fluorescent substrate peptide on an N-[γ-maleimidobutyryloxy]sulfosuccinimide ester (sulfo-GMBS)-modified array surface. These phase transition-based peptide arrays were used to determine kinetic parameters, such as K_m, V_{max}, and IC_{50}, as well as to determine MMP-3 activity in real-time.

2. Materials and methods

2.1. Chemical reagents

The reagents 3-aminopropyltrimethoxysilane, ammonium hydroxide, and hydrogen peroxide were obtained from Sigma-Aldrich (St. Louis, MO). Brij-35 and N-[γ-maleimidobutyryloxy]-sulfosuccinimide ester were purchased from Pierce (Rockford, IL). Tetramethyl-6-carboxyrhodamine (TAMRA)-conjugated MMP-3 substrate peptide (TAMRA-R-P-K-P-V-E-Nva-W-R-K-C-NH_2) was synthesized by Peptron (Daejeon, Korea). GM6001 was purchased from Calbiochem (Darmstadt, Germany).

2.2. Fabrication of peptide arrays

Well-type peptide arrays were fabricated with covalent modification involving the thiol moiety of the fluorescent substrate peptide and the maleimide ring of sulfo-GMBS, a heterobifunctional linker. The N-hydroxysuccinimidyl ester moiety of sulfo-GMBS binds to amine-modified glass arrays, whereas the maleimide moiety of GMBS binds to a cysteine residue of the peptide. Amine-modified glass slides were fabricated according to the procedures of Jung et al. (2009). Briefly, glass slides were cleaned with a solution of NH_4OH:H_2O_2:H_2O (1:1:5, v/v) at 70 °C for 10 min. The slides were immersed in 1.5% 3-aminopropyltrimethoxysilane solution in 95% (v/v) ethanol for 2 h, then rinsed, and baked to dryness at 110 °C. Teflon tapes with arrayed holes of 1.5 mm diameter were attached to the modified glass slides to prepare well-type arrays. The peptide arrays were fabricated by sequential modification with 5 mM sulfo-GMBS in 50 mM sodium bicarbonate buffer (pH 7.0) and various concentrations of the peptide in phosphate buffer (8.1 mM Na_2HPO_4, 1.2 mM KH_2PO_4, pH 7.4).

2.3. Preparation of the catalytic domain of human MMP-3

Catalytic domain (Phe^{100}–Pro^{273}) of human MMP-3 was expressed in *E. coli* as previously reported (Jung et al., 2010). Briefly, the insoluble pellet (inclusion body) of the cell lysate was extensively washed with an inclusion body washing buffer (50 mM Tris-HCl, 2% Triton X-100, 2% sodium deoxycholate, pH 8.0) and solubilized in a resuspending buffer (50 mM Tris-HCl, 6 M urea, 30 mM 2-mercaptoethanol, pH 8.5). Solubilized MMP-3 was diluted to 200 μg/ml with the resuspending buffer containing 150 mM 2-mercaptoethanol and gradually dialyzed using a reaction buffer without detergent (20 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, 0.5 mM $ZnCl_2$, pH 7.5).

2.4. Liquid phase standard curve for quantitation of fluorescence substrate peptide cleaved by MMP-3

For quantitative analysis of fluorescent peptide cleaved by MMP-3, we used a liquid phase standard curve. After applying various concentrations of the substrate peptide in 1 μl of phosphate buffer to array wells, the arrays were immediately analyzed to obtain fluorescence images using a potable fluorescence reader (MAFIS-7000, KPM Tech; Ansan, Korea). The fluorescence reader is designed to simultaneously capture the entire image from peptide arrays without scanning and allows reading of 2-D and 3-D arrays. The light of a specific wavelength (532 nm) provided by a light emitting diode laser is directed to opposing sides of a glass substrate carrying well-type arrays by a pair of optical fiber bundles (Fig. 1). The fluorescence light perpendicularly emitted from arrays was passed through a 593 nm emission filter and is detected by a charge-coupled device camera with 5 μm resolution. Fluorescence signals were accumulated for 5 s and converted to the digital display as an image (8 bit). The fluorescence intensities of the array spots were measured using the embedded software. The cleaved substrate peptide concentration in liquid droplets was determined using a linear liquid phase standard curve of fluorescence intensity vs substrate peptide concentration.

2.5. MALDI-TOF mass spectrometric analysis

Cleavage of substrate peptides by MMP-3 was confirmed by analyzing the cleaved peptide using MALDI-TOF mass spectrometer (Voyager-DE^{TM} STR, Applied Biosystems, foster City, CA) equipped with a 337 nm nitrogen laser. Mass spectra were recorded in reflection mode, using a 20 kV accelerating voltage and a 64% grid voltage. Mass spectra were obtained in the positive mode with an average of 200 laser shots per spectrum. α-Cyano-4-hydroxycinnamic acid was used as a matrix.

2.6. Real-time monitoring of MMP-3 activity using phase transition-based peptide arrays

Monitoring of MMP-3 activity was performed on well-type peptide arrays using a fluorescence reader (MAFIS-7000) as depicted in Fig. 1. After applying various concentrations of MMP-3 from 1 to 20 μg/mL in 1 μl of reaction buffer (20 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, 0.5 mM $ZnCl_2$, 0.002% Brij-35, pH 7.5) to wells of peptide arrays, the arrays were continuously monitored once per minute for 4 min using the fluorescence reader and serial images were obtained. The MMP-3 activity was determined by calculating the concentration of the substrate peptide cleaved by MMP-3 that was measured using a liquid phase standard curve and fluorescence intensities obtained from the serial images.