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Short communication

Inhibitory assay for degradation of collagen IV by cathepsin B with a surface plasmon resonance sensor

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ABSTRACT

We describe a simple method for evaluating the inhibition of collagen IV degradation by cathepsin B with a surface plasmon resonance (SPR) biosensor. The change in the SPR signal decreased with an increase in the concentration of cathepsin B inhibitors. The order of the inhibitory constant (*Ki*) obtained by the SPR method was CA074Me ≈ Z-Phe-Phe-FMK < leupeptin. This order was different from that obtained by benzyloxycarbonyl-Phe-Phe-Fluoromethylketone (Z-Phe-Phe-FMK) as a peptide substrate. The comparison of *Ki* suggested that CA074 and Z-Phe-Phe-FMK inhibited exopeptidase activity, and leupeptin inhibited the endopeptidase activity of cathepsin B more strongly.

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1. Introduction

The cysteine protease cathepsin B is a member of the papain family and an endolysosomal component in cells [1,2], and can degrade the non-helical telopeptide region within collagen molecules [3] which is a fundamental component of the extracellular matrix [4,5]. Levels of cathepsin B are elevated in many human tumors, including brain, liver, breast, lung, prostate and thyroid [6–9]. The digestion of collagen IV by not only intracellular but also extracellular cathepsin B is a crucial step in the process of tumor cell invasion [10–18]. Therefore, the inhibitors of cathepsin B are of interest for therapeutic potential of cancer [19].

The classical cysteine protease inhibitors from micro-organisms origin, such as leupeptin [20] and E-64 [21], are known to be the broad-spectrum cysteine protease inhibitors. These inhibitors bind to cathepsin B covalently, but these reaction are reversible and allows reactivation of the enzyme. The computational studies have been used for designing highly selective cathepsin B inhibitors [22]. Most of them primarily impair exo-peptidase activity of cathepsin B rather than its endo-peptidase activity [23,24]. On the other hand, Mirković et al. have shown that 2A2 monoclonal antibody induces a conformation change in cathepsin B, which leads to inhibition of endo-peptidase activity, potentiating exo-peptidase activity [25]. Schenker et al. have revealed that latching the occluding loop in closed conformation hinders both endo- and exo-peptidase activity of cathepsin B [26]. Mitrović has reported that inhibition of endopeptidase and exopeptidase activity of cathepsin B impairs tumor invasion through inhibiting the degradation of extracellular matrix [27]. These approaches may provide an alternative starting point for the development of clinically active compounds. Therefore, the development of inhibitory assay for cathepsin B is desirable.

In general, peptide substrates, which include amino acid sequences surrounding cathepsin B cleavage sites, are used for evaluating the inhibition of cathepshin B activities [28–33]. The inhibitory assay using microplate titer allows us to evaluate the inhibition effect of multiple samples at small sample volumes [28]. Gold nanoparticles-based colorimetric assay permits the screening of cathepsin B inhibitors with the naked eye [29]. On the other hand, high-throughput screening assay for cathepsin B inhibitors has also been exploited using flow electrospray ionization mass spectrometry [30,31] and high-performance liquid chromatography-electrospray ionization mass spectrometry [32,33]. Most of the peptide substrates used in inhibition assay are degraded by endopeptidase activity rather than exopeptidase activity. This indicates that inhibition of endopeptidase activity of cathepsin B can be evaluated mainly in the inhibition assays using peptide substrates. On the other hand, our previous study has revealed that both endo- and exo-peptidase activity of cathepsin B participates in degradation of collagen IV at acidic pH [34]. This sug-

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gests that the usage of collagen IV as the substrate is important for evaluating inhibitory effects under physiological conditions. However, inhibitory assay using collagen IV as the substrate has not been reported yet.

We have shown that surface plasmon resonance (SPR) biosensors are useful for monitoring the degradation of collagen IV by matrix metalloproteinase-9 [35] and cathepsin B [34]. In the present paper, the SPR sensor using collagen IV is applied to evaluate the inhibitory effect of cathepsin B activity by several kinds of compounds. The inhibitory assay with the SPR sensor is simple and rapid, enabling us to calculate inhibition constants. The inhibition constants obtained by the SPR method are compared with those obtained by using peptide substrates.

2. Experimental

2.1. Reagents

Human collagen type IV was purchased from Cosmo Bio Co., Ltd. (Tokyo, Japan). Cathepsin B and Benzyloxycarbonyl-Phe-Phe-Fluoromethylketone (Z-Phe-Phe-FMK) were obtained from Funakoshi Co., Ltd. (Tokyo, Japan). CA074Me was obtained from Peptide Institute, Inc. (Osaka, Japan). *N*-Succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) was obtained from Thermo Fisher Scientific (St. Worcester, MA, USA). Cysteamine, tris(2-carboxyethyl)phosphine hydrochloride (Tris) and tris(2-carboxyethyl)phosphine (TCEP), leupeptin hemisulfate salt and EA64 were purchased from Sigma Chemical (St. Louis, MO, USA). 1-Ethyl-3-[3-(dimethylamino)propyl]carbodimide hydrochloride (EDC) and 2-[4-(2-hydroxyethyl)-1- piperazinyl]ethansulfonic acid (HEPES) were obtained from Dojindo Laboratories (Kumamoto, Japan). *N*-Hydroxysuccinimide (NHS), citric acid, polyoxyethylene sorbitan monolaurate (Tween 20), ethylenediaminetetraacetic acid (EDTA), sodium acetate trihydrate, sodium chloride and boric acid were from Wako Chemicals Co. (Osaka, Japan). Milli-Q water (Millipore reagent water system, Bedford, MA, USA) was used throughout the experiments.

2.1.1. Apparatus

A BIAcore 2000 (Biacore, GE Healthcare, Berkshire, UK) was used for SPR measurements with sensor chips CM4 (carboxymethylated dextran attached on the gold coated glass surface). The operating temperature was $37 \pm 0.1\,^{\circ}$C. The pH of buffer solutions was adjusted with a glass electrode pH meter model IOL-30 (Denki Kagaku Keiki, Tokyo, Japan).

2.1.2. Conjugation of SPDP with collagen IV

Collagen IV was dissolved in a 10 mM sodium acetate buffer solution (pH 4.0) to give a 2.0 mg/ml solution, and then it was diluted twice with a 0.10 M sodium phosphate buffer (pH 7.4) containing 0.10 M NaCl. A 0.30 ml-portion of the collagen IV solution was mixed with 3.7 μl of 20 mM SPDP in dimethyl sulfoxide (DMSO, anhydrous) and the mixture was incubated for 1 h at room temperature. Unreacted SPDP was removed and replaced by a 10 mM sodium acetate buffer solution (pH 3.0) using ultrafiltration (Microcon YM-50, Millipore). The activated collagen IV hereafter is abbreviated as SPDP-collagen IV.

2.1.3. Immobilization of SPDP-collagen IV on a CM4 sensor chip

SPDP-collagen IV was immobilized on CM4 sensor chips in a flow system through formation of a disulfide crosslink. First, a CM4 sensor chip was equilibrated with a running solution consisting of 0.15 M NaCl, 10 mM HEPES/NaOH (pH 7.4), 3.4 mM EDTA and 0.005% Tween-20 (filtered through 0.22 mm membrane filter, abbreviated as a HBS-EP solution). Then, the CM4 sensor chip was activated by injecting a mixture of 0.1 M NHS and 0.4 M EDC in water for 7 min at a flow rate of 5 μl/min. A 0.1 M borate buffer solution (pH 8.5) containing 40 mM cysteamine was run twice over the activated sensor surface for 7 min (Fig. S1a), followed by injection of 400 μg/ml SPDP-collagen IV in a 10 mM sodium acetate buffer solution (pH 3.0) until the SPR response reach to approximately 1000 RU (Fig. S1b). When regenerating the collagen IV-immobilized sensor chip (collagen IV chip) was necessary, a 0.1 M borate buffer solution (pH 8.5) containing 0.20 M TCEP, a reducing agent, was injected onto the sensor chip at a flow rate of 10 μl/min for 15 min, so that a disulfide bond was cleaved by reduction. The re-immobilization of SPDP-collagen IV were carried out with the injection of 400 μg/ml SPDP-collagen IV in a 10 mM sodium acetate buffer solution (pH 3.0) onto the chip.

2.1.4. Monitoring of cathepsin B activity in the presence of cathepsin B inhibitor

Cathepshin B inhibitors, i.e., leupeptin, CA074Me and Z-Phe-Phe-FMK, were dissolved in DMSO to give a 10 mM solution, respectively. These solutions were prepared immediately before their uses and diluted with a 10 mM citric buffer solution (pH 4.0, abbreviated as a citric solution) if necessary. A 1-μl portion of a cathepsin B solution and a 0.6-μl portion of a 10 mM Tris–HCl buffer solution (pH 7.4, abbreviated as a Tris solution) containing 1.8 mM dithiothreitol (DTT) were mixed and pre-incubated for 1 h at 4 °C. The mixture hereafter is called a DTT-activated cathepsin B solution. The solution was mixed with a 0.6-μl portion of a 10 mM citric buffer solution containing an inhibitor and incubated for a given time. After the surface of a collagen IV chip was equilibrated with a buffer solution at a flow rate of 10 μl/min, the mixture (200 μl portion) was loaded into an autosampler and its 150-μl portion was injected automatically into a flow cell for 15 min. A change in SPR signals was monitored continuously, which reflects the cleavage of collagen IV by cathepsin B.

2.1.5. Evaluation of inhibition constants

An inhibition constant K_i for a cathepsin B inhibitor is given by

$$K_i = \frac{[\mathrm{E}]_{free}[\mathrm{I}]_{free}}{[\mathrm{EI}]} \tag{1}$$

where [EI] is the concentration of a cathepsin B complex with an inhibitor (I), $[\mathrm{I}]_{free}$ and $[\mathrm{E}]_{\mathrm{free}}$ are those of free inhibitor and unbound cathepsin B, respectively. In the presence of the inhibitor (I), the total concentration $[\mathrm{E}]_{\mathrm{total}}$ of cathepsin B is given by

$$[\mathrm{E}]_{\mathrm{total}} = [\mathrm{EI}] + [\mathrm{E}]_{\mathrm{free}} \tag{2}$$

and the total concentration $[\mathrm{I}]_{\mathrm{total}}$ of the inhibitor is given by

$$[\mathrm{I}]_{\mathrm{total}} = [\mathrm{EI}] + [\mathrm{I}]_{\mathrm{free}} \tag{3}$$

A decrease in the SPR signal at time t after the injection of a cathepsin B solution, $\Delta\mathrm{RU}_{\mathrm{CB}}$ (t min), is obtained as shown in Fig. 1a. The concentration of unbound cathepsin B ($[\mathrm{E}]_{\mathrm{free}}$) can be calculated from a $\Delta\mathrm{RU}_{\mathrm{CB}}$ (*15 min*) *vs.* cathepsin B concentration curve (Fig. 1b). Once the $[\mathrm{E}]_{\mathrm{free}}$ is obtained from SPR measurements, the concentration [EI] is calculated from Eq. (2). Finally, the Eq. (1) is expressed as

$$\frac{[\mathrm{EI}]}{[\mathrm{E}]_{\mathrm{free}}} = \frac{[\mathrm{E}]_{\mathrm{total}} - [\mathrm{E}]_{\mathrm{free}}}{[\mathrm{E}]_{\mathrm{free}}} = K_i \frac{1}{[\mathrm{I}]_{\mathrm{free}}} \tag{4}$$

Thus, plotting the ratio $[\mathrm{EI}]/[\mathrm{E}]_{\mathrm{free}}$ against $1/[\mathrm{I}]_{\mathrm{free}}$ yields K_i as a slope of the plot.

2.1.6. Measurements for inhibition constant using Z-Phe-Arg-pNA as a substrate

A 2-μl portion of a cathepsin B solution and a 2-μl portion of a citrate buffer solution containing 5 mM dithiothreitol (DTT) and

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