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Short communication

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

Atsushi Shoji [a,b], Yumiko Suenaga [a], Atsushi Hosaka [a], Yuuki Ishida [a], Akio Yanagida [b], Masao Sugawara [a,*]

[a] Department of Chemistry, College of Humanities and Sciences, Nihon University, Sakurajousui, Setagaya, Tokyo 156-8550, Japan
[b] School of Pharmacy, Tokyo University Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan

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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.



## 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-

* Corresponding author.
*E-mail address:* sugawara@chs.nihon-u.ac.jp (M. Sugawara).

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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 ± 0.1 °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

$$K_i = \frac{[E]_{free}[I]_{free}}{[EI]} \quad (1)$$

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

$$[E]_{total} = [EI] + [E]_{free} \quad (2)$$

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

$$[I]_{total} = [EI] + [I]_{free} \quad (3)$$

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

$$\frac{[EI]}{[E]_{free}} = \frac{[E]_{total} - [E]_{free}}{[E]_{free}} = K_i \frac{1}{[I]_{free}} \quad (4)$$

Thus, plotting the ratio $[EI]/[E]_{free}$ against $1/[I]_{free}$ 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

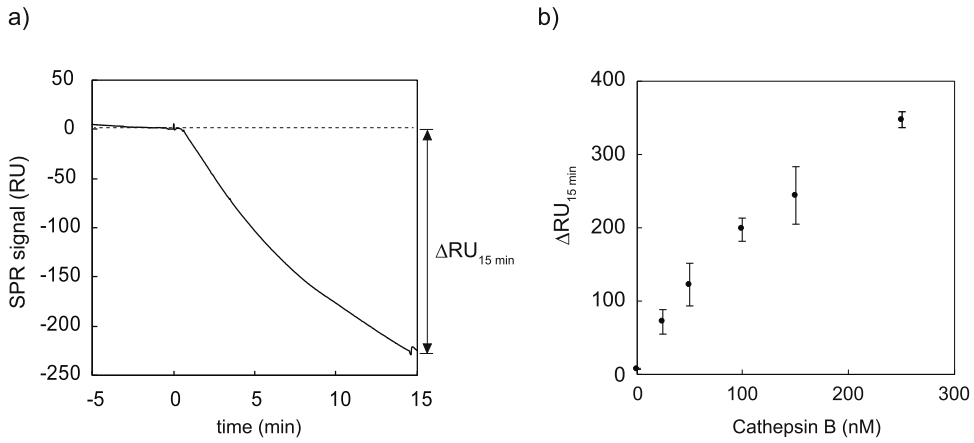


**Fig. 1.** (a) Time course of a SPR signal change caused by degradation of collagen IV. (b) Concentration dependence of $\Delta RU_{(15min)}$ after injection of cathepsin B onto collagen IV chips. Averages of three measurements were plotted. Error bars indicate means ± standard deviations ($n = 3$).

5 mM EDTA were mixed and incubated for 30 min at room temperature. A 3-μl portion of the mixture and a 3-μl portion of a leupeptin, CA074Me or Z-Phe-Phe-FMK solution at a given concentration were mixed and incubated for 60 min (abbreviated as a cathepsin B-inhibitor solution). Z-Phe-Arg-pNA was dissolved in DMSO to give a 15 mM solution and diluted by 5 times with a citrate buffer solution containing 5 mM DTT and 5 mM EDTA. A 2-μl portion of a cathepsin B-inhibitor solution was added into a 0.1 ml portion of a substrate solution. Then, the solution was transferred into a 96-well microplate and its absorbance was measured with a microplate reader SH-9000 (Corona electric Co., Ltd, Japan). The inhibitor constants were calculated by Morison equation [36].

## 3. Results and discussion

### 3.1. Time-dependent inhibition for cathepsin B

The activities of cathepsin B degrading collagen IV on a CM4 sensor chip were measured with or without leupeptin, a slow-tight binding inhibitor [20]. The time profile of the SPR signals in the absence of leupeptin is shown in Fig. 1a. The SPR signal decreased gradually with time, but such a decrease in the SPR signal could not be observed when a 500 μM DTT solution was injected onto the collagen IV chip (Fig. S2a). These indicated that a decrease in the SPR signal resulted from the enzymatic degradation of collagen IV on the sensor surface, rather than the reduction of disulfide bond by DTT. Collagen IV molecules were degraded by both exo- and endpeptidase activity of cathepsin B due to unstable conformation of collagen IV at pH 4.0 [34]. Further, injection of 250 ng/ml cathepsin B without DTT-treatment did not cause the change in the SPR signal (Fig. S2b). The previous report has shown that the SPR signal did not change when 250 ng/ml DTT-activated cathepsin B was injected onto a cysteamine chip [34]. Therefore, specific and non-specific adsorption of cathepsin B on a collagen IV chip did not contribute to the change in the SPR signal significantly. The decrease in the SPR signal at 15 min ($\Delta RU_{15min}$) was plotted against the concentration of cathepsin B as shown in Fig. 1b. The $\Delta RU_{15min}$ increased with an increase in the cathepsin B concentration, indicating that the degradation of collagen IV occurs in a concentration-dependent manner.

When leupeptin (800 nM) was present, the decrease of the SPR signal change became very small (Fig. 2a, curve 1). In contrast, in the absence of leupeptin, the SPR signal markedly decreased with time (curve 2). These results indicate that the degradation of collagen IV by cathepsin B was significantly inhibited by leupeptin. Leupeptin is a slow, tight-binding inhibitor, and the formation of cathepsin B and the inhibitor change gradually with time. In order to investigate steady state time for leupeptin inhibition, the effect of

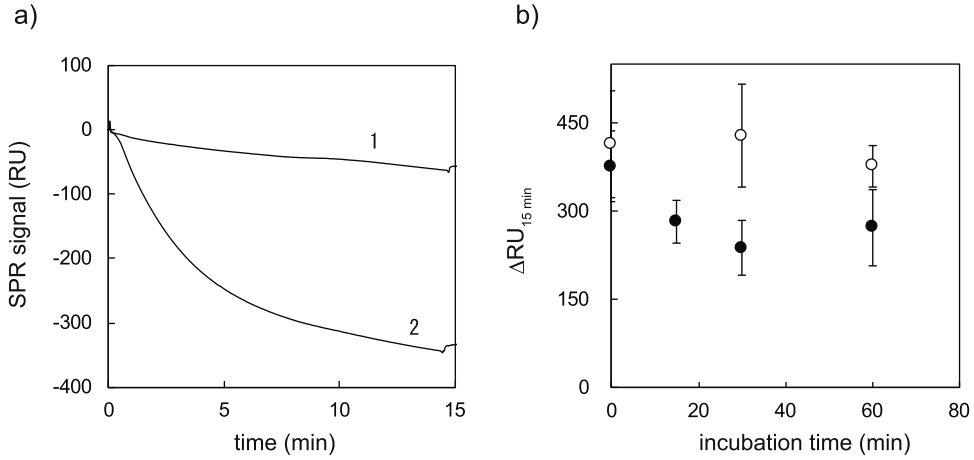


**Fig. 2.** Inhibitory effects of cathepsin B activities obtained by the SPR signals. (a) Time profiles of SPR signals resulting from degradation of collagen IV in the presence of 800 nM leupeptin (1) and its absence (2). After incubation of a mixture containing 250 ng/ml cathepsin B and 220 nM leupeptin for a given time, the mixture was injected on each sensor chip at a flow rate of 10 μl/min. (b) A relationship between $\Delta RU_{(15min)}$ and incubation time. The open circles indicate the $\Delta RU_{(15min)}$ values without leupeptin, and the closed circles indicate those with leupeptin. Averages of three measurements were plotted. Error bars indicate means ± standard deviations ($n = 3$).

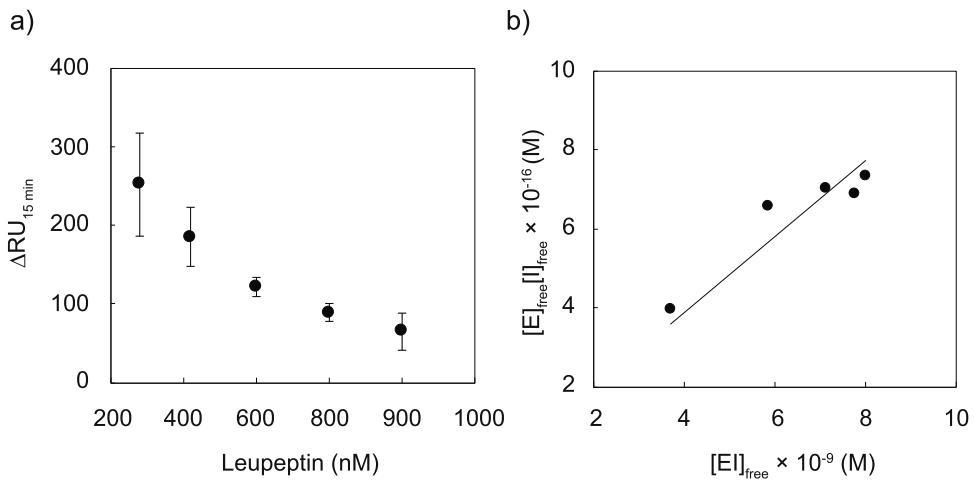


**Fig. 3.** Dependence of inhibitory effect on leupeptin concentration. (a) The $\Delta RU_{(15min)}$ values were plotted against leupeptin concentration. After incubation of a mixture containing 250 ng/ml cathepsin B and 80–800 nM leupeptin for 30 min, the mixture was injected on each sensor chip at a flow rate of 10 μl/min. Averages of three measurements were plotted. Error bars indicate means ± standard deviations ($n = 3$). (b) A $[E]_{free}[I]_{free}$ *vs.* [EI] plot.

**Table 1**
Linearity and $K_i$ values of three inhibitors assayed by SPR method.

| Inhibitors | Regression equation | Correlation ($r$ values) | $K_i$ values (M) | SD | RSD (%) |
|---|---|---|---|---|---|
| Leupeptin | $y = 9.7 \times 10^{-8}\ x$ | 0.889 | $9.7 \times 10^{-8}$ | $1.5 \times 10^{-8}$ | 15.5 |
| CA74Me | $y = 1.1 \times 10^{-9}\ x$ | 0.972 | $1.1 \times 10^{-9}$ | $1.2 \times 10^{-10}$ | 10.9 |
| Z-Phe-Phe-FMK | $y = 2.7 \times 10^{-9}\ x$ | 0.995 | $2.7 \times 10^{-9}$ | $2.4 \times 10^{-10}$ | 9.03 |

incubation time for a mixture containing cathepsin B and leupeptin before injection of the mixture was investigated by monitoring a change in $\Delta RU_{15min}$ (Fig. 2b). The $\Delta RU_{15min}$ values decreased with an increase in the incubation time up to 30 min, suggesting that the rate of the complex formation between leupeptin and cathepsin B is slow [20].

### 3.2. Concentration dependence for cathepsin B inhibition

The inhibition effect of leupeptin at different concentrations for cathepsin B activity was investigated by measuring $\Delta RU_{15min}$. Since the complex formation between cathepsin B and leupeptin was slow, a solution containing cathepsin B and leupeptin was incubated for 30 min before its injection. The $\Delta RU_{15min}$ decreased with an increase in the leupeptin concentration ranging from 80 to 800 nM (Fig. 3a). Considering that leupeptin binds to cathepsin B tightly [20] and the fraction of collagen IV on the chip was much smaller than that of the cathepsin B-leupeptin complex, the presence of collagen IV immobilized on the sensor chip did not affect the complex equilibrium between cathepsin B and the inhibitor in a solution. Therefore, the values of $[E]_{free}[I]_{free}$ and [EI] were able to be calculated from the concentration dependence curve (Fig. 3a). The plot of $[E]_{free}[I]_{free}$ against [EI] is shown in Fig. 3b. The plot showed a linear relationship, from which the $K_i$ value between cathepsin B and leupeptin was calculated to be $9.7 \times 10^{-8}$ M.

### 3.3. Comparison of $K_i$ between collagen IV and a peptide substrate

The inhibition constants $K_i$ of CA074Me and Z-Phe-Phe-FMK, which were irreversible inhibitors of cathepsin B, were evaluated based on a plot of $[E]_{free}[I]_{free}$ against [EI] (Fig. S3a,b). Similarly, the plots obtained from CA074 and Z-Phe-Phe-FMK were linear as shown in Fig. S3 and Table 1. The correlation coefficients of these linear plots were in the range of 0.889–0.995. The relative standard deviations (RSD) of the $K_i$ values were in the range of about 9–15%. The order of $K_i$ was CA074Me ≈ Z-Phe-Phe-FMK < leupeptin (Table 1). On the other hand, the order of *Ki* obtained using Z-phe-Arg-pNA as a peptide substrate was leupeptin ($2.9 \times 10^{-10}$ M) < CA074Me ($1.5 \times 10^{-7}$ M) < Z-Phe-Phe-FMK ($2.3 \times 10^{-6}$ M) (Fig. S4). The optimum pH for cathepsin B activity was about 4.0 when collagen IV was used [34], while for Z-Phe-Phe-FMK, the optimum pH was 6.0 (Fig. S5). Cathepsin B acts as an exopeptidase at pH <5.5 and as an endopeptidase at pH >5.5 [37]. Hence, the *Ki* values obtained from the SPR method were based on the inhibition of both exo- and endo peptidase activity, but the values were affected by inhibition of exopeptidase stronger than endopeptidase. The contribution of exopeptidase activity to degrade collagen IV is larger as compared with Z-phe-Arg-pNA.

The inhibition constants of CA074Me and Z-Phe-Phe-FMK obtained by the SPR method were lower than those obtained by the assay using Z-phe-Arg-pNA. These results suggest that CA074Me and Z-Phe-Phe-FMK prefer to inhibit exopeptidase activity rather than endopeptidase activity of cathepsin B. Yamamoto et al. has revealed that CA074 inhibits the exopeptidase of cathepsin B [23]. On the other hand, the comparison of *Ki* shows that leupeptin inhibits the endopeptidase activity more strongly.

On the other hand, the inhibition effect could not be evaluated by gelatin zymography because cathepsin B activity could not be detected in the absence of above inhibitors (Fig. S6). The zymography necessitates the degradation of numerous gelatin molecules incorporated in acrylamide gel due to its low sensitivity of detecting proteins by Coomassie Brilliant Blue. SPR can detect the degradation of much lower collagen IV molecules on the surface of the sensor chip, leading to rapid analysis, *i.e.,* assay time is under 15 min, for inhibition effect of cathepsin B.

## 4. Conclusion

The inhibitory assay for cathepsin B activity described above shows that the selectivity order is dependent on the peptide substrate used. The current inhibitory assay based on a SPR change uses immobilized collagen IV as a substrate. The proposed method

allows us to calculate inhibition constants with simple procedures and provides the information on the inhibitory effect for cathepsin B to degrade collagen IV.

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