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Evaluation of cathepsin B activity for degrading collagen IV using a surface plasmon resonance method and circular dichroism spectroscopy

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ABSTRACT

Evaluation of cathepsin B activities for degrading collagen IV and heat-denatured collagen IV (gelatin) were performed by surface plasmon resonance (SPR) and circular dichroism (CD) measurements. The optimal pH of cathepsin B activity for degrading each substrate was around 4.0. The $\Delta RU_{(15\,min)}$, which is a decrease in the SPR signal at 15 min after injection of cathepsin B, was smaller for collagen IV than for heat-denatured collagen IV owing to the presence of triple-helical conformation. An unstable nature of the triple-helical conformation of collagen IV at pH 4.0 was shown by the CD study. Degrading collagen IV by cathepsin B was facilitated owing to a local unwinding of the triple-helical conformation caused by proteolytic cleavage of the non-helical region. The concentration dependence of the initial velocity for degrading collagen IV by cathepsin B at pH 4.0 was biphasic, showing that cathepsin B at low concentration exhibits exopeptidase activity, while the enzyme at high concentration exhibits endopeptidase activity. The kinetic parameters for the exopeptidase activity of cathepsin B toward collagen IV and heat-treated collagen IV were evaluated and discussed in terms of the protease mechanism.

1. Introduction

Collagen IV is a fundamental component of the ECM proteins, which provide mechanical strength and structural integrity to various tissues. A collagen IV molecule is composed of three α-chains of primarily repeating Gly-Xaa-Yaa triplets, which induces each α-chain to adopt a left-handed poly-pro-II helix. Three α-chains then intertwine to form a right-handed triple-helical coiled coil [1,2]. Proteases of three classes, including matrix metalloproteinases (MMPs), cathepsin families and members of the urokinase plasmin, can catalyze the hydrolysis of collagen IV [3–5].

The cysteine protease cathepsin B is a member of the papain family and an endolysosomal component in cells [6,7]. The SDS-PAGE gel electrophoresis of digested collagen by cathepsin B has revealed that the proteinase can degrade the non-helical telopeptide region within collagen molecules [8]. The degradation of collagen IV can occur extracellularly by secretion of cathepsin B or intracellularly following the endocytosis of collagen IV [9–17]. Levels of cathepsin B are elevated in many human tumors, including brain, liver, breast, lung, prostate and thyroid [18–21]. Live-cell proteolysis assay of SUM 149 human inflammatory breast cancer cells using quenched-fluorescent derivatives of collagen IV (DQ-collagen IV) have revealed that the invasion of the cancer cells is facilitated by degrading collagen IV [17]. Additionally, an inhibition of the pericellular cathepsin B activities by CA074, which is a cell impermeable, highly selective cathepsin B inhibitor, has reduced both degradation of collagen IV and cell invasion significantly [12,17]. Sameni et al. have shown that inhibition of both extra- and intracellular cathepsin B activities by treatment of living human breast cancer cells (BT20 and BT549) with selective cathepsin B inhibitors reduced cell invasion through a decrease in the degree of digesting collagen IV [9]. These findings indicate that digestion of collagen IV by cathepsin B is a crucial step in the process of tumor cell invasion, and not only intracellular but also extracellular cathepsin B participate in the invasion.

On the other hand, the extracellular pH of microenvironment in tumor cells is known to be around pH 6.5–6.9, which is often lower than that (pH 7.2–7.5) in normal cells [22–24]. In contrast, the lysosomal pH in tumor cells is 2 pH units higher than that of

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normal cells (pH 4.6–5 [25]) [26]. Victor et al. have shown that slight acidification of microenvironments of a variety of tumors, such as melanoma, colon and breast, increases secretion and activity of cathepsin B, leading to an increase in the degree of degrading collagen IV [17]. The SDS-PAGE electrophoretic separation of degraded products of collagen IV by cathepsin B revealed that degradation of collagen IV occurred more rapidly at pH 5.0 than at pH 7.4 [27]. The presence of a large insertion loop, *i.e.*, occluding loop, found in cathepsin B regulates proteolytic mechanism as either exopeptidase or endopeptidase [28]. At acidic condition below pH 5.5, cathepsin B favors exopeptidase [28–31].

Although only a limited number of studies have investigated changes in the triple-helical conformation of collagen IV at acidic pH [32], the result indicates that under acidic condition, loosely packed and unstable region of collagen IV spreads throughout the whole molecule. This suggests that the instability of triple-helical conformation of collagen IV leads to an increase in degradation of collagen IV. Therefore, investigating pH dependence of cathepsin B activity in relation to the conformational stability of collagen IV will be important for understanding the role of cathepsin B in degrading collagen IV. However, the relationship between the stability of triple-helical conformation of collagen IV and cathepsin B activity has not been investigated in detail, probably because that real-time measurement of cathepsin B activity using non-labeled collagen IV as a substrate is difficult.

In our previous paper, we developed a surface plasmon resonance (SPR) method for monitoring the enzymatic activity of MMP-9 for degrading triple-helical collagen IV as substrate [33]. In the present study, on the basis of the SPR method, cathepsin B activities using collagen IV and heat-denatured collagen IV (gelatin) are investigated at different pHs in order to clarify collagenolytic activity of cathepsin B in relation to the stability of the triple-helical conformation of collagen IV, which is measured by circular dichroism (CD) spectroscopy.

2. Experimental

2.1. Reagents

Human collagen type IV was purchased from Cosmo Bio Co. Ltd. (Tokyo, Japan). Cathepsin B was obtained from Funakoshi (Tokyo, 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) were purchased from Sigma Chemical (St. Louis, MO, USA). 1-Ethyl-3-(3-(dimethylaminopropyl)carbodiimide hydrochloride (EDC), and 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethansulfonic acid (HEPES) were obtained from Dojindo Laboratories (Kumamoto, Japan). Citric acid, *N*-hydroxysuccinimide (NHS), polyoxyethylene sorbitan monolaurate (Tween-20), boric acid, 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.

2.2. 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 $37 \pm 0.1\,^{\circ}C$, unless otherwise noted. The pH of buffer solutions was adjusted with a glass electrode pH meter model IOL-30 (Denki Kagaku Keiki, Tokyo, Japan).

2.3. Circular dichroism spectroscopy

Circular dichroism (CD) studies were carried out on a JA750 spectrometer (JASCO Co. Ltd., Tokyo, Japan) equipped with a temperature control unit (JASCO PTC-348). Temperature-induced stability of 1 mg/ml native collagen IV dissolved in a 10 mM phosphate buffer solution (pH 7.4) and a 10 mM citrate buffer solution (pH 4.0 or 5.0) was monitored by far-UV CD spectroscopy using a 1.0-cm path length quartz cell. Thermal transition curves were obtained by recording the molar ellipticity (θ) at 222 nm while temperature was increased continuously from 25 to 65 °C at a rate of 0.2 °C/min.

2.4. Preparation of heat-denatured collagen IV (gelatin)

Collagen IV was dissolved in a 10 mM sodium acetate buffer solution (pH 4.0) to give a 2.0 mg/ml solution, and the solution was incubated overnight at 80 °C. Circular dichroism (CD) spectroscopy was used for characterizing the structure of collagen IV and heat-treated collagen IV. A maximum molar ellipticity (θ) of SPDP-collagen IV at room temperature was observed at 221 nm, where collagen IV species are in triple-helical conformation [32]. On the other hand, the maximum molar ellipticity (θ) at 222 nm was not observed for heat-treated collagen IV, *i.e.*, denatured collagen IV (Fig. S1).

2.5. Conjugation of SPDP with collagen IV and heat-denatured 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 diluted two times with a 0.10 M sodium phosphate buffer (pH 7.4) containing 0.10 M NaCl. Heat-denatured collagen IV was also diluted two times with a 0.10 M sodium phosphate buffer (pH 7.4) containing 0.10 M NaCl. A 0.30 ml-portion of each solution was mixed with 3.7 μl of 20 mM SPDP in dimethyl sulfoxide (DMSO, anhydrous). Then, the mixture was incubated for 1 h at room temperature, and unreacted SPDP was removed and replaced by a 10 mM sodium acetate buffer solution (pH 3.0) using ultrafiltration (Microcon YM-50, Millipopre). The activated collagen IV and heat-denatured collagen IV hereafter are abbreviated as SPDP-collagen IV and SPDP-heat-denatured collagen IV, respectively.

2.6. Immobilization of SPDP-collagen IV and heat-denatured collagen IV on a CM4 sensor chip

SPDP-collagen IV and heat-denatured collagen IV were immobilized on CM4 sensor chips, respectively, 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 μm 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, followed by injection of 400 μg/ml SPDP-collagen IV or SPDP-heat-denatured collagen IV in a 10 mM sodium acetate buffer solution (pH 3.0) until the SPR response reaches to approximately 1000 RU. When regenerating the collagen IV-immobilized sensor chip (collagen IV chip) was necessary, a 0.1 M borate buffer solution (pH 8.5) containing 0.10 M TCEP was injected onto the sensor chip at a flow rate of 5 μl/min for 15 min to cleave a disulfide bond by reduction.