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Inhibition effects of (+)-catechin–aldehyde polycondensates on proteinases causing proteolytic degradation of extracellular matrix

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Abstract

Inhibition effects of (+)-catechin–aldehyde polycondensates against the activity of proteinases, *Clostridium histolyticum* collagenase (ChC) and human neutrophil elastase (HNE) causing proteolytic degradation of extracellular matrix (ECM), have been investigated. In normal tissues, a balance is reached between the formation and destruction of ECM, leading to a state of homeostasis. However, uncontrolled destruction of ECM contributes to tumor invasion and metastasis. In the measurement of the inhibition activity on ChC and HNE, the polycondensates exhibited superior effects compared to the catechin monomer. Kinetic assays of ChC and HNE inhibition by the polycondensate clearly showed a mixed-type inhibition. These data demonstrate that the polycondensates are a new class of proteinase inhibitors useful for a potent therapeutic agent.

Keywords: Catechin; Polycatechin; Collagenase; Elastase; Proteolytic degradation; Inhibitor

Proteolytic degradation of extracellular matrix (ECM) is thought to play a major role in tissue remodeling. This process is known to promote not only the development of diseases such as pulmonary emphysema but also the structural alterations that characterize alveolar and airway fibrosis in interstitial lung diseases [1,2]. Two major proteolytic systems, namely metalloproteinases [e.g., matrix metalloproteinases (MMPs)] and serine proteinases (e.g., neutrophil elastase), have been implicated in several pathophysiological processes involving extensive ECM remodeling [3]. In normal tissues, a balance is reached between the formation and destruction of ECM, leading to a state of homeostasis [4].

Recently, MMPs have been found to be an interesting target in the search of novel types of anticancer, antiarthritis, and other pharmacological agents useful in the treatment of inflammatory processes [5,6]. MMPs are a family of zinc containing metalloproteinases that degrade and remodel structural proteins in ECM, such as membrane collagens, aggrecan, fibronectin, and laminin [7,8]. Their enhanced activities induce tissue degradation, resulting in a wide range of disease processes including cancer and rheumatoid arthritis [9,10]. Like MMPs, bacterial collagenases such as *Clostridium histolyticum* collagenase (ChC) also degrade ECM. ChC belongs to M-9 metalloproteinase family, which is able to hydrolyze triple-helical collagen under physiological conditions, as well as an entire range of synthetic peptide substrates [11–13]. In fact, the crude homogenate of ChC, which contains several distinct collagenase isozymes, is the most efficient system known for the degradation of connective tissue.

Human neutrophil elastase (HNE) belongs to the chymotrypsin family of serine proteinases, whose catalytic site is composed of three hydrogen-bonded amino acid residues, His57, Asp102, and Ser195 [14]. Unlike most proteinases, HNE is able to cleave fibrous elastin, an important ECM protein that has the unique property of elastic recoil, and has therefore a mechanical function in lungs, arteries, skin, and ligaments. In addition to elastin, HNE cleaves many proteins with important biological functions, including collagen and other ECM

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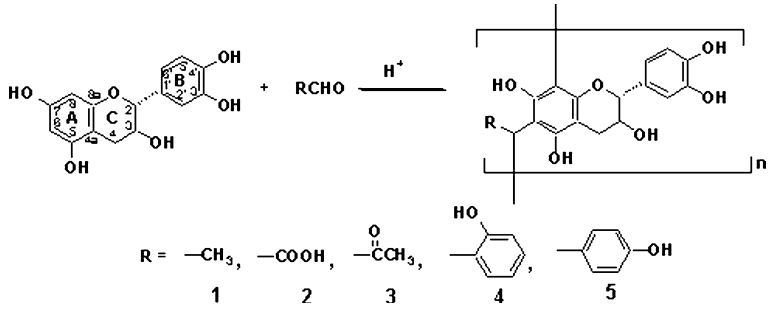

Fig. 1. Schematic diagram of the regioselective synthesis of (+)-catechin–aldehyde polycondensates. Polycondensation of (+)-catechin and aldehydes was carried out in the presence of an acid catalyst [22].

proteins such as fibronectin and laminin. HNE has been implicated in the pathogenesis of a wide range of pulmonary disorders like emphysema, cystic fibrosis, and bronchiectasis. In those diseases the active enzyme is thought to contribute directly to lung by degrading ECM proteins, promoting synthesis of inflammatory cytokines, and undermining the immune response [15]. Active HNE is only detected in the lung when the organ's protective screen of HNE inhibitors is overwhelmed. Therefore, the exploration of HNE inhibitors has been under focus in the search for treatments of neutrophil-mediated lung diseases.

Polyphenols are widely found in plant tissues. For the past several decades, a great number of studies have been conducted to explore antioxidant, anti-mutagenic, anti-carcinogenic, and anti-inflammatory effects of polyphenols [16,17]. Green tea polyphenols have been reported to show antitumor effects due to their ability to inhibit the invasion and metastasis of mouse Lewis lung carcinoma and human fibrosarcoma [18,19]. These effects are thought to be closely related to the inhibition of MMPs because the MMP family seems to play an essential role in the facilitation of tumor invasion and metastasis [20]. Furthermore, it has been found that ester-type catechins of green tea polyphenols strongly suppress the MMP-induced degradation of gelatin [21,22].

In order to improve the biological and physiological activities of catechin, we have designed and synthesized polymerized catechins, (+)-catechin–aldehyde polycondensates (**1**–**5**) (Fig. 1) [23]. This study deals with the inhibition effects and the inhibition mechanism of the polycondensates on ChC and HNE, proteinases causing proteolytic degradation of ECM, on the catechin unit basis compared to monomeric catechin.

Materials and methods

Materials. Clostridium histolyticum collagenase (ChC, EC 3.4.24.3), human neutrophil elastase (HNE, EC 3.4.21.37), *N*-[3-(2-furyl) acryloyl]-Leu-Gly-Pro-Ala (FALGPA, substrate for ChC), *N*-(methoxysuccinyl)-Ala-Ala-Pro-Val 4-nitroanilide (MAAPVN, substrate for HNE), and soybean trypsin inhibitor used for the bioassay were purchased from Sigma Chemical and used as received. Polycondensates were synthesized according to the literature [23]. Their molecular weight (M_n) were 3700 (**1**), 2300 (**2**), 2300 (**3**), 1700 (**4**), and 2000 (**5**). Other reagents and solvents were commercially available and used as received.

Collagenase inhibition activity. The ChC inhibition activities of catechin and polycondensates were measured as described by Van Wart and Steinbrink [24]. The polymer samples were first dissolved in DMSO and used as a 10 times dilution with water. The sample solution and ChC (100 mU/ml) were dissolved in 0.05 M Tricine buffer (with 0.4 M NaCl and 0.01 M $CaCl_2$, pH 7.5) and preincubated at 25 °C for 5 min. Then, FALGPA (300 μM) was added to the mixture to immediately measure the decrease of the optical density (OD) at 324 nm for 20 min for detection of hydrolyzed FALGPA using a Hitachi U-2001 spectrometer.

The ChC inhibition activities were calculated according to the following formula:

$$\text{ChC inhibition activity}(\%) = \frac{\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}}{\text{OD}_{\text{control}}} \times 100,$$

where $\text{OD}_{\text{control}}$ and $\text{OD}_{\text{sample}}$ represent the optical densities in the absence and presence of sample, respectively.

Elastase inhibition activity [14]. The sample solution and HNE (17 mU/ml) were mixed in 0.1 M Tris–HCl buffer (pH 7.5) and preincubated at 25 °C for 5 min. Then, MAAPVN (500 μM) was added to the mixture and incubated at 37 °C for 1 h. Afterward, the reaction was stopped by the addition of soybean trypsin inhibitor (1 mg/ml) and the OD due to the formation of *p*-nitroaniline was immediately measured at 405 nm. The HNE inhibition activities were calculated according to the formula mentioned above in the ChC inhibition activity.

Circular dichroism measurements. Circular dichroism (CD) spectra were recorded on a Jasco J–820 spectropolarimeter at 25 °C in a nitrogen flow rate of 5 ml/min. The CD spectra of samples (100 μM) and the mixtures with ChC in 0.05 M Tricine buffer (with 0.4 M NaCl and 0.01 M $CaCl_2$, pH 7.5) were collected with a bandwidth of 1.0 nm and a resolution of 0.2 nm as a scan speed of 100 nm/min.

Results and discussion

Collagenase inhibition activity

Collagenases are a small group of highly specific proteinases capable of causing hydrolytic cleavage in the triple-helical region of the collagen molecule. In contrast to tissue collagenases, which cleave the collagen helix at a single site, *C. histolyticum* collagenase

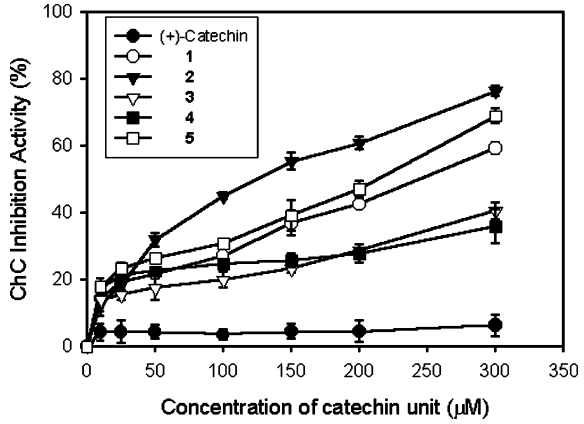

Fig. 2. ChC inhibition activities of (+)-catechin and polycondensates, $n = 3$. The sample solution and ChC (100 mU/ml) were dissolved in 0.05 M Tricine buffer (with 0.4 M NaCl and 0.01 M $CaCl_2$, pH 7.5), and preincubated at 25 °C for 5 min. Then, FALGPA (300 μM) was added to the mixture to immediately measure the decrease of the optical density at 324 nm for 20 min for detection of hydrolyzed FALGPA.

(ChC) known as clostridiopeptidase A or collagenase A may bring about multiple cleavages [25]. In order to evaluate the inhibition activity, the hydrolysis of *N*-[3-(2-furyl) acryloyl]-Leu-Gly-Pro-Ala (FALPGA) by ChC with and without catechin or its polycondensates was monitored by a UV–visible spectrometer at 324 nm. As shown in Fig. 2, the inhibition of catechin against ChC was negligible at concentrations below 300 μM. Green tea polyphenol, (−)-epigallocatechin gallate (EGCG), has been reported to show antitumor effects and suppress gelatin degradation induced by metalloproteinases, whereas (+)-catechin and (−)-epicatechin have little effects [21,22].

On the other hand, the polycondensation of catechin with aldehydes greatly improved its inhibition effects on ChC. All polycondensates (**1**–**5**) showed much amplified inhibition activities on the molar basis of a monomeric repeating unit. It is reported that some collagenase inhibitors attack the zinc site in the catalytic domain of the enzyme, and incorporate a strong zinc-binding function of hydroxamate or carboxylate type, as well as a scaffold that assures favorable interactions with the primed side of the catalytic domain of collagenase [5,26,27]. Polycondensates (**2** and **5**) with the introduction of the carboxyl and 4-hydroxybenzyl groups, respectively, as bridge between the catechin units exhibited higher inhibition effects than other polycondensates. The high activity of **2** is because the carboxyl moiety of **2** is coordinated to the Zn (II) ion, being also hydrogen-bonded to the carboxylate of amino acid residues of the enzyme. In the case of **5**, the 4-hydroxybenzyl group makes extensive hydrophobic interaction with the enzyme. Moreover, **1** also showed relatively high-inhibition effect on ChC. It is well known that high-molecular-weight plant polyphenols such as procyanidin are good chelators that can form precipitates with metal ions and proteins correlating with the degree of polymerization [28,29]. Thus, the higher molecular weight of **1** may enhance the strength of interaction with the enzyme, leading to the higher inhibition activity.

Collagenase inhibition mechanism

To analyze the inhibition type of the present polycondensate for ChC, Lineweaver–Burk plots were used. The reciprocal plots were obtained in the presence of different concentrations of **1** and substrate (Fig. 3). Catechin–acetaldehyde polycondensate (**1**) exhibited dose-dependent inhibition activity on ChC, and Lineweaver–Burk plots clearly showed a mixed-type inhibition. The steady-state analysis for the ChC inhibition by **2** and **5**, which were the most potent inhibitors on ChC among the polycondensates (Fig. 2), also showed a mixed-type inhibition. The kinetic constants for **1** and **2**, dissociating from ChC (K_i) and the ChC–substrate complex (K_i'), were calculated by Dixon plots; the K_i and K_i' values for **1** are 97.5 and 141.8 μM and those for **2** are 74.9 and 144.4 μM, respectively. These data suggest that the polycondensate (**1** or **2**) binds to both ChC and the ChC–substrate complex, and the former takes place more frequently than the latter. Contrary to expectation, the result of the kinetic assay for **5** differed from those obtained with **1** and **2**. The K_i and K_i' values are 133.5 and 93.3 μM, respectively. This means that **5** mainly binds to the ChC–substrate complex.

In order to elucidate the interaction of ChC with catechin or the polycondensate, circular dichroism (CD)

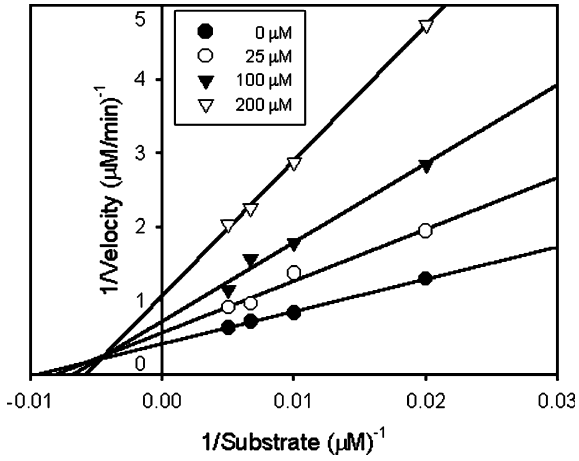

Fig. 3. Steady-state analysis for the inhibition of ChC by polycondensate (**1**) with respect to FALGPA as substrate. The reaction mixtures in 0.05 M Tricine buffer (with 0.4 M NaCl and 0.01 M $CaCl_2$, pH 7.5) contained different concentrations of **1** and FALGPA, and ChC (100 mU/ml).

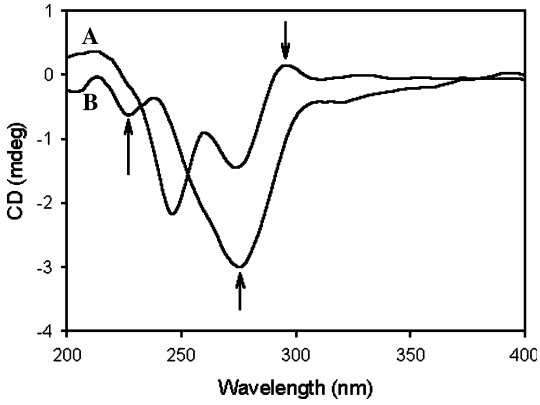

Fig. 4. Difference CD spectra of (+)-catechin (100 μM)–ChC (A) and polycondensate (**1**) (100 μM)–ChC (B) complexes in 0.05 M Tricine buffer (with 0.4 M NaCl and 0.01 M $CaCl_2$, pH 7.5). Each spectrum was obtained by subtracting the spectrum of catechin or **1** in the absence of ChC from that in the presence of ChC.

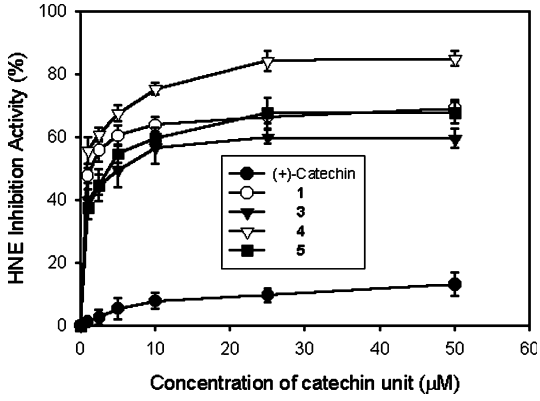

Fig. 5. HNE inhibition activities of (+)-catechin and polycondensates, $n = 3$. The sample solution and HNE (17 mU/ml) were mixed in 0.1 M Tris–HCl buffer (pH 7.5), and preincubated at 25 °C for 5 min. Then, MAAPVN (500 μM) was added to the mixture and incubated at 37 °C for 1 h. Afterward, the reaction was stopped by the addition of soybean trypsin inhibitor (1 mg/ml) and the optical density due to the formation of *p*-nitroaniline was immediately measured at 405 nm.

measurement was carried out. There was a strong negative band at 235 nm corresponding to the peptide backbone absorbance triggered by a mixture type of α-helix (28%), β-sheet (39%), and random coil in a trace of ChC (data not shown) [30,31]. In addition, the negative shoulder band centered at 272 nm and the positive band centered at 290 nm due to three aromatic residues (phenylalanine, tyrosine, and tryptophan) were observed.

In the CD band of a mixture of catechin and ChC, the characteristic change was not found (Fig. 4). On the other hand, the spectrum of a mixture of **1** and ChC was profoundly altered and the fine structure of ChC disappeared. The intensity of the negative band due to the peptide backbone enormously decreased and the shift to 225 nm was observed. The negative shoulder band ascribed to the aromatic residues increased and the small red shift to 275 nm was shown. Additionally, the positive band corresponding to the aromatic residues disappeared. These data suggest that the regular structural pattern of ChC is partially destroyed by the chelation of **1** to the catalytic domain and the side-chain reorientation of ChC; some helixes are converted to β-sheet or random coil [30,31].

Elastase inhibition activity

Human neutrophil elastase (HNE) is released from neutrophils that migrate to the sites of infection or other damaged tissues during the early stages of inflammation, and is consequently a useful marker of inflammation [14]. HNE activates also other proteinases included in the breakdown of matrix proteins, e.g., procollagenase, prostromelysin, and progelatinase, and inactivates a number of endogenous proteinase inhibitors [32,33]. We tested catechin and its polycondensates for an elastase inhibition assay using HNE as enzyme source.

Fig. 5 shows results of the HNE inhibitory effects by catechin and the polycondensates. The effect of catechin was very low in all tested concentrations. Phenolic compounds from plants including the flavonoids and caffeic acid esters have been used for a long time in the treatment of inflammatory disorders. Among the flavonoids, especially the compounds with two hydroxyl groups in 3′- and 4′-position showed a stronger inhibition activity than others, whereas catechin was inactive up to the concentration of 400 μM in the elastase inhibition assay [14,34].

The polycondensates except **2** exhibited greater inhibition activities on HNE in a catechin unit-concentration-dependent manner, compared to the catechin monomer. It was reported that procyanidins, direct condensates of (+)-catechin or (−)-epicatechin linked through C_4–C_6 or C_4–C_8 bonds of catechins, showed higher inhibition activities than catechin [34]. Among the polycondensates, **4** was the most effective inhibitor, suggesting the formation of hydrogen bonds between the phenol moieties of **4** and the protein amino acids [34]. Moreover, the introduced hydroxybenzyl group of **4** enhances the inhibition activity on HNE by the electrostatic interaction. We did not examine the inhibition activity of **2** for HNE, since **2** catalyzed the hydrolysis of the substrate (MAAPVN) for HNE.

To further characterize the inhibition activities of the polycondensates, the kinetic assay with Lineweaver–Burk plots was performed (Fig. 6). The reciprocal plots were obtained with various concentrations of **1** and substrate. Lineweaver–Burk plots showed a mixed-type inhibition with K_i value of 9.2 μM and K_i' value of 16.3 μM, similarly in the ChC inhibition. In addition, the inhibition type of **4** and **5** was also analyzed by Lineweaver–Burk plots (data not shown). The results

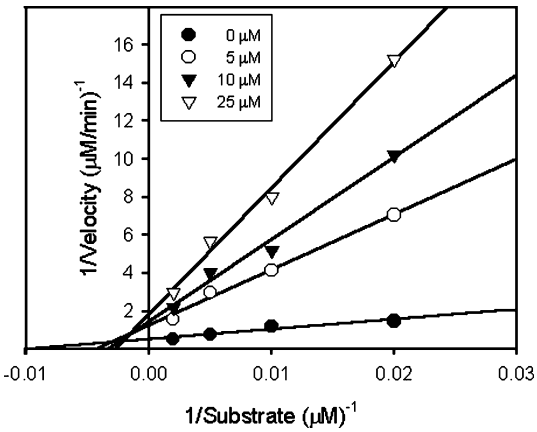

Fig. 6. Steady-state analysis for the inhibition of HNE by polycondensate (**1**) with respect to MAAPVN as substrate. Reciprocal plots obtained at various concentrations of **1** and MAAPVN. The HNE concentration was 17 mU/ml and the reaction mixture was incubated in 0.1 M Tris–HCl buffer (pH 7.5).

present that **4** and **5** are a typical mixed-type inhibitor. The K_i and K_i' values for **4** are 8.5 and 13.7 μM and those for **5** are 9.1 and 15.8 μM, respectively. These data indicate that the polycondensates effectively inhibit the HNE activity by mainly binding to the catalytic site of the enzyme.

Proteinases such as collagenase and elastase are involved in the turnover of matrix proteins during angiogenesis, tissue remodeling, and repair. Since these enzymes play an essential role in the homeostasis of ECM, an imbalance in their expression or activity may have important consequences in various pathologies such as multiple sclerosis, Alzheimer's disease, and cancers. For these reasons, there is a great interest in the discovery of new proteinase inhibitors because of their potential applications. The concern with the importance of regulating these proteinases has been growing in medicinal and pharmacological fields, while no regulating system has been developed because few inhibitors are useful in their applications due to their chemical properties and safety problems.

Previously, we developed polymeric inhibitors for xanthine oxidase by the polymerization or conjugation of catechin [35–38]. For further development of polymeric catechin inhibitors of disease-related enzymes, we have examined the inhibitory effects of (+)-catechin–aldehyde polycondensates against ChC and HNE. These polymers strongly inhibited the ChC and HNE activities, whereas the catechin monomer showed the very low inhibition effects, strongly suggesting that the polycondensates might prevent such proteolytic degradation by limiting the ChC and HNE activities. We believe that the polycondensates are useful for a therapeutic agent to offer protection against proteinase-related diseases including interstitial lung diseases, rheumatoid arthritis, and cancers.

Acknowledgments

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