Inhibitory Effect of Procyanidin Oligomer from Elm Cortex on the Matrix Metalloproteinases and Proteases of Periodontopathogens

Si - Eun Song

The Graduate School

Yonsei University

Department of Dental Science

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Thesis Supervisor: Chong-Kwan Kim

Jung-Kiu Chai

Yun-Jung Yoo

Chang-Sung Kim

Bong-Kyu Choi

The Graduate School

Yonsei University

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Objectives: The purpose of thia studt was to evaluate a partially purified extract (elm extract) from the cortex of *Ulmi macrocarpa* Hance and its active ingredient, a mix of procyanidin oligomers (3 to 12 flavan-3-ol monomers, an average molecular weight of 1,518 with an average polymerization degree of 5.3) for a possible inhibitory effect against protease.

Background: Host-derived matrix metalloproteinases (MMPs) and bacterial proteases have important roles in the gingival tissue destruction that is a characteristic of periodontitis. The inhibitors of this proteases may be developed into therapeutic agents against periodontitis.

Materials & Methods: The inhibitory effects were assessed by gelatin zymography. The MMPs tested were originated from the gingival crevicular fluid (GCF) of adult periodontitis patients and from the conditioned media of cultured periodontal ligament (PDL) cells, which provided the proMMP-2 and activated MMP-2 when treated with a periodontopathogen, *Treponema lecithinolyticum*. Bacterial enzymes tested were secreted forms from two major periodonto pathogens, *Porphyromonas gingivalis* and *Treponema denticola.* In addition, the inhibitory

effects on trypsin-like enzymes from these two periodontopathogens were assayed by the n-benzoyl-DL-arginine-naphthylamide (BANA) test.

Results: The elm extract and the procyanidin oligomer (100-1,000 μg/ml) exhibited potent inhibitory effects on the MMPs in GCF (chiefly MMP-8 and MMP-9), and the pro and active forms of MMP-2, and secreted and trypsin-like enzymes from *Porphyromonas gingivalis* and *Treponema denticola*.

Conclusion: These results suggest that elm cortex should be considered as a potential agent against periodontal diseases, due to its inhibitory action as on MMPs and the proteases of periodontopathogens.

Key Words: elm cortex, Procyanidin oligomer, Matrix Metalloproteinases

Inhibitory Effect of Procyanidin Oligomer from Elm Cortex on the Matrix Metalloproteinases and Proteases of Periodontopathogens

Si-Eun Song, D.D.S., M.S.D.

Department of Dental Science

Graduate School, Yonsei University

(Directed by Prof. Chong-Kwan Kim, D.D.S., M.S.D., PhD.)

I. Introduction

Periodontitis is a polymicrobial infection. Its major pathogenesis is characterized by the destruction of periodontal tissues, often leading to tooth loss. In this respect, host-derived matrix metalloproteinases (MMPs) and collagenolytic enzymes from the periodontopathogens play a central role (Birkedal-Hansen et al., 1993; Sorsa et al., 1992). MMP secretion can be induced when polymorphonuclear leukocytes , gingival fibroblasts , epithelial cells or macrophages respond to cytokines, growth factors or various inflammatory mediators stimulated by periodontopathogens. All MMPs except MT-MMPs are secreted by cells in a latent form and are activated extracellularly. Therefore, overproduction and enhanced activation of MMPs have

important ramifications for various pathological conditions including periodontitis. Elevated activities of collagenases (MMP-1 and MMP-8) and gelatinases (MMP-2 and MMP-9) have been detected in extracts from inflamed gingival tissues and in the gingival crevicular fluid (GCF) of periodontitis patients (Yu et al., 1998; Ingman et al., 1996; Korostoff et al., 2000; Makela et al., 1994; Sorsa et al., 1994) .

Periodontopathogens such as *Porphyromonas gingivalis* and *Treponema denticola* are known to possess strong proteolytic activity, which includes collagenases (Sorsa et al., 1992). Since bacterial collagenases have broad substrate specificity and degrade native collagen molecules into small peptides, they are highly potent. These proteases also participate in the activation of latent MMPs from host cells (DeCarlo et al., 1997; Ding et al., 1997).

It is possible that the inhibition of the proteolytic activity, which originates from the bacterial enzymes and host cells, can prevent tissue destruction in periodontal disease. For the prevention and the efficient treatment of periodontal disease, efforts have been made to identify MMP inhibitors that have potential therapeutic value. Low tetracycline doses are known to inhibit host collagenase activity in the gingival tissue and in the GCF of periodontitis patients, and thus to prevent periodontal attachment loss (Golub et al., 1990; Seymour et al., 1995). Although tetracyclines are used extensively to manage periodontal diseases, their use is limited to those without hypersensitivity to these drugs.

Natural products have received considerable attention in this context, regarding their use as medicinal compounds. Whilst searching for natural collagenase inhibitors, we screened more than 100 plants that have been widely used in traditional medicine.

Among these, elm cortex was found to be the most effective of a few selected plants, and its active ingredient was identified to be procyanidin oligomer. The Procyanidins are a subclass of flavonoids and are comprised of oligomers of catechin. They occur primarily in plants and are known to exert many physiological effects, including antioxidant, anti-inflammatory, analgesic, and enzyme inhibitory effects (Dongmo et al., 2001; Lotito et al., 2000; Moini et al., 2000). The number of catechin units within the procyanidins has been reported to influence their activity (Lotito et al., 2000; Arteel et al., 1999). The possible inhibitory reactions of the procyanidins are known to involve metal chelation, radical trapping, or direct enyme binding (Lotito et al., 2000; Moini et al., 2000).

In Korean traditional medicine, elm cortex from the dried bark of *Ulmus pumila*, *Ulmus macrocarpa*, and *Ulmus davidiana* has been known to act against edema, gastric cancer and inflammation such as arthritis and gastritis (Hong et al., 1990; Kim et al., 1996).

The present study was undertaken to evaluate the effects of the *Ulmus macrocarpa* extract (elm extract) and its active ingredient, the procyanidin oligomer, on the MMPs of host cells and the proteases from the representative periodontopathogens, *P. gingivalis* and *T. denticola*.

II. Materials and Methods

1. Chemicals

All reagents used were of the highest analytical grade and were purchased from the Sigma Chemical Company Inc, St Louis, MO, USA.

2. Extract of elm cortex and procyanidin oligomer

The dried elm cortex of *Ulmus macrocarpa* Hance was pulverized to 10 to 200 mesh and extracted with absolute ethanol at a ratio of 1:5 (w/v) at room temperature for 72 h. The extract was filtered, concentrated under a reduced pressure at 45°C, resuspended in distilled water and then consecutively fractionated with n-hexane, dichloromethane, ethylacetate, and n-butanol. The n-butanol fraction showed the strongest inhibitory effect on collagenase (from *Clostridium histolyticum*) activity and was defined as the 'elm extract' throughout this study. The elm extract contained 20% of the procyanidin oligomer.

The procyanidin oligomer used in this study was isolated from the elm extract and analyzed chemically by LG Household & Health Care/Research, Taejon, Korea. The procyanidin oligomer was composed of 3 to 12 flavan-3-ol monomers connected by single bonds, and found to have an average molecular weight of 1,518 and an average polymerization degree of 5.3. The procyanidin oligomer was purified as follows: The elm extract was further subjected to Sephadex LH-20 column chromatography using a water-methanol mix as eluant with an increasing amount of methanol. The fraction containing the highest inhibitory activity was subjected to liquid chromatography-

mass spectroscopy (LC-MS), MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) mass spectroscopy, HPLC/ESI (high performance liquid chromatography/electron spray ionization), phloroglucinol acidolysis and nuclear magnetic resonance spectroscopy (NMR).

The elm extract and the procyanidin oligomer were dried under vacuum and dissolved in dimethyl sulfoxide (DMSO) at an appropriate concentration for use. The structure of the procyanidin oligomer is shown in Fig. 1.

3. Bacterial culture

T. denticola ATCC 33521 and *Treponema lecithinolyticum* ATCC 700332 were cultured anaerobically in OMIZ-Pat medium for 3-5 days, as described previously (Wyss et al., 1999). *P. gingivalis* ATCC 33277 was cultured anaerobically in brain heart infusion broth* supplemented with hemin (5 μg/ml) and menadione (0.5 μg/ml) for 3 days. Bacterial purity was confirmed by phase contrast microscopy. *T. denticola* and *P. gingivalis* cultures were centrifuged at 5,000 x g for 10 min and the culture supernatants were further centrifuged at 12,000 x g. Supernatants containing the secreted enzymes were collected and used for inhibitory assays.

T. lecithinolyticum whole cell sonicates were prepared and used to activate the MMP-2 secreted from cultured PDL cells as described previously (Choi et al., 2001).

* Difco, Detroit, MI, USA

4. Collection of gingival crevicular fluid (GCF)

GCF samples were collected from deep periodontal pockets (pocket depth >6 mm) of 7 advanced adult periodontitis (AP) patients. Prior to collection, the tooth surface was dried with air and kept dry with cotton wool rolls. Three paper points were inserted into the sulcus for 3 min, and then placed in a vial containing 200 μl of the enzyme reaction buffer (ER buffer: 50 mM Tris-HCl, 0.2 M NaCl, 5 mM $CaCl_2$, pH 7.5). Adsorbed fluid was eluted from the paper points by vigorous vortexing the sample vial and centrifuged at 13,000 x g for 10 min at 4°C. Supernatants were collected and stored at -20°C until required.

5. Cultures of periodontal ligament (PDL) cells

For MMP-2, PDL cells were prepared from the extracted teeth of periodontal healthy donors as previously described (Choi et al., 2001). Briefly, PDL attached to the middle third of the root surface was carefully scraped off with a scalpel, and washed in α-minimal essential medium (MEM) supplemented with antibiotics (100 μg/ml penicillin G, 0.25 μg/ml streptomycin, 85 μg/ml amphotericin-B). The PDL was then minced, placed on the bottom of 25 mm^2 tissue culture flasks and incubated at 37°C in 5% CO_2 in α-MEM containing 10% heat-inactivated fetal bovine serum (FBS) and antibiotics. When the cells grew out grom the explant and formed a confluent layer, they were harvested by trypsinization and subcultured. PDL cells between the 6th and 8th passages were used for the experiments.

To obtain the active form of MMP-2, the PDL cells were seeded at 10^4 cells/well in 96-well plates and grown to confluence in α-MEM with 10% FBS. After incubation

in the serum free medium for 24 h, the cells were treated with whole cell sonicates (50 μg protein/ml) of *T. lecithinolyticum* and further incubated for 48 h. Previously, a part of the latent form of MMP-2 derived from PDL cells was reported to be activated after *T. lecithinolyticum* treatment (Choi et al., 2001).

6. Gelatin zymography

Gelatin zymography was performed to detect protease activity and to examine the inhibitory effects of the elm extract and the procyanidin oligomer. The GCF samples (4 μl) pooled from two patients, the bacterial culture supernatants (15 μl) or the conditioned culture supernatants (15 μl) of the PDL cells were mixed with sample buffer [2.5% (w/v) SDS, 50 mM Tris HCl (pH 6.8), 0.005% bromophenol blue, and 3% sucrose] and loaded onto 8% sodium dodecyl sulfate (SDS)-polyacrylamide gels containing 0.2% gelatin. After electrophoresis, the gels were washed twice in a solution containing 2.5% Triton X-100 and 50 mM Tris-HCl (pH 7.5) for 30 min with gentle shaking. The gel lanes were then dissected and incubated at 37°C for 18 h in ER buffer in the presence of the elm extract or the procyanidin oligomer at final concentrations of 0.01, 0.05, or 0.1% (100-1000 μg/ml). Gels were stained with 0.05% Coomassie brilliant blue R-250 in 10% isopropyl alcohol and 10% acetic acid. After destaining in the same solution without the Coomassie blue dye, clear bands showing gelatinolytic activity were observed. For the control, DMSO, in which the elm extract and the procyanidin oligomer were dissolved, was added instead of the inhibitor. In gelatin zymography, the MMP proforms could also be visualized by a conformational change induced by SDS.

The intensities of the clear bands obtained by zymography were quantified using an image analysis program[†] (TINA2.1), and the band intensities in each lane were compared with those of control lanes.

7. Enzyme-linked immunosorbent assay (ELISA) of MMP-8 and MMP-9

MMP-8 and MMP-9 levels in the GCF samples taken from 7 advanced AP patients were measured using ELISA kits[‡], which had a linear binding curve from 0 to 4 ng/ml and from 0 to 32 ng/ml, respectively.

[†] Neuro-Image Analysis Center, Oxford, UK

[‡] Amersham, Piscataway, NJ, USA

8. Immunoblotting

The identity of the MMP-2 derived from the PDL cells was verified by immunoblotting. The culture supernatants (500 µl) used in gelatin zymography were concentrated using a Centricon 30[§], subjected to 8% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked in PBS containing 5% skim milk for 30 min, and then incubated for 2 h with monoclonal anti-human MMP-2[¶] to detect the pro and active forms of MMP-2. It was then washed in PBS/0.1% Tween 20 and incubated with alkaline phosphatase (AP)-conjugated goat anti-mouse IgG[#]. After washing, 5-bromo-4-chloro-3-indolylphosphate (165 µg/ml) and nitroblue tetrazolium (330 µg/ml) were used as substrates to detect MMP-2.

[§] Amicon, Denvers, CO, USA

[¶] NeoMarkers, 2 µg/ml, Fremont, OH, USA

[#] Sigma, St. Louis, MO, USA

9. BANA test

To evaluate the effects of the elm extract and the procyanidin oligomer on the trypsin-like enzymes of *T. denticola* and *P. gingivalis*, we performed the n-benzoyl-DL-arginine-naphthylamide (BANA) test. A 3-day bacterial culture was adjusted to an optical density of 0.04 at 600 nm in distilled water. Diluted bacterial cultures (48 μl) were mixed with 1 μl of BANA (10 mM) in a total volume of 100 ul of reaction buffer containing 0.05 M Tris-HCl (pH 7.8) and 0.2 M NaCl with or without the elm extract or the procyanidin oligomer. These inhibitors were used at 100-fold concentrations to produce final concentrations of 0.1, 0.05, and 0.01%, respectively. The reaction mixture was incubated at 37°C for 18 h. After addition of 25 μl of 0.1% fast garnet, the change in optical density at 405 nm was measured. Data are presented as % inhibition versus the control values, which were obtained using DMSO instead of the elm extract or the procyanidin oligomer. The culture media (BHI for *P. gingivalis* and OMIZ-Pat for *T. denticola*) were also assayed using the BANA test. No detectable hydrolysis was detected in these media.

10. Effects of elm extract and procyanidin oligomer on cell proliferation

In order to examine the effects of the elm extract and the procyanidin oligomer on cell proliferation, cultured human PDL cells were subjected to the MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide] test, whereby viable cells form formazan crystals intracellularly as a consequence of their metabolic activity. PDL cells were grown to confluence in 96-well plates using the method described by Choi *et al*. (Choi et al., 2001). After treatment with the elm extract or the procyanidin

oligomer at concentrations of 0.01, 0.05 and 0.1%, the plates were further incubated for 48 h. The culture supernatants were then removed and 50 µl of MTT[**] was added to the wells. The plates were incubated at 37°C for 4 h and formazan formation was determined by measuring optical density at 570 nm in an ELISA reader after dissolving the formazan in 100 µl DMSO.

11. Statistical analysis

Statistical differences were determined by Mann-Whiteny U test for comparison between the inhibitor-treated and the non-treated groups. A *P* value of <0.05 was considered to be statistically significant.

[**] Sigma, 1 mg/ml, St. Louis, MO, USA

III. Results

1. Effect on GCF collagenases

Gelatin zymography is a sensitive method for detecting neutral proteases like the MMPs and it is also suitable for determining the effects of protease inhibitors on a target protease. Gelatin zymography was used in this study to evaluate the inhibitory effect of the elm extract and of the procyanidin oligomer on collagenolytic activities. In the GCF samples collected from 7 AP patients, high levels of MMP-8 and MMP-9 were detected by ELISA (Fig. 2). Two samples that showed the highest MMP-8 levels were pooled and subjected to zymography. As shown in Fig. 3, high proteolytic activities were detected on the gelatin zymogram (lane of DMSO). However, these activities were inhibited by both the elm extract and the procyanidin oligomer even at concentrations of 0.01% (100 μg/ml).

2. Effect on MMP-2 derived from PDL cells

Gelatin zymography of the culture supernatants of the PDL cells demonstrated the presence of the latent form of MMP-2 (72 kDa) (data not shown), while the culture of *T. lecithinolyticum*-treated PDL cells produced both the latent and active forms of MMP-2 (62-kDa). As shown in Fig. 4A, the procyanidin oligomer inhibited PDL cell-derived MMP-2 in both the latent and active forms. The elm extract also inhibited both forms of MMP-2, but was less effective than the procyanidin oligomer (data not shown). The identity of MMP-2 was confirmed by immunoblot using anti-MMP-2 antibody (Fig. 4B).

3. Effect on proteases of periodontopathogens

T. denticola and *P. gingivalis* secreted proteases, which showed strong proteolytic activities on a gelatin zymogram. Both inhibitors effectively inhibited the *T. denticola* proteases, whereas the elm extract was less effective on *P. gingivalis* proteases than that of the procyanidin oligomer (Fig. 5A and B).

The strong hydrolysis of BANA by trypsin-like activity was detected in *T. denticola* and *P. gingivalis,* with an average OD at 405 nm of 1.49 and 1.78, respectively. As shown in Fig. 6, the procyanidin oligomer (0.1 - 0.05%) reduced the enzyme activity to 34-58% in *T. denticola* and 39-73% in *P. gingivalis* in a dose-dependent manner, whereas the elm extract reduced enzyme activity to 40-89% in *T. denticola* and 49-91% in *P. gingivalis*.

4. Determination of IC_{50} values

The concentrations of the elm extract or the procyanidin oligomer required to inhibit 50% of the enzyme activity (IC_{50}) were calculated from the zymograms. The IC_{50} values of the elm extract were 29, 45, 200, and 900 μg/ml for GCF collagenases, MMP-2, *T. denticola* proteases, and *P. gingivalis* proteases, respectively. The corresponding IC_{50} values of the procyanidin oligomer were 25, 33, 58, and 60 μg/ml, respectively.

5. Effects on the proliferation of PDL cells

The effects of the elm extract or the procyanidin oligomer on the cell proliferation of PDL cells was determined using the MTT test. Values are presented as percentages of

the control value (100%). As shown in Fig. 7, the procyanidin oligomer (0.01-0.1%) had a statistically insignificant effect on the proliferation of PDL cells. However, at concentrations of 0.05-0.1%, The elm extract was found to inhibit PDL cell proliferation as assessed by microscopy and the MTT test.

IV. Discussion

In this study, the inhibitory effects of the extract of the *Ulmus macrocarpa* cortex and of its active ingredient, the procyanidin oligomer, on various protease activities were evaluated by gelatin zymography. These included the inhibition of the collagenolytic activities in GCF, of MMP-2 secreted from PDL cells, and of the proteolytic enzymes of the major periodontopathogens, *T. denticola* and *P. gingivalis*. In all cases, the procyanidin oligomer exhibited significant inhibitory activity. The elm extract, a partially purified form of elm cortex also showed a strong inhibitory activity against MMPs originating from GCF and PDL cells. However, the effect of the elm extract on bacterial proteases was lower than the procyanidin oligomer, represented by a higher IC_{50} value.

MMP activities are markedly elevated in gingival tissue and in the GCF of periodontitis patients. The collagenases of the GCF are known to be mainly MMP-8 and MMP-9 derived from PMN (Birkedal-Hansen et al., 1993; Ingman et al., 1996; Sorsa et al., 1994; Romanelli et al., 1999). Therefore, GCF MMP-8 and -9 can be potentially used as diagnostic markers of gingival inflammation. We also found high levels of MMP-8 and MMP-9 in the GCF by ELISA. The interstitial collagenases, including MMP-8, can degrade the native type I collagen into 3/4α and 1/4α length fragments, which in turn can act as substrates for other types of MMPs. High levels of MMP-8 in the GCF can initiate the proteolytic connective tissue degradation cascade and inhibiting MMP-8 could be an important regulatory step. Reductions in the levels and activities of MMPs have been found to correlate well with successful periodontal

therapy (Birkedal-Hansen et al., 1993; Sorsa et al., 1994; Golub et al., 1990; Reynolds et al., 2000). Therefore, MMP inhibitors in either synthetic or natural forms have received particular attention.

As natural products, the tea catechins have been reported to inhibit collagenolytic proteases in GCF and in culture supernatants of *P. gingivalis* (Makimura et al., 1993). The authors observed that epicatechin gallate (ECG) and epigallocatechin gallate (EGCG) from green tea completely inhibited collagenase activity, whereas catechin, epicatechin, gallocatechin, and epigallocatechin had no effect. Demeule et al.(Demeule et al., 2000) also reported upon the inhibitory effect of ECG and EGCG from green tea on MMP-2, MMP-9 and MMP-12 activities as well as on proMMP-2 activation. In their studies, the gallate residue appeared to be essential for the inhibitory effect of tea catechin on MMPs. The procyanidin oligomer used in our study possessed 3-12 catechin units and contained no galloyl group, as confirmed by phloroglucinol acidolysis, where phloroglucinol disconnects the bonds between the monomers in polymers, followed by HPLC. Although previous studies have shown that catechin monomers do not possess MMP inhibitory effects, our results show that its oligomeric form exhibited a strong inhibitory effect on MMPs and bacterial proteases. Recent studies have demonstrated the different effects of the procyanidin oligomers versus the monomeric compounds. Lotito *et al*. (Lotito et al., 2000) reported that procyanidin oligomers inhibited the oxidation of the lipid membrane more effectively than catechin monomers. Long chain procyanidins from cocoa have been reported to provide more protection against peroxynitrite-mediated damage than shorter chain procyanidins (Arteel et al., 1999).

Korostoff *et al*. (Korostoff et al., 2000) demonstrated the importance of the role of MMP-2 in tissue destruction. In the present study, the latent and active forms of MMP-2 derived from PDL cells were strongly inhibited by the elm extract and the procyanidin oligomer. The activation of proMMP-2 was illustrate not by 4-aminophenylmercuric acetate, which is usually used for MMP activation, but by a periodontopathogen, *T. lecithinolyticum* (Choi et al., 1999). This method was chosen because it was believed to be more relevant to the *in vivo* processing of periodontal infection.

P. gingivalis and *T. denticola* belong to the major periodontopathogens and possess high level of proteolytic activity. Proteases from these organisms can degrade the interstitial and basement membrane collagens (Sorsa et al., 1992; Sorsa et al., 1994; DeCarlo et al., 1997; Ding et al., 1997; Golub et al., 1990; Fravalo et al., 1996; Ding et al., 1996; DeCarlo et al., 1998). Moreover, a chymotrypsin-like protease of *T. denticola* activated MMP-8 and MMP-9 (Sorsa et al., 1994; Fenno et al., 1997), and a thiol proteinase from *P. gingivalis* was also demonstrated to activate MMP-1, MMP-3 and MMP-9 (Ding et al., 1997). Gelatin zymography showed that whole cell sonicates of *P. gingivalis* and *T. denticola* exhibited strong proteolytic activites (data not shown). In the present study, the culture supernatants of both periodontopathogens were tested, since we believed that enzymes secreted into media are more relevant under *in vivo* conditions. Trypsin-like enzymes of some periodontopathogens, including *T. denticola* and *P. gingivalis* are important virulence factors. The detection of trypsin-like activities in subgingival plaques is correlated with the severity of periodontal diseases, and the removal of anaerobes producing trypsin-like enzymes is

accompanied by a clinical improvement (Loesche et al., 1987). The present study demonstrates the inhibitory effect of the elm extract and the procyanidin oligomer on trypsin-like enzymes using BANA, a specific chromogenic enzyme substrate. The procyanidin oligomer inhibited this enzyme activity more effectively than the elm extract, and the trypsin-like activity of *T. denticola* was slightly more susceptible to these inhibitory effects than *P. gingivalis*.

The elm extract inhibited the proliferation of cultured PDL cells at concentrations of 0.05 and 0.1%, as judged by the MTT test. However, the procyanidin oligomer showed no significant effect on cell proliferation. Takahashi *et al*. (Takahashi et al., 1999) reported that the procyanidin oligomers from apple juice promoted the growth of hair epidermal cells *in vitro* and activated hair follicle growth *in vivo*. Inhibition of the PDL cell proliferation by the elm extract was probably caused by the other components than the procyanidin oligomer.

Our results suggest that the procyanidin oligomer and the elm extract can prevent collagen digestion by various proteases from both eucaryotic and procaryotic origin in periodontal disease. Therefore, the elm cortex should be considered as a potential agent against periodontal disease. However, since its inhibitory effect on diverse enzyme activities, as shown by our present study, was evaluated *in vitro* at neutral pH, several parameters should be addressed for *in vivo* application. These effects include local pH, solubility and the duration time of the substance in tissue, and the concentration required in serum and gingival crevicular fluids. For this purpose, we are now preparing for a large-scale test using an animal model.

V. Conclusion

Host-derived matrix metalloproteinases (MMPs) and bacterial proteases have important roles in the gingival tissue destruction that is a characteristic of periodontitis. In this study, a partially purified extract (elm extract) from the cortex of *Ulmi macrocarpa* Hance and its active ingredient, a mix of procyanidin oligomers (3 to 12 flavan-3-ol monomers, an average molecular weight of 1,518 with an average polymerization degree of 5.3), were evaluated for a possible inhibitory effect against host-derived MMPs and proteolytic enzymes from the major periodontopathogens, *Porphyromonas gingivalis* and *Treponema denticola*.

The MMPs tested were originated from the gingival crevicular fluid (GCF) of adult periodontitis (AP) patients and from the conditioned media of cultured periodontal ligament (PDL) cells, which provided the proMMP-2 and activated MMP-2 when treated with a periodontopathogen, *Treponema lecithinolyticum*. When assessed by gelatin zymography, both the elm extract and the procyanidin oligomer (100-1,000 μg/ml) exhibited potent inhibitory effects on the MMPs in GCF (chiefly MMP-8 and MMP-9), and the pro and active forms of MMP-2. The inhibitory effect on secreted and trypsin-like enzymes from *T. denticola* and *P. gingivalis* were assayed by gelatin zymography and the n-benzoyl-DL-arginine-naphthylamide (BANA) test, respectively. Both the elm extract and the procyanidin oligomer also showed inhibitory effect on these bacterial proteases.

These results suggest that elm cortex should be considered as a potential agent against periodontal diseases, due to its inhibitory action as on MMPs and the proteases of periodontopathogens.

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Figure legends

Figure 1. Structure of the procyanidin oligomer

Figure 2. MMP-8 and MMP-9 levels in the GCF samples. GCF samples from 7 advanced AP patients were assayed for MMP-8 and MMP-9 by ELISA. The results are presented as means± SD which were analyzed in triplicates.

Figure 3. Gelatin zymogram showing the inhibition of GCF collagenases by the procyanidin oligomer and the elm extract. Two GCF samples were pooled and 4 μl (1/100 of original sample volume) were subjected to zymography containing 0.2% gelatin. Each lane of the gel was dissected and incubated at 37°C for 18 h in the ER buffer in the presence of either the procyanidin oligomer or the elm extract at final concentrations of 0.1%, 0.05%, and 0.01%. As a negative control, the same amount of DMSO was added to the reaction mixture instead of the inhibitors.

Figure 4. (A) Gelatin zymogram showing MMP-2 inhibition by the procyanidin oligomer. The culture media (15 μl) of the PDL cells (2-day culture) treated with *T. lecithinolyticum* were obtained and gelatin zymography performed. Each lane of the gel was dissected and incubated at 37°C for 18 h in the ER buffer in the presence of the procyanidin oligomer at final concentrations of 0.1% and 0.01%. The same amount of DMSO was added to the reaction mixture instead of the inhibitors as a negative control. **(B)** Immunoblot showing the MMP-2 identity. The culture media of

the non-treated PDL cells (lane 1) and *T. lecithinolyticum*-treated PDL cells (lane 2) were subjected to 8% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was reacted with anti-MMP-2 antibody, which detected pro (72 kDa) and active forms (62 kDa) of MMP-2.

Figure 5. Inhibition of protease activities of *T. denticola* (A) and *P. gingivalis* (B) by the procyanidin oligomer and the elm extract. Gelatin zymography of the bacterial culture supernatants (15 μl) was carried out. Each lane of the gel was dissected and incubated at 37°C in the ER buffer in the presence of the procyanidin oligomer and the elm extract at final concentrations of 0.1%, 0.05%, and 0.01%. The same amount of DMSO was added instead of the inhibitors as a negative control.

Figure 6. Inhibition of trypsin-like activities by the procyanidin oligomer and the elm extract measured by the BANA test. Fresh cultures of *T. denticola* (A) and *P. gingivalis* (B) were appropriately diluted and mixed with 1 mM BANA in the presence or absence of inhibitors. After incubation of the reaction mixture at 37°C for 18 h, fast garnet was added and the change of optical density at 405 nm was measured. The y axis shows the % inhibition of the control values (100%) for which DMSO instead of inhibitors was used. Experiments were repeated three times and similar results were obtained. The results are presented as means±SD in triplicate samples. * Statistical significance at $P<0.05$.

Figure 7. Proliferation of the cultured PDL cells (2 days) in response to the

procyanidin oligomer (□) and the elm extract (■) measured by the MTT test. The y axis shows the % inhibition of the control value (100%). Experiments were repeated three times and similar results were obtained. The results are presented as means±SD in triplicate samples. * Statistical significance at $P<0.05$.

Figures

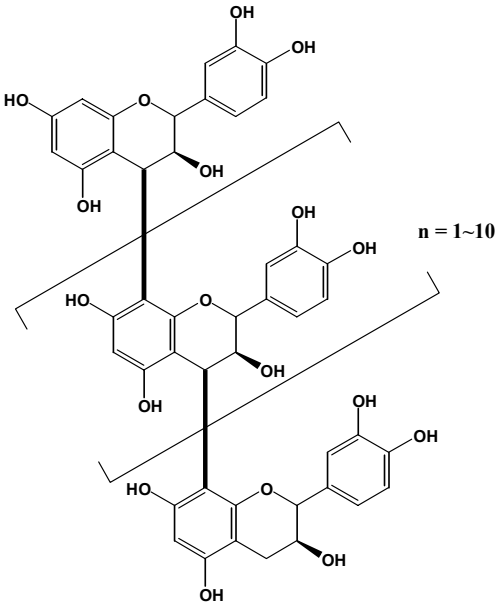
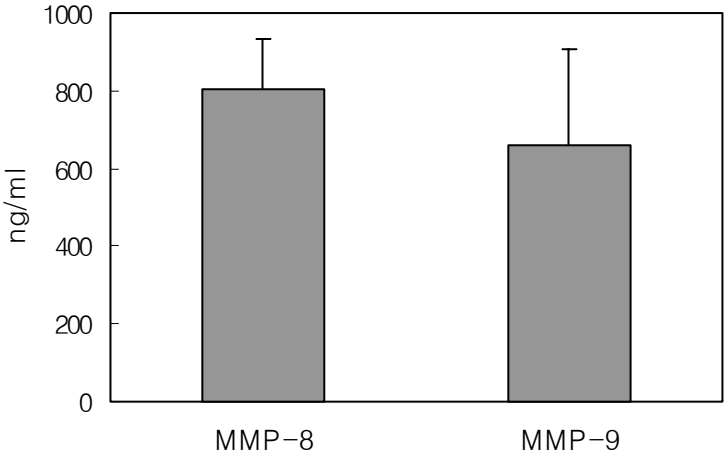


Fig. 1.



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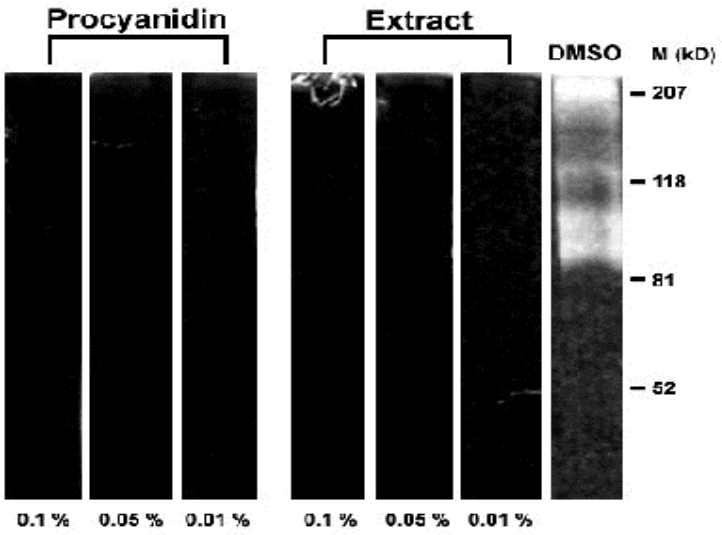
Procyanidin
Extract
DMSO
M (kD)
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81
52
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0.1 %
0.05 %
0.01 %

Fig. 3.

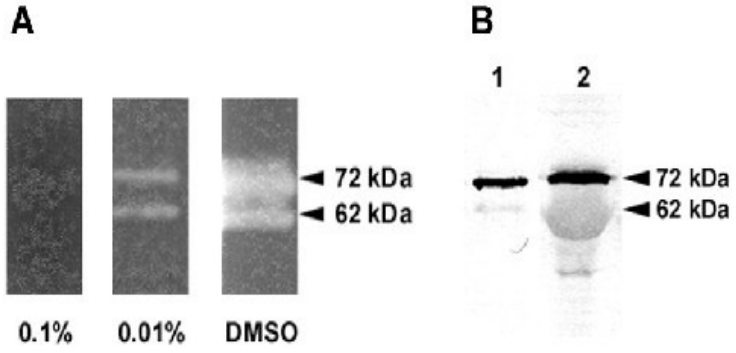
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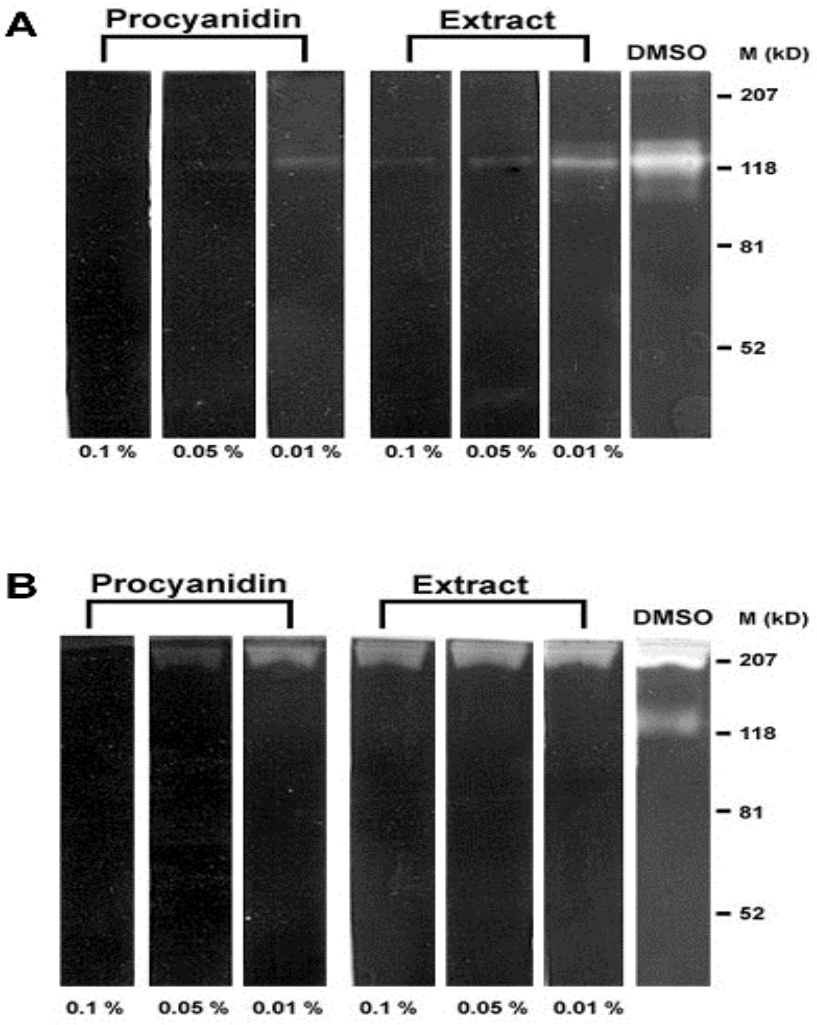
Fig. 4.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Procyanidin
Extract
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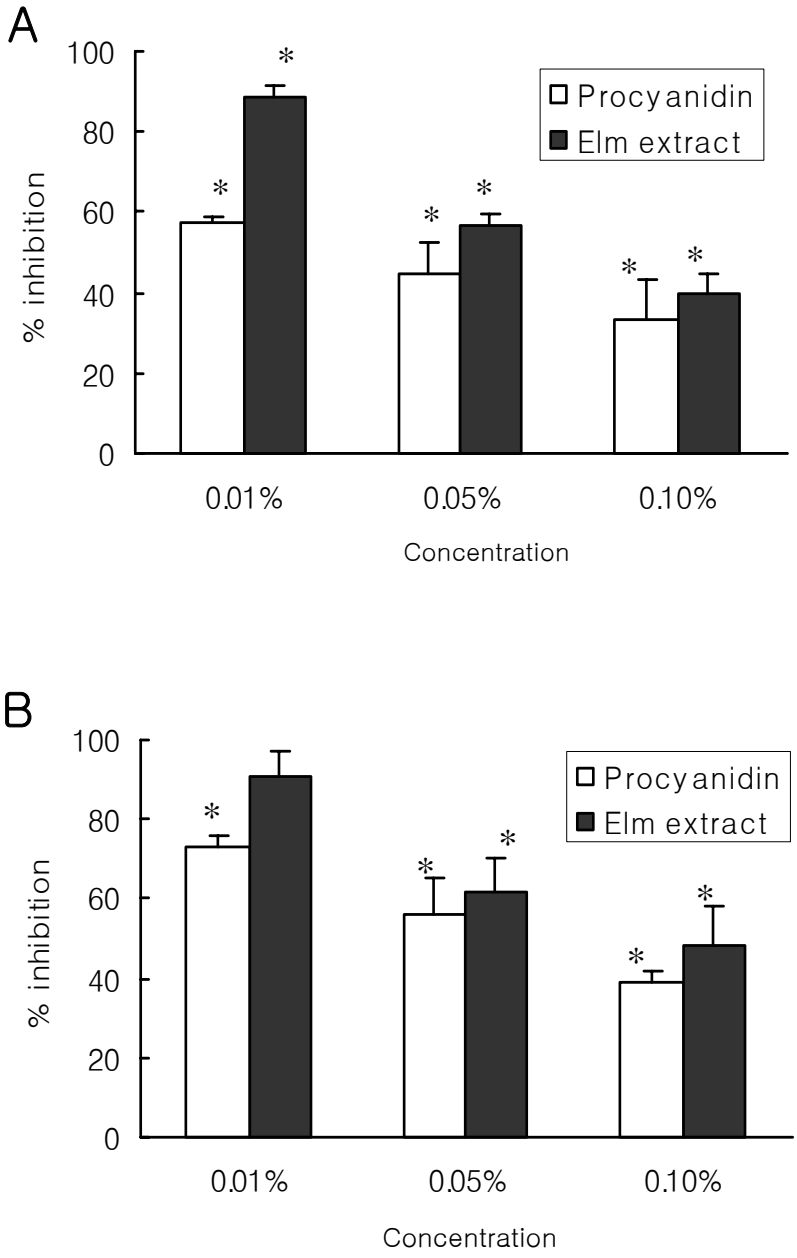
Fig. 5.


A
100
80
60
40
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0
% inhibition
Procyanidin
Elm extract
0.01%
0.05%
0.10%
Concentration
B
100
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% inhibition
Procyanidin
Elm extract
0.01%
0.05%
0.10%
Concentration

Fig. 6.

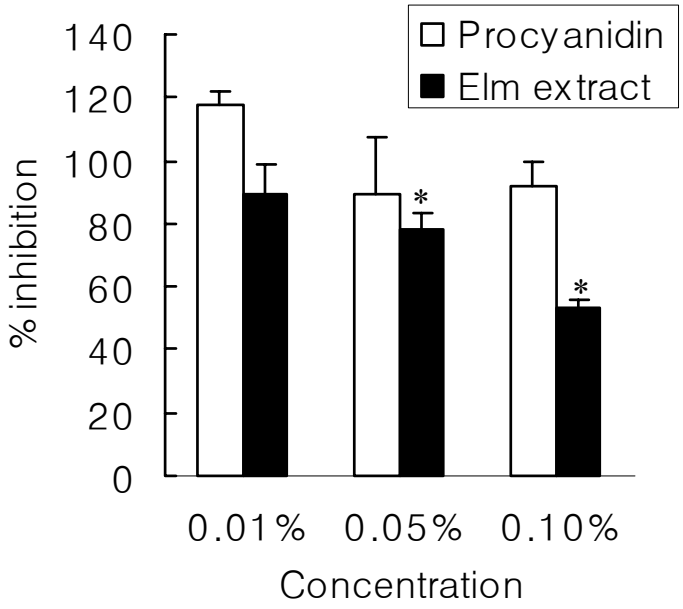
Procyanidin
Elm extract
140
120
100
80
60
40
20
0
% inhibition
*
*
0.01%
0.05%
0.10%
Concentration

Fig. 7.

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국문요약

느릅나무 피질 추출물인 프로시아니딘 올리고머의 매트릭스 메탈로프로테아제와 치주병원균의 단백질 분해효소에 대한 억제효과

< 지도교수 **김 종 관** >

연세대학교 대학원 치의학과

송 시 은

연구목적: *Ulmus macrocarpa* 피질의 부분 정제 추출물인 느릅나무 추출물과 프로시아니딘 올리고머의 단백질 분해효소에 대한 가능한 억제효과를 평가해 보고자 한다.

연구배경: 인체 내에서 분비되는 매트릭스 메탈로프로테아제와 박테리아성 단백질 분해효소는 치주염의 주요 증상인 치주조직 파괴에 중요한 역할을 한다. 이런 단백질 분해효소의 억제제는 치주염에 대한 치료제로 발전시킬 수 있을 것이다.

재료 및 방법: 억제효과는 젤라틴 자이모그라피 방법을 사용하였다. 매트릭스 메탈로프로테아제는 성인성 치주염환자의 치은열구액과 *T. lecithinolyticum* 으로 처리하여 MMP-2 전구체와 MMP-2 활성체가 함께 있는 배양된 치주인대세포의 선택 배지에서 얻은 것을 사용하였다. 박테리아성 효소는 주요 치주 병원균인 *Porphyromonas gingivalis* 와 *Treponema denticola* 로부터 분비된 것을 사용하였다.

또한 두 치주병원균으로부터의 트립신 유사 효소의 억제효과를 보기위해

BANA 실험방법을 사용하였다.

결과: 느릅나무 추출물과 프로시아니딘 올리고머는 치은 열구액 내의 매트릭스 메탈로프로테아제 (주로 MMP-8, MMP-9), 치주인대 세포로부터의 MMP-2 의 전구체와 활성체, *Porphyromonas gingivalis* 와 *Treponema denticola* 로부터 분비된 트립신 유사 효소 모두에게 억제효과를 보였다.

결론: 매트릭스 메탈로프로테아제와 치주병원균의 단백질 분해효소에 대한 억제 작용을 볼 때, 느릅나무 추출물은 치주질환의 가능성 있는 치료제로 고려될 수 있다.

핵심되는 말: 느릅나무 추출물, 프로시아니딘 올리고머, 매트릭스 메탈로프로테아제