Inhibitory Effect of *Astragali* Radix on Matrix Degradation in Human Articular Cartilage

CHOI, SOOIM, SO-RA PARK[1], AND TAE-RYEON HEO*

Department of Biological Engineering, Inha University, Incheon 402-751, Korea
[1]*Department of Physiology, College of Medicine, Inha University, Incheon 402-751, Korea*

Abstract The present study was carried out in order to assess the protective effects of calycosin-7-O-β-D-glucopyranoside, isolated from *Astragali* radix (AR), on hyaluronidase (HAase) and the recombinant human interleukin-1β (IL-1β)-induced matrix degradation in human articular cartilage and chondrocytes. We isolated the active component from the n-butanol soluble fraction of AR (ARBu) as the HAase inhibitor and structurally identified as calycosin-7-O-β-D-glucopyranoside by LC-MS, IR, ^{1}H NMR, and ^{13}C NMR analyses. The IC_{50} of this component on HAase was found to be 3.7 mg/ml by *in vitro* agarose plate assay. The protective effect of ARBu on the matrix gene expression of immortalized chondrocyte cell line C-28/I2 treated with HAase was investigated using a reverse transcription polymerase chain reaction (RT-PCR), and its effect on HAase and IL-1β-induced matrix degradation in human articular cartilage was determined by a staining method and calculating the amount of degraded glycosaminoglycan (GAG) from the cultured media. Pretreatment with calycosin-7-O-β-D-glucopyranoside effectively protected human chondrocytes and articular cartilage from matrix degradation. Therefore, calycosin-7-O-β-D-glucopyranoside from AR appears to be a potential natural ant-inflammatory or antii-osteoarthritis agent and can be effectively used to protect from proteoglycan (PG) degradation.

Key words: Matrix degradation, hyaluronidase, interleukin-1β, *Astragali* radix, calycosin-7-O-β-D-glucopyranoside

Extracellular matrix (ECM) is thought to be involved in cellular activity as a regulatory factor. In addition, ECM may serve as a reservoir of growth factors, thereby modulating cell growth by the interactions between ECM and many growth factors present in ECM [29]. ECM consists primarily of a viscoelastic gel of polyanionic proteoglycans (PGs) immobilized within a dense network of collagen fibrils. Under normal conditions, joint cartilage is maintained on a delicate balance between ECM synthesis and degradation. Osteoarthritis (OA) is a multi-factorial disease characterized by bone remodeling, synovial inflammation, and progressive cartilage degradation that leads to pain and reduced mobility in affected joints [20]. In particular, cartilage degradation is a critical feature of OA and inflammatory joint disease, leading to loss of joint function. Until now, conservative treatment of OA is limited to a few classes of OA drugs, such as paracetamol, nonsteroidal anti-inflammatory drugs (NSAIDs), injectable intra-articular corticosteroids, anti-inflammatory steroids, and hyaluronic acid (HA), which provide general symptomatic relief, and decrease pain and stiffness [7, 9]. However, they are associated with side effects related to the gastrointestinal system, including nausea, vomiting, diarrhea, and drowsiness [8, 23]. Therefore, there exists an urgent need for the development of agents capable of ameliorating the symptoms of OA via modification of the underlying pathological conditions.

Hyaluronidase (HAase) is a mucopolysaccharide-splitting enzyme, which hydrolyses the β-N-acetyl-D-glucosamine (1-4) glycosidic bonds in HA, chondroitin, and chondroitin sulfates [4]. HAase generally exists in an inactive form within subcellular lysosomes and is released in an active form during certain experimental tissue injuries and human diseases, including rheumatoid arthritis. It is well known that cartilage-induced HAase activity is a factor that contributes to cytokine-induced ECM degradation during synovial joint disease, and this reaction, including drastic decreases in PG levels, is induced in the early stages of a cartilage culture [5, 12]. In particular, HA is an abundant component of ECM and is believed to be crucial in tissue remodeling, inflammation, and tumorigenesis. An acute depletion of HA and chondroitin sulfate induced by an intra-articular injection of HAase resulted in a drastic (5–7 fold) increase in synovial permeability [28].

*Corresponding author
Phone: 82-32-860-7511; Fax: 82-32-872-4046;
E-mail: theo@inha.ac.kr

In a recent study, we showed that *Astragali* radix (AR) had the highest inhibitory effect on HAase activity *in vitro* [2]. AR is the root of *Astragalus membranaceus* Bunge, an herbaceous plant that belongs to the Leguminosae family. AR has presently been used as an antiperspirant, diuretic, and tonic in the traditional medicine protocols of many Asian countries. Studies on AR in pharmacology and clinical practice have demonstrated its immunostimulatory, cardiotonic, antioxidative, and anti-inflammatory properties [11, 31]. AR contains various active components, including sucrose, astragaloside, formononetin, calycosin, (3R) 7,2'-dihydroxy-5',6'-dimethoxy-isoflavon-7-*O*-β-D-glucoside, β-sitosterol, palmatic acid, isorhamnetin, quercetin, kaempferol, polyamine, and daucosterol. In particular, flavonoids have been established as one of the most potent beneficial components of AR, because of their various biological effects, including antioxidative effects, immunomodulatory activity, and even anticancer activity [13, 14].

Accordingly, this study was undertaken to ascertain the effects of AR on matrix degradation in chondrocyte cells and cartilage explants. The results showed that AR and calycosin-7-*O*-β-D-glucopyranoside isolated from AR had a strong protective effect on matrix degradation, which was attributed to its inhibitory activity towards HAase as well as cytokines, such as IL-1β, in human chondrocytes and articular cartilage.

MATERIALS AND METHODS

Extraction and Fractionation

Dried AR was purchased from the Kyung-dong herbal market (Seoul, Korea), and 15 kg were then crushed and extracted four times with 95% ethanol at room temperature using a shaking incubator for 1 week. To obtain ethanol extracts of AR, the filtered supernatant was evaporated under a vacuum at 50°C. The crude ethanol extracts (267 g) were then suspended in water and successively partitioned with organic solvents of different polarities to afford n-hexane, chloroform, ethyl acetate, n-butanol, and aqueous fractions.

Isolation of Calycosin-7-*O*-β-D-glucopyranoside

The n-butanol fraction (ARBu, 3.8 g) was subjected to column chromatography on a silica gel using a step-wise gradient solvent system of ethyl acetate containing increasing amounts of methanol (8:2, 6:4, 4:6, 2:8, and 0:1, 800 ml each) to give six subfractions (AE-1, 2, 3, 4, 5, and 6, 240 ml each). Subfraction AE-4 (1.3 g) was then subjected again to C18 column chromatography (Alltech Co., Deerfield, IL, U.S.A.), which was eluted with water containing increasing amounts of methanol (10:1, 5: 1, 1:1, and 0:1) to give four subfractions (AE-4A, B, C, and D). Subfraction AE-4C (148 mg) was further purified using a preparative

HPLC (JAI LC-908, Japan Analytical Industry, Tokyo, Japan) with a JAIGEL GS 310 column (5 μm, 20×500 mm I.D.), which was eluted with 85% methanol to afford five compounds (AE-4C-I, CII, CIII, CIV, and CV). The active component, AE-4C-IV, obtained from the subfraction AE-4C (148 mg) was analyzed by HPLC using a Hypersil ODS column (5 μm, 4.6×250 mm I.D., Phenomenex, CA, U.S.A.). The mobile phase composed of 85% methanol at a flow rate of 0.5 ml/min was used to elute, and the effluent was monitored using an absorbance detector. Each sample was prepared by evaporation or lyphilization to remove the residual solvent, and identification of the active components was carried out, using LC-ESI MS/MS, IR, ^{1}H NMR, and ^{13}C NMR spectra.

Hyaluronidase Inhibitory Assay

HAase activity was determined by the agarose plate method previously described by Richman and Baer [25]. In brief, a plate consisting of 1 mg/ml HA dispersed in 1.5% (w/v) agarose gel was buffered with 0.05 M sodium citrate (pH 5.3, buffer A) containing 0.15 M NaCl and 0.02% (w/v) sodium azide. A stock solution of 3% agarose in buffer A was stored in 9.5 ml aliquots. A stock solution of 2 mg/ml HA in buffer A was prepared by stirring the powder into the buffer overnight at 4°C. A 9.5 ml aliquot of 3% agarose was melted in a boiling water bath, cooled to 60°C, and added to 9.5 ml of 2 mg/ml HA in a 60°C bath with constant stirring. The solution was stirred for approximately 1 min in order to ensure complete mixing, and was then rapidly poured into a 9×9 cm plastic dish on a level surface. After the gel had set, cylindrical holes (2 mm in diameter) were punched into the plate and emptied by suction. Five μl of bovine HAase (5,000 U/ml) was mixed with 5 μl of the test samples (10 mg/ml), and the mixture was then preincubated for 20 min at 37°C. The holes were filled with each treatment group, and then incubated for 20 h at 37°C. After incubation, the gel was covered with 10% (w/v in water) cetylpyridinium chloride as soon as the circles became distinct, and the diameters were measured using calipers and read against a dark background. The means for duplicate determinations of the zone diameter (in millimeters) were then plotted against the logarithm of the HAase activities.

Chondrocyte and Cartilage Explant Cultures

The immortalized human chondrocytes used in this study, the C-28/I2 cell line, were the kind gift of Dr. Mary Golding, at Harvard University. The cultures were maintained in a 5% CO_2 incubator at 37°C in DMEM supplemented with 10% FBS, and continuously passaged at subconfluency. Prior to incubations, the chondrocytes were resuspended in DMEM supplemented with 10% FBS and seeded at a density of 2×10^4 cells/well in 6-well plates for cell proliferation. The human articular cartilage was obtained

from patients over 60 years of age undergoing knee replacement surgery for a knee fracture or OA. The macroscopically healthy-appearing condyle cartilage was dissected under sterile conditions. The cartilage samples were then cut into uniformly sized portions (1–1.5 mm thick) that included superficial, middle, and deep layers and plated into 24-well culture dishes, based on approximately 15–20 mg (wet weight) pieces. Thereafter, the pieces were incubated in DMEM containing 10% FBS, 1% L-glutamine, and penicillin/streptomycin (150 U/ml, 50 mg/ml each) and equilibrated for at least 3 days prior to treatment. After 5 days of culture, the explants were fixed in freshly prepared phosphate-buffered 4% paraformaldehyde. The fixed cartilage samples were then routinely embedded in paraffin and cut into 5 μm-thick frontal sections that included all three layers of cartilage. Thereafter, the sections were stained with the cartilage-specific stains, including hematoxylin, safranin O, and alcian blue, for histopathologic analysis. The culture medium was collected and stored at −20°C to measure the total PG content released into the medium. Each treatment was performed in triplicate.

Determination of Viability of Cell

The cell viability and cytotoxicity were assessed by the trypan blue dye exclusion method. Briefly, C28/I2 chondrocytes were cultured in the presence of 100 to 400 μg/ml ARBu. On Day 10, each sample group was stained with 0.4% trypan blue, and at least 100 cells were counted. The number of viable cells was calculated as percentage of the total cell population.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis

Cells were harvested, and total RNA was obtained using Trizol reagent (Invitrogen, Rockville, MD, U.S.A.). The reverse transcription for the first strand cDNA synthesis was performed using a Superscript preamplification system (Roche, Indianapolis, IN, U.S.A.) according to the manufacturer's instructions. PCR analyses for collagen type II, aggrecan, and the house-keeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), were conducted under the following conditions using a Thermo cycler. After an initial denaturation for 2 min at 95°C, DNA was amplified using 36 cycles of 30 sec at 95°C for denaturation, 30 sec at 58°C for annealing, and 2 min at 72°C for extension of the primers, plus a final extension for 5 min at 72°C, followed by cooling to 4°C. The primers employed were: (antisense) 5'-TCCTTT GGGTTTGCAACGGATTGT-3' and (sense) 5'-CTGGCTCCC AACACTGCCAACGT C-3' for type II collagen, (antisense) 5'-GAGGTGGTAATTG CAGGGAACA-3' and (sense) 5'-TGAGGAGGGCTGG-AACAGTACC-3' for aggrecan, and (sense) 5'-GCTCTC CAGAACATCATCCCTGCC-3' and (antisense) 5'-CGT-TGTCATACCAGGAAATGAGCTT-3' for GAPDH [18].

The PCR products were resolved on a 1.8% agarose gel in a 1× Tris-acetate-EDTA buffer and visualized by ethidium bromide staining and UV transillumination. The relative levels of the PCR products were quantified by densitometric analysis. A cDNA amplification for GAPDH was performed to ensure that an equal amount of RNA had been utilized in the RT-PCR reaction. The results are expressed as the substrate ratio for the GAPDH signal.

Inhibitory Effect on Matrix Degradation in Cartilage Explant

The cartilage was fed with serum-free DMEM for 24 h and then stimulated with or without HAase and sample supplementation in serum-free DMEM containing penicillin/streptomycin. The media were changed with fresh treatment media every day, and the aggrecan degradation products were assessed on Day 5. The culture media were also collected and stored at −20°C to measure the total PG content released into the media. In addition, the cartilage explants were incubated for 48 h in DMEM with IL-1β (20 ng/ml) in the absence or presence of the test samples to determine the inhibitory effect on PG degradation. After 2 days of culture, the total amount of glycosaminogly cans (GAGs) released into each culture medium was measured. Each treatment was performed in triplicate.

Proteoglycan Assay

The amount of PG was measured, based on the 1,9-dimethyl-methylene blue (DMB) method, using a commercially available Blyscan™ kit (Biocolor Ltd, Belfast, N. Ireland) according to the manufacturer's instructions [1]. The concentrations of PG were calculated by comparing the values obtained with those for standard solutions of chondroitin sulfate (1–100 μg/ml of shark cartilage, Sigma, MO, U.S.A.). The standard values were 0, 3.13, 6.25, 12.5, 25, and 50 μg, and the amount of PG in the medium was extrapolated using a plotted graph of these values.

Statistical Analysis

All results were expressed as means±S.D. The statistical analyses were conducted based on student's *t*-test using Sigma Plot® software (SPSS Inc., U.S.A.). Statistical significance was assumed at a level of 0.05.

RESULTS

Isolation and Identification of Calycosin-7-*O*-β-D-glucopyranoside

Through repeated chromatography, the sample was purified and AE-4C-IV, calycosin-7-*O*-β-D-gluco-pyranoside (Fig. 1), identified as the active component by LS-ESI MS, ESI-MS/MS, FT-IR, and ^{1}H/^{13}C-NMR, and the spectroscopic data
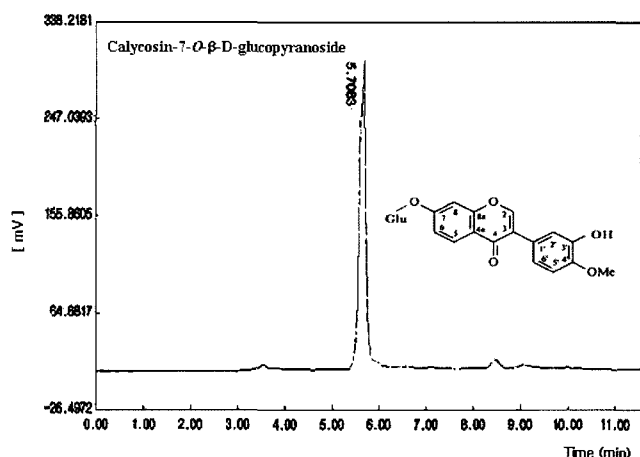
Fig. 1. HPLC chromatogram of calycosin-7-*O*-β-D-glucopyranoside isolated from ARBu.

The active component was analyzed by HPLC using a Hypersil ODS column (5 μm, 4.6×250 mm I.D.). The mobile phase consisted of 85% methanol (in water) at a flow rate of 0.5 ml/min.
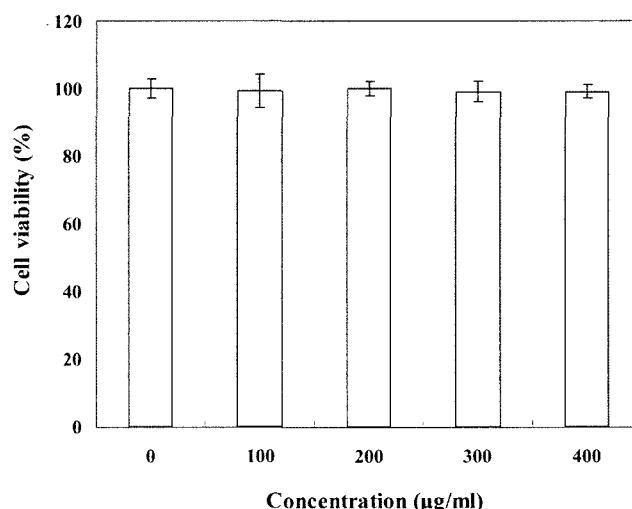

Fig. 3. Cytotoxic effects of ARBu on C28/I2 chondrocyte cells. C28/I2 cells were treated with indicated concentrations of ARBu for 10 days at intervals of 2 days and the cell growth was assessed.

On the 10th day, the treated C28/I2 cells were reacted with 0.4% tryphan blue, and viable cells were counted. Results shown are mean±S.E. (n=3).

were compared with existing data. Calycosin-7-*O*-β-D-glucopyranoside: colorless powder; IR ν_{max}: 1,286, 1,380–1,624, 1,730, 3,422 cm^{-1}; ^{1}H NMR (400 MHz, CD$_3$OD) δ ppm: 3.15–3.75 (6H), 3.98 (3H, s, OMe), 5.15 (1H, anomeric), 6.97 (2H, H-5', H-6'), 7.01 (1H, s, H-2'), 7.23(1H, H-6), 7.67 (1H, d, H-8), 8.15 (1H, d, H-5), 8.23 (1H, s, H-2) ; ^{13}C NMR (400 MHz, CD$_3$OD) δ ppm: 168.1 (C-4), 163.5 (C-7), 159.2 (C-9), 155.3 (C-2), 149.2 (C-4'), 147.5 (C-3'), 128.6 (C-5), 126.0 (C-1'), 121.5 (C-6'), 120.2 (C-10), 117.3 (C-6), 117.0 (C-2'), 112.6 (C-5'), 104.9 (C-8), 101.8 (C-1"), 78.4 (C-5"), 77.5 (C-3"), 76.7 (C-2"), 71.0

(C-4"), 61.0 (C-6"), 50.4 (OMe); ESI MS *m/z* 447 [M$^+$+H]; ESI MS/MS *m/z* 285 (C$_{22}$H$_{22}$O$_{10}$).

Inhibitory Effect of Calycosin-7-*O*-β-D-glucopyranoside on HAase Activity *In Vitro*

HA and its binding protein interact with one another and form a polymeric network that is crucial in tissue remodeling, inflammation, and tumorigenesis. The HAase
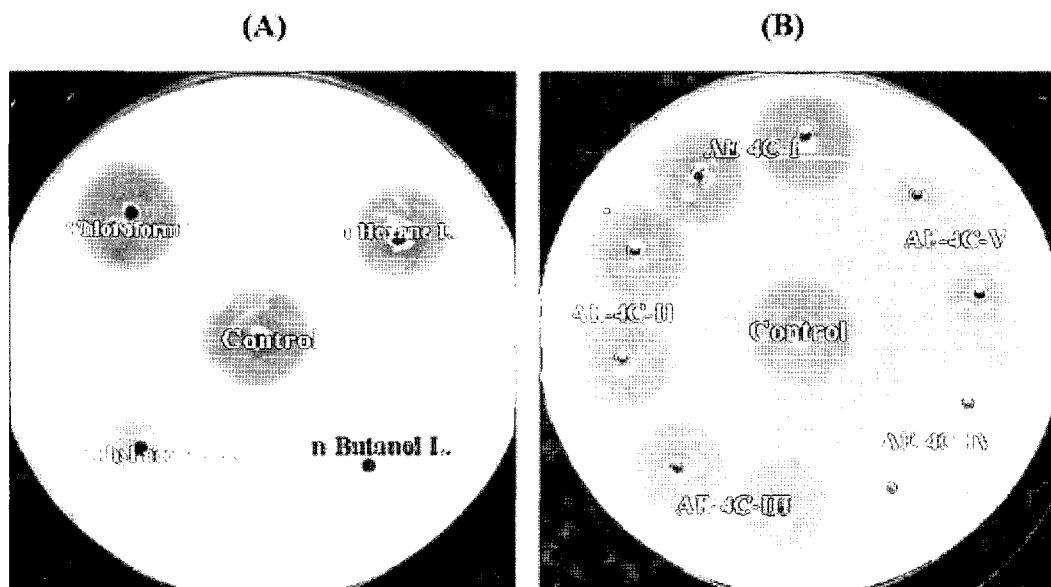
(A)   **(B)**

Fig. 2. HAase inhibitory effects of several fractions from AR ethanol extract, measured by the agarose plate assay *in vitro*.

Samples were each solvent fraction (A) and the peaks isolated from AE-4C (B) by preparative HPLC. The test concentration of each fraction was 10 mg/ml. Commercial HAase (bovine testicular) was treated with 5,000 U/ml.

inhibitory activity of 4 fractions isolated from the ethanol extract of AR was screened by a agarose plate assay. As shown in Fig. 2, the treated group of HAase (5,000 U/ml) showed larger and more visible degradation zones (22 mm) around the wells. However, the ARBu fraction (A) and calycosin-7-O-β-D-glucopyranoside (AE-4C-IV, 10 mg/ml) (B) had the strongest inhibitory effect on HAase with no clear zone, and the ethyl acetate fraction also showed a strong inhibitory activity. The IC_{50} on the HAase activity of calycosin-7-O-β-D-glucopyranoside was 3.7 mg/ml.

Cytotoxic Effect of ARBu on C28/I2 Chondrocytes

The effect of ARBu on toxicity and proliferation was measured in chondrocyte cell cultures and tissue explants using the trypan blue exclusion method and specific staining method, respectively. As shown in Fig. 3, no significant effect on the morphology and viability of chondrocytes was observed with an ARBu concentration of up to 0.4 mg/ml, compared to the control.
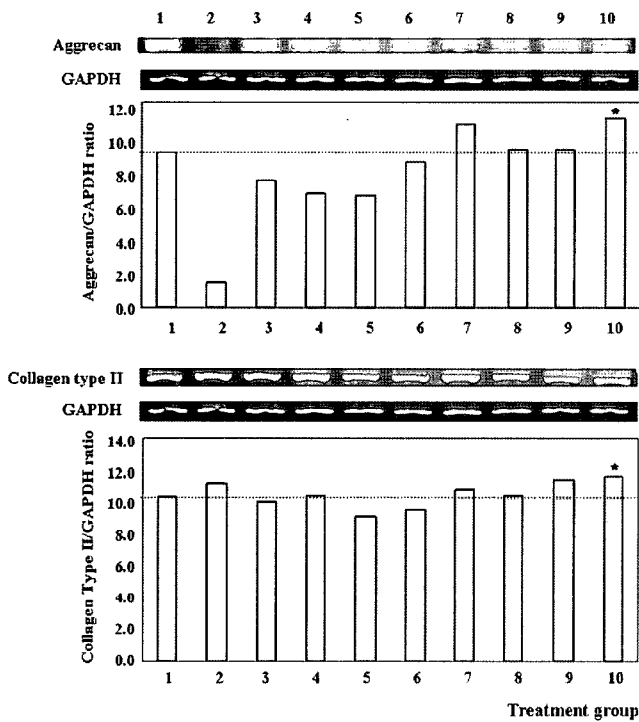

Fig. 4. Representative mRNA of aggrecan and collagen type II expression in HAase-stimulated immortalized chondrocyte cell line C-28/I2 and densitometric analyses.

The total RNA was isolated from each well and used for RT-PCR to detect aggrecan and collagen type II expression. Line 1: control, only supplied with DMEM; Line 2: treated with HAase (100 U/ml); Lines 3, 5, 7, and 9: treated with n-hexane, CHCl$_3$, EtOAc, and n-BuOH, respectively; Lines 4, 6, 8, and 10: treated with HAase (100 U/ml) and each fractionate, respectively. The concentration of each solvent fraction used was 100 μg/ml. The expression of GAPDH was used as the control, and densitometric analyses show the matrix gene ratio of GAPDH.

Effect of ARBu on the Matrix Gene Expression of HAase Stimulated Chondrocytes

We also assessed the effect of ARBu on the matrix gene expression of HAase-treated chondrocyte cell cultures using RT-PCR. As shown in Fig. 4, the matrix expression of aggrecan mRNA was significantly decreased only in the HAase-treated group (Line 2), whereas pretreatment with ARBu maintained aggrecan expression (Lines 9, 10). However, the level of type II collagen mRNA was not influenced by the treatment with HAase (Line 2).

Effect of ARBu on PG Degradation in HAase Stimulated Cartilage Explant

The degeneration of articular cartilage is a key feature of OA, and a low PG content is one of the hallmarks of OA cartilage degeneration [6]. The matrix-specific substrates aggrecan and type II collagen are degraded by proteolytic enzymes; therefore, the enzymatic destruction of aggrecan is an early sign of arthritis. The n-butanol soluble fraction (100 μg/ml) reduced the GAG contents released from the cartilage explant to 9.26±1.51 μg ($P<$ 0.05), when compared with the HAase-treated group (13.82±1.29 μg) ($P<0.05$) (data not shown). To assess the inhibitory effect of ARBu relative to its concentration, the HAase was pretreated with different concentrations of ARBu in the cartilage explants for 5 days (Fig. 5). When compared with the control and ARBu-treated groups (5.0±0.24 μg and 5.1±0.25 μg, respectively), HAase treatment
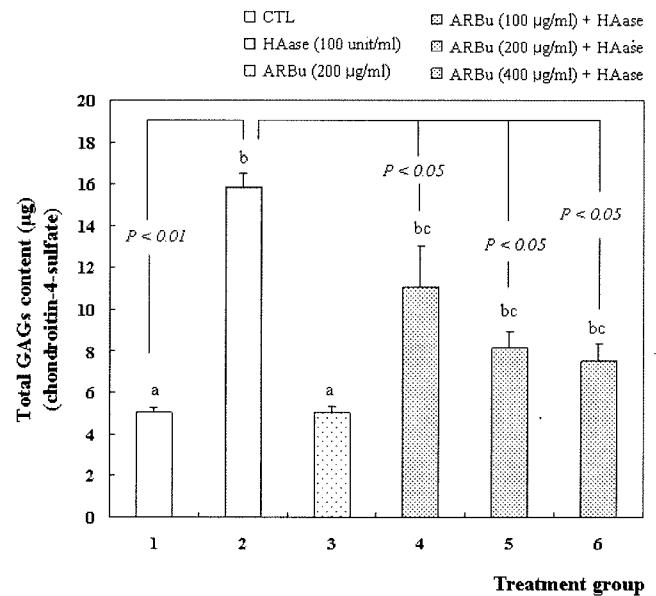

Fig. 5. Inhibitory effect of different concentrations of ARBu on PG degradation in HAase-stimulated cartilage explants.

Total PG concentration in the cartilage culture media was measured on the 5th day using a Blyscan^{TM} kit. The standard calibration curve was prepared with chondroitin-4-sulfate. Results represent mean±S.D. (n=3). P value was compared with the control and HAase-treated groups.

alone produced a significant increase to 15.8±0.68 μg ($P<0.05$), whereas pretreatment with ARBu (100, 200, and 400 μg/ml) and HAase reduced the GAG release to 11.0±1.97, 8.1±0.81, and 7.5±0.80 μg, respectively ($P<0.05$).

Effect of Calycosin-7-*O*-β-D-glucopyranoside on PG Degradation in Cartilage Explant

To investigate the inhibitory effect of the active component on PG degradation, HAase in the cartilage explants was treated with calycosin-7-*O*-β-D-glucopyranoside for 5 days. After HAase treatment, the area of safranin O staining, which indicates metachromasia, had clearly decreased

(Fig. 6A-2); nevertheless, no significant changes of cell density or cellular structure occurred in the cartilage culture. However, calycosin-7-*O*-β-D-glucopyranoside was found to inhibit HAase-induced PG degradation (Fig. 6A-4). Compared to the control and calycosin-7-*O*-β-D-glucopyranoside treated groups (Fig. 6B-1, 3), HAase treatment (Fig. 6B-2) resulted in a dramatic decrease of PG in alcian blue staining; however, the calycosin-7-*O*-β-D-glucopyranoside plus HAase-treated group showed no decease in PG (Fig. 6B-4). Furthermore, the total amount of GAG released into the culture medium from cartilage explants by HAase or calycosin-7-*O*-β-D-glucopyranoside treatment was measured on the 5th day (data not shown): HAase treatment only increased significantly the GAG release to 15.2±0.86 μg, compared with the control levels (5.2±0.21 μg), whereas pretreatment with calycosin-7-*O*-β-D-glucopyranoside (50 μg/ml) reduced the GAG release to 10.26±0.56 μg ($P<0.05$). The group treated with only calycosin-7-*O*-β-D-glucopyranoside (5.3±0.35 μg) exhibited levels similar to the control group ($P<0.05$).

Effect of Calycosin-7-*O*-β-D-glucopyranoside on PG Degradation in IL-1-β-Stimulated Cartilage Explant

The effect of calycosin-7-*O*-β-D-glucopyranoside on cytokine-mediated PG degradation was evaluated as an another

1. Control
2. HAase (100 U/ml)
3. Calycosin-7-O-β-D-glucopyaranoside (50 μg/ml)
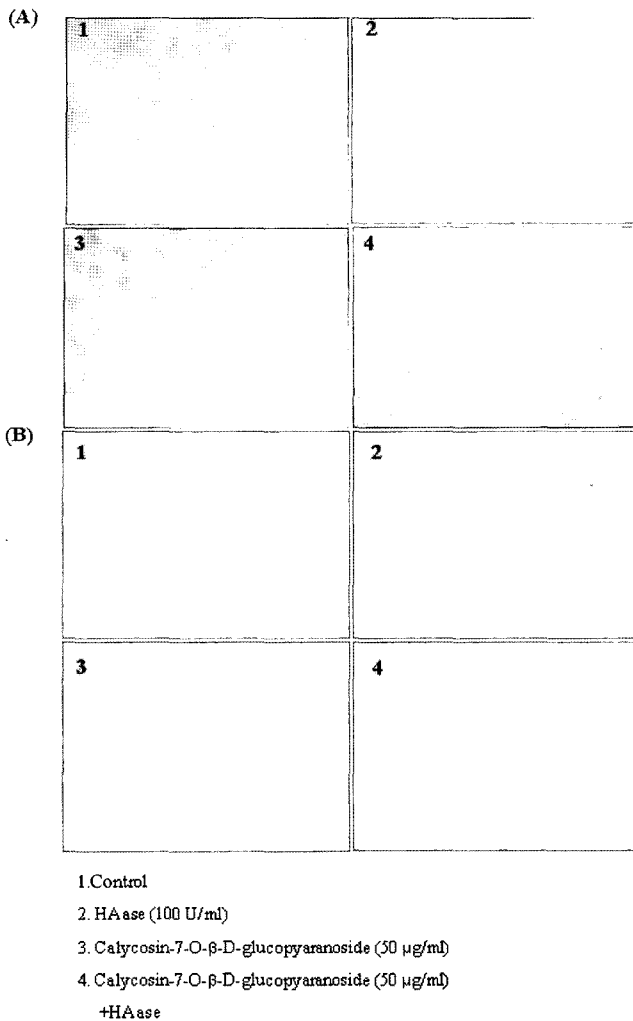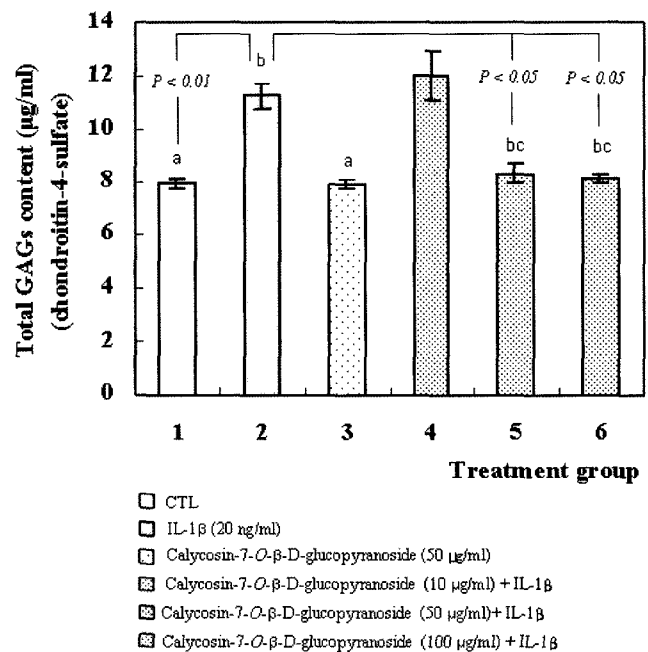4. Calycosin-7-O-β-D-glucopyaranoside (50 μg/ml) +HAase

Fig. 6. Photograph of safranin O (A) and alcian blue (B) staining for representative sections of HAase-stimulated articular cartilage.

Each section was harvested on Day 5. (A): the control group, which was treated with DMEM only; (B): HAase-treated group (100 U/ml); (C): Calycosin-7-*O*-β-D-glucopyranoside (50 μg/ml)-treated group; (D): Calycosin-7-*O*-β-D-glucopyranoside and HAase-treated group. Compared with the control, HAase treatment resulted in a dramatic decrease in PG, shown by staining (original magnification×100).

☐ CTL
☐ IL-1β (20 ng/ml)
☐ Calycosin-7-*O*-β-D-glucopyranoside (50 μg/ml)
☒ Calycosin-7-*O*-β-D-glucopyranoside (10 μg/ml) + IL-1β
☒ Calycosin-7-*O*-β-D-glucopyranoside (50 μg/ml) + IL-1β
☒ Calycosin-7-*O*-β-D-glucopyranoside (100 μg/ml) + IL-1β

Fig. 7. Inhibitory effect of calycosin-7-*O*-β-D-glucopyranoside at different concentrations on PG degradation in IL-1β-stimulated cartilage explants.

The total PG concentration in each section was measured on Day 5 in the cartilage culture media. The control was only supplied with DMEM without any other treatment. The standard calibration curve was calculated using chondroitin-4-sulfate. Results represent the mean±S.D. (n=3).

possible action mechanism. Thus, IL-1β (20 ng/ml) was applied to the cartilage explants, and the total amount of GAG released into the culture medium was measured by the method mentioned above. As shown in Fig. 7, the total amount of degraded GAG increased significantly in the group treated with only IL-1β (11.3±0.53 μg), compared with the control (7.9±0.18 μg). However, calycosin-7-O-β-D-glucopyranoside plus IL-1β treatment inhibited the degradation of GAG by 50 μg/ml (8.3±0.36 μg) and 100 μg/ml (8.1±0.15 μg), respectively.

DISCUSSION

The cartilage-induced HAase activity is a factor that contributes to cytokine-induced ECM degradation during synovial joint disease, and this reaction, including drastic decreases of PG levels, is induced in the early stages of a cartilage culture. In particular, HA and chondroitin sulfate are degraded and acutely depleted by an intra-articular HAase injection [25, 19]. HA and its binding protein interact with one another and form a polymeric network that is crucial in tissue remodeling, inflammation, and tumorigenesis. HA injection is an alternative method for the treatment of OA symptoms, which is currently one of the most popular OA therapies. HA is a precursor of aggrecan synthesized in the articular cartilage and a major component of synovial fluid. Furthermore, HA suppresses the release of PG from ECM within cartilaginous tissue, thereby protecting the surface of the joint cartilage and further preventing cartilage deterioration [10, 27, 30]. Therefore, the inhibition of HAase activity represents a strategy for preventing or even reversing cartilage degradation in patients with OA. Furthermore, HAase treatment has been recently shown to result in a drastic decrease of PG levels, whereas collagenase treatment produces more subtle changes in the intensity of PG staining in the early stage of cartilage culture [11], suggesting that HAase inhibitor was able to enhance the protective response of matrix substrates in chondrocyte or cartilage explants. In the present study, we used an ARBu fraction showing the strongest inhibitory effect on HAase.

We assessed the inhibitory effect of ARBu and calycosin-7-O-β-D-glucopyranoside against HAase in vitro, and the results showed a strong inhibitory effect on HAase. The explant of the group pretreated with HAase and ARBu or calycosin-7-O-β-D-glucopyranoside maintained aggrecan production levels, compared with the HAase-treated group, and the protective effect on the degradation of PG in cell and cartilage explant culture was identified. However, the level of type II collagen mRNA was not influenced by treatment with HAase (Fig. 4, Line 2), concordant with

results from previous research [3, 21] and supports the idea that aggrecan may protect collagen from degradation by interfering with the ability of collagenase to cleave the collagen fibrils in situ within the cartilage matrix. We also investigated the effect of calycosin-7-O-β-D-glucopyranoside on IL-1β-mediated PG degradation, since IL-1β and TNF-α have been identified as cytokines that induce matrix metalloproteinases (MMP) expression in chondrocytes, and subsequent destruction of the cartilage matrix [22]. They are responsible for enhancement of PG degradation and upregulation of MMPs synthesis. In the present study, pretreatment with calycosin-7-O-β-D-glucopyranoside (50 μg/ml) effectively protected the PG degradation of IL-1β (20 ng/ml)-treated human articular cartilage.

The effectiveness and safety of these preparations have been well established, and several natural remedies have been employed to treat OA. Furthermore, the use of such botanicals or nutraceuticals creates multiple downstream events or synergic effects, thereby providing higher efficacy, minimal toxicity, and a wide therapeutic window for effective intervention [15, 16, 24, 32]. Indeed, AR extracts have been found to restore depressed macrophage function [26], enhance cytokine production [9], and modulate immune function [17]. Consequently, two isoflavone glycosides (7,2'-dihydroxy-3' and 4'-dimethoxyisoflavan-7-O-β-D-glycoside) and calycosin-7-O-β-D-glucopyranoside have already been isolated from AR and shown to have strong inhibitory effects on COX-2, an enzyme that exasperates an inflammatory reaction [11]. However, this is the first reported to show that ARBu and calycosin-7-O-β-D-glucopyranoside from AR prevent the matrix substrate degradation induced by inflammatory enzymes or cytokines.

In conclusion, none of the treatment groups revealed any effect on the chondrocyte morphology and viability, or any significant cell death when compared with the control. This suggests that AR, as an HAase or IL-1β inhibitor, was able to enhance protective response of the matrix substrates in chondrocytes and cartilage explants, and possibly had a favorable chondro-protective effect. However, because of complex mechanisms involved in OA, the precise mechanism of calycosin-7-O-β-D-glucopyranoside in relation to matrix degradation still needs to be clarified. Therefore, the action mechanism of the crude extract of AR and isoflavone glycosides of AR, including calycosin-7-O-β-D-glucopyranoside, should be investigated in further studies. Furthermore, the action mechanism of ARBu and active components on MMPs and aggrecanase expression should also be elucidated. At present, the study to identify the effect of calycosin-7-O-β-D-glucopyranoside in OA, particularly in in vivo test using animal model, is in progress, and the results will soon be published.

Acknowledgments

REFERENCES

1. Choi, J. H., J. H. Choi, D. Y. Kim, J. H. Yoon, H. Y. Youn, J. B. Yi, H. I. Rhee, K. H. Ryu, K. Jung, C. K. Han, W. J. Kwak, and Y. B. Cho. 2002. Effects of SKI 306X, a new herbal agent, on proteoglycan degradation in cartilage explant culture and collagenase-induced rabbit osteoarthritis model. *Osteoarthritis Cartilage* **10:** 471–478.

2. Choi, S. I., Y. M. Lee, and T. R. Heo. 2003. Screening of hyaluronidase inhibitory and free radical scavenging activity *in vitro* of traditional herbal medicine extracts. *Kor. J. Biotechnol. Bioeng.* **18:** 282–288.

3. Ellis, A. J., V. A. Curry, E. K. Powell, and T. E. Cawston. 1994. The prevention of collagen breakdown in bovine nasal cartilage by TIMP, TIMP-2 and a low molecular weight synthetic inhibitor. *Biochem. Biophys. Res. Commun.* **201:** 94–101.

4. Feost, G. I., T. Csóka, and R. Stern. 1996. The hyaluronidase: A chemical, biological and clinical overview. *Trends Glycosci. Glycotech.* **8:** 419–434.

5. Flannery, C. R., C. B. Little, C. E. Hughes, and B. Caterson. 1998. Expression and activity of articular cartilage hyaluronidases. *Biochem. Biophys. Res. Commun.* **251:** 824–829.

6. Ghosh, P. and M. Smith. 2002. Osteoarthritis, genetic and molecular mechanisms. *Biogerontology* **3:** 85–88.

7. Hochberg, M. C., R. D. Altman, K. D. Brandt, B. M. Clark, P. A. Dieppe, M. R. Griffin, R. W. Moskowitz, and T. J. Schnitzer. 1995. Guidelines for the medical management of osteoarthritis. Part II. Osteoarthritis of the knee. *Arthritis Rheum.* **38:** 1541–1546.

8. Hochberg, M. C. and M. Dougados. 2001. Pharmacological therapy of osteoarthritis. *Best Pract. Res. Cl. Rh.* **15:** 583–593.

9. Jin, R., L. L. Wan, T. Mitsuishi, S. Sato, Y. Akuzawa, K. Kodama, and S. Kurashige. 1994. Effect of Shi-ka-ron and Chinese herbs on cytokine production of macrophages in immunocompromised mice. *Am. J. Chin. Med.* **22:** 255–266.

10. Kato, Y., Y. Mukudai, A. Okimura, A. Shimazu, and S. Nakamura. 1995. Effects of hyaluronic acid on the release of cartilage matrix proteoglycan and fibronectin from the cell matrix of chondrocyte cultures: Interactions between hyaluronic acid and chondroitin sulfate glycosaminoglycan. *J. Rheumatol. Suppl.* **43:** 158–159.

11. Kim, E. J., O. J. Oh, S. K. Lee, and K. S. Yang. 2001. Inhibitory effect of *Astragali* radix on COX-2 activity. *Kor. J. Pharmacogn.* **32:** 311–315.

12. Kim, H. A., D. I. Suh, and Y. W. Song. 2001. Relationship between chondrocyte apoptosis and matrix depletion in human articular cartilage. *J. Rheumatol.* **28:** 2038–2045.

13. Kim, J. S. and C. S. Kim. 1997. A study on the constituents from the roots of *Astragalus membranaceus* Bunge (α). *Kor. J. Pharmacogn.* **28:** 75–79.

14. Kim, J. S., Y. T. Kim, and C. S. Kim. 2002. A study on the constituents from the roots of *Astragalus membranaceus* Bunge (β). *Kor. J. Pharmacogn.* **31:** 109–111.

15. Lee, H. S., S. Y. Kim, C. H. Lee, and Y. J. Ahn. 2004. Cytotoxic and mutagenic effects of *Cinnamomum cassia* bark-derived materials. *J. Microbiol. Biotechnol.* **14:** 1176–1181.

16. Lee, H. S. 2003. Inhibitory effects of quinizarin isolated from *Cassia tora* seeds against human intestinal bacteria and aflatoxin B1 biotransformation. *J. Microbiol. Biotechnol.* **13:** 529–536

17. Lee, Y. S., O. K. Han, C. W. Park, S. I. Suh, S. W. Shin, C. H. Yang, T. W. Jeon, E. S. Lee, K. J. Kim, S. H. Kim, W. K. Yoo, and H. J. Kim. 2003. Immunomodulatory effects of aqueous-extracted *Astragali* radix in methotrexate-treated mouse spleen cells. *J. Ethonopharmacol.* **84:** 193–198.

18. Lum, Z. P., B. E. Hakala, J. S. Mort, and A. D. Recklies. 1996. Modulation of the catabolic effects of interleukin-1β on human articular chondrocytes by transforming growth factor-β. *J. Cell. Physiol.* **166:** 351–359.

19. Menzel, E. J. and C. Farr. 1998. Hyaluronidase and its substrate hyaluronic acid: Biochemistry, biological activities and therapeutic uses. *Cancer Lett.* **131:** 3–11.

20. Noe, M. C., S. L. Snow, L. A. Wolf-Gouveia, P. G. Mitchell, L. Lopresti-Morrow, L. M. Reeves, S. A. Yocum, J. L. Liras, and M. Vaughn. 2004. 3-Hydroxy-4-arylsulfonyltetrahydropyranyl-3-hydroxamic acids are novel inhibitors of MMP-13 and aggrecanase. *Bioorg. Med. Chem. Lett.* **14:** 4727–4730.

21. Pratta, M. A., W. Yao, C. Decicco, M. D. Tortorella, R. Q. Liu, R. A. Copeland, R. Magolda, R. C. Newton, J. M. Trzaskos, and E. C. Arner. 2003. Aggrecan protects cartilage collagen from proteolytic cleavage. *J. Biol. Chem.* **278:** 45539–45545.

22. Pujol, J. P. and G. Loyau. 1987. Interleukin-1 and osteoarthritis. *Life Sci.* **41:** 1187–1198.

23. Raskin, J. B. 1999. Gastrointestinal effects of nonsteroidal anti-inflammatory therapy. *Am. J. Med.* **106:** 3S–12S.

24. Rhee, S. J., C. Y. Lee, M. R. Kim, and C. H. Lee. 2004. Potential antioxidant peptides in rice wine. *J. Microbiol. Biotechnol.* **14:** 715–721.

25. Richman, P. G., and H. Baer. 1980. A convenient plate assay for the quantitation of hyaluronidase in hymenoptera venoms. *Anal. Biochem.* **109:** 376–381.

26. Rittenhaus, J. R., P. D. Lui, and B. H. Lau. 1991. Chinese medicinal herbs reverse macrophage suppression induced by urological tumors. *J. Urol.* **146:** 486–490.

27. Shimazu, A., A. Jikko, M. Iwamoto, T. Koike, W. Yan, and Y. Okada. 1993. Effects of hyaluronic acid on the release of proteoglycan from the cell matrix in rabbit chondrocyte

cultures in the presence and absence of cytokines. *Arthritis Rheum.* **36:** 247–253.

28. Scott, D., P. J. Coleman, R. M. Mason, and J. R. Levick. 1997. Glycosaminoglycan depletion greatly raises the hydraulic permeability of rabbit joint synovial lining. *Exp. Physiol.* **82:** 603–606.

29. Scully, S. P., J. W. Lee, M.A. Ghert, and W. Qi. 2001. The role of the extracellular matrix in articular chondrocyte regulation. *Clin. Orthop.* **391S:** S72–S89.

30. Sztrolovics, R., A. D. Recklies, P. J. Roughley, and J. S. Mort. 2002. Hyaluronate degradation as an alternative mechanism for proteoglycan release from cartilage during interleukin-1 beta-stimulated catabolism. *J. Biochem.* **362:** 473–479.

31. Zang, Y. D., Y. L. Wang, J. P. Shen, and D. X. Li. 1984. Hypotensive and anti-inflammatory effects of *Astragalus* saponin 1. *Acta Pharm. Sin.* **19:** 333–337.

32. Zheng, H. Z., Y. W. Kim, H. J. Lee, R. D. Park, W. J. Jung, Y. C. Kim, S. H. Lee, T. H. Kim, and K. Y. Kim. 2004. Quantitative change of PR proteins and antioxidative enzymes in response to *Glomus intraradices* and *Phytophthora capsici* in pepper (*Capsicum annuum* L.) plants. *J. Microbiol. Biotechnol.* **14:** 553–562.