Effects of Saponins from the Root Bark of *Aralia elata* on the Transport of Chondroitin Sulfate in Caco-2 Cell Monolayers and Rats

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We investigated the intestinal absorption enhancing effect of the saponins from the root bark of *Aralia elata* (SRBAE) in Caco-2 cell monolayers and rats. SRBAE at concentrations of 0.04% and 0.08% (w/v) decreased the transepithelial electrical resistance (TEER) values and increased the paracellular uptake of chondroitin sulfates (CSs) having different molecular weights (MW 500, 4500, and 18000) in a dose-dependent manner. We also evaluated the cytotoxicity of SRBAE to determine its proper concentration as an absorption enhancer. MTT assay and trypan blue exclusion test indicated that the cytotoxicity of SRBAE at concentrations of 0.04% and 0.08% was negligible. CS (MW 18000) was orally administered with or without SRBAE to rats. The oral administration of SRBAE (250 mg/kg) in 1 h increased the intestinal absorption of CS, by 4.9-fold versus the control (CS alone). Histological examination of the gastrointestinal tissues showed that SRBAE did not cause any damage to tissues. In conclusion, our results suggest that SRBAE acts as an efficient absorption enhancer and makes it easier for hydrophilic molecules to penetrate the intestinal epithelium.

Key words: *Aralia elata*; saponin; Caco-2 cell monolayer; absorption enhancer; chondroitin sulfate

The transport of molecules across the intestinal epithelium occurs by passive diffusion through transcellular or paracellular routes, and through carrier-mediated active or facilitated transport.1 Among these, the paracellular route is a dominant pathway for the passive transepithelial transport of hydrophilic molecules in the small intestine.2 Many hydrophilic drugs are not absorbed by the intestinal epithelium because of the presence of junctional complexes2,3) and their physicochemical characteristics such as hydrophilicity and molecular weight. Among the many approaches used to increase the intestinal absorption of hydrophilic drugs, one involves the use of absorption enhancers, such as surfactants, fatty acids, medium-chain glycerides, steroidal detergents, acylcarbinol, alkaloycholines, N-acetylated α-amino acids, N-acetylated non-α-amino acids, mucosadhesive polymers, and secretory transport inhibitors.4 In the present study, we evaluated the saponins from the root bark of *Aralia elata* (SRBAE) as absorption enhancers.

The root bark of *Aralia elata* Seem. (Araliaceae) has long been used to cure cough,5) cancer,5) diabetes,5—7) gastric ulcer,7,8) hepatitis,8) and rheumatoid arthritis9) in the Korean traditional folk medicines. Chemically, many saponins have been isolated from this root bark.9) Generally, they consist of triterpenoid or steroid aglycones linked to one or more sugar side chains. Most saponins are highly surface-active and form addition-complexes with sterols, including those associated with the plasma membranes of fungi, plants and animal cells.10) Moreover, these saponin properties can affect the integrity of biological membranes. Previous researchers have shown that saponins from different plants can facilitate the uptake of molecules not normally absorbed. Soyasaponins stimulate the uptake of [125I]glycinin into rabbit jejunal mucosa in vitro,11) and *Gypsophila* and *Quinoa* saponins increase the uptake of [3H]polyethylene glycol 4000 in isolated rat jejunum.16,18) Avenacosides A and B from oat saponins enhance the uptake of n-[14C]mannitol, [35Cr]EDTA, and ovalbumin in isolated rat jejunum.19) Moreover, *Quillaja* saponin increases the transepithelial flux of fluorescein,20) and a derivative of this saponin stimulates the permeability of n-[3H]decapeptide and [14C]mannitol through Caco-2 cell monolayers.21) These findings suggest that saponins from plants can be used as absorption enhancers by affecting transport mechanisms in the intestinal epithelium. We have tested several kinds of saponins from *Aralia elata*, *Panax ginseng* (ginsenoside Re, Rc, and Rb 1), *Phytolacca americana* (phytolacoside B, D2, E, F, G, and I), and *Kalopanax pictus*. Among these, SRBAE showed the best ability to reduce TEER values and to enhance hydrophilic macromolecular uptake without inducing severe cytotoxicity in Caco-2 cell monolayers. For these reasons, we have investigated the absorption enhancing activity of SRBAE to increase the permeability of hydrophilic molecules.

Chondroitin sulfate (CS), one of hydrophilic substances, is a glycosaminoglycan composed of an alternating sequence of sulfated and/or unsulfated residues of D-glucuronic (GlcA) and N-acetyl-D-galactosamine (GalNAc) linked by β(1→3) and β(1→4) bonds. Several reports have shown that oral administration of CS is beneficial in the treatment of knee arthritis.23—26) Hence, the number of pharmaceuticals and nutraceuticals containing CS has markedly increased. Nevertheless, a major problem in the successful clinical use of CS is its poor intestinal absorption, resulting from its high molecular weight, charge density, and hydrophilicity.27,28) Thus, the enhancement of intestinal absorption of CS is important in its successful application.

In this paper, in vitro transport experiments were performed on Caco-2 cell monolayers to evaluate the correlation between the molecular weight and transport rate of CS with or without SRBAE. Intact CS (MW 18,000), low molecular weight CS (LMWCS: MW 4500), and CS disaccharides (MW 500) were used as hydrophilic markers. In addition, the absorption enhancing efficacy and the cytotoxicity of

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SRBAE were evaluated in rats.

MATERIALS AND METHODS

Materials CS, originating from the bovine trachea, was provided by New Zealand Pharmaceuticals (Palmerston North, New Zealand). Caco-2 cells were purchased from American Type Culture Collection (Rockville, MD, U.S.A.) and 1,9-dimethyl-methylene blue (DMMB) was from Aldrich (Milwaukee, WI, U.S.A.). Dulbecco’s modified Eagle’s medium (DMEM), Dulbecco’s phosphate buffered saline (DPBS), non-essential amino acids (NEAA), 0.25% of trypsin and 1 mM of EDTA-4Na (trypsin-EDTA), collagen type I, penicillin–streptomycin (10,000 units/ml and 10 mg/ml in 0.9% sodium chloride, respectively), N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid] (HEPES), Hank’s balanced salt solution (HBSS), phenol–chloroform–isoamyl alcohol (25:24:1), chloroform, sodium citrate, chondroitinase ABC from Proteus vulgaris, CS disaccharides (α-ΔUA-[1→3]-GalNAc: ΔDi-0S, α-ΔUA-[1→3]-GalNAc-6S: ΔDi-6S, α-ΔUA-[1→3]-GalNAc-4S: ΔDi-4S), 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT), and Duolite® GT-73 resin were purchased from Sigma (St. Louis, MO, U.S.A.). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT, U.S.A.) and other reagents and chemicals were of the best grade available. Araloside A as a HPLC standard sample was isolated according to a previous report.13)

Extraction and Fractionation The roots of Aralia elata were collected in the plant garden cultivated in Natural Products Research Institute, Seoul National University and a voucher specimen (No. 001005) was maintained.

The dried root barks (1.32 kg) were cut into pieces and re-fluxed twice with 70% methanol solution for 4 h. The solution was filtered, evaporated under vacuum, and then lyophilized to give a powder (360 g). The extract was dissolved in 10% methanol solution and partitioned in succession with n-hexane, chloroform, ethyl acetate, and n-butanol to give a hexane soluble fraction (20.4 g, 1.55%), a chloroform soluble fraction (14.4 g, 1.09%), an ethyl acetate soluble fraction (18.0 g, 1.36%), a butanol (BuOH) soluble fraction (213.6 g, 16.2%), and finally a water soluble fraction (72 g, 5.46%).

Preparation of SRBAE BuOH fraction samples (50 mg) were dissolved in 2 ml of water and applied to Sep-Pak C₁₈ cartridges (Waters, Milford, MA, U.S.A.) pre-washed with methanol (2 ml) and water (5 ml). After washing the column with water (10 ml) and 30% methanol (15 ml), the samples were eluted with methanol (5 ml). The solvent was dried by using the speed-vacuum dryer (N-Biotec, Korea).

Preparation of CS Disaccharides and LMWCs CS (2 g) dissolved in 33 ml of 50 mM sodium phosphate buffer (pH 7.0) was treated with chondroitinase ABC (2 U/17 ml) in the same buffer at 37°C for 48 h. After heating for 5 min, the digestion mixture was freeze-dried and size-fractionated on a Bio-Gel P-10 column (2.5×110 cm) equilibrated with 0.1 M NaCl. The fractions were collected and desalted by Bio-Gel P-2 chromatography and freeze-dried. Their homogeneity was examined by strong anion-exchange HPLC (SAX-HPLC). LMWCs was obtained by using controlled chemical digestion process induced by free radicals from hydrogen peroxide according to a previous report with some modifications.29,30) Briefly, CS (1 g) and copper acetate monohydrate (0.04 g) were dissolved with 10 ml of water in a reaction vessel. The temperature was kept at 60°C and the pH was adjusted to 7.5 during the experiment. A 9% hydrogen peroxide solution was added at a rate of 1.8 ml/h and the reaction was lasted for 180 min. The residual contaminating copper from these products was removed by cation-exchange chromatography using Duolite® GT-73 resin. The pH of fractions was adjusted to 6.0 and then freeze-dried. Dried samples were desalted by Sephadex G-10 gel-permeation chromatography. The average molecular weight and the structure of LMWCs were analyzed by high-performance size-exclusion chromatography (HPSEC) and 1H-NMR spectroscopy, respectively as previously described.29)

Caco-2 Cell Culture Caco-2 cells were maintained in culture medium (DMEM supplemented with 1% NEAA, 10% FBS, and 1% penicillin–streptomycin, pH 7.4) at 37°C in 5% CO₂ and at 90% relative humidity. The cells were harvested with trypsin-EDTA, resuspended in culture medium, and seeded at a density of 3.0×10⁵ cells/ml on collagen coated Transwell® polycarbonate filters (3 µm in pore size, 1 cm² in surface area) from Costar (Cambridge, MA, U.S.A.). The culture medium was changed every other day for 3 weeks. All cells used in the transport experiment were in the range of 30—40 passages and their TEER values were measured using a Milicell® electrical resistance system (Milipore, Bedford, MA, U.S.A.) in the range of 500—550 Ω cm².

In Vitro Transport Experiment Using Caco-2 Cell Monolayers This experiment was performed as previously described.31) To evaluate the effect of SRBAE on the transport of CSs (MW 500, 4500, and 18000), the monolayers were treated with SRBAE (0%, 0.04%, and 0.08%) and incubated for 30 min. After removing SRBAE, CS samples (1 mg/0.5 ml in transport medium) were added to the monolayers. The transport medium (Hank’s balanced salt solution containing 11 mM Na-glucose and 25 mM HEPES) was taken from the basolateral sides of the transwell hourly for 3 h. At each sampling, the inserts were rapidly transferred to another well containing 1.5 ml of the fresh transport medium. The amount of CS disaccharides in collected medium was analyzed by SAX-HPLC and determined by its calibration curve. Intact CS and LMWCs transported across Caco-2 cell monolayers were quantitatively analyzed by DMBB assay.32)

Cytotoxicity of SRBAE in Caco-2 Cell Monolayers Cytotoxicity of SRBAE in Caco-2 cell monolayers was measured at different concentrations (0%, 0.04%, and 0.08%) and evaluated by MTT assay33) and trypan blue exclusion test.34)

Intestinal Absorption of Intact CS in Rats Male Sprague-Dawley rats (250 g) were fasted for 12 h before the oral administration of each intact CS (200 mg/kg) solution containing 50 mg/kg, 125 mg/kg, and 250 mg/kg of SRBAE. After the oral administration, blood (450 µl) was taken from the capillary in the retro-orbital venous plexus at each time (0.5, 1, 2, and 3 h) and immediately mixed with 50 µl of 3.8% sodium citrate. Plasma was prepared by centrifugation at 2500 g for 15 min at 4°C and stored at −80°C until analyzed.

Quantitative Analysis of Absorbed CS in Rat Plasma
One hundred microliters of plasma was added to 100 μl of 50 mM Tris–HCl buffer (pH 8.0) containing 1% actinase (Kaken Pharmaceutical Co., Tokyo, Japan) and incubated at 45 °C for 20 h. The solution was extracted twice with 200 μl of phenol–chloroform–isoamyl alcohol (25 : 24 : 1), and once with 200 μl of chloroform. CS was then precipitated by adding 50 μl of 7 M ammonium acetate and 800 μl of cold ethanol. This solution was centrifuged at 15000 g for 15 min at 4 °C and the ethanol was removed. The dried precipitate obtained was dissolved in 20 μl of water and the CS level in plasma was quantified by the DMMB assay.

**Histological Evaluation of Gastrointestinal Tissues from Rats** After the *in vivo*, transport experiment gastric and intestinal tissues were removed from the rats and fixed in 10% formalin. The tissues were histologically processed as previously described and examined under an optical microscope after staining paraffin-embedded samples with hematoxylin and eosin (H&E).

**Statistical Analysis** Data were analyzed using SPSS software (version 10.0) for Windows, by one-way analysis of variance (ANOVA) with Duncan’s multiple range t-test. Results are expressed as means±standard deviation and were considered significantly at *p* < 0.05.

**RESULTS**

**Preparation of SRBAE** To remove sugars in the butanol extract, Sep-Pak C18 cartridges were used. SRBAE bound to cartridges were eluted with 100% methanol and their yields were 25.30±0.71%. HPLC analysis showed that SRBAE contains 15.83% of araloside A and other saponins (data not shown).

**Preparation of CS Disaccharides and LMWCS** Intact CS was digested with chondroitinase ABC and CS disaccharides from the digestion mixture were isolated (Fig. 1A). F-2 (Fig. 1A) was characterized by using SAX-HPLC, showing that three types of CS disaccharides (ΔDi-0S, ΔDi-6S, ΔDi-4S) were present in this fraction (Fig. 1B). F-1 contained CS oligosaccharides and corresponded to a very minor fraction of the product. The average MW of LMWCS, obtained by the chemical digestion, was determined as 4500 Da by HPSEC and 1H-NMR results showed the same major repeating structure in intact CS and LMWCS.

**Effects of SRBAE on TEER Values** Because TEER is believed to be well correlated with change in paracellular permeability of cell monolayers, the effects of SRBAE on the TEER values across Caco-2 cell monolayers were monitored as a function of time. In the presence of 0.04% and 0.08% SRBAE, the TEER values decreased to 52% and 32% of those of the control and slightly recovered following the removal of SRBAE after the incubation for 30 min (Fig. 2).

**In Vitro Transport Experiment** The cumulative trans-
port of intact CS, LMWCS, and CS disaccharides across Caco-2 cell monolayers was monitored hourly for 3 h (Fig. 3). In the absence of SRBAE, intact CS was not transported through the monolayers; however, transport of intact CS was increased by 0.04% and 0.08% SRBAE, resulting in 0.90±0.32% and 3.74±0.43%, respectively. SRBAE (0.04% and 0.08%) also increased the transport of LMWCS by 1.95- to 4.88-fold and that of CS disaccharides by 1.97- to 6.72-fold versus the control (without SRBAE), respectively (Table 1).

### Cytotoxicity of SRBAE in Caco-2 Cell Monolayers

MTT assay and trypan blue exclusion test were performed to evaluate the cytotoxicity of SRBAE. Mitochondrial dehydrogenase (MDH) activity in the presence of 0.04% SRBAE was similar to that of the negative control (no enhancer). However, 0.08% SRBAE and 0.1% SDS (positive control) decreased MDH activity, to approximately 78% and 11% of the control value, respectively (Fig. 4). By trypan blue exclusion test, 0.1% SDS produced an effect that was approximately 14 times greater than that of the negative control. While 0.04% SRBAE did not affect the trypan blue uptake into the cells, 0.08% SRBAE increased the cellular uptake of trypan blue approximately twice as high as that of the negative control. Although the increased uptake of trypan blue into the cells indicates their plasma membrane damage, the value was approximately 5 times lower than that of the positive control (Fig. 4).

### Effects on SRBAE on the Intestinal Absorption of CS in Rats

The concentrations of CS in rat plasma are shown in Fig. 5. The oral administration of intact CS (200 mg/kg) with SRBAE (50 mg/kg) did not affect the CS level in rat plasma. But SRBAE (125 mg/kg) with intact CS (200 mg/kg) increased the plasma CS level in 2 h by 1.87±0.15 μg/ml (3.3-fold) compared to the control (0.57±0.06 μg/ml). Furthermore, the combination of CS (200 mg/kg) and SRBAE (250 mg/kg) increased the plasma CS level in 1 h by 2.81±0.76 μg/ml (4.9-fold) versus the control, indicating that the intestinal absorption of CS is enhanced by treatment with SRBAE at these concentrations. The area under CS (200 mg/kg) in rat plasma vs. time curve from 0 to 3 h (AUC<sub>0–3h</sub>) in the presence of 125 mg/kg SRBAE was 2.71±0.71 μg·h/ml and in the presence of 250 mg/kg SRBAE was 4.11±1.11 μg·h/ml. The oral administration of CS (200 mg/kg) with SRBAE (50 mg/kg) did not affect AUC<sub>0–3h</sub>.

### Histological Evaluation of Gastrointestinal Tissues

We studied the effects of SRBAE on GI tissues by H&E staining. The morphology of GI tissues, including villi fusion, occasional epithelial cell shedding, and congestion of mucosal capillary, was not visibly affected by the oral administration of SRBAE. In addition, no inflammatory symptoms were detected in the SRBAE treated group (250 mg/kg) (Fig. 6).

### DISCUSSION

In this study, SRBAE increased the transport of CSs both in vitro and in vivo (Figs. 3, 5). Although 0.08% SRBAE reduced MDH activity and increased trypan blue uptake compared to the control in vitro, its cytotoxicity in Caco-2 cells was not severe (Fig. 4). Furthermore, SRBAE did not induce any observable morphological damage on GI tissues in vivo (Fig. 6). These results suggest that the ability of SRBAE to increase CS permeability is not a direct result of its toxicity. In previous reports, saponins have been found to affect the cell membrane at different concentrations, incubation times, and mechanisms, according to their sources and structures. *Gypsophila* saponins act mainly at the level of the plasma membrane and mechanisms, according to their sources and structures.
low concentrations. In many cases, the criterion between rapid depolarization of the small intestinal mucosa at very low concentrations that are not unduly cytotoxic. It seemed that SRBAE allowed the transport of smaller molecules, like CS disaccharides (MW 500), more easily than that of bigger molecules, such as CS oligosaccharide (MW 4500) and intact CS (MW 18000).

Previous studies showed a predominant hypothesis as possible mechanisms that paracellular permeability is modulated via phospholipase-C (PLC) mediated signal transduction. Because of the prominent role played by the intracellular Ca$^{2+}$ in the regulation of tight junctional permeability, it is possible that the change in intracellular Ca$^{2+}$ level may indeed occur in the epithelial cells that have been exposed by saponins. Changes in the intracellular Ca$^{2+}$ level may be due either to passive leakage of extracellular Ca$^{2+}$ through the ubiquitous Ca$^{2+}$ channels and/or leaky plasma membranes or to stimulated release from intracellular stores by inositol 1,4,5-triphosphate (IP$_3$). PLC cleaves phosphatidylinositol 4,5-bisphosphate to form IP$_3$ and diacylglycerol (DAG). IP$_3$ causes Ca$^{2+}$ release from the endoplasmic reticulum and increased intracellular DAG and Ca$^{2+}$ or DAG alone activate the protein kinase C (PKC).

Moreover a rise in the intracellular Ca$^{2+}$ level, activates calmodulin-dependent kinase. PKC and calmodulin-dependent kinase phosphorylate myosin light chain and thereby induces the phosphorylation of myosin light chain, followed by the contraction of the perijunctional actin-myosin ring, which has been reported to open the paracellular route. In other experiments, the action mechanisms of saponins (phytolaccoside B, D$_2$, E, F, G, and I) from Phytolacca americana were investigated through the effects of enzyme inhibitors on TEER values at non-toxic concentrations. Among these, phytolaccoside D$_2$, F, and I showed the absorption enhancing activities through the PLC dependent pathway (unpublished results).

Although a detailed explanation of the TEER reduction, the increase in CS uptake, and the mechanism of action of SRBAE are unclear, it is believed that subtle changes in the cell membrane caused by cytoskeletal changes result in the TEER reduction and enhanced permeability to CS. In conclusion, the use of SRBAE as a pharmaceutical and/or nutraceutical additive with CS can be useful to enhance the efficacy of CS through the increase of intestinal absorption.

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