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RESEARCH NOTE

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Inhibitory Effects of Naringenin and Its Novel Derivatives on Hyaluronidase

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Abstract Naringenin is a bioactive flavanone containing antioxidative, anti-inflammatory, and anticarcinogenic properties. The inhibitory effects on hyaluronidase of naringenin and its novel derivatives were evaluated. Among these flavonoids at 200 μM concentration, 7-*O*-butyl naringenin had the highest inhibitory effect on hyaluronidase with 44.84%. In addition, For naringenin at concentrations of 0, 150, and 190 μM, the apparent Michaelis constants ($_{app}K_m$) were calculated to be 0.60±0.02, 0.43±0.02, and 0.41±0.01 mg/mL of substrate, respectively; for 7-*O*-butyl naringenin at 0, 20, and 30 μM concentrations, those were 0.44±0.03 and 0.27±0.03 mg/mL, respectively. The V_{max} values at 150 and 190 μM naringenin were 0.59±0.02 and 0.56±0.01 mg/mL/min, respectively; and those at 20 and 30 μM 7-*O*-butyl naringenin were 0.50±0.02 and 0.33±0.02 mg/mL/min, respectively. However, the slopes of each inhibitory reaction were not significantly different. Therefore, naringenin and 7-*O*-butyl naringenin were shown to be uncompetitive inhibitors. These results demonstrate the potential use of 7-*O*-butyl naringenin as an anti-inflammatory substance.

Keywords: flavonoid, naringenin, 7-*O*-butyl naringenin, hyaluronidase, anti-inflammatory effect, Lineweaver-Burk plot

Introduction

Flavonoids comprise a large group of naturally occurring compounds widely distributed in the plant kingdom, and found in a variety of food products such as fruits, vegetables, nuts, seeds, and beverages. Several studies have addressed the antioxidative, tissue-protective, and tumoristatic effects of flavonoids (1-4). Flavonoids have also been shown to have anti-inflammatory activities (5-7). Flavonoid structure consists of flavonols, flavones, catechins, anthocyanidines, isoflavones, dihydroflavonols, and chalcones.

Naringenin is abundant in citrus fruits such as grapefruits (*Citrus paradise*) and oranges (*Citrus sinensis*). The role of naringenin and the related citrus flavone, hesperetin, in the prevention and treatment of disease has attracted considerable attention, especially as anticancer and anti-atherogenic compounds (8,9).

Hyaluronidase, an enzyme that depolymerizes the polysaccharide hyaluronic acid in the extracellular matrix of connective tissue, is found both in organs (testis, spleen, skin, eye, liver, kidney, uterus, and placenta) and in body fluids (tears, blood, and sperm) (10).

This enzyme is known to be involved in allergic reactions (11), cancer metastasis (12), inflammation, petechial hemorrhages following its injection in mesentery preparations, and also an increase in the permeability of the vascular system (13). This information suggests that potent hyaluronidase inhibitors might have anti-allergic and anticancer activities, and could become important compounds for the development of new drugs. In particular, some researchers have reported on inhibitory effects of some natural compounds against hyaluronidase such as *Areca catechu* L. extract (14), brown algal phlorotannins (15), and some herbs (16).

The microplate methods have been used for various assays such as the inhibitory effect of flavonoids on the growth of *Helicobacter pylori*, due to its advantages in terms of analyzing many samples at one time, requiring small sample volumes, taking less time, and having lower costs than traditional methods.

Therefore, the objective of this study was to evaluate the inhibitory effects of naringenin and 4 newly derived flavonoids [7-*O*-*tert*-butoxycarbonylmethyl naringenin, 7-*O*-butyl naringenin, 7-*O*-(a-methoxycarbonyl)benzyl naringenin and 7-*O*-(BnO-L-Leu-carbonylmethyl) naringenin] on hyaluronidase using the microplate method. This will provide important information relevant to the pharmaceutical industry for the development of new anti-inflammatory drugs.

Materials and Methods

Chemicals *p*-Dimethylaminobenzaldehyde, sodium hyaluronate, *N*-acetyl glucosamine, and bovine hyaluronidase were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Flavonoids Five kinds of flavonoids were used for this study; naringenin (Sigma-Aldrich), 7-*O*-*tert*-butoxycarbonyl-methyl naringenin, 7-*O*-butyl naringenin, 7-*O*-(a-methoxy-

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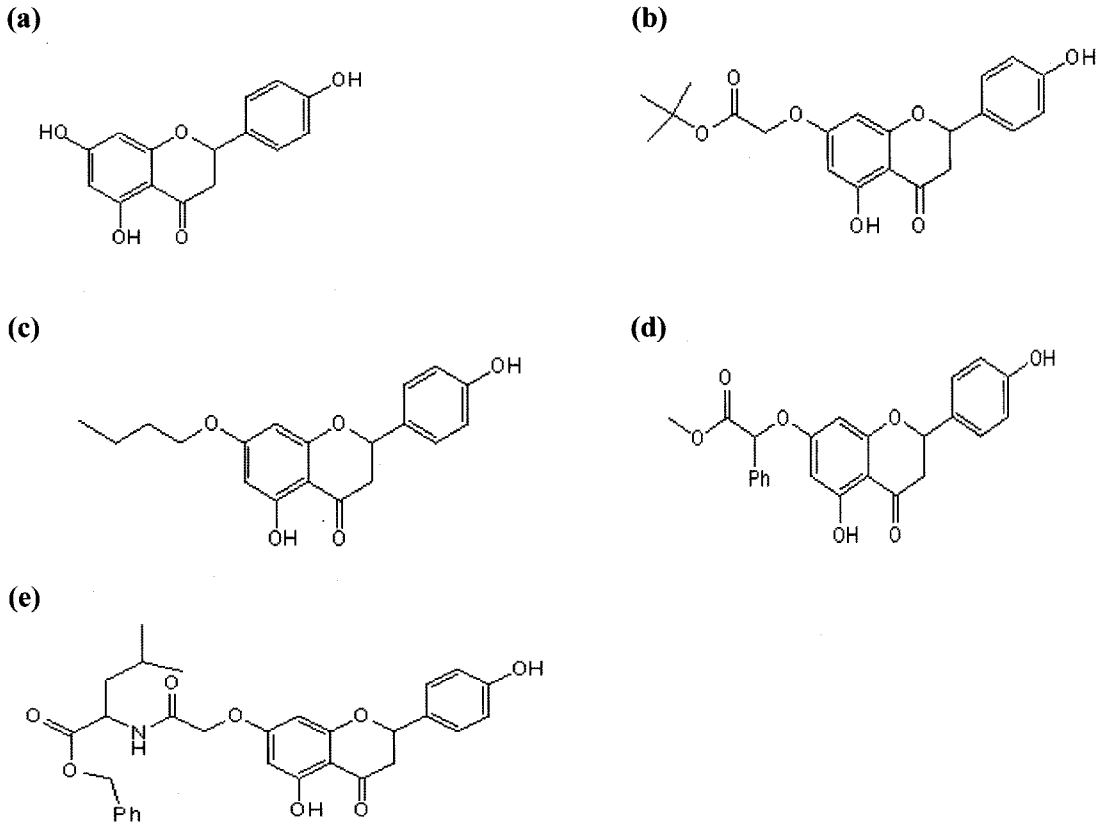

Fig. 1. Chemical structure of flavonoids. (a) Narigenin, (b) 7-*O*-*tert*-butoxycarbonylmethyl naringenin, (c) 7-*O*-butyl naringenin, (d) 7-*O*-(a-methoxycarbonyl)benzyl naringenin, and (e) 7-*O*-(BnO-L-Leu-carbonylmethyl) naringenin.

carbonyl)benzyl naringenin and 7-*O*-(BnO-L-Leu-carbonylmethyl) naringenin (supplied from the Prof. Yong-Sun Park of Chemistry Department, Konkuk University, Seoul, Korea) (Fig. 1). These flavonoids were dissolved in methanol as a stock solution for further studies.

Determination of hyaluronidase inhibition Hyaluronidase activity was determined by measuring the amount of *N*-acetyl-glucosamine formed from sodium hyaluronate with a spectrophotometer (17). One-hundred μL of bovine hyaluronidase (7,420 units/mL, Sigma-Aldrich) dissolved in 0.1 M acetate buffer (pH 3.5) was mixed with 40 μL of a designated concentration of flavonoid sample dissolved in methanol (the final concentration of flavonoids in reaction were controlled to 200 μM). The mixture was then incubated in a water bath at 37°C for 20 min. The samples were treated with 100 μL of 12.5 mM calcium chloride, and then incubated in a water bath at 37°C for 20 min. This Ca^{2+}-activated hyaluronidase was treated with 500 μL of sodium hyaluronidase (5 mg/mL) dissolved in 0.1 M acetate buffer (pH 3.5), and then incubated in a boiling water for 3 min. After cooling to room temperature, 3 mL of *p*-dimethyl-aminobenzaldehyde solution (4 g of *p*-dimethyl-aminobenzaldehyde dissolved in 350 mL of 100% acetic acid and 50 mL of 10 N hydrochloric acid) was added to the reaction mixture. The optical density of the reaction mixture was measured at 570 nm using a microplate reader (Molecular Device, Sunnyvale, CA, USA). All the analytical data in the figures and tables are the means of 3 determinations. The inhibitory effect was expressed as follows;

$$\text{Inhibitory effect (\%)} = [(\text{Absorbance of control} - \text{Absorbance of sample})/\text{Absorbance of sample}] \times 100$$

Kinetic analysis of hyaluronidase with naringenin and 7-*O*-butyl naringenin The inhibition patterns of naringenin and 7-*O*-butyl naringenin were determined under the condition as follows; The concentrations of naringenin and 7-*O*-butyl naringenin were controlled in 20 and 30 μM reaction solution to 150 and 190 μM, respectively. The concentrations of hyaluronidase were controlled to 0.2, 0.4, 0.8, 1.2, 1.6, and 2.0 mg/mL in a reaction solution. The conditions and assay methods for kinetics of hyaluronidase with naringenin and 7-*O*-butyl naringenin were as same as mentioned above.

Statistical analysis Analysis of variance was performed for each group of 3 samples using the SAS program. Duncan's test also was used to verify the significance of differences for each treatment.

Results and Discussion

Inhibitory effects on hyaluronidase by naringenin and its synthetic derivatives Hyaluronic acid is the main component of the extracellular matrix and biological fluids in animal tissue (18). It acts as an important regulator in repairing wounds without scarring (19). However, the degradation of hyaluronic acid to smaller molecules results

Table 1. Inhibitory effects on hyaluronidase by naringenin and its synthetic derivatives

Flavonoids[1)]	Inhibition (%)[2)]
Naringenin	9.58±0.25
7-*O*-*Tert*-butoxycarbonylmethyl naringenin	30.68±0.21
7-*O*-Butyl naringenin	44.84±0.28
7-*O*-(a-Methoxycarbonyl)benzyl naringenin	5.80±0.13
7-*O*-(Bn*O*-L-Leu-carbonylmethyl) naringenin	18.72±0.43

[1)]The concentrations of all flavonoids in reaction were controlled to 200 μM.
[2)]Inhibitory effect (%)=[(Absorbance of control−Absorbance of sample)/Absorbance of sample]×100

in an increase in inflammation, angiogenesis, fibrosis, and collagen deposition in the healing of wounds (20). Hyaluronidase is an endo-hexosaminidase which initiates the degradation of high molecular weight hyaluronic acid. In this study, the inhibitory effects of naringenin and the newly derived flavonoids, 7-*O*-*tert*-butoxycarbonylmethyl naringenin, 7-*O*-butyl naringenin, 7-*O*-(a-methoxycarbonyl) benzyl naringenin, and 7-*O*-BnO-L-Leu-carbonylmethyl) naringenin were evaluated toward hyaluronidase using an *in vitro* assay (Table 1). The inhibitory capacity of the 5 flavonoids in decreasing order was as follows:

7-*O*-Butyl naringenin>7-*O*-*tert*-butoxycarbonylmethyl naringenin>7-*O*-(BnO-L-Leu-carbonylmethyl) naringenin >naringenin>7-*O*-(a-methoxycarbonyl)benzyl naringenin.

Specifically, naringenin was shown to have the lowest inhibitory effect with a value of 9.58% inhibition, whereas 7-*O*-butyl naringenin had the highest value with 44.84% inhibition at 200 μM concentration.

Kinetics of inhibitory effects of naringenin and its synthetic derivatives on hyaluronidase activities With regard to tannin, luteolin, apiotenin, kaempferol, and silybin, it is known that the mode of hyaluronidase inhibition is competitive (21). In this study, the mode of inhibition by naringenin and 7-*O*-butyl naringenin was analyzed using Lineweaver-Burk plots of hyaluronidase activity toward hyaluronic acid in the presence of various concentrations of flavonoid (Fig. 2 and Table 2). For naringenin at concentrations of 0, 150, and 190 μM, the apparent Michaelis constants ($_{app}K_m$) were calculated to be 0.60±0.02, 0.43±0.02, and 0.40±0.01 mg/mL of substrate, respectively. For 7-*O*-butyl naringenin at 20 and 30 μM concentrations, the $_{app}K_m$ for substrate were calculated to be 0.44±0.03 and 0.27±0.03 mg/mL, respectively. In addition, the V_{max} values in presence of 150 and 190 μM naringenin

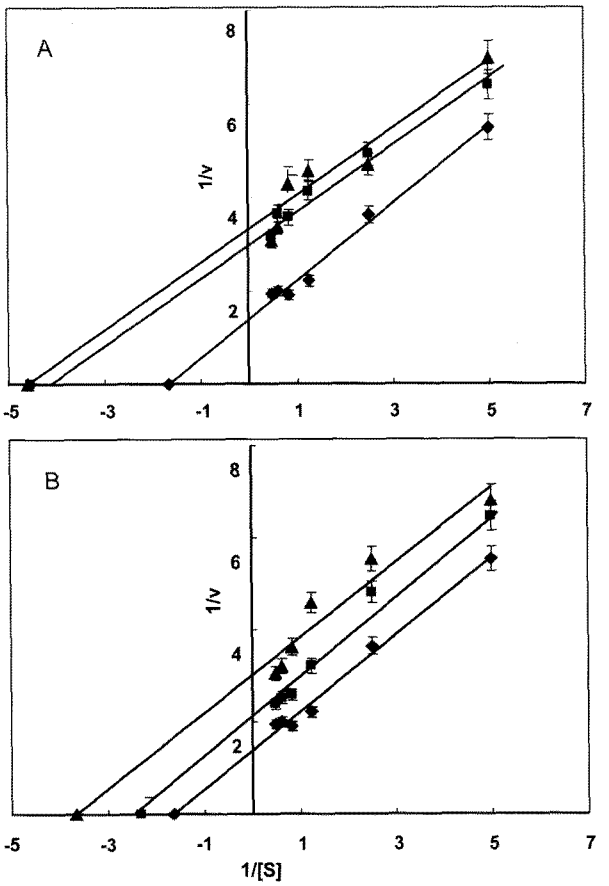

Fig. 2. Lineweaver-Burk plot of hyaluronidase inhibition. A, Naringenin (◆ 0 μM, ■ 150 μM, ▲ 190 μM); B, 7-*O*-butyl naringenin (◆ 0 μM, ■ 20 μM, ▲ 30 μM). The unit of 1/v is mL · min/mg, and [S] is the concentration of hyaluronic acid, mg/mL.

were 0.59±0.02 and 0.56±0.01 mg/mL/min, respectively; and those in presence of 20 and 30 μM 7-*O*-butyl naringenin were 0.50±0.02 and 0.33±0.02 mg/mL/min, respectively. The slopes of each inhibitory reaction were not significantly different. Therefore, naringenin and 7-*O*-butyl naringenin appear to act as uncompetitive inhibitors of hyaluronidase. Furthermore, it appeared that 7-*O*-butyl naringenin is a stronger inhibitor than naringenin. The inhibition patterns observed suggest the modification of a functional group or a conformational change in the enzyme near the catalytic site rather than in the active site, although the precise inhibitory mechanism is still not clear.

In conclusion, these preliminary results confirm the anti-

Table 2. Values of K_m, V_{max}, and slope of Lineweaver-Burk plot of the inhibition on hyaluronidase by naringenin and 7-*O*-butyl naringenin[1)]

	Control	Naringenin		7-*O*-Butyl naringenin	
	0 μM	150 μM	190 μM	20 μM	30 μM
V_{max}(mg/mL/min)	0.72±0.03*	0.59±0.02	0.56±0.01	0.50±0.05	0.33±0.03
$_{app}K_m$ (mg/mL)	0.60±0.02	0.43±0.02	0.40±0.01	0.44±0.03	0.27±0.03
Slope	0.83±0.04	0.87±0.03	0.85±0.02	0.83±0.05	0.82±0.04
R^2 value	0.98±0.01	0.97±0.02	0.93±0.01	0.97±0.01	0.96±0.02

[1)]Values are average±SD of 3 replicates.

inflammatory effects of natural flavonoids and their derivatives. In addition, it is worth noting that the derivatives of flavonoids such as 7-*O*-butyl naringenin may be chemically synthesized from orange or tangerine waste from food processing, thus increasing its industrial value as an antimicrobial agent.

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