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Note

Inhibitive Effects of Alkyl Gallates on Hyaluronidase and Collagenase

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A series of the gallate esters of *n*-alkanols (C_1–C_{12}) was examined to determine their inhibitory activities against hyaluronidase and collagenase. Hexyl, heptyl, octyl, nonyl, and decyl gallates inhibited both hyaluronidase and collagenase, and the most potent inhibitor was octyl gallate against both enzymes. Octyl 3,5-dihydroxybenzoate showed inhibitory effects on hyaluronidase, whereas collagenase was inhibited by octyl 3,4-dihydroxybenzoate.

Key words: alkyl gallates; hyaluronidase inhibitory activity; collagenase inhibitory activity; antioxidant activity

Hyaluronic acid is found in the extracellular matrix of soft connective tissues, such as the umbilical cord, skin, synovial fluid, and vitreous humor. This polysaccharide is involved in cell adhesion and protection, the formation of skin tissue, water retention in tissues, and the maintenance of flexibility in the skin due to its unique hydroscopic, rheologic, and viscoelastic properties.[1)] Hyaluronidase is a mucopolysaccharase that hydrolyzes glycosaminoglycans, including hyaluronic acid, in the extracellular matrix during tissue remodeling.[2)] When the level of hyaluronic acid decreases under conditions in which hyaluronidase activity increases, the moisture and tension of the skin are reduced. Thus hyaluronidase inhibitors are useful cosmeceutical ingredients as they have antiwrinkle and antiaging effects on the skin. In addition, hyaluronidase induces histamine release from mast cells during inflammatory reactions, and some hyaluronidase inhibitors, including disodium cromoglycate, have been used to suppress allergies and inflammation.[3,4)]

Collagen is the major fibrous component of the extracellular matrix in the skin, and the collagen content in the skin decreases greatly during the aging process and due to long-term exposure to ultraviolet radiation.[5,6)] Collagenase (matrix-metalloproteinase, MMP) plays an important role in unbalanced turnover and the rapid breakdown of collagen in human inflamed/ultraviolet-irradiated skin.[7,8)] Hence, collagenase inhibitors, in addition to hyaluronidase inhibitors, have useful functions as cosmetic materials.

Gallic acid (3,4,5-trihydroxybenzoic acid) is a phenolic compound that is found as a constituent of hydrolyzable tannins in many plants. Gallic acid and hydrolyzable tannins have many biological activities, including antioxidation and hyaluronidase inhibition.[9,10)] The gallate esters of *n*-alkanols also act as potent antioxidants, and propyl, octyl, and dodecyl (lauryl) gallates are permitted additives for antioxidation in foods in the United States[11)] although they are not natural compounds. Furthermore, some of these alkyl gallates, including octyl gallate, have been reported to be useful as multifunctional food additives, as they have antibrowning,[12)] antifungal,[13)] and antibacterial[14)] effects. To determine whether alkyl gallates are also useful as cosmeceutical ingredients, in the present study, we have identified their inhibitory activities against hyaluronidase and collagenase.

Methyl gallate, butyl gallate, octyl gallate, and octyl 4-hydroxybenzoate were purchased from Wako Pure Chemical Industries (Osaka, Japan); gallic acid, propyl gallate, and hyaluronidase (from bovine testes) were from Nacalai Tesque (Kyoto, Japan); ethyl gallate, dodecyl gallate, 3,4-dihydroxybenzoic acid, 3,5-dihydroxybenzoic acid, and 3-hydroxybenzoic acid were from Tokyo Chemical Industry (Tokyo); hyaluronic acid sodium salt (from rooster comb) and collagenase (from *Clostridium histolyticum*; type IV) were from Sigma; Pz-peptide (Pz-Pro-Leu-Gly-Pro-D-Arg-OH) was from Bachem (Bubendorf, Switzerland); and recombinant human matrix-metalloproteinase-1 (MMP-1) and Fluorogenic Peptide Substrate IX (Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2) were from R&D systems (Minneapolis, MN).

Hexyl gallate, heptyl gallate, nonyl gallate, decyl gallate, octyl 3,4-dihydroxybenzoate, octyl 3,5-dihydroxybenzoate, and octyl 3-hydroxybenzoate were synthesized as follows: The corresponding phenolic acid (5 g) and *n*-alkanol (50 g) were mixed with H_2SO_4 (approximately 2 ml) and kept at 110 °C for 5 h. After cooling, *tert*-methyl butyl ether (approximately 250 ml) was added to the mixture, and successive washing with saturated aqueous solutions of NaCl and $NaHCO_3$ (5 times each) was done. After removal of the solvent by

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Abbreviations: DPPH, 1,1-diphenyl-2-picrylhydrazyl; DMSO, dimethyl sulfoxide; EC_{50}, 50% effective concentration; IC_{50}, 50% inhibitory concentration; MMP, matrix-metalloproteinase

evaporation, the crude product dissolved in methanol was purified by silica gel column chromatography with a solvent system of chloroform/methanol (98:2).[15] To confirm the structure of the synthesized products, the ^{1}H-NMR spectrum was recorded on a JEOL JNM-AL400 spectrometer (400 MHz) in deuterium methanol and compared with previously reported data.[12,13,15–17]

To evaluate radical scavenging activity against the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, a sample (alkyl gallate) was dissolved in 2 ml of methanol and mixed with 0.5 ml of a DPPH solution (0.4 mg/ml) in methanol. After incubation at room temperature for 30 min in air, the remaining DPPH was determined by measuring the absorbance at 520 nm, and the radical-scavenging activity was estimated as described by Yoshida *et al.*[9]

Hyaluronidase inhibitory activity was examined following Ono *et al.*,[18] with some modifications. Briefly, hyaluronidase (160 units) was incubated with an inhibitor (alkyl gallate) dissolved in 10 µl of 40% dimethyl sulfoxide (DMSO) and 70 µg of compound 48/80 in 0.1 M sodium acetate buffer, pH 4.0, in a total volume of 0.25 ml at 37 °C for 20 min. Then 0.275 mg of hyaluronic acid sodium salt dissolved in 0.25 ml of 0.1 M sodium acetate buffer, pH 4.0, was added to the enzyme solution to start the hyaluronidase reaction, and the mixture was incubated for an additional 40 min.

Collagenase inhibitory activity was determined using the Pz-peptide as a substrate by the method of Hernández *et al.*,[19] with some modifications. The reaction mixture contained 2.5 µg of collagenase from *C. hystolyticum*, an inhibitor (alkyl gallate) dissolved in 25 µl of 10% DMSO, 0.5 mg of Pz-peptide, 20 mM $CaCl_2$, and 0.1 M Tris–HCl buffer, pH 7.1, in a total volume of 0.5 ml. Collagenase inhibitory activity was also evaluated using MMP-1 (mammalian collagenase). The reaction mixture (0.5 ml) contained 10 ng of MMP-1, which had been activated by preincubation with *p*-aminophenylmercuric acetate, an inhibitor (octyl gallate) dissolved in 25 µl of DMSO, 10 µM Fluorogenic Peptide Substrate IX, 10 mM $CaCl_2$, 150 mM NaCl, 0.05% Briji-35, and 50 mM Tris–HCl buffer, pH 7.5, and the enzyme activity was determined by measuring the increase in fluorescence intensity (Ex: 320 nm; Em: 405 nm) at 25 °C.

To determine the antioxidative activity of gallic acid and gallic acid esters of *n*-alkanols with different chain lengths (C_1–C_{12}), the radical scavenging activity, which can be measured on the basis of decolorization following the trapping of unpaired electrons from DPPH, was examined. As shown in Table 1, all of the alkyl gallates examined as well as gallic acid were found to have strong radical scavenging activities against DPPH, and their 50% effective concentration (EC_{50}) values were almost constant (approximately 20 µM) regardless of the alkyl chain length. These results are similar to those of a previous report.[12]

The gallate esters of *n*-alkanols were examined as to their hyaluronidase inhibitory activity, and their 50% inhibitory concentration (IC_{50}) values were compared with that of disodium cromoglycate, a well-known hyaluronidase inhibitor, which is used as an antiinflammation and antiallergic agent[4] (Table 1). Methyl, ethyl, propyl, butyl, and dodecyl gallates as well as gallic acid exhibited virtually no inhibitory activity against hyaluronidase (their IC_{50} values were higher than 1 mM). In contrast, hexyl, heptyl, octyl, nonyl, and decyl gallates exhibited potent hyaluronidase inhibitory activities. It is hence thought that the hyaluronidase inhibitory activity (but not the antioxidant activity) associated with the length of the hydrophobic alkyl chain to a large extent. Octyl gallate was the most potent inhibitor among the alkyl gallates examined, and its IC_{50} value (106 µM) was substantially lower than that of disodium cromoglycate (450 µM). Octyl 3,5-dihydroxybenzoate was a potent inhibitor of hyaluronidase, with an IC_{50} value of 113 µM, comparable to that of octyl gallate; however, other derivatives of octyl benzoate (octyl 3,4-dihydroxybenzozate, octyl 3-hydroxybenzoate, and octyl 4-hydroxybenzoate) exhibited virtually no inhibitory activity. These results suggest that the two hydroxy groups at the 3 and 5 positions in the benzoate moiety are essential for hyaluronidase inhibition. This is in contrast with the catechol structure necessary for antioxidative effects.

Table 1. Hyaluronidase and Collagenase Inhibitory Activities of *n*-Alkyl Gallates and Related Compounds

	Hyaluronidase inhibitory activity IC_{50} (µM)	Collagenase inhibitory activity IC_{50} (mM)	DPPH radical scavenging activity EC_{50} (µM)
Gallic acid	>1,000	2.07	18.5
Methyl gallate	>1,000	>10	18.6
Ethyl gallate	>1,000	>10	21.0
Propyl gallate	>1,000	>10	24.1
Butyl gallate	>1,000	>10	22.7
Hexyl gallate	253	3.43	19.8
Heptyl gallate	112	1.19	21.7
Octyl gallate	106	1.08	20.5
Nonyl gallate	167	1.25	22.8
Decyl gallate	580	2.64	16.6
Dodecyl gallate	>1,000	>10	19.6
Octyl 3-hydroxybenzoate	>1,000	>10	>100
Octyl 4-hydroxybenzoate	>1,000	>10	>100
Octyl 3,4-dihydroxybenzoate	902	3.16	15.4
Octyl 3,5-dihydroxybenzoate	113	>10	>100
Disodium cromoglycate	450	nd	nd
EDTA	nd	0.58	nd

nd, not determined

The kinetics of the hyaluronidase reaction were examined to the presence of octyl gallate. As shown in Fig. 1, the inhibition kinetics, analyzed by Lineweaver-Burk plots, indicated that octyl gallate exhibited uncompetitive inhibition. Thus it is less likely that octyl gallate binds directly to the catalytic domain of hyaluronidase. The inhibition constant, the *K*i value, was estimated to be 45 µM.

Collagenase (from *C. histolyticum*) was inhibited by gallic acid, with an IC_{50} value of 2.07 mM, but gallic acid esters of *n*-alkanols with short chain lengths (C_1–C_4) did not inhibit the enzyme (Table 1). These results suggest that the carboxyl group in gallic acid is essential for collagenase inhibition. However, similarly to the case of hyaluronidase inhibition, the collagenase reaction was strongly inhibited by gallate esters of *n*-alkanols when the alkyl chain length ranged between C_6 and C_{10}, and the most potent inhibitor was octyl gallate. The IC_{50} value of octyl gallate (1.08 mM) was distinctly lower than that of gallic acid although it was

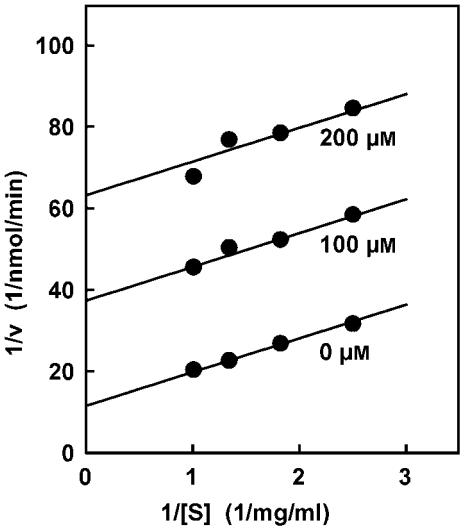

Fig. 1. Lineweaver-Burk Plots of the Hyaluronidase Reaction in the Presence of Octyl Gallate.

The hyaluronidase reaction was done in the presence of octyl gallate at the concentrations cited.

approximately 2-fold higher than that of EDTA (0.58 mM), the most often used collagenase inhibitor.[20] Octyl 3,4-dihydroxybenzoate as well as octyl gallate inhibited collagenase, whereas octyl 3,5-dihydroxybenzoate did not. This result is in contrast with that in the case of hyaluronidase inhibition. In addition to the bacterial collagenase, MMP-1 (a mammalian collagenase) was also inhibited by octyl gallate. The IC_{50} value of octyl gallate for MMP-1 was estimated to be 0.35 mM, lower than that for the bacterial enzyme (1.08 mM).

The data obtained in the present study show that the gallate esters of *n*-alkanols with alkyl chain lengths between C_6 and C_{10} exhibit hyaluronidase and collagenase inhibitory activities, in addition to their antioxidative,[11,12] antibrowning (tyrosinase inhibitive),[12] antifungal,[13] and antibacterial[14] effects. Thus, these alkyl gallates, in particular octyl gallate, which is a permitted food additive for antioxidation in the United States,[11] might be useful cosmeceutical ingredients to improve inflammation and allergic reactions, and to prevent wrinkles and skin aging.

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