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論　文　名	Study on natural and synthesized polyphenolic compounds possessing hyaluronidase and collagenase inhibitory effects （ヒアルロニダーゼおよびコラゲナーゼ阻害作用を有する天然および合成ポリフェノール化合物に関する研究）
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論文要旨

The inhibition of enzyme activity may be as significant as the activity itself in the regulation of biological processes. A lot of enzyme inhibitors, which are found from natural sources or synthesized artificially, are used as therapeutically important tools in several clinical areas. In addition to the medical use, inhibitors against certain enzymes, such as hyaluronidase, collagenase, and urease, are regarded to be useful as functional ingredients of foods and cosmetics.

Hyaluronic acid is found in the extracellular matrix of soft connective tissues, such as the umbilical cord, skin, synovial fluid, and the vitreous humor. This polysaccharide is involved in cell adhesion and protection, formation of skin tissue, water retention in tissues, and maintenance of flexibility in the skin due to its unique hydroscopic, rheologic, and viscoelastic properties. Hyaluronidase is a mucopolysaccharase that hydrolyzes glycosaminoglycans, including hyaluronic acid, in the extracellular matrix during tissue remodeling. When the level of hyaluronic acid decreases under conditions in which the hyaluronidase activity is increased, the moisture and tension of the skin is 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, such as disodium cromoglycate, have been used to suppress allergies and inflammation.

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. Collagenase (matrix-metalloproteinase) plays an important role in unbalanced turn over or rapid breakdown of collagen in human inflamed/ultraviolet-irradiated skin. Therefore, collagenase inhibitors, in addition to hyaluronidase inhibitors, have useful functions as cosmetic materials.

Helicobacter pylori, which is thought to cause gastritis, peptic ulcer disease, gastric adenocarcinoma, and gastric lymphoma, is able to survive in the stomach by releasing an enzyme, urease. Urease converts urea into ammonia, which then counters the stomach acid. This creates a neutralizing environment for protecting *H. pylori* from the acid in the stomach. It is thus suggested that urease inhibitors are useful as therapeutic agents for *H. pylori* infection.

The aim of this study is to search biologically active ingredients useful for functional foods and cosmeceutical products, from natural sources or synthesized compounds. With this intent, preliminarily, I have carried out the screening of some plants found in Okinawa Prefecture for inhibitors against hyaluronidase, collagenase and urease. I found that the leaf extract of *Bischofia javanica* (called "akagi" in Japanese) exhibited potent inhibitory effects against these enzymes, compared with those of the leaf and bark extracts of *Mallotus japonicus* (called "akamegashiwa" in Japanese). Both *B. javanica* and *M. japonicus* are involved in the family of Euphorbiacease, and distributed over Polynesia, Australia, and tropical zone of Asia. *M. japonicus* is one of well-known Chinese medical plants, and its bark, which is commercially available in Japan, is fused and used for the prevention of gastric and duodenal ulcer, and gastric hyperacidity. In contrast, *B. javanica* is meagerly used for folk remedy in the limited area of Polynesia.

The *Bischofia* leaf extract inhibited hyaluronidase with an IC_{50} value of 370 μg/ml, which was comparable to that of disodium cromoglycate (230 μg/ml), which is a well-known hyaluronidase inhibitor used as an antiinflammation and antiallergy agent. In addition, the collagenase inhibitory activity of the *Bischofia* leaf extract was slightly lower than that of EDTA, the most popular collagenase inhibitor (IC_{50} value: 590 μg/ml and 170 μg/ml, respectively). Furthermore, the leaf extract also exhibited the urease inhibitory effect with the almost the same potential as acetohydroxamic acid, which is reported to suppress *H. pylori*-induced gastritis by inhibiting urease (IC_{50} value: 4.9 μg and 2.8 μg/ml, respectively). These results suggest that the Bischofia leaf extract has potential as a novel ingredient for use in a wide range of functional foods and cosmeceutical products.

From 570 g of the crude Bischofia leaf extract, 11.8 mg white powder of compound **1** was purified as a hyaluronidase inhibitor by Diaion HP-20 and Chromatorex column chromatographies followed by repeated HPLC using a Cosmosil C18 column. This compound

was identified as 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose by comparison of ^{1}H- and ^{13}C-NMR spectral data with those reported in the literatures. The IC_{50} value of this compound for hyaluronidase was estimated to be 21.6 μM, which was substantially lower than that of disodium cromoglycate (450 μM). Kinetic analyses indicated that this compound acted as an uncompetitive inhibitor with a *K*i value of 10.5 μM.

1,2,3,4,6-Penta-*O*-galloyl-β-D-glucose (compound **1**) also exhibited a collagenase inhibitory effect with an IC_{50} value of 76 μM, which was also substantially lower than that of EDTA (580 μM). In addition, three collagenase inhibitors, compounds **2** (3.3 mg), **3** (3.1 mg) and **4** (3.3 mg), were isolated from the *Bischofia* leaf extract (570 g). Compounds **2**, **3** and **4** were identified as fisetin, 3,4,8,9,10-pentahydroxy-6H-dibenzo[*b,d*]pyran-6-one and gallic acid, respectively, on the basis of following ^{1}H- and ^{13}C-NMR spectral data by comparison with those reported in literatures. The IC_{50} values of these compounds were estimated to be 205 μM, 150 μM and 2.1 mM, respectively.

Gallic acid is a phenolic compound that is found as a constituent of hydrolyzable tannins, including 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose, in many plants. Gallic acid and hydrolyzable tannins have many biological activities, including the hyaluronidase and collagenase inhibition and antioxidative effects. The gallate esters of *n*-alkanols also act as a potent antioxidant, and propyl, octyl and dodecyl (lauryl) gallates are permitted additives for antioxidation in foods in the United States, although they are not natural compounds. To determine whether *n*-alkyl gallates are also useful as active ingredients of functional foods and cosmeceutical products, I identified their inhibitory activities against hyaluronidase and collagenase.

Gallic acid and its esters of n-alkanols with different chain length (C_1-C_{12}) were examined as to their hyaluronidase inhibitory activity, and their IC50 values were estimated. Methyl, ethyl, propyl, butyl and dodecyl gallates as well as gallic acid exhibited virtually no inhibitory activity against hyaluronidase. In contrast, hexyl, heptyl, octyl, nonyl and decyl gallates exhibited potent hyaluronidase inhibitory activities. It is hence thought that the hyaluronidase inhibitory 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. Kinetic analyses indicated that octyl gallate acted as an uncompetitive inhibitor with a *K*i value of 45 μM. Octyl 3,5-dihydroxybenzoate was a potent inhibitor of hyaluronidase, with an IC_{50} value of 113 μM; however, other derivatives of octyl benzoate (octyl 3,4-dihydroxybeozoate, 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.

Collagenase was inhibited by gallic acid, but gallic acid esters of *n*-alkanols with short chain lengths (C_1-C_4) did not inhibit the enzyme. These results suggest that the carboxyl group in gallic acid is essential for collagenase inhibition. However, similar to the case of the 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 lower than that of gallic acid. 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 the hyaluronidase inhibition.

Thus, the gallate esters of *n*-alkanols with alkyl chain lengths between C_6 and C_{10}, in particular octyl gallate, might be useful ingredients of functional foods and cosmeceutical products to improve inflammation and allergic reactions, and to prevent wrinkles and skin aging.

審査結果の要旨

一般に、酵素活性を阻害する化合物は医薬品として様々な疾患の治療に応用されている。同様に、酵素阻害剤は機能性食品や薬用化粧品の活性成分としても利用されている。本研究は、機能性食品や薬用化粧品の活性成分として有用なヒアルロニダーゼやコラゲナーゼなどに阻害作用を有する新規なポリフェノール化合物の検索することを目的とした。なお、ヒアルロニダーゼ阻害剤は抗炎症・抗アレルギー作用を有する機能性食品素材として有用であり、またコラゲナーゼ阻害剤はヒアルロニダーゼ阻害剤とともに皮膚の保湿等に有効な化粧品素材となりうる。

本研究を行うにあたり最初に、沖縄に自生する様々な植物から調製した抽出物について機能性食品や薬用化粧品として有用な酵素阻害作用を検討した。その結果、アカギ（*Bischofia javanica*）に強いヒアルロニダーゼ、コラゲナーゼ阻害活性を見いだした。アカギは東南アジアやオセアニアに広く分布するトウダイグサ科に分類される常緑高木であり、抗炎症作用を有する民間薬としてポリネシアの限定された地域で僅かであるが利用されている。一方、同様にトウダイグサ科には樹皮や葉が漢方薬として胃潰瘍などの消化器疾患に用いられるアカメガシワ（*Mallotus japonicus*）がよく知られている。そこで、両者を比較したが、アカギ葉抽出物は、アカメガシワ（葉、樹皮）抽出物に比べ、どちらの酵素に対しても強い阻害活性を示した。特に、ヒアルロニダーゼ阻害の50%阻害濃度（IC_{50}）は 370 μg/ml であり、ヒアルロニダーゼ阻害物質であり抗炎症・抗アレルギー剤として利用されている薬剤である disodium cromoglycate（IC_{50}：230 μg/ml; 450 μM）と比較しても遜色のないレベルであった。また、アカギ葉抽出物には胃炎や胃がんの原因となるピロリ菌の増殖抑制に有用なウレアーゼ阻害作用があり、その阻害活性はピロリ菌に起因する胃炎を抑制することが *in vivo* で証明されている acetohydroxamic acid と同レベルであった。

アカギ葉抽出物からヒアルロニダーゼ阻害物質を単離・精製したところ、1,2,3,4,6-penta-*O*-galloyl-β-D-glucose が得られた。本化合物の IC_{50} 値は 76 μM であり、disodium cromoglycate に比べて明らかに低値であった。なお、阻害様式は不競合阻害であり、*K*i 値は 10.5 μM であった。

1,2,3,4,6-penta-*O*-galloyl-β-D-glucose にはヒアルロニダーゼ阻害活性とともにコラゲナーゼ阻害作用が見いだされた。その IC_{50} 値は 76 μM であり、よく知られたコラゲナーゼ阻害剤である EDTA（IC_{50} : 580 μM）より強い阻害作用を示すことがわかった。さらに、アカギ葉抽出物からコラゲナーゼ阻害剤として fisetin（IC_{50} : 205 μM）、3,4,8,9,10-pentahydroxy-6H- dibenzo[*b,d*]pyran-6-one（IC_{50} : 150 μM）および gallic acid（IC_{50} : 2.1 mM）の３種の化合物が単離された。

様々な *n*-alkyl gallate を合成し、ヒアルロニダーゼおよびコラゲナーゼ阻害活性を調べた。その結果、両阻害作用ともに alkyl 基の鎖長によって大きく影響を受け、どちらも octyl gallate に最も強い阻害活性が見いだされた。なお、ヒアルロニダーゼ阻害の IC_{50} 値は 106 μM であり、コラゲナーゼ阻害に対しては 1.08 mM であった。次に galloyl 基の水酸基の特異性について検討した。その結果、ヒアルロニダーゼ阻害については octyl gallate 同様に octyl 3,5-dihydroxybenzoate に強い活性が見られたが、コラゲナーゼ阻害では octyl 3,4-dihydroxybenzoate に強い活性が現れた。以上の結果から、octyl gallate は米国では抗酸化作用を有する食品添加物として利用されているが、さらに機能性食品や薬用化粧品の活性成分としても有用であることが明らかになった。

以上、本研究では、沖縄や東南アジアなどに自生するアカギには強いヒアルロニダーゼ、コラゲナーゼさらにウレアーゼ阻害作用があり、機能性食品素材や化粧品素材として有用であることを明らかにした。さらに、様々な *n*-alkyl gallate を合成し、そのなかで食品の抗酸化剤として利用されている octyl gallate に強いヒアルロニダーゼ、コラゲナーゼ阻害活性を見いだした。これらの成果は、食品科学や天然物化学などの分野に多大な貢献をするものと考えられ、最終試験の結果と併せて、博士（応用生命科学）の学位を授与することを適当と認める。