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Inhibition of Collagenase by Naturally-Occurring Flavonoids

Bo Young Sin and Hyun Pyo Kim

College of Pharmacy, Kangwon National University, Chunchon 200-701, Korea

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We examined the inhibitory activities of various flavonoids, including the flavanones, flavones/isoflavones and flavonols, on collagenase from *Clostridium histolyticum* to establish their therapeutic potential against skin inflammation and photoaging. In general, the flavonols were stronger inhibitors than the flavones/isoflavones, and this indicated the importance of the C-3 hydroxyl substitution. Quercetin was the most active flavonoid among those tested, and it showed an IC_{50} of 286 μM. These novel results suggest that certain flavonoids, particularly the flavonols, may prevent collagen breakdown by inhibiting collagenase in inflamed skin as well as photoaged skin.

Key words: Flavonoid, Quercetin, Collagenase, Skin inflammation

INTRODUCTION

Plant flavonoids are known to possess anti-inflammatory activities both *in vitro* and *in vivo* (Middleton *et al.*, 2000; Kim *et al.*, 2004). Some of them have been claimed as topical anti-inflammatory agents, and certain types of flavonoids, mainly the flavones and flavonols, have been employed as cosmetic preparations in the form of the active constituents of plant extracts. One of the flavonoids' cellular mechanisms of action against skin inflammation is the inhibition of arachidonic acid metabolizing enzymes such as lipoxygenase, and this reduces the eicosanoid concentrations in the inflamed lesions. It has also been shown that some flavones/flavonols, when applied topically, down-regulate the expression of proinflammatory molecules such as cyclooxygenase-2 and tumor necrosis factor-α in experimental skin inflammation (Chi *et al.*, 2003).

Recent studies have demonstrated that certain matrix metalloproteinases (MMP) are highly induced in inflamed skin as well as in photoaged skin, and they breakdown the dermal matrix proteins such as collagen and elastin; this possibly leads to the prolonged skin damage and wrinkle formation (Fisher and Voorhees, 1998). Therefore, the agents that inhibit collagenase and/or elastase activity may have beneficial effects for maintaining healthy skin by preventing dermal matrix degradation. In this respect, the effects of flavonoids on matrix metalloproteinases (MMP) have been previously examined. For instance, delphinidine (flavan) and several other flavonoids were reported to inhibit gelatinases (MMP-2 and MMP-9) (Nagase *et al.*, 1998; Ende and Gebhardt, 2004). Some flavonoids such as quercetin, kaempherol and hyperoside inhibit neutrophil elastase (MMP-12) over a micromolar range of concentrations (Melzig *et al.*, 2001). When different types of flavonols and catechins were examined, the flavonoids having polyhydroxyl groups, i.e. delphinidine, morin, myricetin, and taxifolin, and the catechins having a galloyl moiety showed inhibitory activity against MMP-2, -9, and -12 (Demeule *et al.*, 2000; Sartor *et al.*, 2002). However, to date, the direct modulatory effect of flavonoids on collagenase or MMP-1 (mammalian collagenase-1) has rarely been demonstrated, despite the importance of MMP-1 and collagen breakdown in inflammatory skin diseases and photoaging. It has only been described that (-)-epicatechin gallate and (-)-epigallocatechin gallate isolated from tea inhibited collagenase at high concentrations (Makimura *et al.*, 1993).

Therefore, we investigated the inhibitory activity of some different types of flavonoids against collagenase from *Clostridium histolyticum* to elucidate their therapeutic potential against skin inflammation. We found that several derivatives, particularly the flavonols, demonstrated significant inhibition of collagenase. We also present some of the structure-activity relationships of these compounds.

Correspondence to: Hyun Pyo Kim, College of Pharmacy, Kangwon National University, Chunchon 200-701, Korea
Tel: 82-33-250-6915, Fax: 82-33-255-9271
E-mail: hpkim@kangwon.ac.kr

MATERIALS AND METHODS

Materials

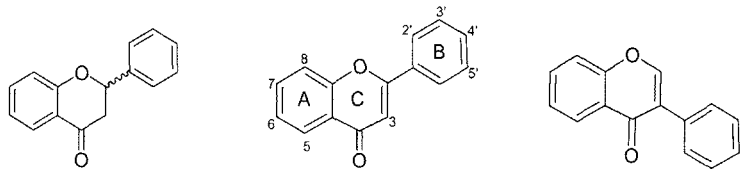
Collagenase (clostridiopeptidase A, EC 3.4.24.3) from *Clostridium histolyticum* type H, PZ-peptide (4-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg monohydrate), 1,10-phenanthroline (a MMP inhibitor) and flavonoid derivatives including flavanone, naringenin, apigenin, luteolin, galangin, kaempferol, quercetin, morin, and myricetin were purchased from Sigma-Aldrich. Chrysin, oroxylin A and wogonin were isolated from *Scutellaria baicalesis*, and tectorigenin was purified from *Belamcanda chinensis* according to the previously reported (You *et al.*, 1999). Genistein and daidzein were obtained from *Pueraria lobata* (Lee *et al.*, 1994).

Collagenase assay

The collagenase activity was measured using a previously described procedure (Sawabe *et al.*, 1998) with a slight modification. An assay tube contained collagenase (5 μg) and PZ-peptide (0.5 mg) in 0.1 M Tris buffer (pH 7.1) containing 20 mM $CaCl_2$ in the presence or absence of test compounds (total volume of 1.7 mL). The tube was incubated at 37°C for 30 minutes, and 25 mM citric acid solution (1 mL) was then added to terminate the reaction. Immediate after, ethylacetate (5 mL) was added, and the solution was vortexed and centrifuged. The absorbance of the organic layer was measured at 320 nm. The test compounds, including the flavonoids, were initially dissolved in DMSO and then they were diluted in the same buffer to the appropriate concentrations. The control tube also contained the same amount of DMSO. The percent inhibition was calculated according to the following formula:

% inhibition = $(OD_{control}-OD_{sample}) \times 100/OD_{control}$, where $OD_{control}$ and OD_{sample} represented (OD of the control with collagenase - OD of the control without collagenase) and (OD in the presence of the test compound with collagenase - OD in the presence of the test compound without collagenase), respectively. All the measurements were duplicated and the values presented are the arithmetic means.

Table I. Inhibition of collagenase by the naturally-occurring flavonoids



Flavanones Flavones and Flavonols Isoflavones

compound	3	5	6	7	8	2'	3'	4'	5'	% inhibition	
										50 μM	100 μM
Flavanone	H	H	H	H	H	H	H	H	H	-[a]	-
Naringenin	H	OH	H	OH	H	H	H	H	H	-	-
Flavone	H	H	H	H	H	H	H	H	H	-	-
Chrysin	H	OH	H	OH	H	H	H	H	H	-	11
Apigenin	H	OH	H	OH	H	H	H	OH	H	-	15
Luteolin	H	OH	H	OH	H	H	OH	OH	H	-	10
Oroxylin A	H	OH	OMe	OH	H	H	H	H	H	-	-
Wogonin	H	OH	H	OH	OMe	H	H	H	H	-	-
Flavonol											
Galangin	OH	OH	H	OH	H	H	H	H	H	-	11
Kaempferol	OH	OH	H	OH	H	H	H	OH	H	22	27
Quercetin	OH	OH	H	OH	H	H	OH	OH	H	31	34
Morin	OH	OH	H	OH	H	OH	H	OH	H	-	13
Myricetin	OH	OH	H	OH	H	H	OH	OH	OH	25	30
Isoflavone											
Genistein	H	OH	H	OH	H	H	H	H	H	-	12
Tectorigenin	H	OH	OMe	OH	H	H	H	OH	H	-	14
Daidzein	H	H	H	OH	H	H	H	OH	H	-	-
1,10-Phenanthroline										26	40

[a]less than 10% inhibition.

RESULTS AND DISCUSSION

When the various flavonoids were incubated at 50 and 100 μM, most of the flavones, flavonols, and isoflavones that were tested were revealed to have, more or less, an inhibitory action on the collagenase obtained from *C. histolyticum*, while the flavanones without the C-2,3-double bond did not show significant inhibition at concentrations up to 100 μM (Table I). In general, the flavonols were stronger inhibitors than the flavones/isoflavones, and this implied that the C-3-hydroxyl group is important for the compounds to have higher inhibitory activity (apigenin vs. kaempferol and luteolin vs. quercetin). Particularly, kaempferol, quercetin, and myricetin showed considerable inhibition. In contrast, galangin that has no hydroxyl substitution on the B-ring and morin that has the B-ring catechol moiety (2',4'-dihydroxyl) showed much reduced activity, and this strongly suggested that the hydroxylation pattern in the B-ring is also an important determinant. A methoxyl substitution at C-6 or C-8 was not a favorable structure for enhancing the inhibitory activity of the flavones (chrysin vs. oroxylin A or wogonin). The concentration-dependent inhibition of kaempferol and quercetin is shown in Fig. 1. The IC_{50} value of quercetin was approximately 286 μM. As expected, 1,10-phenanthroline inhibited collagenase activity with an IC_{50} of 157 μM.

To the best of our knowledge, this is the first report showing the collagenase inhibitory activity by the flavone/flavonol derivatives. However, it should be mentioned that the inhibitory potency on collagenase by the flavonoids is not strong compared with those activities against other related enzyme systems, including neutrophil elastase (the IC_{50}'s of the various flavonoids were 0.3-84 μM) (Melzig *et al.*, 2001). Under the experimental conditions of the present study, the range of concentrations of quercetin that showed significant collagenase inhibition were revealed to be 25-500 μM. These concentrations of flavonoids in the dermal area may not be easily maintained *via* oral administration of the compound, but the concentration ranges of 25-100 μM are possible to obtain by topical application on the inflamed area. In addition, several flavonoids such as genistein, quercetin, nobiletin, and baicalein were previously found to down-regulate the MMP-1 expression (Kang *et al.*, 2003; Song *et al.*, 2001; Lin *et al.*, 2003; Choe *et al.*, 2003). These previous results and our findings from the present investigation suggest that certain topically applied flavonoids, especially quercetin, may protect against collagen degradation by collagenase inhibition and/or down-regulation of collagenase induction. These activities of the flavonoids may contribute, at least in part, to reduced destruction of the dermal tissue and reduced damage of inflamed or photoaged skin. The effects on the mammalian collagenases and the effects on animal skin via topical application need to be further elucidated to clearly establish the pharmacological potential of flavonoids for treating humans.

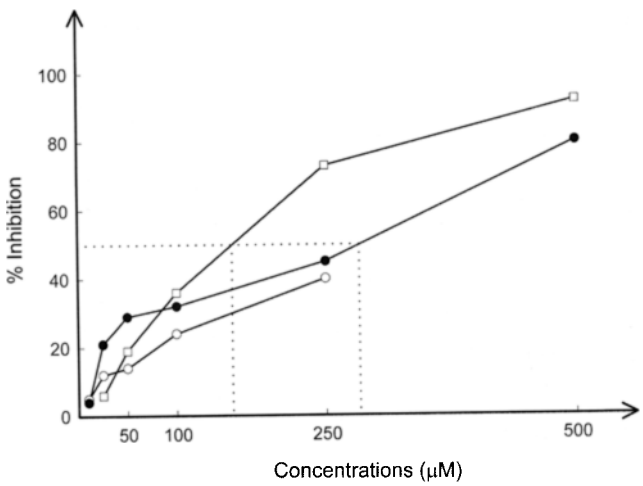


Fig. 1. The concentration-dependent inhibition of collagenase Kaempferol (○), quercetin (●), 1,10-phenanthroline (□). Note: The maximum solubility of kaempferol in the reaction buffer was 250 μM.

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