Suppression of Degranulation of RBL-2H3 Cells by Means of Flavonoids in Natural Plants

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ABSTRACT

Some of common flavonoids, phytochemicals that may be rich in various vegetables and fruits showed a great suppression of degranulation of RBL 2H3 cells. The activity is determined by the hexsosaminidase release rate that is coupled with the release of histamine, one of chemical mediators from the RBL 2H3 cells. The activities of flavonoids were comparatively studied by IC_{50}. Isorhamnetin showed the highest suppression of degranulation at 3.1 µmol/L. Flavones and flavonols such as isorhamnetin, luteolin and quercetin bearing double bond between C_2 and C_3 positions showed high level (IC_{50}: 3.1 µmol/L to 3.7 µmol/L) of the activities in suppression. As antioxidant activities of flavonoids was related with anti-inflammatory activity, chemical structure-activity relationship of flavonoids was investigated by TBA tests for antioxidant activity and RBL 2H3 tests for anti-allergic activity. As the results, aromatic ring on C-ring was indispensable for the anti-allergic activity. However, aromatic ring with orth diols on B-ring was essential for antioxidant activity. Therefore the active sites of flavonoids to anti-allergic activity and antioxidant activity were found to be different.

Key words: RBL 2H3, Allery, Degranulation, Flavonoids

INTRODUCTION

Food allergies have been serious problems for many people in the world (Hadley, 2006). Especially the incidence of the patients who suffer any kinds of allergies increased in big cities. Food additives, air pollution, and some changes of food habits are believed to directly or indirectly influence the high incidence of food allergies. Therefore, now-a-days, food ingredients that may frequently cause immunological inflammatory diseases are required to be indicated on the food packaging in Japan. Histamine analogues that inhibit immune reactions as antagonists, and new drug designs that protect the releasing of the histamine are still under investigation as medicinal drugs.

Flavonoids are common secondary products. The diverse benefits such as anti-tumor activity (Chowdhury et al., 2002), anti-obesity (Kamisoyama et al., 2008), anti-microbial (Cushnie and Lamb, 2005), antioxidants (Pietta, 2000, Tamura and Yamagami, 1994) and other effects were reported on flavonoids isolated from fruits and vegetables. Their daily intakes from fruits and vegetables are quite high and the amounts are compatible to other dietary antioxidants like vitamin C and vitamin E. The antioxidant activities of flavonoids were due to their molecular structure, especially existence of the number of polyol group on aromatic ring. The aimed flavonoid compounds (Figure 1) are rich in hops, berries, teas, beans and others. It is well-known that catechins are rich in green tea and grape seeds, anthocyanins are abundant in grapes, berries, eggplant and black beans. Isoflavones are mainly found in soy bean, and then flavonols are in green tea and onion. Moreover, flavanone are found in some fruits such as lemon and oranges. Recently, some catechins and their derivatives from green teas such as epigallocatechin 3-*O*-(3-*O*-methylgallate) and epigallocatechin

3-*O*-(4-*O*- methylgallate) were found as anti-allergic active compounds (Maeda-Yamamoto et al., 2004). Those compounds were rich in some Oolong teas and Benifuki and some green teas.

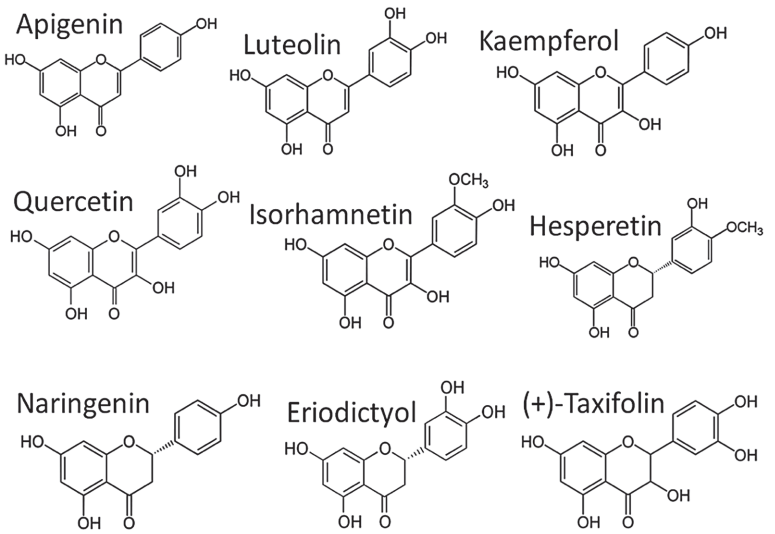

Figure 1. Typical flavonoid aglycone in plants

It was reported that a great suppression of degranulation of RBL 2H3 cells is related with anti-allergic activity (Shimoda et al., 2006). The activity was determined by the release rate of hexosaminidase that coupled with the release of histamine from the RBL 2H3 cells. The anti-inflammatory activity of flavonoids in liquorice leaves (*Glycyrrhiza glabra* L.) was comparatively studied with antioxidant activity of flavonoids (Siracusa et al., 2011). Inflammatory disorder is one of allergic reactions such as iching, sneezing and so on (Holgate et al., 2005). However, direct discussion of anti-allergic activity and antioxidant activity is rare in literatures.

In this research work, we introduce what types of favonoids are useful for anti-degranulation of histamine. Moreover, useful or common functional groups of flavonoids for anti-allergic activity and antioxidant activity were discussed. This kind of information is quite useful for the selection of target plants that may produce value added foods.

MATERIALS AND METHODS

Reagent

Dulbecco's modified Eagle's medium (DMEM), PBS (—) and triton X-100 were obtained from Wako (Osaka, Japan). Fetal bovine serum (FBS) was obtained from Bio-west. Antibiotic-Antimycotic 100X was obtained from GIBCO (Tokyo, Japan). Mouse monoclonal anti-dinitrophenol, albumin dinitrophenyl (HSA), Tyrode's salt solution, and *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside (*p*-NAG) were obtained from SIGMA (Tokyo, Japan). Bovine Serum Albumin (BSA) was obtained from Jackson Immuno Research Laboratories, Inc. (PA, USA).

Preparation of the media and samples

DMEM including 10% FBS and 1% Antibiotic-Antimycotic 100X was used as a base medium. Modified tyrode (MT) buffer was prepared by dissolving HEPES (4.76 g/L) and BSA (1 g/L) in tyrode's salt solution. Anti-DNP IgE solution (anti-body) was prepared by diluting mouse monoclonal anti-dinitrophenol with base medium (50 ng/ml). HSA solution (antigen) was prepared by dissolving HSA in MT buffer (2.5 μg/ml). The tested flavonoid samples were dissolved in DMSO, and the solutions were added to MT buffer (final DMSO concentration was adjusted at 0.1%).

Anti-allergic activity on RBL-2H3 cell line

Rat Basophilic leukemia RBL-2H3 cells are mucosal mast cell type, which is a major model for the study of anti-allergenic activity of foodstuffs (Sugiura et al., 2009; Shimoda et al., 2006). RBL-2H3 cells (RCB2782, RIKEN BIORESOURCE CENTER, Ibaraki, Japan) in base medium were dispensed into 24 well plates at a cell density of 2×10^5 cells/well and were cultured overnight at 37°C in an atmosphere of 5% CO_2. The cells were washed by PBS (1 ml), anti-DNP IgE solution (500 µl) was added to negative control well and sample well, base medium was added into blank well, and cells were incubated for 2 hrs for sensitization of the cells. To remove excess IgE, cells were washed 2 times with MT buffer. MT buffer was added into blank well and negative control well, the tested solution (490 µl) was added to sample well, and cells were incubated for 10 min. The HSA solution (10 µl) was added to each well, and cells were incubated for 30 min to evoke allergic reactions (degranulation). The reaction was stopped by cooling it in an ice bath for 10 min. The supernatant was collected, and the cells were lysed with 500 µl of 0.1% (W/W) Triton X-100. The supernatant (50 µl, SN well) or the cell lysate (50 µl, CL well) was transferred into a 96-well plate, and incubated for 5 min. One hundred microliters of 0.1 M citrate buffer (pH 4.5) containing 3.3 mM *p*-NAG as substrate was added into the X well and incubated for 25 min at 37°C. The reaction was stopped by adding 100 µl of 2 M glycine buffer (pH 10.0, stop buffer) into all X and Y series of wells. As blank tests, to the Y series of wells, 100 µl of 0.1 M citrate buffer (pH 4.5) with 3.3 mM *p*-NAG was added after addition of the stop buffer as described above. The absorbance (OD) of each well was measured at 405 nm using a micro plate reader (Biorad Model 550, Bio-Rad Laboratories). The β-hexosaminidase release was calculated by subtracting the colorization of the sample solution.

$$\beta\text{-hexosaminidase release rate (\%)} = \frac{SX - SY}{(SX - SY) + (CLX - CLY)} \times 100$$

OD at 405 nm of
- S: supernatant well, CL: cell lysate well
- X: first addition is substrate and then stop buffer solution
- Y: first addition is stop buffer solution and then substrate

Lipid peroxidation

The lipid peroxidation was determined by using Fe-linoleic acid method described by Tamura and Shibamoto (1991). Linoleic (17.8 mmol) was poured into a 30-mL test-tube and then diluted with a 4.85 ml trizma-buffer solution (0.25 mM, pH 7.4) containing 0.2% SDS (w/v) and 0.75 mM potassium chloride. Trizma-buffer was prepared by diluting 6.057 g tris(hydroxymethyl) aminomethane and 11.184 g potassium chloride with pure water filtered by milipore filter to 1 L after adjusting the pH of the solution to 7.4. Lipid peroxidation was initiated by adding 20 mM ferrous sulfate (0.05 ml). The total volume of the reacting solution was adjusted to 5 ml. Incubation was continued for 16 h at 37ºC in a dark place. The reaction was stopped by adding 90.8 mM BHT alcoholic solution (0.1 ml) in the tube. Each tube contained 2 mg BHT. The obtained reacted solution (0.2 ml) was used for TBA assay. Antioxidant (5 mmol) was diluted with 0.5% trifluoroacetic acid-ethanol and then the solution was filled to 5 ml. The solution (0.1 ml) containing antioxidants was mixed with the trizma-buffer solution (4.9 ml) mentioned above when necessary.

TBA assay

The antioxidative activity was determined by using the thiobarbituric acid (TBA) method described by Tamura and Yamagami (1994). The reacted solution (0.2 ml) mentioned above was derivatized to thiobarbituric acid reactive substances by incubation with 0.67% (w/v) thiobarbituric acid (1.0 ml) and 0.05 N HCl (3.0 ml) for 30 min in a 95ºC water bath. The solution was then cooled in ice for 5 min. The colored substances were extracted by 4.0 mL of 1-butanol. The absorbance of the 1-butanol layer was measured at 535 nm. The results are expressed in terms of

malonaldehyde production; a calibration curve was constructed by using malonaldehyde bis-diethyl acetal (1,1,3,3-tetraethoxypropane) as a standard. Malonaldehyde quantitatively prepared by heat-assisted acid hydrolysis (mentioned above) from an aliquot of 1,1,3,3-tetraethoxypropane (10 µmol/ml) was immediately derivatized to a thiobarbituric acid reactive substance to make the standard calibration curve.

RESULTS AND DISCUSSION

The β-hexosaminidase release by flavonoids

The β-hexosaminidase release of flavonoid aglycons (Figure 1) and their glycosides was calculated by following the equation shown in "Materials and Methods" section. The anti-allergic activity was expressed as the IC_{50}, meaning the concentration of 50 % inhibition against the total release of β-hexosaminidase as shown in Table 1.

IC_{50} of twenty compounds listed in Table 1 showed a wide spectrum in the values (500 times difference or it was greater than that). The detail discussion of IC_{50} had been done by each class of the chemicals as shown below.

Table 1. Anti-allergic activity of typical flavonoids and the related compounds.

Sample	IC_{50} [µmol/L]
Isorhamnetin	3.11
Luteolin	3.39
Quercetin	3.63
Apigenin	3.71
Kaempferol	10.8
Eriodictyol	94.6
Homoeriodictyol	159
(+)-Taxifolin	165
Hesperetin	182
Rutin	250
Silymarin	272
Naringenin	321
Luteolin 7-glucoside	470
Rhoifolin	1130
Catechol	1160
Apigenin 7-glucoside	1260
Naringenin 7-glucoside	1500
Diosmin	Unlimited
Hesperidin	Unlimited
Flavanone	Unlimited

Comparison of the anti-allergic activity between naringenin and flavanone

The chemical difference between naringenin and flavanone is only the existence of three hydroxy groups at 5, 7 and 4' position of ring A and ring B of naringenin. Naringenin showed IC_{50} at 320 µmol/L. However, IC_{50} of flavanone could not show any activity. Hydroxy group on the aromatic rings should increase the electron density of the rings. So, affinity of flavonoids with receptors (Porat et al., 2006) of sensitized mast cells might be increased.

Comparison of the activity among apigenin, kaempherol and naringenin and also among luteolin, quercetin and taxifolin

The chemical difference among apigenin, kaempherol and naringenin is only the existence of hydroxy group and double bond at C_2-C_3 position of the C-ring. Apigenin, kaempherol and naringenin showed IC_{50} at 3.71 μmol/L, 10.8 μmol/L and 321 μmol/L, respectively. On the other hand, IC_{50}s of luteolin, quercetin and taxifolin were 3.39 μmol/L, 3.63 μmol/L, and 165 μmol/L, respectively. Both data supported that anti-allergic activity was arranged to be flavone > flavonol > flavanone in the greater order. Double bond between C_2-C_3 should be essential for the activity. Even though both flavonoids have aromatic rings in A-ring and B-ring, conjugation of both electron delocalization (Rice-Evans et al., 1995) of A-ring and B-ring may be essential for the activity. Flavonoids bearing ortho dihydroxy group showed a bit higher contribution to the activity than those bearing monohydroxy group. Hydroxy groups on the aromatic rings also increased the electron density of the rings.

Comparison of the activities of apigenin, apigenin 7-glucoside, apigenin 7-*O*-neohesperidoside (Rhoifolin) in flavones

The difference in chemical structures among apigenin and other two derivatives listed in Table 1 is the number and kinds of glycosides at 7 position. Rhoifolin, a type of flavonoid isolated from *Boehmeria nivea*, China grass or ramie (leaf), from *Citrus limon*, from *Citrus x aurantium* has 2-(α-L-rhamnosyl)-β-D-glucoside at the 7 position. Rhofolin showed IC_{50} at 1130 μmol/L. On the other hand, the anti-allergic activity of apigenin and apigenin 7-glucoside showed 3.71 μmol/L and 1260 μmol/L, respectively. A great difference was observed apigenin and its glycosides. The effect of the difference of the number and kind of glycosides was small. The glycosidation of flavonoids apparently weaken the power of anti-allergic activity. The same tendency in the activity was observed between luteolin and luteolin 7-glucoside. Anti-allergic drugs must penetrate the cell membranes of mast cells. Glycosides bearing higher polarity inhibit easy penetration of the cells. When we intake flavonoids with glycosides from plant resources as foods, the glycoside linkages may be hydrized (Boyer et al., 2005; Murota and Terao, 2003) and the activity will be created after the hydrolysis. So, we should focus on the activity after absorption and metabolization in a near future. On the other hand, these glycosides may be useful for transportation of flavonoids in human bloods.

Comparison of the activities of naringenin, eriodictyol, hesperetin, and homoeriodictyol in flavanones

For flavanones, lower anti-allegric activities were expected when compared with corresponding flavone derivatives. As we expected, lower IC_{50}s of naringenin, eriodictyol, hesperetin, and homoeriodictyol were observed at 321 μmol/L, 94.6 μmol/L, 182 μmol/L, 159 μmol/L, respectively. The number of functional groups such as hydroxy and methoxy groups on B-ring influenced to the activity and then it was concluded that ortho hydroxy group, or orho hydroxy and methoxy group improved the activities. However, 4'-methoxy group may not improve it so much.

Comparison of the effect of rhamnosylglucoside and functional groups on B-ring of flavonoids

Diosmin, rhoifolin, hesperidin and rutin were compared the activity. All of these flavonoids have a rhamnosylglucoside group on the molecules. Hesperidin is one kind of flavanone. So, the anti-allergic activity was unliminted as we expected from the data described above. Diosmin and rhoifolin have similar types of rhamnosylglucosides at 7 position of the flavones but the number and position of hydroxy and methoxy group of both chemicals are quite different. IC_{50}s of anti-allergic activities were unlimited for diosmin and 1130 μmol/L for rhoifolin, respectively. 4'-Methoxy group of diosmin might intensively suppress the anti-allergic activity. On the other words, 4'-hydroxy group of rhoifolin contributed the anti-allergic activity.

Rutin, a flavonol rhamnosylglucoside showed rather high activity (IC_{50}: 250 µmol/L) than rhoifolin (IC_{50}: 1130 µmol/L) because rutin is in a group of flavonol and also has diol on the B-ring.

Antioxidant activity of some flavonoids

Flavonoids that have orth diol on the flavonoid B-rings had tested the antioxidant activity by means of malondialdehyde formation from linoleic acid oxidation system as shown in Figure 2. Luteolin, eriodictyol and taxifolin were the major chemicals as potent antioxidants as α-tocopherol. C_2-C_3 Double bonds of C-ring of eriodictyol and taxifolin are saturated. Quercetin did not showed higher activity but it was still high in the activity as same as tea polyphenol, epicatechin gallate (data not shown). In consequence, ortho diol on B-ring of individual flavonoids should be important (Pietta, 2000) for antioxidant activity. Antioxidant activity of anthocyanins supported the tendancy of importance of ortho diol (Tamura and Yamagami, 1994).

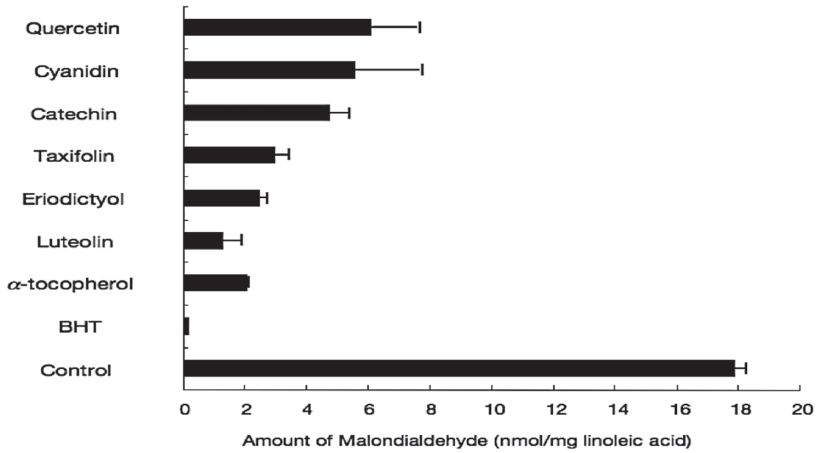

Figure 2. Comparison of antioxidative activity among flavonoids in the linoleic acid system at pH 7.4 (n = 4).

However, conjugated double bond at 2 position of flavonoids with A-ring and B-ring were not indispensable for higher antioxidant activity of the flavonoids. By DPPH method, Okawa et al. (2001) also reported high antioxidant activity of flavanols such as catechin and epicatechin which did not have conjugated double bond at the 2 position. Their DPPH method also gave a consistent data with our results. Aromatic ring with ortho diols on B-ring was essential but aromaticity of C-ring is not important for antioxidant activity.

CONCLUSION

The anti-allergy activities of flavonoids were comparatively studied by IC_{50}. Isorhamnetin showed the highest suppression of degranulation at 3.11 mmol/L. Flavones and flavonols such as isorhamnetin, luteolin, quercetin and apigenin bearing double bond between C_2 and C_3 positions showed high activities (IC_{50}: 3.11 mmol/L to 3.71 mmol/L) for the allergy suppressions. Especially, importance of flavonols was clarified in this paper. Ortho hydroxy groups and hydroxy-methoxy group on B-ring assisted the increase of the activity except 4'-methoxy group. On the other hand, antioxidant activities of flavonoids also required ortho diols on aromatic ring of B-ring of flavonoids. In consequence, aromatic ring on C-ring was indispensable for the potent anti-allergic activity. However, aromatic ring with ortho diols on B-ring was essential for antioxidant activity. Therefore the active sites and acting mechanisms of flavonoids for anti-allergic activity and antioxidant activity was found to be a bit different even though polyphenolic character of flavonoids are required in both activities.

This kind of information is quite useful for the selection of target plants that may contain valuable flavonoids as nutraceutical ingredients.

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