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Phenolic constituents isolated from *Fragaria ananassa* Duch. inhibit antigen-stimulated degranulation through direct inhibition of spleen tyrosine kinase activation

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ABSTRACT

We isolated eight phenolic constituents from *Fragaria ananassa* Duch. (strawberry) and determined their structures using 1D, 2D-NMR. Among the isolated compounds, linocinnamarin (**LN**), 1-*O*-*trans*-cinnamoyl-β-D-glucopyranose (**CG**), and cinnamic acid (**CA**) exhibited antigen (Ag)-stimulated degranulation in rat basophilic leukemia RBL-2H3 cells. In order to reveal the underlying mechanisms, we examined the effects of **LN** and **CA** on cellular responses induced by antigen stimulation. Treatment with both **LN** and **CA** markedly inhibited antigen-stimulated elevation of intracellular free Ca^{2+} concentration and reactive oxygen species (ROS). Both **LN** and **CA** suppressed Ag-stimulated spleen tyrosine kinase (Syk) activation. These results indicate that inhibition of antigen-stimulated degranulation by **LN** and **CA** is mainly due to inactivation of Syk/phospholipase Cγ (PLCγ) pathways. Our findings suggest that **LN** and **CA** isolated from *F. ananassa* Duch. (strawberry) could be beneficial agents for alleviating symptoms of type I allergy.

1. Introduction

Berry species belonging to the Rosaceae family include a variety of economically significant horticultural fruits cultivated in many countries. Strawberry (*Fragaria ananassa* Duch.), raspberry (*Rubus ideaus* L.), blackberry (*Rubus fruticosus* L.), and cloudberry (*Rubus chamaemorus* L.) plants provide delicious fruits that can be consumed in fresh forms and as processed foods including jams, juices and liquors. Plants of the Rosaceae family contain polyphenols, anthocyanins, flavonoids, ellagic tannins, and condensed tannins. These phenolic compounds are abundant in highly colored berries, and due to their popularity and wide consumption, these berries serve as one of our most important dietary sources of phenolics.[1,2] In recent years, it has been reported that strawberry has anti-oxidant,[3] anti-tumor,[4] anti-diabetes,[5] and anti-obesity activity.[6]

Fragaria ananassa Duch. is highly popular among berries, and chemical studies of the berry have been gradually conducted. For example, *trans*- or *cis*-tiliroside, which are flavonol glycoside phenylpropanoids isolated from *F. ananassa* Duch., showed inhibitory activity of the drug-metabolizing enzyme CYP3A4.[7] 5-Carboxypyranopelargonidin 3-*O*-β-glucopyranoside, a unique anthocyanin isolated from acidic methanol extract of *F. ananassa* Duch., demonstrated potential as a colorant in solution at around pH 5.[8] A new anthocyanin, taxfolin 3-arabinoside,[9] and a new dimeric anthocyanin, pelargonidin 3-glucoside (covalently linked to four flavan-3-ols),[10] have recently been identified.

Recently, we also investigated *F. ananassa* Duch. (strawberry 'Nohime') and isolated the four flavonoids of chrysin, kaempferol, 7-*O*-cinnamoylchrysin, and *trans*-tiliroside. These compounds suppressed the antigen-stimulated degranulation of the high-affinity immunoglobulin E receptor (FcεRI) in RBL-2H3 cells. It was suggested that inhibition of degranulation by the flavonoids was mainly due to the inhibition of $[Ca^{2+}]_i$ elevation and spleen tyrosine kinase (Syk) inactivation.[11]

In this paper, we used *F. ananassa* Duch. (strawberry 'Minomusume'), which was developed for forced culture at the Gifu Prefectural Agricultural Technology Center. As a continuation to our previous study, we were interested in other anti-allergic compounds derived from the strawberry plant. In this study, we endeavored to isolate the said compounds.

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2. Results and discussion

2.1. Structures of isolated compounds

The juice and methanol extract of *F. ananassa* Duch. were separated by column chromatography (Sephadex LH-20, Amberlite XAD-7HP, and silica gel) and preparative thin-layer chromatography (PTLC) using a silica gel to give eight phenolic compounds. These isolated compounds included two phenylpropanoid glycosides (linocinnamarin; **LN** and 1-*O*-*trans*-cinnamoyl-β-D-glucopyranose; **CG**), two phenylpropanoids (*p*-coumaric acid; **CU** and cinnamic acid; **CA**), and four flavonoids (chrysin; **CH**, kaempferol; **KF**, catechin; **CT**, and *trans*-tiliroside; **TL**) (Fig. 1).

Compound (**LN**) was obtained as a colorless syrup. FABMS of this compound revealed a molecular ion peak at *m/z* 341 $[M+1]^+$. The ^{1}H NMR spectrum showed the signals of a methoxy group at δ_H 3.79, an olefin at δ_H 7.63 and 6.40, a *para*-substituted aromatic ring at δ_H 7.54 and 7.10, and a sugar moiety at δ_H 4.95, 3.89–3.84, 3.75–3.66, and 3.51–3.41. The geometry of the olefin of a phenylpropanoid moiety was determined to be *E* based on the proton coupling constant (J = 16.0 Hz). The value of the proton coupling constant of the anomeric glucose proton resonance (J = 7.3 Hz) showed that the sugar moiety adopts a β-configuration.[12] The ^{13}C NMR and DEPT spectra confirmed these structural moieties and indicated the existence of a carbonyl carbon of a carboxylate ester at δ_c 168.1. The glycoside was identified as β-glucopyranoside based on comparisons of its ^{13}C NMR data to those reported for hexosides.[13] In addition, the HMBC spectrum showed that δ_H 4.95 (Glc-anomeric proton) was correlated with δ_c 159.6 (quaternary carbon of aromatic ring), indicating that the β-glucopyranosyl group was attached to the aromatic ring. On the basis of the spectral data obtained from ^{1}H, ^{13}C NMR, DEPT, COSY, HMQC, and HMBC experiments, we identified compound (**LN**) as the phenylpropanoid glycoside, linocinnamarin.[14]

Compound (**CG**) was also obtained as a colorless syrup. FABMS of this compound revealed a molecular ion peak at *m/z* 311 $[M+1]^+$. The ^{1}H and ^{13}C NMR spectra exhibited signals similar to those of compound (**LN**) with the exception of a methoxy group. Accordingly, these results suggested that compound (**CG**) was a phenylpropanoid glycoside. The HMBC spectrum showed that δ_H 5.61 (Glc-anomeric proton) was correlated with δ_c 164.9 (carbonyl carbon), indicating that the β-glucopyranosyl group was attached to a carboxylate ester. The spectral data were compared with those in the literature, and compound (**CG**) was identified as 1-*O*-*trans*-cinnamoyl-β-D-glucopyranose.[15] Other isolated compounds were also identified based on reference data from the literature.[16–18]

There are only a few reports about the isolation of linocinnamarin (**LN**) from natural sources.[19–21] This is the first report indicating that the Rosaceae family contains linocinnamarin (**LN**). Since then, this compound has been isolated from plants of three other families, that is, Linaceae, Pinaceae, and Apocynaceae. From a chemotaxonomic point of view, the limited distribution of linocinnamarin (**LN**) in these four families is quite interesting. Linocinnamarin (**LN**) is reported to exhibit biological activities, and has been shown to have a weak effect in increasing peripheral blood flow in mice.[22]

2.2. LN and CA suppress antigen-stimulated degranulation in RBL-2H3 cells through the inactivation of Syk

In type I allergy, the binding of antigen to the IgE bound-FcεRI on the surface of mast cells and basophils is the first event leading to the release of chemical mediators such as histamine, arachidonic acid metabolites, and cytokines which mainly cause asthmatic and inflammatory responses.[23–25] Thus, mast cells are key effector cells in IgE-mediated immune responses. Recently, we reported the inhibitory effects of flavonoids (chrysin; **CH**, kaempferol; **KF**, *trans*-tiliroside; **TL** and 7-*O*-*trans*-cinnamoylchrysin) isolated from *F. ananassa* Duch. on Ag-stimulated degranulation in RBL-2H3 cells.[11] In the present study, we further examined the effects of two phenylpropanoid glycosides (**LN** and **CG**), two phenylpropanoids (**CU** and **CA**), and **CT** isolated from *F. ananassa* Duch. on Ag-stimulated degranulation from RBL-2H3 cells. In our assay system, approximately 60% of β-hexosaminidase was released from the RBL-2H3 cells in absent of each sample by determination of the total β-hexosaminidase activity after sonication of the cell

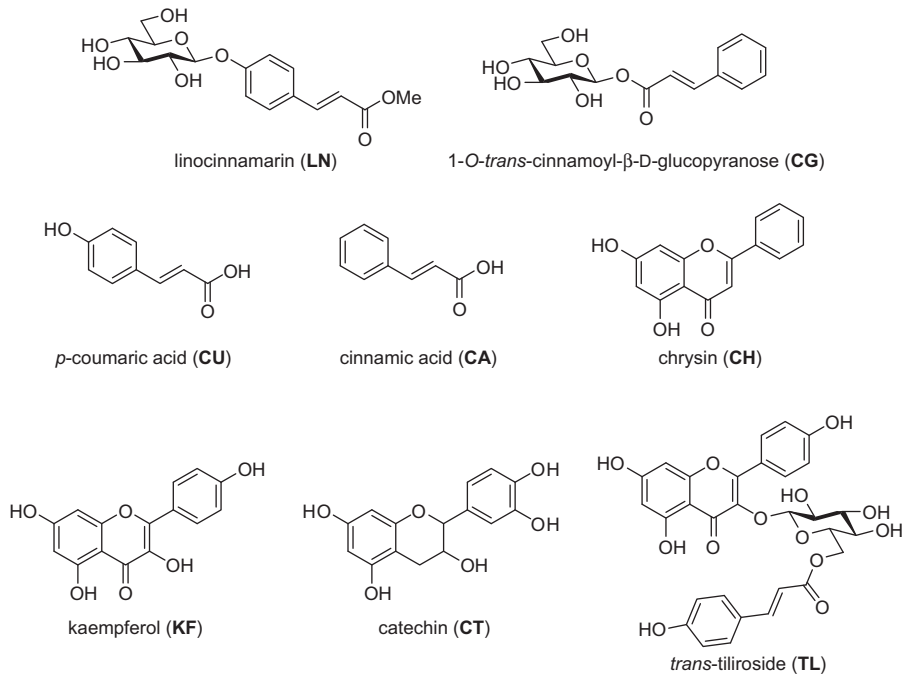

Figure 1. Structures of isolated constituents from *Fragaria ananassa* Duch.

suspension. Among these, **LN** and **CA** markedly suppressed Ag-stimulated degranulation in a dose-dependent manner (Fig. 2). It is also of interest to note that the level of Ag-stimulated degranulation suppression induced by **CG**, which is a glycoside of **CA**, was significantly lower than that induced by **CA**. In contrast, **CU** had no effect on Ag-stimulated degranulation.

To reveal the mechanisms underlying the inhibitory effects of **LN** and **CA** on Ag-stimulated degranulation in RBL-2H3 cells, we further examined the elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), production of intracellular reactive oxygen species (ROS), and early intracellular signaling events following Ag stimulation. Upon Ag-stimulated degranulation, $[Ca^{2+}]_i$ immediately increase through IP_3-mediated Ca^{2+} release of from the endoplasmic reticulum and influx from the extracellular medium.[26,27] Intracellular ROS generated by NADPH oxidase plays a crucial role in Ag-stimulated Ca^{2+} influx.[28–31] Both **LN** and **CA** significantly suppressed Ag-stimulated $[Ca^{2+}]_i$ elevation (Fig. 3) and ROS production (Fig. 4A). However, the suppression of ROS production following Ag stimulation by **LN** and **CA** were not due to their radical scavenging activities (Fig. 4B).

Ag-mediated aggregation of FcεRI on mast cells leads to the activation of multiple signal transduction pathways which play crucial roles in degranulation and cytokine production.[32] As shown in Figure 5, treatment with **LN** and **CA** suppressed the activation of spleen tyrosine kinase (Syk) and its downstream targets phospholipase Cγ1 and -2 (PLCγ1/2). On the other hand, neither **LN** nor **CA** showed inactivation of Lyn, a molecule located upstream of Syk. Thus, it was inferred that both **LN** and **CA** treatments attenuate Ag-stimulated Syk activation and thereby inhibit Ca^{2+} mobilization and ROS production.

Syk is one of the most important signaling molecules in Ag-stimulated degranulation.[33–35] Denyer and Patel also verified that the Syk inhibitor R-112 alleviates tree pollen-induced rhinitis.[36] In the present study, **LN** treatment also suppressed the signaling molecules downstream of Syk including phosphatidylinositol 3-kinase (PI3K)/Akt kinase (Akt), extracellular signal-regulated kinase-1 and -2 (ERK1/2), c-Jun *N*-terminal kinase (JNK), p38 mitogen-activated protein (MAP) kinase (p38), and cytosolic phospholipase A_2 (cPLA$_2$). On the other hand, although **CA** treatment inactivated Syk and PLCγ1/2, these downstream signaling molecules were not substantially suppressed (Fig. 5). In this study, we could not demonstrate in detail the mechanisms by which **CA** treatment regulates the downstream signaling molecules of Syk. Further study is needed to clarify these signaling pathways.

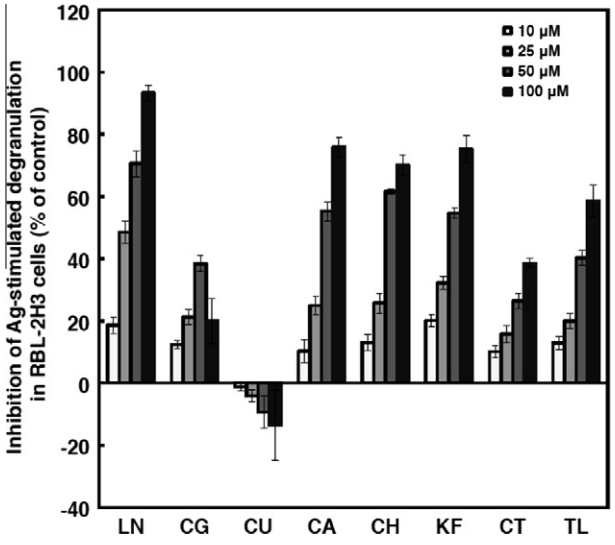

Figure 2. Effects of phenolic constituents and flavonoids isolated from *F. ananassa* Duch. on Ag-stimulated degranulation in RBL-2H3 cells. IgE-sensitized RBL-2H3 cells were treated with each compound (10–100 μM) and stimulated with antigen (DNP-BSA), and then subjected to β-hexosaminidase release assays. Values are means ± SEM (n = 10).

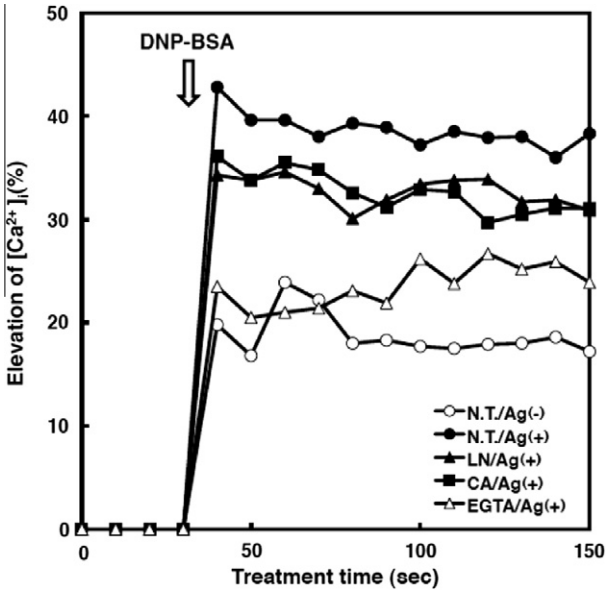

Figure 3. Effects of **LN** and **CA** on Ag-stimulated elevation of intracellular $[Ca^{2+}]_i$ in RBL-2H3 cells. IgE-sensitized RBL-2H3 cells were treated with or without 50 μM of **LN** or **CA** for 30 min. The cells were then stimulated with or without DNP-BSA, after which intracellular Ca^{2+} levels were measured. Values are means ± SEM (n = 12). Arrow, time at which DNP-BSA was added; filled circle, Ag-treated cells; open circle, non-Ag-treated cells; filled triangle, Ag- and **LN**-treated cells; filled square, Ag- and **CA**-treated cells; open triangle, Ag- and EGTA-treated cells. NT, non-treatment.

Taken together, our findings suggest that the inhibitory effect of **LN** and **CA** isolated from *F. ananassa* Duch. on Ag-stimulated degranulation is mainly due to the inactivation of Syk/PLCγs pathways (Fig. 6). Since we have demonstrated in a previous report that **KF** isolated from *F. ananassa* Duch. also significantly suppresses Syk activation following Ag stimulation, strawberry could be an effective food for alleviating symptoms of type I allergy.

3. Materials and methods

3.1. Extraction and isolation procedures

Strawberries ('Minomusume'; 9.62 kg) were squeezed and separated into juice (7.2 L) and residual substance. The juice (3.0 L) was concentrated under reduced pressure, and then subjected to an Amberlite XAD-7HP column chromatography (CC) eluted with distilled water and methanol. The methanol fraction was concentrated and the concentrate (74 g) was partitioned with ethyl acetate. The concentrated ethyl acetate-soluble phase (1.0 g) was separated by a Sephadex LH-20 CC, and four fractions (Fr. 1–4) were obtained upon elution with methanol. Fr. 2 (565 mg) was applied sequentially on a silica gel CC eluted with chloroform–methanol (1:0 to 10:1; v/v) in a step-wise manner to give three fractions (Fr. 2-1 to 2-3). The purification of Fr. 2-1 (39 mg) by a PTLC using chloroform–methanol (5:1) yielded cinnamic acid (**CA**; 3.7 mg). Further purification of Fr. 2-2 (122 mg) by PTLC using chloroform–methanol (10:3) afforded chrysin (**CH**; 2.2 mg). Fr. 2-3, upon purification by PTLC under the same conditions yielded 1-*O*-*trans*-cinnamoyl-β-D-glucopyranose (**CG**; 3.0 mg). Fr. 3 (123 mg) was purified by PTLC using chloroform–methanol (5:1) to give kaempferol (**KF**; 2.3 mg). The purification of Fr. 4 (126 mg) by PTLC using ethyl acetate–methanol (10:1) yielded catechin (**CT**; 8.0 mg). The

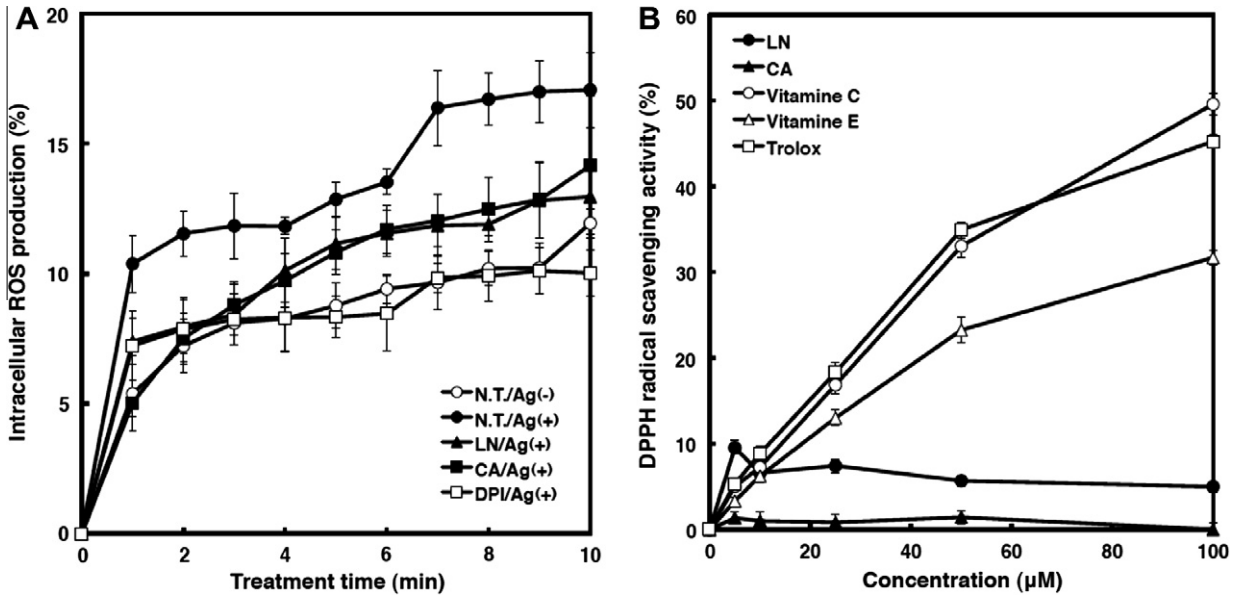

Figure 4. Effects of **LN** and **CA** on Ag-stimulated production of intracellular ROS in RBL-2H3 cells. (A) IgE-sensitized RBL-2H3 cells were treated with CM-H_2DCF-DA and incubated with or without 50 μM of **LN** or **CA**, or 200 nM of diphenyleneiodonium chloride (DPI) for 30 min. The cells were then stimulated with or without DNP-BSA, after which ROS-mediated DCF oxidation was measured. Values are means ± SEM (n = 10). NT, non-treatment. (B) Antioxidant activity of **LN** and **CA**. Vitamin C, E, and Trolox were used as potent antioxidative references. Values are means ± SEM (n = 12).

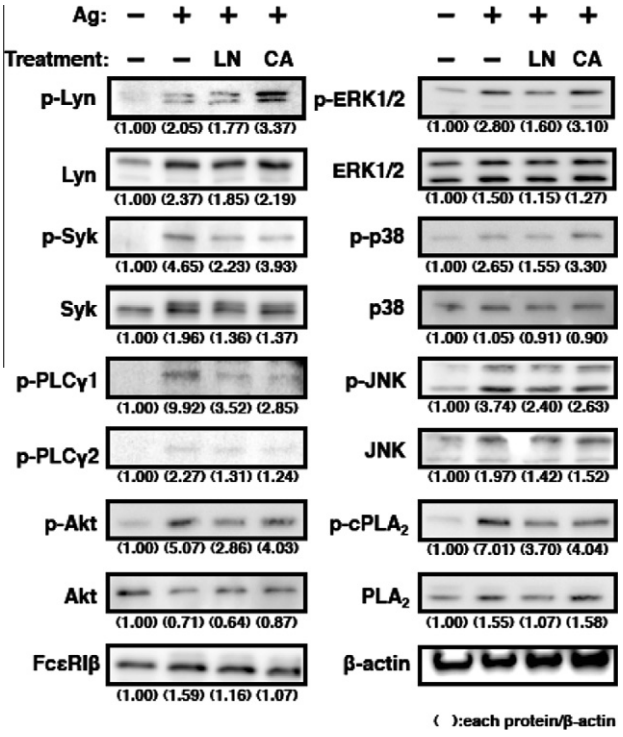

Figure 5. Effects of **LN** and **CA** on Ag-stimulated activation of intracellular signaling pathways in RBL-2H3 cells. IgE-sensitized RBL-2H3 cells were treated with or without 50 μM of **LN** or **CA** for 30 min. The cells were then stimulated with or without DNP-BSA. Cell lysates were subjected to Western blot analysis for the indicated proteins. A representative blot from three independent experiments is shown.

water layer after partitioning was concentrated and completely dried, and methanol was added to the residual substance to divide it into methanol-soluble and -insoluble phases. The concentrated methanol-soluble phase (14.9 g) was separated by a Sephadex LH-20 CC, and five fractions (Fr. 5–9) were obtained upon elution with methanol. Fr. 6 (291 mg), upon purification by PTLC using chloroform–methanol (5:1), yielded linocinnamarin (**LN**; 4.8 mg). The residual substance was extracted with 0.5% trifluoroacetic acid in methanol. The filtrate was concentrated under reduced pressure to give methanol extract (431 g), and then subjected to an Amberlite XAD-7HP CC eluted with distilled water and methanol. The methanol fraction was concentrated and further separated by a Sephadex LH-20 CC to obtain four fractions (Fr. 10–13) upon elution with methanol. Fr. 11 (10 g) was applied sequentially on a silica gel CC eluted with ethyl acetate–acetone–methanol (1:0:0 to 0:10:1; v/v) in a step-wise manner to give three fractions (Fr. 11-1 to 11-3). The purification of Fr. 11-2 (309 mg) by PTLC using chloroform–methanol (10:3) yielded *p*-coumaric acid (**CU**; 2.9 mg), kaempferol (**KF**; 3.2 mg), and *trans*-tiliroside (**TL**; 2.2 mg).

3.1.1. Linocinnamarin (LN)

Colorless syrup; FABMS: m/z 341 $[M+H]^+$; ^{1}H NMR (600 MHz, CD_3OD): δ 7.63 (1H, d, J = 16.0 Hz, H-7), 7.54 (2H, d, J = 8.7 Hz, H-2 and H-6), 7.10 (2H, d, J = 8.7 Hz, H-3 and H-5), 6.40 (1H, d, J = 16.0 Hz, H-8), 4.95 (1H, d, J = 7.3 Hz, H-1′), 3.89–3.84 (2H, m, H-6′α and H-6′β), 3.76 (3H, s, –COOCH_3), 3.75–3.66 (1H, m, H-3′), 3.51–3.41 (3H, m, H-2′, H-4′, and H-5′); ^{13}C NMR (150 MHz, CD_3OD): δ 168.1 (C-9), 159.6 (C-4), 144.5 (C-7), 129.5 (C-2 and C-6), 128.5 (C-1), 116.6 (C-8), 115.4 (C-3 and C-5), 100.5 (C-1′), 76.9 (C-5′), 76.6 (C-3′), 73.5 (C-2′), 69.9 (C-4′), 61.1 (C-6′), 50.8 (–COOCH_3).

3.1.2. 1-*O*-*trans*-Cinnamoyl-β-D-glucopyranose (CG)

Colorless syrup; FABMS: m/z 311 $[M+H]^+$; ^{1}H NMR (600 MHz, CD_3OD): δ 7.76 (1H, d, J = 15.8 Hz, H-7), 7.71–7.67 (2H, m, H-2 and H-6), 7.44–7.41 (3H, m, H-3, H-4, and H-5), 6.55 (1H, d, J = 15.8 Hz, H-8), 5.61 (1H, d, J = 8.5 Hz, H-1′), 3.80 (1H, dd, J = 13.0, 2.0 Hz, H-6′α), 3.67 (1H, dd, J = 13.0, 2.0 Hz, H-6′β), 3.51–3.48 (1H, m, H-3′), 3.43–3.39 (3H, m, H-2′, H-4′, and H-5′); ^{13}C NMR (150 MHz, CD_3OD): δ 164.9 (C-9), 157.8 (C-7), 145.7 (C-1), 130.6 (C-4), 129.1 (C-3 and C-5), 128.4 (C-2 and C-6), 117.7 (C-8), 94.7 (C-1′), 77.7 (C-3′), 77.1 (C-5′), 73.1 (C-2′), 70.3 (C-4′), 61.7 (C-6′).

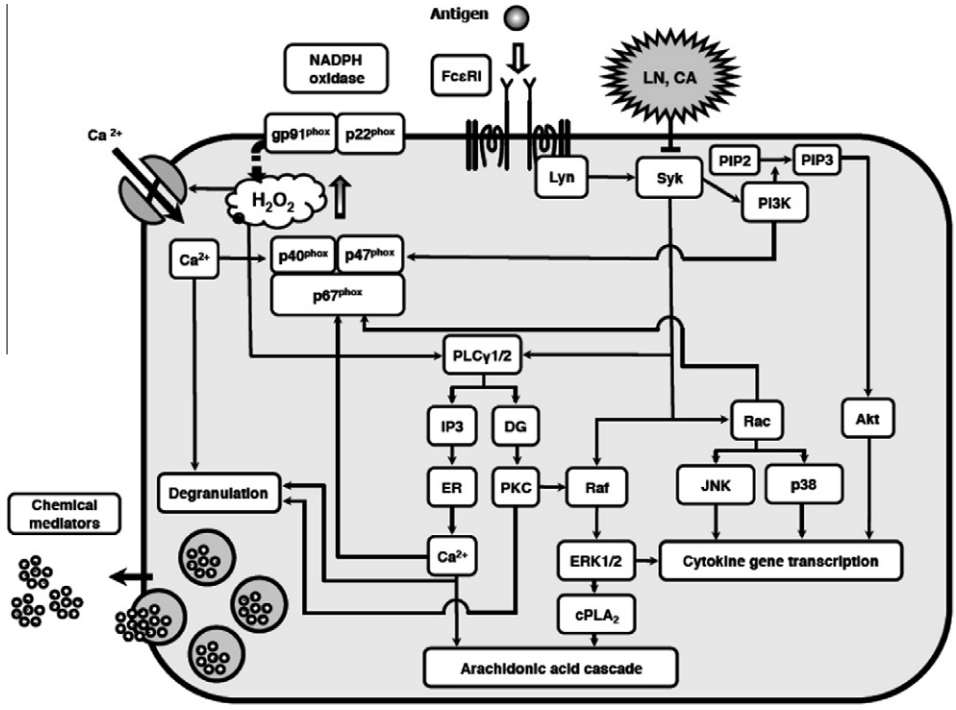

Figure 6. A scheme showing the inhibitory effects of **LN** and **CA** on Ag-stimulated degranulation in RBL-2H3 cells. **LN** and **CA** treatments inhibit Ag-stimulated degranulation through direct inhibition of Syk activation.

3.2. Bioassay methods

3.2.1. Reagents and materials

Mouse anti-dinitrophenol (DNP) monoclonal IgE was purchased from Yamasa (Tokyo, Japan). The 25× Complete®, a mixture of protease inhibitors, was obtained from Roche (Penzberg, Germany). The phosphatase Inhibitor Cocktails® 1 and 2 were from Sigma (MO, USA). The antibodies to Akt, phospho-Akt, p44/42 MAP kinase (ERK), phospho-p44/42 MAP kinase (Thr202/Tyr204) (p-ERK), SAPK/JNK (JNK), phospho-SAPK/JNK (Thr183/Tyr185) (p-JNK), p38 MAP kinase (p38), phospho-p38 MAP kinase (Thr180/Tyr182) (p-p38), Lyn, phospho-Lyn, $cPLA_2$, phospho-$cPLA_2$, phospho-PLCγ1, phospho-PLCγ2, phospho-Syk were from Cell Signaling Technology (MA, USA). The antibody to Syk was obtained from Santa Cruz Biotechnology (CA, USA). Anti-β-actin antibody was purchased from Sigma (MO, USA). The FcεRIβ antibody was kindly provided by Dr. J. Rivera (NIH, Molecular Immunology and Inflammation Branch). Anti-rabbit and -mouse antibodies conjugated with horseradish peroxidase and the ECL chemiluminescence kit were obtained from GE Healthcare (NJ, USA).

3.2.2. Cell culture

RBL-2H3 cells were obtained from the Health Science Research Resource Bank (Tokyo, Japan). Cells were grown in Eagle's minimum essential medium (Gibco, MD, USA) containing 10% heat-inactivated fetal bovine serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C.

3.2.3. Degranulation assay

β-Hexosaminidase release assay: We used β-hexosaminidase as a marker of degranulation. RBL-2H3 cells (2×10^4 cells/well) were seeded onto 24-well plates and cultured for 1 h. The cells were then treated with anti-DNP IgE at a concentration of 0.45 μg/ml and incubated for 24 h. After washing twice with Siraganian buffer A (NaCl 119 mM, KCl 5 mM, $MgCl_2$ 0.4 mM, PIPES 25 mM, and NaOH 40 mM, pH 7.2), 160 μl of Siraganian buffer B (5.6 mM glucose, 1 mM $CaCl_2$, and 0.1% BSA) was added to each well. After incubation at 37 °C for 10 min, the cells were treated with each sample at 37 °C for 30 min. Subsequently, 20 μl of DNP-labeled bovine serum albumin (DNP-BSA) was added to the culture medium at a concentration of 10 μg/ml. Ten minutes later, the cells were put on ice for 10 min to terminate the reaction, after which the supernatants were harvested by centrifugation at 300*g* at 4 °C for 10 min. The supernatants (50 μl) were transferred into 96-well microplates and reacted with 50 μl of 0.1 M citrate buffer (pH 4.5) including 1 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (CB-PNAG) at 37 °C. An hour later, the reaction was terminated by adding stop buffer (0.1 M Na_2CO_3/$NaHCO_3$, pH 10.0). The absorbance was measured at 405 nm using a colorimetric microplate reader.

β-Hexosaminidase inhibitory activity: On 96-well microplates, each sample (5 μl) and conditioned medium prepared from Ag-stimulated RBL-2H3 cells (45 μl) were reacted with 50 μl of 0.1 M CB-PNAG at 37 °C for 1 h. After terminating the reaction, the absorbance at 405 nm was measured. The inhibition of degranulation was calculated as follows:

$$\begin{aligned}&\text{Inhibition of degranulation } (\%)\\&= [1 - (A_{405\text{nm of sample}}\\&\quad - A_{405\text{nm negative control}})/(A_{405\text{nm positive control}}\\&\quad - A_{405\text{nm negative control}})] \times 100\\&\quad - \beta\text{-hexosaminidase inhibitory activity } (\%).\end{aligned} \tag{1}$$

$$\begin{aligned}&\beta\text{-Hexosaminidase inhibitory activity } (\%)\\&= [1 - (A'_{405\text{nm of sample}}\\&\quad - A'_{405\text{nm negative control}})/(A'_{405\text{nm positive control}}\\&\quad - A'_{405\text{nm negative control}})] \times 100.\end{aligned} \tag{2}$$

3.2.4. Measurement of intracellular Ca^{2+} concentrations

Intracellular Ca^{2+} levels were determined with Calcium Kit-Fluo 3™ (Dojindo Laboratories, Kumamoto, Japan). RBL-2H3 cells

(5×10^4 cells/well) were seeded onto 96-well microplates and incubated for 1 h. The cells were then treated with anti-DNP IgE (0.45 μg/ml) and incubated for 24 h. After washing twice with PBS, 100 μl of loading buffer containing Fluo-3AM (Calcium Kit-Fluo 3™) was added to the culture medium. An hour later, the cells were washed with PBS and incubated in 90 μl of loading buffer containing 50 μM of sample. After incubation for 30 min, the cells were stimulated by DNP-BSA (10 μg/ml). Fluorescence was measured using a fluorometric imaging plate reader (excitation; 490 nm, emission; 530 nm).

3.2.5. Measurement of intracellular ROS levels

Intracellular ROS levels were measured using CM-H_2DCFDA. IgE-sensitized RBL-2H3 cells (5×10^4 cells/well) were incubated with 10 μM CM-H_2DCFDA for 30 min at 37 °C. After washing twice, the cells were incubated with 50 μM of sample for 30 min. The cells were then stimulated by DNP-BSA (10 μg/ml), and fluorescence was measured using a fluorometric imaging plate reader (excitation; 490 nm, emission; 530 nm).

3.2.6. Measurement of DPPH radical scavenging activity

To measure in vitro antioxidant activity, a DPPH radical-scavenging assay was carried out as described previously.[37] Briefly, 0.1 ml of a 0.5 mM DPPH radical solution, 0.8 ml of 99% ethanol, and 0.1 ml of sample were rapidly mixed. Subsequently, the decrease in absorbance at 517 nm was monitored. The DPPH free radical scavenging activity (%) was calculated using the following formula: $[(A_{517\text{nm of control}} - A_{517\text{ nm of sample}})/A_{517\text{ nm of control}}] \times 100$. Vitamin C, E, and Trolox, which are potent antioxidants, were used as positive controls.

3.2.7. Immunoblot analysis

Cell lysates were prepared as described previously.[38] Protein samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and then transferred onto polyvinylidene fluoride (PVDF) membranes. After blocking for 1 h in 5% nonfat milk, the membrane was incubated with a primary antibody at 4 °C overnight, followed by incubation with a horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. Immunoreactive proteins were detected with the enhanced ECL kit and chemiluminescence detector (LAS-4000, Fujifilm, Japan).

4. Conclusions

We performed a chemical study of *F. ananassa* Duch. and identified eight phenolic constituents. Among these, linocinnamarin (**LN**) was isolated from the Rosaceae family for the first time. Furthermore, we demonstrated the inhibitory effects of phenylpropanoid glycosides and other phenolics isolated from *F. ananassa* Duch. on antigen (Ag)-stimulated degranulation in rat basophilic leukemia RBL-2H3 cells. Linocinnamarin (**LN**) and cinnamic acid (**CA**) suppressed Ag-stimulated degranulation through direct inactivation of Syk. Our findings suggest that linocinnamarin (**LN**) and cinnamic acid (**CA**) isolated from *F. ananassa* Duch. could be beneficial for alleviating symptoms of type I allergy.

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