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Ishizuchi dark tea suppresses IgE-mediated degranulation of RBL-2H3 cells and nasal rubbing behavior of pollinosis in mice

Michiyo Kondo ^a, Kosuke Nishi ^a, Takuya Sugahara ^{a,b,c,*}

^a Faculty of Agriculture, Ehime University, Matsuyama, Ehime 790-8566, Japan

^b Food and Health Sciences Research Center, Ehime University, Matsuyama, Ehime 790-8566, Japan

^c South Ehime Fisheries Research Center, Ehime University, Ainan, Ehime 798-4205, Japan

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ABSTRACT

Ishizuchi dark tea is a microorganism-fermented tea produced in Ehime, Japan. We herein describe the anti-allergic effect of Ishizuchi dark tea. The water extract from Ishizuchi dark tea suppressed degranulation of RBL-2H3 cells in a dose-dependent manner with no apparent cytotoxicity. Fractionation and β -hexosaminidase release assay of the tea components revealed theabrownins as the major bioactive substance in Ishizuchi dark tea. Phosphorylation of Syk, a tyrosine kinase, tended to be suppressed by treating RBL-2H3 cells with theabrownins. In addition, Ishizuchi dark tea suppressed an allergic symptom in the Cry j1-induced pollinosis model mice. Taken together, these data indicated that Ishizuchi dark tea would be a beneficial functional food with an anti-allergic effect.

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1. Introduction

Allergy is a hypersensitivity reaction in response to a certain allergen, resulting in various symptoms such as asthma, hay fever, hives, and life-threatening anaphylaxis (Galli, Tsai, & Piliponsky, 2008; Simons, 2010). Approximately 25% of the population in developed countries is estimated to suffer from allergic symptoms (Galli et al., 2008). Immediate-type allergy is a disorder of the immune system provoked by the excessive activation of mast cells, a critical effector involved in allergic reactions, via an allergen-specific immunoglobulin E (IgE). Stimulation by an allergen via IgE bound on the high-affinity IgE

receptor (FcεRI) expressed on the surface of mast cells leads to initiation of the release of chemical mediators such as histamine, eicosanoids, and prostaglandins stored in cytoplasmic granules by fusing with the plasma membrane, which is termed degranulation, and to synthesis of leukotrienes, chemokines, and cytokines in the process of mast cell degranulation (Oliver, Kepley, Ortega, & Wilson, 2000). Released chemical mediators are involved in various allergic symptoms. Thus, prevention of mast cell degranulation is one of the most effective approaches to attenuate allergic symptoms.

Tea, an extract from leaves of the plant *Camellia sinensis*, is one of the most widely consumed beverages all over the world. The health benefits of tea have been confirmed in a huge

* Corresponding author. Faculty of Agriculture, Ehime University, 3-5-7 Tarumi, Matsuyama, Ehime 790-8566, Japan. Tel.: +81 89 946 9863; fax: +81 89 946 9863.

E-mail address: mars95@agr.ehime-u.ac.jp (T. Sugahara).

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number of biological, nutritional, and epidemiological studies and summarized in several reviews (da Silva Pinto, 2013; Dufresne & Farnworth, 2001; McKay & Blumberg, 2002; Namal Senanayake, 2013). Depending on the manufacturing process, tea is classified into four major types: non-fermented tea (e.g. green tea), semi-fermented tea (e.g. oolong tea), fermented tea (e.g. black tea), and post-fermented tea (e.g. pu-erh tea and dark tea) (Chen, Shurlknight, Leung, & Sang, 2012). Post-fermented tea is further classified into three types: aerobically fermented tea, anaerobically fermented tea, and both aerobically and anaerobically (two-step) fermented tea (Miyagawa, Otsubo, & Katafuchi, 1989). Ishizuchi dark tea, a traditional pickled tea produced in Ehime, Japan, is manufactured by aerobic fermentation with fungi such as *Aspergillus*, *Debaryomyces*, and *Candida*, followed by anaerobic fermentation with bacteria such as *Lactobacillus*. Most of post-fermented tea is manufactured through the single-step fermentation, and hence the two-step-fermented tea, including Ishizuchi dark tea, is quite unique in the world.

Several bioactive compounds have been found in tea. Catechins are well known compounds contained in green tea and account for approximately 30% of the dry weight of tea leaves (Balentine, Wiseman, & Bouwens, 1997). During the oxidative processing, catechins are oxidized to theaflavins (TF) and thearubigins (TR) and these compounds are responsible for the color in oolong and black teas (da Silva Pinto, 2013). Pu-erh tea, a post-fermented tea, contains theabrownins (TB) that can be dissolved in water but not in ethyl acetate, *n*-butanol, or other organic solvents. The TB content in pu-erh tea ranges from 10 to 14% with an average of 12% (Liang, Zhang, & Lu, 2005). Although the chemical composition of TB is still unknown, TB has some biological activities such as a serum lipid-lowering effect (Gong, Peng, Chen, Gao, & Zhou, 2010) and a suppressive effect on proliferation and differentiation of 3T3-L1 preadipocytes (Yang et al., 2014).

We have screened various dietary ingredients involved in attenuation of allergic symptoms, and consequently found Ishizuchi dark tea with the anti-allergic functionality. The objectives of the present study were to assess the anti-allergic effect of Ishizuchi dark tea and to elucidate the class of bioactive substances that exhibit a beneficial anti-allergic effect. Experiments were designed to examine the anti-allergic effect of Ishizuchi dark tea using rat basophilic leukemia RBL-2H3 cells that have been commonly used for the screening of substances inhibiting mast cell degranulation *in vitro* (Ikawati, Wahyuono, & Maeyama, 2001) and in a murine model of polinosis *in vivo*. Our data suggest that Ishizuchi dark tea would be involved in attenuation of allergic symptoms. The findings demonstrated in the present study would be of significance in providing a new dimension to the tea functionality.

2. Materials and methods

2.1. Materials

Dried leaves of Ishizuchi dark tea and green tea were kindly provided by Satsuki-kai (Ehime, Japan), the manufacturer of Ishizuchi dark tea. Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, thapsigargin, bovine serum albumin (BSA), mouse anti-dinitrophenol (DNP) monoclonal IgE,

DNP-human serum albumin (HSA) conjugates, fetal bovine serum (FBS), Triton X-100, and HPLC grade acetonitrile were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultra pure water (18.2 MΩ) was prepared in a Milli-Q system (Millipore, Billerica, MA, USA) and used for HPLC analysis. Epigallocatechin gallate (EGCG) was purchased from Mitsui Norin (Tokyo, Japan) and dissolved in dimethyl sulfoxide at 100 mM. Goat anti-actin antibody and horseradish peroxidase (HRP)-labeled anti-goat IgG antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HRP-labeled anti-rabbit IgG antibody and rabbit antibodies against phosphoinositide 3-kinase (PI3K) p85, phosphorylated PI3K p85/p55, Syk, phosphorylated Syk, phospholipase C (PLC) γ1, phosphorylated PLCγ1, PLCγ2, and phosphorylated PLCγ2 were purchased from Cell Signaling Technology (Danvers, MA, USA). All other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan), unless otherwise noted.

2.2. Sample preparation

Dried leaves of Ishizuchi dark tea were suspended in deionized distilled water at 0.25 g/mL and extracted at 121 °C for 20 min. After centrifugation at 10,000 × *g* for 20 min at 4 °C, the supernatant was collected and dialyzed against 10 mM sodium phosphate buffer (NaPB, pH 7.4) at 4 °C for 4 days using a dialysis membrane (Wako Pure Chemical Industries) with molecular weight cutoff of 14 kDa. The Ishizuchi dark tea (IDT) extract was then filtrated through a 0.22 μm membrane filter and used for the experiments described below. A water extract from dried leaves of green tea, abbreviated as GTE, was also prepared by the same method as described above for IDT preparation and used for the β-hexosaminidase release assay described below. Dry weight was measured by weighing a portion of freeze-dried samples.

2.3. Cells and cell culture

RBL-2H3 cells were obtained from American Type Culture Collection (Rockville, MD, USA) and cultured in DMEM supplemented with 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 5% FBS at 37 °C under a humidified 5% CO₂-95% air atmosphere.

2.4. β-Hexosaminidase release assay

2.4.1. Antigen-induced β-hexosaminidase release assay

The assay was performed as previously described (Ishida, Nishi, Watanabe, & Sugahara, 2013) with some modifications. RBL-2H3 cells were seeded at 4.0×10^4 cells/well in a 96-well culture plate (BD Falcon, Franklin Lakes, NJ, USA) and cultured at 37 °C overnight. The cells were subsequently sensitized with the anti-DNP IgE (50 ng/mL) diluted in the medium for 2 h. After washing with the modified Tyrode's (MT) buffer (20 mM HEPES, 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, and 0.05% BSA, pH 7.4) twice, the cells were treated with various concentrations of samples or vehicle (control) diluted in the MT buffer and incubated at 37 °C for 10 min. The cells were then stimulated with DNP-HSA at the final concentration of 50 ng/mL. After incubation for 30 min, the medium was collected from each well, and the cells were sonicated in 130 μL

of the MT buffer containing 0.1% Triton X-100 for 5 s on ice. Both the media and cell lysates were transferred to a new 96-well microplate at 50 μ L/well and incubated at 37 °C for 5 min. Then, 100 μ L of 3.3 mM 4-nitrophenyl 2-acetamido-2-deoxy- β -D-glucopyranoside dissolved in 0.1 M citrate buffer (pH 4.5) were added to each well and incubated at 37 °C for 25 min. The enzyme reaction was terminated by adding 100 μ L of 2 M glycine buffer (pH 10.4), and the absorbance was measured at 405 nm using a microplate reader (SH-8000Lab, Corona Electric, Ibaraki, Japan). A β -hexosaminidase release rate was calculated as:

$$100 \times \left[\frac{A_{\text{supernatant}} - A_{\text{blank of supernatant}}}{\{(A_{\text{supernatant}} - A_{\text{blank of supernatant}}) + (A_{\text{cell lysate}} - A_{\text{blank of cell lysate}})\}} \right]$$

in which “A” is the absorbance of each well.

2.4.2. Thapsigargin-induced β -hexosaminidase release assay
RBL-2H3 cells were seeded at 4.0×10^4 cells/well in a 96-well culture plate and cultured at 37 °C overnight. After washing with the MT buffer twice, the cells were treated with various concentrations of samples or water (control) diluted in the MT buffer and incubated at 37 °C for 10 min. The cells were subsequently stimulated by adding 10 μ L of thapsigargin diluted in the MT buffer at the final concentration of 0.5 μ M to each well, and the cells were incubated at 37 °C for 30 min. Then, the β -hexosaminidase release rate was measured as described above.

2.5. Cell viability assay

Cytotoxicity of IDT in RBL-2H3 cells was examined using a WST-8 assay kit (Kishida Chemical, Osaka, Japan) as described previously (Onishi et al., 2014), with some modifications. In brief, RBL-2H3 cells sensitized with the anti-DNP IgE as described above were treated with various concentrations of IDT or with NaPB diluted in the MT buffer and then stimulated with DNP-HSA as above. After washing wells of the 96-well culture plate with phosphate-buffered saline (PBS, pH 7.4) once, 100 μ L of DMEM containing 10% WST-8 solution were added to each well, and the cells were incubated for 15 min at 37 °C. The absorbance was then measured at 450 nm using a microplate reader (Model 650, Bio-Rad Laboratories).

2.6. HPLC analysis

2.6.1. Reversed-phase chromatography

Reversed-phase HPLC analysis was performed using an Acquity UPLC system (Waters, Milford, MA, USA). Chromatographic separations were conducted using an Acquity UPLC BEH C_{18} column (1.7 μ m, 2.1×50 mm, Waters). A linear gradient elution program was applied as follows: 0–1.5 min (10% acetonitrile + 0.1% formic acid); 1.5–5 min (10–35% acetonitrile + 0.1% formic acid); 5–6 min (35% acetonitrile + 0.1% formic acid); 6–8 min (35–100% acetonitrile + 0.1% formic acid); 8–10 min (100% acetonitrile + 0.1% formic acid). Flow rate was maintained at 0.5 mL/min, and temperature of the column was set at 35 °C. An injection volume was set up in 10 μ L, and the chromatogram monitored at a wavelength of 280 nm.

2.6.2. Size exclusion chromatography

Size exclusion chromatography analysis was performed on a LaChrom Elite HPLC system (Hitachi, Tokyo, Japan) with an TSK-gel G4000SW_{XL} column (7.8 $\times$ 300 mm, Tosoh, Tokyo, Japan). The column was pre-equilibrated with PBS, and temperature of the column was set at 15 °C. Two hundred μ L of samples were applied to the column and eluted with PBS with the flow rate at 1.0 mL/min. The chromatogram was monitored at a wavelength of 280 nm. A gel filtration molecular weight markers kit (Sigma-Aldrich) was used to acquire a calibration curve.

2.7. Sample fractionation

IDT was fractionated as shown in Fig. 1 according to a couple of previous papers (Chitpan, Wang, Ho, & Huang, 2007; Halder, Pramanick, Mukhopadhyay, & Giri, 2006; Peng, Liu, Liu, Zhou, & Gong, 2013), with some modifications. At first, 20 mL of IDT (3.1 mg/mL) was concentrated to 1/4 of its original volume under reduced pressure and partitioned with ethyl acetate. The collected organic layer was used as a TF fraction. The aqueous layer was next partitioned with *n*-butanol, and the collected organic layer was used as a TR fraction. Four volumes of ethanol were added to the aqueous layer and centrifuged at $2340 \times g$ for 10 min. Both, the supernatant and precipitate were collected each, and the precipitate was used as a TB fraction. Each organic layer was evaporated and all fractions were reconstituted in 20 mL of deionized distilled water with heating at 70 °C for 1 to 6 h.

2.8. Immunoblot analysis

RBL-2H3 cells were seeded at 1.8×10^6 cells in 50 mm culture dishes (BD Falcon), and cultured overnight. After sensitizing with the anti-DNP IgE at the same concentration as described above, the cells were washed with the MT buffer and treated with 1.2 mg/mL of TB or with water (control) diluted in the MT buffer for 10 min. The cells were subsequently stimulated for 5 min with DNP-HSA at the final concentration of 50 ng/mL. After removing the added reagents, cells were lysed

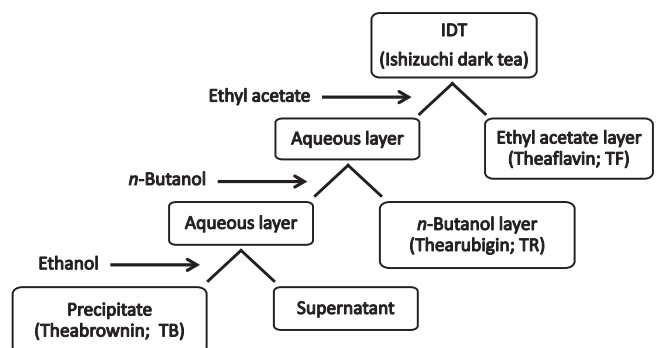


Fig. 1 – A flowchart depicting the fractionation of IDT. IDT was first partitioned with ethyl acetate. After transferring the ethyl acetate fraction (TF), the aqueous layer was further partitioned with *n*-butanol. After transferring the *n*-butanol fraction (TR), four volumes of ethanol were added to the aqueous layer and centrifuged. Supernatant and precipitate (TB) were obtained.

and centrifuged at $14000 \times g$ for 15 min at 4°C . After each supernatant was equalized to the same protein concentration, proteins in a total cell lysate were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane (Hybond-P, GE Healthcare, Buckinghamshire, UK). Immunoblotting with various antibodies was performed as previously described (Nishi et al., 2014).

2.9. Murine pollinosis model

A total of 22 female BALB/c mice (5-week-old) were purchased from Japan SLC (Shizuoka, Japan) and kept in a temperature-controlled facility ($24 \pm 1^\circ\text{C}$) with a 12-h light/12-h dark cycle. Animals received standard chow and water *ad libitum*. Following a 1-week adaptation period, mice were randomly divided into 3 groups of eight or seven mice as follows: control group (7 mice), IDT-treated group (7 mice), and intact group (8 mice). The intact and control groups were given water *ad libitum*, while the IDT-treated group was given IDT (3.1 mg/mL) instead of water *ad libitum*. A mouse model of Japanese cedar pollinosis was developed according to Nomiya et al. (2008), and treatment of mice with Cry j1 was conducted as scheduled in Fig. 2. Mice in the control and IDT-treated groups were sensitized with $1.0\ \mu\text{g}$ of the purified Cry j1 (Hayashibara, Okayama, Japan) dissolved in $10\ \mu\text{L}$ of PBS, while the intact group was treated with PBS only, by the intranasal administration using a $10\ \mu\text{L}$ glass microsyringe (Hamilton, Reno, NV, USA) on days 0, 7, and 14. Next, mice in the control and IDT-treated groups were challenged by intranasal administration of $0.2\ \mu\text{g}$ of Cry j1 in $10\ \mu\text{L}$ of PBS for 6 consecutive days on days 21 to 26, while the intact mice were given PBS only instead of the antigen. On day 27, all mice including the intact group were treated with $0.2\ \mu\text{g}$ of Cry j1. Immediately after the final challenge, nasal rubbing frequency was counted for 30 min. All animal experiments described herein were carried out in accordance with the protocol approved by the Laboratory Animal Care Committee of Ehime University. Mice were maintained in accordance with the Guidelines for the Care and Use of Laboratory Animals of Ehime University.

Control and IDT-treated groups

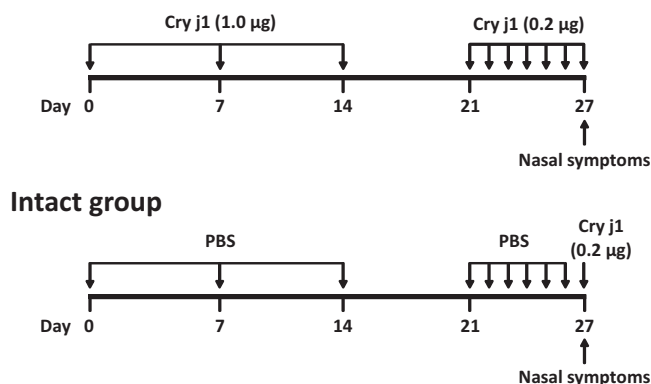


Fig. 2 – Schematic depiction of the experimental protocol used for induction of pollinosis using Cry j1 in BALB/c mice. PBS, phosphate-buffered saline.

2.10. Enzyme-linked immunosorbent assay (ELISA)

Serum IgE and IgG₁ levels were measured by in-house-developed ELISA as described in Nishimoto et al. (2009) with some modifications. In brief, $100\ \mu\text{L}$ of rat anti-mouse IgE antibody (BD Pharmingen, San Diego, CA, USA) or rat anti-mouse IgG antibody (MP Biomedicals, Santa Ana, CA, USA) diluted in 50 mM sodium carbonate-bicarbonate buffer (pH 9.6) was added at $1.0\ \mu\text{g/mL}$ to each well of a 96-well microtitre plate (Nunc, Roskilde, Denmark) and incubated at 4°C overnight. After washing with 0.05% Tween 20-PBS (PBS-T) 3 times, each well was blocked with 5% skim milk in PBS for 2 h at 37°C . After washing with PBS-T 3 times, each well was treated with $50\ \mu\text{L}$ of standards or mouse sera diluted in 5% skim milk-PBS for 1 h at 37°C . After washing with PBS-T 3 times, each well was treated with $100\ \mu\text{L}$ of biotinylated rat anti-mouse IgE antibody (BD Pharmingen) or biotinylated rat anti-mouse IgG₁ antibody (Molecular Probes, Carlsbad, CA, USA) diluted 1000 times in 5% skim milk-PBS for 1 h at 37°C . After washing with PBS-T 3 times, $100\ \mu\text{L}$ of streptavidin-HRP conjugate (Molecular Probes) diluted in 1% BSA-PBS were added to each well and incubated for 1 h at room temperature. After washing with PBS-T 3 times, $0.6\ \text{mg/mL}$ of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) dissolved in a 0.03% H_2O_2 -0.05 M citrate buffer (pH 4.0) was added at $100\ \mu\text{L/well}$, and the absorbance at 415 nm was measured after adding $100\ \mu\text{L/well}$ of 1.5% oxalic acid for termination of the coloring reaction. Ig concentrations were quantitatively determined by a standard curve using the mouse IgE or IgG₁ standards. Assays were conducted in triplicate.

2.11. Statistical analysis

Data obtained were expressed as the mean $\pm$ standard deviation (SD). Dunnett's test or Tukey–Kramer test was used to assess the statistical significance of the difference. *p*-Values < 0.05 or < 0.01 were considered statistically significant.

3. Results

3.1. Effect of IDT on degranulation of RBL-2H3 cells

First, we investigated whether IDT possesses an anti-allergic activity by assessing its impact on mast cell degranulation using RBL-2H3 cells. Anti-DNP IgE-sensitized RBL-2H3 cells were treated with various concentrations of IDT or with NaPB (control), and degranulation was induced by treating the cells with DNP-HSA. β -Hexosaminidase release was used as a marker of mast cell degranulation. As shown in Fig. 3A, the β -hexosaminidase release from intracellular granules in RBL-2H3 cells was significantly reduced by treating cells with IDT depending on its concentration, indicating that IDT suppresses degranulation of RBL-2H3 cells in a dose-dependent manner. The β -hexosaminidase release rate was decreased by 77% compared with the control, when cells were treated with $0.39\ \text{mg/mL}$ of IDT. Significant differences were observed against the control in the β -hexosaminidase release rate at $97\ \mu\text{g/mL}$ or higher concentrations of IDT. An effect of IDT on the viability of RBL-2H3 cells was assessed by the WST-8 assay. As

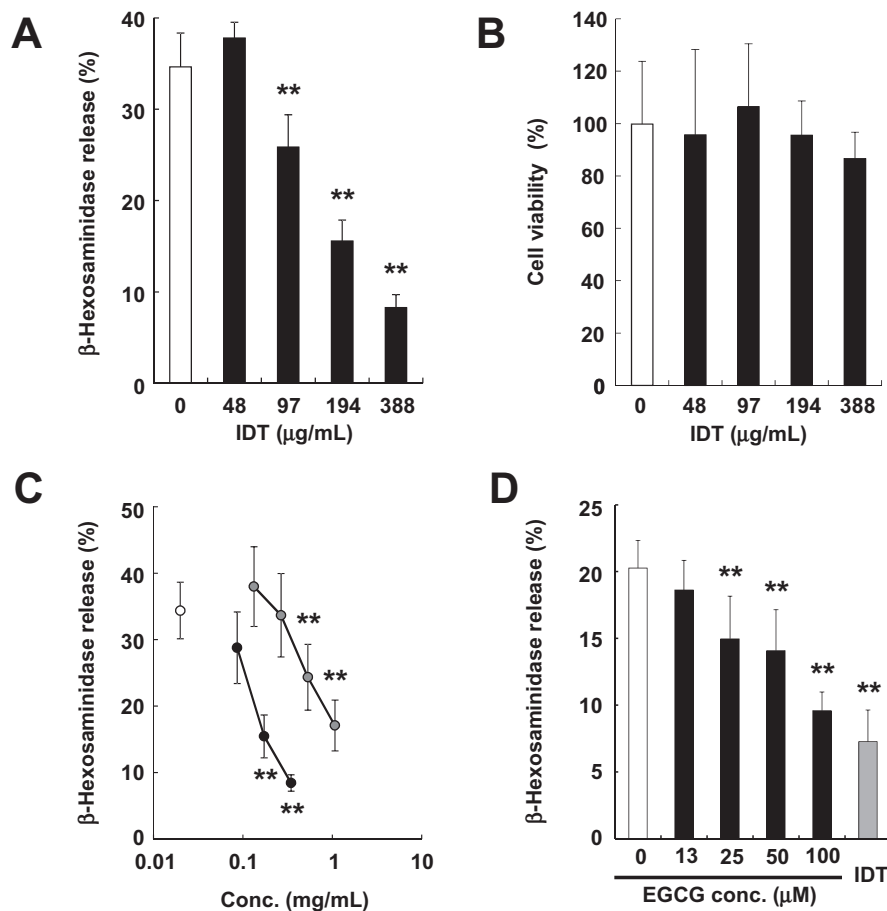


Fig. 3 – Effects of IDT and GTE on degranulation and viability of RBL-2H3 cells. All data are represented as the mean $\pm$ SD ($n = 6$). All data were assessed using a Dunnett's test for significance of difference vs. control; $**p < 0.01$. (A) Effect of IDT on the Fc ϵ RI-mediated β -hexosaminidase release from RBL-2H3 cells. Anti-DNP IgE-sensitized cells were treated with indicated concentrations of IDT or with NaPB (control), and degranulation was induced with DNP-HSA. Released β -hexosaminidases were used as a marker of degranulation. (B) Effect of IDT on the viability of RBL-2H3 cells. Viability of RBL-2H3 cells was assessed using the WST-8 solution after the anti-DNP IgE-sensitized cells were treated with indicated concentrations of IDT or with NaPB (control), followed by stimulation with DNP-HSA. Absorbance was then measured at 450 nm. (C) Effect of tea extracts on the Fc ϵ RI-mediated β -hexosaminidase release from RBL-2H3 cells. Anti-DNP IgE-sensitized cells were treated with the indicated concentrations of IDT (closed circle) or GTE (gray circle) or with NaPB (open circle), and degranulation was induced with DNP-HSA. Released β -hexosaminidases were used as a marker of degranulation. (D) Effect of EGCG on the Fc ϵ RI-mediated β -hexosaminidase release from RBL-2H3 cells. Anti-DNP IgE-sensitized cells were treated with the indicated concentrations of EGCG or IDT (0.48 mg/mL) or with NaPB, and degranulation was induced with DNP-HSA. Released β -hexosaminidases were used as a marker of degranulation. IDT, Ishizuchi dark tea; EGCG, epigallocatechin gallate.

shown in Fig. 3B, cytotoxicity of IDT in RBL-2H3 cells was not noted at all concentrations tested, indicating that the suppressive effect of IDT on degranulation of RBL-2H3 cells was not caused by cell death. The effect of GTE, a water extract of green tea, on mast cell degranulation was also assessed to compare the anti-allergic effect with that of IDT. Granule release from RBL-2H3 cells caused by the antigen-induced degranulation was also significantly decreased by treating cells with GTE, although its suppressive activity was weaker than that of IDT (Fig. 3C). From these results, both IDT and GTE were found to have a potential to inhibit mast cell degranulation at the concentrations indicated in Fig. 3C. The effect of EGCG on degranulation of RBL-2H3 cells was also examined. Because EGCG is the most well-known substance contained in tea leaves,

which displays anti-allergy activities (Inoue, Suzuki, & Ra, 2010), there was a possibility that EGCG is a bioactive substance in IDT. As shown in Fig. 3D, EGCG exhibited a suppressive effect on degranulation in a dose-dependent manner. However, IDT (0.39 mg/mL) suppressed degranulation stronger than EGCG even at 100 μ M, indicating that EGCG is not the main bioactive substance in IDT.

3.2. Measurement of the EGCG amount in IDT

The amount of EGCG in IDT was measured by reversed-phase HPLC analysis. Retention time of EGCG was observed at 2.7 min (Fig. 4A). The peak of EGCG was observed in both IDT and GTE at the corresponding retention time (Fig. 4B and 4C),

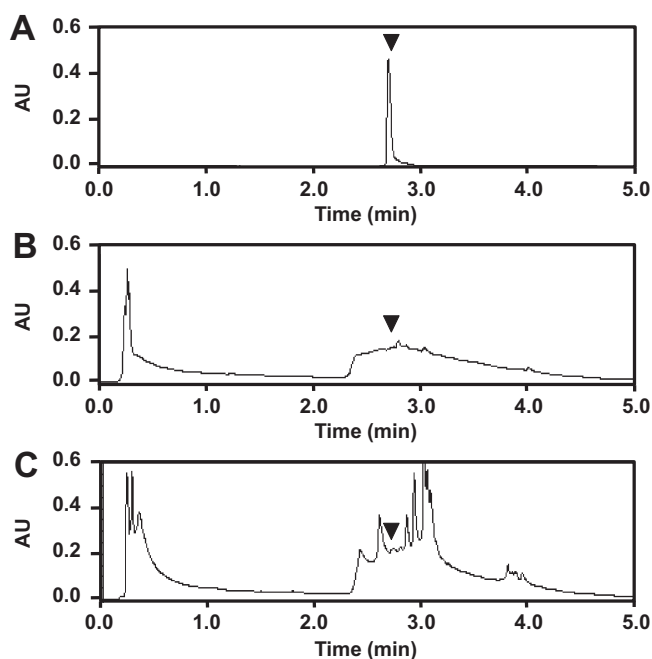


Fig. 4 – Reversed-phase HPLC analysis of EGCG in IDT. Arrows indicate the peak of EGCG. A representative chromatogram from three independent experiments is shown. (A) A chromatogram of EGCG (1 nmol). (B) A chromatogram of IDT (28 µg). (C) A chromatogram of GTE (77 µg).

indicating that EGCG was present in IDT. The EGCG concentration was estimated to be around 30 µM in 2.8 mg/mL of IDT, while GTE (7.7 mg/mL) contained approximately 50 µM. We also prepared an ethanol extract from the leaves of Ishizuchi dark

tea as well as the water extract and found that the ethanol extract does not suppress degranulation of RBL-2H3 cells at all (data not shown).

3.3. Effect of pigment components in IDT on degranulation of RBL-2H3 cells

Polyphenols such as TF, TR, and TB are the most common pigment compositions in fermented tea. IDT was fractionated into these three components by liquid–liquid distribution, and the fractions obtained were subjected to the β -hexosaminidase release assay. The result showed that TR and TB significantly suppressed degranulation, whereas TF and supernatant did not (Fig. 5A). In particular, TB strongly inhibited degranulation of RBL-2H3 cells. These findings suggested that TB is the major bioactive substance in IDT.

The effect of TB on the Fc ϵ RI-independent degranulation was examined using thapsigargin. As shown in Fig. 5B, all tested samples including IDT showed no suppressive effect on degranulation of RBL-2H3 cells, indicating that TB would affect the early signaling events involved in mast cell degranulation evoked through Fc ϵ RI.

3.4. Molecular weight of the bioactive substance

To examine the molecular weight of the bioactive substances in IDT, the size exclusion chromatography analysis was conducted. Fig. 6A shows that only a single peak was observed in IDT. According to a calibration curve obtained with the gel filtration molecular weight makers, a size distribution pattern of the major substance in IDT was shown to be 24 ± 3 kDa. In addition, TB was also subjected to the size exclusion chromatography analysis. Again, there was only a single peak in the chromatogram and the estimated molecular weight was 24 ± 3 kDa, suggesting that the major component in IDT would be TB.

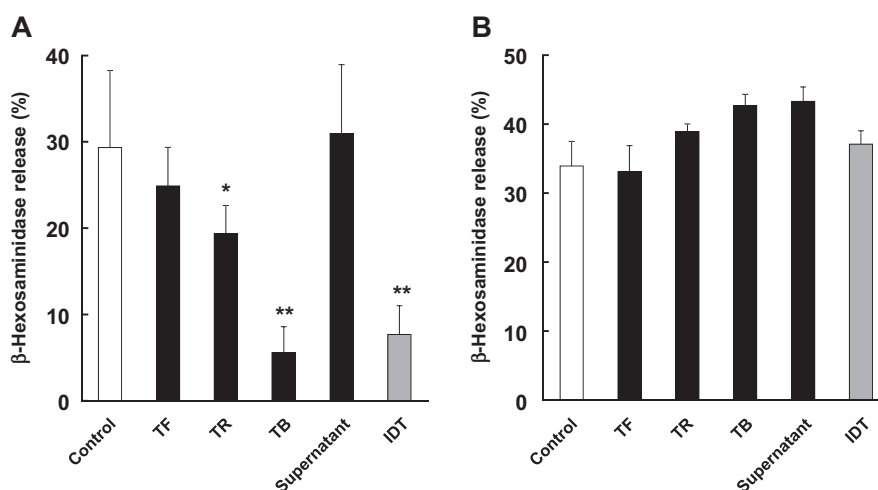


Fig. 5 – Effect of IDT components on degranulation of RBL-2H3 cells. Data are represented as the mean $\pm$ SD. All data were assessed using a Dunnett's test for significance of difference vs. control; * $p < 0.05$, ** $p < 0.01$. (A) Anti-DNP IgE-sensitized cells were treated with TF, TR, TB, supernatant, IDT, or water (control), and degranulation was induced with DNP-HSA. Released β -hexosaminidases were used as a marker of degranulation. $n = 5$ –7. (B) RBL-2H3 cells were treated with TF, TR, TB, supernatant, IDT, or water (control), and degranulation was induced with 0.5 µM thapsigargin. Released β -hexosaminidases were used as a marker of degranulation. $n = 6$. TF, theaflavins; TR, thearubigins; TB, theabrownins; IDT, Ishizuchi dark tea.

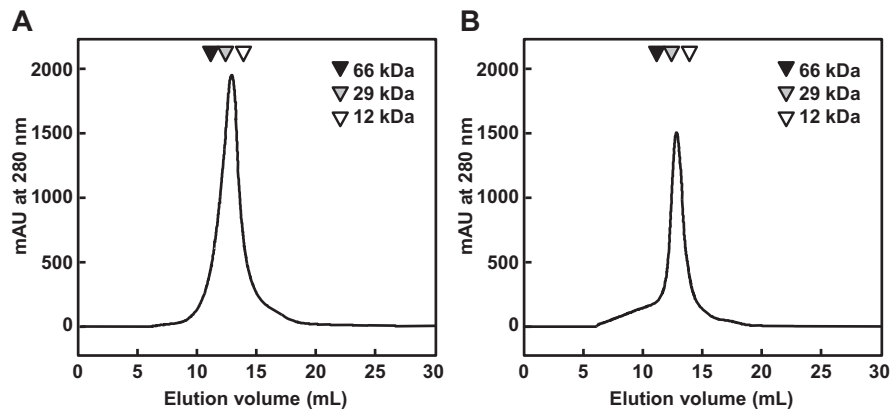


Fig. 6 – Size exclusion chromatography analysis of IDT and TB. A representative chromatogram from three independent experiments is shown. Chromatographic analysis was monitored at 280 nm. Black, gray, and white triangles indicate the elution volumes of albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (MW: 12 kDa), respectively. (A) A chromatogram of IDT (620 µg). (B) A chromatogram of TB (920 µg).

3.5. Effect of TB on the signaling pathways involved in degranulation of RBL-2H3 cells

To elucidate the molecular mechanism underlying the suppressive effect of IDT on degranulation of RBL-2H3 cells, we examined whether TB affects phosphorylation of kinases and PLC γ in the signal pathways mediated by Fc ϵ RI in RBL-2H3 cells

by immunoblot analysis. As seen in Fig. 7, phosphorylation of Syk, a tyrosine kinase, was decreased by treating cells with TB, although there was no statistical significance against the antigen-treated control cells. In addition, phosphorylation of PLC γ 1/2 was also slightly decreased along with that of Syk (Fig. 7). On the other hand, TB did not affect phosphorylation of PI3K (Fig. 7). These results implied that TB suppresses the

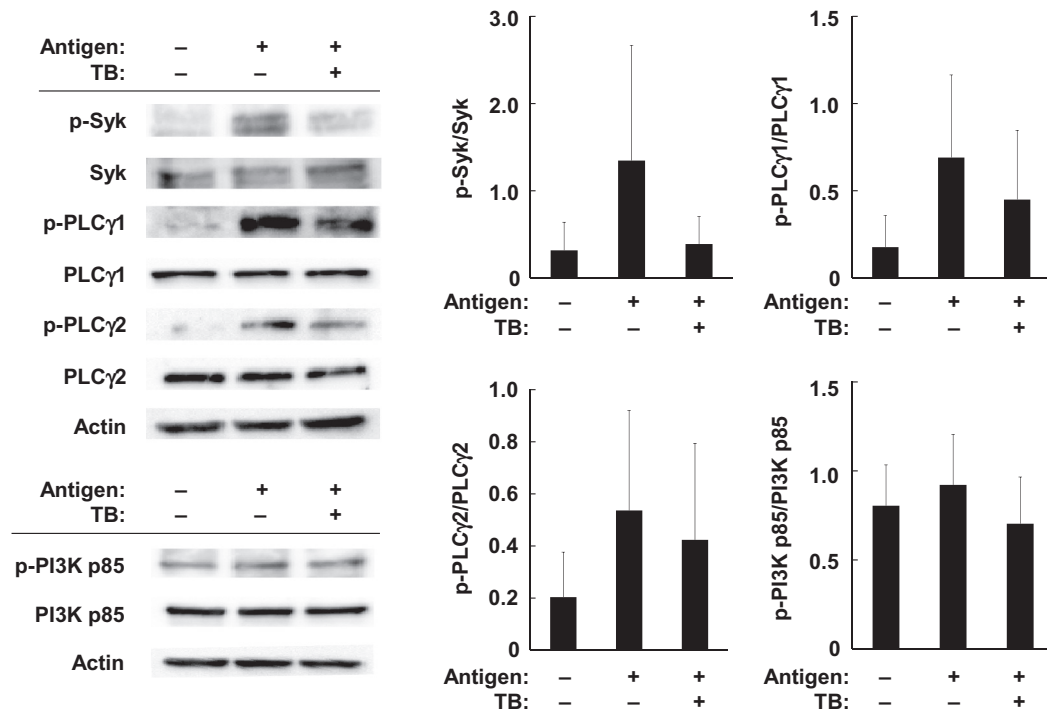


Fig. 7 – Effect of TB on the signaling pathways involved in degranulation of RBL-2H3 cells. Anti-DNP IgE-sensitized RBL-2H3 cells were treated with TB (1.2 mg/mL) or water for 10 min. The cells were subsequently stimulated with or without DNP-HSA for 5 min. Cell lysates were then prepared, and immunoblot analyses were performed. The p-Syk, p-PLC γ 1, p-PLC γ 2, and p-PI3K represent phosphorylated Syk, phosphorylated PLC γ 1, phosphorylated PLC γ 2, and phosphorylated PI3K, respectively. A representative blot from three independent experiments is shown. The results of densitometric analysis were expressed as the ratio of (phosphorylated protein amount)/(whole protein amount). Data were represented as the mean $\pm$ SD ($n = 3$). TB, theabrownins; PLC, phospholipase C.

FcεRI-mediated degranulation in RBL-2H3 cells via inhibition of Syk and PLCγ cascades. Cells were also treated with IDT and the results were similar to those with TB (supplemental data).

3.6. Effect of IDT in the Cry j1-induced pollinosis mouse model

The effect of IDT on the IgE-mediated allergic reaction was examined *in vivo* using the Cry j1-induced pollinosis mouse model. Cry j1 is the major allergen in Japanese cedar (*Cryptomeria japonica*) pollen. The application amount of Cry j1 and administration schedule were followed as described in Nomiyama et al. (2008). Mice were sensitized and challenged with Cry j1 as shown in Fig. 2. The IDT-treated mice were given IDT (3.1 mg/mL) instead of water with free access for 27 days. Free intake of IDT did not affect the body weight of mice (data not shown). Fig. 8A shows the average drinking volumes per mice in each group measured every 3 days. Although the drinking volumes differed greatly among the groups in the first 9 days, there was no such a big difference since then. The average drinking volumes per day were 3.5, 4.1, and 3.7 mL/mouse/day

for the intact, control, and IDT-administered groups, respectively. Because the concentration of IDT used for administration was 3.1 mg/mL, the amount of daily intake of IDT was 11.5 mg/mouse/day. On day 27, all mice were treated with Cry j1 to evaluate its exposure in the sensitized (control and IDT-administered groups) and unsensitized (intact group) mice. Fig. 8B showed that the rubbing frequency was significantly increased in the control group compared with that in the intact group ($p < 0.01$). In addition, the rubbing frequency was significantly decreased in the IDT-treated group compared with that in the control group ($p < 0.01$). There was no statistical significance between the intact and IDT-administered groups. This result suggests that IDT has a potential to attenuate allergic symptoms *in vivo*. Biochemical analysis was also performed by measuring the total serum IgE and IgG₁ levels in mice, because the humoral immune response results in the increased production of IgE and IgG₁ in rodents caused by the activation of the Th₂ type of T helper lymphocytes. As shown in Fig. 8C and 8D, there was no statistical significance among the groups, indicating that the administration of IDT does not suppress the production of these antibodies.

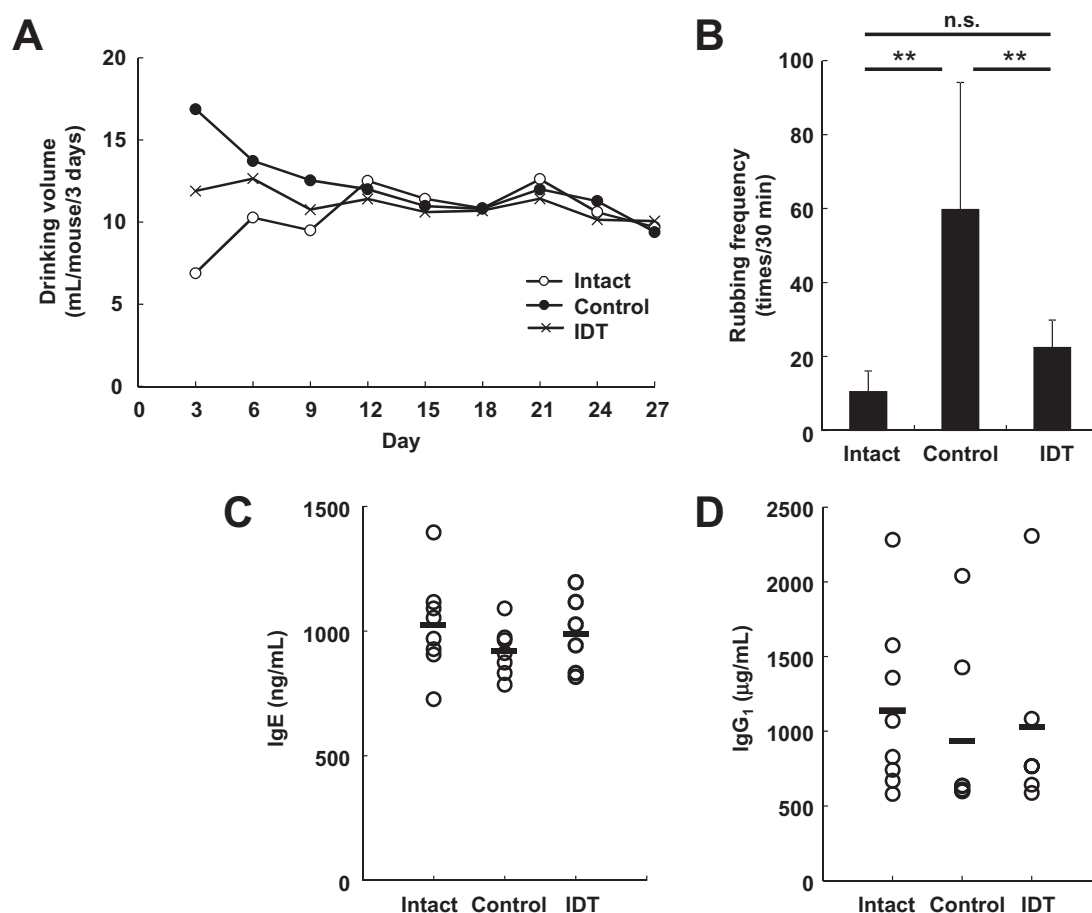


Fig. 8 – Effect of IDT on pollinosis in mice. Intact group: $n = 8$; control group: $n = 7$; IDT-administrated group: $n = 7$. (A) Drinking volume of water or IDT per mouse in each group. Drinking volume was measured every 3 days. (B) Nasal rubbing frequency was counted for 30 min after the final challenge with Cry j1. A Tukey–Kramer test was used to assess significance of difference. $**p < 0.01$. n.s. indicates no significance of difference between two groups. (C) Serum IgE levels in each mouse. Bars mean the average IgE levels of each group. (D) Serum IgG₁ levels in each mouse. Bars mean the average IgG₁ levels of each group. IDT, Ishizuchi dark tea.

4. Discussion

Ishizuchi dark tea is a highly unique microorganism-fermented tea, because it is manufactured through the two-step fermentation process. Goishi tea, another two-step microorganism-fermented tea manufactured similarly to Ishizuchi dark tea, has been so far reported to display a suppressive effect on diet-induced obesity (Jobu et al., 2013). However, the anti-allergic effect of the two-step fermented tea has not been reported yet. In the present study, we demonstrate that IDT inhibits mast cell degranulation using RBL-2H3 cells (Fig. 3A) without any cytotoxic influences (Fig. 3B). In addition, IDT exerted stronger suppressive effect on mast cell degranulation than GTE (Fig. 3C). These data suggested that Ishizuchi dark tea has a potency to attenuate allergic symptoms by inhibiting mast cell degranulation and that the potency is enhanced by fermentation. Fermentation may modify the structure of bioactive substances, such as polymerization of catechins and formation of novel polyphenols, resulting in the enhancement of biological functions (Sogawa et al., 2009).

The biological effects of tea are mainly attributed to polyphenols such as catechins. For example, EGCG, a catechin abundantly contained in green tea, is known to exert anti-allergic effect (Inoue et al., 2010). Although we confirmed the suppressive effect of EGCG on degranulation of RBL-2H3 cells, IDT (0.48 mg/mL) showed stronger effect than EGCG even at 100 μ M (Fig. 3D). Reversed-phase chromatography revealed the presence of 30 μ M EGCG in IDT (2.8 mg/mL). Thus, EGCG concentration in IDT used for the β -hexosaminidase release assay was 4 μ M at most. According to Inoue et al. (2010) and our data (Fig. 3D), this concentration is not enough to suppress mast cell degranulation. In addition, we confirmed that GTE contained more EGCG than IDT. Nevertheless, the suppressive effect of IDT on mast cell degranulation was stronger than that of GTE (Fig. 3C). Sogawa et al. (2009) found that Awa-ban tea, a microorganism-fermented tea, exhibited the anti-obesity activity as strong as green tea, although the amount of catechins was lower in Awa-ban tea than in green tea, which is known to contribute to the anti-obesity activity. Thus, multimeric polyphenols produced by fermentation were thought to have a similar effect to catechins. An ethanol extract from the leaves of Ishizuchi dark tea was also prepared and showed no suppressive effect on degranulation of RBL-2H3 cells (data not shown). All these data suggested to us that EGCG is not a major bioactive substance in IDT.

Catechins are oxidized by polyphenol oxidases in leaves during black tea production. On the other hand, microorganism-fermented tea is more strongly oxidized by microorganisms and environmental oxygen than black tea. In the course of fermentation of tea by microorganisms, TF, TR, and TB are yielded by catechin oxidation (Peng et al., 2013). To elucidate the class of bioactive substances, IDT was fractionated into TF, TR, TB, and others by liquid–liquid distribution, and then these samples were assessed by the β -hexosaminidase release assay. The result raised the possibility that the major bioactive substance in IDT is TB (Fig. 5A). Although TR also showed a suppressive effect on degranulation of RBL-2H3 cells, it was assumed that part of TB remained in the *n*-butanol-soluble fraction (TR), which caused a weak suppression of mast cell

degranulation by TR. The effect of TB on the Fc ϵ RI-independent degranulation of RBL-2H3 cells was examined using thapsigargin that is a specific inhibitor of the Ca²⁺-dependent ATPase pumping Ca²⁺ from the cytosol into endoplasmic reticulum. By inhibiting the Ca²⁺-dependent ATPase, the intracellular Ca²⁺ elevates and mast cell degranulation is induced. Because TB or IDT did not exert any suppressive effect on degranulation of RBL-2H3 cells, the bioactive substance in IDT was thought to interact with the early signaling events involved in degranulation. Size exclusion chromatography also suggested that the major bioactive substance in IDT is TB, because there was only a single peak in IDT even when the analysis was monitored at 210 nm (data not shown) and its profile closely resembled that of TB (Fig. 6). TR has been reported not to be over 2100 Da (Chen et al., 2012). TB has been reported to be produced by enzymes contained in microorganisms during fermentation of Pu-erh tea (Wang, Peng, & Gong, 2011).

The mechanism underlying the suppressive effect of TB on degranulation of RBL-2H3 cells was investigated. Fc ϵ RI is composed of four subunits: an α subunit, a β subunit, and diametric γ subunits (Metcalf, Peavy, & Gilfillan, 2009). After interacting with antigen, the Fc ϵ RI signaling pathway initiates to activate protein tyrosine kinases Lyn and Syk by phosphorylation. The activated Syk in turn induces phosphorylation on the downstream molecules such as LAT, PLC γ , and PI3K, leading to mast cell degranulation (Gilfillan & Rivera, 2009). We assessed the phosphorylation levels of these proteins by immunoblotting and found a suppressed tendency of the phosphorylation levels of Syk, PLC γ 1, and PLC γ 2 by treating cells with TB (Fig. 7), implying that TB affects the early signaling events by inhibiting phosphorylation of Syk. The suppressive effect of TB on degranulation of RBL-2H3 cells was found to be caused by inhibiting activation of Syk that plays an important role in the amplification of mast cell signaling (Gilfillan & Rivera, 2009).

We lastly investigated whether the outcomes of the *in vitro* studies might be involved in those of *in vivo* studies. The anti-allergic effect of IDT was examined in a murine model of pollinosis. After the final challenge with Cry j1, nasal rubbing was counted for 30 min. Free feeding of IDT instead of water significantly reduced the frequency compared with the control group mice, indicating that IDT has an effect to reduce the symptoms of cedar pollen allergy. The *in vivo* data seemed to reflect an impact of IDT on the degranulation of RBL-2H3 cells as was noted *in vitro* as described above. Total serum IgE and IgG₁ levels in mice were also measured (Fig. 8C and 8D). Because there was no statistical significance among the groups, the intake of IDT does not contribute to suppression of the IgE production. In the present study, total IgE and IgG₁ levels were measured. Thus, a different trend in IgE and IgG₁ productions might be observed, if the serum antibody levels specific for the antigen (Cry j1) were measured.

5. Conclusion

In the present study, the water extract from the leaves of Ishizuchi dark tea was shown to inhibit degranulation of

RBL-2H3 cells without any cytotoxic influences. The activity of IDT was stronger than that of GTE. The bioactive substance in Ishizuchi dark tea was thought to be TB with the molecular weight of 24 ± 3 kDa. The phosphorylated level of Syk tended to be decreased by treating RBL-2H3 cells with TB. In addition, IDT palliated allergic symptoms of the Cry j1-induced pollinosis model mice. Overall, these results suggested a potential that Ishizuchi dark tea might be effective in the alleviation of allergic symptoms. This is the first demonstration that the microorganism-fermented tea has an anti-allergy effect, and thus the anti-allergic activity of Ishizuchi dark tea would provide a new dimension to the functionality of the microorganism-fermented tea.

Acknowledgments

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Appendix: Supplementary material

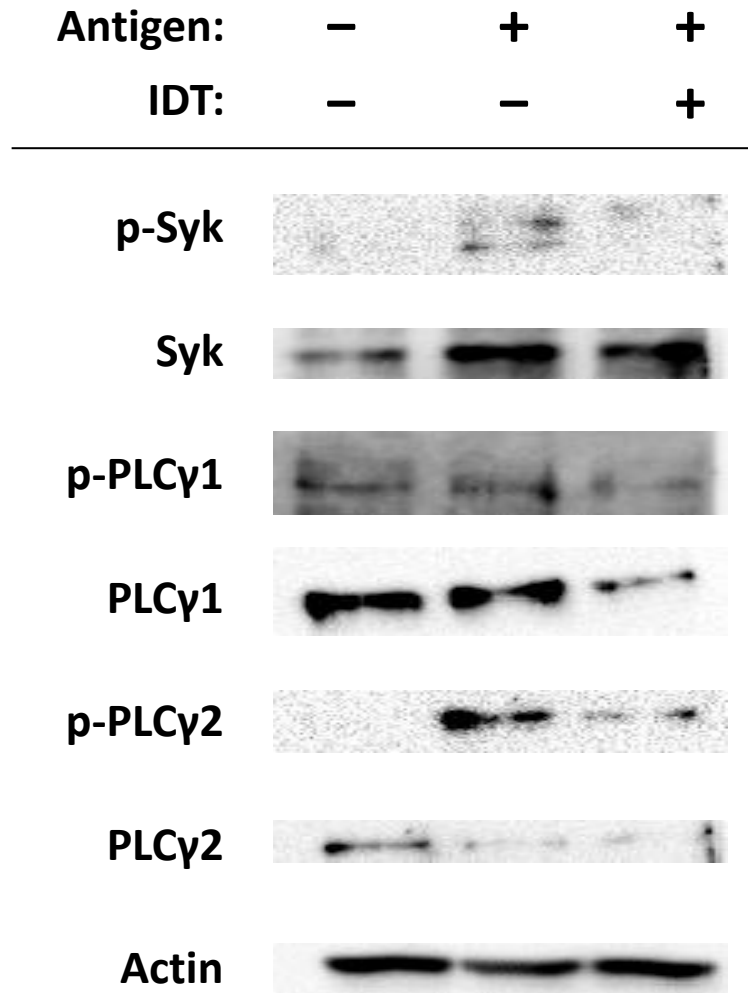
Supplementary data to this article can be found online at doi:10.1016/j.jff.2015.02.045.

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RBL-2H3 cells were seeded at 1.8×10^6 cells in 50 mm culture dishes (BD Falcon), and cultured overnight. After sensitizing with the anti-DNP IgE at the same concentration as described above, the cells were washed with the MT buffer and treated with 0.39 mg/mL of IDT or with NaPB (control) diluted in the MT buffer for 10 min. The cells were subsequently stimulated with DNP-HSA at the final concentration of 50 ng/mL for 5 min. After removing the added reagents, cells were lysed and centrifuged at $14000 \times g$ for 15 min at 4°C . After each supernatant was equalized to the same protein concentration, proteins in a total cell lysate were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane (Hybond-P, GE Healthcare, Buckinghamshire, UK). Immunoblotting with various antibodies was performed as previously described (Nishi et al., 2014). A representative blot from two independent experiments is shown.