The development of a suitable manufacturing process for ‘Benifuuki’ green tea beverage with anti-allergic effects

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Abstract: Epigallocatechin-3-O-(3-O-methyl) gallate (EGCG3′′Me) has been reported to inhibit type I allergy better than epigallocatechin gallate (EGCG), a major catechin in tea leaves (Camellia sinensis L). We examined the effects of extraction and sterilization on the catechin content and histamine release from mast cells, as a representative reaction of early phase allergy, in the manufacture of ‘Benifuuki’ green tea beverage. Among various varieties of tea, the cultivar ‘Benifuuki’ contains approximately 2% of EGCG3′′Me. Ester-type catechins and their epimers increased with the increased extraction temperature of the tea. A tea infusion, extracted at 90 °C, strongly inhibited histamine release from mast cells. Furthermore, sterilization affected the catechin content in the manufactured green tea beverage. Sterilization at high temperature promoted the isomerization of catechins and the sterilized green tea beverage had a strong inhibitory effect. When EGCG3′′Me, EGCG, epicatechin-3-O-gallate (ECG) and their epimers, GCG3′′Me (gallocatechin-3-O-(3-O-methyl) gallate), GCG (gallocatechin-3-O-gallate) and CG (catechin-3-O-gallate) were compared, the anti-allergic effect of GCG3′′Me was strongest, and the order of activity was GCG3′′Me > EGCG3′′Me > GCG > EGCG. We consequently suggest that it was necessary to extract components from tea at the highest temperature possible, and to pasteurize under retort conditions (118.1 °C, 20 min), to manufacture functional green tea beverage with an anti-allergic action.

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Keywords: galloatechin-3-O-(3-O-methyl) gallate (GCG3′′Me); epigallocatechin-3-O-(3-O-methyl) gallate (EGCG3′′Me); gallocatechin-3-O-gallate (GCG); epigallocatechin-3-O-gallate (EGCG); tea polyphenol; catechin; histamine release; anti-allergic activity; tea; retort; manufacturing process; epimerization

INTRODUCTION

Allergies are diseases of excess immunity. Mast cells play a critical role in the effector phase of IgE-dependent immediate hypersensitivity and allergic diseases. Cross-linking of high-affinity IgE receptors (FceRI) with IgE and allergens (cedar pollen, house dust, mites and food proteins) initiates the activation process, leading to the release of preformed and de novo synthesized vasoactive amines (histamine or serotonin), proteases, leukotrienes, cytokines, and chemokines.1–3 These chemical and polypeptide agents elicit various allergy-associated pathophysiological changes locally and systemically: for instance, amines, histamine and serotonin enhance vascular permeability, and cytokines such as TNF-α recruit inflammatory cells to the site of allergen exposure. The chemical mediators may cause inflammation in the body and may trigger a critical allergic reaction.4

The number of patients having allergosis, such as pollinosis, atopic dermatitis, allergic rhinitis, asthma and food allergies, has increased rapidly in recent years in Japan. It is said that more than 30% of Japanese have some allergy,5 and 13 million people have pollinosis.

Tea (Camellia sinensis L) is consumed all over the world, and in particularly large quantities in Japan and China where it has been used for medicinal purpose for centuries. It has been reported that tea has various bioregulatory activities, such as those that are anti-carcinogenic,6–11 anti-metastatic,12–16 anti-oxidative,17–20 anti-hypertensive,21 anti-hypercholesterolemic,22–24 anti-caries,25,26 anti-bacterial27 and intestinal flora amelioration.28

The major mechanism for these activities has been shown to involve catechins, a group of polyphenolic compounds. Tea leaves contain approximately...
10–20% dry weight of catechins; ie (−)-epigallocatechin-3-O-gallate (EGCG; approximately 50% of total catechins), (−)-epigallocatechin (EGC; 25%), (−)-epicatechin-3-O-gallate (ECG; 12%) and (−)-epicatechin (EC; 12%). In particular, EGCG is a rare catechin, found in no other plants.

It has been reported that the flavonoids in 27 kinds of herb tea and green tea infusions prevent allergic disease. Moreover, catechins have been reported to inhibit rat or mouse type I allergies or histamine release from rat basophils. There is evidence that two O-methylated catechins, epigallocatechin-3-O-(3-O-methyl) gallate (EGCG3'Me) and epigallocatechin-3-O-(4-O-methyl) gallate (EGCG4'Me), in tea leaves in special tea varieties such as ‘Benifuuki’, ‘Benifuji’ or ‘Seishin-taipan’, have stronger anti-allergic activities than EGCG. Furthermore, it was found that EGCG3'Me suppressed the FcεRI expression by inhibiting ERK1/2 phosphorylation or mast cell activation by inhibiting multiple protein kinases. The structural formulae of the catechins used are shown in Fig 1.

Catechins have two asymmetric carbon atoms in the C-ring, and epimers of carbon at the C-2 position of the ring were detected in tea infusions. Analyses of catechins in tea infusions have shown that amounts of most catechins in tea infusions decreased by heat processing, but (±)-catechin was particularly increased, because of the epimerization of (−)-epicatechin.

In Japan, canned and bottled teas are becoming remarkably popular. It was supposed that we could manufacture bottled tea with an anti-allergic action by using tea leaves containing anti-allergic substances. However, the relationship between the manufacturing process of this tea beverage and the anti-allergic activity was unclear. In this paper, we therefore tried to clarify the changes in catechin content and anti-allergic activity of ‘Benifuuki’ green tea beverage during the manufacturing process.

**MATERIALS AND METHODS**

**Reagents**

The catechins EGCG, galloatechin-3-O-gallate (GCG), EGCG3'Me and GCG3'Me were kindly provided by Drs M Sano and T Miyase of the School of Pharmaceutical Science, University of Shizuoka, Japan. ECG and CG, o-phthalaldehyde, and N-acetylcysteine were purchased from Kurita Kougyo (Tokyo, Japan), Nacalai tesque (Kyoto, Japan) and Wako Chemical (Osaka, Japan), respectively.

**Preparation of green tea beverage**

‘Benifuuki’ fresh tea leaves harvested in May 2001 at the plantation of the National Research Institute of Vegetables and Tea Sciences in Kanaya, Shizuoka, Japan. Tea leaves were immediately steamed for 35 s and then dried using a 35-kg scale auto green tea manufacturing machine (Terada-Seisakusho, Shizuoka, Japan).

To differentiate among the extraction conditions, ‘Benifuuki’ green tea was extracted at 50, 70, 90°C for 6 min with 30 times the volume of distilled water. After filtration, 600 mg l⁻¹ of L-ascorbic acid sodium salt and 70 mg l⁻¹ of sodium bicarbonate were added to these tea infusions, which were then sterilized with retort sterilization.

Another tea infusion extracted at 90°C was sterilized as follows: non-sterilized (hot pack; control), ultra-high temperature sterilization (UHT: 138°C, 30 s) or retort sterilization (118.1°C, 20 min). For the investigation, the concentration of polyphenols in each extract was standardized as follows.

**Analysis of polyphenol content**

Generally, the uniform quality of green tea beverage is controlled by the polyphenol concentration. To standardize each sample under various sterilized conditions to the same level of polyphenol, the polyphenol content in the infusion was measured by a colorimetric method using ferrous tartrate. Briefly, an A solution (100 mg dl⁻¹ of ferrous sulfate and 500 mg dl⁻¹ of potassium sodium tartrate), a B solution (Sorensen buffer; 1/15 M Na₂HPO₄ solution–1/15 M KH₂PO₄ solution 84:16), and an ethyl gallate standard solution were prepared. Five milliliters of infusion sample (standard solution or distilled water as blank) was taken into a 25-ml flask, 5 ml of A solution was added and mixed completely, then B solution was added to make up to 25 ml. The absorbency of the reaction was measured by a spectrophotometer at the wavelength of 540 nm (SmartSpecPlus, Bio-Rad, CA, USA). Ethyl gallate was used as polyphenol standard. Standard solution was prepared 0, 20, 40, 60, 80 and 100 mg dl⁻¹ to make a linear calibration curve. Polyphenol content (mg dl⁻¹) was calculated by multiplying ethyl gallate content by a coefficient of 1.5.

**Analysis of catechins**

Tea beverage was diluted fivefold with distilled water and 20μl of the sample was filtered through
a membrane filter (DISMIC-13HP-PTFE, pore size 0.45 μm, ADVANTEC, Tokyo, Japan) and injected with an autosampler (SIL-10Avp, Shimadzu, Kyoto, Japan) into the HPLC apparatus (Shimadzu class VP HPLC system). HPLC was performed with a Shimadzu LC-10A pump coupled with a photodiode-array detector (UV 280 nm) (SPD-M10Avp, Shimadzu, Kyoto, Japan) using a reverse-phase MightySil RP-18 column (4.6 mm id × 150 mm, particle size; 5 μm, Kanto Kagaku Chemical, Tokyo, Japan), which was eluted as described below, at a flow rate of 1 ml min⁻¹ at 40°C.

HPLC analysis was performed using a linear gradient system with mobile phase A (H₂O–acetonitrile–H₃PO₄ 400:10:1) and mobile phase B (methanol–mobile phase A 1:2). The linear gradient elution was maintained at 100% A for 2 min, programmed to 20% mobile phase A over 43 min, maintained at 20% mobile phase A for 5 min, and then returned to 100% mobile phase A for 10 min. Quantification was carried out using the external standard method. Quantification of catechins was performed using data acquisition and processing system of LC workstation (Class VP system, Shimadzu).

Cells and culture
Bone marrow cells from the femur of NC/Nga mice (Charles River Japan, Kanagawa, Japan) were cultured in 4 ng ml⁻¹ murine recombinant IL-3 (Peprotec (NJ, USA)) containing RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen Life Technologies, CA, USA), 2 mM glutamine and 50 μM 2-mercaptoethanol in humidified 95% air 5% CO₂ at 37°C.47 More than 95% pure mast cells were obtained as bone marrow derived mast cell (BMMC) after four weeks of culture.

Degranulation by FcεRI cross-linking
BMMC cells were passively sensitized at a density of 2 × 10⁶ cells ml⁻¹ with 1 μg ml⁻¹ anti-dinitrophenyl (DNP) mouse monoclonal IgE antibody Sigma-Aldrich (MO, USA) at 37°C overnight. After washing in Tyrode buffer (Ca²⁺-free; 10 mM HEPES pH 7.4 (Wako chemical, Osaka, Japan) with 800 mg dl⁻¹ NaCl, 20 mg dl⁻¹ KCl, 5.6 mg dl⁻¹ NaH₂PO₄, 100 mg dl⁻¹ glucose, 50 mg dl⁻¹ gelatin and 1 μM MgCl₂·6H₂O), they were re-suspended in Tyrode buffer at a density of 1 × 10⁶ cells ml⁻¹, and incubated for 20 min with samples at 37°C, then stimulated by 300 ng ml⁻¹ of DNP-HSA (human serum albumin) (LSL Cosmo Bio, Tokyo, Japan) with 300 μM CaCl₂ for 10 min at 37°C. The reactants were added to the 4 mM EDTA/Tyrode solution and cooled on ice to stop the reaction. To measure the histamine, the reactions were centrifuged at 15 000 × g for 5 min at 4°C, the equivalent volume of 0.1 M hydrochloride was added to the supernatant, and the released histamine was measured by on-column HPLC.48 HPLC was performed using a Shimadzu LC-10A pump coupled with a fluorescent photometric detector (excitation 330 nm, emission 430 nm) (RF-10AxL, Shimadzu, Kyoto, Japan) with a reverse-phase Asahipak-ODP-50-4E column (4.6 mm id × 250 mm, particle size; 5 μm, Showa Denko, Tokyo, Japan). The reaction was eluted with 50 mM sodium borate–acetonitrile (80/20) buffer containing 1 mM o-phthalaldehyde and 1 mM N-acetylcyesteine, at a flow rate of 0.5 ml min⁻¹ at 37°C.

Statistics
The results are shown as the means ± SD in triplicate experiments. Statistical analysis was performed using Tukey’s multiple-range test. A value of p < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION
We aimed to construct a more effective manufacturing process to commercialize an anti-allergic beverage made from ‘Benifuuki’ tea cultivar. It was reported that heating process induces the epimerization of tea catechins,42,43,49,50 and the inhibitory effect on histamine release of the epimerized catechins, GCG, is greater than that of EGCG.33 There are two processes affecting the catechin content in a manufacturing process: the extraction from tea leaves and sterilization. To clarify the changes in catechin composition and anti-allergic activity (histamine release inhibitory effect) during the beverage manufacturing process, we used HPLC to measure the catechin content of tea extracted at various temperature.

Tea infusions extracted at 50, 70, 90°C for 6 min were sterilized with retort sterilization, and the catechin content was analyzed. As shown in Fig 2, a rise in extraction temperature tended to increase...
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Figure 3. Histamine-release inhibitory effects of tea samples prepared with various temperature extractions. Tea infusions extracted at 50, 70, 90 °C for 6 min were sterilized with retort sterilization, diluted twofold and added to BMMC assay. Each value represents the mean ± SD, n = 3. *Significantly different from 50 °C value, p < 0.05.

Figure 4. Catechin content of ‘Benifuuki’ tea samples prepared under different sterilized conditions. Tea infusions extracted at 90 °C for 6 min were sterilized under three conditions. These samples received 2 or 50 µg ml⁻¹ as polyphenol content in a BMMC assay. Control: non-sterilization; UHT: ultra-high temperature sterilization (138 °C, 30 s); Retort: retort sterilization (118.1 °C, 20 min).

Figure 5. Histamine-release inhibitory effects of tea samples prepared with different sterilized conditions. Tea infusions extracted at 90 °C for 6 min were sterilized under three conditions. These samples received 2 or 50 µg ml⁻¹ as polyphenol content in a BMMC assay. Control: non-sterilization; UHT: ultra-high temperature sterilization (138 °C, 30 s); Retort: retort sterilization (118.1 °C, 20 min). Each value represents the mean ± SD, n = 3. *Significantly different from hot pack value, p < 0.05.

the content of the catechins and their isomers. The catechin content extracted at 50 °C was lower than that extracted at 70 or 90 °C. It was clear that the increased extraction temperature resulted in a higher concentration of catechins.

We then examined whether the extraction temperature affected the anti-allergic action. As shown in Fig 3, tea extracted at 90 °C showed a strong inhibitory effect on histamine release. Extracts at 70 and 90 °C significantly inhibited histamine release compared with 50 °C extraction. It was reported that a temperature of more than 80 °C caused an acceleration of catechin isomerization, and thermal treatment at 120 °C for 30 min in a pH 5 solution was effective in forming these isomers in quantity. This study indicated that a high extraction temperature increased the level of histamine release from BMMC was temperature-dependent. This result suggested that the extraction efficiency of catechins was promoted with a rise in extraction temperature and an accelerated anti-allergy effect.

We next investigated the relationship between the sterilization condition and the amount of remaining catechins. Figure 4 shows the varying catechin content of tea under three sterilized conditions. Hot-pack (control; non-sterilization), the shortest heating time among the three conditions, produced 20% of epimers by isomerization. In contrast, approximately 50% or more isomers were produced by UHT or retort sterilization, respectively. There was a tendency for levels of EGCG3’Me to decrease and GCG3’Me to increase under retort sterilization compared with the UHT sterilization. It was therefore suggested that isomerization required longer heating time rather than higher heating temperature.

Since it was surmised that the intensity of anti-allergic activity of EGCG3’Me was different from the epi-form catechin, the effects of the sterilized conditions on histamine release from BMMC were investigated. Each tea sample was administered 2 or 50 µg ml⁻¹ as polyphenol content in a BMMC assay. As shown in Fig 5, the intensity of the inhibitory effect of these teas on histamine release from BMMC was: retort sterilization > UHT sterilization > control, in that order, and there was statistical significance.
Comparison of histamine-release inhibitory effects of ECG, Figure 6. Furthermore, GCG3* (H Nagai et al. [17]) showed more than half of epi-form catechins converted to their isomer by retort processing. The intensity of the conversion rate of EGCG3 Me to GCG3 Me was retort > UHT sterilization > and control, in that order.

In this paper, it has been shown that the process of sterilization was important for increased anti-allergic activity in the manufacture of the beverage. Retort or UHT sterilization caused a rise of inhibitory activity by the promotion of isomerization. Furthermore, it has been reported that GCG, an isomer of EGCG, had strong anti-allergic activity, and this paper showed that GCG3 Me had a stronger inhibitory effect on histamine release.

As a result, the epimers of epi-form catechins that were presented in tea leaves were more effective and the effect was GCG3 Me > EGCG3 Me > GCG > EGCG > CG = ECG. It has been reported that the inhibitory effect of GCG and EGCG on histamine release was stronger than that of ECG. ECG was also more active than EC and/or C.

When manufacturing green tea beverage, it is estimated that there is a 7–8% degradation of catechins by retort sterilization. However, it is interesting that high temperatures may result in efficient epimerization and promotion of anti-allergic activity.

CONCLUSION
This research indicated that the manufacturing process could promote the anti-allergic action of tea using ‘Benifuuki’ green tea. To manufacture functional ‘Benifuuki’ green tea with an anti-allergic effect, tea should be extracted at the highest temperature possible and sterilized under retort conditions (118.1 °C, 20 min).

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![Figure 6. Comparison of histamine-release inhibitory effects of ECG, CG, EGCG, GCG, EGCG3 Me and GCG3 Me. Each sample received 25 µg ml⁻¹.](image-url)
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