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Determination of Mechanism of Flock Sediment Formation in Tea Beverages

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The mechanism of sediment formation during the storage of green tea beverage was investigated. Green tea extract was separated by Diaion HP-20 column chromatography, and a sediment-formation test was performed. Results showed that at least one compound of the substance causing flock sediment was contained in each of the HP-20 nonadsorbed and adsorbed fractions. From the following fractionations and structure analyses, the substance in the HP-20 adsorbed fraction was determined to be 1-*O*-galloyl-4,6-*O*-(*S*)-hexahydroxydiphenoyl-*β*-D-glucose (strictinin), which is one of the ellagitannins. Strictinin was hydrolyzed to ellagic acid by heat-sterilization processes such as retort sterilization or the ultra-high temperature processing used during the manufacturing of tea beverages. Ellagic acid combined with proteins in the HP-20 nonadsorbed fraction to form an irreversible sediment of green tea beverage; ellagic acid and proteins were confirmed to be present in that sediment. The HP-20 adsorbed fraction contained little strictinin and formed hardly any sediment, suggesting that control of the strictinin content is significant in avoiding sediment formation during the manufacturing process of tea beverages.

KEYWORDS: Tea beverage; green tea; jasmine tea; sediment formation; strictinin; protein

INTRODUCTION

Nowadays, tea beverages such as green (nonfermented) tea, oolong (semifermented) tea, black (fermented) tea, and jasmine (light-fermented) tea, tightly packed in cans or plastic bottles, continue to be manufactured in Japan. Green and jasmine tea beverages show traces of flock, floating, or turbid white suspended matter or of sediment that occurs during storage, all of which pose problems in the manufacture of these beverages. This sediment is occasionally found even when these teas are processed with a filter below 1 μm. When such sediment occurs, especially in tea beverages in clear bottles, their marketability will suffer due to the unpleasant visual impression. However, no established consensus exists on the mechanisms underlying sediment formation in green and jasmine tea beverages. Therefore, in manufacturing the tea beverage, materials are selected which had been proven by preliminary testing not to cause sedimentation in the beverage.

The sediments resulting from infusions of oolong (*1*, *2*) and black (*3*−*7*) tea have reportedly been associated with oxidized polyphenols, catechins, caffeine, and proteins and are called tea cream. The major monomeric catechins of tea leaves are (−)-epigallocatechin gallate (EGCG), (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC), (−)-epicatechin (EC), and (−)-gallocatechin gallate (GCG), comprising about 10−20% of the product's dry weight. This tea cream differs from the sediments of green and jasmine tea beverages generated when they are stored above room temperature since tea cream occurs when the teas are processed in concentrated form. It has been originally suspected that some oxidized polyphenols gradually bound with caffeine, proteins, pectin, or other polysaccharides by the catalytic action of metal ions to form complexes.

The objective of this study was to determine the precise substances causing flock sediment formation following the heat sterilization of green tea beverages. To determine the relevant components, green tea extract was separated and the responsible phenolic compound was then isolated and identified.

MATERIALS AND METHODS

Materials. The green tea used in this study was a commercially refined tea (high-grade tea; Itoen, Ltd., Tokyo, Japan). Ellagic acid was purchased from Wako Pure Chemical Industries, (Osaka, Japan) and bovine serum albumin (BSA) from Sigma-Aldrich Co., (St. Louis, MO). All chemicals were of reagent grade.

Extraction and Manufacture of Green Tea Beverage. A 20 g amount of refined green tea (*Camellia sinensis*, first flush) was extracted in 800 mL of distilled water at 70 °C for 3.5 min with moderate agitation. Leaves were removed by a mesh sieve of 100 μm pore size and quickly cooled to 30 °C. The resulting extract was centrifuged at 3000*g* for 10 min to remove the insoluble components. The supernatant was added to ascorbic acid (final concentration, 500 μg/mL), adjusted to pH 6.0 with $NaHCO_3$, poured into a heat-resistant bottle, and heated in an autoclave at 121 °C for 7 min.

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Processing of Green Tea Extract. After the above centrifugation, the supernatant was separated by passing through a column (160 × 52 mm i.d.) filled with a highly porous polystyrene gel (Diaion HP-20, Mitsubishi Chemical Co., Tokyo, Japan). Nonadsorbed fraction was used for the experiments. The column was washed first with distilled water and successively eluted with 20, 40, 60, and 100% methanol. The fractions producing significant levels of sediment were then passed through a column (80 × 55 mm) filled with Cosmosil 75C18opn reverse-phase resin (Nacalai Tesque Inc., Kyoto, Japan) and successively eluted with 10, 20, and 30% methanol.

These fractions were further fractionated by preparative HPLC with a 250 × 20 mm i.d. Wakosil-II 5C 18HG Prep reverse-phase column (Wako). The mobile phase was 22% methanol containing 0.1% acetic acid with a flow rate of 8 mL/min at room temperature. The target compound was identified using FAB MS and ^{1}H and ^{13}C NMR. Mass spectra (MS) were obtained using a JEOL JMS-SX 102 mass spectrometer (JEOL Ltd., Tokyo, Japan). ^{1}H and ^{13}C NMR spectra were recorded on a JEOL JNM-A 400 (400.0 and 100.4 MHz; JEOL), and chemical shifts are given in δ (ppm) with tetramethylsilane used as an internal standard.

Sediment-Formation Test. The brix value of extracts was measured by a DD-7 differential refractometer (Atago Co., Ltd., Tokyo, Japan). A final volume of 250 mL of the mixture containing 100 mL of the HP-20 nonadsorbed fraction (brix 0.4%), 0.125 g of ascorbic acid, and each eluate, respectively, was adjusted to pH 6.0 with $NaHCO_3$, poured into a heat-resistant bottle, and heated in an autoclave at 121 °C for 7 min. Samples were stored at 37 °C and observed for the formation of flock sediment.

HPLC Conditions. The determination of phenolic compounds and the derivatives was carried out using a Hitachi Model D-7000 HPLC equipped with a L-7100 pump, a programmable L-7250 autosampler, and D-7000 chromatography data station software (Hitachi, Ltd, Tokyo, Japan). Separation was done on a 250 × 4.6 mm i.d. Wakosil-II 5C18 HG reverse-phase column (Wako). Mobile phase A was 15% methanol containing 0.1% phosphoric acid, and mobile phase B was 45% methanol containing 0.1% phosphoric acid. The gradient elution system was as follows: 0−18 min, 100% A; 18.1−33 min, 100% B; 33.1−45 min, 100% A. All analyses were carried out at 40 °C. The flow was kept constant at 0.6 mL/min at 40 °C, and the eluents were monitored by a Hitachi L-7420 UV/vis detector at 280 nm.

Collection of Flock Sediment, and Hydrolysis. The flock sediment of green tea beverage was collected using the method of Garrido et al. (*8*) with a slight modification. Briefly, it was collected by filtering green tea beverage through a 0.45 μm cellulose acetate filter (Advantec MFS Inc., Tokyo, Japan) with a vacuum. The filter was washed with distilled water to remove water-soluble compounds, immersed in methanol in a heat-resistant bottle, and sonicated until the sediment peeled off. The methanol extract was brought to a final volume of 100 mL, acidified with 100 μL HCl, and autoclaved at 121 °C for 7 min. Phenolic compounds were analyzed using the above HPLC conditions.

SDS−PAGE. The proteins in the green tea extract were precipitated with 80% ethanol, and the precipitate was resolved with distilled water. The resulting solution was treated with 15% trichloroacetic acid (*9*) removed when the residues were washed with acetone dissolved with a sample buffer containing 20% glycerol, 50 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol, 0.01% bromophenol blue, 1% sodium dodecyl sulfate (SDS), and 1% 2-mercaptoethanol; pH 6.8. After the hydrolysis of methanol extracts with HCl, the flock sediment was concentrated by a rotary evaporator and washed with methanol to remove HCl. The residues were dissolved with the sample buffer. Each sample was monitored by SDS−polyacrylamide gel electrophoresis (SDS−PAGE) using a 5−20% gradient gel (Pagel NPG-520L, Atto Co., Tokyo, Japan). The proteins were visualized by staining with a silver-stain kit (2D-Silver Stain II; Daiichi Pure Chemicals, Tokyo, Japan).

RESULT AND DISCUSSION

Extraction and Fractionation with HP-20. The refined green tea was extracted and the supernatant liquid passed through a column filled with Diaion HP-20 to obtain the HP-20 adsorption fraction. Then, the column was eluted with 80% methanol to obtain the HP-20 80% methanol fraction. The sediment-formation test was carried out as in the above-described experiments.

Table 1. Sediment-Formation Test of HP-20 Fractions

samples	1 week	2 weeks	3 weeks	4 weeks
nonadsorbed fraction (A)	−	−	−	−
80% methanol fraction (B)	−	−	−	−
(A) + (B)	+	+	+	+

[a] −, no sediment; +, sediment formed.

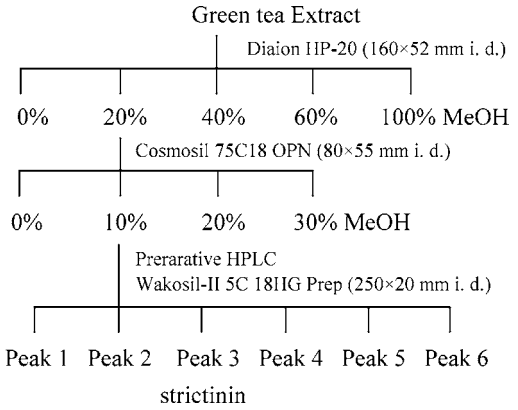

Figure 1. Extraction and isolation of green tea extract to determine the relevant components of flock sediment.

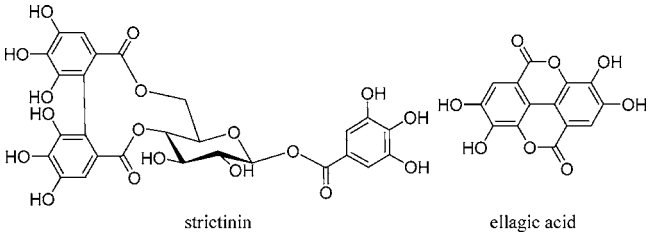

Figure 2. Chemical structures of strictinin and ellagic acid.

As shown in Table 1, the occurrence of flock sediment was observed only in the HP-20 nonadsorbed fraction added to the 80% methanol fraction. These findings showed that at least one component of the substance causing flock sediment was contained in each of the HP-20 nonadsorbed fractions and the HP-20 adsorbed 80% methanol fraction.

The sediment component contained in the HP-20 adsorbed fraction was isolated. An outline of the working procedure of this isolation is shown in Figure 1. The formation of flock sediment was observed in the HP-20 20 and 40% methanol fractions. The main catechin component of the 20% methanol fraction was EGC. Other major catechins (EGCG, ECG, EC, and GCG) and caffeine abounded in the 40% methanol fraction. Since the formation amount of sediment formed, especially in the former, was high, the 20% methanol fraction was then separated by the ODS column and preparative HPLC. The sediment-formation test was repeated for each of the six peaks obtained by preparative HPLC, and sediment formation was observed in peak 3. This compound was purified and obtained as an amorphous white powder. From the ^{1}H and ^{13}C NMR and FAB MS, its structure was found to correspond to that of 1-*O*-galloyl-4,6-*O*-(*S*)-hexahydroxydiphenoyl-β-D-glucose (strictinin), which was consistent with the previously reported data (*10*−*12*). Strictinin (Figure 2) is one of the ellagitannins extracted from tea, comprising about 0−1.0% of its dry weight.

As for the 40% methanol fraction of the HP-20 column, when the same identification as the above was obtained, it also

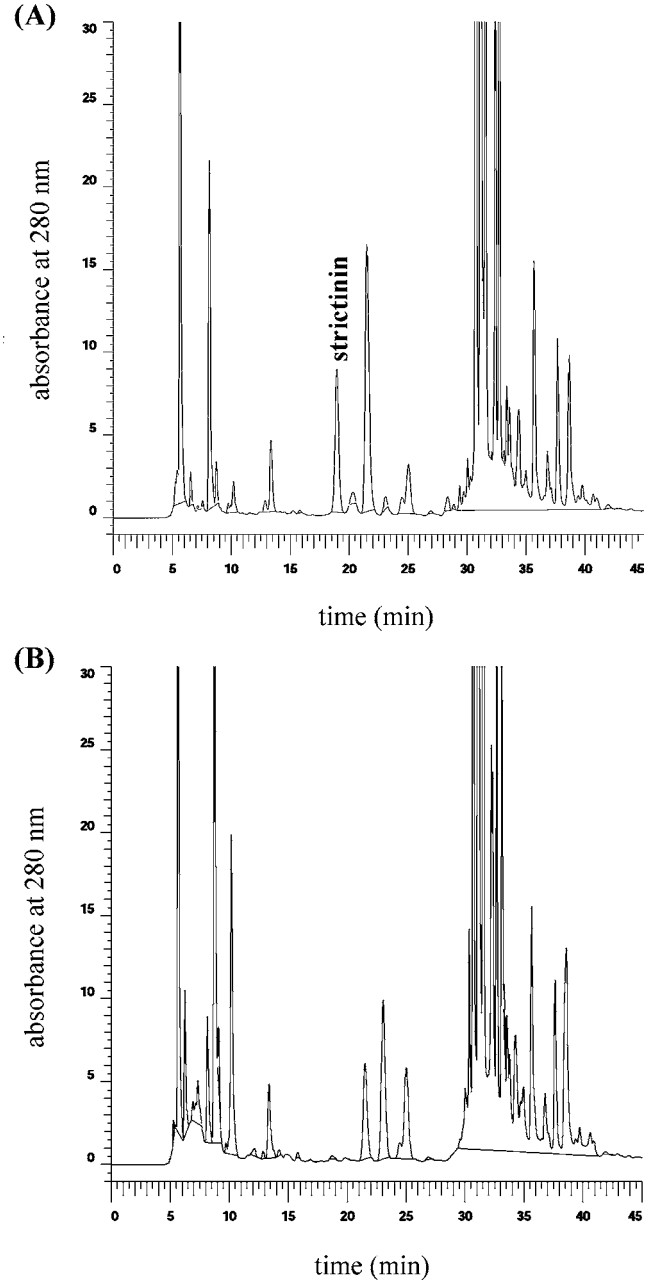

Figure 3. HPLC profiles of strictinin in green tea extract. Chromatograms showing changes in the strictinin peak before (**A**) and after (**B**) heat sterilization. A peak (15.2 μg/mL) appeared at a retention time of about 19 min (**A**). This peak was eliminated by heat sterilization.

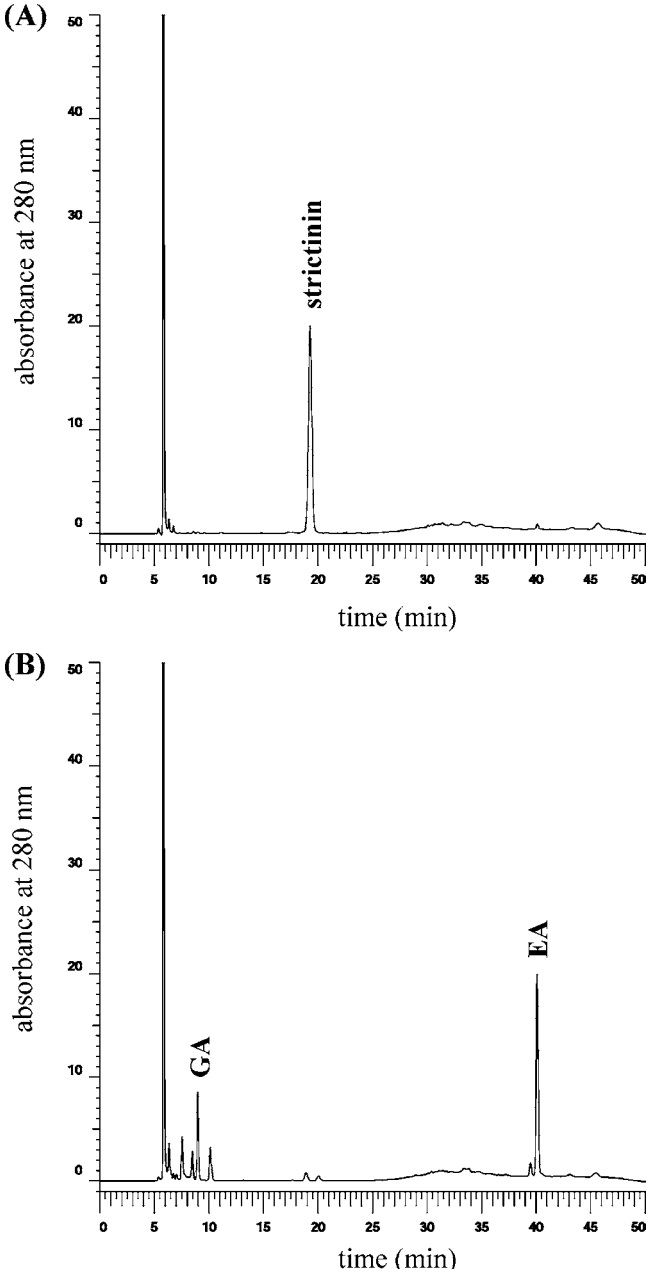

Figure 4. HPLC profiles of strictinin aqueous solution after heat sterilization. Chromatograms showing changes in the strictinin solution (9.5 μg/mL) before (**A**) and after (**B**) heat sterilization. Strictinin was eliminated, and ellagic acid (2.8 μg/mL) appeared after heat sterilization. GA, gallic acid; EA, ellagic acid.

contained strictinin. In addition, no components other than strictinin in that fraction were involved in sediment formation.

Characteristic Property of Strictinin in Beverage Processing. Green tea extract (brix 0.3%) containing 500 ppm ascorbic acid was sterilized at 121 °C for 7 min. Strictinin concentrations before and after heat sterilization were analyzed by HPLC. The peak appearing at a retention time of about 19 min was determined by the absolute calibration method with the purified strictinin.

As shown in Figure 3, the content of 15.2 μg/mL strictinin was supposedly decomposed or precipitated by heat sterilization. Since the strictinin contained in the tea extract was completely decomposed under a heat-sterilization process such as retort or ultra-high temperature that is typically involved in the manufacture of tea beverages, hardly any strictinin would remain in beverages sold publicly.

Hydrolysis of Strictinin with Heat Sterilization. Ellagitannins are known to be hydrolyzed by treatment with an acid, a base, or heat, thus liberating ellagic acid (*8*, *13*−*19*). Many fruits such as strawberries, raspberries, and grapes have been reported to contain ellagitannins, and the resulting ellagic acid was found to be the substance forming the juice's sediments (*8*, *15*−*19*). The purified strictinin was dissolved with distilled water and heat-sterilized at 121 °C for 7 min. The chromatograms before and after heat sterilization are shown in Figure 4. Strictinin was eliminated after heat sterilization, and ellagic acid appeared at a retention time of about 40 min. On the other hand, ellagic acid was hardly detectable after heat sterilization of the HP-20 nonadsorbed fraction containing purified strictinin (Figure 5). Thus, when a tea extract is sterilized, strictinin decomposes to form ellagic acid, which binds with components contained in

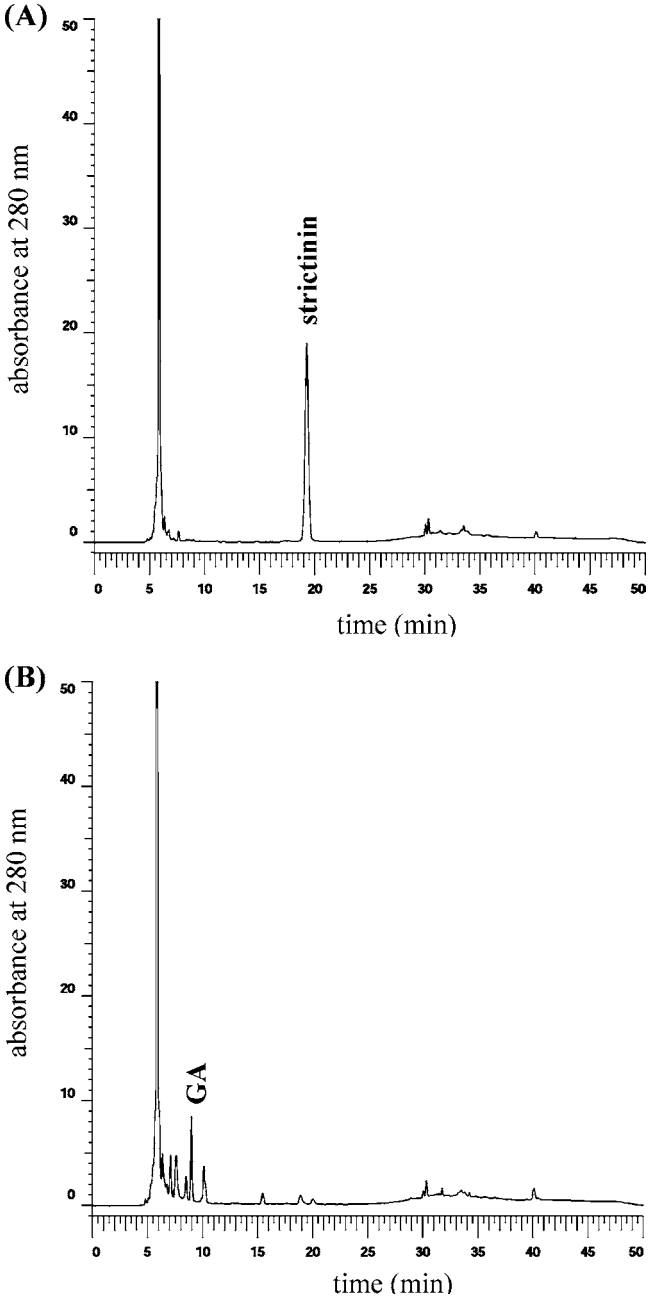

Figure 5. HPLC profiles of a mixed solution of strictinin and the HP-20 nonadsorbed fraction. Chromatograms showing the changes in the mixed solution of strictinin (9.1 μg/mL) and the HP-20 nonadsorbed fraction before (**A**) and after (**B**) heat sterilization. Strictinin was eliminated, and ellagic acid was hardly detectable after heat sterilization. GA, gallic acid.

the HP-20 nonadsorbed fraction to form a precipitate, i.e., the flock sediment.

Identification of Ellagic Acid in Flock Sediment. The flock sediment of 250 mL of green tea beverage was collected with a 0.45 μm cellulose acetate filter to identify the concentration of ellagic acid by HPLC (*8*). This sediment consisted of a dark green amorphous substance that proved to be insoluble in water, methanol, or ethanol. The ellagic acid in the sediment extracted by 100 mL of methanol had a concentration of only 0.3 μg/mL. Since it was thought to be insoluble in methanol, this solvent was acidified with 100 μL of HCl. More ellagic acid was liberated by methanol−HCl hydrolysis from the sediment at a concentration of 10.0 μg/mL. However, there was no further remarkable increase following severe hydrolysis by autoclave at 121 °C for 7 min (12.0 μg/mL). In each stage of this study, insoluble materials appeared, indicating that some insoluble materials were also constituents of flock sediment.

Identification of Protein in Flock Sediment. Some kinds of proteins in green tea beverage and its flock sediment were identified with SDS−PAGE. This precipitation, which removed ellagic acid by acid hydrolysis, could be dissolved in the sample buffer for SDS−PAGE, whereas the flock sediment of green tea beverage could not. The content of proteins decreased after heat sterilization, showing that some ellagic acid combined with proteins immediately after manufacturing.

In addition, both strictinin and BSA (each at a final concentration of 10 μg/mL) were dissolved in distilled water and heat-sterilized at 121 °C for 7 min. A large amount of flock sediment appeared immediately after heat sterilization, whereas each individual component failed to appear.

The beverage industry has been faced with the occurrence of flock sediment while preserving tea beverages, especially in green and jasmine teas. It is desirable to avoid the formation of flock sediment in these beverages since it can be mistaken for mold propagating. To address this problem, the components of green tea extracts were fractionated and sediment-formation tests were performed. It was determined for the first time that strictinin and proteins in tea beverages were the substances causing flock sediment. It should be noted that strictinin is hydrolyzed to yield ellagic acid under heat-sterilization treatment (e.g., by a retort or ultra-high temperature processing) during the manufacture of tea beverages. This liberated ellagic acid then combines with proteins to form an irreversible sediment. The same mechanism was also confirmed in the manufacture of jasmine tea beverage. When green tea leaves involve a strictinin amount of about 0.43% or more of its dry weight, flock sediment tended to occur, and jasmine tea leaves tended to precipitate about 0.9% or more of its dry weight. The reason for the difference in the amount of strictinin when flock sediment is formed with green tea beverage and jasmine tea beverage is that in the jasmine tea leaf manufacturing process there is an odor-emitting phase from several hours' accumulation of the green tea leaves and budding jasmine flowers at 45 °C or less, in which process protein is possibly denatured. The tea cream resulting from oolong and black tea beverages is associated mainly with catechins and caffeine, being different from flock sediment. There is only a little strictinin in most oolong and black teas. These beverages failed to form flock sediment. Green tea is manufactured by steaming or roasting immediately after plucking the raw tea leaves to inactivate the oxidative enzyme, and oolong and black teas are manufactured by fermentation with the enzymes of raw leaves. Strictinin in the oolong and black tea was dissolved in the process of fermentation. The reason for the failure to form flock sediment in oolong and black tea beverages was thought to be that the strictinin, and its derivatives were not extracted. Pectin, which is the main polysaccharide in tea extract, has been thought to be one of the substances causing flock sediment (*20*). Polysaccharide in flock sediment was changed to methylglucoside by methanol−HCl hydrolysis at 95 °C for 5 h, and these trimethylsilyl derivatives were analyzed with GC-MS (*21*). However, since there were few trimethylsilyl derivatives detected, we concluded that polysaccharides were not among the substances causing this sediment. Based on the sediment-formation test, none of the components in the fraction (except for strictinin) were involved in sediment formation, indicating that ellagic acid derived from strictinin was the main component of flock sediment. Moreover, green tea beverage manufactured from leaves that contained hardly any strictinin also failed to form flock sediment.

The content of hydrolyzable tannins has been shown to undergo seasonal changes in the leaves of *Liquidambar formosana* (*22*) and *Betula pubescens* (*23*, *24*). The strictinin contents of tea leaves also undergo changes depending on factors such as the time of the harvest season and their growth level (data not shown). This result strongly suggested that such factors may control the sediment formation in tea beverages.

Since the ellagitannin in the tea leaves was only a small amount of strictinin, it was considered not to contribute to sedimentation in the tea beverage (*12*). In this study, strictinin was found at a level of 0.6% in the first-flush tea leaves used. Other hydrolyzable tannins in the leaves of *Camellia sinensis* have been identified as 1,4,6-tri-*O*-galloyl-*β*-D-glucose, 3-galloylquinic acid, 3-*p*-coumaroylquinic acid, 3-caffeoylquinic acid, 5-caffeoylquinic acid, and 1-*O*-galloyl-*β*-D-glucose. Recently, a metabolic pathway of ellagitannins has been proposed (*25*), and strictinin has been synthesized via the pathway of 1,2,3,4,6-penta-*O*-galloyl-*β*-D-glucose and tellimagrandin II. However, the strictinin in tea leaves may derive from 1,4,6-tri-*O*-galloyl-*β*-D-glucose by the oxidative coupling of two gallic acid moieties enzymatically. In fact, the 1,4,6-tri-*O*-galloyl-*β*-D-glucose content was found to be significantly higher in some kinds of first-flush leaves containing very little strictinin.

The precipitation observed in wine and grape juice was found to contain ellagic acid and was seen to be particles or needles rather than flock precipitation in many cases (*17*, *19*). This is considered to be due to the fact that there are few proteins in the precipitation of grape juice or wine since both are acid beverages under pH 4.0 (*8*). Interestingly, this kind of precipitation also slightly appeared in acid green tea beverage (pH 3.5) that was acidified with ascorbic acid.

The current study showed that strictinin in tea extracts was hydrolyzed to yield ellagic acid under heat-sterilization and that flock sediment occurred when ellagic acid combined with proteins. High-temperature, high-pressure sterilization (e.g., by a retort or ultra-high temperature processing) is indispensable in the manufacture of neutral beverages (pH 5.0−7.0) to prevent the propagation of microbes. It is also necessary to control the strictinin and protein contents of tea beverages so as to avoid the formation of flock sediment.

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