Original Article

**In vitro assay to estimate tea astringency via observing flotation of artificial oil bodies sheltered by caleosin fused with histatin 3**

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**A B S T R A C T**

Astringency, a sensory characteristic of food and beverages rich in polyphenols, mainly results from the formation of complexes between polyphenols and salivary proteins, causing a reduction of the lubricating properties of saliva. To develop an in vitro assay to estimate the astringency of oolong tea infusion, artificial oil bodies were constituted with sesame oil sheltered by a modified caleosin fused with histatin 3, one of the human salivary small peptides. Aggregation of artificial oil bodies was induced when they were mixed with oolong tea infusion or its major polyphenolic compound, (−)-epigallocatechin gallate (EGCG) of 100 μM as observed in light microscopy. The aggregated artificial oil bodies gradually floated on top of the solution and formed a visible milky layer whose thickness was in proportion to the concentrations of tea infusion. This assay system was applied to test four different oolong tea infusions with sensory astringency corresponding to their EGCG contents. The result showed that relative astringency of the four tea infusions was correlated to the thickness of floated artificial oil bodies, and could be estimated according to the standard curve generated by simultaneously observing a serial dilution of the tea infusion with the highest astringency.

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1. **Introduction**

Tea is one of the most widely consumed beverages around the world, and its major ingredients, flavonols and polyphenols, have been demonstrated to provide a variety of health benefits [1–5]. Oolong tea possessing a taste and color somewhere between green and black teas is the most popular tea in Taiwan [6,7]. The taste quality of oolong tea infusion...
In this study, we aimed to develop a practical assay to estimate the astringency of oolong tea in vitro. The assay reagent was designed to comprise artificial oil bodies constituted with salivary proteins, such as histatins [9]. The basic and aromatic residues of histatins were found to be essential for the interaction of polyphenols (tannins) with salivary proteins [13–16]. The tannin–protein complexes consequently aggregate and precipitate, causing the loss of lubricity in the oral cavity, and thus induce astringent sensation in the mouth. Among the salivary proteins, histatins, such as histatin 3 and histatin 5, have been shown to possess potent tannin-binding ability under slightly alkaline environment [17]. In an nuclear magnetic resonance (NMR) study, the basic and aromatic residues of histatins were found to complex with tannins via hydrophobic interactions [18].

In the past decades, several methods were developed and aimed to estimate astringency by quantitating phenolic compounds in the beverage, e.g., Bate–Smith method, vanillin assay and gallic acid equivalence method using the Folin–Ciocalteu reagent [19–21]. These oversimplified methods were practically unsatisfactory as the astringency thresholds of different phenolic compounds varied significantly, and thus the total contents of phenolic compounds detected by these methods were not reliably correlated to the astringency of the beverage samples [22]. To overcome the dead-end of the phenolic quantity methods, tannin–protein binding assays were developed recently, such as salvia precipitation method and peptide adsorption technique [23,24]. Though these binding assays adequately mimic human bioresponse, they are unsuitable for frontline users as their operations are laborious and professional machines are required in the detection.

Seed oil bodies are storage organelles composed of neutral lipids (mainly triacylglycerols) surrounded by a monolayer of phospholipids embedded with unique integral proteins, oleosin, caeleosin, and steroleosin [25]. Stable artificial oil bodies have been technically reconstituted with the three essential components of oil bodies, triacylglycerols, phospholipids, and oil-body proteins (oleosin or caeleosin) [26]. Several application platforms have been developed on the basis of artificial oil bodies, including a protein expression system, an oral delivery system for hydrophobic drugs, a new enzyme-fixation technique, and a hapten presentation system for producing mono-specific antibodies against small molecules [27–30].

In this study, we aimed to develop a practical assay to estimate the astringency of oolong tea in vitro. The assay reagent was designed to comprise artificial oil bodies constituted with sesame oil sheltered by a modified caeleosin fused with histatin 3. After incorporation with oolong tea infusion, artificial oil bodies in the assay reagent aggregated and gradually floated on top of the mixture solution. Relative astringency of oolong tea infusions was estimated on the basis of observing flotation of aggregated artificial oil bodies that formed a visible milky layer with thickness in proportion to tea astringency.

2. Materials and methods

2.1. Chemicals and materials

High-performance liquid chromatography (HPLC) grade acetonitrile and methanol were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Acetic acid (99.7%) was obtained from J.T. Baker (Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA). Phosphoric acid (85%) was brought from Merck Millipore (Gibbstown, NJ, USA). Purified water was afforded by a Millipore Direct-Q purification system (Billerica, MA, USA). 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) and EGCG were purchased from Sigma (St Louis, MO, USA). Sesame oil was purchased from a local market. Oolong teas, prepared from tea plants (Camellia sinensis L., Chin-shin oolong) cultivated in four different altitudes of the mountain area of Center Taiwan, Chu Shan (600 m), Lu Shan (1200 m), Yu Shan (1600 m), and Ta Yu Ling (2200 m), were gifts or purchased from local manufacturers.

2.2. Plasmid construction

The cDNA fragment encoding a sesame caeleosin of 245 amino acid residues (accession number AF109921) was constructed in the nonfusion expression vector pET29a (Novagen, Madison, WI, USA), using an Ndel site at the initial methionine position and a Xhol site in the polylinker of the vector [31]. The construct of a modified caeleosin lacking the amphipathic α-helix (residues 101–115) was generated and described in a previous study [32]. Histatin 3 DNA fragment (GATAGCCATGCAGACGGATCATCAGCGC-ATAAGCGCAATTCCAT-GAATACATCACAGCCATCGTGGTTATCGTAGCAACTATAAA-GAAAACATCACAGCCATCGTGGTTATCGTAGCAACTATAAA-

TACGATAACTGAGTGA), containing a Scal restriction site at the initial position and an Xhol restriction site at the terminal position, was synthesized by Genewiz Inc. (South Plainfield, NJ, USA) and constructed in pUC57-Amp. The plasmid was purified by the Gene-Spin MiniPrep Plasmid Purification Kit (Promech, Taipei, Taiwan), restricted by Scal and Scal (NEB, England), and ligated with the modified caeleosin fragment by T4 DNA ligase (Promega, Madison, WI, USA).

2.3. Querexpression of recombinant proteins and sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis

The recombinant plasmids encoding the modified caeleosin with or without histatin 3 were transformed to Escherichia coli BL21 (DE3). Overexpression of the two recombinant fusion proteins were induced by adding 1mM isopropyl β-D-thiogalactoside (IPTG) in a bacteriophage T7 RNA polymerase/ promoter system. After induction for 3 hours, E. coli cells were harvested, lysated by sonication in the presence of 100mM potassium phosphate buffer, pH 7.0, and fractionated into supernatant and pellet by centrifugation (10,000g). Proteins extracted from the supernatant and pellet of E. coli cells were mixed with the sample buffer containing 62.5mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 0.02% bromophenol
blue, 10% glycerol, and 5% β-mercaptoethanol, and resolved by SDS-polyacrylamide gel electrophoresis (SDS–PAGE) using 12.5% acrylamide. Following electrophoresis, the gel was stained with Coomassie blue R-250. The recombinant fusion proteins eluted from the SDS–PAGE gel according to the method described by Chen et al [33], were quantitated by using ImageJ 1.41 program (http://rsb.info.nih.gov/ij/), and used to generate artificial oil bodies.

2.4. Constitution of artificial oil bodies

Artificial oil bodies were generated with 40 mg of sesame oil, 150 μg of DSPC, and 750 μg of the recombinant fusion protein (modified caleosin fused with or without histatin 3) in 1 mL of 100 mM potassium phosphate buffer plus with 30 mM potassium chloride, pH 7.0 [27]. DSPC dissolved in chloroform was placed at the bottom of an Eppendorf tube, and the chloroform was allowed to evaporate in a chemical hood overnight. After evaporation, the sesame oil and the recombinant fusion protein suspended in 1 mL of the potassium phosphate buffer were incorporated, followed by sonication with a 3-mm-diameter probe in a Sonics & Materials VCX750 ultrasonic processor (Newtown, CT, USA) with 30% amplification for 20 seconds, and samples were cooled down in an ice bucket for 5 minutes. The sonication was repeated two more times to generate artificial oil bodies. Artificial oil bodies of approximately 40 mg oil/mL were stored at 4°C and used as the assay reagent for the estimation of astringency.

2.5. Preparation of tea infusions

Infusions of oolong teas were prepared according to the protocol suggested by the Taiwan Tea Research and Extension Station officially used for the tea evaluation in Taiwan. Each oolong tea of 3 g was added to boiling reverse-osmosis (RO) water of 150 mL. After 6 minutes, the brew was collected in an appraisal bowl, cooled down to 40°C in a water-bath, and used for the following analysis. For a serial dilution of Chu Shan oolong tea infusion, RO water was added to adjust the tea concentrations to 0%, 20%, 40%, 60%, 80%, and 100%. Similarly, the serially diluted infusions were kept in the 4°C water-bath prior to further analysis.

2.6. Light microscopy of artificial oil bodies

Artificial oil bodies incorporated with oolong tea infusion, EGCG or RO water were examined in a Portable 2-in-1 Microscope (Forever Plus Corp., Taiwan). Two types of artificial oil bodies (sheltered by the modified caleosin fused with or without histatin 3) of 100 μL were firstly diluted with 400 μL of the potassium phosphate buffer, and then mixed with 500 μL of Chu Shan oolong tea infusion, EGCG (200 μM) or RO water used for the preparation of oolong tea. The samples were kept at room temperature for 60 minutes, and the floated artificial oil bodies on top of the solutions were collected and observed for their aggregation under the light microscope.

2.7. HPLC analysis of tea infusions

Tea infusions were filtered through a 0.45-μm polyvinylidene difluoride (PVDF) membrane filter (PALL Corporation, Glen Cove, NY, USA). Chemical constituents in the tea infusions were analyzed on a liquid chromatography system coupled to a Model 600E photodiode array detector (Waters Corporation, Milford, MA, USA) and performed using a 250 mm × 4.6 mm internal diameter (i.d.), 5 μm, Mightysil RP-18 GP HPLC column (Kanto Chemical Co., Inc., Japan). The mobile phase consisted of (A) acetonitrile and (B) distilled water containing 0.5% acetic acid. The linear gradient started with 5% (A) and increased to 25% (A) in 100 minutes. The column was maintained at room temperature and the injection volume was 10 μL at a flow rate of 1 mL/min. The UV absorbance detection wavelength was set at 270 nm. Phenolic compounds in tea infusion were assigned according to the analysis described previously [34].

2.8. Sensory evaluation of tea astringency

Sensory evaluation of tea astringency was performed in the Tea Research and Extension Station, Wunshan Branch (New Taipei City, Taiwan). Five experts constantly serving as professional panelists in the local tea competitions and five volunteers with no history of known taste disorders were invited for sensory evaluation of astringency in infusions prepared from the four different oolong tea samples. The relative astringency was scored on a 5-point scale (1, 3, and 5 points representing weak, intermediate and strong astringent taste). The sample infusions

![Figure 1 – SDS–PAGE of the recombinant modified caleosin fused with or without histatin 3 in E. coli. Total proteins of E. coli with the modified caleosin (mCal) alone or fused with histatin 3 (mCal–Hst 3) overexpressed before or after isopropyl β-D-1-thiogalactopyranoside (IPTG) induction were resolved in SDS–PAGE. Soluble (sup.) and insoluble (ppt) proteins extracted from E. coli cells containing the two recombinant proteins were also analyzed. Labels on the left indicate the molecular masses of commercial marker proteins (Genemark, Taichung, Taiwan). E. coli = Escherichia coli; hst3 = histatin 3; IPTG = isopropyl β-D-thiogalactoside; mCal = modified caleosin; ppt = insoluble; SDS–PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis; Sup = soluble.](http://example.com/figure1.png)
were swirled around in the mouth, and then expectorated. Data were expressed as mean ± SEM of scores from the 10 subjects.

2.9. In vitro assay for the relative astringency of tea infusions

To estimate relative astringency in vitro, each oolong tea infusion of 750 μL was mixed with an equal volume of artificial oil bodies (40 mg oil/mL) in a disposable plastic cuvette of 1.5 mL-capacity. Gradual formation of a visible milky layer on top of the mixture solution for 60 minutes. The thickness of the milky layer for each sample solution at 0 minutes, 15 minutes, 30 minutes, and 60 minutes was photographed by a digital camera, and quantitated with the program in the Microsoft Powerpoint 2013. Relative astringency of oolong tea infusions was estimated according to the relative thickness of the milky layer recorded at the same time (30 minutes). The statistics are calculated by Microsoft Excel 2013.

3. Results

3.1. Production of recombinant caleosin fused with or without histatin 3

Two recombinant proteins containing a modified caleosin fused with or without histatin 3 were successfully overexpressed in E. coli cells (Figure 1). As expected, the fusion of histatin 3 resulted in the increase of the molecular mass (approx. 25 kDa) of the recombinant modified caleosin by approximately 4 kDa. Both recombinant proteins were insoluble and predominantly found in the pellet of cell extracts.

3.2. Generation of artificial oil bodies sheltered by caleosin fused with or without histatin 3

Stable artificial oil bodies were generated with sesame oil sheltered by the modified caleosin fused with or without...
histatin 3. The particle sizes (mostly 1–2 μm) of the two types of artificial oil bodies (with or without fusion of histatin 3) were found similar as observed in a light microscope (Figures 2A and 2B). These artificial oil bodies were observed as individual particles in a suspension buffer of pH 7.0. Artificial oil bodies sheltered by the modified caleosin fused with histatin 3 were found to aggregate in oolong tea infusion or in the presence of EGCG (100 μM) at pH 7.0 (Figures 2C and 2E); by contrast, those sheltered by the modified caleosin (without histatin 3 fusion) remained as individual particles or slightly aggregated in the same conditions (Figures 2D and 2F). Presumably, fusion of histatin 3 to the modified caleosin played a key role for the complex formation with polyphenols in oolong tea infusion, and thus induced the aggregation of artificial oil bodies.

3.3. Visible flotation of aggregated artificial oil bodies in tea infusions

The aggregated artificial oil bodies sheltered by the modified caleosin fused with histatin 3 gradually floated and formed a milky layer on the top of the sample solutions in cuvettes.

Figure 3 — Flotation of artificial oil bodies in different concentrations of oolong tea. Chu Shan oolong tea infusion of different concentrations (0%, 20%, 40%, 60%, 80%, and 100%) was mixed with artificial oil bodies sheltered by the modified caleosin fused with histatin 3, and then loaded into cuvettes. Aggregated artificial oil bodies that floated and formed milky layers on the top of sample solutions in cuvettes were photographed 0 minutes, 15 minutes, 30 minutes, and 60 minutes after mixing with different concentrations of tea infusion.

Figure 4 — Comparison of the HPLC profiles of four oolong tea infusions. HPLC profiles of oolong teas prepared from the same tea plant cultivar (Chin-shin oolong) grown in four different attitudes of the mountain area of Center Taiwan, Chu Shan (600 m), Lu Shan (1200 m), Yu Shan (1600 m), and Ta Yu Ling (2200 m), were analyzed and compared. Caffeine and the major catechin, EGCG were labeled in each HPLC profile of the tea infusion. To reveal more polyphenols of relatively minor abundance, enlargement profiles were shown in the inserted panels between 55 and 75 minutes. In the enlarged profiles, three relatively abundant peaks at 62.9 minutes, 65.5 minutes, and 71.2 minutes were identified as quercetin-3-O-glucosyl-rhamnosyl-glucoside (Q3G), epicatechin-3-O-gallate (ECG) and kaempferol-3-O-glucosyl-rhamnosyl-glucoside (K3G), respectively. EGCG = (−)-epigallocatechin gallate; HPLC = high performance liquid chromatography.
visible milky layer on top of the tea infusion within 30 minutes, and the accumulated thickness of the milky layer seemed to reach a plateau in 60 minutes (Figure 3). The visible milky layer formed on top of the tea infusion after 60 minutes was found to be very stable, and remained nearly unchanged thereafter for at least 6 hours (data not shown). Moreover, the thickness of the milky layer was observed to be in proportion to the concentration of tea infusion, and thus in proportion to the content of polyphenols in tea infusion. Therefore, measurement of the thickness of the floated artificial oil bodies was used to estimate the relative astringency of different oolong tea infusions in the following assay.

3.4. Estimation of relative astringency of four oolong tea infusions

It has been well-recognized that the higher the altitude the tea plants are cultivated in the mountain area of Center Taiwan, the less astringency the infusion of their consequent oolong tea is perceived. This empirical sensation is partly explained by the observation that the polyphenols in oolong tea infusions are inversely correlated to the cultivation altitude [35]. Accordingly, four representative oolong teas produced from Chu Shan (600 m), Lu Shan (1200 m), Yu Shan (1600 m), and Ta Yu Ling (2200 m) were found to contain polyphenols inversely correlated to the cultivation altitude (Figure 4).

Visible flotation and milky layers of aggregated artificial oil bodies sheltered by the modified caleosin fused with histatin 3 were also observed for all the four oolong tea infusions (Figure 5). The thickness of the milky layer on top of the tea infusion was found inversely correlated to the cultivation altitude of oolong tea, and thus inversely correlated to the content of polyphenols (EGCG). A standard curve was generated by simultaneously observing the thickness of the milky layer in the serial dilution of Chu Shan oolong tea infusion (as shown in Figure 3) that possessed the highest polyphenolic content and astringency in the four oolong tea infusions (Figure 6A). Taking the astringency of Chu Shan oolong tea infusion as 100%, relative astringency of Lu Shan, Yu Shan, and Ta Yu Ling oolong tea infusions were semiquantitatively calculated as 78%, 57% and 45%, respectively (Figure 6B). Moreover, the relative astringency of the four oolong tea infusions estimated by this in vitro assay and oral astringency scores evaluated by sensory evaluation (Figure 6C) followed the same trends, corresponding to their relative contents of EGCG, the major catechin in oolong tea (Figures 4 and 6D). Therefore, it is suggested that the assay system is suitable to estimate relative levels of astringency in oolong tea infusions.

4. Discussion

In this study, we successfully developed an in vitro assay to estimate the relative astringency of oolong tea. The assay reagent contained artificial oil bodies constituted with sesame oil cuvettes were photographed 0 minutes, 15 minutes, 30 minutes, and 60 minutes after mixing with different tea infusions.
sheltered by an integral oil-body protein, caleosin fused with a human salivary peptide, histatin 3. Relative astringency of oolong tea was estimated on the basis of observing flotation of aggregated artificial oil bodies that formed a visible milky layer with thickness in proportion to tea astringency. It is expected that this in vitro assay system is applicable to estimate relative astringency of food and beverages, such as red wine, provided it undergoes necessary minor adjustment.

To promote the local marketing as well as to grade the quality of oolong tea, more than one hundred competitions for freshly prepared oolong teas are held by many official organizations and private associations every year in Taiwan. In some large competitions, several thousands of tea samples have to be scored for aroma, luster, and flavor (umami, bitterness, and astringency) by sensory evaluation in a few days; in general, the evaluation is first screened by local experts and finally examined by three to five professional panelists from the Taiwan Tea Research and Extension Station. The heavy sample loading in the tea competitions has been a stressful burden and challenge for the referees of sensory evaluation as it is not easy to maintain the mouth tasting condition identically for huge sample amounts in a limited time. Therefore, there is an urgent demand for reliable scientific detections to assist the sensory evaluation in tea competitions. According to this study, the assay system based on observation of floated artificial oil bodies putatively provides a fast, friendly, reproducible, and inexpensive method to estimate the relative astringency of tea infusions. It is promising that this assay system should be a helpful tool to assist and verify the relative astringency of oolong tea infusions scored by sensory evaluation in tea competitions. Realistically, it is impossible for general users to prepare artificial oil bodies due to the lack of the recombinant caleosin fused with histatin 3 and the unavailability of sonicator apparatus. Practically, ready-to-use artificial oil bodies should be commercially produced in solid form (powder or tablet) via the well-established protocols [36], and thus general users are able to take advantage by simply mixing tea infusion with the commercial powder or tablet, and observe the thickness of floated artificial oil bodies for the estimation of relative astringency of the tea samples.

The astringent taste of tea is mainly caused by phenolic compounds, such as catechin and flavonid glycoside [17]. However, it has been shown that the astringency thresholds of Chu Shan oolong tea infusion as 100\%, relative astringency of Lu Shan, Yu Shan, and Ta Yu Ling oolong tea infusions was calculated according to the equation (n = 3); (C) astringency of Chu Shan, Lu Shan, Yu Shan, and Ta Yu Ling oolong tea infusions was evaluated by a sensory panel of 10 subjects on a 5-point scale (1, 3, and 5 points representing weak, intermediate, and strong astringent taste). Data are mean ± SEM (n = 10); (D) taking the EGCG content of Chu Shan oolong tea infusion as 100\%, relative EGCG content of Lu Shan, Yu Shan and Ta Yu Ling oolong tea infusions was calculated according to the HPLC analysis as shown in Figure 4 (n = 3). EGCG = (−)-epigallocatechin gallate; HPLC = high performance liquid chromatography; SEM = standard error mean.
of different types of catechin and flavonoid derivatives varied significantly [22]. In this regard, knowing the relative astringency of these tea phenolic compounds should be useful for the improvement of current tea manufacture processes as well as for the development of further refinement protocols in terms of reducing astringency of oolong tea products. Puteatively, the in vitro assay developed in this study is suitable to be employed to estimate the relative astringency of all the detectable phenolic compounds, at least the abundant ones, found in oolong tea infusions.

As saliva contains diverse proteins and small peptides [9], the utilization of histatin 3 for the complex formation with polyphenols in the astringency assay seems to be oversimplified for mimicking the sensation of astringency in mouth. For the follow-up improvement of this assay system, the major proteins, such as α-amylase, lactoferrin, and mucins as well as other abundant peptides should be included in proportions similar to those found in saliva. Practically, each saliva protein or peptide should be individually linked to caesinoid and used to generate artificial oil bodies, respectively. Mixtures of different artificial oil bodies linked with major saliva proteins and peptides in proper composition is expected to be established in the next generation of this astringency assay system.

Conflicts of interest

All authors declare no conflicts of interest.

Acknowledgments

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