Journal of Food and Nutrition Research, 2013, Vol. 1, No. 6, 164-173
Available online at http://pubs.sciepub.com/jfnr/1/6/8

DOI:10.12691/jfnr-1-6-8

Concurrent Accumulation of Myricetin and Gallic Acid Putatively Responsible for the Umami Taste of a Specialized Old Oolong Tea

Feng-Yin Li[1], Wei-Hung Yang[2,3], Chi-I Chang[4], Sin-Jie Lee[2], Chih-Chien Hung[1], Ying-Jie Chen[2], Tzyy-Rong Jinn[5,*], Jason T.C. Tzen[1,5,6,*]

[1]Department of Chemistry, National Chung-Hsing University, Taichung, Taiwan, R.O.C
[2]Graduate Institute of Biotechnology, National Chung-Hsing University, Taichung, Taiwan, R.O.C
[3]Taichung Hospital Department of Health, Taichung, Taiwan, R.O.C
[4]Graduate Institute of Biotechnology, National Pingtung University of Science and Technology, Pingtung, Taiwan, R.O.C
[5]School of Chinese Medicine, China Medical University, Taichung, Taiwan, R.O.C
[6]Agricultural Biotechnology Research Center, Academia Sinica, Taipei, Taiwan, R.O.C
*Corresponding author: tctzen@dragon.nchu.edu.tw; jinn@mail.cmu.edu.tw

Received November 20, 2013; Revised December 08, 2013; Accepted December 15, 2013

Abstract Strong umami taste was perceived in a specialized old oolong tea reproducibly converted from a fresh oolong tea by fierce baking biannually for five years. Phenolic compounds in this umami tea and those in an initial fresh oolong tea were analyzed and compared. The results implied that the abundant catechin derivatives and flavonol glycosides were significantly reduced while three compounds were evidently accumulated after the tea conversion. These three compounds were chemically identified as gallic acid and two correlated flavonols, myricetin and quercetin. Molecular modeling suggested that myricetin and gallic acid might serve as ligand and enhancer to activate the umami receptor synergistically. Binary docking of myricetin and gallic acid induced the gate closure of the binding cavity in the umami receptor, in a manner similar to that of glutamate and its enhancer, inosine 5′-monophosphate. Regardless the induced fitness, the detailed molecular interactions between these two sets of binary components and the receptor were drastically different. In this binary docking modeling for umami taste, myricetin could not be equivalently replaced with any of the major tea phenolic compounds.

Keywords: *gallic acid, myricetin, old oolong tea, quercetin, umami*

Cite This Article: Feng-Yin Li, Wei-Hung Yang, Chi-I Chang, Sin-Jie Lee, Chih-Chien Hung, Ying-Jie Chen, Tzyy-Rong Jinn, and Jason T. C. Tzen, "Concurrent Accumulation of Myricetin and Gallic Acid Putatively Responsible for the Umami Taste of a Specialized Old Oolong Tea." *Journal of Food and Nutrition Research* 1, no. 7 (2013): 164-173. doi: 10.12691/jfnr-1-6-8.

1. Introduction

Tea has been consumed as a healthy beverage around the world for several thousand years. Oolong tea possessing a taste and color somewhere between green and black teas is manufactured predominantly in Fujian and Guangdong of China as well as in Taiwan. For the preparation of oolong tea, young leaves are freshly harvested and allowed to undergoing a semi-fermentation process, where the term 'fermentation' refers to natural browning reactions induced by oxidative enzymes in the leaf cells [1]. The degree of fermentation is practically controlled during the tea preparation, usually ranging from 20 to 80% according to the appetite of customers. In response to the growing market as well as the demand of quality advancement, versatile process conditions have been attempted to generate variable products of oolong tea by local manufacturers in the past few decades.

Traditionally, old oolong tea is named for those oolong teas baked by professional drying processes and stored for more than five years [2]. It is normally consented that the longer oolong tea is stored and further oxidized progressively, the better it is in terms of taste and beneficial effects to human health. A major type of old oolong tea commonly practiced in Taiwan utilizes periodical baking refinement at least annually during the aging process. Aging and baking processes are generally regarded as two major factors for the quality control of old oolong teas. As expected, different combinations of aging and baking processes have led to the commercial production of diverse old oolong teas with distinct tastes and different healthy effects empirically [3].

Umami, a Japanese term originally defined for delicious flavor, is now known as the fifth basic taste [4]. Monosodium glutamate, a prototypical umami ligand, is frequently added in Asian cuisine for the improvement of food taste. This savory taste induced by glutamate can be synergistically amplified with an enhancer, such as inosine 5′-monophosphate (IMP) [5]. Several G protein-coupled receptors in the taste bud on the tongue are proposed to be involved in the umami sensing [6]. Among them, a

heterodimer receptor (T1R1/T1R3) is assumed to be the binding target of the binary components (ligand and enhancer) for the synergistic umami taste. This heterodimer umami receptor as well as the sweet receptor (also a heterodimer, T1R2/T1R3) has been regarded as the target of natural and artificial sweeteners [7].

It has been suggested that the umami taste of green tea is mainly caused by the presence of free amino acids [8]. Among free amino acids in green tea, glutamate and theanine are exceptionally abundant and account for two-thirds of total amino acid content; therefore, these two abundant amino acids are regarded as key ingredients responsible for the umami taste of green tea. Several nucleotides, e.g., IMP, which have been known as adequate enhancers for the umami taste of glutamate, are present in negligible amounts in the tea infusion, and thus unlikely contribute to the savory taste of green tea [9]. However, two small phenolic compounds, gallic acid and theogallin (a gallic acid molecule estered with a quinic acid molecule), were identified as umami-enhancing constituents in green tea as they were able to elevate the umami intensity of glutamate proportionally [10]. It was assumed that these two phenolic compounds might serve as enhancers and synergistically amplify the umami taste induced by glutamate.

In an attempt to inspect diverse old oolong teas in Taiwan, a specialized old oolong tea was identified for its strong umami taste. In the present study, we aimed to identify the potential constituents responsible for the umami taste by comparing the phenolic compounds in this tea, tentatively named umami tea, with those in an initial fresh oolong tea. Chemical structures of the compounds apparently enriched in the umami tea were determined. Molecular modeling was employed to evaluate the possible umami constituents by docking candidate compounds to the umami receptor. Detailed molecular interactions of the possible umami compounds were mimicked and compared with those of glutamate and its enhancer, IMP within the binding cavity of the umami receptor.

2. Methods

2.1. Chemicals and Materials

Gallic acid was purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Acetonitrile (HPLC grade) and acetic acid (99.7%) were obtained from Merck KGaA (Darmstadt, Germany) and J. T. Baker (Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA), respectively. Purified water was afforded by a Millipore clear water purification system (Direct-Q, Millipore, Billerica, MA, USA). A specialized old oolong tea (umami tea) and an initial fresh oolong tea were gifts from a local tea producer, Mr. Ching-Hwei Kao in Hong-Shang Village, Nantou County, Taiwan. Fresh oolong tea was prepared with young green shoots of tea plant (*Camellia sinensis* L., Chin-Shin oolong) harvested from Hong-Shang Village (altitude around 1800 meter) in May 2008, following the traditional semi-fermentation process with a final fermentation degree of approximately 50%. The umami tea was prepared by baking a fresh oolong tea intensely (ranging from 120 to 150°C for 10 h), and the baking was repeated biannually for five years. Ten preparations of the umami tea were produced during the years of 2001-2008 by the same baking process operated by Mr. Kao.

2.2. Preparation of Tea Infusions and HPLC/UV Analysis

Tea infusions were prepared by adding 20 ml of boiling water to 1 g of tea preparations (ground in powder) and kept in a 95°C water bath [11,12]. After 15 min, the brew was filtered through a 0.45 μm polyvinylidene difluoride (PVDF) membrane filter (PALL Corporation, Glen Cove, NY, USA), and used for the following analysis. For HPLC/UV analysis, 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 i.d., 5 μm, C 18 reversed-phase column (Thermo Electron Corporation, Waltham, MA, USA). The mobile phase was consisted of (A) water containing 0.025% phosphoric acid and (B) acetonitrile. The gradient was as follows: 0-60 min, linearly gradient from 10% to 30% B; 60-70 min, 30% B; and 70-100 min, linear gradient from 30% to 10% B. In all experiments, the injection volume was 10 μl and the flow rate was 1 ml/min at room temperature. The UV absorbance detection wavelength was set at 280 nm. Gallic acid abundantly accumulated in the umami tea was identified by comparing its retention time and mass fragmentation patterns with those of an authentic standard in a similar manner as reported in our previous studies [12,13].

2.3. Isolation of Two Flavonols Accumulated in the Umami Tea

The umami tea of 600 g was powdered and extracted three times (7 days each) with methanol (4 l) at room temperature. After removal of the solvent under reduced pressure, the extract was suspended in H_2O (600 ml), and then partitioned sequentially using EtOAc and *n*-BuOH (600 ml × 3). The EtOAc-soluble fraction (80 g) was chromatographed over a Sephadex LH-20 column (7 × 90 cm, Pharmacia Biotech) eluting with mixtures of water, methanol, and acetone of reducing polarity as eluents. Twelve fractions were collected as follows: fr. 1 [3000 ml, water], fr. 2 [3000 ml, water - methanol (9:1)], fr. 3 [2500 ml, water - methanol (8:2)], fr. 4 [2000 ml, water - methanol (7:3)], fr. 5 [2000 ml, water - methanol (6:4)], fr. 6 [2500 ml, water - methanol (5:5)], fr. 7 [2500 ml, water - methanol (4:6)], fr. 8 [2500 ml, water -methanol (3:7)], fr. 9 [2500 ml, water - methanol (2:8)], fr. 10 [2000 ml, water - methanol (1:9)], fr. 11 (3000 ml, methanol), and fr. 12 [4000 ml, methanol - acetone (5:5)]. Fraction 7 (11.4 g) was rechromatographed on a silica gel column (5 × 45 cm, 230-400 mesh ASTM, Merck) and eluted with CH2Cl2-MeOH (30:1 to 0: l) to obtain eight fractions (~800 ml for each), fr. 7A-fr. 7H. Fr. 7E (310 mg) was separated by semi-preparative HPLC on a Merck Lichrosorb Si 60 column (5 μm, 250 × 10 mn) with CH_2Cl_2-acetone (3:1) as eluent, 2 ml/min, to yield myricetin (33.8 mg, t_R = 31.5 min). Fraction 8 (360 mg) was further chromatographed on the silica gel column, eluted with CH_2Cl_2-MeOH (20:1 to 0:1) to obtain seven fractions (~700 ml for each), 8A-8G. Fraction 8E (360 mg) was purified by semi-

preparative HPLC on the Merck Lichrosorb Si 60 column with CH_2Cl_2-acetone (7:3) as eluent, 2 ml/min, to yield quercetin (41.5 mg, t_R = 32.8 min).

2.4. Determination of Chemical Structures of the Two Flavonols by NMR

NMR spectra were acquired on a Varian Mercury plus 400 NMR instrument at 400 MHz (^{1}H) and 100 MHz (^{13}C) in deuterated acetone ($(CD_3)_2CO$) at a constant temperature controlled and adjusted to around 300 K, using the residual solvent resonance as internal shift reference. For the ^{13}C NMR spectra, multiplicities were determined by a Distortion-less Enhancement by Polarization Transfer (DEPT) experiment.

Quercetin: yellow amorphous powder; ^{1}H NMR (400 MHz, acetone-d_6): δ 6.25 (1H, d, *J* =1.6 Hz, H-6), 6.51 (1H, d, *J* = 1.6 Hz, H-8), 6.99 (1H, d, *J* = 8.8 Hz, H-5'), 7.70 (1H, dd, *J* = 2.4, 8.8 Hz, H-6'), 7.81 (1H, d, *J* =2.4 Hz, H-2'), 12.16 (1H, s, 5-OH); ^{13}C NMR (100 MHz, acetone-d_6): δ 94.6 (C-8), 99.3 (C-6), 104.3 (C-10), 115.9 (C-2'), 116.3 (C-5'), 121.6 (C-6'), 123.9 (C-1'), 136.9 (C-3), 146.0 (C-3'), 147.1 (C-2), 148.5 (C-4'), 157.9 (C-9), 162.5 (C-5), 165.1 (C-7), 176.7 (C-4) [14,15].

Myricetin: yellow amorphous powder; ^{1}H NMR (400 MHz, acetone-d_6): δ 6.25 (1H, d, *J* =2.0 Hz, H-6), 6.50 (1H, d, *J* =2.0 Hz, H-8), 7.41 (2H, s, H-2′,6′), 12.16 (1H, s, 5-OH); ^{13}C NMR (100 MHz, acetone-d_6): δ 94.5 (C-8), 99.3 (C-6), 104.2 (C-10), 108.4 (C-2′, 6′), 122.8 (C-1'), 136.5 (C-3), 137.1 (C-4'), 146.5 (C-3′, 5′), 147.0 (C-2), 157.9 (C-9), 162.5 (C-5), 165.2 (C-7), 176.7 (C-4) [15,16].

2.5. Analysis of the Content of Glutamate in Tea

The content of glutamate in tea was analyzed according to the method described previously [17]. Briefly, tea sample of 2 g was extracted with 100 ml of hot water for 6 min, cooled to room temperature, and then filtered through a 0.45 μm nylon filter membrane. After filtration, 10 ml of tea infusion was titrated to pH 3 with HCl and partitioned with an equal volume of ethyl acetate twice. The water layer was adjusted to pH 8.4 with $NaHCO_3$, and partitioned with an equal volume of ethyl acetate again. The water layer (50 μl) containing amino acids was subjected to dabsylation by mixing with 100 μl of dabsyl chloride (1 mg/ml in acetone) at 70°C for 10 min. Determination of the dabsyl-amino acids derived from tea samples was carried out by HPLC at 467 nm. The HPLC system consisted of Waters In-Line Degasser AF, Waters 600 Controller, Waters 717 plus Autosampler, and Waters 2996 Phctodiode Array Detector. A poroshell 120 EC-C 18 column (Agilent Technologies, 4.6 mm × 100 mm i.d., 2.7 μm particle size) coupled with a poroshell 120 EC-C 18 guard column (Agilent Technologies, 4.6 mm × 5 mm i.d., 2.7 μm particle size) was used.

2.6. Molecular Modeling

The 2D structures of tea phenolic compounds selected for molecular modeling were constructed by using the ChemDraw program, and their corresponding 3D structures were converted by the Chem3D program (http://www.cambridgesoft.com/). The obtained structures were further energy minimized with B3LYP/6-31G*, a density functional theory method, in Gaussian 03 package (http://www.gaussian.com/) and verified with frequency check.

On the basis of homologous conservation, the umami receptor is assumed to possess an equivalent ligand-triggering mechanism through a venus fly trap (VFT) domain as proposed for the activation of the sweet receptor [18]. Having an architecture of two lobe structures connected by a three-stranded flexible hinge, the binding cavity of the VFT domain in the umami receptor seems to be open or closed in the absence or presence of ligand, in a manner similar to those of the VFT domains in metabotropic glutamate receptors (mGluRs). The closed form induced by binding with a ligand, such as glutamate, is the active form that initiates the downstream signaling pathway for umami sensation. Except where explicitly mentioned, all the modeling processes were performed by using Discovery Studio 2.1 platform (http://accelrys.com/). The open form structure of the VFT domain in the umami receptor was constructed through homology modeling with the available structures of mGluR family, 3KS9, 2E4Z, 1EWK, 1EWV, and 1EWT as templates [19,20]. The closed forms of the VFT domain were obtained by molecular dynamics-based simulated annealing when docking with ligands and enhancers.

The molecular modeling scheme was designed according to a two-step mechanism, i.e., docking and locking, as proposed in the dynamics of flytrap closure measured for the VFT domain of iGluR2, an ionotropic glutamate receptor [19]. A potential umami ligand and its enhancer were introduced into the umami receptor during the docking process with LigandFit, a shape-directed rapid docking method, and the hotspot defined as the ligand-binding site of the VFT domain as reported previously [20]. The obtained structures were subjected to an energy minimization process to remove any existing strain or overlap between neighboring atoms and followed by a structural optimization process under CHARMm force field [21]. Among the candidate structures generated by LigandFit, the docked structure with the highest Ligscore2 value computed by the score ligand pose module was selected to undergo the locking process, in which a molecular dynamics-based simulated annealing was used to optimize the final configuration. Afterward, the obtained structures were validated through Profile-3D and Ramachandran plot.

3. Results

3.1. Identification of An Umami Tea in Taiwan Old Oolong Teas

Among diverse old oolong teas in Taiwan, a specialized old oolong tea was identified for its strong umami taste. Tracking for 10 preparations of this umami tea produced in different years by the same manufacturer, we found that the umami taste was reproducibly generated under a biannual baking process of five years (totally, 10 times of periodical baking intensely). According to the manufacturer, the umami taste was slightly perceived after 5 times of baking (2-3 years) and subsequently enhanced to a strong level in the following 5 baking processes. To

examine the effects of the intense baking processes on the outward appearance of the umami tea, its tea leaves and infusions were compared with those of an initial fresh oolong tea Figure 1. It seemed that tea granules became compact and transformed from yellow green to carbonized gray brown after the baking conversion. Moreover, the tea infusion color seemed to change from golden yellow to dark red while the carbonized tea leaves turned black and could no longer expand to their original sizes as the initial fresh tea leaves did when they absorbed hot water in a regular tea preparation.

3.2. Comparison of Phenolic Compounds in the Umami Tea and A Fresh Oolong Tea

To explore the changes of phenolic compounds in the preparation of umani tea, infusion of the umami tea was analyzed and compared with that of an initial fresh oolong tea. For a better comparison with our previous analyses of various tea infusions [12,13], the current analysis was performed under the same condition. The results indicated that the abundant catechin derivatives, such as (−)-epigallocatechin gallate (EGCG) and (−)-epicatechin gallate (ECG), as well as flavonol glycosides, such as kaempferol-3-*O*-glucosylrhamnosyl- glucoside (K-3G), seemed to be significantly reduced after the tea conversion Figure 2. In contrast, three compounds were evidently accumulated in the umami tea compared with the fresh oolong tea, and they were chemically identified as gallic acid and two correlated flavonols, myricetin and quercetin. Significant accumulation of these three compounds was consistently observed in 10 different preparations of this umami tea reproducibly generated by the same baking protocol (data not shown).

Figure 1. Tea leaves and infusions of a fresh oolong tea and the umami tea. Tea leaves were shown before and after tea preparation. After tea preparation (95°C for 15 min), the tea infusions were kept at room temperature for 30 min prior to photographing

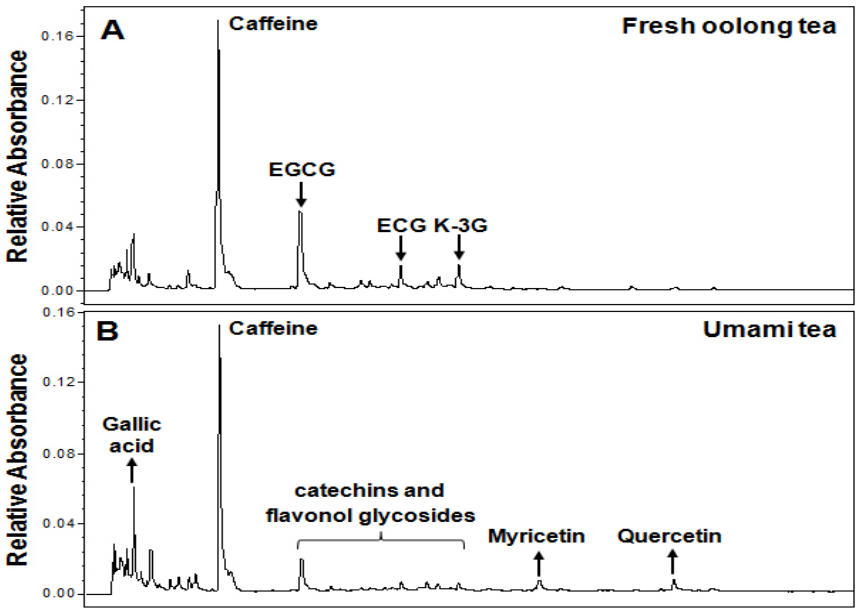

Figure 2. Liquid chromatography profiles (0-75 min) of infusions of the fresh oolong tea (A) and the umami tea (B) at 280 nm; In comparison with the fresh oolong tea, major phenolic compounds apparently decreased and increased in the umami tea were indicated by down and up arrows, respectively

To examine the occurrence of the three identified compounds during the preparation process, the intermediate umami teas after baking for 2, 3 and 4 years were subjected to the same HPLC analysis. The results showed that gallic acid was moderately accumulated after baking for 2 years, and significantly increased in the following baking; whereas, the contents of myricetin and quercetin were relatively low after baking for 2 years, but substantially elevated in the following baking Figure 3. Coincidentally, the contents of myricetin and quercetin in the intermediate umami teas were found proportional to the progressive enhancement of umami taste during the preparation of this specialized tea as experienced by the manufacturer. In contrast, the contents of glutamate, the major amino acid responsible for the umami taste of green tea [10], were found moderately reduced after baking for 2 years, but nearly depleted in the following baking Figure 4. As expected, similar to glutamate, all the free amino acids in the tea were nearly depleted after baking for more than 3 years (data not shown).

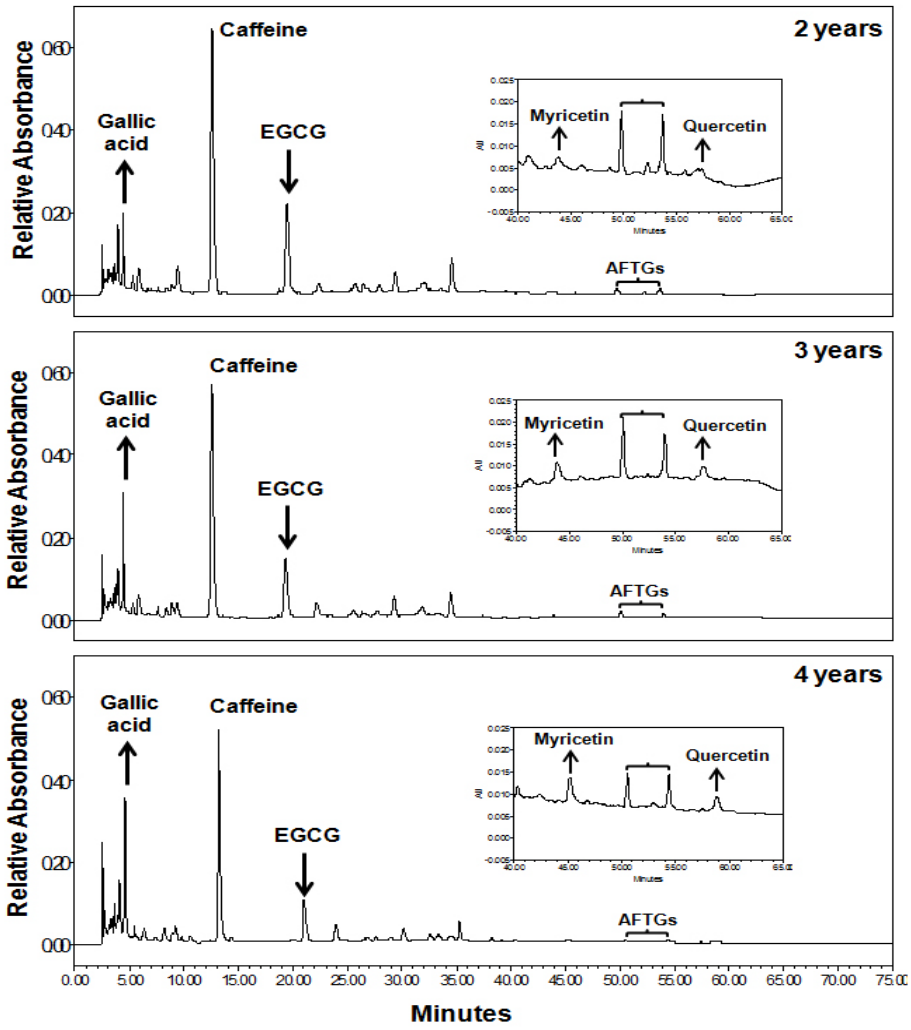

Figure 3. Liquid chromatography profiles (0-75 min) of infusions of the intermediate umami teas after baking for 2, 3 and 4 years at 280 nm, Amplification of each profile from 40 to 65 min is shown in an inserted panel within the diagram; AFTGs represents acylated flavonol tetraglycosides identified previously [12]

3.3. Molecular Modeling of Possible Umami Compounds in Oolong Tea

To assist the evaluation of the potential constituents responsible for the umami taste, molecular docking of candidate compounds to the umami receptor were mimicked and compared with that of glutamate and IMP. The tea phenolic compounds selected for the docking modeling are gallic acid, myricetin, quercetin, (−)-gallocatechin (GC), (−)-epigallocatechin (EGC), EGCG, K-3G, and myricetin-3-O-galactose (M-Gal), and their chemical structures are shown in Figure 5.

In our docking results, the combination of myricetin and gallic acid induces the gate closure of the binding cavity in the umami receptor, in a manner similar to that of glutamate and IMP Figure 6. The distance between the two lobes of the binding cavity reduces from originally 15.1 Å to 6.1 or 7.8 Å upon binary docking with myricetin and gallic acid or glutamate and IMP. Correspondingly, myricetin occupies the glutamate (ligand) binding site while gallic acid resides in the IMP (enhancer) binding site. Equivalent to glutamate, myricetin binds close to the hinge region of the binding cavity, and presumably triggers the hinge-bending motion to activate the gate closure along with the interaction with the lower lobe. Similar to IMP, gallic acid binds near to the opening of the bi-lobed architecture to stabilize the closed conformation. The modeling results support that myricetin and gallic acid are suitable ligand and enhancer, presumably superior to glutamate and IMP, to activate the umami receptor synergistically.

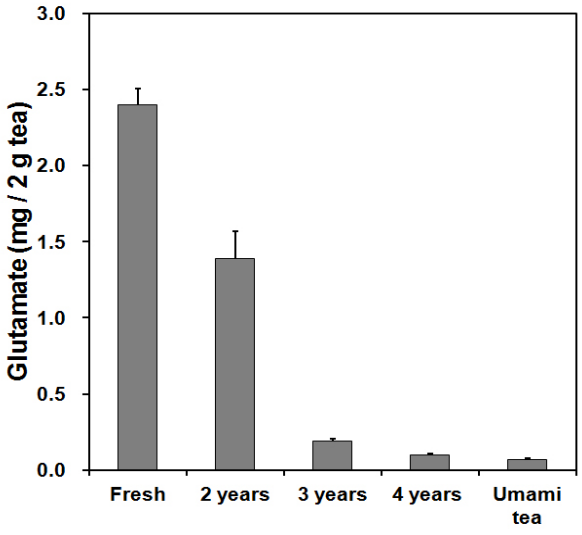

Figure 4. Contents of glutamate in the tea samples during the conversion of umami tea; Contents of glutamate in the fresh oolong tea, umami tea and intermediate umami teas after baking for 2, 3 and 4 years were analyzed and compared. Data are mean ± SEM (n = 3)

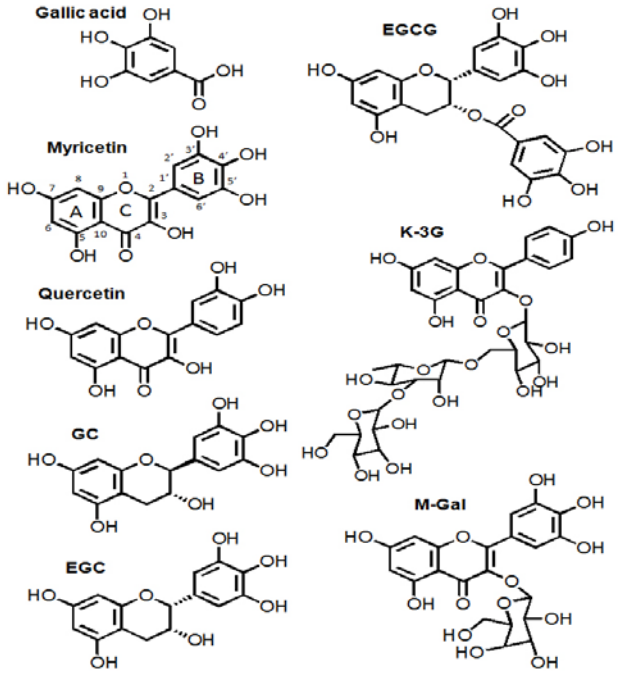

Figure 5. Chemical structures of tea phenolic compounds used for molecular modeling; Gallic acid, myricetin, quercetin, three catechins (GC, EGC and EGCG), and two flavonol glycosides (K-3G and M-Gal) were selected for molecular docking to the umami receptor in this study

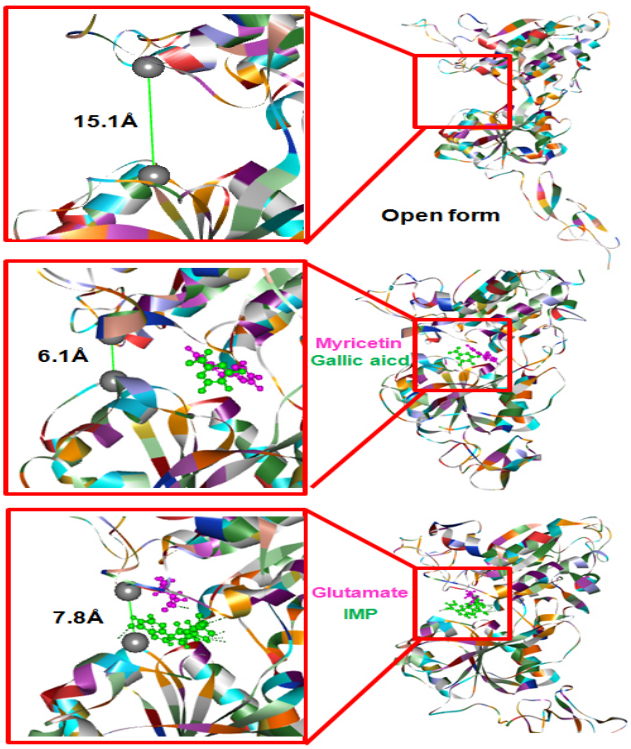

Figure 6. Binary docking of myricetin and gallic acid to the umami receptor compared with that of glutamate and IMP, the known umami ligand and enhancer, respectively. The right panels show the modes of the umami receptor (in ribbon structure) with or without the docking of ligands and enhancers (in pink and green ball-and-stick, respectively). The magnified docking sites are shown in the left panels along with the labeled distance between the two lobes of the binding cavity

Regardless the induced fitness for the gate closure of the binding cavity, the detailed molecular interactions between these two sets of binary components and the binding cavity of the umami receptor were drastically different Figure 7. Glutamate and IMP are charged molecules and interact with the receptor mainly through pure charge-charge interaction and ionic hydrogen bonding with limited hydrophobic interaction. In contrast, myricetin and gallic acid are neutral molecules and interact with the receptor through hydrogen bonding and hydrophobic interaction. Usually the charge-charge interaction and ionic hydrogen bonding are stronger than the regular hydrogen bonding interaction. This may explain why glutamate, being a relatively small molecule in comparison with myricetin, can also act as an effective agonist for umami sensation.

Regarding the detailed molecular interactions in the binding cavity of the umami receptor, all the hydrophilic functional groups of gallic acid are involved in hydrogen bonding while the only aromatic ring is involved in π-cation interaction with Arg277 Figure 7A. Similarly, all the hydroxyl groups and the only carbonyl group of myricetin are involved in hydrogen bonding. Ring B of myricetin is close to the hinge region with the three hydroxyl groups interacting with six residues, Ser172, Gln195, Asp192, Glu301, Asn388, and Ser384, as an anchor to stabilize the closed conformation. Ring A and C of myricetin are involved in π-π stacking interaction, a kind of hydrophobic interaction while the ether oxygen faces the opening of the binding cavity without forming any hydrogen bonding. As to the detailed molecular interactions of glutamate in the binding cavity of the umami receptor, the backbone amine group enters near the hinge region with hydrogen bonding, the α-carboxylate group forms hydrogen bonding to stabilize the closed conformation, and the γ-carboxylate group points toward the opening of the binding cavity Figure 7B. These results indicate that Ring B of myricetin acts like the backbone of glutamate while Ring A and C of myricetin act like the side chain of glutamate for the molecular interactions within the binding cavity of the umami receptor.

3.4. Docking of Catechins to the Umami Receptor

To explore why the strong umami taste is not perceived in fresh oolong teas, three representative catechins, GC, EGC and EGCG Figure 5 are first used to replace myricetin in the docking modeling for umami sensation. EGCG is the most abundant catechin in tea; EGC, possessing a chemical structure highly resemble to that of myricetin, is the pyrolytical product of EGCG by removing the gallic acid moiety; and GC is the epimer of EGC. The modeling results indicate that none of the catechins induce the gate closure of the binding cavity in the umami receptor as effectively as myricetin Figure 8. As to the single docking of EGCG, which may be regarded as the enhancer (gallic acid) covalently attached to Carbon 3 of Ring C in EGC, the effectiveness for gate closure of the binding cavity is the worst. Although the orientation of EGC or GC inside the binding cavity resembles to that of myricetin, its Ring B is not as close to the hinge region as that found in myricetin. Furthermore, less hydrogen bonding and no π-π interaction were found in the molecular interaction patterns of these catechin docking conformations in comparison with the docking of myricetn with gallic acid. According to the above structural analyses of docking modeling, all the catechins found in oolong tea seem to be inadequate to act as a umami ligand.

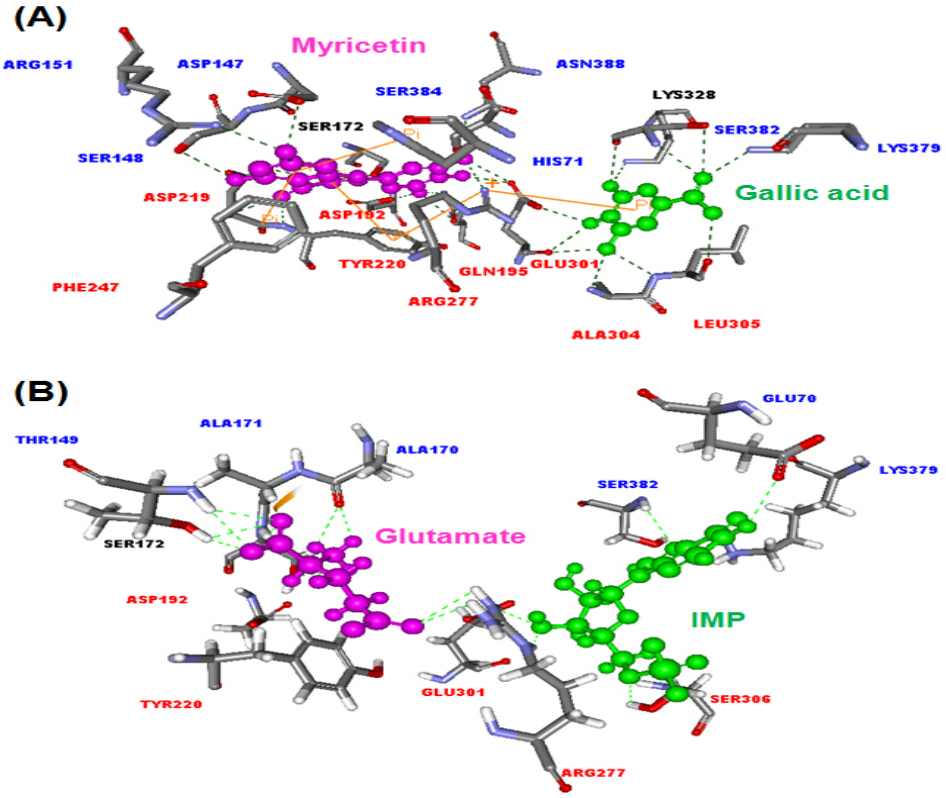

Figure 7. Detailed molecular interactions of myricetin-gallic acid (A) or glutamate-IMP (**B**) within the binding cavity of the umami receptor; Molecular interactions between the two sets of binary docking molecules (ligand and enhancer) and the umami receptor shown in Figure 6 are illustrated in more details. Ligands and enhancers are depicted in pink and green ball-and-stick, respectively. Residues belonging to the upper lobe, the lower lobe and the hinge region are labeled in blue, red and black. Hydrogen bonding is shown in green dashed lines, and π-π stacking or π-cation interaction is designated in orange lines

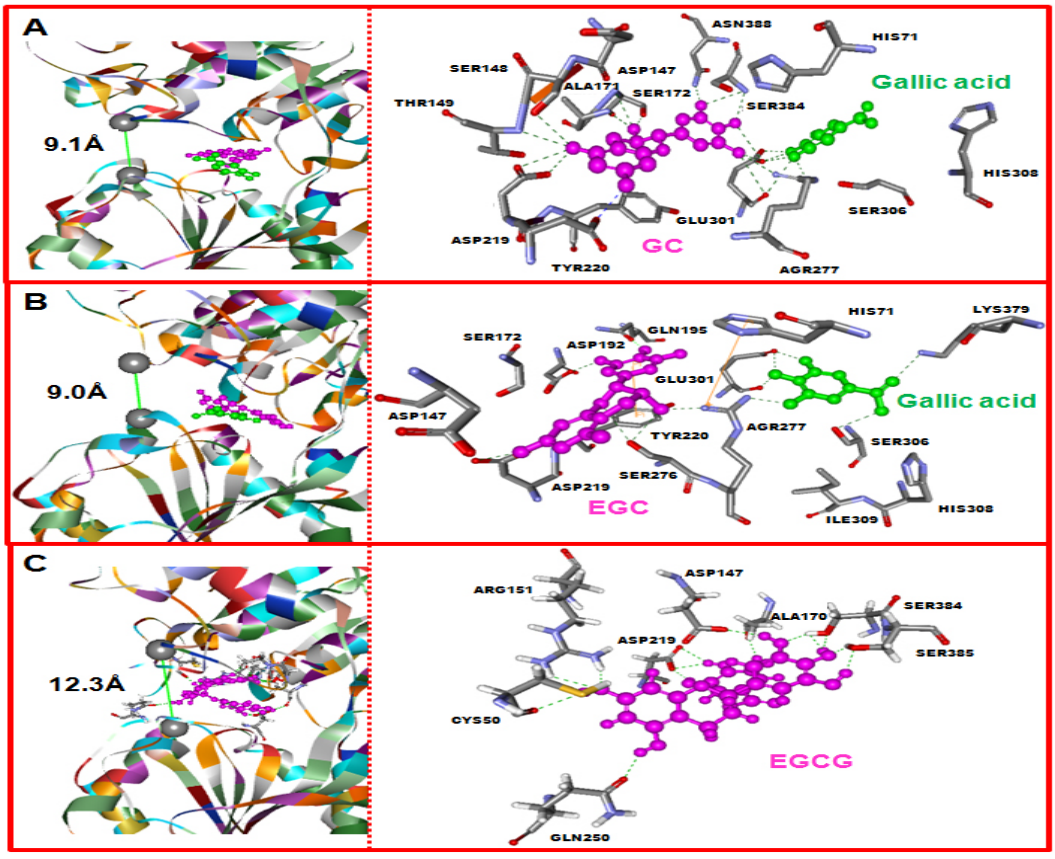

Figure 8. Docking of major catechins to the binding cavity in the umami receptor along with their corresponding molecular interactions; Three representative catechins, GC (A), EGC (B) and EGCG (C), depicted in pink ball-and-stick, are selected for the docking modeling in company with gallic acid, depicted in green ball-and-stick, except for EGCC. Detailed molecular interactions of the three catechins within the binding cavity of the umami receptor are shown in the right panels along with the distance between the two lobes of the binding cavity labeled in the left panels

3.5. Docking of tea Flavonol Compounds to the Umami Receptor

Besides catechins, flavonol derivatives represent another major group of phenolic compounds in oolong teas. Being a flavonol, myricetin is replaced with three tea flavonol derivatives, quercetin, K-3G and M-Gal Figure 5 in the docking modeling for umami sensation. Quercetin, found also in the umami tea but not in fresh oolong teas, differs from mercetin by missing the hydroxyl group in Carbon 5' of Ring B; K-3G is the most abundant flavonol derivative in the fresh oolong tea Figure 2; and M-Gal is the smallest myricetin glycoside found in fresh oolong teas. The modeling results indicate that the distance between the two lobes of the binding cavity reduces to 8.4 Å upon binary docking with quercetin and gallic acid, a little wider than that (7.8 Å) found in the binary docking of glutamate and IMP Figure 4 and Figure 9A. The number of hydrogen bonding interaction in Ring B of quercetin with the umami receptor reduces to only one compared to six found in Ring B of myricetin. It is because that there are only two hydroxyl groups of Ring B in quercetin and these two hydroxyl groups cannot provide sufficient interaction to sustain Ring B close to the hinge region. Nevertheless, there is one extra π-π interaction between Ring B of quercetin and Trp220, which is not found in the myricetin case. Taken together, it is likely that concurrence of quercetin and gallic acid may lead to a subtle umami sensation.

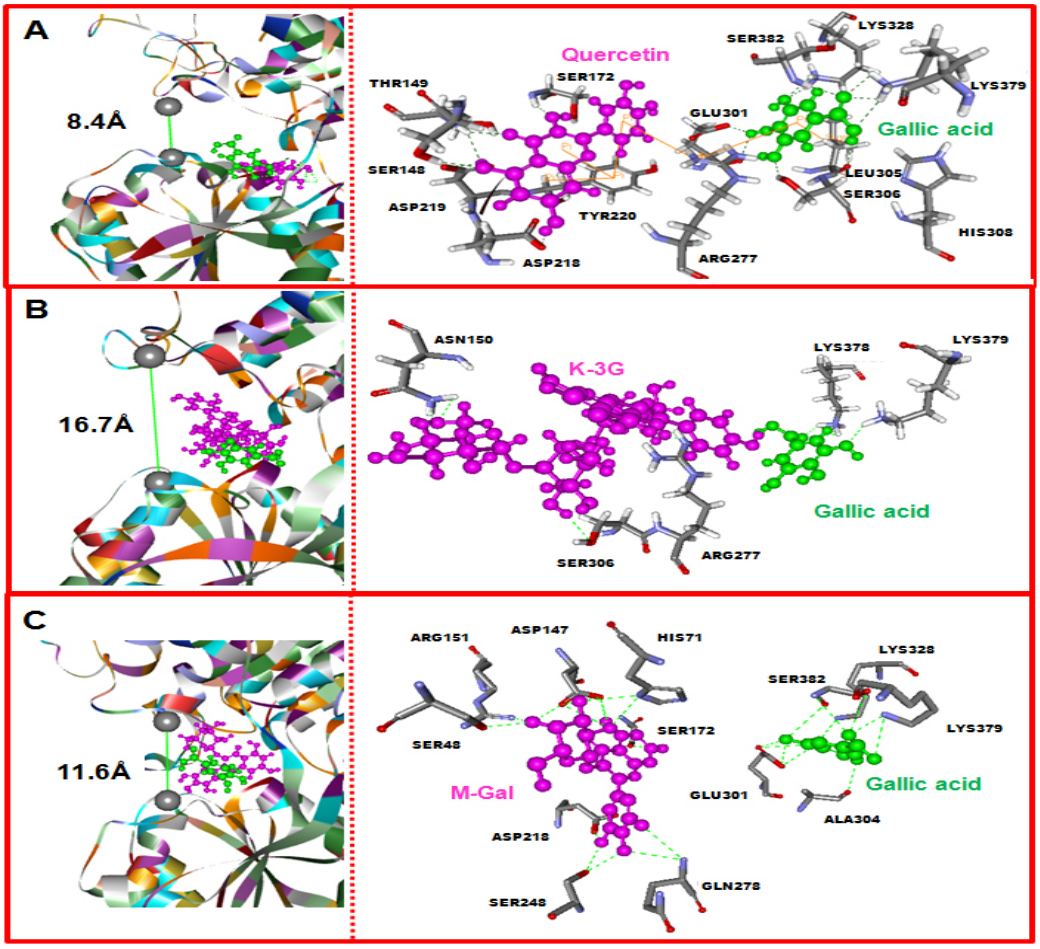

Figure 9. Docking of tea flavonol compounds to the binding cavity in the umami receptor along with their corresponding molecular interactions; Three representative flavonol compounds, quercetin (A), K-3G (B) and M-Gal (C), depicted in pink ball-and-stick, are selected for the docking modeling in company with gallic acid, depicted in green ball-and-stick. Detailed molecular interactions of the three flavonol compounds within the binding cavity of the umami receptor are shown in the right panels along with the distance between the two lobes of the binding cavity labeled in the left panels

As for the docking of K-3G and gallic acid, the triple glucose moiety extends out of the binding cavity and thus severely prevents the gate closure of the two lobes Figure 9B. Obviously, the three sugar moieties of K-3G are too long to accommodate into the binding cavity of the umami receptor. As for the docking of M-Gal and gallic acid, the gate closure of the binding cavity cannot be effectively induced Figure 9C. It indicates that M-Gal is not an adequate ligand and even worse than quercetin. There are two possible reasons for this observation. Firstly, the galatose moiety pulls the whole structure of M-Gal a little bit toward to the opening of the binding cavity preventing the Ring B of myricetin backbone entering deeply into the hinge region, and consequently reducing the interaction strength between the hydroxyl groups of Ring B and the hinge residues. Secondly, the galatose moiety also changes the orientation of myricetin backbone within the binding cavity. Originally, it is Oxygen 1 of the Ring C in myricertin pointing toward the opening, but this oxygen atom in M-Gal points inward causing the plane of myricertin upside down with Ring A moving to a different location in the binding cavity. This orientation change reduces the interaction strength between M-Gal and the binding cavity of the umami receptor. It is likely that no sugar attachment is allowed for myricertin to serve as a ligand for the activation of umami sensation.

4. Discussion

In this work, a specialized old oolong tea was identified with strong umami taste, and thus used to screen for possible umami constituents. Three phenolic compounds were significantly accumulated in this umami tea when compared with an initial fresh oolong tea, and chemically determined as gallic acid, myricetin and quercetin. Our modeling results suggest that myricetin (or quercetin) and gallic acid may be suitable ligand and enhancer to activate the umami receptor synergistically. According to the molecular docking, myricetin seems to be a better ligand than quercetin for the activation of the umami receptor. Of course, whether the combination of myricetin (or quercetin) and gallic acid can induce appropriate umami taste remains to be further evaluated scientifically including sensory evaluation. Moreover, we evaluated the docking of major catechins and flavonol derivatives to explain why the umami taste is not perceived in fresh oolong teas. Taken together, the current investigation seems to shed a new light on the structural rules for the umami ligand and enhancer, including size, shape and type of binding interaction, and may provide a route to search for alternative sources other than monosodium glutamate for the improvement of food taste.

Chemical conversion of phenolic compounds in the umami tea, i.e., major catechin derivatives and flavonol glycosides were evidently reduced while gallic acid was substantially elevated, was similar to that observed in our previous studies of typical old oolong teas [12,22] The massive accumulation of gallic acid was assumed to be originated from the decomposition of EGCG and some other catechins [23]. Meanwhile, myricetin and quercetin as well as kaempferol were consistently found as minor constituents in typical old oolong teas, and the occurrence of myricetin and quercetin was presumably resulted from deglycosylation of their glycoside derivatives during the baking processes[12]. Relatively, the contents of myricetin and quercetin in the umami tea were substantially higher than those in typical old oolong teas. This substantial difference, indicating a higher extent of deglycosylation of flavonol glycoside derivatives, was probably caused by the relatively intense baking conditions in the preparation of umami tea.

Interestingly, both myricetin and gallic acid are conjugated molecules, and therefore are planar in structure. The planar structures of these two molecules have at least two advantages for their docking to the umami receptor. Firstly, the planar structures allow myricetin and gallic acid to penetrate deeply into the hinge region of the bi-lobed structure without much steric hindrance. Secondly, these two molecules may act as double-sided tapes to ensure better closure of the two lobes during the locking process. In contrast, glutamate and IMP are not planar in structure, and thus some maneuver within the binding cavity of the umami receptor is unavoidable during their docking.

The relatively poor docking interactions of catechins in the binding cavity of the umami receptor may be explained by their shapes. Unlike myricetin, catechins are not planar as the double bond between Carbon 2 and 3 of Ring C is replaced with a single bond and the carbonyl oxygen atom in Ring C is missing. Due to the bending in Ring C, Ring A of EGC or GC is more close to the upper lobe but away from the lower lobe, and Ring B of EGC or GC cannot contact with the hinge region as much as that of myricetin to fully activate the hinge-bending motion. Consequently, myricetin acts as a thin plate sticky in both sides to glue the two lobes of the binding cavity, whereas GC or EGC acts as a hard-bended plate with a bump hindering the closure of the two lobes. As to EGCG, the constrained gallic acid moiety is unable to occupy the enhancer site, and thus the closure of the two lobes of the binding cavity is severely hindered. Furthermore, the docking orientation of EGCG inside the binding cavity is upside down compared to that of myricetin since the ether oxygen atom faces inward.

5. Conclusion

In this study, three phenolic compounds were found massively accumulated in a specialized old oolong tea with strong umami taste. Putative umami constituents were screened with the assistance of molecular modeling and docking simulation. Our results suggest that myricetin and gallic acid are putatively responsible for the umami taste through the same molecular mechanism conducted by glutamate and IMP. With this finding, we further evaluated the docking of major catechins and tea flavonol derivatives to explain why the umami taste is not perceived in fresh oolong teas. This investigation seems to shed a new light on the structural rules for the umami ligand and enhancer, including size, shape and type of binding interaction. We believe that this study opens a new route to develop natural sweeteners that may provide a route to search for alternative sources other than monosodium glutamate for the improvement of food taste

Acknowledgement

We thank Mr. Ching-Hwei Kao for kindly providing the valuable tea samples. The work was supported by a grant from the National Science Council, Taiwan (NSC 100-3114-B-005-001 to JTC Tzen).

References

[1] Haslam, E. "Thoughts on thearubigin" *Phytochemistry*, 64(1), 61-73, 2003.

[2] Lee, V. S. Y., Chen, C. R., Liao, Y. W., Tzen, J. T. C., and Chang, C. I. "Structural determination and DPPH radical scavenging activity of two acylated flavonoid tetraglycosides in oolong tea (*Camellia sinensis*)" *Chemical & Pharmaceutical Bulletin*, *56*(6), 851-853, 2008.

[3] Sang, S., Lambert, J. D., Ho, C. T., and Yang, C. S. "The chemistry and biotransformation of tea constituents" *Pharmacological Research*, 64(2), 87-99, 2011.

[4] Chandrashekar, J., Hoon, M. A., Ryba, N. J., and Zuker, C. S. "The receptors and cells for mammalian taste" *Nature*, 444, 288-294, 2006.

[5] Ghirri, A., and Bignetti, E. "Occurrence and role of umami molecules in foods" *International Journal Food Sciences and Nutrition*, 63, 871-881, 2012.9.

[6] Mouritsen, O. G. and Khandelia, H. "Molecular mechanism of the allosteric enhancement of the umami taste sensation" *the FEBS Journal*, 279, 3112-3120, 2012.

[7] Behrens, M., Meyerhof, W., Hellfritsch, C., and Hofmann, T. "Sweet and umami taste: natural products, their chemosensory

targets, and beyond" *Angewandte Chemie (International ed. in English)*, 50 (10), 2220-2242, 2011.

[8] Nakagawa, M. "Contribution of green tea constituents to the intensity of taste element of brew" *Journal of Food Science and Technology*, 22, 59-64, 1975.

[9] Horie, H., Ujihara, T., and Kohata, K. "Analysis of tasty nucleotides in green tea infusions (in Japanese)" *Tea Research Journal*, 93, 55-61, 2002.

[10] Kaneko, S., Kumazawa, K., Masuda, H., Henze, A., and Hofmann, T. "Molecular and sensory studies on the umami taste of Japanese green tea" *Journal of Agricultural and Food Chemistry*, 54(7), 2688-2694, 2006.

[11] Kuo, P. C., Lai, Y. Y., Chen, Y. J., Yang, W. H., and Tzen, J. T. C. "Changes in volatile compounds upon aging and drying in oolong tea production" *Journal of the Science of Food and Agriculture*, 91, 293-301, 2011.

[12] Lee, V. S. Y., Dou, J., Chen, R. J. Y., Lin, R. S., Lee, M. R., and Tzen, J. T. C. "Massive accumulation of gallic acid and unique occurrence of myricetin, quercetin and kaempferol in preparing old oolong tea" *Journal of Agricultural and Food Chemistry*, 56(17), 7950-7956, 2008.

[13] Dou, J., Lee, V. S., Tzen, J. T. C., and Lee, M. R. "Identification and comparison of phenolic compounds in the preparation of oolong tea manufactured by semifermentation and drying processes" *Journal of Agricultural and Food Chemistry*, 55(18), 7462-7468, 2007.

[14] Yang, S. C., Fang, J. M., and Cheng, Y. S. "Chemical constituents from the root and aerial parts of *Rosa taiwanensis*" *Journal of the Chinese Chemical Society*, 42, 573-577, 1995.

[15] Sawai, Y., Moon, J. H., Sakata, K., and Watanabe, N. "Effects of structure on radical-scavenging abilities and antioxidative activities of tea polyphenols: NMR analytical approach using 1,1-diphenyl-2-picrylhydrazyl radicals" *Journal of Agricultural and Food Chemistry*, 53(9), 3598-3604, 2005.

[16] Matsuda, H., Higashino, M., Chen, W., Tosa, H., Iinuma, M., and Kubo, M. "Studies of cuticle drugs from natural sources. III. Inhibitory effect of *Myrica rubra* on melanin biosynthesis" *Biological & Pharmaceutical Bulletin*, 18(8), 1148-1150, 1995.

[17] Syu, K. Y., Lin, C. L., Huang, H. C., and Lin, J. K. "Determination of theanine, GABA, and other amino acids in green, oolong, black, and Pu-erh teas with dabsylation and high-performance liquid chromatography" *Journal of Agricultural and Food Chemistry*, 56, 7637-7643, 2008.

[18] Zhang, F., Klebansky, B., Fine, R. M., Liu, H., Xu, H., Servant, G., Zoller, M., Tachdjian, C., and Li, X. "Molecular mechanism of the sweet taste enhancers" *Proceedings of the National Academy of Science of the United States of America*, 107(10), 4752-4757, 2010.

[19] Kunishima, N., Shimada, Y., Tsuji, Y., Sato, T., Yamamoto, M., Kumasaka, T., Nakanishi, S., Jingami, H., and Morikawa, K. "Structural basis of glutamate recognition by a dimeric metabotropic glutamate receptor" *Nature*, 407(6807), 971-977, 2000.

[20] Muto, T., Tsuchiya, D., Morikawa, K., and Jingami, H. "Structures of the extracellular regions of the group II/III metabotropic glutamate receptors" *Proceedings of the National Academy of Sciences of the United States of America*, 104(10), 3759-3764, 2007.

[21] Brooks, B. R., Bruccoleri, R. E., Olafson, B. D., States, D. J., Swaminathan, S., and Karplus, M. "CHARMM: A program for macromolecular energy, minimization, and dynamics calculations" *Journal of Computational Chemistry*, 4(2), 187-217, 1983.

[22] Chen, Y. J., Kuo, P. C., Yang, M. L., Li, F. Y., and Tzen, J. T. C. "Effects of baking and aging on the changes of phenolic and volatile compounds in the preparation of old Tieguanyin oolong teas" *Food Research International*, 53, 732-743, 2013.

[23] Dou, J., Lee, V. S., Tzen, J. T. C., and Lee, M. R. "Rapid identification of acylated flavonol tetraglycosides in oolong teas using HPLC-MSn" *Phytochemical Analysis*, 19(3), 251-257, 2008.