Glycyrrhetic acid monoglucuronide: sweetness concentration–response and molecular mechanism as a naturally high-potency sweetener

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Received: 13 August 2018 / Revised: 3 December 2018 / Accepted: 14 January 2019 / Published online: 6 February 2019
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Abstract Glycyrrhetic acid monoglucuronide (GAM) is obtained from the natural sweetener glycyrrhizin through enzymolysis. Its sweetness concentration–response (C–R) behavior in room-temperature in water was determined using two-alternative forced choice discrimination tests. The C–R equation of resultant hyperbolic curve relating sucrose equivalent (SE, %) to GAM concentration ([GAM], mg/L) was $SE = 19.6 \times [GAM] / (194.8 + [GAM])$. From the C–R function, Pw (2) of GAM, relative to a 2% (w/v) sucrose reference, is more than 900, which has much higher potency than its precursor glycyrrhizin. Molecular modeling showed that GAM is finely bound into protein 1EWK through conventional hydrogen bonds, π -Alkyl interactions and Van der Waals

bonds, which exhibited better protein inclusion than Glycyrrhizin. Thus, GAM could be developed as a new zero-calorie, naturally high-potency sweetener.

Keywords Glycyrrhetic acid · Sweetener · Sensory evaluation · High-potency · Molecular modeling

Introduction

With the increase in overweight, obese, and diabetic patients, consumers are conscious of their health and pay attention to food labels to ensure that the calorie content is in the recommended range. This promotes the continuous growth of global sweetener market as a result of increased focus on low-calorie sweeteners (Fowler, 2016; Piernas et al., 2014). Conventional sweeteners like sugar and high-fructose corn syrup are high in calories. The sweetener industry and people in homes are increasingly substituting sugar with low-calorie, intense sweeteners (Drewnowski and Rehm, 2016; Popkin and Hawkes, 2016). Although there are thousands of available low-calorie food and beverage products, most are not commercially viable. In the United States, only seven intensely sweet sugar substitutes have been approved for use, which are stevia, aspartame, sucralose, neotame, acesulfame potassium, saccharin, and advantame. Compared to artificial sweeteners, natural sweeteners have hardly any side effects to gain more popularity (Kim et al., 2017; Mooradian et al., 2017; Roberts, 2016). In short, natural non-calorie sweeteners are always the research hotspot.

Since rebaudioside A, a high-potency and low-caloric sweetener, was first permitted as a general purpose sweetener in the United States, natural non-caloric sweeteners are gaining popularity (Espinoza et al., 2014;

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Reynolds et al., 2017). Many consumers express interest in natural non-caloric, high-potency sweeteners (DuBois and Prakash, 2012; O'Brien, 2011). The sensation of sweetness is sometimes notably different from sucrose, so they are often used in complex mixtures that achieve the most natural sweet sensation. And a high commercial potential sweetener must be accepted in all six metrics as follows: safety, taste quality, stability, solubility, cost, and patentability (DuBois, 2008). Therefore, to develop a potential sweetener is an extreme challenge.

Glycyrrhizin (glycyrrhizic acid), the main sweet-tasting constituent of *Glycyrrhiza glabra* (licorice) root, could be transformed via enzymolysis or be metabolized in the liver to glycyrrhetic acid monoglucuronide (Fiore et al., 2005; Kim et al., 2000). The latter exhibited benign anti-inflammatory, antitumor, and antiviral activities (Li et al., 2017; Park et al., 2004; Tang et al., 2014; Yang et al., 2014). Due to fine stability, solubility and sweetness, it may be developed as a natural high-potency sweetener. The molecule is a natural triterpenoid saponin containing one 18 β -H-oleanane-type aglycone and one glucuronic acid. Its chemical formula is C₃₆H₅₄O₁₀ and its character is white crystalline powder (Yang et al., 2014). The structure has been elucidated using ¹H-, ¹³C-NMR and mass spectroscopy and reported to be 20 β -carboxy-11-oxo-30-nor-olean-12-en-3 β -D-glucopyranosiduronic acid (Fig. 1).

Glycyrrhizin is a sweet natural product that is extracted from licorice root, which isn't used as a sweetener but is used as a flavor. GAM, zymolytic prosapogenin of the natural sweetener glycyrrhizin, needs to carry out systematic experiments to study the complete sensory properties GAM as a sweetener. The detailed studies on GAM's sweetness quality and temporal profile are being carried out, but a key first step is to set up the sweetness potency. As a new sweetener, our study indicates the relative sweetness of pure GAM is 900–1000 times sucrose at 2% sucrose equivalent. Therefore, this key study can provide the sweetness concentration–response (C–R) relationship of GAM. Sensory studies designed to supply this information have now been completed and the results are reported here.

It is well-known that the human sweetener receptor is a single heterodimeric G protein coupled receptor (GPCR). The metabotropic glutamate receptors (mGluRs) are the most studied members of the Class C GPCRs, in which large Nterminal Venus Flytrap-like Domain of mGluR1 closes on binding glutamate (Kunishima et al., 2000). The extracellular ligand-binding region of mGluR1 showed that 'active' and 'resting' conformations of disulphide-linked homodimers are modulated through the dimeric interface by a packed α -helical structure. The bi-lobed protomer architectures flexibly change their domain arrangements to form an open or closed conformation with dynamic equilibrium (Muto et al., 2007; Zhang et al., 2010). As a template, systematic homology modeling yielded reliable models of all possible heterodimers of the human T1R2 and T1R3 sequences with the open and closed conformations of mGluR1 (DuBois, 2016; Temussi, 2007). Based on the mGluR's activating mode, molecular modeling is used to explain how the sweet receptor binds GAM, which is very important for its intrinsic biological significance.

Materials and methods

Tasting solutions

GAM was provided by Elion-Nature Biological Technology Co., Ltd. It comprised 96.2% potassium salt of glycyrrhetic acid monoglucuronide (the relative content, determined with area normalization method by HPLC at 250 nm). GAM is a white powder, odorless, soluble in water, glycerin, propylene glycol, slightly soluble in anhydrous ethanol. As a dry powder at ambient temperature and with controlled humidity, GAM is stable for at least three years. Sucrose was purchased from Energy Chemical (the content: 99.5%). Water was directly obtained from tap water through pure water instrument (Smart-RO30 Reverse Osmosis Unit, Shanghai Hetai instrument Intl. Co., Shanghai, China).

Fig. 1 Chemical structures of Glycyrrhizin and glycyrrhetic acid monoglucuronide (GAM)

Sensory evaluation

According to previous report (Fry et al., 2012), testing was conducted by means of 2-AFC (two-alternative forced choice) discrimination tests. Each panel comprised a minimum of 70 tasters; otherwise they numbered 72–81. Although all volunteers were Elion-Nature employees and untrained, almost half were regular volunteers and familiar with sensory testing routines. According to the standards of normal taste and odor acuity, our subjects of this project are trained to have the ability to recognize and discriminate many different types of taste stimuli. The final panels of this project were selected for their ability to: correctly discriminate the tastes of sweet (2.0% sucrose), salty (0.2% NaCl), sour (0.015% citric acid), and bitter (0.025% caffeine); accurately give four concentrations of each taste stimulus in order; in triangle tests, can identify the odd sample correctly. The purpose of the training was to make the panel familiar with taste characteristics of sweeteners. The panel's ability of recognizing, description and quantification of the taste profiles was trained in techniques. As consumer responses were required, no prescreening was done. Panelists were informed consent for information on the nature of GAM and participants were paid.

Treatment of panel data

Panel responses were counted as percentage of the panel finding the sucrose sample sweeter. For a given concentration of GAM, the percentage responses were plotted against sucrose concentration for the range of sucrose levels tested. Panel responses were plotted on a probability scale to obtain a straight line (Fry et al., 2012).

Molecular modeling

To visualize the possible binding model of interactions between a protein (sweet taste receptor) and small molecules (ligands) were the molecular docking techniques used. Molecular modeling in this study was conducted by using CDocker protocol in Discovery Studio 3.5 (Discovery Studio 3.5, Accelrys, Inc. San Diego, CA) (Wu et al., 2003; Yang et al., 2016). The CDocker Interaction Energy of glycyrrhizin in ligand8, ligand6 and ligand9 sites of the sweetener receptor protein 1EWK was smaller than that of GAM, which indicating that GAM and 1EWK had better binding stability. Both 2D and 3D maps of GAM and Glycyrrhizin most potent ligands with 1EWK were depicted in Figs. 4 and 5, respectively.

Results and discussion

Tasting solutions

Solutions for tasting were made on the same day and stored for six to eight hours. During the study, GAM was dissolved in water and made 10 solutions of known exact concentration in the range of 10–210 mg/L. According to previously reported C–R curves, the sucrose concentrations were estimated before preliminary, informal tasting. Therefore, the concentrations were selected as detailed under ‘Sensory evaluation’ to provide a fairly even spread of data points across the range of panel response from 10% to 90% of panel saying sucrose was sweeter.

Sensory evaluation

Two tastings were carried out each day, one in the morning and the other in the afternoon. Samples were provided as pairs of solutions, about 30 mL of each in 60 mL (2.0 oz) plastic cups. All solutions were conserved at room temperature (25 ± 1 °C). At each time before the beginning of test, the panel must be calibrated with the standardized agent of many different kinds of tastes. In addition, the panel also received tests of their tastes acuity to qualify them in future experiments. Solutions containing a known concentration of GAM and one of sucrose were provided to each panel. Samples were coded with 3-digit random numbers and presented in a balanced sequence. Panelists were required to rinse their mouths well with purified water before tasting, and re-taste is allowed. Solutions were swallowed after at least 10 s stay in mouth. Tasters were required to select one solution as being sweeter. Data were collected by means of using Origin 8 as data analysis tool.

Fig. 2 Concentration-response curve for dry GAM potassium in water by means of 2-AFC discrimination tests, which was obtained from 10 isosweet concentrations of sucrose for GAM concentrations from 10 to 210 mg/L

A different concentration of sucrose was used at each subsequent panel. This procedure was repeated until a minimum of 5 comparisons at different levels of sucrose had been collected for each concentration of GAM. As shown in Fig. 2, the C–R curve for the 10 isosweet determinations represents the information accumulated from over 3600 judgments.

Fig. 3 GAM potassium in water: linear dependency of potency on equivalent sweetness

Treatment of panel data

Straight lines were fitted to the probit plots by linear regression. The line helped select the spread of sucrose concentration used as underway testing. Equations for regression lines were used to determine the sucrose concentration at which 50% of the panel found the sucrose sweeter. This was believed as the concentration isosweet with the concentration of GAM under test.

It is clear that the C–R curve tends to a maximum, meaning that GAM potency varies with the range of testing concentrations. For a high-potency sweetener, the C–R function can be described as a hyperbolic function analogous to the Michaelis–Menten equation for enzyme–substrate interaction. Such an equation is shown as follows:

$$R = R_m \times C / ((1/K) + C)$$

where R is the observed response, R_m is the maximum response, C is the sweetener concentration, and K is the apparent receptor-sweetener association constant. $(1/K)$ is the apparent dissociation constant. This relationship is the known Beidler equation (Beidler, 1954; DuBois et al., 1991).

The constants R_m and $1/K$ can be obtained from a plot of potency (= response/concentration) against sweetness

Fig. 4 Molecular docking 2D mode of interaction of GAM (left) and glycyrrhizin (right) with the sweetener receptor protein (PDB code: 1EWK) analyzed by Discovery Studio 3.5. Conventional Hydrogen

Bond and Carbon Hydrogen bond and Alkyl as well as Pi-Alkyl are shown by green, light green and pink, respectively

Fig. 5 3D mode of interaction of GAM (above) and glycyrrhizin (below) with the sweetener receptor protein (PDB code: 1EWK) analyzed by Discovery Studio 3.5

response (Figs. 2, 3), where R_m is the intercept on the abscissa and $1/K$ the concentration at which the response is $R_m/2$.

The resultant equation of GAM for room temperature is $R = 19.6 \times [\text{GAM}] / (194.8 + [\text{GAM}])$

where R is the response in % sucrose equivalent and $[\text{GAM}]$ is the concentration of dry GAM (potassium salt) in mg/L. The correlation coefficient was $r^2 = 0.9985$.

The results show that GAM (potassium salt) has a potency of about 750 at 5% sucrose equivalent (SE) and 590 at 8% SE making it one of the most potent naturally occurring sweeteners known. The C–R curve tends to a maximum at a relatively high sucrose equivalent ($R_m = 19.6\%$ SE). This implies that sweetness equivalent to 10% w/v sucrose, and even higher, should be readily achievable using GAM as the sole sweetener.

As precursor of GAM, glycyrrhizin's C/R function in water is $R = 7.3 \times C / (210 + C)$ (DuBois et al., 1991). Given the low R_m of 7.3% SE, glycyrrhizin applications are only limited to blends with other sweeteners and where the glycyrrhizin contribution is limited to < 5% SE. From the C/R function, Pw (5) is only 110. Compare to glycyrrhizin, GAM could be developed as a high-potency sweetener.

It is well known that intense sweeteners which tightly bind to sweet-taste receptor also can bind to bitter-taste receptor and they usually have negative attributes such as bitter, astringent, and artificial sweetness flavors. Further, compared with sucrose, the sweet tastes of GAM are accompanied by slightly bitter, metallic and astringent tastes as well as a delayed onset of sweetness and pronounced sweetness linger. However, GAM doesn't exhibit undesirable licorice-like “off” taste.

Molecular modeling

In terms of chemical bonds, compound GAM formed well combination to 1EWK protein with Glu325, His374, Tyr74, His55, Asn403, Lys409, Val405, these amino acids bind with 1EWK protein enhances the binding activity of compound GAM. As showed from Fig. 4, GAM formed conventional hydrogen bonds with Asn403 and Glu325, while Glycyrrhizin formed with Tyr74 and Lys409. GAM formed π -Alkyl interaction with His55 and Tyr74, while Glycyrrhizin formed the π -Alkyl with Val405 and His374. Arg71, Glu72, Val405 formed Van der Waals bonds with GAM, for another, Glycyrrhizin formed it with Arg323, Ala321, Asp322. It can be seen that the combination of GAM with 1EWK is more stable and the binding energy of

the site is higher than that of Glycyrrhizin, which indicates that GAM is more reasonable in combination with the molecules, and the stability of binding with protein molecules is better. Moreover, in molecular docking 3D modeling of the docking analysis (Fig. 5), compound GAM has a better protein inclusion with 1EWK protein through the closer binding interactions which indicates GAM enhances the proximity in compare with Glycyrrhizin. The explanation of how GAM and Glycyrrhizin are bound into the sweet receptor is that there are, apparently rather than multiple receptors, multiple sites on the single sweet taste receptor (Belloir et al., 2017; Cui et al., 2006; Sanematsu et al., 2014).

Therefore, compared to glycyrrhizin, GAM could be suitable to formulating beverages (the key application for high-potency sweeteners) with low or zero energy content, which have sweetness intensities of those customary in full-calorie, full-sugar versions (Baker et al., 2016; Goryakin et al., 2017; Wölwer-Rieck et al., 2010).

In conclusion, the sweetness C–R behavior of GAM (potassium salt) in the range of 0–210 mg/L (0–20% sucrose equivalent) at room temperature is described by the equation: $R = 19.6 \times [\text{GAM}]/(194.8 + [\text{GAM}])$. As proapogenin of glycyrrhizin, GAM could be developed as a zero-calorie, high-potency sweetener. Molecular modeling showed that GAM is finely bound into protein 1EWK through conventional hydrogen bonds with Asn403 and Glu325; π -Alkyl interactions with His55 and Tyr74 and Van der Waals bonds with Arg71, Glu72, Val405, which implied that GAM has better protein inclusion than Glycyrrhizin. As a novel sweetener, we have measured the sweetness of GAM over a range of concentrations, which will help to develop zero-, low- and reduced-calorie products formulate with GAM.

Acknowledgements This study was supported by the Natural Science Foundation of Jiangsu Province under Grant [BK20160570].

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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