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A kinetic study of bitter taste receptor sensing using immobilized porcine taste bud tissues

Lihui Wei[a], Lixin Qiao[a], Guangchang Pang[a,b,*], Junbo Xie[a,b,*]

[a] Biotechnology & Food Science College, Tianjin University of Commerce, Tianjin 300134, PR China
[b] Tianjin Key Laboratory of Food Biotechnology, Tianjin 300134, PR China

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ABSTRACT

At present, developing an efficient assay method for truly reflecting the real feelings of gustatory tissues is of great importance. In this study, a novel biosensor was fabricated to investigate the kinetic characteristics of the receptors in taste bud tissues sensing bitter substances for the first time. Porcine taste bud tissues were used as the sensing elements, and the sandwich-type sensing membrane was fixed onto a glassy carbon electrode for assembling the biosensor. With the developed sensor, the response currents induced by sucrose octaacetate, denatonium benzoate, and quercetin stimulating corresponding receptors were determined. The results demonstrated that the interaction between the analyst with their receptors were fitting to hyperbola (R^2=0.9776, 0.9980 and 0.9601), and the activation constants were 8.748×10^{-15} mol/L, 1.429×10^{-12} mol/L, 6.613×10^{-14} mol/L, respectively. The average number of receptors per cell was calculated as 1.75, 28.58, and 13.23, while the signal amplification factors were 1.08×10^{4}, 2.89×10^{3} and 9.76×10^{4}. These suggest that the sensor can be used to quantitatively describe the interaction characteristics of cells or tissue receptors with their ligands, the role of cellular signaling cascade, the number of receptors, and the signal transmission pathways.

1. Introduction

The traditional measurements of taste are performed with the taste test, which usually exhibit large differences (different people have different feelings) and limitations (traditional methods cannot be used for toxic substances). At present, electronic tongue is the most commonly used instrument for measuring taste, which also has some disadvantages, i.e. inconvenience of carrying, high cost, poor accuracy and stability (Funasaki et al., 2006; Toko, 1996; Ivarsson et al., 2005; Zheng and Keeney, 2006). Although surface plasmon resonance (SPR) techniques can be used to implement non-standard measurement of the interactions between the receptor and ligand (Kovacic and Somanathan, 2012), they cannot reveal the status of signal output (its biological effects) produced by intracellular signal amplification and transmission after binding of the receptor and the taste substances. In summary, the above methods cannot truly reflect the real feelings of gustatory tissues to the different taste substances.

G protein coupled receptors (GPCRs) are the most important receptors for taste, smell, chemotaxis, immunity, endocrine, and neurotransmitters, which play a crucial role in substance metabolism, energy metabolism, and signal exchange of cells (Overington et al., 2006; Hollenstein et al., 2013). T2Rs are the receptors for various bitter substances (Shi et al., 2003). As a member of the GPCR superfamily, its extracellular domains are mainly responsible for binding to different receptors, while intracellular domains are responsible for activating different signaling pathways (Audet and Bouvier, 2012). There have been many reports demonstrating that the signal transduction of bitter substances mainly rely on the T2Rs (Lindemann, 2001; Gilbertson et al., 2000; Margolskee, 2002). However, the kinetic characteristics of the receptors sensing bitter substances have never been investigated due to the absence of an efficient assay method.

Cells can sense the environmental signals through the interactions between the receptors and ligands. Through binding to the corresponding receptors, ligands facilitate the linkage of extracellular domain, transmembrane domain and intracellular domain, which can alter the ion-channel switches by various intracellular signaling cascades and signal transmission networks. Then, the electrochemical signals are generated to stimulate the nervous system, or auto-signaling molecules (including hormones, cytokines, and chemokines) are secreted, to regulate immune, metabolism, and endocrine network in the body (Venkatakrishnan et al., 2013). Therefore, when the ligands with different concentrations are bound to receptors, the corresponding signals (X, independent variable) are input into the body. The body will generate the output signals (Y, dependent variable) through the above

* Corresponding authors at: Biotechnology & Food Science College, Tianjin University of Commerce, Tianjin 300134, PR China.
E-mail addresses: pgc@tjcu.edu.cn (G. Pang), xjbo@tjcu.edu.cn (J. Xie).

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signaling processes. As long as X and Y have a definite correlation, a certain functional relationship exists between X and Y. Moreover, this functional relationship reflects the interactions between the receptor and the ligand, which in turn produces intracellular signaling cascades as well as overall biological effects (Fig. S1). As a result, we can quantitatively determine the cellular signaling pathways and its biological effects through detecting the output signal Y (Pang et al., 2016). Furthermore, the response of cells, tissues, and organs to the surrounding environment and their causal relationship (including the taste sense) can be quantitatively described.

In the taste sensing system, the peeling taste mucosal epithelium can maintain the inherent community and biological microenvironment of the taste receptor cells in the taste buds, which can be effectively activated by taste substances (Qiao et al., 2015). Therefore, this study fixed porcine taste bud tissues between two microporous membranes to prepare taste sensing membrane, then fixed the membrane to glassy carbon electrode to develop a novel biological sensor. With the developed sensor, three bitter substances (sucrose octaacetate, denatonium benzoate, and quercetin) were determined. The interaction kinetic parameters between the ligands and receptors, as well as biological effects, were investigated through the mathematical model.

2. Materials and methods

2.1. Materials and equipment

Soluble starch was purchased from Yingdaxigui Chemical Reagent Factory (Tianjin, China). Sodium alga acid was from Guangfu Fine Chemical Research Institute (Tianjing, China). Glutaraldehyde was from Bodi Chemical Co., Ltd. (Tianjing, China). Microporous membrane was from Whatman (GE Healthcare). $CaCl_2$, Collagenase II, Dispase II, sucrose octaacetate, denatonium benzoate, and quercetin were all from Sigma-Aldrich. All reagents were analytical pure, and ultrapure water was used in this study.

CHI600E electrochemical workstation (Shanghai Chenhua Instruments Co. Ltd., China) was used in the electrochemical experiments, and equipped with a three-electrode system: glassy carbon electrode (GCE Φ=3 mm) as working electrode, Ag/AgCl electrode as reference electrode, and platinum wire electrode as counter electrode.

2.2. Separation of taste bud tissue

The mixed enzymes solution of collagenase I (1 mg/mL) and dispase II (3 mg/mL) for separating the porcine tongue epithelium was warmed up for 15 min (37 °C) to maximize the enzyme activity. The porcine tongue was cropped from the tip to the edge of foliate papilla, and rinsed with anatomical solution to remove the residual blood. After 0.3–0.5 mL of pre-warmed solution was injected into the layer between tongue epithelium and muscles, the specimen was placed in anatomical solution and incubated for 6–8 min at room temperature. Then, the epithelium was stripped from the muscle layer, and immediately placed in anatomical solution at 0 °C (Stone et al., 2002).

2.3. Preparation of taste biosensor

According to the reported methods (Wee and Gombotz, 1998; Zactiti and Kieckbusch, 2009; Reiss et al., 1998), the aldehyde starch gel solution was prepared. 1 g Soluble starch was dissolved in 100 mL 1% glutaraldehyde solution, and then heated and stirred at 80 °C for 30 min. After being placed over night at room temperature, the aldehyde starch gel solution was then mixed with 2 g/100 mL sodium alga acid solution at 1:1 ratio. 10 μL of this mixed solution was evenly smeared onto two polycarbonate microporous membranes (diameter 25 mm, pore size 0.22 μm). Then 9 mm^2 of the epithelial tissue was placed in the center of one membrane and covered with the other to form a “sandwich” type membrane. Apart from increasing the amount of taste bud tissue fixed on the biosensor, the sandwich membrane could also prevent the tissue from flowing or falling off, in the premise that ligands could pass through it freely to contact the receptors.

The prepared sandwich membrane was soaked in 5 g/100 mL $CaCl_2$ for 10 s to form a stable chelate complex (as fixing agent) via ion exchange between sodium alga acid and $CaCl_2$ (Bierhalz et al., 2014; Rhim, 2004; Rhim et al., 2006). After being rinsed with physical saline solution to remove residual Cl^- and Ca^{2+}, the membrane was fixed to the surface of glassy carbon electrode by overlapping the tissue with the electrode core. Then the bitter taste biosensor was obtained (Lindemann et al., 1996).

2.4. Determination of bitter substances by biosensors

In this study, a glassy carbon electrode fixed with taste measurement membrane was used as a working electrode, an Ag/AgCl electrode was used as the reference electrode , and a platinum wire electrode was used as counter electrode. Ultrapure water was used for the blank measurement. Under a certain voltage, the response currents of sucrose octaacetate, denatonium benzoate, and quercetin (with different concentrations) were determined through current-time measurement. The change rate of the response currents was used as the detection index, and calculated with the formula as follows:

$$\Delta I/\% = \frac{I_1 - I_2}{I_1} \times 100 \quad (1)$$

I_1 and I_2 represent the steady-state current values at the same time point before and after the determination of bitter substances, respectively.

3. Results and discussion

3.1. Characterization of the electrode and potential optimization of current-time measurement

To investigate the electrode's characterization in process of the biosensor preparation, cyclic voltammetry (CV) was performed under the condition of 50 mV/s (scan rate) and −0.1–0.6 V (scan range), and 1×10^{-3} mol/L $K_3Fe(CN)_6$ (including 0.2 mol/L KNO_3) was used as test solution (pH=7.0) (Wei and Pang, 2016). The currents of bare electrode's redox peaks were 9.75×10^{-6} A and -1.03×10^{-5} A. Compared to bare electrode, the redox peak currents decreased obviously after the electrode was fixed with microporous membrane, which was 3.38×10^{-6} A and -3.67×10^{-6} A respectively. This is resulted from the fixed microporous membrane increasing the resistance of the electrode's surface. Likewise, after the electrode was further fixed with taste bud tissue, the redox peak currents were decreased to be 2.54×10^{-6} A and -2.69×10^{-6} A respectively. All of this indicating that the taste biosensor was assembled successfully (Fig. 1A).

As for the detection of the analytes, we used current-time measurement (amperometric i-t curve) to perform the analysis. With the assembled sensor, current-time measurement was performed under different potentials. Differences of steady state currents before and after adding 10–5 mol/L of sucrose octaacetate, denatonium benzoate, and quercetin were used to evaluate the effect of different potentials on the electrochemical response of the sensor. The results showed that current change was the highest at −0.36 V (Fig. 1B). Therefore, −0.36 V was selected as the constant potential to study the response characteristics of bitter biosensor to sucrose octaacetate, denatonium benzoate, and quercetin.

3.2. Action regularity of bitter substances with their receptors

When the taste substances come into contact with taste bud tissues, they cause hyperpolarization of taste cells to generate bio-currents

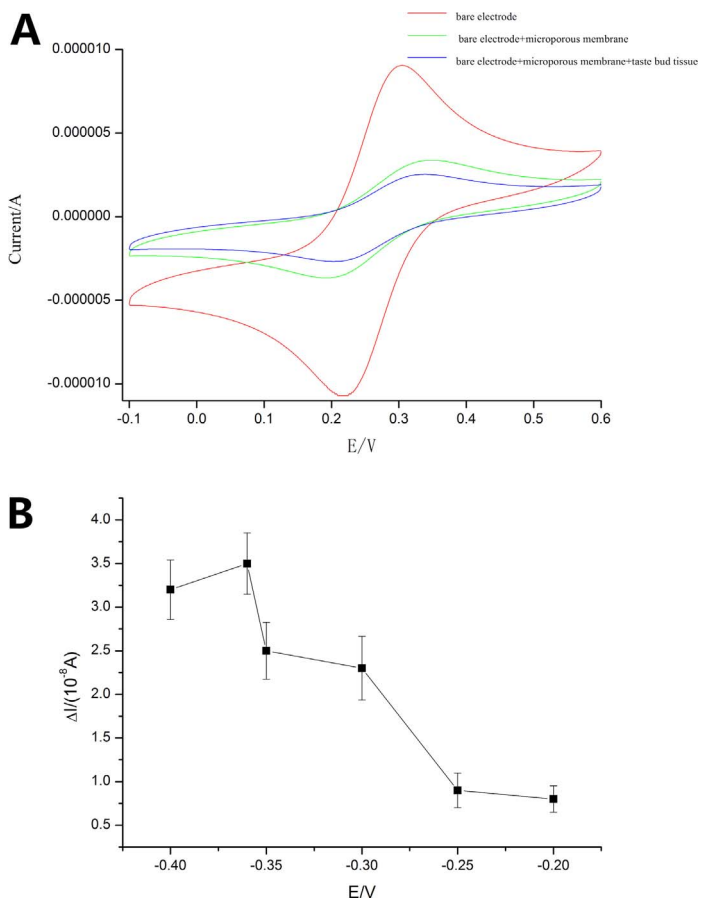

Fig. 1. (A) Description of electrode's characterization in process of taste biosensor preparation by cyclic voltammetry. (B) The change of current responses of the biosensor at different potentials.

through different ion-channel switches. These output signals can be detected with the electrochemical workstations (Qiao et al., 2015; Katsuragi et al., 1996). Since the range of the concentrations of bitter substances (sucrose octaacetate, denatonium benzoate, and quercetin) was very wide, we plotted the line graphs with the lg(C) as X-axis and the change rate of the response current ($\Delta I/\%$) as Y-axis. (a, b, c represents sucrose octaacetate, denatonium benzoate, and quercetin respectively). As shown in Fig. 2, the change rate of the currents showed a good linear relationship with the concentrations (lg(C)) of sucrose octaacetate, denatonium benzoate, and quercetin in the range of 10^{-14}-10^{-12} mol/L, 10^{-13} –10–8 mol/L, and 10^{-14}-10–9 mol/L, and the minimum detection concentration was 1×10^{-14} mol/L, 1×10^{-13} mol/L, 1×10^{-14} mol/L, respectively.

3.3. Kinetic curve of the interaction of bitter substances and their receptors

In the concentration ranges mentioned above, the line graphs were replotted with the original concentration (further partitioned) as X-axis and the change rate of the response current ($\Delta I/\%$) as Y-axis (Fig. 3). According to the law of receptor-ligand binding kinetics (more details are in the Supplementary material part 2), hyperbolic curve fitting was performed, and the corresponding diagrams are embedded in Fig. 3 respectively. The fitting formulae are as follows:

$$\Delta I_a/\% = \frac{8.3123\times10^{-13}\,C}{0.7774+10^{-14}\,C}\ (R^2=0.9776)\quad \Delta I_b/\% = \frac{9.9613\times10^{-12}\,C}{44.7227+10^{-13}\,C}$$
$$(R^2=0.9980)\quad \Delta I_c/\% = \frac{5.2419\times10^{-13}\,C}{20.1050+10^{-14}\,C}\ (R^2=0.9601) \qquad (2)$$

As shown in embedded diagrams of Fig. 3, the curve of interaction between bitter substances (sucrose octaacetate, denatonium benzoate, and quercetin) and their receptors were hyperbolic, indicating it has substrate saturation effects. This is similar to the kinetics of the interaction of enzyme with substrate.

According to the Lineweaver-Burk equation (double reciprocal equation), the following diagrams were plotted using reciprocal of the bitter substances concentrations as X axis, and reciprocal of the change rate of response current ($\Delta I/\%$) as Y axis (Fig. 4).

The linear regression equations are fitted by the above functions as follows:

$$\frac{1}{\Delta I_a/\%} = 1.041\times10^{12}\frac{1}{C}+0.0119\ (R^2=0.9874)\frac{1}{\Delta I_b/\%}$$
$$= 2.760\times10^{12}\frac{1}{C}+0.0193\ (R^2=0.9610)\frac{1}{\Delta I_c/\%}$$
$$= 1.585\times10^{13}\frac{1}{C}+0.0240\ (R^2=0.9764) \qquad (3)$$

3.4. Activation constants

In this study, the activation constant (K_a) was calculated with the kinetic Eq. (3). The activation constant was similar to K_m, which is defined as: the concentration of ligand at half of the maximum output signals of cells. The smaller the K_a value was, the higher the efficiency of the signal output generated by the ligand-receptor interaction. When the output signals of all cells are maximized, all the receptors on cells (or domain) are saturated. Therefore, the activation constants (K_a) essentially reflect the number of receptors (or domains) that are capable of binding to the ligands per cell on average. The smaller the K_a was, the less the number of receptors (or domains) was on per cell. The activation constants of the

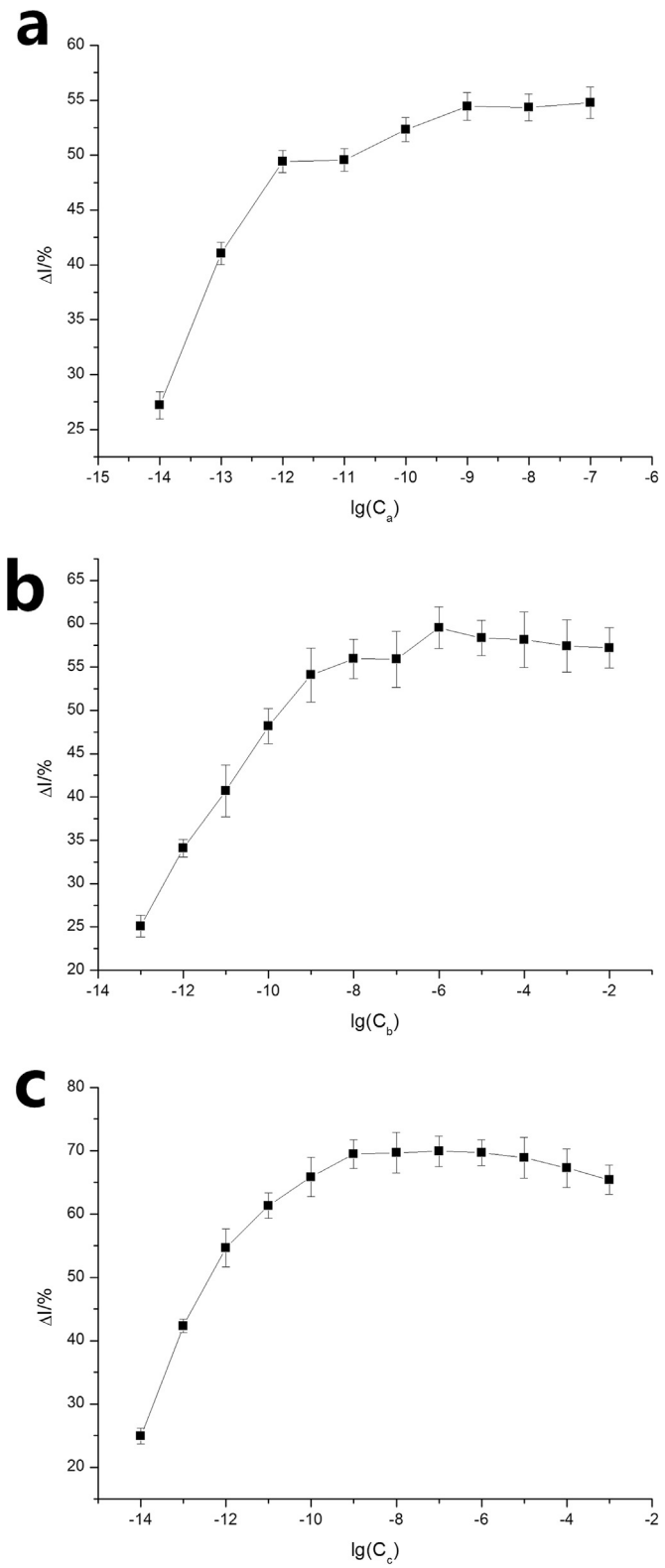

Fig. 2. Current response values of bitter substances in the detection range (lg(C)): a, b, c represent sucrose octaacetate, denatonium benzoate, and quercetin respectively.

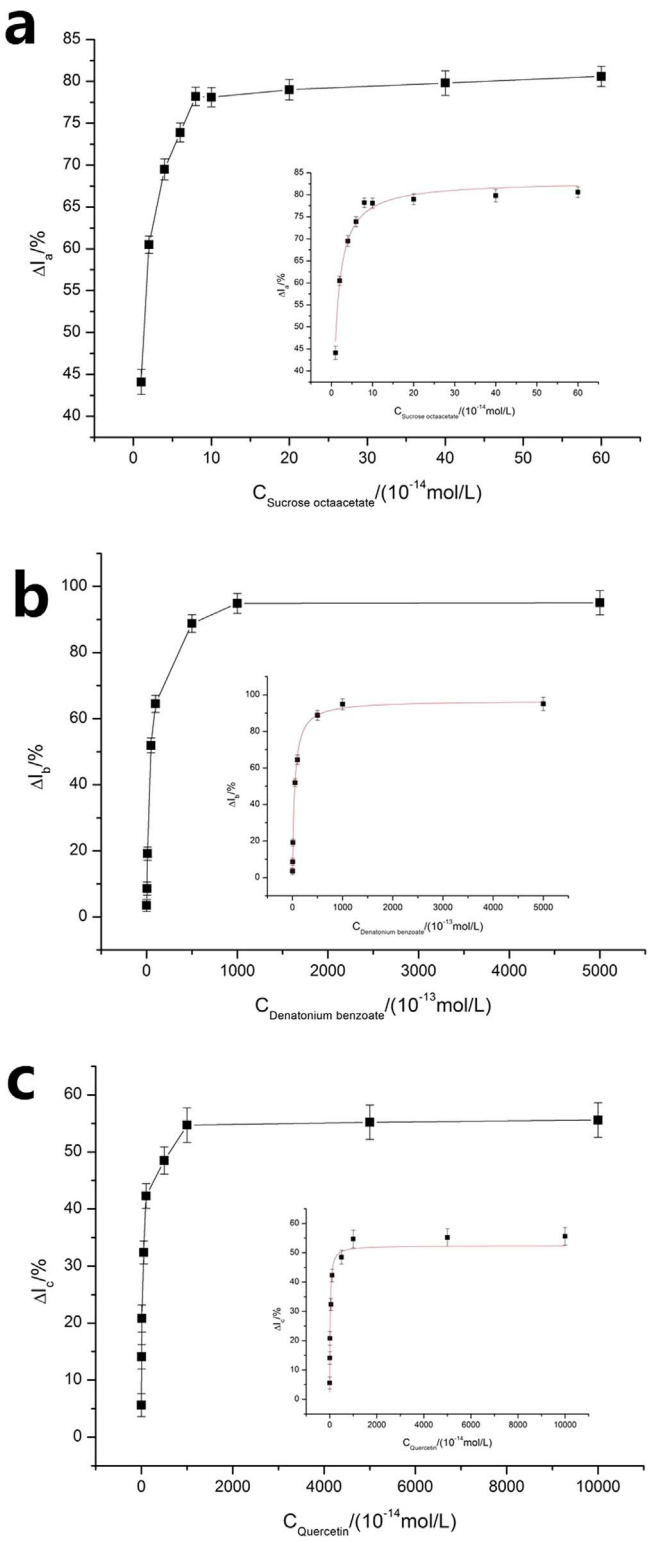

Fig. 3. The curve of interaction between bitter substances (sucrose octaacetate, denatonium Benzoate, and quercetin) and their receptors: a represents sucrose octaacetate (10^{-14} mol/L-10^{-12} mol/L), and the embedded diagram represent the hyperbola fitting of sucrose octaacetate; b represents denatonium benzoate (10^{-13} mol/L-10^{-8} mol/L), and the embedded diagram represent the hyperbola fitting of denatonium benzoate; c represents quercetin (10^{-14} mol/L-10^{-9} mol/L), and the embedded diagram represent the hyperbola fitting of quercetin.

interaction of sucrose octaacetate, denatonium benzoate, and quercetin with their receptors were $K_{a1}=8.748\times10^{-15}$ mol/L, $K_{a2}=1.429\times10^{-12}$ mol/L, and $K_{a3}=6.613\times10^{-14}$ mol/L, respectively. For porcine taste buds, sucrose octaacetate was the most efficient, followed by quercetin and denatonium benzoate. The number of receptors (or domains) of sucrose octaacetate on porcine taste buds were the least, while denatonium benzoate had the most. They may act on different receptors or different domains of the same receptor.

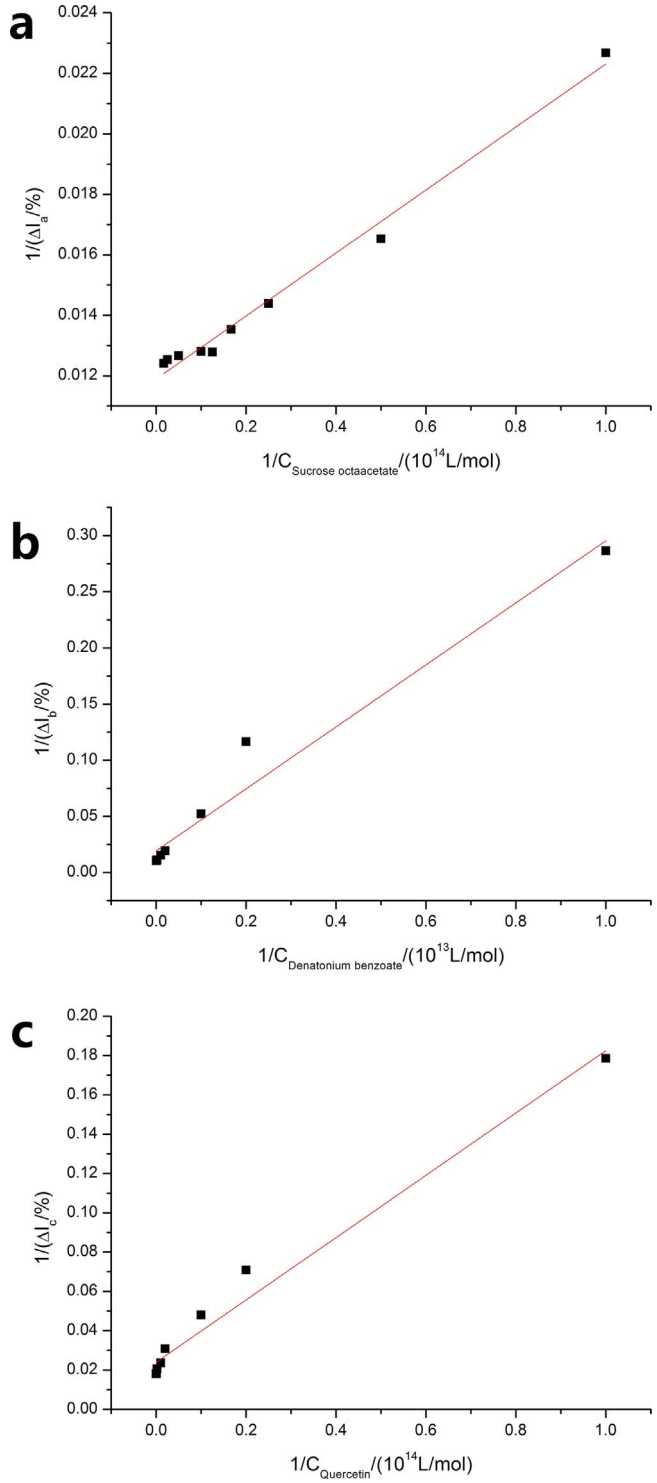

Fig. 4. Plotting with double reciprocal: a represents sucrose octaacetate in the concentration range of 10^{-14} mol/L to 10^{-12} mol/L; b represents denatonium benzoate in the concentration range of 10^{-13} mol/L to 10^{-8} mol/L; c represents quercetin in the concentration range of 10^{-14} mol/L to 10^{-9} mol/L.

3.5. The average number of receptors per cell

When there is only one receptor on a cell, the signal response curve exhibits a square wave pattern (Fig. S2-**a in blue**) (either 0 or 1). It means that the maximum response signal is obtained once binding the ligand, implying that the maximum response signal is at the lowest detection limit. When more than one receptor is present on a cell, there is a certain cumulative effect for the generated signal due to combining different numbers of receptors. The mutual accumulation of the signals shows a hyperbolic type in the signal response curves (Fig. S2-**b in green**). When there are no fixed tissues on the electrode, the signal intensity increases linearly with the increase of the analyte concentration (Fig. S2-**c in red**), implying that there is no cumulative effect between the response signal and the base fluid in the detection. The number of receptors on each cell is defined as follows:

$$n = \frac{C_M}{C_L} = \frac{2K_a}{C_L} \quad (4)$$

C_M is the concentration of the maximum response signal when the number of receptors on the cell is not 1. C_L is the concentration of the maximum response signal when there is only one receptor on the cell.

The number of receptors of denatonium benzoate and quercetin were 28.58 and 13.23, respectively, which was far greater than that of sucrose octaacetate (1.75). Moreover, the kinetic curves of quercetin and denatonium benzoate were similar, which may be due to the similarities in their molecular structures. Sucrose octaacetate was a polyhydroxy compound and had significant differences in structure, resulting in a major difference in the binding sites with GPCR extracellular domains. After all, the binding of extracellular domains and the corresponding changes in its spatial structures can influence the signal pathways and amplification effect.

These demonstrated properties in this kinetic study verified the structural adaptability and similarity of the ligands binding with the extracellular domains of receptors. Due to the different signal pathways activated by binding with their receptors, different bitter substances varied in bitterness degree and style. According to the K_a values, the bitterness sensitivity strength of the 3 analytes is as follows: sucrose octaacetate > quercetin > denatonium benzoate.

Given that a receptor (or domain) may bind to multiple ligands, and a ligand may also bind to multiple receptors (or domains) (Venkatakrishnan et al., 2013), the receptor-ligand titers be more accurate to evaluate the effect. Receptor-ligand titer refers to the total number of the determinants (domains) of receptor-ligand that bind the ligand to the corresponding receptor. The determinants (domain) of receptor-ligand included both the sites of the ligand and different domains of the ligand that can be bound by the receptor.

3.6. Signal amplification factors of the biosensor for bitter substances

In order to reveal the relationship between the ligand and the receptor more accurately, we compared the current response values generated by the bare electrodes under different concentrations of ligand solution to eliminate the influence of the bare electrodes. Using the logarithmic value of the analyte concentration as X axis and the change rate of response current ($\Delta I/\%$) as Y axis, the straight line fitting was performed (Fig. 5**A**). A good linear relationship was found between the current change and the logarithm of concentration. The equations for the action of sucrose octaacetate, denatonium benzoate, and quercetin with bare electrodes are as follows:

$$\Delta I_a/\% = 5.0843\lg(C_a) + 75.2705 \ (R^2 = 0.9824) \Delta I_b/\% = 5.7635\lg(C_b) + 83.3418 \ (R^2 = 0.9869) \Delta I_c/\% = 5.5029\lg(C_c) + 79.5430 \ (R^2 = 0.9882) \quad (5)$$

Signal amplification factor of the biosensor is the ratio of the ligand concentrations binding with the biosensor and the bare electrode respectively, as the change rate of response current ($\Delta I/\%$) is at half of the maximum value (Fig. S2).

Signal amplification factors can be calculated by the formula:

$$N_a = 10^{[\lg(C_a) - \lg(K_a)_a]} N_b = 10^{[\lg(C_b) - \lg(K_a)_b]} N_c = 10^{[\lg(C_c) - \lg(K_a)_c]} \quad (6)$$

From Fig. 5**B** and the formula (6), the signal amplification factors of sucrose octaacetate, denatonium benzoate and quercetin were deduced as 1.08×10^4, 2.89×10^3 and 9.76×10^4, respectively. It can be seen that

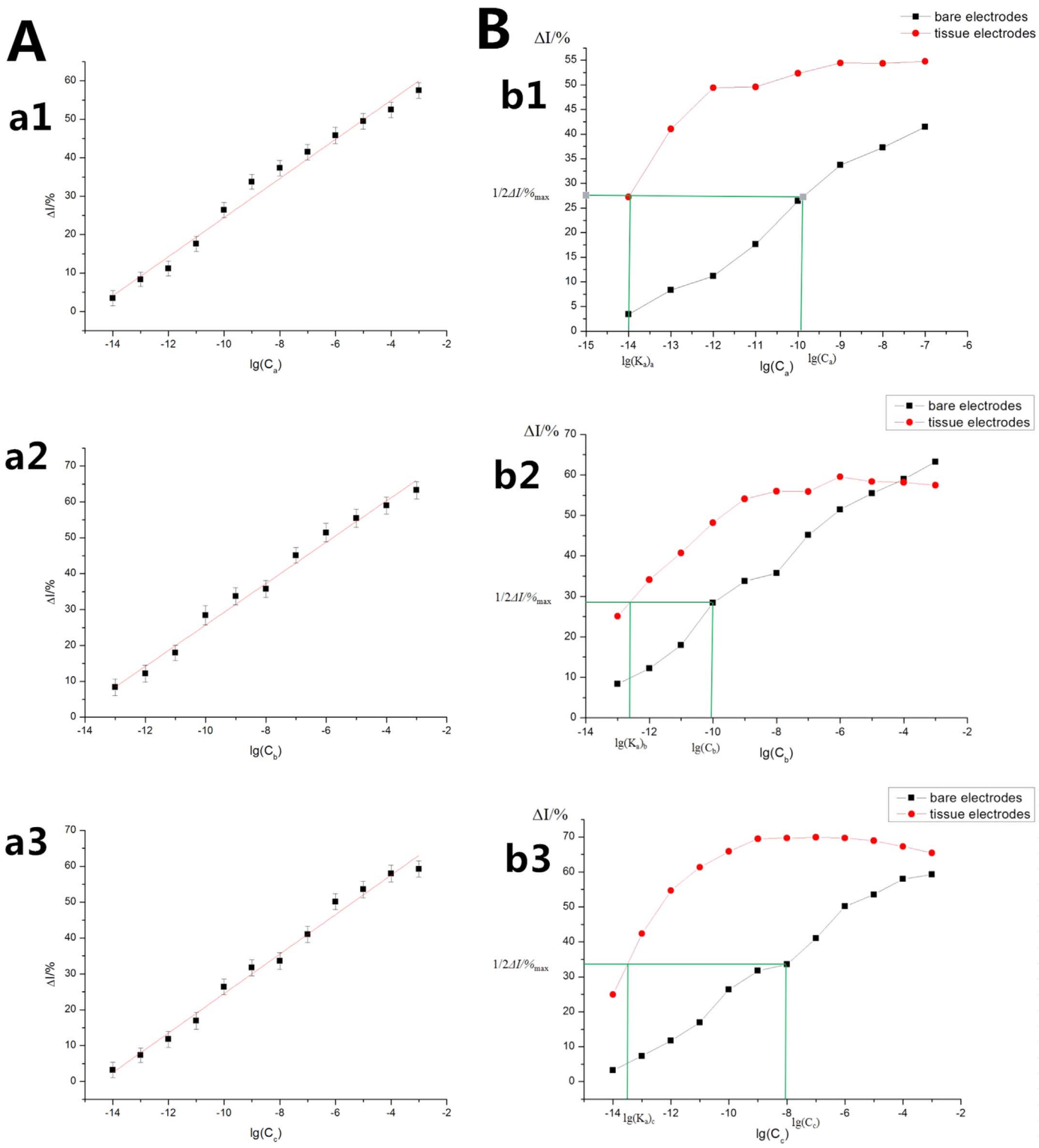

Fig. 5. (A)Curve of the actions of bitter substances with bare electrodes: a1, a2, a3 represents sucrose octaacetate, denatonium benzoate, and quercetin respectively ; (B) Diagram of the signal amplification times calculation: b1, b2, b3 represents sucrose octaacetate, denatonium benzoate, and quercetin respectively.

the signal amplification effect of cellular system is dramatic. This is the first report on assessing the effect of cell signal amplification, and still needs further validation.

3.7. Stability and reproducibility of the biosensor

The prepared biosensor was continuously (n=10) used to measure 10^{-5} mol/L sucrose octaacetate. The relative standard deviation of the measurement results was 5.34%, suggesting that the stability of this taste tissue sensor was good. This biosensor (stored in saline at 4 ℃) was then used to measure sucrose octaacetate of the same concentration every day (n=7). In the first two days, the change rate of current was almost constant, but was 94.68% of the initial determined value at the third day, and 90.09% of the initial determined value at the seventh day, suggesting that this sensor can be stably stored for at least 7 days (Table 1).

Table 1
The reproducibility of the biosensor.

Sensor number	Blank current (μA)	Responsive current (μA)	Δ*I*/%
1	−0.74 ± 0.01	−0.42 ± 0.01	43.14
2	−0.69 ± 0.01	−0.39 ± 0.01	43.65
3	−0.92 ± 0.01	−0.51 ± 0.01	44.11
4	−0.73 ± 0.01	−0.42 ± 0.01	42.47
5	−1.20 ± 0.01	−0.70 ± 0.01	42.39

Five electrochemical tissue sensors prepared in different batches were used to measure the same concentration of sucrose octoacetate solution. The relative standard deviation of the measurement results (Δ*I*/%) was 1.72% (Table 1), indicating that reproducibility of the taste tissue sensor was good.

4. Conclusions

In this study, a novel biosensor was prepared with immobilized porcine taste buds and used to investigate the kinetics of the interactions between bitter substances (sucrose octaacetate, denatonium benzoate, and quercetin) and their receptors. The activation constants, signal amplification factors, as well as the average number of receptors per cell were determined for the first time. The results demonstrated that pigs were more sensitive to sucrose octaacetate, and the number of receptors for denatonium benzoate was the most on porcine taste buds. Compared with the bare electrodes, the signal amplification of the taste bud cells was significant. In addition, the stability and reproducibility of the sensor was good, which can be stably stored at 4 °C for at least seven days. It provides a new idea for the development of taste sensors, especially for the study of the kinetics of receptor and ligand.

Acknowledgements

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bios.2017.01.064.

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