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Investigation of the interaction for three Citrus flavonoids and α-amylase by surface plasmon resonance

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Abstract

Flavonoids，a class of natural drugs with broad biological activity, exhibit inhibitory effect on α-amylase. Citrus peel is a good source of flavonoids. The real-time interactions between three Citrus flavonoids (naringin, neohesperidin, hesperidin) and α-amylase were investigated by surface plasmon resonance biosensor, and were compared with the α-amylase inhibitors, acarbose. These results showed the binding affinities of naringin, neohesperidin and hesperidin with α-amylase reach the highest at pH 6 with K_D values of 2.27±0.18, 3.09 ± 0.20 mM and 3.51 ± 0.09 mM, and can be reinforced with 0.2 M NaCl and 0.1 M $CaCl_2$, respectively. The results of 1, 1-diphenyl-2-picryl hydrazyl radical assay indicate that the antioxidant activities of naringin, neohesperidin and hesperidin are significantly inhibited by interacting with α-amylase, and the inhibition percentage are 47.61±0.034%, 22.81±0.037% and 21.01±0.051%, respectively. Additionally, it is found that both the number and the position of hydroxyl group play an important role in the interaction of three Citrus flavonoids and α-amylase. These results provide useful information for rapid screening inhibitors of α-amylase from plant-based food.

Keywords: Citrus flavonoids, α-Amylase, Interaction, Surface plasmon resonance biosensor, DPPH

Chemical compounds studied in this article

Naringin (PubChem CID: 442428); Neohesperidin (PubChem CID: 442439);

Hesperidin (PubChem CID: 10621); Acarbose (PubChem CID: 122172882); Atrazine (PubChem CID: 2256);

1. Introduction

The incidence of diabetes is increasing dramatically worldwide because of changes in lifestyle and diet. Postprandial hyperglycemia is considered as the most dangerous factor, which causes the onset and gradual deterioration of diabetes (Yang, He, & Lu, 2014). Inhibition of the digestive enzymes activity is an effective way to mitigate and prevent the diabetes. Although acarbose has been proved to be effective to act as the inhibitor of digestive enzymes, it has been reported to cause some adverse effects, such as liver disorders, diarrhea, and abdominal pain (Hsiao, Liao, Cheng, & Wu, 2006). α-Amylase is a pivotal digestive enzyme, usually acting on starch or glycogen. Therefore, α-amylase inhibitors from plant-based food have received increasing attention, because they are free of major undesirable side effects.

Flavonoids, a class of natural drugs with broad biological activity (Tomásbarberán, & Andréslacueva, 2012), exhibit inhibitory effect on α-amylase (Tsujita, Shintani, & Sato, 2013; Cao, & Chen, 2012; Hargrove, Greenspan, Hartle, & Dowd, 2011), and exist abundantly in various fruit and vegetables. Citrus peel, a well known traditional Chinese herbal medicine, is a good source of flavonoids (Liu, & Deng, 2007), which can be added to foods, such as soups, salads, chicken or fish to experience their potential benefits due to its easily accessibility and low cost. Generally, citrus flavonodes are categorized into two groups, including flavanone

glycosides (naringin, neohesperidin, hesperidin, etc.) and polymethoxylated flavones (nobiletin, sinensetin, tangeretin, etc.), which may regulate blood sugar, and reduce the level of serum triglycerides and cholesterol in human body (Lu, Zhang, Bucheli, & Wei, 2006). However, the interactions between Citrus flavonoids and α-amylase have not been well understood. Although fluorescence spectrum has been successfully used to study the binding constants (K_A) and binding sites of three flavonoid compounds from tartary buckwheat bran on α-amylase (Li, Gao, Gao, Shan, Bian, & Zhao, 2009), it can not give the precise kinetic process for the interaction between flavonoids and α-amylase.

Surface plasmon resonance (SPR) biosensor is a powerful tool to analyze the molecular interactions, by which the association and the disassociation of molecules can be monitored in real-time without labels (Tiwari et al., 2014; Wilson, 2002). These label-free interactions provide direct and detailed feedback about the kinetics and the affinity of the complex formation. Consequently, SPR biosensors have been widely used to study the biospecific interaction of low-molecular weight compounds (Vachali, Nelson, & Bernstein, 2012; Thompson, Singh, & Dalgleish, 2010; Tan et al., 2014).

In order to find out the real-time binding behavior of three Citrus flavonoids (naringin, neohesperidin, hesperidin, Fig.1) with α-amylase, the effects of such binding on Citrus flavonoids antioxidant activity, and whether such binding behavior and effect is related to the number and position of hydroxyl group in flavonoids

molecules, we investigated the real-time interaction kinetics of the three Citrus flavonoids and α-amylase in detail using SPR biosensor, and acarbose and atrazine were selected as the contrast (Fig.1).Their binding constants (K_D) were calculated and compared. The effects of extrinsic factors, including pH and salt concentration, on the binding affinity of three Citrus flavonoids with α-amylase were also examined. The evolution of antioxidant activities of three Citrus flavonoids were also revealed during the interaction with α-amylase based on the 1, 1-diphenyl-2-picryl hydrazyl (DPPH) radical assay. These results illustrate how the SPR biosensor can be used to provide detailed kinetics information about the Citrus flavonoids and α-amylase interactions, which indicates SPR may serve as a useful tool for rapid screening α-amylase inhibitors.

2. Experimental

2.1. Materials and reagents

Hesperidin (purity ≥ 95%), neohesperidin (purity ≥ 99%), naringin (purity ≥ 95%) were purchased from National Institutes for Food and Drug Control (NIFDC Beijing, China). Bacilus subtilis α-amylase was purchased from Shanghai Ryon Biological Technology Co. Ltd. (Shanghai China). DPPH and pyrogallic acid were purchased from Changsha LongHe chemical and glass experimental materials limited Co. Ltd. Acarbose (purity ≥ 98%), atrazine, 3-mercaptopropionic acid (MPA), n-hydroxysuccinimide (NHS), and 1-ethyl-3-(3-dimethylami-nopropyl) carbodiimide hydrochloride (EDC) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Other reagents were analytical-reagent grade from local suppliers, and used without further purification. α-Amylase was dissolved with 0.01M phosphate buffer solution (PBS, pH 6), and stored at 4℃. Hesperidin, neohesperidin and naringin were prepared by dissolving in ethanol as stock solutions, and then diluted with PBS (pH 6) as standard solutions. Thoroughly degassed 0.01 M PBS (pH 6) was used as the running buffer in this work.

2.2. Apparatus

The SPR instrument and the preparation of gold film used in our study can be referred to our previous published paper (Liu et al., 2015). Unless otherwise stated, a standard flow rate of 10 μL/min was used for all experiments. The ultraviolet–visible (UV–vis) spectrum was recorded on UV-2450 spectrophotometer (Shimadu, Japan).

2. 3 SPR measurements of three Citrus flavonoids and α-amylase interactions

The real-time interaction analysis of three Citrus flavonoids and α-amylase were performed on a two-channel BI 2000-SPR instrument (Biosensing Instrument Inc., Tempe, AZ) at room temperature. α-Amylase was immobilized on the chip surface by a standard amine coupling method. The Au chip was firstly immersed into the solution of 10 mM MPA (dissolved with ethanol) for overnight, and then the resulted Au chip was washed with ultrapure water to remove the physically adsorbed MPA molecules, and thoroughly dried with N_2 flow. Finally, the Au chip was placed on the prism with the index-matching oil. After the achievement of the stable baseline, 100 μL freshly prepared EDC/NHS (0.4 M/0.1 M) solution was injected into the SPR flow cell to

activate the carboxyl on the chip surface. Then, 100 μL α-amylase solution (160 μM) was injected into the flow cell to immobilize α-amylase on the chip surface, after excess reactive groups on the surface were deactivated by a 7-min liquid pulse of 1.0 M ethanolamine (pH 8.5). Finally, different concentrations of Citrus flavonoids (40-800 μM) were injected over the α-amylase coated surface, respectively. The bound Citrus flavonoids can be desorbed from the α-amylase coating surface by acid and alkali. To enable reuse of the same chip, the chip surface could be regenerated by a 100 μL injection of 2 mM NaOH after each measuremen, with the regeneration time of at least 12 times. The SPR response was traced as the function of time, showing the kinetics of the binding events occurring at the chip surface. For kinetic analysis, the SPR curves were fitted using BI-SPR 2000 control software (version 2.2.0) according to 1:1 langmuir-binding model (Jolie et al.,2010), from which the association and dissociation rate constants were estimated. To ensure the accuracy of the experiments, binding experiments were performed in triplicate. The data are expressed as the mean ± relative standard deviation (RSD).

2.4 Effect of pH and salt concentration on the interactions of three Citrus flavonoids and α-amylase

The effect of pH on the interaction of three Citrus flavonoids and α-amylase were investigated by the SPR within the pH range 5.0-8.0. According to the method described in section 2.3, after the chip surface modified with MPA was activated, α-amylase was immobilized on the chip surface by injection 100 μL α-amylase

solution of 160 μM. Then 400 μM Citrus flavonoids solution was flowed over the chip surface. The pH of α-amylase, Citrus flavonoids and the corresponding running buffer are the same for each measurement. To estimate the effect of NaCl and $CaCl_2$ concentration on the binding affinities of three Citrus flavonoids with α-amylase, a series of 200 μM Citrus flavonoids with different concentrations NaCl (0.01 M-0.8M) or $CaCl_2$ (0.005 M-0.2 M) solutions were flowed over the chip surface modified with α-amylase, respectively. The kinetic analysis was the same as described in 2.3.

2.5 Effect of α-amylase on antioxidant activity of three Citrus flavonoids

The antioxidant activities of three Citrus flavonoids were measured using the DPPH radical assay, with and without α-amylase, respectively (Sharma, 2008). α-Amylase (160 μM) and three Citrus flavonoids of different concentration (20-300 μM) were prepared with PBS (pH 6) and ethanol, respectively. 1 mL Citrus flavonoids solution (1 mL Citrus flavonoids and 0.2 mL α-amylase) and freshly prepared 1 mL DPPH were simultaneously added to the test tube, and mixed for 5 min, after which, 3 mL ethanol (2.8 mL ethanol) was added to the mixture. Then, the mixture was incubated in the dark for 20 min, at room temperature. The absorbance was measured at 517 nm by a UV-VIS spectrophotometer. DPPH radical scavenging activity was calculated as follows:

$$\text{DPPH radical scavenging activity}(\%) = [1 - (A_i - A_j)/A_0] \times 100 \quad (1)$$

where A_0 is the absorbance of the DPPH (1 mL DPPH and 4 mL ethanol), A_i is the absorbance of the DPPH and sample (1 mL DPPH, 1 mL Citrus flavonoids and 3 mL

ethanol, or 1 mL DPPH, 1 mL Citrus flavonoids, 0.2 mL α-amylase and 2.8 mL ethano1), and A_j is the absorbance of the blank sample (1 mL Citrus flavonoids and 4 mL ethanol). All the measurements were conducted in triplicate. These results were expressed as IC_{50} (half maximal inhibitory concentration), which were obtained by plotting the percentage of DPPH radical scavenging activity against the Citrus flavonoid concentration.

2.6 Statistical Analysis

All experiments were repeated three times. The data were expressed as means ± standards errors. Statistical analysis was carried out by using one-way analysis of variance in SPSS 18.0 (SPSS, Chicago, IL, USA); $p < 0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. Enzyme immobilization

The amine coupling method is a good strategy for immobilizing α-amylase on the chip surface. The –COOH group reacted with NHS under the catalysis of EDC for only 15 min. To obtain the optimized α-amylase concentration to form the compact monomolecular layer on the chip surface, different concentration α-amylase solutions were separately injected into the flow cell to perform their binding on the activated chip surface. Fig. 2 shows the α-amylase concentration dependent SPR response tendency. A remarkable SPR response can be observed due to the binding of α-amylase, which increases with increasing the concentration of α-amylase. SPR

response reached about 501.548 ± 26.92 mDdeg when the chip was exposed to the 160 μM α-amylase solution. The α-amylase surface coverage was determined according to Jung's formula using SPR angular shift (Jung, Chinowsky, Mar, Yee, & Campbell, 1998; Gouzy, Kess, & Krämer, 2008). The surface coverage of α-amylase was 9.65×10^{-14} mol/mm^2. Usually, the standard error of the high capacity surface is larger than that of the low capacity surface for binding event (Zhou, Li, Zhang, & Liu, 2008), resulted from the mass transport, steric hindrance, crowding and aggregation of high capacity surface (Roden, & Myszka, 1996; Myszka, 1999; Gomes, Giralt, & Andreu, 2000). Therefore, the immobilized α-amylase concentration of 160 μM was chosen for the subsequent assays.

3.2 SPR analysis of the interaction of three Citrus flavonoids and α-amylase

To estimate the binding affinities of three Citrus flavonoids and α-amylase, the association and disassociation of different concentration Citrus flavonoids (40 to 800 μM) with the α-amylase covered chip surface were analyzed, respectively. The response signal of SPR biosensor may provide the kinetic information on the amount of complex formed on the chip surface at each Citrus flavonoid compound concentration. Periodically throughout each experiment, the PBS buffer was injected into the cell to serve as a baseline reference for removing any systematic drift over time. These results indicate that three Citrus flavonoids can all bind with α-amylase to form a complex. The plateau responses reached rapidly for each Citrus flavonoids during the association phase, and each of them was dissociated from α-amylase in 3

min (Fig. 3 A-C). Fig. 3 D shows the SPR response for each Citrus flavonoids, acarbose and atrazine with 400 μM flowed over the α-amylase-modified chip surface, respectively. Fig. 3 E shows kinetic curve of SPR chip regeneration using 2mM NaOH. The binding parameters for the three Citrus flavonoids, contrast and α-amylase were given in Table 1.

As shown in Fig. 3 D, the binding ability between three Citrus flavonoids, acarbose and α-amylase were significant higher than that of atrazine and α-amylase ($p < 0.05$). The order of the binding affinity was acarbose > Citrus flavonoids > atrazine (Table 1). These results suggest that the binding ability was affected by the number of hydroxyl group (Fig. 1). The interactions of three Citrus flavonoids and contrast with α-amylase are driven by hydrophobic force, belonging to noncovalent bonding weak forces (Yan, Feng, Fei, Bian, & Fang, 2009), which is usually enhanced with increasing the number of hydroxyl (Li, Yang, Gao, Zhang, & Wu, 2011). Although all of the three Citrus flavonoids have the same number of hydroxyls group (Fig.1), they have different binding ability with the order of naringin > neohesperidin > hesperidin, which suggests that the position of the hydroxyl group in Citrus flavonoids may also affect their binding with α-amylase. As shown in Fig. 1, the main difference of the structure among neohesperidin, hesperidin and naringin is located at the B-ring, where a methoxy group is in the ortho-position of the hydroxyl group for both neohesperidin and hesperidin. Apparently, this methoxy group will decrease the activity of hydroxyl group in B-ring due to the steric hindrance. Consequently, the binding affinities of

both neohesperidin and hesperidin with α-amylase are lower than that of naringin. The structural difference between neohesperidin and hesperidin is located at 6-membered heterocyclic D-ring, where the hydroxyl group (red in Fig.1) in neohesperidin has more flexibility, making it much easier to bind with α-amylase with higher stability comparing to hesperidin. This agrees well with the result that K_D of neohesperidin and hesperidin are 3.09 ± 0.20 mM and 3.51 ± 0.09 mM, respectivily. These results clearly demonstrate that these SPR conditions can be applied to study the interaction kinetic of three Citrus flavonoids with α-amylase.

3.2 Effect of pH and salt concentration on the three Citrus flavonoids and α-amylase interactions

PH of the buffer has a significant influence on the activity of all the Citrus flavonoids and α-amylase, and thereby affects their interaction. As shown in Fig. 4 A, similar trends were observed for three Citrus flavonoids when they bound to α-amylase within the pH range 5.0-8.0, which shows that the strongest binding ability is obtained at pH 6. This may be ascribed to the α-amylase isoelectric point (5.04) (Li, Guo, Yue, Li & Jiao, 2005) effect, and the associated structure change in α-amylase.

The activity of the α-amylase is the decisive factor for the interaction of flavonoids and α-amylase. α-Amylase is a metalloenzyme (Gupta, Gigras, Mohapatra, Goswami, & Chauhan, 2003), and Cl^- is its activator (Kuriki, & Imanaka, 1999). So, α-amylase activity is influenced by Cl^- and metal ion, especially Ca^{2+}. Moreover，most of the foods themself contain Na^+ and Ca^{2+}. NaCl and $CaCl_2$ are also used as

preservatives and thickeners etc in the food industry. Fig. 4 B and 4 C give the effects of NaCl and $CaCl_2$ concentration on the interactions between three Citrus flavonoids and α-amylase. As shown in Fig. 4 B, when NaCl concentration is less than 0.2 M, the SPR response increases with increasing NaCl concentration, while it decreases with increasing NaCl concentration when NaCl concentration is above 0.2 M. These indicate that the binding ability of three Citrus flavonoids to α-amylase is the strongest at 0.2 M NaCl. K_D is 1.45 mM, 1.94 mM and 2.03 mM for the interaction of naringin, neohesperidin, and hesperidin with α-amylase, respectively. Similarly, it was found that the binding ability of three Citrus flavonoids to α-amylase is the strongest at 0.1 M $CaCl_2$ (see Fig. 4C). K_D is 1.23 mM, 2.01 mM and 2.43 mM for the interaction of naringin, neohesperidin, and hesperidin with α-amylase, respectively. This may be ascribed to that lower concentration salt may improve the stability of α-amylase activity. Therefore, the binding affinity of the three Citrus flavonoids with α-amylase can be reinforced by optimal concentration NaCl or $CaCl_2$.

3.3 Effect of α-amylase on antioxidant activity of flavonoids

The DPPH radical assay is a fast, simple, stable and economic method to measure the antioxidant capacity of flavonoids (Jabbari, & Jabbari, 2015). It is based on the scavenging of DPPH radical through the action of an antioxidant, which decreases the absorbance of the DPPH solution in the visible spectrum. The decrease of the absorbance is considered as the measurement for the antioxidant activity (Chen, Wang, Zeng, Yuan, & Zhou, 2011). To assess if α-amylase affects the antioxidant activity of

three Citrus flavonoids, the immediate DPPH radical scavenging activities of the tested samples were detected. As seen from Fig. 5, α-amylase has no the scavenging activity of DPPH radical, and three Citrus flavonoids show the higher scavenging activity of DPPH radical. Moreover, the three Citrus flavonoids- α-amylase samples show the lower the radical scavenging activity than that of three Citrus flavonoids alone. These results reveal that the DPPH radical scavenging activities of three Citrus flavonoids are inhibited significantly in the presence of α-amylase ($p < 0.05$), and the inhibition percentage of antioxidant activity for naringin, neohesperidin and hesperidin are 47.61±0.034%, 22.81±0.037% and 21.01±0.051%, respectively (see Table 2). As seen from Table 1 and Table 2, when the three Citrus flavonoids bind with α-amylase, the order of the inhibition percentage of antioxidant activity is the same as that of the binding affinity. This is because the antioxidant activity of three Citrus flavonoids is closely related to their hydroxyl groups (Labuckas, Maestri, Perelló, Martínez, & Lamarque, 2008; Rawel, Ranters, Rohn, & Kroll, 2004). Actually, some of the hydroxyl groups in the three Citrus flavonoids will be masked by binding with α-amylase, which decreases the antioxidant activity of the Citrus flavonoids.

4. Conclusions

The binding properties of three Citrus flavonoids, including naringin, neohesperidin and hesperidin, with α-amylase were evaluated by a label-free SPR detection system. The results show that naringin, neohesperidin and hesperidin can

bind with α-amylase to respectively form a new stable complex, with the binding affinity order of naringin > neohesperidin > hesperidin. The binding affinities of three Citrus flavonoids with α-amylase reach the highest at pH 6, and can be reinforced with 0.2 M NaCl and 0.1 M $CaCl_2$, respectively. Moreover, the antioxidant activities of three Citrus flavonoids are significantly decreased, when they interact with α-amylase. Additionally, it is found that the interaction between three Citrus flavonoids and α-amylase depends strongly on both the number and position of hydroxyl group in Citrus flavonoids. These indicate that the SPR-based method may not only reduce assay complexity and time, but also rapidly screen potential inhibitors of the α-amylase from plant-based food.

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Table.1 Kinetic parameters for three Citrus flavonoids, acarbose, atrazine and α-amylase interaction.

	k_a（$mol^{-1}·L·s^{-1}$）	k_d(s^{-1})	K_D(mM)
Naringin	7.05±0.08	0.0159±0.10	2.27±0.18
Neohesperidin	5.49±0.12	0.0167±0.08	3.09±0.20
Hesperidin	4.94±0.09	0.0157±0.03	3.51±0.09
Acarbose	11.12±0.07	0.0088±0.04	0.78±0.12
Atrazine	1.32±0.09	0.0167±0.05	12.65±0.13

Table.2 Effect of binding to α-amylase on the antioxidant activity of three Citrus flavonoids (DPPH radical assay).

	Inhibition (%)	not add α-amylase IC_{50} (μM)	add α-amylase IC_{50} (μM)
Naringin	47.61±0.034	84.52±0.039	124.51±0.020*
Neohesperidin	22.81±0.037	114.61±0.029	140.3±0.045*
Hesperidin	21.01±0.051	119.63±0.034	144.1±0.024*

Inhibition(%) = (add α-amylase IC_{50} - not add α-amylase IC_{50})/not add α-amylase IC_{50}

*Represents for significant difference comparing with three Citrus flavonoids alone ($p < 0.05$).

Figure Captions

Fig.1. Molecular structures of naringin, neohesperidin, hesperidin, acarbose and atrazine.

Fig.2. SPR response graph for α-amylase of different concentrations immobilized onto sensor chip surface.

Fig.3. SPR kinetic curves of binding process between α-amylase and (A) naringin, (B) neohesperidin, (C) hesperidin. Experimental data (black lines) are shown together with fits (Red lines). (D) SPR response for (a) acarbose, (b) naringin, (c) neohesperidin, (d) hesperidin and (e) atrazine of 400μM flowed over the chip surface modified with α-amylase, respectively. *Represents for significant difference comparing with atrazine alone ($p<0.05$) (E) The kinetic curve of SPR chip regeneration using 2 mM NaOH.

Fig.4. SPR response for interaction of three Citrus flavonoids (200 μM) and α-amylase (160 μM) with various pH values (A), various concentration of NaCl (B) and various concentration of $CaCl_2$ (C) at room temperature.

Fig.5. Absorbance values of (a) DPPH, (b) DPPH + α-amylase, (c) DPPH + naringin, (d) DPPH + naringin + α-amylase, (e) DPPH + neohesperidin, (f) DPPH +neohesperidin + α-amylase, (g) DPPH + hesperidin and (h) DPPH + hesperidin+ α-amylase. The concentrations of three Citrus flavonoids and α-amylase were 150 μM, and 160 μM, respectively.

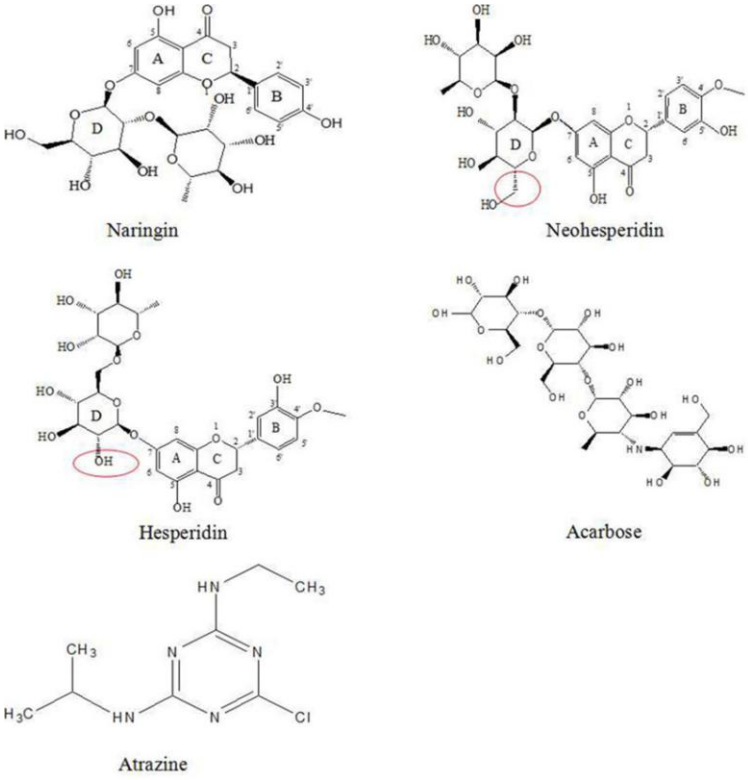


Fig.1

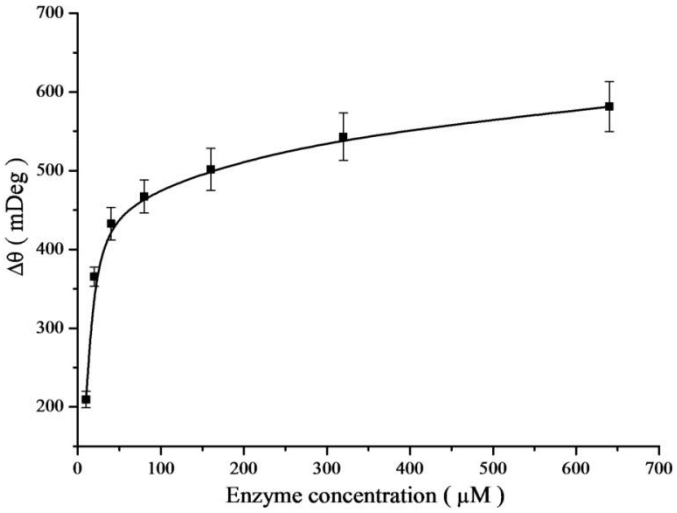
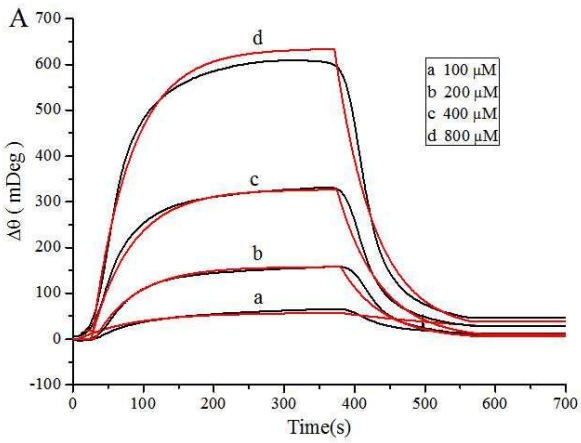
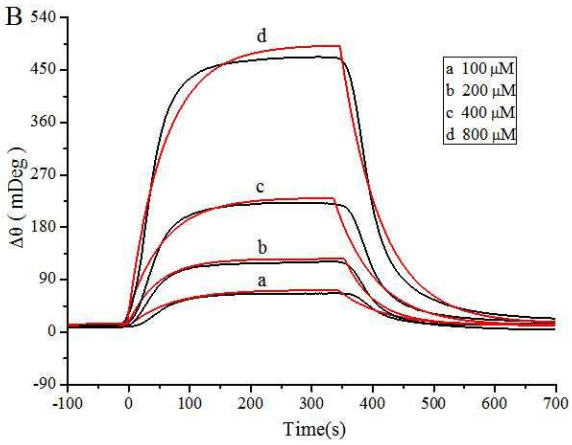
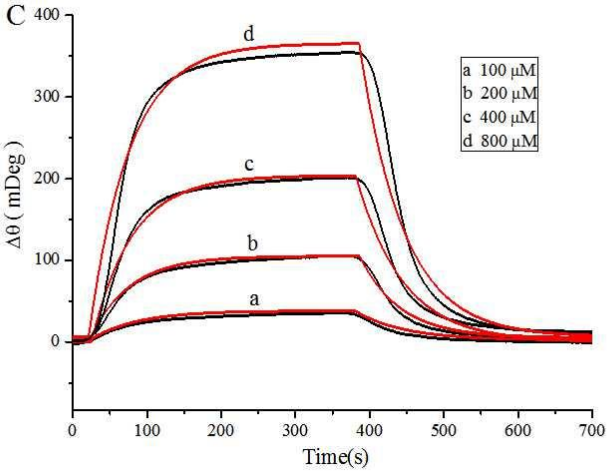
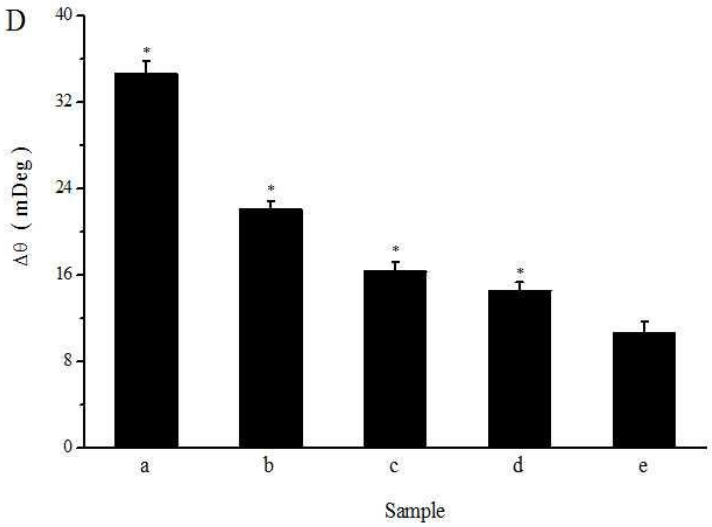


Fig.2









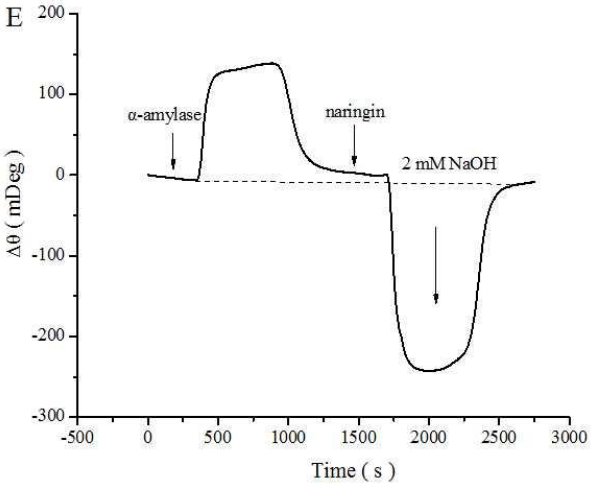


Fig.3

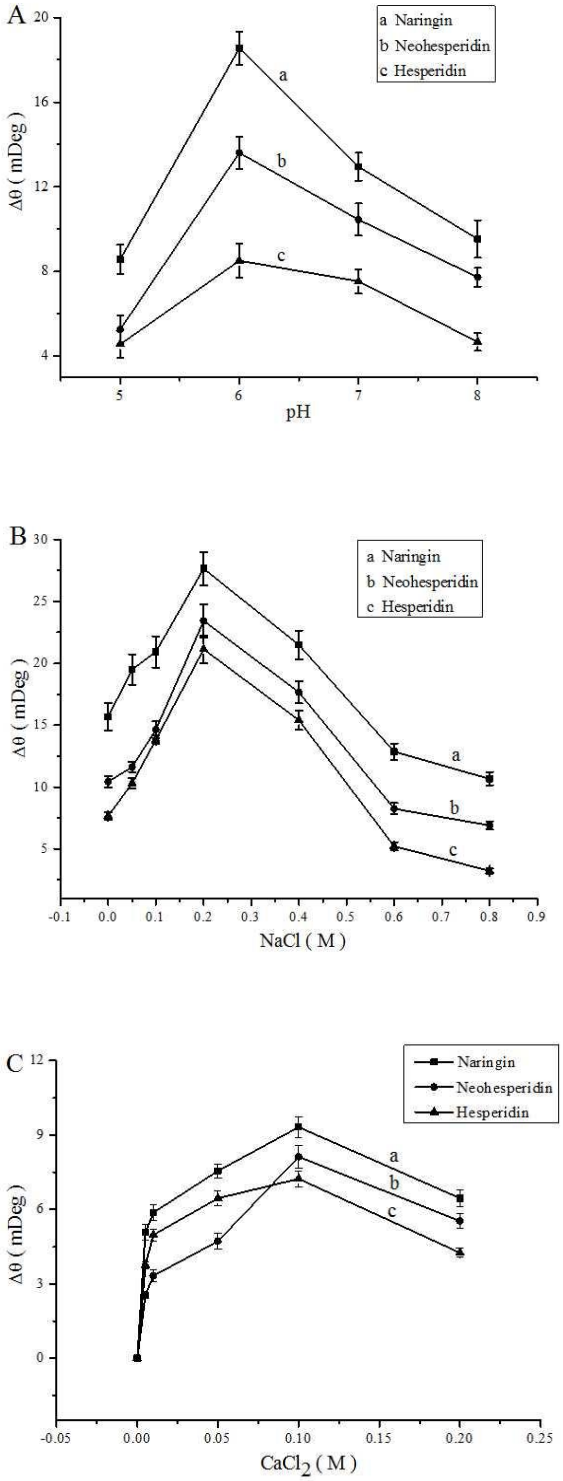


Fig.4

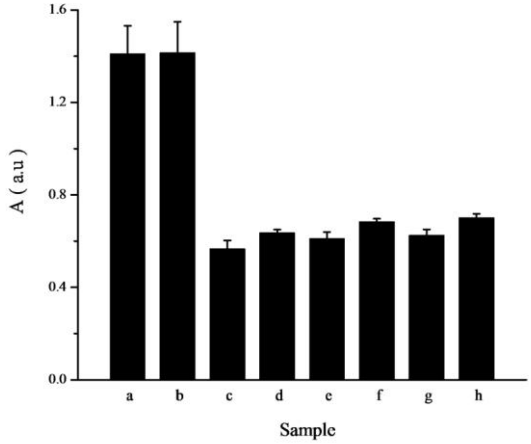
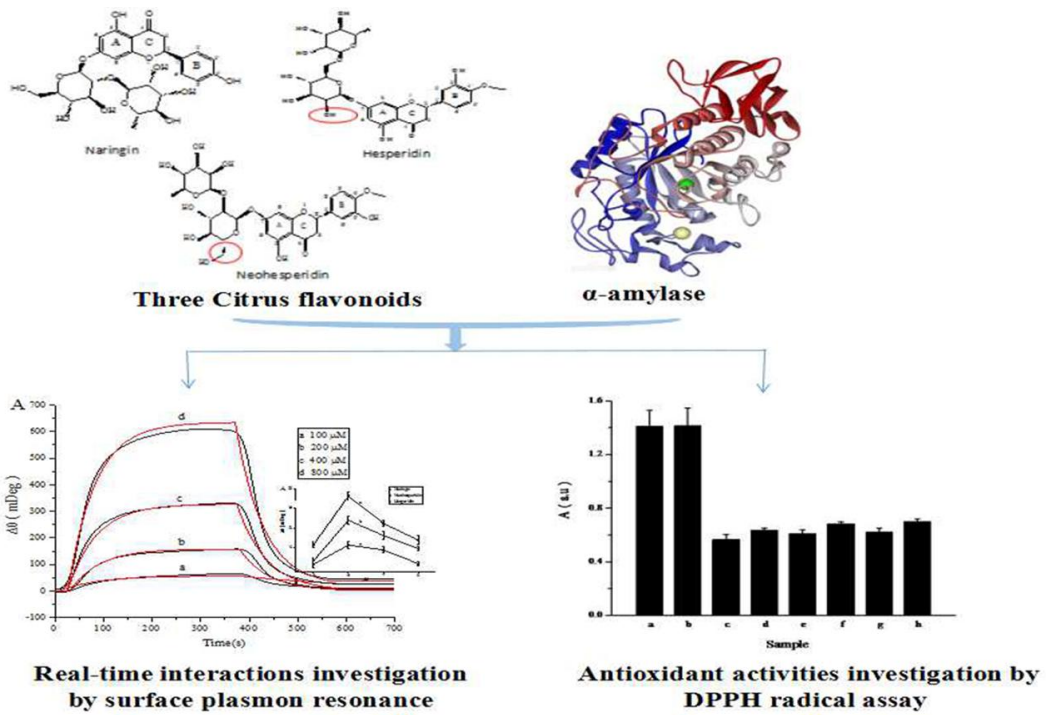


Fig.5



Graphical Abstract

Highlights

- The real-time interaction kinetics between three Citrus flavonoids (hesperidin, neohesperidin, naringin) and α-amylase were investigated using SPR biosensor.
- Binding affinities of three Citrus flavonoids to α-amylase reached the highest at pH 6, and can be reinforced with 0.2 M NaCl and 0.1 M $CaCl_2$.
- Binding of three Citrus flavonoids to α-amylase induced the decrease of the antioxidant activities of three Citrus flavonoids.
- Binding of three Citrus flavonoids to α-amylase depends strongly on both the number and position of hydroxyl group.