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Monitoring the binding processes of (–)-epigallocatechin gallate and theaflavin-3,3'-digallate to alpha-casein surface using quartz crystal microbalance with dissipation

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Abstract

Quartz crystal microbalance with dissipation monitoring (QCM-D) has been employed to study the interactions between (–)-epigallocatechin gallate (EGCG) - the powerful green tea antioxidant polyphenol - and theaflavin-3,3'-digallate (TF-3) - the black tea polyphenols, which gives black tea its unique color and taste with α-casein, surface. The adsorbed mass and thickness of EGCG and TF-3 adlayer on α-casein surface at various concentration, pH, and sodium chloride concentrations have been determined by QCM-D using Voigt model. The results show that the Freundlich model can be used to describe the adsorption isotherm of both EGCG and TF-3 on α-casein surface, suggesting that the adsorption on α-casein surface are dominated by hydrophobic nonspecific interactions, which are supported by stronger adsorption at the isoelectric point (pI) of α-casein for both EGCG and TF-3. The addition of salt slightly reduces the EGCG binding to protein surfaces. The shifts in the positions of both amides I and II bands in the FTIR spectra of the α-casein surface with EGCG adsorption indicate the presence of the hydrogen bonding.

Introduction

Tea - consumed by over two-thirds of the world's population - is now the world's second most popular beverage, second only to water (Graham, 1992). It's also classified as a functional drink as it contains polyphenol compounds - especially flavonoids - which promote health benefits. While EGCG is a major flavanol and the most powerful antioxidant in green tea, TF-3 is a unique flavanol, and the most powerful health benefit in black tea. Their structures are registered in Figure 1. With their antioxidative ability anti-cancer and anti-inflammatory properties, both EGCG and TF-3 are shown to be the key active compounds for prevention of degenerative diseases such as heart disease, cancer and diabetes (Dong *et al.*, 1997; Liang and Lin, 2000; Pan *et al.*, 2000; Higdon and Fri, 2003).

There are a number of studies indicating that tea polyphenols have the ability to interact non – covalently or covalently with proteins, especially proline-rich proteins such as bovine serum albumin, human salivary protein, casein and gelatin (Brown and Wright, 1963; Wroblewski *et al.*, 2001; de Freitas *et al.*, 2004; Jobstl *et al.*, 2006). Many studies have indicated that hydrophobic and hydrogen bonding play a crucial role for tea polyphenols and protein

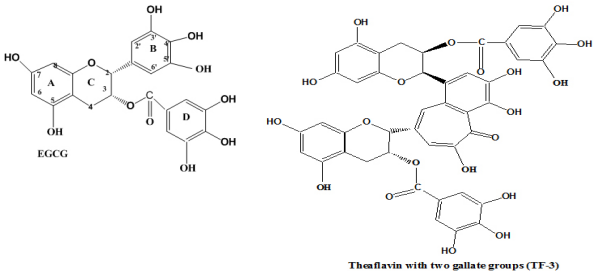

Figure 1. Structures of (–)-epigallocatechin gallate (EGCG) and theaflavin-3,3'-digallate (TF-3).

interaction (Brown and Wright, 1963; Wroblewski *et al.*, 2001; Jobstl *et al.*, 2006; Wang *et al.*, 2007; Chitpan *et al.*, 2007). There is some evidence showing that tea polyphenol and protein interaction have an adverse impact on biological activity like on antioxidative property of tea polyphenols, on nutritive value of protein itself and on some digestive enzymes (Brown and Wright, 1963; Hertog *et al.*, 1977; Rohn *et al.*, 2002; He *et al.*, 2006).

Milk is generally added to tea to reduce the astringency caused by the flavonoid-like tannin substance, which then appears to precipitate in the tea cup. Proteins in milk are composed of 30-35 g protein L^{-1}, and 76-86% of these proteins are caseins. Caseins are hydrophobic, non-globular molecules and high charge. Milk caseins are made up of several components, and the major molecule components

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are α_{S1}- casein, α_{S2}- casein, β-casein, and κ-casein (Walstra *et al.*, 1999; Huppertz, 2013). All types of casein are small phosphoprotein 19-25 KDa in size, and strongly hydrophobic. The ratio content of α-casein is generally higher than other forms of casein, which exist in proportions of 4:1:4:1 for α_{S1}, α_{S2}, β and κ-casein, respectively (Marchesseao *et al.*, 2002; Phadungath, 2005; Liu and Guo, 2007). The isoelectric point of casein is at pH 4.6. Milk is added to black tea and green tea as milk tea products which are now available in the global market. Brown and Wright (1963) showed that tea polyphenols mainly interact with the α-casein complex and the β-casein of the milk (Brown and Wright, 1963), and some studies have demonstrated that the interaction of milk protein and tea polyphenol have a negative effect on the antioxidant potential of tea polyphenols (Serafi *et al*.,1996; Hertog *et al*.,1997).

Serafi *et al*. (1996) using TRAP evaluated a total reduction in antioxidant capacity in the blood of human volunteers who drank black tea with and without 25% milk, and found that the consumption of tea with milk inhibited *in vivo* antioxidant activity because of the complexation of tea polyphenol by milk proteins. In a later study, Hertog *et al.* (1997) found that the consumption of tea was not associated with a lower mortality of ischemic heart disease, even for heavy tea drinkers in Wales, UK (Hertog *et al.*, 1997). The data from this study does not coincide with some epidemiological studies which have indicated an inverse relation between tea consumption and the risk of cardiovascular and other chronic diseases due to the antioxidant properties of tea polyphenols. They also suggested that the flavonoid binding capacity of milk proteins result in the reduction of absorption of tea polyphenols from the gastrointestinal tract.

Previous studies using Quartz Crystal Microbalance with Dissipation revealed that non-covalent binding like electrostatic, hydrophobic and hydrogen bonding plays a key role for interaction of protein bovine serum albumin and green tea EGCG and black tea polyphenols including thearubigins (Wang *et al.*, 2007; Chitpan *et al*., 2007). The aim of this study is to monitor the interaction between both green tea polyphenol, EGCG, and black tea polyphenol, TF-3, with milk casein, α-casein using QCM-D at different condition

Materials and Methods

α-casein from bovine milk (BSA, ≥ 98% pure by gel electrophoresis) was purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. 11-Mercaptoundecanoic acid (11-MUA) (Aldrich, St. Louis, MO), N-hydroxysuccinimide (NHS) (Aldrich), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (Sigma), acetic acid (Aldrich), sodium acetate (Fisher, Fair Lawn, New Jersey), ammonia hydroxide (NH_4OH) (VWR, West Chester, PA), hydrogen peroxide (H_2O_2) (Aldrich), sodium chloride (NaCl) (Fisher), and absolute ethanol (Fisher) were all used as received.

Preparation of EGCG and TF-3 solution

(–)-Epigallocatechin gallate (EGCG, ≥ 95%) were purchased from DSM (Basel, Switzerland). The EGCG solution was then prepared in 0.01 M acetate buffer at pH 4.6 with concentration of 0.23% (5 mM), 0.69% (15 mM), 0.92% (20 mM), 1.37% (30 mM) and 2.29% (50 mM), respectively. Theaflavin-3,3'-digallate (TF-3) was extracted from black tea extract by ethyl acetate, and was vacuum evaporated to remove solvent. The fraction of ethyl acetate was then subjected to a Sephadex LH-20 column and eluted with acetone solution (40% v/v). According to its elution sequences, TF-3 was collected. The extracted TF-3 then was dissolved in 0.01 M acetate buffer at pH 4.6 with concentration of 0.005% (0.058 mM), 0.02% (0.23 mM), 0.032% (0.368 mM), 0.05% (0.576 mM) and 0.08% (0.921 mM).

Preparation of α-casein Surface

AT-cut gold-coated quartz crystals (fundamental frequency of 5 MHz) were purchased from Q-Sense AB (Sweden). The immobilization of α-casein on the gold-coated quartz crystal surface was carried out using the procedure modified from a previously-published paper (Wang *et al*., 2007; Chitpan *et al*., 2007). Gold-coated quartz crystals were first cleaned in an UV/ozone chamber for 10 mins, followed by immersion in a 1:1:5 mixture of ammonia hydroxide (NH_4OH, 25%), hydrogen peroxide (H_2O_2, 30%), and Milli-Q water for 5 mins at 75°C, and finally cleaned in an UV/ozone chamber for another 10 mins. These gold-coated quartz crystals were then rinsed with a large quantity of Milli-Q water and dried with nitrogen gas (N_2), and subsequently soaked in 10 mM 11-mercaptoundecanoic acid (11-MUA)/ absolute ethanol solution at 60°C for at least 24 hours. The excess amount of 11-MUA was rinsed off with absolute ethanol, and the modified quartz crystal surfaces were dried under N2 flow. Just before the immobilization of protein, 11-MUA-coated quartz crystal surfaces were activated by a mixed solution containing 1:1 (v/v) of 100 mg mL^{-1} 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and 100 mg mL^{-1} N-hydroxysuccinimide (NHS) in Milli-Q water for 1 hour. The solution of

10 mg mL^{-1} α-casein in phosphate buffer (pH 7.0) was used to incubate the activated surfaces at 4°C for at least 24 hours. The quartz crystal surfaces were finally rinsed thoroughly by phosphate buffer, followed by Milli-Q water, and dried under N_2 flow.

FTIR Measurements

Infrared spectrum of thearubigin powder was measured using Attenuated Total Reflectance Fourier Transform Infrared (ATR-FT-IR) spectrometer, while the infrared spectra of α-casein-modified quartz crystal surfaces before and after EGCG and TF-3 adsorption were collected with a Thermo Nicolet Smart Apertured Grazing Angle (SAGA) accessory operating at the grazing angle of incidence of 80°. Both ATR and Grazing Angle measurements were carried out using a Fourier Transform Infrared spectrometer (Thermo Nicolet 670, Madison, WI). The resolution was set to 4 cm^{-1}, and 1024 scans were collected for each sample.

QCM-D Measurements

The interactions between tea polyphenol, EGCG and TF-3n and α-casein functionalized quartz crystal surface were studied using a commercial QCM-D apparatus (Q-Sense AB, Sweden) with a Q-Sense D300 electronic unit. A polypropylene pipette tip connecting to the temperature-controlled chamber was initially filled with deionized water of pH 6. By opening the valve, distilled water was exchanged in the QCM-D chamber via the gravitational flow. After a stable baseline was established, polyphenol in buffer solution was exposed to α-casein-modified crystal surface. At the same time, the adsorption was monitored as a function of time by recording the shifts in both frequency (ΔF) and energy dissipation (ΔD) simultaneously at the fundamental resonant frequency along with the third, fifth, and seventh overtones until the steady state of the adsorption was reached. The long-term stability of the frequency was within 1 Hz, and this drift was negligible compared to the frequency shifts due to the adsorption.

Traditionally, Sauerbrey mass was calculated from Sauerbrey equation (Wang *et al*., 2007).

$$M = -\frac{C}{n}\Delta F \qquad (1)$$

ΔF, M, and n represent the frequency change, adsorbed mass per unit area, and overtone number, respectively. C is the mass sensitivity constant(17.7 ng cm^{-2}Hz). The Q-Sense software determines the resonance frequency and the decay time τ_0 of the exponentially damped sinusoidal voltage signal over the crystal, and the dissipation factor D can be obtained from the following equation:

$$D = \frac{1}{\pi f_0 \tau_0} = \frac{2}{\omega \tau_0} \qquad (2)$$

Where f_0 is the resonance frequency and τ_0 is the decay time.

Results and Discussion

Effect of EGCG and TF-3 concentrations

The adsorption of EGCG and TF-3 on α-casein surface at pH 4.6, which is close to the isoelectric point (pI) of α-casein, was measured with QCM-D by recording changes in frequency and dissipation with time. Figure 2 displays the typical time-resolved resonance frequency shifts (ΔF) and energy dissipation shifts (ΔD) for the third overtone upon the addition of various concentrations of EGCG (see Figure 2 a, b) and TF-3 (see Figure 2 c). Prior to the introduction of EGCG and TF-3 solution into the chamber, a steady baseline was acquired using pH 4.6 sodium acetate buffer. Right after the injection of each solution, there was often a rapid decrease in ΔF and a marked increase in ΔD, followed by gradual changes of ΔF and ΔD until steady states were reached. These changes in ΔF and ΔD indicated the adsorption of EGCG and TF-3 on α-casein surface. During the process of EGCG adsorption on α-casein surface, mass and thickness, as in Figure 3a, of the adsorbed EGCG adlayer were calculated by software (QTools) based on Voigt model using the responses at third, fifth, and seventh overtones. Increasing concentration of EGCG from 0.23% to 2.29%, both thickness and mass of EGCG adlayer increase from 6 to 70 nm and from 15 to 175 ng cm-2 respectively. The data also showed that the adsorption of EGCG on protein α-casein is higher than on BSA protein surfaces as shown in Figure 3 (c).

Previous research using the same technique revealed that TF-3 - the key polyphenol in black tea - had interaction with bovine serum albumin through hydrophobic and hydrogen bonding. Recent research investigated the adsorption of TF-3 on α-casein surface, and the result showed that increasing TF-3 concentrations, from 0.005 g % to maximum concentration 0.08 g %, both thickness and mass of adlayer increased from 3 to 13.89 nm and 136 to 628 ng cm^{-2}, respectively, as shown in Figure 3b. Compared with TF-3 adlayer on BSA surface in the previous study by Chitpan *et al.* (2007) maximum thickness and mass at maximum concentration were 10.07 nm and 479.97 ng cm^{-2} respectively. The result from this study indicated both adsorption of TF-3 and EGCG adlayer on α-casein surface are higher

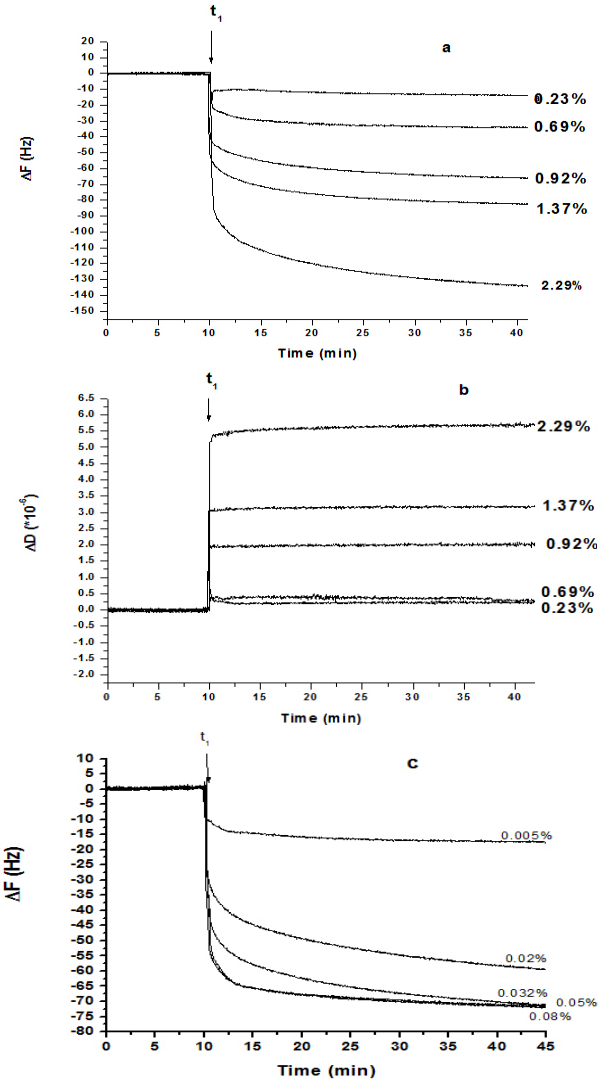

Figure 2. Time-dependent frequency shifts and energy dissipation shifts for EGCG (a, b) and frequency shifts for TF-3 (c) adsorption on α-casein-modified quartz crystal surface at various concentrations. The arrows indicate the time for the injection of tea polyphenol molecules (t_1).

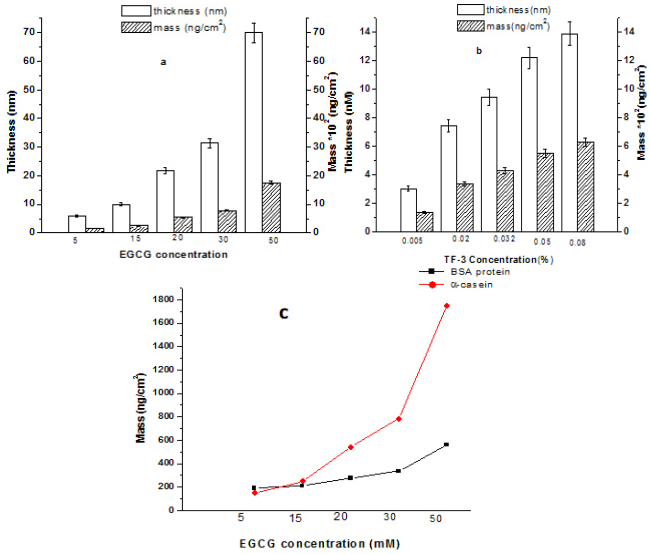

Figure 3. Changes of mass and thickness of EGCG (a) and TF-3 (b) adlayer on α-casein surface at various polyphenol concentrations. (c) mass change at various concentration of EGCG adlayer on α-casein surface compared with BSA protein.

than on BSA surfaces, which also indicated the effect of protein types on binding process of both EGCG and TF-3. This phenomenon can be explained thus, that polyphenols bind most strongly to proline-rich proteins. The enhanced ability of interaction with phenolic compounds is related to their flexible secondary structure, and the greater extent of hydrogen bonding is due to the increased accessibility of the peptide bond. In addition to proline, other amino acids involved in the polyphenol protein interaction include arginine, cysteine, methionine, tryptophan, phenylalanine and tyrosine (Murray *et al*., 1994; O'Connell and Fox, 2001; Wroblewski *et al*., 2001; Poncet *et al*., 2007). Proline, arginine and tyrosine component of α-casein (6%, 10% and 7% respectively) are more than BSA protein (5%, 4% and 3%, respectively), leading to the stronger binding interaction of EGCG and TF-3 to α-casein compared to BSA protein.

The adsorption isotherm of EGCG and TF-3 onto α-casein surface can be determined from the changes of the adsorbed mass of these polyphenols against tea polyphenol concentration. The Langmuir and Freundlich models were used to predict the performance of an adsorption isotherm system in this study. The Langmuir model assumes the monolayer coverage of adsorbate over a homogenous adsorbent surface, and a saturation point will finally be reached according to the following equations

$$\frac{C}{M} = \frac{1}{KM_m} + \frac{1}{M_m}C \qquad (3)$$

where C is the concentration of adsorbate solution, M is the amount of adsorbate on the adsorbent, K is a direct measure for the intensity of the adsorption process, and M_m is a constant related to the area occupied by a monolayer of adsorbate, reflecting the adsorption capacity. The Freundlich model, an empirical exponential equation which assumes that the mass of adsorbates on the adsorbent surface increases as the adsorbate concentration increases. The Freundlich equation can be expressed as follows:

where n and K_f are constants, and C is the concentration of the solution.

$$\log M = \log K_f + 1/n \log C \qquad (4)$$

The result in figure 4 indicates that EGCG adsorption fits well with the Freundlich model, with

Table 1. Freundlich and Langmuir constants

Polyphenols	Freundlich model			Langmuir model		
	Kf	*n*	R^2	*K*	M_m	R^2
EGCG	23.3292	0.94	0.98	-	-	-
TF-3	723.936	1.76	0.99	3	833	0.99

Freundlich and Langmuir constants were obtained from the linear equation depicted in (3) and (4) equation

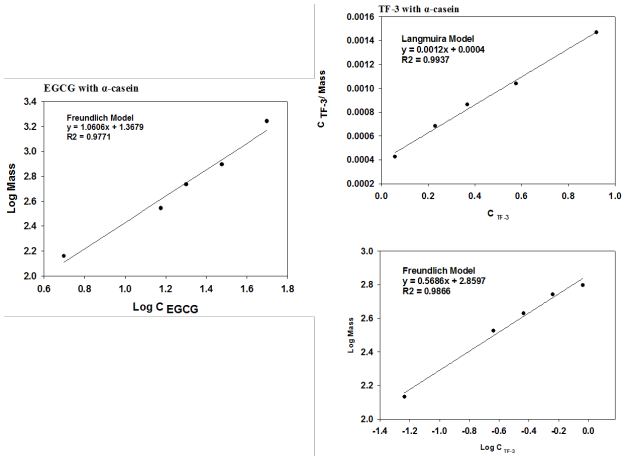

Figure 4. The adsorption isotherm of EGCG and TF-3 onto α-casein surface fitted to the Langmuir model (top) and the Freundlich model (bottom and left).

a correlation coefficient of 0.97 but it does not fit with the Langmuir model. This proves that there is a formation of multilayer or aggregation of protein molecules, and indicates that the EGCG adsorption onto the α-casein surface may be mainly governed by nonspecific hydrophobic interactions. The experimental data of TF3 adsorption on α-casein surface showed that it fit well with both the Freundlich model and Langmuir model, with a correlation coefficient of 0.99.

The Huang *et at.* (2007) study suggests that gallate group has significant impact on the interaction between EGCG and protein BSA. For the Freundlich model, higher affinity constant (*n*) and a constant relating to bonding energy (K_f) of TF-3 (see Table 1) than EGCG also might indicate that adsorption of TF3 onto casein is stronger than EGCG due to the structure difference in particular at gallate group possessing as shown in Figure 1.

Effect of pH

Since protein has ionizable properties in different pH, this unique property can be used to explain the effect of pH on the interaction. In addition to the experiments done at pI of α-casein, pH 4.6, the adsorption process was measured at pHs 7.0 and 3.0, where protein carries net negative and positive charges, respectively. The mass and thickness of the adsorbed EGCG and TF-3 adlayer on α-casein surface at pH 7.0, 4.9, and 3.0 are compared in Figure 5 (a, b). Like the adsorption of these tea polyphenols on BSA surface in previous studies (Wang *et al.*,

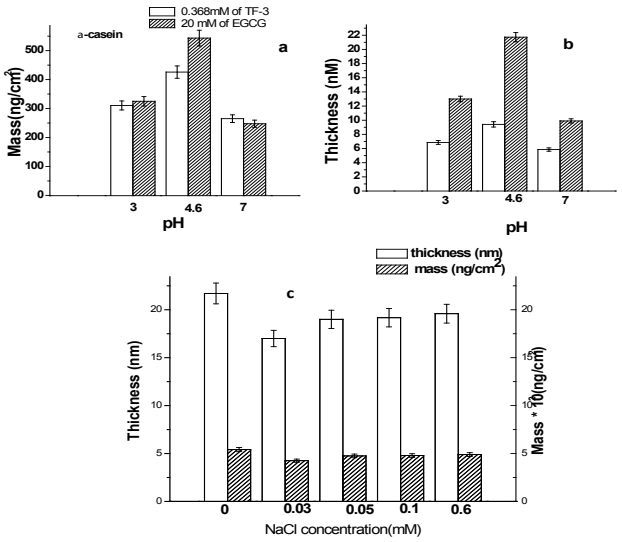

Figure 5. Changes of mass (a), and thickness (b) of EGCG at 20mM and TF-3 at 0.368mM adlayer on α-casein surfaces at pH 3, 4.6, and 7 (c) Changes of mass and thickness of EGCG adlayer on α-casein surface at various sodium chloride concentrations

2007; Chitpan *et al.*, 2007), the significantly highest adsorbed EGCG and TF-3 at pI which can be indicated by mass and thickness suggests that the contribution from hydrophobic interaction between protein surface and polyphenols both EGCG and TF-3. The adsorption above and below the pI may not be the main factor that causes EGCG adsorption. The higher electrostatic repulsion between charged protein molecules will be responsible for the lower EGCG adsorption capacities at both pH 7.0 and pH 3.0, leading to smaller values of mass and thickness of EGCG adlayer and TF-3 at either these two pHs than that at pH 4.6.

Salt effect

Salt concentration has a slight impact on the EGCG adsorption onto α-casein surface, with a 11% decrease in the adsorbed mass when CNaCl increases from 0 to 0.6 M, as shown in Figure 5 (c). Technically, salt usually either reduces the electrostatic interaction or enhances the hydrophobic interaction; the present salt-reduced effect seems contrary to our above conclusion that hydrophobic interaction is dominant in EGCG/α-casein interaction. The salt effect data here is accordant with the EGCG/BSA protein interaction (previous study) regarding the addition of salt from 0 to 0.1% which caused a 20% reduced mass of EGCG adlayer onto BSA surface. This disagreement can be explained. At pH values close to the isoelectric point (pI), protein molecules have very low surface charges and are capable of self-association through the electrostatic interactions between positively- and negatively-charged patches on their surface. The small amount of metal ions from salt can reduce the

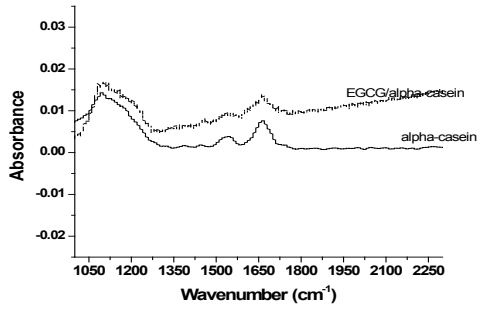

Figure 6.FTIR spectra of pure α-casein surface (bottom) and α-casein surface with adsorbed EGCG molecules (top).

attraction between the protein molecules and the decrease in protein molecular size and existence of a certain critical distance for the formation of the inter-protein complexes consequently lead to suppress EGCG adsorption onto protein.

Hydrogen bonding

FTIR spectra of α-casein surface with and without the adsorption of EGCG were used to investigate the hydrogen bonding between the adsorbed EGCG molecules and protein surfaces as recorded in Figure 6. Since the phenolic groups of polyphenol have been considered to form hydrogen bonding with carbonyl groups in proteins, amount and differences in the spatial positions of gallate groups were supposed to have a great influence on the interaction (Loomis, 1974). The FTIR spectrum of pure α-caseins surface shows two characteristic bands at 1666 cm^{-1} and 1542 cm^{-1}. The 1666 cm^{-1} (amide-I) band indicate the protein amide C=O stretching vibrations, and the 1542 cm^{-1} (amide-II) band is due to the amide N-H bending vibrations and C-N stretching vibrations (Hertog *et al.*, 1997). The position of amide band I in EGCG adsorbed α-casein surface has a slight shift from 1666 to 1668 cm^{-1}. Amide II band has a small change from 1542 to 1549 cm^{-1} after EGCG adsorption. The peak position changes observed in the amide I and amide II bands may be attributed to hydrogen bonding between EGCG and protein, suggesting that the hydrogen bonding may occur between the phenolic hydroxyl groups in EGCG and the functional groups (i.e. amide groups) of protein. Compared to protein BSA from previous research (Wang *et al.*, 2007), the hydrogen bonding may involve more in the EGCG/BSA interaction than in the EGCG/α-casein interaction. Unlike bovine serum albumin, globular structure protein, casein is linear amphiphilic and strongly hydrophobic proteins, and the relatively stronger adsorption of EGCG/casein than BSA may interfere with the amount of hydrogen bonding between EGCG phenolic hydroxyl groups and the functional groups (i.e. amide groups) of casein.

Conclusion

In summary, the adsorption of (–)-epigallocatechin gallate (EGCG) and theaflavin-3,3'-digallate (TF-3) molecules on α-casein surface was monitored in real time through the simultaneous measurements of the shifts in both resonance frequency and energy dissipation using QCM-D. The adsorbed EGCG and TF-3 masses and thicknesses under different physicochemical conditions were calculated using Voigt model. The result suggest that EGCG adsorption onto the α-casein surface are mainly governed by nonspecific hydrophobic interactions, while specific interactions, such as electrostatic interaction and hydrogen bonding are the dominant forces in TF-3-α-casein interactions. The significantly highest adsorbed EGCG and TF-3 at pI suggests that the contribution from hydrophobic interaction between protein surface and polyphenols both EGCG and TF-3. The existence of electrostatic interaction is also confirmed by the complex salt concentration effects. Hydrogen bonding is another dominant force in the EGCG- α-casein interactions, as evidenced by the large casein band position shifts for both amide I and amide II before and after EGCG adsorption in FTIR spectra. The adsorption isotherm for EGCG adsorption on α-casein can be better described by the Freundlich model, while the adsorption isotherm for TF-3 adsorption on casein can be described by the Langmuir model and the Freundlich model. This research will help us better understand the mechanism that controls the polyphenols binding to proteins, and can be extended to the studies of bindings between drugs and receptor proteins.

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