MONITORING OF THE BINDING PROCESSES OF BLACK TEA POLYPHENOLS TO BOVINE SERUM ALBUMIN SURFACE USING QUARTZ CRYSTAL MICROBALANCE WITH DISSIPATION

by

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ABSTRACT OF THE DISSERTATION

Monitoring the Binding Processes of Black Tea Polyphenols to Bovine Serum Albumin Surface using Quartz Crystal Microbalance with Dissipation Monitoring by MONTHANA CHITPAN

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Among three major types, green, oolong, and black tea, black tea is the most popular of tea consumption as indicated by almost 80% in the industry of tea production with 3 billion kilograms. Theaflavins and thearubigin are two main polyphenols that give black tea its characteristic color and taste. Theaflavins are classified as the mixtures of four compounds including theaflavin (TF-1), theaflavin-3-gallate (TF-2a), theaflavin-3'gallate (TF-2b), and theaflavin-3, 3'-digallate (TF-3). There are numerous epidemiological studies both in vitro and vivo have indicated that black tea polyphenols including, theaflavins, and thearubigin, have ability to be antioxidative, antiinflammatory, anti-mutagenic, and anticancer properties, consequently reducing the risk of non-communicable degenerative diseases such as cancer, and coronary heart disease. Tea polyphenols have been reported to interact with proteins such as salivary proline-rich protein, bovine serum albumin, and milk protein. Some studies reported that the interactions between polyphenols in tea and proteins might lead to the loss of bioavailability of polyphenols and their bioactive capacity.

The structure difference between thearubigin and theaflavins in terms of amount of gallic acid and hydroxyl functional groups could provide the different interaction with proteins. This study aims to monitor the binding processes of thearubigin and theaflavins including theaflavin (TF-1) and theaflavin-3,3'-digallate (TF-3), with bovine serum albumin(BSA) surface using quartz crystal microbalance with dissipation monitoring QCM-D at different environment such as concentration, pH, ionic strength and temperature.

BSA protein was immobilized on the surface of self-assemble monolayer of quartz crystal electrode. The mass and thickness of black tea polyphenols adlayer on BSA surfaces had been determined by QCM-D using Voigt model. Our results showed that the adsorption isotherm of thearubigin on BSA surface can be better described by the Langmuir model than the Freundlich model, suggesting that the thearubigin adsorption on BSA surface was dominated by electrostatic interactionas evidenced by the stronger thearubigin adsorption at pH below the isoelectric point (pI) of BSA. On the other hand, TF-3 had stronger adsorption at the isoelectric point (pI) of BSA and the adsorption isotherm of both TF-3 and TF-1 on BSA surface can be better described by Freundlich model, suggesting that the TF-3 adsorption on BSA surface is dominated by hydrophobic interactions. The much higher adsorption capacity on BSA surface of TF-3 than TF-1 indicates the importance of galloyl group in polyphenol/protein interactions. The addition of salt influenced the thearubigin and theaflavins binding to BSA surfaces. The shape and shifts in the positions of both amides I and II bands in the FTIR spectra of the BSA surface indicated the presence of the hydrogen bonding.

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DEDICATION

To my dear parent, Mr. Pinit and Mrs. Thipphawan Chitpan and my beloved aunts, Dee,

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1.INTRODUCTION

Compared with green and oolong tea, black tea is the most consumed beverage. The most potent group of tea component, which influence human health benefits are polyphenols, in particular the catechins. Black tea contains about 200 mg flavonoids per cup. During the manufacture of back tea, the fermentation (enzymatic oxidation) processes cause green tea catechins to oxidize and polymerize to form oligomeric flavanols, including theaflavins, thearubigins, and other oligomers. Theaflavins and thearubigins are classified as two major polyphenols that give black tea its unique color and taste. While theaflavins are responsible for orange red and astringent taste thearubigins are responsible for rusty red brown and richness taste. Black tea contains about 10-20% thearubigins and 1-2% theaflavins in dried weight.

Black tea polyphenols have numerous health benefits, including the prevention of chronic diseases such as cancer and cardiovascular diseases. It is now clearly evidence support the protective ability to reduce the risk of coronary heart disease (CHD) of black tea, where an intake of \geq 3 cups per a day. Apart from antioxidative activity, abundant epidemiological studies, which both *in vitro* and *in vivo* showed that black tea polyphenols particularly theaflavins have ability to act as anti-carcinogenesis.

There are a number of studies indicating that polyphenols including tea polyphenols have ability to interact non – covalently or covalently with proteins. A lot of studies indicated that the hydrophobic and hydrogen bonding play crucial role in the interactions. The tea polyphenol and protein interaction can generate the adverse effect on biological activity like antioxidative property of tea polyphenols, on nutritive value of protein itself, and on the digestive enzyme *in vivo*.

For instance, adding milk to tea, especially black tea, is a tea drinking habits which usually practices in some countries particularly in UK. Milk is generally added to tea to reduce the astringency caused by flavonoid-like tannin substance, which then appear to precipitate in the tea cup. Some studies have demonstrated that milk protein have effect on antioxidant potential of tea polyphenols.

The interaction of protein-polyphenol can provide the positive health benefit. Apart from, the scavenging free radical properties, one of the reasons why tea poly phenol can protect certain degenerative diseases might because they can bind to enzyme or protein involving in human degenerative disease generation. For instance, EGCG show the cell growth inhibitory activity through binding to the cell surface of cancer known as the 67-kDa receptor (67LR) which is associated with tumor invasion and metastasis.

There are many techniques used to determine the polyphenol-protein interaction such as Isothermal tritation calorimetry, Dynamic light scattering (DLS), and Fluorescence spectra. Among other methods, Quartz crystal microbalance (QCM) is considered to be new technique for studying the polyphenol-protein interaction, since it provides the simplicity, real time monitoring, and the sensitivity in nanoscale. QCM-D is an extremely sensitive mass sensor capable of measuring the mass changes of a material at the level of 0.4 ng/cm². The detection signals and calculated adsorbed mass in the conventional QCM are solely based on the assumption that the changes of the fundamental frequency of piezoelectric is proportional to the change of adsorbed mass on the surface of quartz crystal calculated from the classical Sauerbrey equation.

We hypothesize that the different structure of black tea polyphenols, thearubigin and theaflavins in terms of amount of gallic acid and hydroxyl functional groups cause the different interaction with protein which can be studied using Quartz Crystal Microbalance with Dissipation Monitoring. The results can be used to explain not only the loss of bioactivity of tea polyphenols but also clarify the tea bioactivity on degenerative diseases such as cancer. The aims of this research, therefore, are

- To determine the suitability of using QCM-D to study the interaction between bovine serum albumin as protein model and black tea polyphenol, therubigin
- To determine the interaction between bovine serum albumin proteins and theaflavins including TF-1 and TF-3

2. LITERATURE REVIEW

2.1 Black tea

Tea refers to the plant *Camellia sinesis*, its leaves, and the extracts and infusion thereof. Tea is now the second most popular beverage, next only to water, consumed by over two-third of the world's population. It was drunk in the UK for 350 years and in Asia for more than 4000 years (Gardner *et al.*, 2007). There are three types of tea, classified by the degree of fermentation, referred natural browning reaction induced by enzymatic oxidation in plant cell. Green (unfermented) is produced from fresh tea leaves and is inhibited enzymatic oxidation whether using steaming or panfried. Oolong (partially-fermented) tea is made by wilting fresh leaves by sun, then slightly bruising. Black (fully fermented) tea is made by crushing tea leaves to release the polyphenol oxidase for catalyzing the enzymatic oxidation and polymerization of tea catechin (Lydia *et al.*, 2001; Haslam., 2003; Chung *et al.*, 1999).

Among three types of tea, black tea is the most popular tea produced and consumed worldwide which is 78%, following 20% of green tea, and less than 2% of oolong tea (Bode and Dong., 2002). While black tea is preferential in the United State, England, and other Western countries, green tea is favored consumed primarily in Asian and Northern African countries and oolong tea is popular in China and Taiwan. Yang *et al* indicated that around 100 ml cup (3 oz) of tea pack with about 250-350 mg tea solid (Yang *et al.*, 2000)

The most potent group of tea component, which influence human health benefits are polyphenols, in particular the catechins (Art *et al.*, 2002; Ferruzzi and Green., 2006; Liang *et al.*, 1999). During the manufacture of back tea, the fermentation (enzymatic

oxidation) processes cause green tea catechins to oxidize and polymerize to form oligomeric flavanols, including theaflavins, thearubigins, and other oligomers (Liang et al., 2000). Theaflavins and thearubigins are two main polyphenols that give black tea its characteristic color and taste (Haslam., 2003). While theaflavins are responsible for orange red and astringent taste thearubigins are responsible for rusty red brown and richness taste (Haslam et al., 2003; Yao et al., 2006). The structure of thearubigins (Figure 1), the most abundant phenolic fraction in black tea with molecular weight ranging from 1000 to 40000, has not yet been well characterized (Yao et al., 2006). Black tea contains about 10-20% thearubigins and 1-2% theaflavins in dried weight (Dong et al., 1997). Apart of these two main polyphenols, tea also contains small amounts of flavonals like kaempferol, quercetin and myricitin, in the form of glycosides as in Table 1 (Gardner., et al., 2007). A cup of green tea, 2.5g of dried green tea leaves brewed in 200 ml of water, contains around 90mg of EGCG, the most powerful anitioxidant and aniticancer among green tea catechin). On the other hand, a cup of black tea contains 12-15 mg of theaflavins (Dong et al., 1997).



Galloyl

Theaflavins	R1	R2
Theflavin (theaflavin-1;TF-1)	Н	Н
Theaflavin-3-gallate (theaflavin-2a; TF-2a)	G	Н
Theaflavin-3'-gallate (theaflavin-2b; TF-2b)	Н	G
Theaflavin-3,3-digallate (theaflavin-3; TF-3)	G	G

Figure 1 Theaflavin structure and thearubigin possible structure

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Flavonoid	Component	% Dry weight
Catechin	Epigallocatechin gallate (EGCG)	10-12
Theaflavins	From oxidation of catechin	3-6
Thearubigins		12-18
Flavonols	Quercetin	6-8
	Keampherol	
	Rutin	
Methylxanthines	Caffeine	8-11
Phenolic acid	Caffeic acid	Not available
	Quinic acid	
	Gallic acid	
Amino acid	Theanine	Not available

Table 1 Key flavonoid and their components as a percentage of dry weight

This provides a mean caffeine level of 40 mg of caffeine per 235 ml cup (Gardner et al, 2007)

2.2 Health benefit of black tea

The abundant epidemiological studies, which both *in vitro* and *vivo*, have indicated that consumption of tea polyphenols including catechins, theaflavins, and thearubigins is associated with reducing risk of chronic noncommunicable diseases (NCD), caused 60% global death such as coronary heart disease, stroke, cancer (Hertog *et al.*, 1993a; Yang and Wang., 1993; Geleijnse *et al.*, 1999; Art *et al.*, 2001). Recent studies show that black tea polyphenols have numerous health benefits, including the prevention of degenerative diseases such as cancer and cardiovascular diseases (Shiraki *et al.*, 1994; Leung *et al.*, 2001; Miller *et al.*, 1996; Lin *et al.*, 1999; Yoshino *et al.*, 1994; Gupta *et al.*, 2002). One of the health benefits of tea are generally attributed to the

antioxidant properties of major phenolic compounds (catechin, theaflavins, thearubigins), when consumed, may act as free radical scavengers which remove endogenously generated superoxide, peroxyl, and hydroxyl radicals (Rice-Evans., 1999; Yen and Chen., 1995; Higdon and Fri., 2003). Tea polyphenols and their metabolites also posses the antibacterial properties against pathogenic bacteria such as *Clostridium perfringen*, Clostridium difficile, Escherichia Coli, Salmonella, and Pseudomonas and enhance the intestinal beneficial bacterial like *Bifidobacterium* spp and *Lactobacillus* sp which are classified as probiotic by improving the intestinal microbial balance (Lee *et al.*, 2006; Cho et al., 2007). The evidence from experimental and clinical studies has indicated that tea exerts antioxidative, anti-inflammatory, and vasodilating effects. In vitro studies have shown that green and black tea possess considerable radical scavenging potential, inhibiting oxidation of Low-density lipoproteins (LDLs) and plasma (Nanjo et al., 1999; Ishikawa et al., 1997; Lotito and Fraga., 1998). The oxidation of LDLs appears to be an important step in the formation of atherosclerotic plaques and subsequent cardiovascular disease (Steinberg et al., 1898). Cao et al investigated the effect of EGCG on angiogenesis in chick chorioallantic membrane assay, and indicated that EGCG suppresses endothelial cell growth, and the formation of new vessels, required for tumor growth and metastasis (Cao et al., 1999).

Leung *et al* (2001) showed that theaflavins of black tea equally possessed antioxidant potency as catechins of green tea by inhibition of LDL oxidation *in vitro* (Leung *et al.*, 2001). Using tert-butyl hydroperoxide-induced lipid peroxidation of rat liver homogenates, Tomita reported that antioxidant activity of both theaflavins and thearubigins was higher than that of glutathione, L-ascorbic acid, and α -tocopherol (Yoshino *et al.*, 1994).

Gardner *et al* reviewed the health benefit of black tea from the considerable evidence of studies. It is clearly evidence support the protective ability to reduce the risk of coronary heart disease (CHD) of black tea, where an intake of \geq 3 cups per a day. Drinking black tea also has the positive impact on dental health, anti-cariogenic properties; through suppress salivary amylase activity, which in turn slowly release source of fermented carbohydrate such as starch. Black tea consumption has positive effect on bone mineral density (BMD) in particular older women through increasing in BMD. Moreover, the amino acid found in black tea (theanin) acts as a neurotransmitter, improving memory and learning ability. However, Gardner et al concluded that the epidemiological evidence available is still not enough to draw the conclusion on the benefit of black tea on anticancer (Gardner *et al., 2007*). Although, it seem still not clear for black tea consumption on positive effect on cancer, there are huge studies on the mechanism process of black tea polyphenols especially, theaflavins, as an anti-cancer.

2.2.1 Anti-cancer of black tea through signal transduction pathways

Among the health benefit of black tea, anti-cancer and anti-inflammatory have been the topic of considerable interest. There are several possible mechanisms to explain the anticancer activities of black tea For instance, their potent antioxidant activity which is important in alleviating cancer-associated oxidative stress, their ability to mediate metabolizing or detoxification enzymes, and their potential to induce cell cycle arrest and apoptosis and one that is very important and widely studied is the cell signal pathway, referred to signal transduction, responsible for regulating cellular proliferation or apoptosis. This process is stimulating a cellular response by which information from an extracellular signal is transmitted from the plasma membrane into the cell following signal the intracellular molecule. Then a cell responds by stimulating acting gene transcription through proteins known as transcription factor. AP-1 and NF- κ B are two transcription factor known as extremely crucial in tumor promoter-induced transformation, and both of them are influence differentially by the MAP kinase pathway, respond to extracellular mitogens and various cellular activities, such as gene expression, differentation, and cell survival/apoptosis. Effect of black tea polyphenol on inhibition AP-1 and NF- κ B show in Figure 2 **Effect of tea components on; (a) AP-1activation induced by**.

2.2.1.1 Black tea and AP-1

A number of recent researches have been presented the data of black tea polyphenol induced cellular signal transduction pathway associated with AP-1, NF- κ B, and MAP kinase. There are three classes of MAPK family include the c-Jun N-terminal kinases (JNKs/SAPKs), so called stress activation protein kinase, p38, and the extracellular signal-regulated protein kinase (ERKs). In the JB6 mouse epidermal, theaflavin inhibited AP-1 transactivation and AP-1 DNA binding activity through JNKs/SAPKs inactivation but not ERKs pathway (Dong *et al.*, 1997). However, Chung et al found that theaflavin-3,3'-digallate inhibited ERK and p 38 kinase phosphorylation. They also indicated that theaflavin-3,3'-digallate decrease level of other AP-1 protin component, c-Jun and Fra-1 (Chung *et al.*, 1999). Chung et at also found that it has ability to decrease level Raf-1 protein which play important role in hematopoietic cell growth and activated by phosphorylation after mitogen or growth factor stimulation (Chung *et al.*, 2001). Black tea polyphenols are more effective than EGCG, green tea polyphenol, in inhibiting AP-1 binding activity (Chen *et al.*, 1999). Epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) strongly activate ERKs, so they are considered as initiator of tumor promoters. Compared with EGCG, theaflavin-3,3'-digallate is more effective in inhibiting EGF- and PDGF-induced phosphorylation of EGF receptor (Liang *et al.*, 1999).

2.2.1.2 Black tea and NF-кВ

The mechanism of NF- κ B activation is well understood. The inactivation form of NF- κ B, found in the cytosol, is bond to inhibitory kappa B (I κ B) protein. Activation of NF- κ B is started when I κ B is phosphorylated to release NF- κ B, and then translocated into nuclease, following transcription by binding to DNA sequence in specific gene. NF- κ B activation is associated with acceleration of tumorigenesis. Lin et al found that theaflavin-3,3'-digallate blocked the activation of NF- κ B, phosphoralation of I κ B, and reduced lipopolysacchride-induced nuclear accumulation of transcription factor NF- κ B (Lin *et al.*, 1999). Pan et al also indicated that theaflavin-3,3'-digallate was shown to inhibited IKK1, and IKK2, two putative I κ B α kinases involved in NF- κ B activation, and its effect was stronger than other tea polyphenol like EGCG (Pan *et al.*, 2000). Nitric oxide and the prostaglandin-synthesizing enzyme cyclooxygenase-2 (*COX-2*) play an important role in inflammation and in multiple stages of carcinogenesis and both of them are regulated by NF- κ B activation. Lin also showed that theaflavin-3,3'-digallate

inhibited nitric oxide generation, key role in carcinogenesis, and inducible nitric oxide synthase (iNOS) protein in lipopolysaccharide-activated macrophages more effectively than EGCG (Lin *et al.*, 1999).

Lu et al found that theaflavin-3-monogallate and theaflavin-3'-monogallate mixture at 50 μ m totally inhibited the serum-induced *Cox-2* gene expression at both mRNA and protein level (Lu *et* a., 2000).



Figure 2 Effect of tea components on; (a) AP-1activation induced by tumor promoters, including TPA, EGF, and UV irradiation, (b) NF-κB activation induced by tumor promoters, including TNF-α, LPS, and TPA. (Bode *et al.*, 2003)

2.3 Metabolism of black tea

The most abundant polyphenols in diet are not necessarily leading to the highest content of active metabolites in target tissue because the bioactive properties, strong antioxidative, anti-inflammatory and anti-cancerous ability, of polyphenols not only count on the amount consumed but their metabolite and bioavailability as well. When absorbed, most polyphenols are conjugated to glucoronide, sulphate, and methyl groups in the gut mucosa and inner tissue by enzymes in the intestinal mucosa and in the liver and the non-conjugated one are nearly absent in plasma. This mechanism facilitates excretion, influencing the restriction of their toxicity potential (Yang *et al.*, 1998; Feng., 2006; Rietveld *et al.*, 2003).

Many metabolism process including intestinal metabolism, microbial metabolism, hepatic metabolism and chemical degradation are involved in the fate of green tea, and to be responsible for its low availability in animals, and in humans. Tea flavonoids are found to be absorbed from the upper intestine. A few percent of tea flavanols are directly absorbed in the small intestine and excreted in the urine, and many of them were found to conjugate (Van *et al.*, 2006). Bacterial flora in the colon metabolizes flavonoids, not absorbed in the small intestine, or excreted into bile, and then microbial metabolized flavonoid are absorbed from colon (Kohri *et al.*, 2003; Meng *et al.*, 2002; Wang *et al.*, 2001). Although the plasma antioxidant activity potential of both green tea and black tea are not significant different, the uptake of theaflavin found in black tea is comparatively low compared with catechin and very small amount of theaflavin in plasma and urine were also reported (Mulder *et al.*, 2001; Rietveld *et al.*, 2003). Tons of researches have

been conducted green tea metabolism, biotransformation, and bioavailability of green tea, but there is few data for metabolism of black tea (Kohri *et al.*, 2003; Meng *et al.*, 2002; Mulder *et al.*, 2005; Lamber *et al.*, 2007; Vaidyanathan *et al.*, 2002; Vaidyanathan *et al.*, 2001; Vaidyanathan *et al.*, 2003; Lee *et al.*, 2002). The reason might be because it is hard to detect the black tea polyphenol like theflavins in plasma. **Figure 3** shows the major biotransformative pathways for the green tea catechins.

Daykin et al investigate metabolism of green and black tea after drinking using¹H nuclear magnetic resonance (NMR) spectroscopy. The resulted showed that hippuric acid was the major urinary black tea metabolite and they also identified unknown metabolite as 1,3-dihydroxyphenyl-2-O-sulfate (sulfate conjugate of pyrogalloyl) which was suspected that came from gallic acid (Daykin *et al.*, 2005). This result is consistent with Clifford's study that revealed that hippuric acid, the conjugate of benzoic acid with glycine were identified as the major metabolite of black tea using the same instrument and found this compound increase a 3-fold in the urine(Clifford *et al.*, 2000). The structure of tea polyphenol metabolites showed in **Figure 4**. Mulder *et al* reported that the major metabolite of both green tea and black tea was hippuric acid after they found this compound in urine (Mulder et al., 2005). Recently, van Dorsten *et al* confirm that hippuric acid and 1,3-dihydroxyphenyl-2-O-sulfate are major metabolites of both green tea and black tea and both compound found were the end products of flavonoid degradation by colonic bacteria (Van *et al.*, 2006).

Since the structure of thearubigins is still not well characterized, the bioavailability of thearubigins has not been studied.



Figure 3 Biotransformation of the green tea catechins. Abbreviations: 4'-MeEGC, 4'-*O*-methyl-(–)-epigallocatechin; 4',4"-di-*O*-methylEGCG, 4',4"-di-*O*-methyl-(–)-epigallocatechin-3-gallate; COMT, catechol-*O*-methyltransferase; EGC, (–)-epigallocatechin; EGCG, (–)-epigallocatechin-3-gallate; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; SULT, sulfotransferase; UGT, UDP-glucuronosyltransferase. (Lamber *et al.*, 2007)



Figure 4 Basic structure of the flavonoid (-)-epicatechin (1; epicatechin R1 and R2 = H; epicatechin gallate R1 = gallate and R2 = H; epigallocatechin R1 = H and R2 = OH; and epigallocatechin gallate R1 = gallate and R2 = OH), gallate (2), 1,3-dihydroxyphenyl-2-*O*-sulfate (3), and hippuric acid (4). (Daykin *et al.*, 2005)

2.4 Polyphenol protein interaction.

There are a number of studies indicating that polyphenols can interact non covalently or covalently with proteins as shown in Table 2. Both ways can lead to precipitation of proteins, via either multisite interaction (several phenolics bond to one protein molecule) or multidentate interactions (one phenolic bond to several protein sites or protein molecules). A number of amino acids that involve in the polyphenol protein interaction include proline, arginine, cysteine, methionine, tryptophan, phenylalanine, tyrosine, histidine, lysine as their structures shown in Figure 5. The type of the nature of protein-polyphenol complex formation depends both on the concentrations of protein and polyphenol and on their ratio. When the situation in which the number of polyphenol ends equals the number of protein binding sites, the largest network, and largest particles are formed as being explained by Siebert et al in Figure 6 (Siebert et al., 1996). In contrast, the small aggregate or protein dimer is formed when there are a large excess of either protein or polyphenols. With excess protein relative to polyphenol, each polyphenol molecule bridges between two protein molecules, so proteins can not further bridge to others. With the excess polyphenol relative to proteins, all of protein binding sites are occupied and free polyphenol have a small chance finding a free binding site (Prigent et al., 2003; Siebert et al., 1996). The complexation between polyphenols and proteins can be reversible or irreversible leading to soluble or insoluble complex (Luck et al., 1994)



Arginine





Figure 5 Amino acids involving in the phenolic compound protein interaction.

The non-covalent interactions including hydrogen bonds and hydrophobic forces are normally formed under non-oxidizing condition (Chen and Hagerman 2004, Hagerman *et al.*, 1998). Hydrogen, hydrophobic, and ionic bonding can be formed owing to the structure of polyphenols and many different functional groups in the proteins (Hagerman *et al.*, 1998; Sarni-Manchado *et al.*, 1999; Kroll *et al.*, 2000; Suryaprakash *et al.*, 2000; Rawel *et al.*, 2003, 108).



http://pubs.acs.org/isubscribe/journals/jafc

au/44/i01/figures/jf9502459f00002.html

Figure 6 Model for protein-polyphenol interaction. Polyphenol are depiced as having two ends that can bind to protein. Proteins are depicted as having a fixed number of polyphenol binding sites

2.4.1 Non-covalent interaction between protein and phenolic compounds

2.4.1.1 Hydrogen bonding

Hydrogen bond is important in the complex formation because of its strength. The isolated phenolic hydroxyl groups, excellent hydrogen bond donor, can form strong hydrogen bonds with the carbonyl functions of the amino acids or peptide backbone, (Hagerman *et al.*, 1998; de Freitas and Mateus., 2001a; Kroll *et al.*,2000). The interaction between polyphenol and protein is affected by the shape of polyphenol, projection and number of phenolic hydroxyl groups, addition of galloyl groups, position of peripheral groups imposed by stereochemistry of the pyranic ring of polyphenol molecule the nature of protein, and the medium that interaction take place (Kroll *et al.*, 2000; De Freitas and Mateus, 2001a; Rawel *et al.*, 2002a). The phenolic hydroxyl groups on both A and B rings (Figure 7) in the polyphenol molecule play an important role in protein-phenol complex formation (Kawamoto *et al.*, 1996; Simon *et al.*, 2003). Phenolic hydroxyl groups that are located close to other hydrogen bonding groups (1,2-dihydroxy and 1,2,3-trihydroxy groups) generally form hydrogen bonds with proteins.

The nature of protein but not the structure and flexibility of polyphenols has an effect on binding mechanism. The structure and flexibility of polyphenols have effect on

the energetic and stoichiometry of the interaction (Frazier *et al* 2003). The aromatic groups of polyphenols are supposed to be involved in a face to face stacking with amino acid residue of linear proteins, whereas the interaction with globular proteins such as BSA, probably involves only surface exposed residues (Carvalho *et al.*, 2004).

Polyphenols bind most strongly to proteins with high proline rich proteins. The enhanced ability of the proline rich proteins to interact with phenolic compounds is related to their flexible secondary structure and the greater extent of hydrogen bonding due to the increases accessibility of the peptide bond. In addition the carbonyl group of tertiary amides is a better hydrogen bond acceptor than the carbonyl group of primary or secondary amides (Loomis, 1974; Luck *et al.*, 1994; O'Connell and Fox, 2001; Wroblewski *et al.*, 2001). Poncet-Legrand et al showed that there was a relatively high affinity of galloylated flavanols toward poly(L-proline), and the coexistence of both enthalpy as well as entropy drove the flavan-3-ols and poly(L-proline) interaction (Poncet-Legrand *et al.*, 2007). In addition proline residues in proteins, arginin can form hydrogen bond donating guanido group situated at the terminus (Murray *et al.*, 1994).



Structure of major polyphenol in green tea ((-)-epigallocatechin gallate)

Figure 7 Structure showing the A, B, C, D ring of tea polyphenol (EGCG)

The smaller polyphenols can bind to proteins but they can not cross-link them to generate larger complexes. Larger polyphenols which are more aryl rings and more phenolic groups especially ortho groups are able to bind and precipitate proteins without any major conformational restriction (Baxter et al., 1997; de Freitas and Mateus, 2001). Protein structure has an influence to which pH it can form complexs with polyphenols through hydrogen bonding (Hagerman and Bulter, 1981; Naczk et al., 1996; Hagerman et al., 1998; de Freitas and Mateus, 2001b). Hagerman and Bulter have found that the proteinpolyphenol complex formation is usually strongest just below the isoelectric point of proteins where the protein-protein electrostatic repulsion is minimized (Hagerman and Bulter, 1981). Interaction between proteins and polyphenols can be influenced by various factors, such as the solution conditions including solvent composition, ionic strength, and pH, temperature, and time (Prigent et al., 2003). The enthalpy is the driven the exothermic interaction. Frazier et al found that there is the non-covalent epicatechin–BSA complex formed by hydrogen bonding, and an enthalpy driven exothermic interaction, indicated by the stability of the free energy (ΔG) and negative entropy (Frazier *et al.*, 2006)

2.4.1.2 Hydrophobic interactions

The non-covalent interactions between phenolic compounds and protein are created by hydrophobic association established by hydrogen bonding. (Prigent *et al.*, 2003) In hydrophobic bonding, the aliphatic (basic) and aromatic side chains (hydrophobic regions) of the protein amino acids and the aromatic nuclei of the
polyphenol would be possible stabilize the complexes formed. It has been proposed that the hydrophobic and hydrogen interaction can be predicted by the polarity of polyphenol. The hydrogen bonding is the dominant force for the more polar phenolic compound, while the hydrophobic forces dominate the reaction for the nonpolar polyphenolic compound (Kawamoto *et al.*, 1996; Siebert *et al.*, 1996; Baxter *et al.*, 1997; Lu and Bennick, 1998; Hagerman *et al.*, 1998; Wroblewski *et al.*, 2001).

Hydrophobic are more important for the stabilization of the complexes between proteins and polyphenols formed in which proline residues play a key role. Among the protein that bind polyphenols most strongly are member of the family of proline rich proteins. The pyrrolidine rings of proline residues are good hydrophobic binding sites because they posses an open flat and rigid, hydrophobic surface favorable for association with other flat, hydrophobic functions, such as aromatic rings in the polyphenols (Luck et al., 1994; Murray et al., 1994; Baxter et al., 1997; Charlton et al., 2002b; Simon et al., 2003). The hydrophobic interaction usually occurs between phenolic rings available for intermolecular interaction and the least sterically hindered amino acids including aromatic side chain of phenylalanine, tyrosine, the imidazole ring of histidine, and hydrophobic section of the side chain arginine and lysine (Murray et al., 1994; Baxter et al., 1997; Suryaprakash et al., 2000; Wroblewski et al., 2001; Charton et al., 2002b; Prigent et al., 2003). Larger polyphenols form complexes with the proline-rich peptide more effectively due to a greater number of phenolic rings, resulting in increasing in hydrophobicity (Sarni-Manchado et al., 1999; de Freitas and Mateus., 2001a; Baxter et al., 1997).

The regular increase in the number of aromatic and pyranic rings with the molecular weight of polyphenol (such as procyanidin) provides a multiplicity of sites of potentially hydrophobic nature to participate in interactions, presumably stabilized by hydrogen bonds from the o-dihydroxylphenol group (de Freitas and Mateus, 2001b). Especially the phenolic rings A and D (Figure 7) are important for face-to-face stacking with proline residues (Charlton *et al.*,2002b). Galloyl group of polyphenol was found to play important role with protein. He et al reported proved that galloyl groups of gallotannins were hydrophobic sites and can interact with aliphatic side chains of amino acids through hydrophobic association (He *et al.*, 2006).

2.4.2Covalent interaction between proteins and phenolic compounds

The covalent binding of phenolic compounds to proteins occurs through quinine, oxidized phenolic substance. Generally, phenolic substances may be readily oxidized in alkaline solution or in presence of polyphenol oxidase (Chen and Hagerman., 2004; Rawel *et al.*, 2002b). Quinone are formed when 1, 2-dihydroxy or 1,2,3-trihydroxy phenolic groups are oxidized (**Figure 8**) (Loomis, 1974). It is now generally accepted that two reaction steps are involved. The first step consists of the hydroxylation of monophenols into *o*-diphenols followed by oxidation of *o*-phenols into *o*-quinones (Rawel *et al* 2001).

Quinones represent a species of highly reactive substance that normally react further with other quinones (Kroll *et al.*, 2002). In addition, electrophilic quinone can bind covalently with nucleophiles such as amino or thiol groups of protein nucleophilic functional groups including lysine, methionine, histidine, cysteine, tyrosine, and tryptophan residues, limiting the digestibility of the protein molecules (Rawel et al., 2001a, O'Connell and Fox., 2001; Kroll et al., 2002; Chen and Hagerman., 2004). These result in deterioration of flavor, color, polyphenol bioactive activities, and nutritional quality of food (Rawel et al., 2001a; Reidl et al., 2001).. The protein quinone interaction can change the isoelectric point of protein to the lower pH values due to the introduction of carboxylic groups following the covalent attachment of phenolic acids, and by the parallel blocking of the lysine residues in protein (Rawel et al., 2001a; Rawel et al., 2002b).



Ortho - di-hyydroxy/tri-hidroxy

Ortho quinone derivative (o-quinone)

Figure 8 Quinone formation

Tuble 2 The Sta	ales on protein polypi			
STUDY	PROTEINS ¹	POLYPHENOLS ²	INTERACTION TYPE	
Sahoo et al 2008	BSA	Curcumin(IOC, DAC)	Hydrophobic, hydrogen bonding	
He et at, 2006	Histone, BSA Casein, Gelatin	Gallotannin	Hydrophobic, hydrogen bondong	
Frazier et al, 2006	BSA	EGCG	Hydrogen bonding	
Richard et al, 2005	Neurotensin	Reveratrol	Hydrophobic, hydrogen bondig	
Poncet-Legrand et al, 2007	Poly(L-proline)	Flavan-3-ols	Hydrophobic, hydrogenbonding	
Chen and Hagerman, 2004	BSA	PGG, PGG _{ox}	Hydrophobic, hydrogen and covalent bonding	
Kroll et al, 2000	Myoglobin	Chlorogenic, caffeic and quinic acids, <i>p</i> -quinone	Covalent binding	
Siebert et al, 1996	Different peptides	Tannicacid, catechin	Hydrophobic hydrogen bonding	
Hagerman et al, 1998	BSA	PGG, EC ₁₆ -C	Hydrophobic Hydrogen bonding	
Sarni et al, 1999	HSP	Tannins	Hydrophobic Hydrogen bonding	
Suryaprakash et al, 2000	Sunflower seed	Caffeic and quinic acid	Hydrophobic, hydrogen, Ionic bonding	
Rawel et al, 2003	WPI, β-Lg	Quercetin, rutin	Covalent binding	
Prigent et al, 2003	BSA, lysozyme, α-La	Chlorogenic acid	Non-covalent interactions	
Luck et al, 1994	Casein, gelatin, PRPs	PGG, 4GG, 3GG, tea polyphenol	Hydrophobic and Hydrogen bonding	
De-Freitas and Mateus, 2001ab	BSA, PRPs	Procyanidin	Hydrophobic and Hydrogen bonding	
Rawel et al, 2002a	SG, STI	Chlorogenic, caffeic and gallic acid, flavone, kaempferol, quercetin, myricetin	Covalent binding	
Simon et al, 2003	PRPs	B ₃ procyanidin	Hydrogen bonding	
Charlton et al,	PRPs and	EGCG, ECG, PGG,	Hydrophobic and	
2002 ab	peptides	3GG, 4GG	hydrogen bonding	
Frazier et al, 2003	BSA, gelation	Tannins	Hydrophobic interaction	
Wroblewski et al, 2001	HSTS	EGCG, PGG	Hydrophobic bonding	

Table 2 The studies on protein-polyphenol interactions

Baxter et al, 1997	PRPs	Tannins	Hydrophobic bonding		
Hagermanan and	Gelatin, PRPs,	Sorghum condensed	Hydrogen bonding		
Bulter, 1981	Polyamino acid	tannins			
⁻¹ BSA, bovine serum albumin; HSP, human salivary protein; WPI, whey protein isolate; β -Lg, β -lactoglobulin α -La, α -					

lactalbumin; PRPs, proline-rich proteins; SG, soy glycine; STI, soy trypsin inhibitor; HSTS, human histatin ² PGG, pentagalloylglucose; PGG ox, oxidized PGG; EC16-C, epicatechin $_{16}(4\rightarrow 8)$ catechin; 4GG, tetragalloylglucose; 3GG, trigalloylglucose; EGCG, epigallocatechin gallate; ECG, epicatechin gallate; IOC, isoxazolcurcumin; DAC, diacetylcurcumin;

2.5 Effect of tea polyphenols on proteins

2.5.1 Health benefit impact

Plant polyphenols are considered as safe natural compounds. The complex binding of polyphonols in particular tea polyphenol such as tannin, catechin, theaflavin to protein brings about both adverse and great positive factors to human health. As the beneficial side, tea polyphenol have been found to inhibit some cancer related protein that control DNA replication and transformation, and are well known to inhibit the large multi-catalytic protease (proteasome) and metaloproteionase responsible to tumor survival and metastasis. Besides, the scavenging free radical properties, one of the reasons why tea poly phenol can protect certain degenerative diseases might because they can bind to enzyme or protein involving in human disease generation.

Tachibana et al found that using surface plasma resonance, EGCG from green tea can bind to the cell surface of cancer known as the 67-kDa receptor (67LR) with predicted K_d Value of 39.9 nM. Expression level of the 67-kDa receptor protein was associated with risk of tumor invasion and metastasis. The result indicated that the cell growth inhibitory activity of EGCG correlated with the binding strength of EGCG to this receptor (Tachibana *et al.*, 2004). Green tea catechin particularly EGCG also have ability to inhibit bovine pancreatic ribonuclease A (RNase A), which are responsible for cleavage RNA in cell, inhibit protein systheses and consequently leading to human disease process. FTIR, circular dichroism (CD), and ultraviolet (UV) revealed that green tea polyphenol caused changing of RNase A secondary structure. The power of ribonucleolytic inhibitory effect depended on the association constant value for binding. Among green tea catechin, ECG, EGC, and EC, EGCG was found to form the most stable complex with RNase, and found to be the most potent ribonucleolytic inhibitory effect (Ghosh et al., 2004; Ghosh et al., 2007). Abe et at pointed that lowering of cholesterol as well as cancer prevention properties of green tea polyphenols were attributed to the ability to bind to squalene epoxidase (SE), a late-limiting enzyme of cholesterol biogenesis, consequently leading to potent inhibition enzyme activity (Abe et al., 2000). Bertoldi et al strongly support that green tea polyphenols had been shown to inactivated Dopa decarboxylase(DDC), key enzyme involved in the biosynthesis of biogenic amine, and associated with Parkinson's disease, through the covalent modification binding to DDC (Bertoldi et al., 2001). Recently, Bode et al proposed that since tea polyphenols have a high binding affinity to proline rich protein and many protein kinase in tumor promoter-induced pathway are proline-rich protein, the chemopreventive properties of tea polyphenol is an outcome of their ability to bind to protein kinase in signal transduction pathway (Bode *et al.*, 2002).

2.5.2 Adverse impact on human health

The ability of polyphenols to interact and form the insoluble complex with both macromolecule such as protein, enzyme, and essential mineral like Fe, Zn, and Cu have long been associated with reduction in nutritive value and their bioavailability (Hurrell *et*

al., 1982; Hurrell et al., 1984; Lazaro et al., 1995; Matheis et al., 1984; Hurrell et al., 1999; Temme et al., 2002; Rohn et al., 2001; Rohn et al., 2002a; Rohn et al., 2002b). The nutritional of food in particular macromolecules is regulated by the enzyme such as trypsin, chymotrypsin, pepsin, and α -amylase through the hydrolysis during digestion. Number of studies indicated plant phenolic compounds react with enzyme, influencing their physicochemical and structure properties following and generating the adverse effect on nutritive value of food. Rohn *et at* reported that plant polyphenols, caffeic acid, chlorogenic acid, ferulic acid, gallic acid, m-, o-, and p-dihydrobenzenes, quinic acid, and p-benziquinone had adverse effect on proteolytic digestion enzyme of food protein including, α -amylase, trypsin, and lysozyme, after forming complex in the reaction. The slower of hydrolysis of selected food protein and decrease in the affinity of these enzymes to the substrate indicate that both physicochemical ability and their activity of these enzymes are totally changed by phenolic conpounds (Rohn et al., 2001; Rohn et al., 2002a; Rohn et al., 2002b). The enzyme inhibition effect of these phenols is because of the covalent attachment of the phenolic to reactive nucleophilic sites in the enzymes.

Whereas tea polyphenols possess various beneficial properties such as reducing the risk of cancer and heart diseases, and acting as the natural antioxidant in food industry, they might inhibit certain enzyme especially biological digestive enzymes and reduce food digestibility, consequently leading to loss of food nutritive value. Rawel *et al* concluded that reaction of plant phenolic substances and digestive enzyme, lysozyme, influenced their physicochemical and enzymatic degradation. The reaction occurs at lysine side chains and at the indole ring of tryptophan residues of the lysozyme. (Rawel *et al.*, 2001).

Kim and Miller's study clearly showed that proline-rich proteins whether from salivary glands or diet, since it can bind to tea polyphenol, can protect against the inhibition of iron absorption (Kim et al., 2005). Honda, M reported that theaflavins from black tea and galloyl catechin from green tea showed potent inhibitory impact on salivary alpha-amylase, intestinal sucrase and maltase by *in vivo* experiment. After taking tea extracted polyphenol, the intestinal α -amylase activity decrease, and the blood glucose level was found to be suppressed (Honda., 1994). Hara et al investigated the inhibition effect of four types of green tea catechin, EC, EGC, ECG, and EGCG, and theflavin from black tea *in vitro* study. After the interaction, the result showed that EC and EGC did not have significant effect on the α -amylase activity, but theaflavin, TF3, TF2, and TF1 possessed more potent inhibitory effect on α -amylase activity than green tea catechin did (Hurrell *et al.*, 1982). It had been proved that green tea polyphenol were able to bind and precipitate protein, implying a potential ability of tea polyphenol to denature digestive enzyme (He et al., 2006). The inhibitory effect power of green tea polyphenols with concentration of 0.05 mg/ml on four typical digestive enzyme involving α -amylase, pepsin, trypsin, and lipase were reported, respectively, 61%, 32%, 38%, and 54%, indicating that green tea polyphenols owing antinutritional properties. (He et al., 2006). Huang and Zhao suggested that after induced by complexation with green tea polyphenols, trypsin in secondary structure was changed, increasing of α -helix content, decrease of random coil content, and unchanged β -sheet and β -turn content, measured by far-UV circular dichroism (CD) spectra. Besides the structure change, the biological activity of trypsin was found to be inhibited of 47 % of regular trypsin activity, at 0.75:1 ratio of tea polyphenol/Trypsin (Huang and Zhao., 2008).

2.6 Effect of proteins on tea polyphenols bioactivity

2.6.1 Effect of antioxidant activity of phenolic compound

The interaction with protein decreases the ability of phenolic compounds to quench the free radicals (Riedl and Hagerman, 2001). Wang and Goodman (1999) showed that phenolic compounds with no affinity to proteins might not be able to inhibit LDL oxidation since they are hard to be transferred to the location where oxidation occurs.

2.6.1.1 Effect of milk protein on tea polyphenol antioxidant activities

There are some evidences showing that tea polyphenols possess high binding affinity for proline rich proteins such as casein (Luck *et al.*, 1994). Firstly, Brown and Wright (1963) using electrophoretic methods investigating the interaction between milk proteins and tea polyphenols showed that tea polyphenols mainly interact with the α -casein complex and the β - casein of the milk. Jobstl *et al.* (2006) indicated that there is the non-covalent cross-linking of EGCG by casein, emphasizing the interaction of tea polyphenols with proteins. A recent study, Huang *et al.* (2007) uses Quartz Crystal Microbalance with Dissipation Monitoring to investigate the interaction between EGCG and globular protein, BSA (bovine serum albumin). This study has shown that there is aggregation of BSA through EGCG bridges and mainly from the galloyl group.

Black tea is commonly consumed in Western countries, while consumption of green tea is confined mainly to Asia and Middle East, where it is generally taken without the addition of milk. Adding milk to tea especially black tea is a tea drinking habits usually practices in UK. Milk is generally added to tea to reduce the astringency caused by flavonoid-like tannin substance, which then appear to precipitate in the tea cup. While some studies have demonstrated that milk protein have effect on antioxidant potential of tea polyphenols, others have shown that milk protein does not reduce the antioxidant activities of tea polyphenols as in Table 3. It has been reported that the interaction between tea polyphenols and protein such as milk protein affects tea polyphenols antioxidant potential in vitro (Art *et al.*, 2002, 2001a/b; Langley-Evans., 2000). The effect of black tea consumption with or without milk on the antioxidative status in vivo remains to be established unequivocally and has been the issue of much debate.

Starting 1996, Serafi *et al* using TRAP evaluated a total reduction in antioxidant capacity in blood of human volunteer who drank black tea with and without 25 % milk and found that consumption of tea with milk inhibited in vivo antioxidant activity because of the complexation of tea polyphenol by milk proteins (Serafini *et al.*, 1996). Later study in 1997, Hertog et al found that the consumption of tea was not associated with a lower mortality of ischemic heart disease even heavy tea drinker in a Welsh population in UK (Hertog *et al* 1997a,b). The data from this study is not following the 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. This study suggested that flavonoid binding capacity of milk proteins resulting in reduced in absorption of tea polyphenols from gastrointestinal tract.

On the other hand, in 1998, Van het Hof *et al* studied the effect of added 16 % milk to black tea polyphenols by assessing blood concentration of catechins and indicated

that addition of milk to black tea has no effect on the bioavailability of catechins as indicated no significantly difference after consumption of black tea with milk compared to black tea alone. However, the absorption of the main black tea polyphenols including theaflavins and thearubigins had not been studied (Van het Hof *et al.*, 1998). Two years later, Langley- Evan 2000 using the *in vitro* ferric reducing ability of plasma (FRAP) assay determined the impact of adding 7 % milk in black tea on tea antioxidant potential. The result indicated that the addition of milk to tea did reduce an antioxidant potential of black tea in vitro by as much as 28%. In the same year, Langley-Evan *et al* at also studied the in vivo by evaluating the contribution of black tea flavonoid to circulating antioxidant potential using FRAP assay. The data shown that the consumption of black tea without milk elevated plasma FRAP in subjects (ELangley-Evans., 2000 ab). At the same year, Leenen et al investigated the effect of both green and black teas in vivo study using the same method, FRAP assay. In contrast to the previous studies, the result shown that addition of 20 % milk did not affect the plasma antioxidant activity in human.

Besides antioxidant potential effect, Krull Cyrille *et al* (2001) determined the effect of 50 % milk on the antimutagenic properties and antioxidant capacity of green and black tea extract after passage through the in vitro gastrointestinal model. Their study indicated that the addition of milk had only a small inhibiting effect on the antimutagenic activities and the antioxidant capacities as indicated by TEAC assay was reduced to the same extent as the reduction of antimutagenic activities. They suggested that the milk protein-polyphenol complex become resistant to gastric hydrolysis so the amount of antimutagenic compound available for absorption was reduced (Krul *et al.*, 2001). Reddy *et al* 2005 assessed the effect of addition of 20 % milk to black tea on its ability to

modulate oxidative stress and antioxidant status using FRAP assay and found that although addition of milk may affect the absorption or bioavailability of tea catechins, it may not adversely affect the antioxidant capacity of black tea and its ability to prevent in vivo oxidation (Reddy *et al.*, 2005). Lorenz M *et al* 2006 investigated the effect of tea with and without 10% milk on endothelial function as sensitive parameter of vascular wall homeostasis as indicated by Flow-mediated dilation, representing an early marker of vascular dysfunction. They shown that black tea significantly improved endothelial function in human as indicated by increasing FMD whereas the addition of milk completely prevented this effect (Lorenz *et al.*, 2006).

Study	n	Design ¹	Tea ²	Dose	e	Milk ³	%	Parameter ⁴	Result
_				g	ml		milk		
Serafini, 1996	15	Р5	GT,BT	6	300	WM	25	TRAP	Inhibit effect
Hertog, 1997	-	FL	-	-		-	-	IHD incidence	-
Van het Hof,1998	12	СО	BT	3	600	SSM	10	Blood catechins	Not effect bioavailab ility
Langley- Evan SC, 2000(a)	-	Vitro	BT	3.5	200	WM, SSM, SM, BM	7	FRAP	Reduce FRAP
Langley- Evan SC,2000(b)	9	СО	BT	3.25	200	SSM	10	FRAP	Reduce FRAP
Leenen R, 2000	21	СО	GT, BT	2	300	WM	20	FRAP	Not reduce FRAP
Krul C, 2001	-	Vitro	GT, BT	10	300	WM, SSM, SM	50	TEAC AA	Reduce TEAC Reduce AA
Reddy VC, 2005	9	СО	BT	7	350	WM	20	FRAP TBARS	Not reduce FRAP& TBARS
Lorenz M, 2006	16	CO	BT	5	500	SM	10	FMD	Reduce FMD

Table 3 Studies of effect of milk on bioactive activity of tea polyphenol

¹ P#, parallel study, number per group; FL, follow up study; CO, Crossover study
² GT, green tea; BT, black tea
³ WM, whole milk; SSM, semi - skimmed milk; SM, skimmed milk; BM,

⁴ TRAP, total radical trapping antioxidant parameter; IHD, ischemic heart disease ; FRAP, ferric reducing antioxidant power; TEAC, trolox equivalent antioxidant capacity; AA, Antimutagenic activity; TBARS, thiobarbituric acid reactive substances;FMD, flow mediated dilation

2.7 Protein BSA

Bovine serum albumin (BSA), a protein with a molecular weight of 66300 Da, is separated form plasma using electrophoresis or ionic-exchange techniques. They are the most abundant protein found in animal blood plasma, and they have ability to bind various biological molecules involving hydrophobic, hydrophilic, cationic and anionic substances .They have been widely used as model protein to investigate and understand the protein-ligand interaction. BSA is composed of 607 amino acids and its sequence contains 17 disulfide bridges and one free cysteine in position 34. Their structures are homologous to HSA containing three homologous all- α domaims. The BSA content of glycine and isoleucine is lower than in average proteins. Since BSA is an albumin it is characterized by a low content of tryptophan and methionine, and a high content of cystine and charged amino acids such as aspartic, glutamic, lysine, and arginine, which are 7%, 10%, 10%, 4% respectively. BSA has two tryptophans (Trp 134 and Trp 213) which Trp 213 is located in hydrophobic fold while the additional tryptophan residue, Trp 134, has been proposed to be on the surface of molecules. BSA is considered as proline rich protein which composed of 28 proline residues, 5% (Giancola et al., 1997; Sahoo et al., 2008). Figure 9 show the amino acid sequence of BSA.

1 mkwvtfisll llfssaysrg vfrrdthkse iahrfkdlge ehfkglvlia fsqylqqcpf

61 dehvklvnel tefaktcvad eshagceksl htlfgdelck vaslretygd madccekqep 121 ernecflshk ddspdlpklk pdpntlcdef kadekkfwgk ylyeiarrhp yfyapellyy 181 ankyngvfqe ccqaedkgac llpkietmre kvltssarqr lrcasiqkfg eralkawsva 241 rlsqkfpkae fvevtklvtd ltkvhkecch gdllecaddr adlakyicdn qdtissklke 301 ccdkplleks hciaevekda ipenlpplta dfaedkdvck nyqeakdafl gsflyeysrr 361 hpeyavsvll rlakeyeatl eeccakddph acystvfdkl khlvdepqnl ikqncdqfek 421 lgeygfqnal ivrytrkvpq vstptlvevs rslgkvgtrc ctkpesermp ctedylslil 481 nrlcvlhekt pvsekvtkcc teslvnrrpc fsaltpdety vpkafdeklf tfhadictlp 541 dtekqikkqt alvellkhkp kateeqlktv menfvafvdk ccaaddkeac favegpklvv 601 stqtala

Pattern	Time found	Percentage
Ala(A)	47	8
Cys(C)	35	6
Asp(D)	40	7
Glu(E)	59	10
Phe(F)	30	5
Gly(G)	17	3
His(H)	17	3
Ile(I)	15	2
Lys(K)	60	10
Leu(L)	65	11
Met(M)	5	1
Asn(N)	14	2
Pro(P)	28	5
Gln(Q)	20	3
Arg(R)	26	4
Ser(S)	32	5
Thr(T)	35	6
Val(V)	38	6
Trp(W)	3	0
Tyr(Y)	21	3

Figure 9 Amino acid sequence of bovine serum albumin

2.8 Adsorption

Adsorption is a physical-chemical process of binding molecule to the surface. Whereas the molecule that bind to the surface are called the adsorbate, while the substance the support the adsorbate is generally called the adsorption. Since the adsorption is the first step in a reaction on a surface, it is the important step of overall reaction. There are two types of adsorption based on the nature of bonding between adsorbate and adsorbate. The direct chemical bond between the adsorbate and adsorbate or the surface is called chemisorption, while the process where no direct bond is referred to physisorption. In fact, instead of chemical bond, physisorption is governed by the physical force including van der Walls, ionic bonding, and polar interaction (Masel, 1996). Yoon *et al* reported that the major interactions involved in protein adsorption are hydrophobic, electrostatic interaction, and hydrogen bonding (Yoon *et a.*, 1997).

The adsorption process will continue until the thermodynamic equilibrium of the adsorbate concentration is reached. Measuring the amount adsorbed as a function of the partial pressure or concentration at certain temperature of adsorption equilibrium is refered to adsorption isotherm. The adsorption isotherm is used to predict the performance of adsorption system. There are several empirical adsorption isotherms such as lattice gas, multisite, tempkin fowler, but both Langmuir and freundlich isotherms have been widely used to explain the protein adsorption on the surface (Masel., 1996; Cussler., 1997).

2.8.1 Langmuir Isotherms

Langmuir isotherm model was created by Irving Langmuir in 1916 for gases adsorbed on solids (Langumiur, 1918). This simplest model is based on the four following assumption.

- 1. At the maximum adsorption, only a monolayer is formed
- 2. The surface of the adsorbent is uniform, that is, all the adsorption sites are equivalent.
- 3. Adsorbed molecules do not interact.
- 4. All adsorption occurs through the same mechanism.

This model is used when there is a strong specific interaction between the surface and the adsorbate so that a single adsorbed layer forms and no multilayer adsorption occurs. The driving force, which is the concentration in fluid times an area, which is the amount of the bare surface should be proportional to the rate of attachment to the surface.

Langmuir adsorption isotherm can be derived by the following mechanism at equilibrium

$$S + R \leftrightarrow SR$$

where S is the adsorbete solution, R is the adsorbent, SR represents the adsorbed state of adsorbete on the surface. The equilibrium constant of this reaction can be written as following

$$K = [SR] / [S] [R]$$

After mass balance is used to calculate the adsortion isotherm, the derivation linear form of Langmuir equation can be described by the following equation

$$M = \frac{M_m KC}{1 + KC} \tag{1}$$

$$\frac{C}{M} = \frac{1}{KM_m} + \frac{1}{M_m}C \tag{2}$$

where *C* is the equilibrium adsorbate concentration in solution (mol/L), *M* is the amount of adsorbate on the adsorbent (surface) (mol/g or mol/cm²), *K* is a direct measure for the intensity of the adsorption process, and $M_{\rm m}$ is maximum concentration of adsorbate, reflecting the adsorption capacity. Langmuir constants, K and M_m can be calculated from the slope and intercept of the plot

of
$$\frac{C}{M}$$
 versus $\frac{C}{M_m}$ from linear equation

2.8.2 Freundlish Isotherm

While the assumptions of the most popular adsorption model, the Langmuir mode, for a single solute system is that surface is homogeneous, and adsorption energy is constant over all sites, Freundlish model is the most crucial multisite adsorption isotherm for rough or heterogeneous surfaces. This model is an empirical equation based on the distribution of solute between the solid phase and aqueous phase at equilibrium. The basic Freundlich equation is :

$$Q_e = K_F C_e^{-1/n} \tag{3}$$

The equation can be rearranged into a linear form:

$$\ln q_e = \ln K_F + \underline{1} \ln C_e \tag{4}$$

where K_F is a constant for the Freundlich system, relating to bonding energy. K_F can be defined as the adsorption or distribution coefficient and represents the quality of the adsorbate on the surface for a unit equilibrium concentration (Ce = 1 mol/L). The slope 1/n, ranging between 0 and 1, is a mearure of adsorption intensity or surface heterogeneity. A larger value for 1=n indicates a larger change in effectiveness over different equilibrium concentrations. Also, when 1=n is >1.0, the change in adsorbed concentration is greater than the change in the solute concentration. For an X–Y plot of this empirical rquation, where X= ln q_e, and Y= ln C_e , K_F and 1/n can be determined from the intercept and slope.

2.9 Measurement of protein-polyphenol interactions

There are a number of techniques for the determination of interaction between protein and plant polyphenols. Most studies noemally use two and more method to prove the protein-polyphenol interaction. The principles of popular method always use is as the following;

2.9.1 Isothermal tritation calorimetry (ITC)

ITC is a thermodynamic technique, and is used for determine affinity for proteinprotein or protein-ligand. The principles are that the aliquot titrant whether protein or small molecule typically at concentration ≥ 0.5 mM, are injected into the cell containing protein solution typically at concentration 20 to 100µM. During titration, the amount of heat released, exothermic reaction or absorbed, endothermic reaction, is measured. Since this quantitative measurement directly allows accurate determination of binding constants (K_B), reaction stoichiometry (n), enthalpy (Δ H) and entropy (Δ S), a single experiment provides a complete thermodynamic profile of the molecular interaction. The major disadvantage of this technique is that it requires relatively high concentrations of samples (Heerklotz *et al.*, 1999; Frazier *et al.*, 2006).

2.9.2 Dynamic light scattering (DLS)

DLS is also known as Photon Correlation Spectroscopy. The experiment's theory, first discovered by microbiologist, Brown, is based essentially on the assumptions that the movement of small particles, 0.5 to 1.0 using a microscope at a magnification of 200 - 400X, are in Brownian motion (random walk). Compared with small particles, larger one, usually above approximately 250 nm diameters, moves more slowly under the same temperature. By shining a monochromatic light beam like a laser onto a solution containing particles in Brownian motion, moving particles are hit by the light, leading to a Doppler Shift. Since the molecules give a secondary source of light and subsequently scatter light in all direction, this will cause changing the wavelength of the incoming light, frequency shifts, the angular distribution, the polarization. The intensity of the scatter light is associated with the size, shape and

molecular interactions in the scattering material. This theory brings about two important measurements involving turbidimetric and nephelometric measurement. Whereas nephelometric method is detection of scattered light at an angle to the light incident like an angle of 90°, 70°, 75°, turbidimetric measurement is detection at 0°. Both assays are developed to measure the scattering of light by the assay solution rather than the changes in color, and they are similar in that the sample interacts with a specific reagent to form a suspension of particles in assay solution. The difference is that turbidimetric method measure the intensity of light transmitted through solution while nephelometric assay measure the amount of incident light scattered by the suspension of particles (Denis *et al.*, 1921; Kakmunee *et al.*, 2003)

2.9.3 Fluorescence spectra

Fluorescence is depended on the three-stage process that occurs in fluorophores or fluorescent dyes, molecule that absorbs energy and emission energy at a specific wavelength. These three stages involve excitation, excited-state lifetime, and fluorescence emission. When electron in an atom or molecules is exited from its electronic ground state to high energy level, it will absorb energy for example a photon. As the electron returns to its ground state, original energy level, due to reaching the lowest vibrational state of the excited electronic state, it will emit energy or photon absorbed as light and fluorescence process occurs. The emitted photons have different energies, leading to different frequencies. Using different wavelengths of excitation, the structure of molecule can be determined by measurement the different frequencies of fluorescent light emitted by a sample along with their relative intensities. The wavelength at which absorption at excitation and emission occur is specific to the certain molecule. Fluorescence is good technique for studying aromatic organic compounds because of containing the energy sharing, unpaired electron structure of the carbon ring in their structure (Hudson *et al.*, 2007; Hidrovo *et al.*, 2001).

2.9.4 Surface plasma resonance (SPR)

SPR was first identified early in the 20th century. This standard method is for measuring adsorption of material onto planar metal like gold and silver, surfaces or onto the surface of other metal nanoparticles. To investigate the measure the interaction, one of interactant molecule is immobilized onto the sensor surface and the analyte, binding partner, is injected in aqueous solution through the flow cell. The accumulation of protein on the surface, attributed to binding of analyte to immobilized ligand, cause an increase in the refractive index near (within ~ 300 nm) a sensor surface. A polarised laser light is directed through a medium with high refractive index to a thin layer of gold with low refractive index. The surface plasmons, then, are generated at the surface of the gold layer. Absorbing the light is visible as a decrease in the intensity of reflected light. This change in refractive index is measured in real time monitoring, and the result plotted as response or resonance units (RUs) versus time (a sensorgram). This analysis is used for biomolecular interaction, interactions between DNA - DNA, DNA - protein, lipid protein and hybrid systems of biomolecules (Besenicar et al., 2006; Wikstrom et al., 2007).

2.9.5 Circular dichroism (CD)

Circular dichroism (CD) spectroscopy is widely used for characterize all type and size of the chiral molecules and large biological molecules, but a primary use is for analysing the secondary structure or conformation of macromolecules, particularly proteins. Circular dichroism can be used to observe how secondary structure changes with environmental conditions or on interaction with other molecules since the secondary structure of protein is sensitive to its environment like temperature or pH. It is considered as a excellent technique for determining the secondary structure of protein.

The principles is that the beams of light and left circularly polarized light travel with different speeds through L-amino acids in protein in particular the amides of the polypeptide backbone of proteins , leading to rotating the polarized light. It is the technique that measures the difference or unequal in absorbance of right- and left-circularly polarized light. The secondary structure can be determined by CD spectroscopy in the "far-uv" spectral region (190-250 nm) and tertiary structure of a protein are sensitive to the "near-uv" spectral region (250-350 nm). The a-helical proteins have negative bands at 222 nm and 208 nm and a positive band at 193 nm while the antiparallel b-pleated sheets have negative bands at 218 nm and positive bands at 195 nm (Creighton *et al.*, 2002; Greenfield., 2006; Bulheller *et al.*, 2007)

2.9.6 Matrix-assisted laser desorption/ionization (MALDI)-TOF(time-of-flight mass spectrometer)-MS

MALDI-TOF MS was introduced by Karas and Hillenkamp in 1988. This soft ionization method was established on the purpose of high molecular involatile biomolecule determination, even a mass range above 100,000 Da. It is considered as the fast and simple technique for detecting molecular ions and requires a very little sample. The sample is not necessary to be in form of solution. The matrix composed of crystallized molecules, normally 3,5-dimethoxy-4-hydroxycinnamic acid, α -cyano-4-hydroxycinamic acid, 2,5-dihydroxybenzoic acid (DHB), mixed with sample(analyte) such as protein. The excitation of the matrix by pulsed laser light results in the desorption and ionization of intact molecular ions. The matrix transfer part of its charge to the sample, consequently not only ionizing them but protecting them from the disruptive energy of the laser. The charged species are then propelled into the flight tube where they are separated on the basis of mass (Zhu *et al.*, 2007; Griffin *et al.*, 1999; Dopke *et al.*, 1998)

2.9.7 Nuclear magnetic resonance (NMR)

This technique is a powerful tool for determination molecular structure including relative configuration, relative and absolute concentrations, and intermolecular interactions of organic compound without the destruction of sample. NMR theory occur when the molecule is placed in static magnetic field, the some neclei in atom exist in discrete nuclear spin states, and Nuclear magnetic resonance spectroscopy detects the energy difference between the two spin states (the transitions between these spin states). This small energy difference (ΔE), depending on the magnetic field strength and the specific nucleus being studied, is usually given as a frequency in units of MHz (Wade., 1999)

2.10 Immobilization of protein on gold surface

Protein can be physically and chemically attached on the gold surface. Physical linking process includes hydrophobic or electrostatic interactions between the protein and the surface. However, the adsorption can be reversible, causing removing protein out of the surface by certain buffers or detergents. On the other hand, chemically adsorption process involves the covalent bonding of protein to the surface. The resultant irreversible binding has produced high level of surface coverage, make this method is more popular (William et al., 1998). For covalent immobilization of proteins on self-assembled monolayer (SAMs), the procedure involves the formation of either a disulphide or an amide. One method for amide bond formation utilizes the production or termination of SAMs with an N-hydroxysuccinimide (NHS) ester (Parker et al., 1996, 1995). Side chain amino groups of lysine residues on the protein surface displace the terminal HNS group, resulting in covalent immobilization of protein. Frey et al have shown that a monolayer of poly(L-lysine) is attached covalently via amide bonds to an alkanethiol self assembled monolayer (SAM) on gold surface. The amide bond is formed in two steps. First, the terminal carboxylate groups of an 11-mercaptoundecanoic acid (MUA) SAM are activated to the N-hydroxysulfosuccinimide (NHSS) ester followed by the reaction this MUA-NHSS ester monolayer with the amino groups of PL to create multiple amide bond linkages to the surface as shown in Figure 10(Frey and Corn., 1996). A relatively high pH was used to deprotonate the lysine residues on the surface to enable reaction with NHS eater (William et al., 1998).



Figure 10 Reaction scheme for forming amide bonds with a self-assembled monolayer of 11-mercaptoundecanoic acid (MUA) on a gold surface.

2.11 Quartz crystal microbalance with dissipation (QCM-D)

Quartz crystal microbalance (QCM), a piezoelectric-based biosensor, is an extremely sensitive mass sensor capable of measuring the mass changes of a material at the level of 0.4 ng/cm². The detection signals and calculated adsorbed mass in the conventional QCM are solely based on the assumption that the changes of the fundamental frequency of piezoelectric is proportional to the change of adsorbed mass on the surface of quart crystal calculated from the classical Sauerbrey equation (Sauerbrey., 1959). This linesr relationship between frequency change (Δf) and mass adsorbed (Δm) is given by:

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

However, it is now known that the changes of the fundamental frequencies of quartz crystals depend not only on the masses adsorbed on the quartz crystal surfaces, but also others factors, such as temperature and the viscoelastic properties of the adlayers (Carrigan *et al.*, 2005).

In many systems involving biomolecules, the adsorbed film is not rigid, making the Sauerbrey relation invalid. A film that is "soft" (viscoelastic) will not fully couple to the oscillation of the crystal, which dampens the crystal's oscillation. The conventional QCM has limits for precise mass detection in liquid environment as the mass obtained is very often overestimated (Janshoff *et a*l., 1997; Muramatsu *et al.*, 1987). The improved QCM with dissipation monitoring, also called QCM-D, has shown to provide a better tool to investigate viscoelastic soft materials since it also takes into account the energy dissipation factor. Previously, conventional QCM was used to monitor the binding kinetics between bovine serum albumin and tannic acid (Liu *et al.*, 2006). Very recently, we employed QCM-D to study the interactions between (–)-epigallocatechin gallate (EGCG) and bovine serum albumin (BSA) surface (Huang *et al.*, 2007). Our results showed that the EGCG adsorption on BSA surface was dominated by nonspecific hydrophobic interaction.

2.12 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectroscopy gives the information of protein conformation in the secondary structure. It is a technique that measures the infrared intensity versus wavelength (wavenumber) of light. By shining infrared radiation on sample protein, polypeptide backbone absorbs light and cause the vibrational spectra band at different

frequencies depended on wavelengths of radiation in the infrared region of the spectrum are absorbed by the sample. The amide I, II, and III are the most noticeable, easily measured, and are sensitive to conformational of backbone that links the amino acids. Amide I band represent stretching vibration of the C=O bond while amide II band provides primarily to bending vibrations of the N-H bond. Amide I band share with inplane NH bending and C-N stretching, so the frequency of this vibration depends on the hydrogen bonding between the C=O and NH which varies for the different secondary structure of polypeptides. Since amide III band is relatively weak and is affected by other vibration, most studies have focused on amide I and II band (Creighton., 2002; Ng *et al.*, 2002; Ge *et al.*, 2006).

FTIR is the technique capable of quantitative and qualitative analysis of chemical characteristics on the surfaces. FTIR spectra were collected with a FTIR spectrometer (Thermo Nicolet 670, Medison, WI), using a pure gold or Si wafer as the background, depending upon the substrate used. A thermo Nocolet Attenuated Total Reflectance (ATR) accessory, or Smart Apertured Grazing Angle (SAGA) accessory with a grazing angle of 80° was used to collect reflection absorption IR spectra. The resolution was set to 4 cm⁻¹, and 256 scans.



Figure 11 The principle of FTIR

3. MATERIALS and METHODS

3.1 Materials

Bovine serum albumin (BSA, \geq 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 (NH₄OH) (VWR, West Chester, PA), hydrogen peroxide (H₂O₂) (Aldrich), and absolute ethanol (Fisher) were all used as received. Sodium phosphate monobasic, and sodium phosphate dibasic were obtained from Sigma-Aldrich Co., MO. Milli-Q water was used throughout the study for preparation of aqueous solution, otherwise stated. AT-cut quartz crystal coated with gold (fundamental frequency of 5 MHz) was obtained from Q-Sense AB (Sweden).

3.2 Preparation of thearubigins

5 g black tea water soluble powder (a gift from Unilever Company) was dissolved in 200 mL water. It was extracted with 200 mL ethyl acetate for three times. The water layer was then extracted with 200 mL butanol for three times. The combined butanol extract was dried using a rotary evaporate under vacuum. 1.2 g of thearubigins was obtained. The extracted thearubigin was then dissolved in 0.01 M acetate buffer at pH 4.9 to prepare thearubigin solutions with concentration of 0.002%, 0.005%, 0.02%, 0.032%, 0.08%, 0.15%, and 0.30% respectively

3.3 Preparation of theflavins

Theaflavins were extracted form black tea extract by Ethyl acetate and were vacuum evaporated to remove solvent. The fraction of ethyl acetate then was subjected to a Sephadex LH-20 column and eluted with acetone solution (40%; v/v). According to their elution sequences, three fractions were collected. They are TF1, TF2 and TF3. The TF2s was a mixture of theaflavin-3-monogallate (TF2) and theaflavin-3'-monogallate (TF2'). They cannot be isolated to pure compounds under LH-20 chromatography. Their composition ratio for TF2: TF2' was 3:1 after quantified by HPLC method (Huang *et al.*, 2006). Molecular weight of TF1, and TF3 are 564, 868.70, and 458.30 g/mol. The extracted theaflavins (TF1) was then dissolved in 0.01 M acetate buffer at pH 4.9 at different concentration of 0.02%, 0.032%, 0.05%, 0.065%, and 0.08% respectively. The extracted theaflavin 3,3'- digallate (TF3) was also prepared at the same condition.

3.4 Preparation of BSA Surface

The linkage of BSA protein onto the gold-coated crystal was produced using the procedure adopted from elsewhere (Huang *et al.*, 2007). In brief, 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 (NH₄OH, 25%), hydrogen peroxide (H₂O₂, 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 crystals were then rinsed with a large quantity of Milli-Q water and dried with nitrogen gas (N₂), and subsequently soaked in 10 mM 11-mercaptoundecanoic acid solution (11-MUA) in absolute ethanol 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-ethyl-3-(3dimethylaminopropyl)carbodiimide hydrochloride (EDC) and 100 mg/mL Nhydroxysuccinimide (NHS) in Milli-Q water for 40 mins. The solution of 10 mg/ml BSA in phosphate buffer (pH 7.4) was used to incubate the activated surfaces at 4 °C for at least 24 hours. The quartz crystal surfaces were finally rinsed out with phosphate buffer followed by Milli-Q water, and dried under N₂ flow.

3.5 QCM-D Measurements

The interactions between black tea polyphenols and BSA chemically-linked quartz crystal surface were studied using a commercial QCM-D apparatus (Q-Sense AB, Sweden) with a Q-Sense D300 electronic unit, while QSoft 301 program was used in data collection. All QCM-D measurements were performed at 25 ± 0.02 °C and temperature is controlled through an interior petelier element. A 5 ml polypropylene pipette tip connecting to the temperature-controlled chamber as a sample reservoir was initially filled with studied buffer. By opening the control valve, buffers were exchanged in the QCM-D chamber via the gravitational flow. The setup of the device is shown in Figure 3.1. After a stable baseline was established at least 10 mins, polyphenol solutions in buffer were exposed to BSA-modified crystal surface. At the same time, the adsorption was monitored as a function of time by recording the shifts in the frequency (ΔF) and in the 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. Changes in frequency and energy dissipation were simultaneously obtained at the 3rd, 5th and 7th overtone, corresponding to the fundamental frequency of the quartz crystal at 15, 25, and 35 MHz, respectively. The long-term stability of the frequency was within 1 Hz, and this drift was negligible compared with the frequency shifts due to the adsorption.

Normalized data obtained from different overtones were used in the calculation of mass load, thickness, and shear viscosity of adsorbed layers using Voigt model.

Sauerbrey mass was calculated from Sauerbrey equation (Sauerbrey, 1959).

$$M = -\frac{C}{n}\Delta F \tag{5}$$

 ΔF , *M*, and *n* represents the frequency change, adsorbed mass per unit area, and overtone number, respectively. *C* is the mass sensitivity constant (17.7 ng/cm²Hz). The Q-Sense software (QTools) 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 equation:

$$D = \frac{1}{\pi f_0 \tau_0} = \frac{2}{\omega \tau_0} \tag{6}$$

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

The Sauerbrey equation is in fact derived from uniform ultrathin rigid films with material properties indistinguishable from those of the quartz crystal. For an ideal rigid film, the ratios of the frequency shift with the overtone number ($\Delta F_n/n$) at different

overtones should be overlapped with each other ($\Delta F_3/3 = \Delta F_5/5 = \Delta F_7/7$). However, a film that is "soft" (viscoelastic) will not fully couple to the oscillation of the quartz crystal, which dampens the crystal's oscillation. Therefore, the Sauerbrey equation will no longer be valid in the calculation of the adsorbed mass and thickness. Viscoelastic properties changes in the adsorbed materials on the electrode surface would create additional frequency shifts besides the ones due to the mass load on the electrode surface (27). The decreases in ΔF and the increases in ΔD indicate the mass increase of the adsorbed black tea polyphenol adlayers and the formation of loose polyphenol adlayers on BSA surface, respectively.

By taking into account the viscoelastic properties of the system, Voigt model can allow a more accurate estimation of mass changes using QCM-D responses (28). In this model, ΔF and ΔD of an adsorbed layer can be concisely expressed using the following equations:

$$\Delta F \approx -\frac{1}{2\pi\rho_0 h_0} \left\{ \frac{\eta_3}{\delta_3} + h_1 \rho_1 \omega - 2h_1 \left(\frac{\eta_3}{\delta_3} \right)^2 \frac{\eta_1 \omega^2}{\mu_1^2 + \omega^2 \eta_1^2} \right\}$$
(7)

$$\Delta D \approx \frac{1}{\pi f \rho_0 h_0} \left\{ \frac{\eta_3}{\delta_3} + 2h_1 \left(\frac{\eta_3}{\delta_3} \right)^2 \frac{\eta_1 \omega^2}{\mu_1^2 + \omega^2 \eta_1^2} \right\}$$
(8)

where ρ_0 and h_0 are the density and thickness of the crystal, respectively. η_3 is the viscosity of the bulk fluid, $\delta_3 [= (2\eta_3/\rho_3\omega)^{1/2}]$ is the viscous penetration depth of the shear wave in the bulk fluid, ρ_3 is the density of the bulk fluid, and ω is the angular frequency of the oscillation. Here, four unknown parameters of the adsorbed layer including the thickness, density, viscosity, and elastic shear modulus, are represented by

 h_1 , ρ_1 , η_1 , and μ_1 , respectively. Because the adsorbed layer exhibit different penetration depth of harmonic acoustic frequencies, ΔF and ΔD are measured simultaneously at the fundamental resonant frequency along with the third, fifth, and seventh overtones. As a result, up to eight experimental values of ΔF and ΔD are available. The Q-Sense software, which is on the basis of Voigt model, has been used to model the responses at third, fifth, and seventh overtones during the process of black tea polyphenol adsorption on BSA surface, and provide the mass and thickness of the adsorbed polyphenol adlayer on BSA surface.



Figure 12 Illustration of the setup for QCM-D experiment.
3.6 FTIR measurement

Infrared spectrum of black tea polyphenol powder were measured using Attenuated Total Reflectance Fourier Transform Infrared (ATR-FT-IR) spectrometer, while the infrared spectra of BSA-modified quartz crystal surfaces before and after black tea polyphenol 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.

4. RESULTS and DISCUSSION

4.1 Binding of thearubigin to BSA surface

Many studies show that the non-covalent bonding plays a key role in the protein polyphenol interaction (Kawamoto *et al.*, 1996; Simon *et al.*, 2003; Hagerman *et al.*, 1998; de Freitas and Mateus., 2001a; Kroll *et al.*, 2000; Luck *et al.*, 1994; Murray *et al.*, 1994; Baxter et al., 1997; Charlton *et al.*, 2002b; Simon *et al.*, 2003). Therefore, temperature, pH, and ionic strength known to influence the non-covalent interaction, should have significant impact on the protein polyphenol interaction.

4.1.1. Effect of thearubigin concentration.

The typical adsorption process of thearubigin on bovine serum albumin (BSA) surface was monitored in real time by simultaneously measuring resonance frequency shifts (ΔF) and energy dissipation shifts (ΔD). Figure 13 displays the typical time-resolved resonance frequency shifts (ΔF) and energy dissipation shifts (ΔD) for the fifth overtone upon the addition of 0.08% thearubigins onto bovine serum albumin (BSA) surfaces at acetate buffer pH 4.9, which is close to the isoelectric point (pI) of BSA protein. Right after the injection of each thearubigin solution, there was often a rapid decrease in ΔF and a marked increase in ΔD , followed by much gradual changes of ΔF and ΔD until steady state were reached. These changes in ΔF and ΔD indicate the adsorption of thearubigins on BSA surface. Figure 14a and 14b show ΔF and ΔD changes as a function of time upon the addition of 0.032% thearubigin in pH 3.0 and 0.01 M acetate buffer solution. The ΔF obtained at three different overtones (n=3, 5, 7) were normalized by their overtone number. The arrows indicated the injection time of

thearubigin solution (t_1) , and several times of rinsing with buffer solutions (t_2, t_3) respectively.

The adsorption of thearubigin on BSA surface consists of reversible and irreversible adsorption processes. We use the data that were obtained after two times of rinsing to explain the reversible adsorption process. During the rinsing processes (t_2 , t_3), small amount of adsorbed thearubigin mass decreased, as indicated by the small increase in ΔF . At the same time, ΔD decreased slightly, suggesting that the thearubigin molecules were loosely adsorbed on BSA surface, and the loss of small amount of thearubigin adlayer more rigid. Although the reversible thearubigin adlayer can be washed out by the buffer rinsing processes, the irreversible adsorption may be the main driving force that causes the loss of antioxidant activities of thearubigin. The irreversible adsorption of thearubigin on BSA surface is mainly caused by specific interactions, such as hydrogen bonding and electrostatic interaction, and will be discussed in detail in the following sections of this paper.



Figure 13 Time-dependent frequency shifts and energy dissipation shifts for thearubigin adsorption on BSA-modified quartz crystal surface at 0.08% thearubigin in buffer 4.9: a;frequency change, b; dissipation change



Figure 14 (a) Frequency shift (ΔF) and (b) energy dissipation shift (ΔD) induced by the adsorption of 0.032% thearubigin acetate buffer solution at pH = 3.0 and I = 0.01 M on bovine serum albumin –coated quartz crystal surface. ΔF and ΔD are measured simultaneously at three overtones (n = 3, 5, 7) and normalized by their overtone number. The arrows indicate the time for the injection of thearubigin molecules (t₁) and two times of rinsing steps (t₂ and t₃).



Figure 15 Time-dependent frequency shifts (ΔF) for thearubigin adsorption on BSA-modified quartz crystal surfaces for thearubigin solutions of various concentrations: (a) 0.005%; (b) 0.02%; (c) 0.032%; (d) 0.08%; and (e) 0.15%. The thearubigin solutions were prepared in 0.01M acetate buffer at pH = 4.9.

Figure 15 displays the time-resolved frequency shifts (ΔF) for the fifth overtone upon the addition of thearubigin solutions of different concentrations (ranging from 0.005% to 0.15%) onto BSA surface at 0.01M and pH 4.9 acetate buffer solution. With the increase of thearubigin concentration, ΔF increases, suggesting the increase of adsorbed mass on the basis of the Sauerbrey equation. To obtain a more precise picture of thearubigin adsorption behaviors, the thearubigin adsorbed mass and the thickness of thearubigin adlayer on the basis of Voigt model were calculated and shown in Figure 16. Increasing thearubigin concentration from 0.002% to 0.300% leads to the increases in both thickness (i.e., from 2.1 nm to 11.3 nm) and mass (i.e., from 134.7 to 711.1 ng/cm²) of the adsorbed thearubigin adlayer.



Figure 16 Changes of mass and thickness of thearubigin adlayer on BSA surfaces at various thearubigin concentrations:(a) 0.002%; (b) 0.005%; (c) 0.02%; (d) 0.032%; (e) 0.08%; (f) 0.15%; and (g) 0.30%. The thearubigin solutions were prepared in 0.01M acetate buffer at pH = 4.9.

The equilibrium of adsorption is a fundamental property of the solute-surface interaction because the adsorption process will continue until a thermodynamic equilibrium of the solute concentration is reached.

The equilibrium at a given temperature is usually presented with an isotherm, which is the plot of the mass of the adsorbed solute versus the solute concentration. The adsorption isotherm is useful in predicting the performance of an adsorption system. Either Langmuir isotherm or Freundlich isotherm may be used to describe the adsorption isotherm of thearubigin onto BSA surface, which can be determined from the changes of the adsorbed mass of thearubigin against thearubigin concentration. Langmuir model assumes that (1) the adsorbed layer should be monolayer; (2) there are finite numbers of identical adsorption sites on the surface; and (3) the adsorption ability of a solute to each of these sites is independent of the occupation of neighboring sites (Langmuir., 1918). This model is useful when there are strong specific interactions between the surface and the adsorbate. The driving force for the adsorption is the concentration of the solute, and the Langmuir isotherm can be described by the following equations (Langmuir., 1918).

$$M = \frac{M_m KC}{1 + KC} \tag{9}$$

$$\frac{C}{M} = \frac{1}{KM_m} + \frac{1}{M_m}C \tag{10}$$

where *C* is the concentration of adsorbate solution, *M* is the amount of adsorbate on the adsorbent, *K* is a direct measure of the intensity of the adsorption process, and $M_{\rm m}$ is a constant related to the area occupied by a monolayer of adsorbate, reflecting the adsorption capacity.

The experimental data of thearubigin adsorption on BSA surface give a satisfactory fit to the Langmuir model with a correlation coefficient of 0.9735, as shown in **Figure 17a**, indicating that the Langmuir model can be used to describe the thearubigin adsorption on BSA surface. M_m, which indicates the absorption capacity, and K, which reflects the intensity of adsorption process, can be estimated from the slope and intercept of the plot of $\frac{C}{M}$ versus $\frac{C}{M_m}$, and are equal to (690 ± 51) ng/cm² and 38.0 ±

5.6 respectively.

For comparison, we also fit the thearubigin adsorption isotherm with the Freundlich model, which can be described as

$$M = K_f C^{1/n} \tag{11}$$

The results of K_f and *n* estimated from the fitting to eq. (7) are 975 ± 97 and 3.23 ± 0.42 respectively (**Table 4**). The fit to experimental data using the Freundlich model is less satisfactory compared with the Langmuir model, as shown in **Figure 17b**.

The Freundlich model is an empirically model, and predicts infinitely adsorption at infinite concentration. At low thearubigin concentration, although the distribution of thearubigin on the BSA may not be homogeneous due to the broad molecular weight dispersity of thearubigin which ranges from 1,000 to 40,000 Daltons, the better fit of thearubigin adsorption isotherm using the Langmuir model than the Freundlich model may indicate that the thearubigin adsorption onto the BSA surface is mainly governed by specific and strong interactions, and the Langmuir adsorption process may be less disturbed by the heterogeneity of binding of thearubigin on BSA surface.

Thearubigin is a polar molecule containing a large amount of hydroxyl groups as well as some carboxyl groups, which can form specific and strong interactions with BSA molecules through both hydrogen bonding and electrostatic interaction. However, at higher thearubigin concentration, large molecules may occupy more than one adsorption site than small ones. Two competing effects, the heterogeneity of the thearubigin molecules bound on BSA surface and the specific interactions between thearubigin and BSA, may cause good fits of the data by the Freundlich model at higher thearubigin concentration.



Figure 17 The adsorption isotherm of thearubigin onto BSA surface fitted to the Langmuir model (top) and the Freundlich model (bottom).

To prove the existence of hydrogen bonding between the adsorbed thearubigin molecules and BSA surfaces, FTIR spectra of BSA surfaces with and without the thearubigin adsorption were collected, as shown in **Figure 18.** The FTIR spectrum of pure BSA surface displays two characteristic bands at 1666 cm⁻¹ and 1546 cm⁻¹. The 1666 cm⁻¹ (amide-I) band arises predominantly from the protein amide C=O stretching vibrations, and the 1546 cm⁻¹ (amide-II) band is due to the amide N-H bending vibrations and C-N stretching vibrations (Huang *et al.*, 2007). The positions of amide bands I and II in thearubigin adsorbed BSA surface show a remarkable shift from 1667.3 cm⁻¹ to 1561.3

cm⁻¹ ($\Delta v = 106.0 \ cm^{-1}$) and from 1536.0 to 1445.2 cm⁻¹ ($\Delta v = 90.8 \ cm^{-1}$) respectively after thearubigin adsorption. The significant peak position shifts observed in the amide I and amide II bands may be attributed to hydrogen bonding between thearubigin and BSA molecules, suggesting that the hydrogen bonding may occur between the phenolic hydroxyl groups in thearubigin and the functional groups (i.e. amide groups) of the BSA.



Figure 18 FTIR spectra of pure BSA surface (the dash line) and BSA surface with adsorbed thearubigin molecules (the solid line).

4.1.2 Effect of pH

To investigate the possible role of electrostatic interaction involved in the binding between thearubigin and BSA, in addition to the experiments performed at pH 4.9, the experiment also conducted QCM-D measurements at pH values of 7.0 and 3.0, where BSA carries negative and positive charges, respectively. The mass and thickness of the adsorbed thearubigin adlayer on BSA surface at pH 7.0, 4.9, and 3.0 are compared in **Figure 19**. The significantly higher adsorbed thearubigin and thickness at pH3 suggests the contribution from electrostatic interaction between BSA surface and thearubigin. The protein molecules near their pIs, whose total net charges are nearly zero and their intermolecular electrostatic repulsion forces are minimized, usually assume a more compact globular conformation. The significantly higher or lower adsorbed thearubigin mass and thickness at pH 3 or pH 7 suggest that the electrostatic interaction between BSA surface charges and charges existing in thearubigin play a significant role in their interactions. At pH 3, the maximum adsorbed thearubigin mass and thickness indicate the existence of strong electrostatic attraction between positively charged BSA surface and negatively charged thearubigin mass and thickness at pH 7 may arise from the electrostatic repulsion between negatively charged protein surface and negatively charged thearubigin molecules. One notes from the possible dimeric structure of thearubigin molecules shown in **Figure 1** that thearubigin molecules carry negatively charged carboxyl group (Haslam., 2003).



Figure 19 Changes of mass and thickness of 0.032 % thearubigin adlayer on BSA surfaces at various pH values

4.1.3 Effect of Salt Concentration

To further understand the nature of interactions between thearubigin and BSA, we also study the effects of salt concentration on the binding of thearubigin molecules on BSA surfaces.

Figure 20 displays the changes of adsorbed mass and thickness of thearubigin adlayer on BSA surfaces as a function of salt concentration. At pH 4.9, when the acetate buffer concentration increases from 0.001 M to 0.100 M, both adsorbed mass and thickness increase. On the other hand, as the salt concentration increases above 0.1 M, both adsorbed mass and thickness decrease. These results indicate that salt concentration has complex effects on the electrostatic interaction between protein BSA and thearubigins. Similar salt concentration effects were also observed in the electrostatic interaction between some proteins and certain polyelectrolytes (Hattori *et al.*, 2002; Seyrek *et al.*, 2003).

The protein molecules are essentially amphoteric polyelectrolytes, containing both positive and negative charges. Therefore, there simultaneously exists electrostatic attraction and electrostatic repulsion between the charges in protein molecules and thearubigin molecules. The electrostatic attraction and electrostatic repulsion may be related to the average distance between the protein's positive sites and the thearubigin's negative sites (R_+), the average distance between the protein's negative sites and the thearubigin's negative sites (R_-), and the Debye length (R_d) through the following equation:

$$U = -\frac{Q_{\rm p}}{2\varepsilon} \left(\frac{Q_{\rm +}}{R_{\rm +}} e^{-R_{\rm +}/R_{\rm d}} - \frac{Q_{\rm -}}{R_{\rm -}} e^{-R_{\rm -}/R_{\rm d}} \right)$$
(8)

where U is the potential energy for the electrostatic interaction, Q_p is the charges of thearubigin associated with the protein molecule which contains Q₊ positive charges and Q. negative charges, and ε is the dielectric constant. If Q₊, Q₋, R₊, and R₋ are independent from salt concentration (I), the increase of salt concentration leads to Coulombic screening through influencing $R_d (R_d \approx 0.3/\sqrt{I})$. During the thearubigin adsorption on BSA surface, at lower salt concentrations, there may be $R_+ < R_d < R_-$, and the increase of salt concentration is mainly to screen the electrostatic repulsion, but not disturb the electrostatic attraction between thearubigin and BSA surface. Consequently, the total interactions will be enhanced with increasing salt concentration. This salt concentrationenhanced effect thus increases thearubigin adsorbed mass on BSA surface when I increases from 0.001 M to 0.1 M. On the contrary, when I is above 0.1 M, there may be $R_d < R_+ < R_-$, and the added salt can screen both electrostatic attraction and repulsion significantly owing to the higher salt concentration. Therefore, the increase in I beyond 0.1 M leads to gradually-reduced amount of thearubigin molecules adsorbed on BSA surface. However, when I is further increased to 0.4 M, the addition of thearubigin may promote the formation of certain type of complex with BSA, resulting in an increase in the amount of thearubigin adsorbed on BSA surface.



Figure 20 Changes of mass and thickness of 0.032 % thearubigin adlayer on BSA surfaces at various salt concentration in acetate buffer concentrations (pH = 4.9).

4.1.4 Effect of temperature

Temperature-dependent QCM-D measurements at 25 °C, 30 °C, and 35 °C for the adsorption of 0.032% thearubigin solution on BSA surface have also carried out to see

whether hydrophobic involved in the thearubigin and BSA adsorption. Increasing temperature enhance the hydrophobic interaction. However, we find that, even at temperature below the critical denaturing temperature of BSA (i.e., 60 °C), the increase of temperature actually decreases the amount of both thickness and mass of the thearubigin adlayer, as shown in **Figure 21**. This result indicates that the hydrophobic interaction is not the dominant factor associated with the binding between thearubigin and BSA, simply because the hydrophobic interaction usually increases with the increase of temperature (Byler and Susi., 1986), which is opposite to the experimental results obtained regarding the changes of the adsorbed mass and thickness of thearubigin with temperature.



Figure 21 Changes of mass and thickness of TR adlayer on BSA surface at various temperatures.

4.2 Binding of theaflavins to BSA surface

4.2.1 Effect of Theflavin (theaflavin-1;TF-1)concentrations

The typical adsorption process of various TF1 on bovine serum albumin (BSA) surface was monitored through the real time-resolved resonance frequency shifts (ΔF) and energy dissipation shifts (ΔD) as shown in **Figure 22**. **Figure 23** a show ΔF and ΔD changes as a function of time upon the addition of 0.576 mM TF1 in pH 4.9 and 0.01 M acetate buffer solution. The ΔF obtained at three different overtones (n=3, 5, 7) were normalized by their overtone. The arrows indicated the injection time of TF-1 solution (t₁), and several times of rinsing with buffer solutions (t₂, t₃) respectively. The adsorption of TF-1 on BSA surface consists of reversible and irreversible adsorption processes as indicating by the data obtained after two times of rinsing which can explain the reversible adsorption process.

Obviously, orderly increasing the theaflavin (TF1) concentrations from 0.230 mM to 0.921mM causes the frequency change increase from 14 to 102 Hz as shown in **Figure 23 b**, suggesting the increasing of adsorbed mass on the principle of Sauerbrey equation.



Figure 22 Time-dependent frequency shifts and energy dissipation shifts for TF1 at 0.576 mM at fifth overtone



Figure 23 (a)Time-dependent frequency shifts (ΔF) for TF1 adsorption on BSA-modified quartz crystal surfaces for various concentrations. The theaflavin solutions were prepared in 0.01M acetate buffer at pH = 4.9. (b). ΔF for 0.576 mM is measured simultaneously at three overtones (n = 3, 5, 7) and normalized by their overtone number. The arrows indicate the time for the injection of TF1 molecules (t₁) and two times of rinsing steps (t₂ and t₃).

The software (QTools) based on Voigt model has been used to calculate the thickness and mass of the adsorbed TF1 adlayers on BSA surface and the result obtained were plotted in **Figure 24**. As expect, increasing TF1 concentrations from 0.230 mM to maximum 0.921 mM both thickness and mass of TF1 adlayer increase from 1.11 to 5.75 nm and 50.23 to 260.19 ng/cm⁻²



Figure 24 Changes of mass and thickness of theaflavin without gallate (TF1) adlayer on BSA surfaces at various theaflavin concentrations.

Langmuir model and Freundlich model were used to predict the performance of an adsorption system in this study. The result in **Figure 25**, indicates that TF1 adsorption completely satisfactory fit well with Freundlich model with a correlation coefficient of

0.94(**Table 4**), but it does not fit with Langmuir model. This prove that there are the formation of multilayer or aggreagation of protein molecules



Figure 25 The adsorption isotherm of TF1 onto BSA surface fitted to the Freundlich model.

4.2.2 Effect of pH

The effects of pH on the absorption were studied in the pH form 3, 4.9, and 7 $(25^{\circ}C)$. Mass and thickness obtained are plotted in

Figure 26. The adsorption extent of TF1 on BSA surface at pH 7, where protein is carrying negative charges, is higher than the adsorption at pH 3, and 4.9, where protein is carrying positive charge and neutral. The significantly higher or lower adsorbed TF1 mass and thickness at pH 3 or pH 7 suggest that the electrostatic interaction between BSA surface charges and charges existing in TF1 play a significant role in their interactions. At pH 7, the maximum adsorbed TF1 mass and thicknesses indicate the existence of strong electrostatic attraction between negatively charged BSA surface and positive charged TF1 molecules. On the other hand, the minimum adsorbed TF1 mass and thickness at pH 3 may arise from the electrostatic repulsion between positively charged protein surface and positively charged TF1 molecules. This result implies that some functional group of TF1 structure will give charge at pH 3 and 7.

Figure 27 indicates that pH has no impact on degree of reversible TF1 adlayer. When comparing the frequency change after rinsing at different pH, the result show that after rinsing, the amount of reversible TF1 adlayer are almost the same in pH3, 4.9, and 7.



Figure 26 Changes of mass and thickness of 0.368 mM theaflavin without gallate adlayer on BSA surfaces at various pH 3, 4.9, and 7



Figure 27 Time-dependent frequency shifts (ΔF) for TF1 adsorption on BSA-modified quartz crystal surfaces for 0.576 mM at pH 3, 4.9, and 7. ΔF is measured simultaneously at fifth overtones. The arrows indicate the time for the injection of TF1 molecules (t₁) and two times of rinsing steps (t₂ and t₃)

4.2.3 Effect of salt and ionic strength

To better understand the nature of interactions between TF1 and BSA, we also study the effects of salt concentration and ionic strength on the binding processs. **Figure 28** a, and b displays the changes of adsorbed mass and thickness of TF1 adlayer on BSA surfaces as a function of salt concentration and ionic strength. At pH 4.9, when the acetate buffer concentration increases from 0.001 M to 0.100 M, both adsorbed mass and

thickness increase. On the other hand, as the salt concentration increases above 0.01 M, both adsorbed mass and thickness decrease. These results indicate that salt concentration has complex effects on the electrostatic interaction between protein BSA and TF1. Similar salt concentration effects were also observed in the electrostatic interaction between some proteins and certain polyelectrolytes including thearubigins and BSA protein as above study. The explanation for this phenomenon can be better explained as the reason used to explain thearubigins and BSA protein above. Briefly, increasing salt concentration leads to Coulombic screening effect on the adsorption between TF1 and BSA protein.





Figure 28 Changes of mass and thickness of TF1 adlayer on BSA surface at various sodium chloride concentrations (a), and at various phosphate buffer concentration (pH 4.9) (b).

4.3 Binding of Theaflavin-3, 3-digallate (theaflavin-3; TF-3) to BSA surface

4.3.1 Effect of Theaflavin-3, 3-digallate concentrations

The binding process of TF3 on BSA surface are conducted be monitoring the typical time-resolved resonance frequency shifts (ΔF) and energy dissipation shifts (ΔD) as shown in **Figure 29**. **Figure 29** b show ΔF and ΔD changes as a function of time upon the addition of 0.921 mM TF3 in pH 4.9 and 0.01 M acetate buffer solution. The ΔF obtained at three different overtones (n=3, 5, 7) were normalized by their overtone

number. The arrows indicated the injection time of TF3 solution (t_1), and several times of rinsing with buffer solutions (t_2 , t_3) respectively. Right after the injection of TF3 solution, there was often a rapid increase in ΔF and a marked increase in ΔD , followed by much gradual changes of ΔF and ΔD until steady states were reached. The changes in ΔF and ΔD indicate the adsorption of TF3 on BSA surface.

Increasing the TF3 concentration causes increasing change of frequency as in **Figure 30**. The software (QTools) based on Voigt model has been used to model the responses at third, fifth, and seventh overtones during the process of TF3 adsorption on BSA surface, and provide the mass and thickness of the adsorbed TF3 adlayer on BSA surface, as shown in **Figure 31**.

Increasing TF3 concentrations from 0.058 mM (0.050 g/L) to maximum 0.921 mM (0.800 g/L) both thickness and mass of adlayer increase from 2.66 to 10.07 nm and 120.5 to $479.97 \text{ ng/cm}^{-2}$.



Figure 29(a). Time-dependent frequency shifts (ΔF) and energy dissipation shifts(ΔD) for TF3 adsorption on BSA-modified quartz crystal surface for Theaflavin-3, 3-digallate (TF-3) adsorption on BSA-modified quartz crystal surfaces at seventh overtone. The TF3 solutions were 0.291mM prepared in 0.01M acetate buffer at pH = 4.9. (b). ΔF for 0.921 mM is measured simultaneously at three overtones (n = 3, 5, 7) and normalized by their

overtone number. The arrows indicate the time for the injection of TF3 molecules (t_1) and two times of rinsing steps $(t_2 \text{ and } t_3)$.



Figure 30Time-dependent frequency shifts (ΔF) for theaflavin-3,3'-digallate (TF-3)

adsorption on BSA-modified quartz crystal surfaces for various concentrations.



Figure 31 Changes of mass and thickness of theaflavin-3,3'-digallate (TF3) adlayer on BSA surfaces at various theaflavin concentrations. The TF3solutions were prepared in 0.01M acetate buffer at pH = 4.9.

Huang *et at* (2007)'s study suggested that gallate group had the significant impact on the interaction between EGCG and protein BSA. TF1 and TF3 structures are different at gallate group possessing as shown in **Figure 1**. Consequently, the adsorption of TF3 adlayer on the BSA surface indicated by mass of TF3 adlayer is much higher than TF1 as shown in **Figure 32**. Higher affinity constant (n) of TF3 (**Table 4**) than TF1 also confirm this phenomenon.



Figure 32 Change of mass of theaflavin-3,3'-digallate (TF3) comparing with theaflavin (TF1)

The experimental data of TF3 adsorption on BSA surface give unsatisfactory fit to the Langmuir model as shown in **Figure 33**, indicating that the Langmuir model is not ideal for TF3 adsorption behavior. On the other hand, the result shows that it is fitting well with the Freundlich model, an empirical exponential equation, with a correlation coefficient of 0.995 (**Table 4**). The better fit of TF3 adorption isotherm using the Freundlich model than using the Langmuir model prove that the TF3 adsorption isotherm onto the BSA surface prove the formation of multilayer or aggreagatation of protein molecules, and the binding is mainly governed by nonspecific hydrophobic interactions, in agreement with some previous studies of polyphenol/protein interactions in solutions (Jöbstl *et al.*, 2004; Huang *et al.*, 2007).

Huang *et al* found that the adsorption isotherm of EGCG on BSA surface can be better described by the Freundlich model than the Langmuir model, indicating that EGCG adsorption on BSA surface is dominated by nonspecific hydrophobic interactions. TF3, TF1 and EGCG provide the same fit of absorption isotherms on BSA surface with Freundlich model, while thearubigins had the better fit of the absorption isotherm on the BSA surface to Langmuir model (our previous result). These suggest that thearubigin adsorption on BSA surface is dominated by specific interaction such as electrostatic interaction but the absorption of theaflavins and EGCG are governed by nonspecific interaction such as hydrophobic interactions. These may because the structures of EGCG, TF1, and TF3 are much more similar to each other comparing with thearubigin **Figure 1** (Huang, 2007).

Polyphenols	Freundlich model			Langmuir model		
	Kf	n	R^2	K	M_m	R^2
TR	975 ± 97	3.23 ± 0.42	0.9480	38.0 ± 5.6	690 ± 51	0.9735
TF3	1730± 87	2.01 ± 0.10	0.9917	42.5 ± 2.1	588 ±29	0.9840
TF1	290 ± 17	0.91 ± 0.06	0.9486	-	-	-

Table 4 Freundlich and lagmuir model parameters with correlation coefficient



Figure 33 The adsorption isotherm of theaflavin-3,3'-digallate(TF3) onto BSA surface fitted to the Langmuir model (top) and the Freundlich model (bottom).

4.3.2 Effect of pH

To support that TF3 adsoption on BSA surface is govern by non specific hydrophobic interaction, the effects of pH on the absorption were studied at 3, and 7 despite of pH 4.9, by QCM-D measurements. Mass and thickness obtained are plotted in **Figure 34**. The adsorption extent of TF3 on BSA surface at pH 3 and 7

where BSA carries positive and negative charges, respectively, is almost the same while the significantly higher adsorbed TF3 mass and thickness is at pI of protein (pH 4.9), indicating that hydrophobic interaction plays role in the interaction between TF3 and BSA protein. The protein molecules near their pIs, whose total net charges are nearly zero and their intermolecular electrostatic repulsion forces are minimized, usually assume a more compact globular conformation. The maximum binding of a protein by polyphenols at pH near its isoelectric point (pI) has also been reported by many other investigators (Nack *et al.*, 1996; Van *et al.*, 1969) This suggests that the contribution from electrostatic interaction between BSA surface and EGCG both above and below the pI may not be the main factor that causes EGCG adsorption.



Figure 34Changes of mass and thickness of 0.368 mM theaflavin-3,3'-digallate (TF3) adlayer on BSA surfaces at various pH values

pH has also have effect on the amount of reversible TF3 adlayer as shown in Figure 35.

During the rinsing steps, the recoveries of frequency change at pH 7 were rarely recovered comparative to pH3, suggesting that the adsorption of TF3 at pH 7 is comparatively stronger than one at pH 3.



Figure 35 Δ *F* at seventh overtone of 0.368 mM TF3. The arrows indicate the time for the injection of theaflavin-3,3'-digallate (TF3) molecules (t₁) and two times of rinsing steps (t₂ and t₃).
4.3.3 Effect of salt and ionic strength

The influence of added salt and ionic strength is shown in **Figure 36**. The changes of mass and thickness of TF-3 adlayer on BSA surface at different ionic strength and sodium chloride concentrations decrease with the increase of both ionic strength from 0 to 0.4 M and salt concentration from 0 to 0.2 M. The TF-3 adsorption onto BSA surface is very sensitive to salt concentration. As a matter of fact, salt usually either reduces the electrostatic interaction or enhances the hydrophobic interaction; the effect of salt here seems contrary to our above conclusion that the hydrophobic interaction is dominant the interaction between TF-3 and BSA. This result is similar to the EGCG in Huang 's study which can be explained as following. The addition of small amount of salt which reduces the attraction between the protein molecules, cause BSA molecules exist as monomers following decrease in protein molecular size, leading to suppression TF-3 adsorption and inhibit the TF bridge function for BSA aggregation owing to the existence of a certain critical distance of the inter-protein complexes (Huang *et al.*, 2007; Chitpan *et al.*, 2007).

The ionic strength was varied by varying the concentration of phosphate buffer pH 4.9. Further increase in ionic strength form to 0 to 0.4 M caused a slightly decrease in the absorption. This result is different form the BSA and thearubigin adsorption in previous study (Jobstl *et al.*, 2006). This also indicate that the electrostatic interaction do not involve in the TF-3 and BSA protein interaction.



Figure 36 Changes of mass and thickness of 0.368 mM theaflavin-3,3'-digallate (TF3) adlayer on BSA surfaces (a) at various salt concentration ; (b) at different acetate buffer concentrations (pH = 4.9)

4.2.4 Effect of temperature

Herein, the absorption of TF3 to BSA was examined at 25, 30 and 35 °C, respectively as shown in **Figure 37**. The absorption of TF3 to BSA was virtually not affected by variation in temperature. Basically, the driving force for hydrophobic interaction may be attributed to the relatively nonpolar structure of polyphenol since higher temperatures (even below the critical denature temperature of BSA) can cause partial denaturation of proteins (Boye *et al.*, 1997) leading to unfolding of BSA molecules and increase the exposure of the hydrophobic surfaces of the protein. The result obtained here appears to be contrary to the conclusion that hydrophobic interaction play role in the interaction between TF3 and BSA protein. The short range temperatures studied can be used to explain the result.



Figure 37. Changes of mass and thickness of 0.368 mM theaflavin-3,3'-digallate (TF-3) adlayer on BSA surface at various temperatures, pH4.9.

4.4 Hydrogen bonding

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 (Hayashi et al., 1987). FTIR is also used to investigate the hydrogen bonding between the adsorbed theaflavins molecules and BSA surfaces, FTIR spectra of BSA surfaces with and without the theaflavins adsorption were collected, as shown in Figure 38. The FTIR spectrum of pure BSA surface displays two characteristic bands at 1660 cm⁻¹ and 1540 cm⁻¹. The 1660 cm⁻¹ (amide-I) band indicate the protein amide C=O stretching vibrations, and the 1546 cm⁻¹ (amide-II) band is due to the amide N-H bending vibrations and C-N stretching vibrations (Chitpan et al., 2007). The positions of amide bands I and II for both TF-1 and 3 adsorbed BSA surface were shifted. The amide I of TF-1 and TF-3 were shifted from 1660 cm⁻¹ to 1657 cm⁻¹ and 1626 cm⁻¹ respectively. The amide II band of both theaflavins were shifted form 1550 cm⁻¹ to 1541 cm⁻¹ and 1536 cm⁻¹ for TF-1 and TF-3 respectively. The significant peak position shifts observed in the amide I and amide II bands may suggest the attribution of hydrogen bonding between the phenolic hydroxyl groups of theaflavins and the functional groups such as carbonyl group of the BSA. It is well accepted that Fourier transform infrared spectroscopy (FTIR), using amide I and II band which are in range 1700-1500 cm⁻¹, can be used to determine the changes in protein secondary structure (Byler and Susi., 1986; Tanaka et al., 1987; Raussens et al., 2006; Chu et al., 2003; Langer et al., 1999). Normally, the absorbance in the range from 1650 to 1658 cm-1 and 1545 cm-1 are associated with presence of α -helix while the

absorbance between 1640 and 1620 cm-1 as well as 1530 cm-1 are associated with β sheet structure (Raussens *et al.*, 2006). The shape and the shifted peak of amide I and II band also reveal that the secondary structure of protein BSA including α -helix and β sheet structure may be changed (Chu *et al.*, 2003; Langer *et al.*, 1999).



Figure 38 FTIR spectra of pure BSA surface and BSA surface with adsorbed theaflavin without gallate (TF-1) and theaflavin-3,3'-digallate (TF-3) molecules

5. CONCLUSION

In summary, the strength of interaction between protein BSA and black tea polyphenols including, thearubigin, and theaflavin depend on depends on many physicochemical factors such as pH, salt concentration, and temperature. The adsorption of black tea polyphenol including thearubigin, TF3, and TF1, molecules on BSA 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 polyphenols masses and thicknesses under different physicochemical conditions were calculated using Voigt model. The differences of theaflavin-3,3'-digallate (TF-3) and theaflavin (TF-1) structure particularly in the galloyl functional groups are attributed to the different interaction with protein BSA.

The results here suggest that there is electrostatic attraction and repulsion between thearubigins and TF1 with BSA protein, which are indicated by the maximum and minimun interaction at pH below or lower than pI of protein. TF-3's maximum adsorption is at the pI of protein, 4.9, suggesting that nonspecific hydrophobic interaction is found to be dominated in BSA protein TF3 interaction

Salt has hinder effect on thearubigin and TF1 adsorption, and reduce the TF3 adsorption on BSA surface. The adsorption behavior of TF3 and TF1 are well fitted with Freundlish model whereas the adsorption of thearubigin is fitted with Langmuir model. These behaviors imply that the interaction of protein BSA with TF-3 is the nonspecific interaction while protein BSA thearubigin interaction is specific.

Hydrogen bonding is another dominant force in the black tea polyphenol-BSA interactions, as evidenced by the large BSA band position shifts for both amide I and

amide II before and after black tea polyphenol adsorption in FTIR spectra. The adsorption capacity of TF1 on BSA surface was much lower than TF3, introducing the importance part of gallate functional group of black tea polyphenol involved in the polyphenol/protein interaction. QCM-D is considered as a good technique for investigating interaction of plant polyphenols with proteins. The different structures among black tea polyphenols result in differential interaction behaviors with proteins which may help to explain the difference in bioactivity of tea polyphenols.

6. SUGGESTED FUTURE STUDY

1. To explain the electrostatic interaction of TF1 and BSA protein, it is recommended to analyse the charge of TF1 at different pH by measure zeta potential

2. CD is recommended to futher use to understand the amount of protein secondary structure change of BSA including α -helix, and B-sheet

3. The method used in this study can be applied to investigate the interaction of other proteins such as milk casein with tea polyphenols.

4. Apart from investigating protein with plant polyphenols, QCM-D is recommended to determine the interaction of some bioactive protein like enzymes with plant polyphenols to explain the bioactive mechanism.

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