EGG PROTEIN INTERACTIONS WITH PHENOLIC COMPOUNDS: EFFECT ON PROTEIN PROPERTIES

By

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A thesis submitted to McGill University in partial fulfillment of
the requirements of the degree of Master of Science

December, 2012

Suggested Short Title:

EGG PROTEIN-PHENOLIC INTERACTIONS

ABSTRACT

Interactions of egg proteins (ovalbumin, conalbumin, egg white proteins, and egg yolk proteins) with selected phenolic compounds (flavone, chrysin, quercetin, and rutin) in aqueous media were examined by electrophoresis and fluorescence spectrophotometry. SDS-PAGE and native-PAGE results showed variable changes on the electrophoretic behaviour of egg white proteins in the presence of quercetin, while conalbumin-phenolic reaction products showed interactions under non-reducing conditions only. Fluorescence quenching technique was used to investigate the nature of egg protein-phenolic interactions and to estimate the effect of glycosylation and hydroxylation of phenolic compounds on the affinity to egg proteins. Stern-Volmer data revealed that the mechanism of egg protein-phenolic interactions is the static quenching and suggest that the diffusion does not play a role in fluorescence quenching in egg protein-phenolic interactions; the binding data analysis suggests that glycosylation and hydroxylation of phenolic compounds lower the affinity to egg proteins. Fluorescence quenching results showed that fluorescence intensity of egg proteins decreased with increasing concentration of phenolics. Enzymatic hydrolysis of egg protein-phenolic products assessed by using trypsin-chymotrypsin mixture and bacterial protease revealed that in vitro egg protein digestion was adversely affected by the interaction of phenolics. Proteins extracted from muffin mixture with added phenolics were investigated by electrophoresis techniques and enzymatic hydrolysis. SDS-PAGE results showed changes in electrophoretic patterns of ovalbumin. In vitro enzymatic hydrolysis of proteins extracted from the muffin was adversely affected by the addition of phenolics.

RESUME

Les interactions de protéines d'œuf (ovalbumine, conalbumine, protéines de blanc d'oeuf, protéines de jaune) avec des composés phénoliques sélectionnés (flavone, chrysine, quercétine et rutine) dans des milieux aqueux ont été examinées par électrophorèse et spectrofluorométrie. Les résultats de native-PAGE et SDS-PAGE ont montré des changements variables sur le comportement électrophorétique des protéines du blanc d'œuf en présence de la quercétine, tandis que les produits de réaction entre conalbumine et phénoliques ont montré des interactions dans des conditions non réductrices seulement. La technique extinction (*quenching*) de la fluorescence a été utilisé pour étudier la nature des interactions protéines d'oeuf et phénoliques, et pour estimer l'effet de la glycosylation et l'hydroxylation de composés phénoliques sur l'affinité à la protéine d'oeuf. Les données de Stern-Volmer ont révélé en utilisant un quencher « desactivateur » que le mécanisme d'interactions entre la protéine d'œuf et les composées phénoliques est de type électrostatique et suggère que la diffusion ne joue pas un rôle dans l'extinction de la fluorescence en présence d'interactions protéines d'œuf et composés phénoliques. L'analyse des données « *binding* » soit des liaisons, suggère que la glycosylation et l'hydroxylation des composés phénoliques réduit l'affinité pour les protéines d'oeuf. Les résultats de l'extinction de la fluorescence ont montré que l'intensité de fluorescence des protéines de l'œuf diminue avec l'augmentation de la concentration des composés phénoliques. Les produits d'hydrolyses enzymatiques des complexes protéines d'oeuf-composés phénoliques évaluées suite à une protéolyse par un mélange trypsine-chymotrypsine et de la protéase bactérienne ont révélé que la digestion *in vitro* des protéines d'oeuf a été affectée négativement par l'interaction des composés phénoliques. Les protéines extraites du mélange à muffins enrichis en composés phénoliques ont été étudiés par des techniques d'électrophorèse et d'hydrolyse enzymatique. Les résultats du SDS-PAGE ont montré des changements dans le modèle électrophorétique de l'ovalbumine. L'hydrolyse enzymatique *in vitro* des protéines extraites du muffin a été affectée négativement par l'ajout de composés phénoliques.

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CHAPTER 1

INTRODUCTION

1.1 General Introduction

Food contains both macro-components such as proteins, carbohydrates and lipids, and micro-components such as vitamins, minerals, enzymes and polyphenols (Gaonkar and McPherson, 2006; Sikorski, 2007). These macro- and micro-components can interact especially during food processing, handling and storage, leading to desirable and undesirable changes in foods. An example of these interactions is protein-phenolic interactions that have generated interest in recent years due to their influence on properties of polyphenols and proteins which, in turn, reflects adversely or positively on food quality (Gaonkar and McPherson, 2006; Sikorski, 2007).

In many studies, phenolic compounds showed antioxidant, antimicrobial, antimutagenic, and anticarcinogenic properties, besides having a role as food ingredients in the food industry. Polyphenols have radical-scavenging capacity and exhibit inhibitory activity against many foodborne pathogens as well as may prevent cancer, osteoporosis, atherosclerosis, brain dysfunction and coronary heart diseases. Moreover, polyphenols can be used as natural colorants and preservatives in food (Scalbert and Williamson, 2000; EL Gharras, 2009; Ignat *et al*., 2011; Perumalla and Hettiarachchy, 2011). However, these properties can be affected through protein-phenolic interactions as Xiao and Kai (2011) mentioned. The authors mentioned that protein-phenolic interactions can diminish the antioxidant activity of polyphenols and this may result in weakening the resistance system against free radicals formation which is considered one of the causes of cancer and other health problems, within the body. In addition, the interactions between polyphenols and blood proteins can affect bioavailability of polyphenols (D'Archivio *et al*., 2007; Xiao and Kai, 2011).

The interaction between phenolics and proteins can affect food quality where it is responsible for a stringency perception (resulting from tannin-protein interactions), for formation of haze and aggregation in beverages such as wine, grape and apple juice, and for reducing the digestibility of dietary proteins (EL Gharras, 2009; Siebert, 2009). However, these interactions can develop organoleptic properties of food and may prevent

enzymatic browning and enzyme-catalyzed lipid oxidation by inhibiting polyphenol oxidase and lipase (Raghavendra *et al*., 2007; Noor-Soffalina *et al*., 2009; Altunkaya, 2011). Polyphenols also improve the foaming properties of both β-lactoglobulin and egg white proteins (O'Connell and Fox, 2001). Thus, investigating and understanding of protein-phenolic interactions are highly recommended to enhance and improve the quality and safety of food products.

Limited studies and scanty information are gained about interactions of polyphenols and egg proteins especially ovalbumin that has an extensive use in food industry because of its functional and nutritional properties (Lu *et al*., 2009).

1.2 Research Objectives

The overall objective of this research is to study the interactions of selected polyphenols with egg proteins; the specific objectives are to investigate

(i) Characterization of egg protein-phenolic interactions using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), native polyacrylamide gel electrophoresis (native-PAGE) and fluorescence quenching.

(ii) Effect of glycosylation and hydroxylation of selected polyphenols on binding with egg proteins using fluorescence quenching.

(iii) Effect of egg protein-phenolic interactions on the digestibility of egg proteins *in vitro*.

CHAPTER 2

LITERATURE REVIEW

2.1 Phenolic Compounds

2.1.1 Definition of Phenolic Compounds

From a chemical point of view, the term 'phenolic' or 'polyphenol' can be defined as a substance which consists of an aromatic ring linked to one or more hydroxyl groups, including functional derivatives (esters, methyl ethers, glycosides etc.) (Ho *et al*., 1992).

2.1.2 Classification of Phenolic Compounds

Phenolic compounds are usually divided into four different groups according to the number of phenol rings that they contain and the structural elements that bind these rings together (Manach *et al*., 2004). These groups are phenolic acids (hydroxybenzoic acids and hydroxycinnamic acids) (Manach *et al*., 2004; Manach *et al*., 2005), flavonoids that can be subdivided into six classes: flavonols, flavones, flavanols, flavanones, isoflavones and anthocyanidins (Scalbert and Williamson, 2000; Manach *et al*., 2004; EL Gharras, 2009), stilbenes and lignans (Manach *et al*., 2004; Scalbert and Williamson, 2000).

2.1.3 Properties of Food Phenolic Compounds

Polyphenols show a range of properties, depending on their chemical nature that may vary from simple to highly polymerized compounds. Phenolic compounds exhibit organoleptic, physical, antoxidant, antimicrobial properties as well as the ability to interact with food compounds such as proteins (Cheynier, 2005; EL Gharras, 2009).

Anthocyanins, the most abundant phenolic pigments in plants, show red, purple, or blue color whereas flavonols and flavones show the yellow color (Cheynier, 2005; EL Gharras, 2009) and these colors can play a role in acceptability of food especially beverages. Polyphenol oxidation, such as enzymatic browning that takes place in some fruits and vegetables during food processing, can cause undesirable brown pigments (Altunkaya, 2011); polyhpenols are also responsible for characteristic odor in some foods such as vanillin and eugenol in cloves (Cheynier, 2005; EL Gharras, 2009), ice cream, and yoghurt (O'Connell and Fox, 2001) and for the bitter and astringent taste in food (Cheynier, 2005; EL Gharras, 2009).

Polyphenols can absorb in the UV and visible regions at different wavelengths depending on their particular structures. B-ring in most flavones and flavonols show

absorption at 320-385 nm, while A-ring absorption is at 250-285 nm, taking into account the number and arrangements of hydroxyl groups present in phenolic compounds (Rice-Evans *et al.*, 1996). For example, the absorbance of kaempferol (with hydroxyl groups at positions 3,5,7,4') is at 367 nm, quercetin (3,5,7,3',4',) at 371 nm, myricetin (3,5,7,3',4',5') at 374 nm, whereas flavanones (naringenin, 5,7,4', and taxifolin, 3,5,7,3',4') are at 289 and 290 nm, respectively (Rice-Evans *et al.*, 1996).

Solubility of polyphenols depends on many factors such as chemical structure, the nature of the solvent used in extraction of polyphenols, pH, temperature, and the ability to complex other substances such as carbohydrates and proteins. Hence, it is hard to develop an extraction procedure appropriate for extraction of all plant polyphenols (Naczk and Shahidi, 2006; Chebil, *et al.*, 2007). The solubility of flavonoids (quercetin, isoquercitin, rutin, chrysin, naringenin, and hesperetin) in acetonitrile, acetone, and tert-amyl alcohol was investigated. Hesperetin and naringenin exhibit the highest solubility in acetonitrile (85 and 77 mmol/L) respectively, while quercetin in acetone (80 mmol/L) and the lowest solubility of rutin was observed in acetonitrile (0.50 mmol/L) (Chebil, *et al.*, 2007). Anthocyanins dissolve highly in methanol (Naczk and Shahidi, 2006). Water is considered as a weak solvent of some flavonoids (rutin, naringin, quercetin, and neohesperidine dihydrochalcone) (Chebil, *et al.*, 2007). At pH 8, the solubility of hesperetin and naringenin was four times higher than at pH 1.5 and higher temperature increased the solubility (Chebil, *et al.*, 2007).

Phenolic compounds exhibit potential antioxidant and antimicrobial properties. Green tea extract can inhibit lipid oxidation by interacting with free radicals (Perumalla and Hettiarachchy, 2011). Tea catechins display higher antioxidant activity against lipid oxidation of cooked patties in comparison with ginseng, mustard, rosmary, sage, butylated hydroxyanisole (BHA), butylated hydroxyl toluene (BHT), and vitamin C and E (Perumalla and Hettiarachchy, 2011). Gram-positive and Gram-negative bacteria had been inhibited by green tea extract especially foodborne pathogens such as *E. Coli* O157:H7*, Campylobacter jejuni , L. Monocytogenes, Staphylococcus epidermidis, Vibrio cholera, Salmonella typhimurium, Shigella dysenteriae, Salmonella Enteriditis, Staphylococcus aureus, and Shigella flexneri* (Perumalla and Hettiarachchy, 2011).

2.1.4 Distribution of Phenolic Compounds in Foods

Polyphenols are commonly found in the plant kingdom (Manach *et al*., 2004; EL Gharras, 2009). They are widely distributed in plant foods (vegetable, cereals, legumes, fruits, nuts, etc) and beverages (wine, cider, beer, tea, cocoa, juices, etc) (Bravo, 1998).

2.1.4.1 Phenolic Acids

Hydroxycinnamic acids and hydroxybenzoic acids represent phenolic acids in foods (Manach *et al*., 2004). Hydroxycinnamic acids such as p-coumaric, caffeic, ferulic and sinapic acids are more prevalent than the hydroxybenzoic acids (gallic acid and protocatechuic acid) in foods (Manach *et al*., 2004; EL Gharras, 2009). Caffeic acid represents 75% - 100% of the total hydrxycinnamic acid content in most fruits (Manach *et al*., 2004; EL Gharras, 2009). Ferulic acid is the major hydroxycinnamic acid form in wheat bran (5 mg/g) (Scalbert and Williamson, 2000), barley seeds and barley brans (Naczk and Shahidi, 2006). Hydroxybenzoic acids especially gallic acid is about 4.5 g/Kg fresh weight of tea leaves (Manach *et al*., 2004).

2.1.4.2 Flavonoids

Flavonoids are low molecular weight components, basically consisting of two aromatic rings A and B attached by three carbon atoms (Figure 2.1) (Manach *et al*., 2004; Ignat *et al*., 2011). They are commonly found in plant kingdom as glycosides (EL Gharras, 2009). More than 6400 flavonoids have been identified in plants, and the list is constantly growing (Harborne and Williams, 2000; Ignat *et al*., 2011); these are the most common phenolics in our diets, and are classified into six subclasses including flavonols, flavones, flavanols, flavanones, isoflavones, and proanthocyanidins (Scalbert and Williamson, 2000; Manach *et al*., 2004; EL Gharras, 2009; Ignat *et al*., 2011).

2.1.4.2.1 Flavonols

Flavonols are the most common flavonoids in many fruits, vegetables and beverages; kaempferol, quercetin and myricetin represent the largest percentage of flavonols in foods (Scalbert and Williamson, 2000; Manach *et al*., 2004). The richest sources of flavonols are onions (up to 1.2 g/Kg), tomato skin, curly kale, leeks and broccoli (Manach *et al*., 2004; Naczk and Shahidi, 2006). In addition, myricetin, quercetin and kaempferol are found in blueberries (Naczk and Shahidi, 2006).

2.1.4.2.2 Flavones

Flavones consist essentially of glycosides of luteolin and apigenin which are found in sweet red pepper and celery, respectively. Parsley is considered the main edible source of flavones to date (240-1850 mg/Kg) (Manach *et al*., 2004). Skin of citrus fruit and cereals such as millet and wheat also contain significant amount of flavones (Scalbert and Williamson, 2000; Manach *et al*., 2004; EL Gharras, 2009).

2.1.4.2.3 Flavanols

The main flavanols are catechins which are found in different fruits such as apricot, cherry, apple and blueberries (Manach *et al*., 2004; Naczk and Shahidi, 2006). Green tea, chocolate and beans have the highest levels of flavanols (800, 610, 550 mg/Kg respectively) (Manach *et al*., 2004). Gallocatechin, epigallocatechin, and epiggallocatechin gallate are found in seeds of legumes, in grapes, and in tea (Manach *et al*., 2004; EL Gharras, 2009).

2.1.4.2.4 Flavanones

Citrus fruits have the highest level of flavanones especially naringenin and hesperetin which are the major flavanones found in grapefruit and sour orange respectively (Manach *et al*., 2004; Naczk and Shahidi, 2006; EL Gharras, 2009). Grapefruit juice contains between 100-650 mg flavanones/L whereas lemon juice contains 50-300 mg flavanones/L (Manach *et al*., 2004); flavanones are also found in tomatoes and mint (Manach *et al*., 2004; EL Gharras, 2009).

2.1.4.2.5 Isoflavones

Soybean and its products have a high concentration of the three isoflavones genistein, daidzein, and glycitein (Scalbert and Williamson, 2000; Manach *et al*., 2004). Fresh soybeans contain between 580-3800 mg isoflavones/Kg and dry bean contains 1 mg of genistein and daidzein/g while soymilk contains between 30-175 mg/L (Scalbert and Williamson, 2000; EL Gharras, 2009).

2.1.4.2.6 Proanthocyanidins

Proanthocyanidins are responsible for the color of the fruits, which are considered as an excellent source of proanthocyanidins, such as blackberry 4000 mg/Kg, black grape 7500 mg/Kg and cherry 4500 mg/Kg (Scalbert and Williamson, 2000; Manach *et al*., 2004; EL Gharras, 2009). In addition, cranberries, apple, pear, tea, chocolate serve as a

good source of proanthocyanidins (Scalbert and Williamson, 2000; Naczk and Shahidi, 2006).

2.1.4.3 Lignans

Flaxseed and flaxseed oil have significant amounts of lignans; flaxseed has 3.7g lignans/Kg dry weight (Scalbert and Williamson, 2000; Manach *et al*., 2004). Lentils, triticale, wheat, rye, sesame seed, garlic, asparagus, carrots, pears, prunes are considered as common dietary sources of lignans (Manach *et al*., 2004; Naczk and Shahidi, 2006; EL Gharras, 2009).

2.1.4.4 Stilbenes

Stilbenes are found in relatively small quantities in food; resveratrol is found in very low level in red wine (0.3-7 mg/L), grape and wilting berries (Manach *et al*., 2004; Naczk and Shahidi, 2006; EL Gharras, 2009).
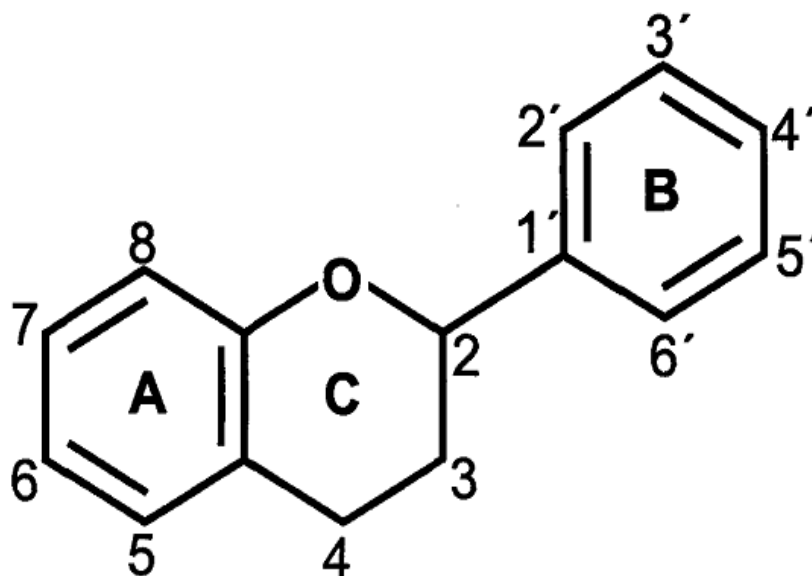
Figure 2.1 Basic structure and numbering system of flavonoids (Bravo, 1998).
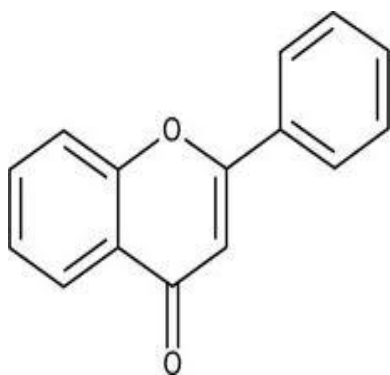
Flavone
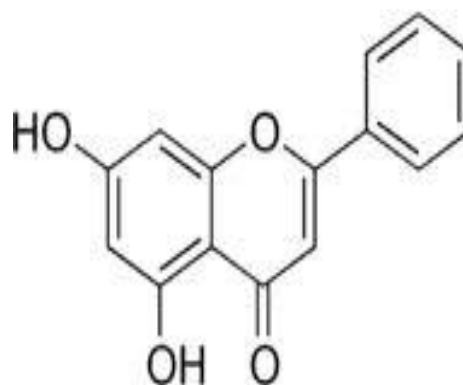
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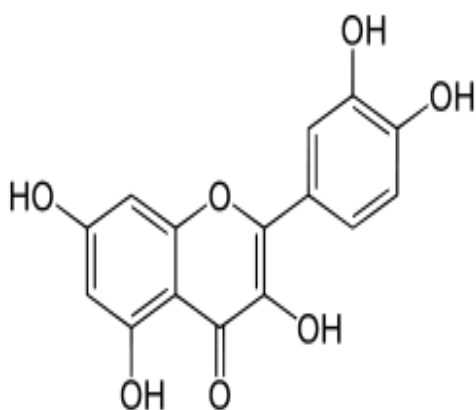
Quercetin
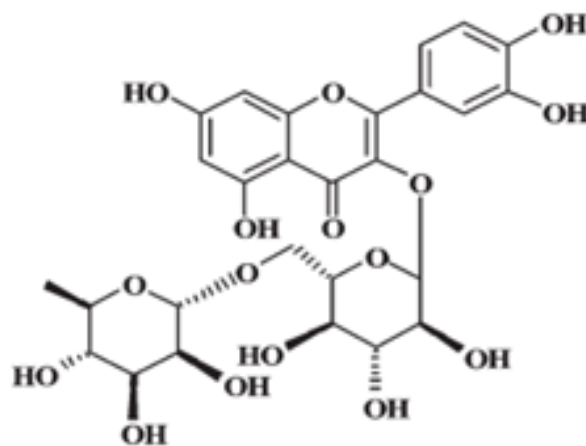
Rutin

Figure 2.2 The structure of selected phenolic compounds (Xiao *et al.*, 2008).

2.2 Protein-Phenolic Interactions

2.2.1 Nature of Protein-Phenolic Interactions

Although the nature of protein-phenolic interactions is not fully understood (Yuksel *et al*., 2010), it is suggested that polyphenols can complex proteins by four potential mechanisms (Rawel *et al*., 2001; Yang *et al*., 2009). Phenolics contain hydroxyl groups (a hydrogen bond donor) that can form strong hydrogen bonds with the carbonyl groups (the carbonyl group of tertiary side chains is a better hydrogen bond acceptor than that of primary or secondary side chains) in proteins (O'Connell and Fox, 2001 Rawel *et al*., 2001; Chen and Hagerman, 2004; Rawel and Rohn, 2010). Hydrophobic interactions also play a significant role in protein-phenolic interactions (Rawel *et al*., 2001; Rhon *et al*., 2006; Staszewski *et al*., 2011). The amphiphilic compounds such as hydroxycinnamate derivatives have an affinity for hydrophobic side chains in proteins (Rawel and Rohn, 2010). The ionized carboxyl of polyphenols (e.g., hydroxycinnamates) can interact with basic groups of arginine and lysine residues in proteins through ionic binding. Ionic interactions represent 50% of linkages formed in canola protein-phenolic complexes (Rawel and Rohn, 2010). Oxidized phenolics can bind lysine, tryptophan, methionine, histidine, tyrosine and cysteine residues by covalent linkages which form between free nucleophilic functional groups (e.g., sulfhydryl, amine, amide, indole, and imidazole) and electrophilic groups in quinines (Rawel *et al*., 2000; Bittner, 2006; Rawel and Rohn, 2010). Oxidized caffeic acid bind covalently to cysteine (thiol group) and this covalent attachment can be either reversible or irreversible (Bittner, 2006). van der Waals forces can also play a role in protein-phenolic interactions (Yang *et al*., 2009).

2.2.2 Factors Affecting on Protein-Phenolic Interactions

2.2.2.1 Chemical Structure

Protein-phenolic interactions are influenced by the chemical structure of polyphenols. Generally, high molecular weight and flexibility of polyphenols increase the affinity for complexing proteins (Deaville *et al*., 2007; Soares *et al*., 2007; Pignataro, 2008). In addition, B-ring hydroxylation of flavonols has a significant effect on protein affinity; the more hydroxyl groups on the B-ring, the stronger binding between protein and polyphenols (Xiao *et al*., 2008). Glycosylation of flavonoids influences adversely binding to proteins (e.g., bovine serum albumin (BSA) and whey proteins) (Rawel *et al*., 2003;

Cao *et al.*, 2009; Xiao *et al.*, 2009). Flavonoid polyglycoside such as rutin and flavonoid monoglycosides (baicalin, quercitrin, daidzin, puerarin, and genistin) lower the affinity for BSA by 5-10 and 5600 times compared with flavonoid aglycones (baicalein, quercetin, daidzein, and genistein) (Xiao *et al.*, 2009).

Protein structure can also affect protein-phenolic interactions. Protein binding to polyphenols can be affected by protein conformation (flexible secondary structure, carbonyl group of tertiary amides is a better hydrogen bond acceptor than the carbonyl group of primary or secondary amides), charge and size of protein, and amino acid composition of protein (O'Connell and Fox, 2001; Prigent *et al.*, 2003; Pignataro, 2008; Siebert, 2009).

2.2.2.2 pH and Ionic Strength

O'Connell and Fox (2001) and Pignataro (2008) reported that protein-phenolic interactions are dependent on pH; maximal level of the interactions is at or close to the protein isoelectric point (pI). Rawel *et al.* (2005) reported that the binding of polyphenols (chlorogenic, ferulic, and gallic acids, guercetin, rutin, and isoquercetin) to different proteins (human serum albumin, BSA, albumin, soy glycinin, and lysozyme) was decreased by decreasing pH. Highly acidic and alkaline conditions dissociate tannic acid-BSA complexes (Osawa and Walsh, 1993) while pH 3.0 show no effect on chlorogenic acid-BSA binding compared to pH 7.0 (Prigent *et al.*, 2003).

Ionic strength also influences differently protein-phenolic interactions. Increasing ionic strength diminish the aggregation and precipitation of proteins (α-amylase and BSA) by tannins (Rawel *et al.*, 2005; Carvalho *et al.*, 2006; Pignataro, 2008). The presence of potassium, copper, cobalt, nickel, manganese and zinc ions decrease the affinity between hesperitin and BSA (Hegde *et al.*, 2010). These results contradict other findings which show that calcium, magnesium, sodium and potassium ions enhance the formation of insoluble tannin-protein complexes at pH 5.5-7.0 with ratio 1:1 pure tannin: protein (Perez-Maldonado *et al.*, 1995).

2.2.2.3 Temperature

In general, increasing temperature cause a decrease in the binding of polyphenols to proteins. Heating to 60 ^{o}C and 90 ^{o}C lowered the ability of BSA to complex chlorogenic acid and quercetin, respectively (Prigent *et al.*, 2003, Rawel *et al.*, 2005).

2.2.2.4 Polysaccharides

Glucose, β-cyclodextrin, and arabinogalactan can inhibit protein-tannin interactions (Pignataro, 2008). Carvalho *et al*. (2006) reported that wine pectic polysaccharides also inhibit the formation of aggregates between grape seed tannins and α-amylase and IB8c, a proline-rich protein with 41% proline, 23% glycine, and 15% glutamine residues. Two mechanisms have been suggested to explain the influence of polysaccharids on the formation of aggregates between tannins and proteins: (i) polysaccharides such as pectin, xanthan, polygalacturonic acid, and gum arabic could form ternary complexes with the protein-tannin aggregate due to their ionic character, and enhance the solubility of the complexes in aqueous medium or (ii) some polysaccharides have the ability to develop gel-like structures e.g., xanthan gum and polygalacturonic, or hydrophobic pockets e.g., cyclodextrin could encapsulate tannins, restraining them from binding to proteins (Carvalho *et al*., 2006; Pignataro, 2008).

2.2.3 Caseins-Phenolic Interactions

The casein fractions, α-, β-, and κ-caseins, can interact with polyphenols found in beverages particularly coffee and tea (Yuksel *et al*., 2010); the interactions of α- and β-caseins with tea polyphenols have been reported. A study showed that tea polyphenols ((+)-catechin, (-)-epicatechin, (-)-epigallocatechin and (-)-epigallocatechin gallate) weakly bind to both α-casein and β-casein in solution; this binding occurs due to both hydrophilic and hydrophobic interactions between polyphenols and casein (Hasni *et al*., 2011). Another study reported that β-casein has more hydrophobic nature than α- casein because β-casein has five phosphoserine residues and this difference in hydrophobic nature results in higher stability of β-casein complexes with polyphenols than α- casein complexes; insoluble complexes can be formed between catechins and β-casein (Phadungath, 2005; Hasni *et al*., 2011). Casein conformation is altered in the presence of polyphenols with a decrease in α-helix and β-sheet (secondary structure) and an increase in random coil and turn structure, suggesting that casein-phenolic interactions can cause protein unfolding (Kanakis *et al*., 2011).

2.2.4 Whey Proteins-Phenolic Interactions

β-Lactoglobulin (β-LG), a globular protein, is the major protein of milk whey (1g/L) (Kanakis *et al*., 2011); it occurs usually as a homodimer and binds hydrophobic and

amphiphilic compounds, including fatty acids, retinol, phospholipids, vitamin D, and aromatic compounds (Riihimaki *et al*., 2008; Kanakis *et al*., 2011). Plant phenolic compounds interact with globular proteins; hence, many studies have been designed to investigate interactions between β-LG and phenolic compounds. One study examined the interactions of β-LG with tea polyphenols (+)-catechin, (-)-epicatechin, (-)-epicatechin gallate and (-)-epigallocatechin gallate) and showed that polyphenols bind β-LG by hydrophopic and hydrophilic interactions, and that, in contrast to casein, the effect of polyphenols on β-LG stability and secondary structure lead to an increase in α-helix and β-sheet, suggesting protein structural stabilization (Kanakis *et al*., 2011). Another study observed that the interaction between the monomeric phenolic compound, 5-*o*-caffeoylquinic acid (5-CQA), and β-LG had no effect on the solubility of β-LG and 5-CQA had a low affinity for β-LG (Prigent *et al*., 2003). In terms of physicochemical characters of β-LG, the isoelectric point of β-LG (pI 5.1-5.5) decreased (pI 3.9-4.9) as a result of reaction of chlorogenic acid, caffeic acid, gallic acid and p-quinone with whey proteins; the molecular weight of β-LG influenced interaction with chlorogenic acid whereas quinic-, and ferulic acid interaction with whey protein was not affected (Rawel *et al*., 2001).

2.2.5 Soy Proteins-Phenolic Interactions

Many studies have investigated the interaction between soy glycinin (SG), the most abundant protein in soybeans (Rawel *et al*., 2002), and phenolic compounds. It has been shown that polyphenols react differently with the side chains of SG. The reactivity of quercetin and myricetin with tryptophan residues are greater than with chlorogenic-, caffeic-, and gallic acid, whereas flavone and apigenin showed no significant reactivity towards the tryptophan in SG (Rawel *et al*., 2002; Kroll *et al*., 2003). Caffeic acid is more reactive with the free amino groups and lysine side chains of SG than the other flavonoids (Rawel *et al*., 2002) while quercetin is the more reactive with cystein side chains of SG (Kroll *et al*., 2001). Structural changes of SG-phenol derivatives have been observed; the secondary structure (α-helix and β- sheet) was slightly decreased by flavones while apigenin, kaempferol and quercetin caused an increase in α-helix and β- sheet (Rawel *et al*., 2002). The tertiary structure of SG has been influenced by chlorogenic acid which produced a decrease in the molecular ellipicity, while quercetin, myricetin, caffeic- and

gallic acid produced an increase, and flavone as well as apigenin had no effect on SG (Rawel *et al*., 2002). These structural changes affected the surface properties of SG making it hydrophilic in nature (Rawel *et al*., 2002). SG exhibited a high affinity with isoflavones (genistin and malonylgenistin) compared with β- conglycinin (Speroni *et al*., 2010) and the strength of binding of SG with other phenolics can be ordered as follows: gallic acid > chlorogenic acid ≈ quercetin > myricetin > caffeic acid > kaempferol > apigenin > flavone (Rawel *et al*., 2002).

2.2.6 Muscle Proteins-Phenolic Interactions

Actin and myosin are the most known muscle proteins; however, limited information is available on muscle protein-phenolic interactions compared to other food proteins. One of the few studies reported that the affinity of flavonoids (kaempferol, quercetin, fisetin, genistein, epigallocatechin, and taxifolin) to actin was similar, and that hydrophobic interactions are far more relevant to actin binding than hydrophilic interactions (Bohi, *et al*., 2007). Flavonoids affected the conformation of actin, forming stable complexes with the actin (Bohi, *et al*., 2007). Investigation of myosin-tannins complexes showed that the largest part of tannins are liberated from the complexes, indicating unstable myosin-tannins complexes and a weak ability to form the complexes (Amarowicz, *et al*., 2009).

2.2.7 Cereal Proteins-Phenolic Interactions

Cereal proteins have great structural diversity that affects the ability to bind various phenolic compounds. Kafirin is the major protein found in sorghum (*Sorghum bicolor*), representing about 50-60% of total protein (Emmambux and Taylor, 2003). Ferulic acid and catechin as well as extracted polyphenols from condensed tannin-free sorghum showed no ability to complex with kafirin; on the contrary, tannic acid and sorghum-condensed tannins showed high affinity to complex kafirin (Emmambux and Taylor, 2003).

Wheat gluten protein-phenolic interactions have been also studied; Labat *et al*. (2000) and Kaewtatip *et al*. (2010) showed that ferulic acid and coumaric acid hampered re-formation of disulfide bridge in gluten in dough. Tannic acid also influenced negatively gluten cross-linking but formed a complex with gluten (Zhang *et al*., 2010).

2.2.8 Interactions of Phenolic Compounds with Proteins in Oilseeds

Canola proteins have shown the ability to interact with polyphenols. Phenolic compounds (phenolic acids and condensed tannins) complex canola proteins predominantly by ionic bonding; however, binding between phenolic compounds and canola proteins by hydrophobic interaction, hydrogen bonding or covalent bonding is much weaker (Xu and Diosady, 2000). Sinapic acid complexed 12S canola globulin, the main protein in canola isolate, by electrostatic interactions at pH4.5 while thomasidic acid formed a complex with 12S canola globulin mainly by hydrophobic interactions at pH 7.0 and 8.5 (Rubino *et al*., 1996).

Helianthinin represents approximately 60% of the total sunflower protein; chlorogenic acid (CGA) and caffeic acid (CA) are the main polyphenols in sunflower (Suryaprakash and Prakash, 1995). CGA and CA, as well as quinic acid (QA) bind helianthinin mainly by hydrophobic and ionic/hydrogen bonding interactions (Kunanopparat *et al*., 2008; Tang and Wang, 2010). The maximum binding of CGA with helianthinin occurs at pH 6.0 (Suryaprakash and Prakash, 1995), 7.0 and 3.0 (Saeed and Cheryan, 1989).

2.2.9 Interactions of Phenolic Compounds with Enzymes

Several studies have investigated the interactions of polyphenols with enzymes. Reactions of polyphenols (caffeic acid, chlorogenic acid, ferulic acid, gallic acid, *m*-, *o*-, p-dihydroxybenzenes, quinic acid, *p*-benzoquinone, and cyaniding-3-glucoside) with α-amylase, trypsin and lipase illustrated that the activity and the conformational changes of the enzymes are adversely influenced by phenolic compounds, and that both hydrogen bonding and hydrophobic interactions take place (Rohn *et al*., 2002; Rhon *et al*., 2006; Raghavendra *et al*., 2007; Wiese *et al*., 2009).

2.2.10 Interactions of Phenolic Compounds with Collagen and Gelatin

Collagen the major compound of connective tissue, presenting in skin, tendons, bone, and membrane was investigated with quercetin in terms for binding and conformational changes; quercetin binds to collagen weakly within the hydrophobic pockets of collagen and surround the protein by hydrogen bondings (Yang *et al*., 2009). The conformation of protein was not influenced although there was a slight decrease in the helix structure content of collagen (Yang *et al*., 2009).

Gelatin interactions with tannic acid and sorghum tannins stronger complex formation compared with olive oil- extracted phenolics, grape seed proanthocyanidins, mimosa 5-deoxy proanthocyanidins, and tea catechins; hydrogen bonding plays a major role in formation of the complexes (Pripp *et al.*, 2005; Frazier *et al.*, 2010).

2.2.11 Egg White Proteins-Phenolic Interactions

Egg white proteins, representing more than 80% of egg white, include 54-65% ovalbumin, 12-13% conalbumin, 11% ovomucoid, 1.5-3.5% ovomucin, 3.4-3.5% lysozyme, 2% G2 and G3 ovoglobulins, and other minor proteins e.g., avidin and cystatin (Stevens, 1991; Kovacs-Nolan *et al.*, 2005; Wu and Acero-Lopez, 2011). These proteins play an important role in egg-containing foods due to their nutritional and functional properties; where egg white proteins have high bioavailability and content of essential amino acids, and show foaming and gelling properties (Stevens, 1991; Guerrero-Legarreta and Hui, 2010; Hoppe, 2010). Selected properties of egg white proteins are listed in Table 2.1.

Table 2.1 Selected properties of major egg white proteins

Protein	%(w/w)	pI	M_w(kDa)	T_d(^{o}C)	Cysteines	-SH	S-S	% of carbohydrate
Ovalbumin [a,b,c]	54	4.5-4.9	45	75-84	6.0	4	1.0	3.05
Conalbumin [a,b,c]	12	6.0-6.1	77.7	61-65	30		15	2.6
Ovomucoid [a,b,c]	11	4.1	28.0	77	18		9	16.5-32.6
Ovomucin [a,b,c]	3.5	4.5-5	5000-8000		2.0			13
Lysozyme [a,b,c]	3.4	10.7	14.3-14.6	69-77	6.0		4	0
G2 ovoglobulin [a,c]	1.0	4.9-5.3	47-49					
G3ovoglobulin [a,c]	1.0	4.8-5.8	49-50					
Ovoflavoprotein [a,c]	0.8	4.0	32-35		5.0		2	11
Cystain [a,c]	0.05	5.1, 6.5	12-13.1					0
Avidin [a,c]	0.05	10.0	55-68.3		2.0		1	7

Source:
a- (Guerrero-Legarreta and Hui, 2010)
b- (Hoppe, 2010)
c- (Stevens, 1991)

The major functional protein of egg albumin is ovalbumin that comprises the majority of egg white protein (Figure 2.3) (Guerrero-Legarreta and Hui, 2010; Hoppe, 2010). Ovalbumin is widely used as a standard preparation in protein studies (Huntington and Stein, 2001). It is a monomeric phosphoglycoprotein with sequence of 385 amino acids which form the secondary structure (41% α-helix, 34% β-sheet, 13% random coils, and 12% β-turns) and the tertiary structure (Stevens, 1991; Huntington and Stein, 2001; Guerrero-Legarreta and Hui, 2010; Hoppe, 2010). It is suggested that two kinds of oligosaccharide (high mannose-type and hybrid-type) are linked to asparagine (293 and 317) located in amino acids sequences of ovalbumin, whereas two phosphorylation sites are located at serines 69 and 345 (Stevens, 1991; Huntington and Stein, 2001). Although ovalbumin belongs to the serpin superfamily, it shows inhibitory inactivity toward protease (Huntington and Stein, 2001). Peptides of ovalbumin inhibit growth of *Bacillus subtilus*, *Candida albicans, E. coli,* and other microbes, and also control hypertension, enhance immune responses for cancer immunotherapy, and diminish lipid oxidation (Kovacs-Nolan *et al*, 2005).

Ovalbumin has also the ability to bind polyphenols and shows high affinity toward quercetin through hydrogen bonding, hydrophobic interactions, and electrostatic forces; ovalbumin-quercetin complexes are unstable with increasing temperature and the binding changes the conformation of ovalbumin (Lu *et al*., 2009).

Conalbumin is the second major protein, 12-13%, in egg white with molecular weight 77-80 kDa (Stevens, 1991) (Figure 2.4). It is a glycoprotein with 686 amino acids, containing 15 disulfide linkages. It has the ability to bind ferric ions and it has two globular lobes which contain an iron-binding site for each lobe (Wu and Acero-Lopez, 2011). Besides its ability to bind and transport iron ions, conalbumin shows antimicrobial effects against *E. coli*, *Salmonella* spp and *Candida albicans* growth (Kovacs-Nolan *et al*., 2005; Wu and Acero-Lopez, 2011).
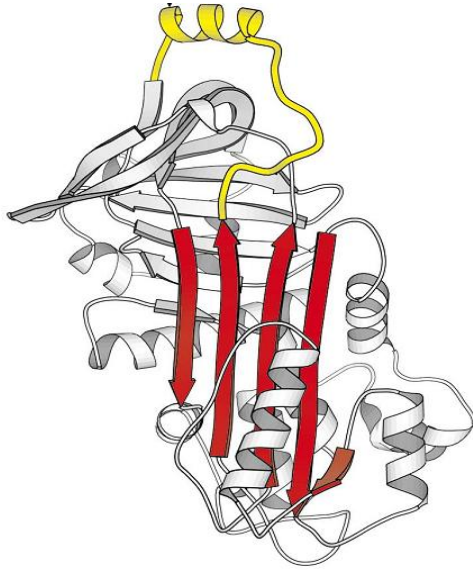
Figure 2.3 The 3-D structure of ovalbumin (Huntington and Stein, 2001).
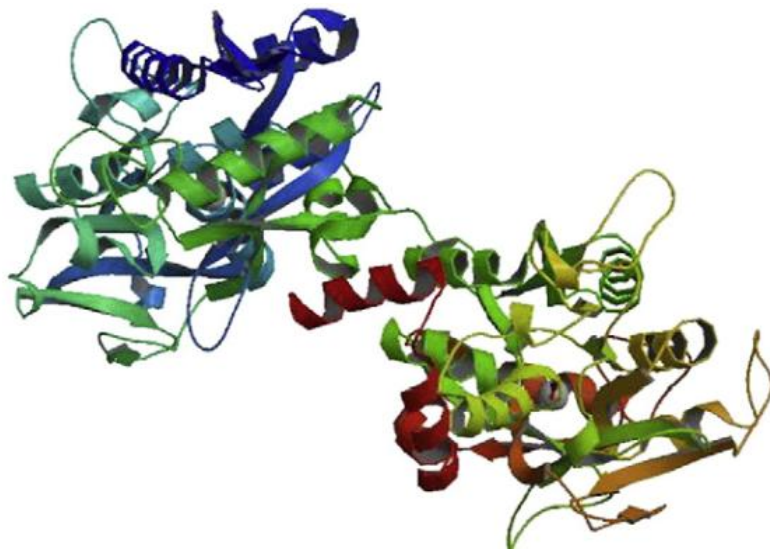
Figure 2.4 The 3-D structure of conalbumin (Wu and Acero-Lopez, 2011)

2.2.12 Effect of Protein-Phenolic Interactions on Food Quality

Food manufacturers continuously try to improve food quality (e.g. sensory attributes, nutritional, and microbial quality) through either preventing or allowing formation of substances that can reduce or enhance food quality. Polyphenols are examples of substances that can influence negatively or positively food quality. Polyphenols, in some fruits and vegetables, can be converted enzymatically to brown pigments, and this enzymatic browning leads to loss of nutritional, functional and organoleptic quality of food (Rawel *et al*., 2001; Altunkaya, 2011). In addition, conversion of ferulic acid to guaiacol can cause an off–flavor in fruit juices and in some milk products (in-bottled sterilized milk, and Gouda cheese) due to *p*-cresol produced by some bacteria (O'Connell and Fox, 2001). It has been suggested that at high levels, polyphenols may be responsible for enzyme-catalyzed discoloration in different cheeses such as Gorgonzola, Camembert, Brie, Emmental, Provolone, Romano, and Parmesan, and for undesirable flavour, e.g., sharp, medicinal and sheepyard (O'Connell and Fox, 2001). Ferulic acid reduces bread volume produced from hard wheat (Koh and Ng, 2009). Polyphenols also have positive effect on food quality. Perumalla and Hettiarachchy (2011) reported that green tea extract (tea catechins) can inhibit lipid oxidation in various foods such as raw and cooked meat, poultry products and fish, whereas grape seed extract that contains oligomeric proanthocyanidins reduce rancid flavour development in some meat products. Phenolic compounds have the ability to protect proteins from oxidation; thiol groups oxidation has been inhibited by caffeic acid in fish (Lund *et al*., 2011) while pink bark phenolic compounds, anthocyanins, and cranberry proanthocyanidins protect tryptophan oxidation by formation protein-phenolic complexes (Salminen and Heinonen, 2008). Polyphenols can enhance the flavour quality of cocoa beans at pH 7.0-7.5 (Noor-Soffalina *et al*., 2009). Whey proteins can prevent enzymatic browning in fresh-cut lettuce and apples by competing against polyphenols oxidase for phenolics or by interacting with phenol oxidation products such as o-quinones produced by polyphenols oxidase (Altunkaya, 2011). The ability of polyphenols to complex milk proteins enhances dairy products quality where the heat stability of milk was enhanced by addition of caffeic acid (O'Connell and Fox, 1999). Storage stability of concentrated milk, sterilized milk, milk powder, and canned milk-shakes was improved by using polyphenols such as hesperidin,

quercetin, catechol, rutin, naringin and hesperidin methyl chalcone (O'Connell and Fox, 2001). Inhibition of oxidative rancidity in margarine, milk powder, and ghee by catechin, catechol, resorcinol, quercetin, and kaempferol has been reported (O'Connell and Fox, 2001). Good quality rice bran oil can be achieved using chlorogenic acid and caffeic acid which prevent oxidation by inhibition of lipase (Raghavendra *et al*., 2007). In addition, the interaction between tannic acid and gluten protein produced from Chinese wheat, delays bread staling and enhance dough and bread quality (Zhang *et al*., 2010). Polyphenols can also enhance microbial quality of food products; O'Connell and Fox (2001) reported that polyphenols e.g., ferulic acid, tea catechins, oleuropein, ellagic acid, and p-coumaric acid inhibit growth of various foodborne pathogens including *Salmonella enteritidis, Listeria monocytogenes, Staphylococcus aureus,* as well as fungi in milk.

CHAPTER 3
MATERIALS AND METHODS

3.1 Materials

Ovalbumin (from chicken egg white 62-88%, 9006-59-1), conalbumin (from chicken egg white, 1391-06-6), flavone (2-phenyl-1-benzopyran-4-one, 525-82-6), chrysin (5,7-Dihydroyflavone, 480-40-0), quercetin (3,3',4',5,7-pentahydroxyflavone, 117-39-5), rutin (quercetin-3-rutinosidehydrate, 207671-50-9), protease (from *Bacillus Licheniformi*, 9014-01-1), trypsin type XI, E.C. 3.4.21.4 (from bovine pancrease, 9002-07-7) and chymotrypsin type II, E.C. 3.4.21.1 (from bovine pancrease) were purchased from Sigma-Aldrich Chemical Canada, Ltd.

Hen's egg produced at Pat McDiarmid et Fils Inc. farm was purchased from a local grocery store and used within 24 hours. All other chemicals were of analytical-reagent grade without further purification.

3.2 Egg Protein-Phenolic Interactions

Working solutions of ovalbumin and conalbumin (1.0×10^{-4} mol/L) were prepared by dissolving appropriate amounts of ovalbumin and conalbumin in phosphate buffer pH 7.4. Appropriate amounts of phenolics (flavone, chrysin, quercetin and rutin) were dissolved in methanol-water solution (1:4, v/v) to obtain 10×10^{-4} mol/L. The phenolic solutions were diluted to 2.5, 5, 7.5 $\times10^{-4}$ mol/L and 1ml of egg protein solutions was added to 2 ml of phenolic solutions for the reactions between the phenolics and ovalbumin and conalbumin derivatives.

Egg white was separated from egg yolk manually; 1 g of egg white and egg yolk were diluted in 4 ml phosphate buffer pH 7.4, and 1 ml of egg white and egg yolk suspension was added to 2 ml phenolic solutions. Control samples of egg proteins were prepared by adding 2 ml of phosphate buffer pH 7.4 to 1 ml of egg protein solutions with no phenolics. All samples were incubated at 37 ℃ for 24 h.

3.3 Egg Protein- Phenolic Interactions in a Muffin Model

3.3.1 Preparation of Muffin

The following recipe was used to prepare muffin: 43 g butter, one egg ($\approx$ 45 g), 111 g sugar, 164 g wheat flour, 140 ml milk and 10 g backing powder (Titlisbusykitchen, 2009).

The selected phenolics (flavone, chrysin, quercetin, and rutin) were added to muffin dough at a ratio of 100, 400, 1000 mg phenolics/Kg muffin. The selected phenolics were mixed manually with the whole egg for 3 min, then the other ingredients were added sequentially with continuous mixing during the addition each ingredient. The dough was cooked at 150 ℃ for 30 min. Control muffin was prepared under the same conditions without addition of the selected phenolics.

3.3.2 Extraction of Muffin Proteins

The cooked muffin was crushed manually and 20 g of the crushed muffin was added to 45 ml Tris-HCl, pH 6.8 and stirred for 3 h at room temperature. The mixture was centrifuged at 8000 rpm for 30 min. Figure 3.1 shows the extraction steps for muffin proteins.

20 g of cooked muffin

↓

Extract with Tris-HCl, pH 6.8 for 3 h at 25 ℃

↓

Centrifugation for 30 min

Precipitate   Supernatant (muffin protein extract)

↓

Analysed by electrophoresis
and protein digestibility
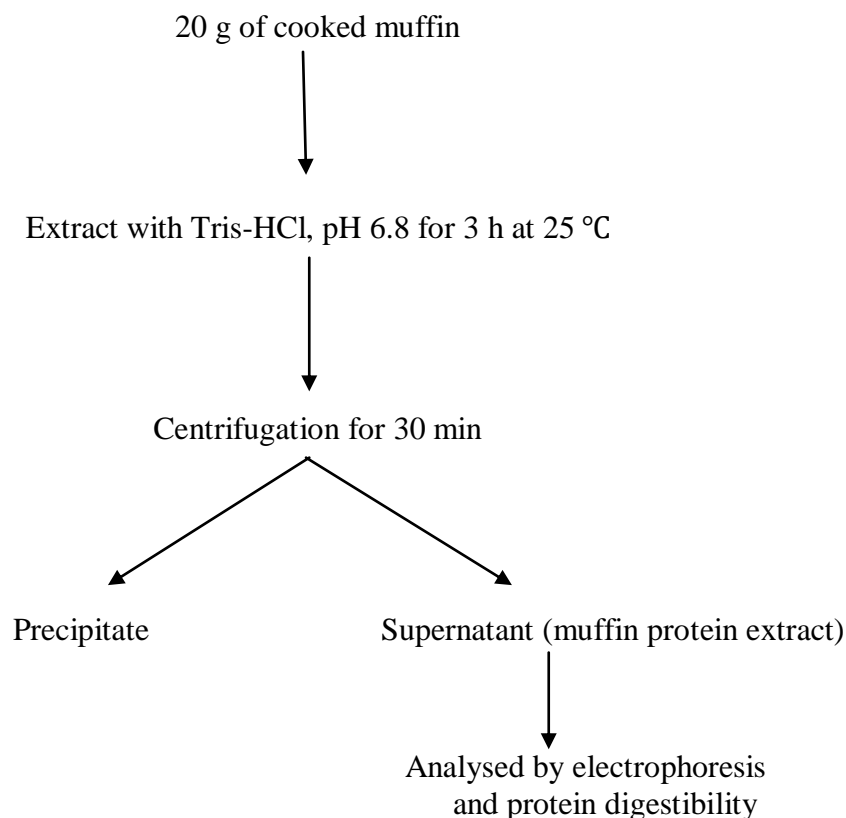
Figure 3.1 Extraction steps of muffin proteins

3.4 Characterization of Egg Protein-Phenolic Products and Muffin Protein Extract

3.4.1 Polyacrylamide Gel Electrophoresis (PAGE)

Sodium dodecyl sulfate-PAGE (SDS-PAGE), and native-PAGE were performed according to the procedures described by Laemmli (1970) and Davis (1964), respectively.

3.4.1.1 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

3.4.1.1.1 Sample Preparation

100 µl egg protein-phenolic reaction mixtures and 400 µl muffin protein extract were diluted in SDS reducing sample buffer (glycerol, 0.5% bromophenol blue, 2-mercaptoethanol, 0.5 M Tris-HCl, pH 6.8 and 10% SDS, and distilled water). Samples were heated at 95 ^{0}C for 5 min before loading 15 µl into sample wells. SDS-PAGE high-range protein standard (BIO-RAD 161-0317, Hercules, CA) was diluted in SDS reducing

sample buffer by ratio 1:20 respectively, and heated at 95 ^{0}C for 5 min before loading 10 µl into a sample well; the SDS-PAGE high-range protein standard contained myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (22 kDa), lysozyme (14 kDa) and aprotinin (7 kDa).

3.4.1.1.2 Gel Preparation

Tris- acrylamide gel consisting of 12% separation gel was prepared with the following reagents: distilled water, 30% acrylamide, 1.5 M Tris-HCl, pH 8.8, 10% SDS solution, 10% ammonium persulafate (APS) and tetramethylethylene-diamine (TEMED). 4% acrylamide in stacking gel was prepared with the same reagents except using Tris-HCl, pH 6.8 (instead of Tris-HCl, pH 8.8). A gel sandwich was assembled into a casting stand according to manufacturer's instructions. 12% separating gel was poured into gel sandwich and allowed to polymerize within 10 min, followed by 1-2 ml of 4% stacking gel to fill gel sandwich, followed by insertion of a comb into the sandwich. 15 µl egg protein reaction mixtures, 20 µl of muffin protein extract samples and 10 µl of SDS-PAGE high-range protein standard was loaded into the wells.

3.4.1.1.3 Electrophoresis Running and Staining and Destaining Conditions

The gels were run in SDS Tris-glycine buffer pH 8.3 for 1-1.5 h, at a constant voltage of 120 V/gel using a mini protein III electrophoresis cell unit (Bio-Rad, Hercules, CA). The gels were stained for 16 h in Coomassie Brilliant Blue R-250 solution, then destained by fixing solution (70% distilled water, 20% methanol and 10% acetic acid solution) and scanned after removing the blue background colour.

3.4.1.2 Native- Polyacrylamide Gel Electrophoresis (Native-PAGE)
3.4.1.2.1 Sample Preparation

100 µl ovalbumin-phenolic and conalbumin-phenolic mixtures, 80 µl egg white-phenolic and egg yolk-phenolic mixtures and 400 µl muffin protein extract were diluted in Native sample buffer (glycerol, 0.5% bromophenol blue, 0.5 M Tris-HCl, pH 6.8 and distilled water) before loading 20 µl into sample wells. High-molecular weight protein standard for native electrophoresis (GE Healthcare Ltd. 17-0445-01, UK) was diluted in

100 µl native sample buffer and loaded into a sample well. High-molecular weight protein standard for native electrophoresis contains the following proteins: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa) and bovine serum albumin (66 kDa).

3.4.1.2.2 Gel Preparation

10% polyacrylamide gradient separation gel was prepared with the following reagents: distilled water, 30% acrylamide, 1.5 M Tris-HCl, pH 8.8, 10% ammonium persulafate (APS) and tetramethylethylene-diamine (TEMED). 4% acrylamide in stacking gel was prepared with the same reagents except using Tris-HCl, pH 6.8 instead of Tris-HCl, pH 8.8. A gel sandwich was assembled into a casting stand according to manufacturer's instructions. 10% separating gel was poured into gel sandwich and allowed to polymerize within 10 min, followed by 1-2 ml 4% stacking gel to fill gel sandwich and inserting a comb into the sandwich to form sample wells. 20 µl egg protein-phenolic reaction mixtures and muffin protein extract samples and 10 µl of high molecular weight protein standard for native electrophoresis were loaded into the wells.

3.4.1.2.3 Electrophoresis Running and Staining and Destaining Conditions

The gels were run in Tris-glycine buffer pH 8.3 for 1.5-2 h at a constant voltage of 120 V/gel using a mini protein III electrophoresis cell unit (Bio-Rad, Hercules, CA). The gels were stained for 18 h in solution of Coomassie Brilliant Blue R-250 then destained by fixing solution (70% distilled water, 20% methanol and 10% acetic acid solution) and scanned after removing the blue background colour from the gels.

3.5 Investigation on Mechanism of Egg Protein-Phenolic Interactions

3.5.1 Fluorescence Quenching

Fluorescence quenching was carried out according to the method of Xiao *et al*., (2008). Appropriate quantities (1×10^{-4} mol/L) phenolics solution were added to 1.0 ml egg protein solutions (ovalbumin solution, conalbumin solution, egg white protein solution, egg yolk protein solution). The resultant mixture was subsequently incubated at pH 7.4 and 37 ℃ for 24 h. Ovalbumin and conalbumin concentrations were fixed at

1×10^{-6} mol/L and the fluorescence emission spectra were recorded in the range of 300-450 nm following an excitation at 279 nm. The fluorescence quenching data were analyzed using the Stern-Volmer equation 3.1 (Lu *et al*., 2009), while binding data analysis was calculated by double-logarithm equation 3.2 (Xiao *et al*., 2009).

$$F_0/F = 1+ Ksv[Q]……………………………(3.1)$$

where:
F_0 and F are fluorescence intensities in the absence and presence of quencher respectively
Ksv = Stern-Volmer quenching constant
[Q] = Concentration of quencher

$$\text{Log} \frac{F0-F}{F} = \log Ka + n \log [Q]…………….. (3.2)$$

where:
F_0 = the fluorescence intensity in the absence of the quencher.
F = the fluorescence intensity in the presence of the quencher.
Ka = the binding constant.
n   = the number of binding sites.
[Q] = the quencher concentration.

3.6 Preparation of Enzymatic Hydrolysates from Egg Proteins and Muffin Proteins

3.6.1 Enzymatic Hydrolysis of Egg Protein-Phenolic Products and Muffin Protein Extracts

Egg protein-phenolic reaction products and muffin extract were digested by two-enzyme mixture (trypsin and chymotrypsin) and protease (from *Bacillus licheniformis*) according to the method of Adebiyi *et al*., (2008). Egg protein-phenolic products and muffin protein extracts were adjusted to pH 8.0 using sodium phosphate buffer (pH 8.5) and incubated at 37 ℃ (enzyme: substrate ratio = 1:20). At time intervals between 0 to 120 min, 0.5 ml of incubation mixtures were removed after 0, 30, 60, 90, 120 min digestion and the enzymatic reaction was stopped by heating at 95 ℃ for 10 min, followed by centrifugation by (Beckman centrifuge, model J2-21) and filtration.

3.6.2 Determination of Degree of Hydrolysis

The degree of hydrolysis of egg protein-phenolic products and muffin protein extracts was measured by the procedure of Ayad (2010). 50 µl hydrolysates was mixed with 2 ml of o-phthadialdehyde (OPA) reagent prepared using 0.1 M sodium tetraborate, 20% SDS, β- mercaptoethanol, 40 mg OPA dissolved in 1 ml methanol. The mixture was incubated for 2 min at room temperature and the absorbance was measured at 340 nm (Beckman Coulter, model DU 800 spectrophotometer). The degree of hydrolysis (DH) of egg protein-phenolic products and muffin extracts was calculated by the equation 3.3 (Ayad, 2010).

DH (%) = (MW Δ_{340nm}) / (d. e. p) × 100………………………….. (3.3)

where:
 MW = Average molecular weight of amino acids (120)
Δ_{340nm} = Absorbance at 340 nm
d = Dilution factor
e = Average molar absorption of amino acids (6000 M^{-1} cm^{-1})
p = protein concentration

Protein concentration of muffin protein extract, egg white and egg yolk were determined by the Dumas method, AOAC method 968.06 (AOAC. 2005), using a TruSpec nitrogen analyzer (LECO Co). 0.1 g freeze-dried samples was weighed in nitrogen-free combustion boat and inserted into automated sample loader. After combustion process, nitrogen gas (N_2) was released, and measured by a conductivity cell. Nitrogen content was converted to crude protein by the conversion factor 6.25.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Egg Protein-Phenolic Interactions in Aqueous Media

4.1.1 Electrophoresis and Fluorescence Quenching

4.1.1.1 Ovalbumin

SDS-PAGE and native-PAGE results of ovalbumin-phenolic products at 5×10^{-4} molL^{-1} phenolic concentration are shown in Figures 4.1A and 4.1B, respectively. The interaction between ovalbumin and selected phenolic compounds flavone, chrysin, querctin, and rutin in lanes 2, 3, 4, and 5 respectively did not show any change in the electrophoretic behaviour compared to ovalbumin only. Ovalbumin and the ovalbumin-phenolic reaction products SDS-PAGE gave two bands: conalbumin with molecular weight 77KDa and ovalbumin 45KDa, while native-PAGE showed four bands: conalbumin 78 kDa and b1, b2, b3 which are three ovalbumin isoforms. Desert *et al.,* (2001) reported that these ovalbumin isoforms differ in its phosphorylation degrees: the di-phosphorylated form b1, mono-phosphorylated form b2, and non-phosphorylated form b3. At phenolic concentration of 10×10^{-4} molL^{-1}, ovalbumin-phenolic products analyzed by SDS-PAGE and native-PAGE showed no differences in their electrophoretic behaviour (Figure 4.2A and 4.2B) compared to 5×10^{-4} molL^{-1} phenolic concentration (Figure 4.1A and 4.1B). The elecrophoretic results are in agreement with Rawel *et al.*, (2002) who reported that flavone and apigenin have no effect on elecrophoretic behaviour of soy glycinin, and in contrast with Rawel *et al.*, (2002) who reported in the same study that quercetin and kaempferol changed the elecrophoretic behaviour of soy glycinin.

The quenching effect of phenolics at 5×10^{-4} molL^{-1} phenolics concentration observed from fluorescence spectra of ovalbumin is shown in Figure 4.1C. Rutin showed the highest fluorescence intensity quenching, followed by querctin, chrysin, and flavone. Rutin resulted in 24% quenching, whereas querctin quenched 20%, flavone and chrysin quenched only 7.5% and 14% of ovalbumin fluorescence respectively. These results

suggest that the phenolic compounds structure have an effect on the environment of aromatic amino acids residues (Lu *et al*., 2009).

At phenolics concentration of 10×10^{-4} molL^{-1}, fluorescence spectra of ovalbumin-phenolic reaction products (Figure 4.2C) was quenched approximately 1.5-fold compared to the results obtained at 5×10^{-4} molL^{-1} phenolic concentration (Figure 4.1C). Rutin resulted in 39% quenching, whereas querctin quenched 33%, flavone and chrysin quenched 16% and 21% of ovalbumin fluorescence, respectively. Increase in phenolics concentration resulted in changes of the environment of tryptophan and tyrosine residues suggesting that the ovalbumin protein structure changed slightly as a result of the interaction with the phenolics (Lu *et al*., 2009); these changes cannot be observed in the electrophoretic behaviour of ovalbumin.

The effect of phenolic compounds on fluorescence spectra of ovalbumin is similar to that documented by Cao *et al*., (2009) who reported that baicalein, quercetin, baicalin and quercitrin decreased fluorescence intensity of bovine serum albumin.
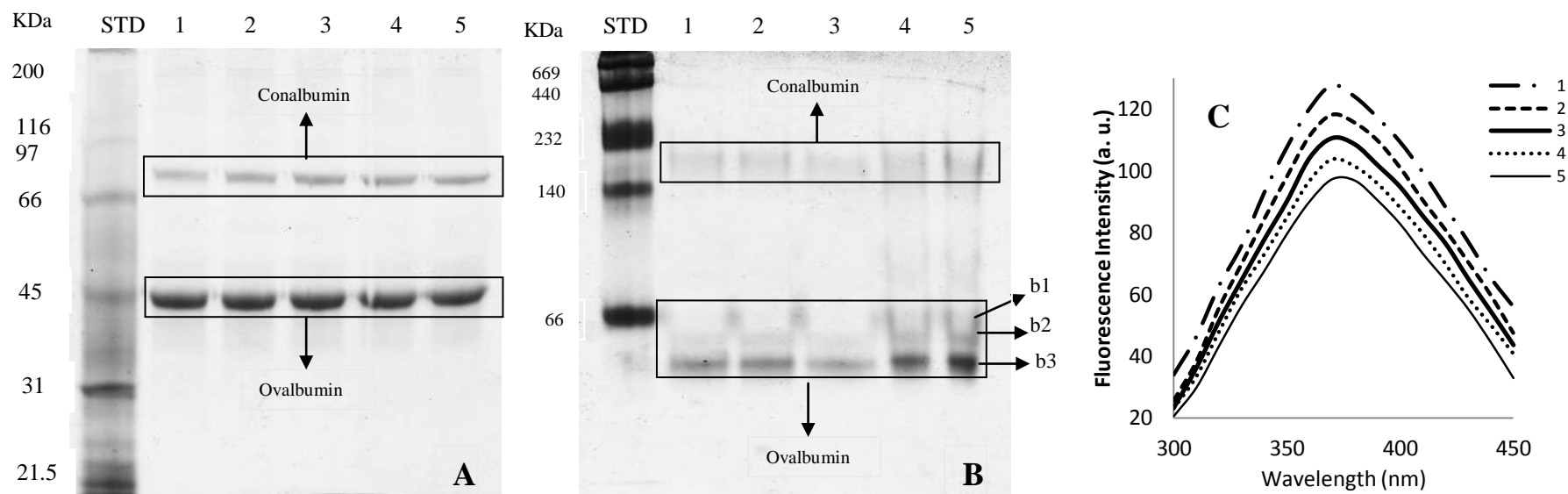
Figure 4.1 SDS-PAGE (**A**) and native-PAGE (**B**) ovalbumin-phenolic products; (**C**) the quenching effect of phenolics on fluorescence spectra of ovalbumin. STD= standard proteins; lanes: 1= control ovalbumin; 2= ovalbumin-flavone products; 3= ovalbumin-chrysin products; 4= ovalbumin-quercetin products; 5= ovalbumin-rutin products. Phenolics concentration is 5×10^{-4} mol L^{-1}.
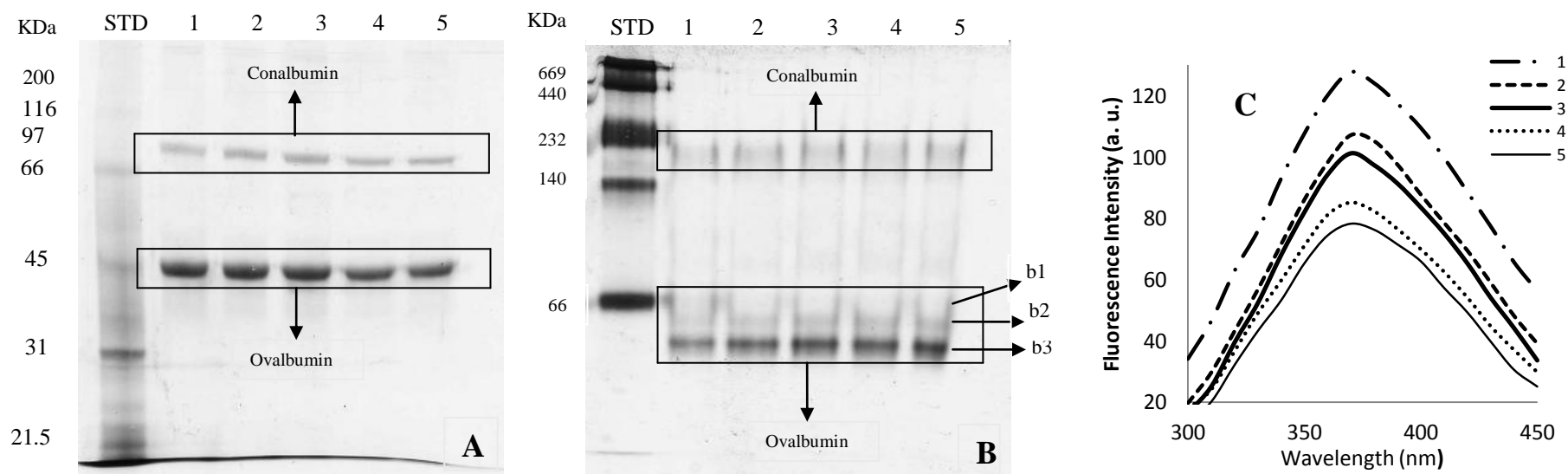
Figure 4.2 SDS-PAGE (**A**) and native-PAGE (**B**) of ovalbumin-phenolic products; (**C**) the quenching effect of phenolics on fluorescence spectra of ovalbumin. STD= standard proteins; lanes: 1= control ovalbumin; 2= ovalbumin-flavone products; 3= ovalbumin-chrysin products; 4= ovalbumin-quercetin products; 5= ovalbumin-rutin products. Phenolics concentration is 10×10^{-4} mol L^{-1}.

4.1.1.2 Conalbumin

SDS-PAGE and native-PAGE results of conalbumin-phenolic products at 5×10^{-4} molL^{-1} phenolics concentration are shown in Figure 4.3A and 4.3B, respectively. The results show one band which is conalbumin with molecular weight 78 kDa. SDS-PAGE results showed that the interaction of conalbumin with phenolic compounds flavone, chrysin, quercetin and rutin in lanes 2, 3, 4, and 5 respectively did not result in any change in the electrophoretic pattern compared to conalbumin (lane 1), while native-PAGE showed differences in electrophoretic behavior of the conalbumin-phenolic products. At phenolics concentration of 10×10^{-4} molL^{-1}, conalbumin-phenolic products analyzed by SDS-PAGE showed no differences in their electrophoretic behaviour (Figure 4.4A), while native-PAGE showed significant changes in electrophoretic behaviour of the conalbumin-phenolic products (Figure 4.4B). SDS-PAGE results are in agreement with Rawel et al., (2000) who reported that lysozyme derivitized with chlorogenic acid at pH 7.0 showed no changes in its electophoretic behaviour. However, Kroll et al., (2003) reported that the electrophoretic behaviour of myoglobin was influenced by quercetin and myricetin.

The quenching effect of 5×10^{-4} molL^{-1} phenolics concentration on fluorescence intensity of conalbumin is shown in Figure 4.3C. Rutin showed the highest fluorescence intensity quenching, followed by quercetin, chrysin and flavone. Rutin quenched 31% of conalbumin spectra, whereas flavone, chrysin and quercetin quenched only 11%, 17% and 24%, respectively. At phenolic concentration of 10×10^{-4} molL^{-1}, fluorescence intensity of conalbumin-phenolic reaction products was quenched approximately 2-fold (Figure 4.4C) compared to the results obtained at 5×10^{-4} molL^{-1} phenolics concentration. Rutin caused 58% quenching, whereas quercetin quenched 45%. Flavone and chrysin quenched 19% and 32% of conalbumin florescence, respectively. The fluorescence quenching results are similar to those documented by Hasni *et al*., (2011) who reported that catechin, epicatechin, epigallocatechin and epigallocatechin gallate quenched fluorescence intensity of casein.

Figure 4.3 SDS-PAGE (**A**) and native-PAGE (**B**) of conalbumin-phenolic products; (**C**) The quenching effect of phenolics on fluorescence spectra of conalbumin. STD= standard proteins; lanes: 1= control conalbumin; 2= conalbumin-flavone products; 3= conalbumin-chrysin products ; 4= conalbumin-quercetin products; 5= conalbumin-rutin products . Phenolics concentration is 5×10^{-4} mol L^{-1}.

Figure 4.4 SDS-PAGE (**A**) and native-PAGE (**B**) of conalbumin-phenolic products; (**C**) The quenching effect of phenolics on fluorescence spectra of conalbumin. STD= standard proteins; lanes: 1= control conalbumin; 2= conalbumin flavone products; 3= conalbumin-chrysin products ; 4= conalbumin-quercetin products; 5= conalbumin-rutin products. Phenolics concentration is 10×10^{-4} mol L^{-1}.

4.1.1.3 Egg White Proteins

Figure 4.5A and 4.5B show the SDS-PAGE and native-PAGE of egg white protein-phenolic reaction products at 5×10^{-4} molL^{-1} phenolics concentration, respectively. Egg white protein-phenolic products SDS-PAGE gave two bands corresponding to conalbumin (78 kDa) and ovalbumin (45 kDa), while native-PAGE showed four bands: conalbumin 78 kDa and b1, b2, and b3 which are three ovalbumin isoforms (Desert et al., 2001). SDS-PAGE of egg white proteins reacted with phenolic compounds flavone, chrysin and rutin in lanes 2, 3, and 5 respectively showed no differences in electrophoretic patterns of conalbumin and ovalbumin, whereas querctin induced a change in electrophoretic pattern of conalbumin (lane 4). The egg white protein-quercetin reaction products analyzed by native-PAGE showed a change in its electrophoretic behaviour compared to the other phenolics, while no change in the ovalbumin isoforms was observed. At phenolics concentration 10×10^{-4} mol L^{-1}, egg white protein-phenolic products analyzed by SDS-PAGE and native-PAGE showed similar electrophoretic behaviour (Figure 4.6A and 4.6B), respectively compared to 5×10^{-4} molL^{-1} phenolics concentration (Figure 4.5A and 4.5B). SDS-PAGE observations of egg white proteins are similar to those of whey proteins; Rawel *et al*., (2003) reported that quercetin influenced electrophoretic behaviour of whey proteins, while rutin showed no effect.

The quenching effect of phenolics on fluorescence spectra of egg white proteins at 5×10^{-4} mol L^{-1} phenolics concentration is shown in Figure 4.5C. Rutin showed the highest fluorescence intensity quenching, followed by quercetin, chrysin, and flavone. Rutin caused 25% quenching, whereas quercetin quenched 20%. Flavone and chrysin quenched 5.5% and 13%, respectively. At 10×10^{-4} mol L^{-1} phenolics concentration (Figure 4.6C), rutin, quercetin, chrysin and flavone quenched 41%, 35%, 22% and 13% of egg white proteins fluorescence spectra, respectively.

Figure 4.5 SDS-PAGE (**A**) and native-PAGE (**B**) of egg white protein (EWP)-phenolic products; (**C**) The quenching effect of phenolics on fluorescence spectra of EWP. STD= standard proteins; lanes: 1= control (EWP); 2= EWP-flavone products; 3= EWP-chrysin products; 4= EWP-quercetin products; 5= EWP-rutin products. Phenolics concentration is 5×10^{-4} mol L^{-1}.
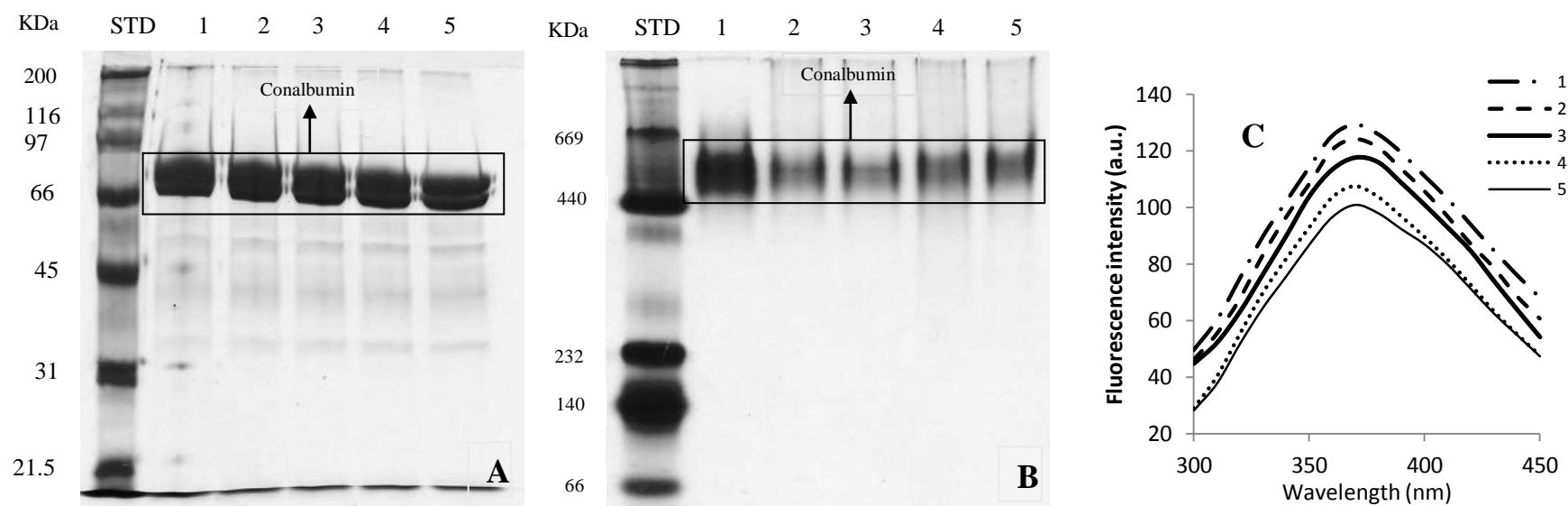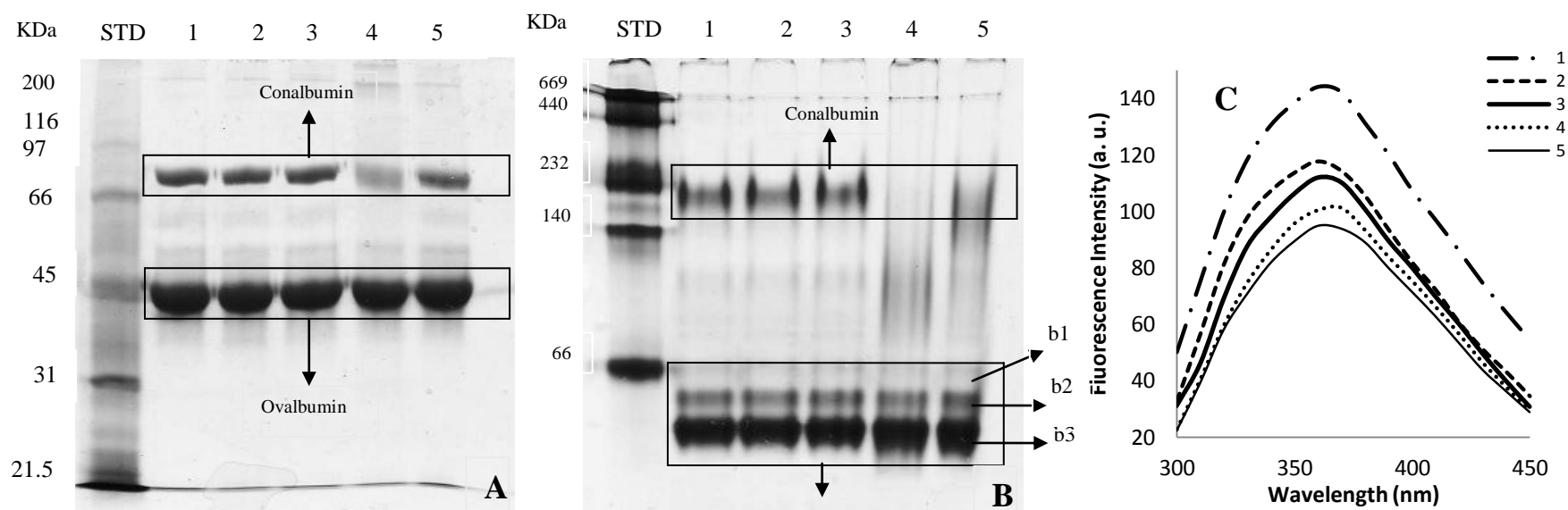
Figure 4.6 SDS-PAGE (**A**) and native-PAGE (**B**) of egg white proteins (EWP)-phenolic products. (**C**) The quenching effect of phenolics on fluorescence spectra of EWP. STD= standard proteins; lanes: 1= control (EWP); 2= EWP-flavone products; 3= EWP-chrysin products; 4= EWP-quercetin products; 5= EWP-rutin products. Phenolics concentration is 10×10^{-4} mol L^{-1}.

4.1.1.4 Egg Yolk Proteins
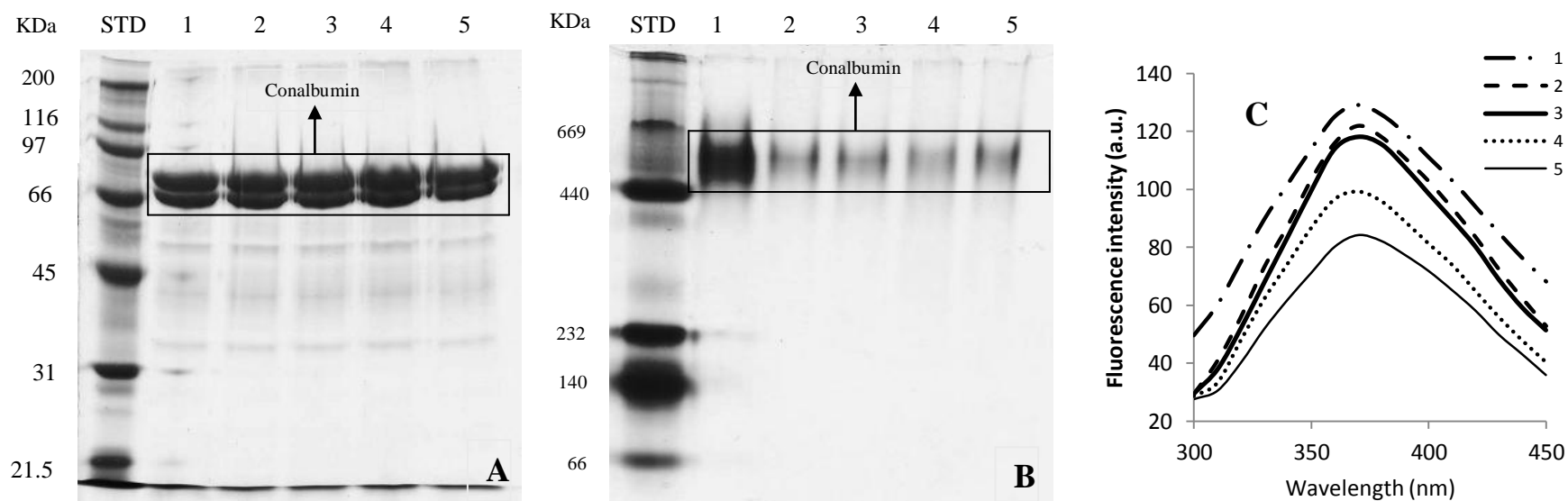
SDS-PAGE and native-PAGE results of egg yolk protein-phenolic products at 5×10^{-4} mol L^{-1} phenolic concentration are shown in Figures 4.7A and 4.7B, respectively. Egg yolk protein-phenolic reaction products SDS-PAGE showed the following subunits: band (a) apo-LDL with molecular weight 175 kDa, (b) apo-LDL 137 kDa and apo-HDL 105 kDa, (c) α-livetin 83 kDa, apo-HDL 79kDa, γ-livetin and apo-LDL 60-70 kDa, (d) apo-HDL 53 kDa, (e) phosvitin 46 kDa, (f) β-livetin 38-40 kDa and (g) apo-HDL 32 kDa (Laca *et al.*, 2010). The interaction of egg yolk proteins with phenolic compounds flavone, chrysin, quercetin and rutin in lanes 2, 3, 4 and 5, respectively did not show any change in the electrophoretic behavior compared to egg yolk proteins only. At phenolic concentration of 10×10^{-4} mol L^{-1}, egg yolk protein-phenolic products showed no differences in their electrophoretic behaviour (Figures 4.8A and 4.8B) compared to 5×10^{-4} mol L^{-1} phenolic concentration (Figures 4.7A and 4.7B).

The quenching effect of phenolics at 5×10^{-4} mol L^{-1} phenolic concentration on fluorescence spectra of egg yolk proteins is shown in Figure 4.7C. Rutin showed the highest quenching, followed by querctin, chrysin, and flavone. Rutin caused 34% quenching, quercetin quenched 32%; flavone and chrysin quenched 9% and 26% of egg yolk proteins florescence, respectively. However, at 10×10^{-4} mol L^{-1} phenolic concentration, EYP fluorescence was quenched to 25%, 48.5%, 37% and 39% by flavone, chrysin, quercetin and rutin, respectively (Figure 4.8C).
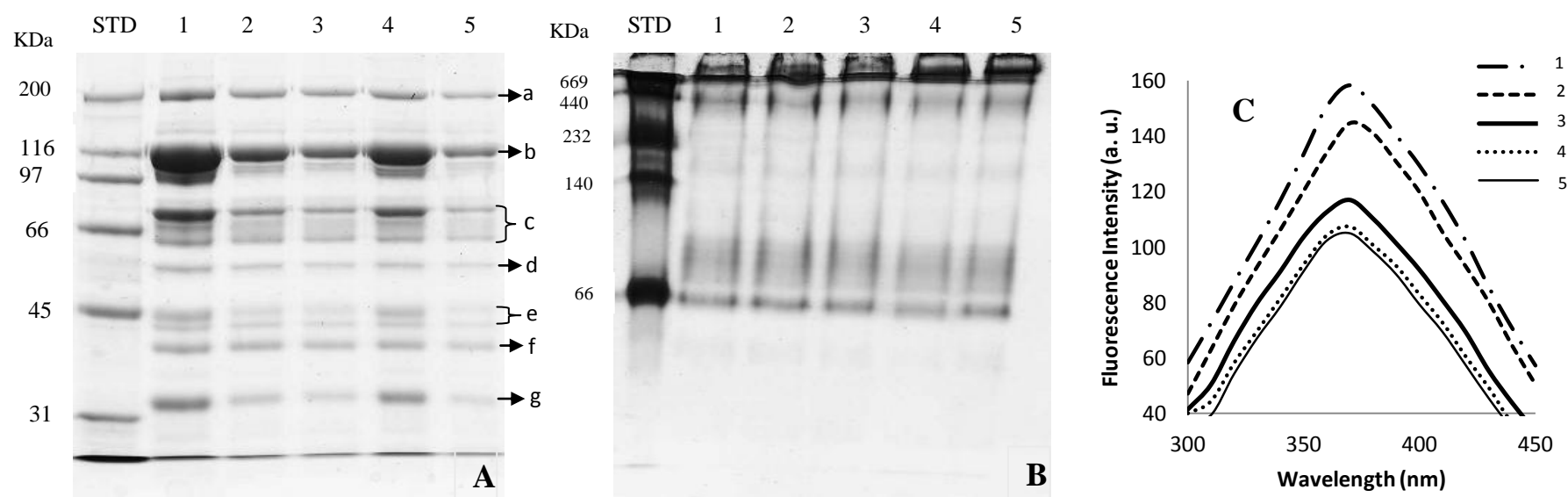
Figure 4.7 SDS-PAGE (**A**) and native-PAGE (**B**) of egg yolk proteins (EYP)-phenolic products. (**C**) The quenching effect of phenolics on fluorescence spectra of EYP. STD= standard proteins; lanes: 1= control (EYP); 2= EYP-flavone products; 3= EYP-chrysin products; 4= EYP-quercetin products; 5= EYP-rutin products. Phenolics concentration is 5×10^{-4} mol L^{-1}.
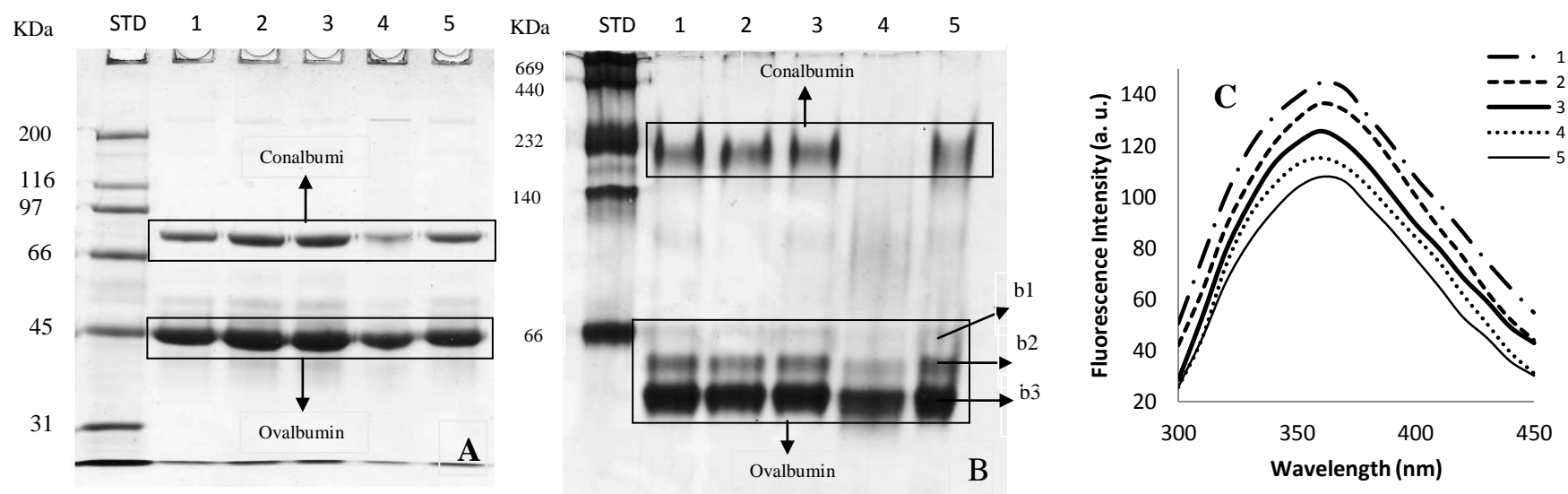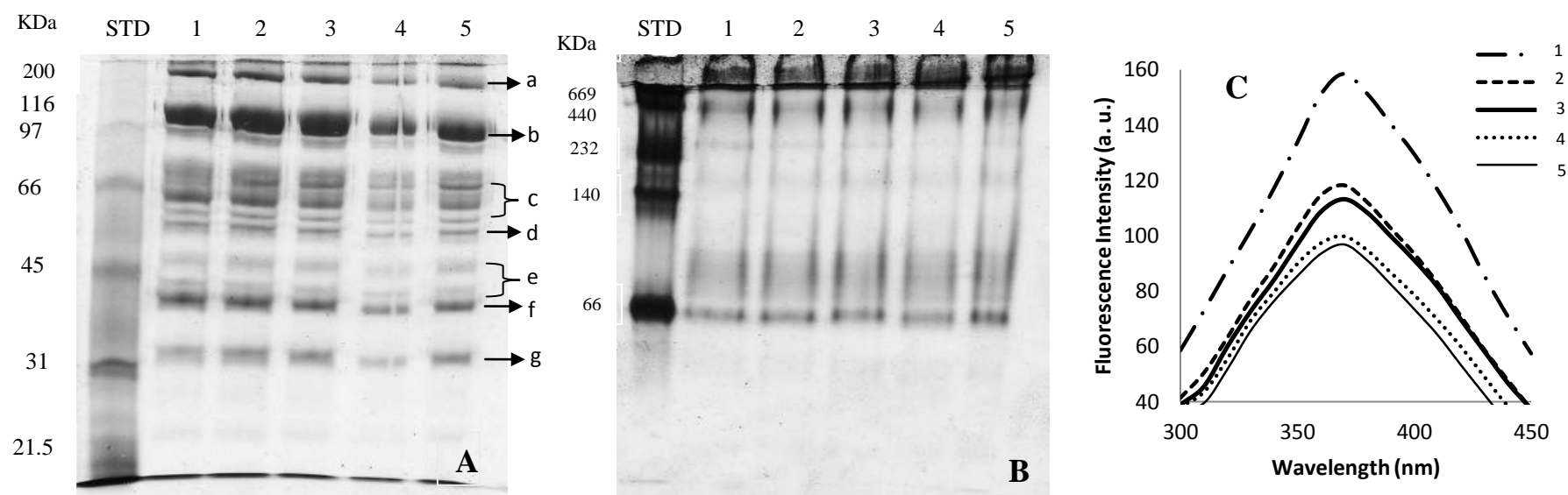
Figure 4.8 SDS-PAGE (**A**) and native-PAGE (**B**) of egg yolk proteins (EYP)-phenolic products. (**C**) The quenching effect of phenolics on fluorescence spectra of EYP. STD= standard proteins; lanes: 1= control (EYP); 2= EYP-flavone products; 3= EYP-chrysin products; 4= EYP-quercetin products; 5= EYP-rutin products. Phenolics concentration is 10×10^{-4} mol L^{-1}.

**4.1.2 Mechanism of Egg Protein-Phenolic Interactions**

4.1.2.1Fluorescence Quenching of Egg Protein-Phenolic Products

Figure 4.9 shows the Stern-Volmer curves (F/F$_0$ versus [phenolics]) for ovalbumin, conalbumin, egg white proteins and egg yolk proteins fluorescence quenching by (a) flavone, (b) chrysin, (c) quercetin, and (d) rutin. The Stern-Volmer quenching constant (Ksv) for phenolics ranges from 1.7×10^4 L/ mol to 5.3×10^4 L/ mol. The linear plots are not sufficient to define the fluorescence quenching mechanism. Hence, the fluorescence quenching mechanism was further confirmed by calculating the bimolecular quenching rate constant (K$_q$) using equation 4.1 (Lu *et al*., 2009):

K_q= Ksv/ τ_0 …………………(4.1)

where τ_0 denotes the average fluorescence lifetime of molecule without quencher, which is approximately 10^{-8} s (Lu *et al*., 2009). In Table 4.1, the bimolecular quenching rate constant (K$_q$) calculated was 100- 250 fold greater than the maximum value possible for limiting diffusion quenching constant of the biomolecule (K$_q$= 2×10^{10} L mol^{-1} s^{-1}) (Lu *et al*., 2009); this suggests that the quenching mechanism for egg proteins fluorescence by phenolics is of a static quenching, and the complexes formed by these interaction could be stable. These results are in agreement with the findings of other researchers; Lu *et al*., (2009) reported that fluorescence quenching of ovalbumin by quercetin is a static type. In addition, the nature of fluorescence quenching of bovine serum albumin by baicalin, baicalein, quercetin, quercitrin and puerarin is static (Xiao *et al*., 2007; Cao *et al*., 2009); however, the quenching mechanism of collagen by quercetin was dynamic quenching (Yang *et al*., 2009).
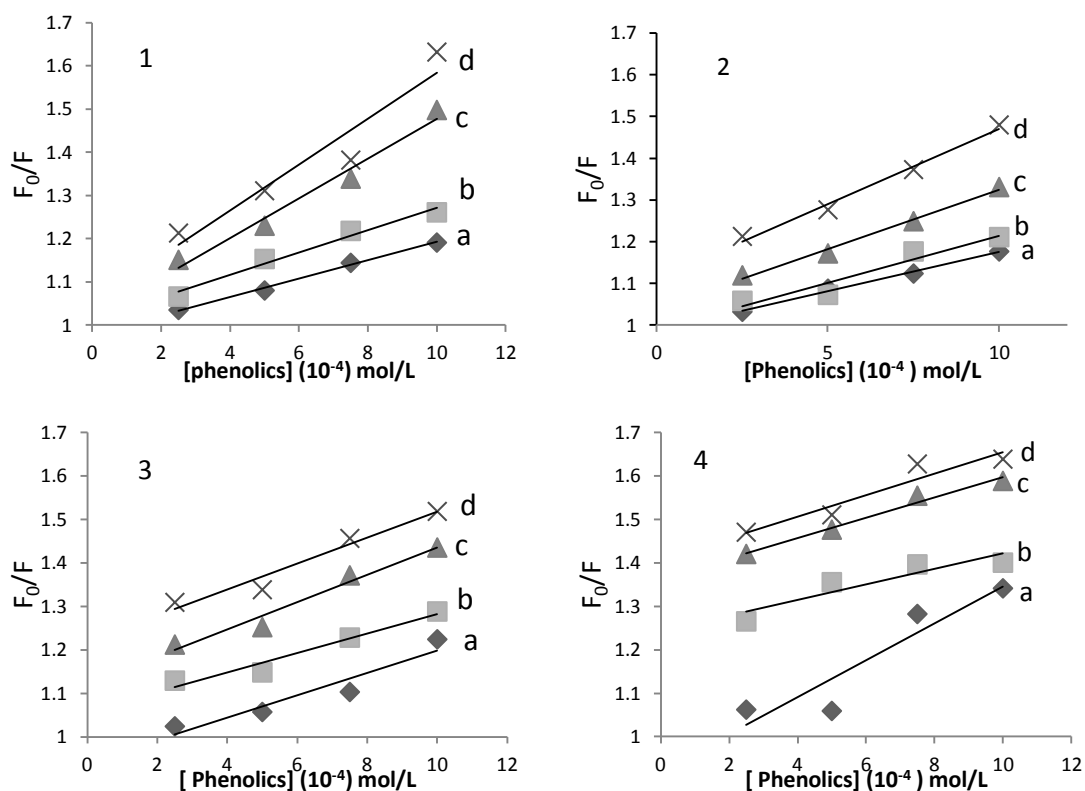
Figure 4.9 Stern-Volmer plots of fluorescence quenching of (1) ovalbumin (2) conalbumin (3) egg white proteins (4) egg yolk proteins by (a) flavone (b) chrysin (c) quercetin (d) rutin at 37 ^{0}C and pH 7.4.

Table 4.1 Stern-Volmer quenching constants, for egg proteins interaction with phenolics at 37 ^{0}C and pH 7.4

Egg proteins	Phenolics	Ksv (10^6 L mol^{-1})	Kq (10^{12} L mol^{-1} S^{-1})
Ovalbumin	Flavone	0.0213	2.13
	Chrysin	0.0260	2.60
	Quercetin	0.0460	4.60
	Rutin	0.0531	5.31
Conalbumin	Flavone	0.0188	1.88
	Chrysin	0.0225	2.25
	Quercetin	0.0285	2.85
	Rutin	0.0359	3.59
Egg white proteins	Flavone	0.0258	2.58
	Chrysin	0.0223	2.23
	Quercetin	0.0298	2.98
	Rutin	0.0315	3.15
Egg yolk proteins	Flavone	0.0424	4.24
	Chrysin	0.0178	1.78
	Quercetin	0.0233	2.33
	Rutin	0.0258	2.58

4.1.2.2 Binding Constant and Structure-Affinity Relationship

The double-logarithm curves of egg proteins-phenolic reaction products shown in Figure 4.10 and Table 4.2 give the apparent binding constants (K_a) and the binding site values (*n*). K_a and *n* values decreased with increasing hydroxylation (number of hydroxyl groups of phenolics) and glycosylation (sugar moiety at C-ring of rutin). The n values obtained (0.7-1.3) showed the high affinity sites, while the n values (0.2-0.4) showed the lowest high affinity sites (Xiao *et al*., 2009). These results suggests that hydrophobic interactions are dominant in egg protein- phenolic reactions due to presence of hydroxyl groups in chrysin and quercetin, which showed lower K_a and *n* values compared to flavone which has no hydroxyl group. These results are consistent with those obtained by other studies. Cao *et al*., (2009), Xiao *et al*., (2009) and Papadopoulou *et al*., (2005) reported that phenolic glycosides such as rutin, quercitrin, baicalin, genistin, daidzin and quercitrin lower the affinity for bovine serum albumin compared to phenolic aglycones such as quercetin, baicalein, genistein and myricetin, and that sugar moieties of phenolics tested may cause a steric hindrance or increasing polarity which prevent phenolics penetrating into hydrophobic region of bovine serum albumin. However, Xiao *et al*., (2008) and Hasni *et al*., (2011) suggested that hydroxylation of flavonoids (increasing number of hydroxyl groups) decrease the affinity for bovine serum albumin and caseins.
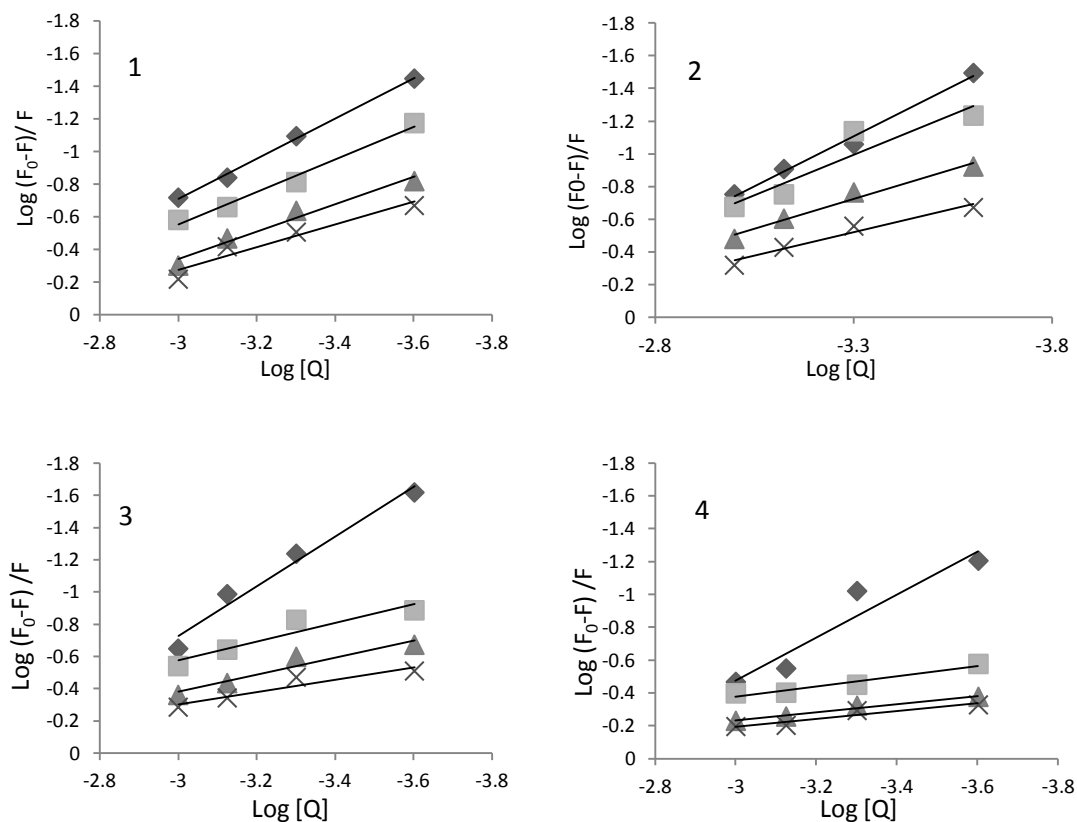
Figure 4.10 Double-logarithm Curves of ($\diamond$) flavone ($\blacksquare$) chrysin ($\triangle$) quercetin ($\times$) rutin effect on fluorescence of (1) ovalbumin (2) conalbumin (3) egg white proteins (4) egg yolk proteins at 37 ^{0}C , pH 7.4.

Table 4.2 The binding parameters for the system of phenolic-egg proteins at 37 ^{0}C, pH 7.4

Egg proteins	Phenolics	OH groups	Log Ka(L mol^{-1})	n
Ovalbumin	Flavone	Zero OH	2.9	1.2
	Chrysin	2 OH	2.4	0.9
	Quercetin	5 OH	2.1	0.8
	Rutin	4 OH	1.8	0.6
conalbumin	Flavone	Zero OH	2.9	1.2
	Chrysin	2 OH	2.2	0.9
	Quercetin	5 OH	1.6	0.7
	Rutin	4 OH	1.3	0.5
Egg white proteins	Flavone	Zero OH	3.9	1.5
	Chrysin	2 OH	1.2	0.5
	Quercetin	5 OH	1.1	0.5
	Rutin	4 OH	0.8	0.3
Egg yolk proteins	Flavone	Zero OH	3.4	1.3
	Chrysin	2 OH	0.7	0.4
	Quercetin	5 OH	0.6	0.3
	Rutin	4 OH	0.5	0.2

4.1.2.3 Effect of Phenolics Concentration on Ovalbumin and Conalbumin Fluorescence Spectra

Figure 4.11 shows the effect of increasing phenolics concentration on fluorescence intensity of ovalbumin. In all cases, the fluorescence intensity of ovalbumin decreased gradually with the increasing concentration of phenolics; these results indicate that phenolics can interact with ovalbumin and suggest that there are some changes in aromatic amino acids environment (Lu *et al*., 2009). Rutin produced the highest fluorescence quenching followed by quercetin, chrysin and flavone.

The effect of phenolic concentration on fluorescence intensity of conalbumin is shown in Figure 4.12. Fluorescence of conalbumin was quenched gradually with increasing concentration of phenolics; the effect was lower with flavone and chrysin compared to quercetin and rutin. Rutin caused the highest quenching for conalbumin fluorescence spectra followed by quercetin, chrysin and flavone. These results are in agreement with Cao *et al*., (2009), Lu *et al*., (2009) and Hasni *et al*., 2011 who reported that increasing concentration of phenolics resulted in a decrease in fluorescence intensity of ovalbumin, caseins and bovine serum albumin, suggesting that protein-phenolic complexes are affected by both the concentration and structure of phenolic compounds.
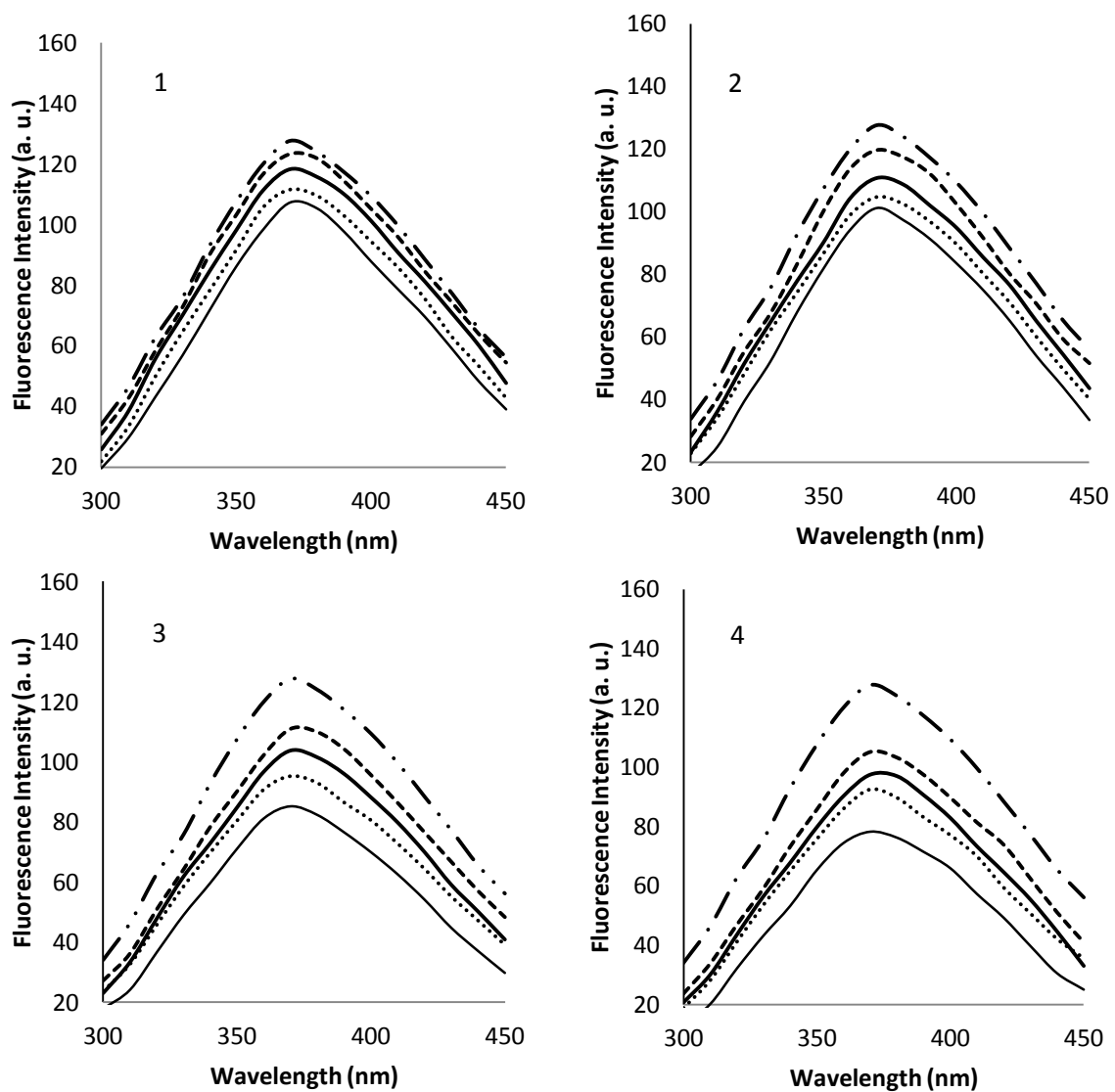
Figure 4.11 Effect of increasing concentration of phenolics on fluorescence spectra of ovalbumin; (—·—·) 0.00, (----) 2.5, (———) 5, (......) 7.5, (——)10 ($\times 10^{-4}$ mol/L) of (1) flavone (2) chrysin (3) quercetin (4) rutin.
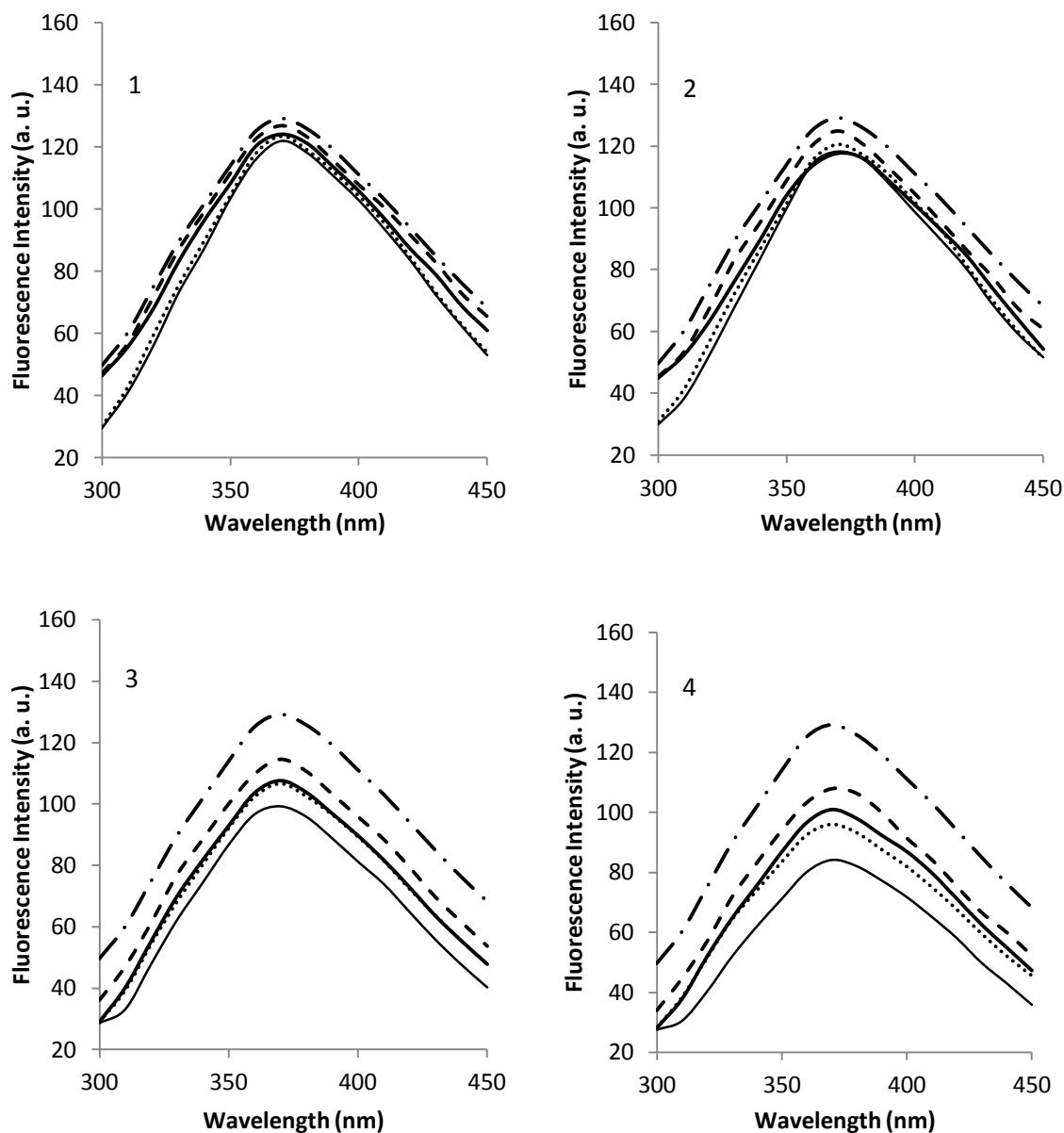
Figure 4.12 Effect of increasing concentration of phenolics on fluorescence spectra of conalbumin; (━ ・ ━ ・) 0.00, (- - - -) 2.5, (━━) 5, (......) 7.5, (——)10 ($\times 10^{-4}$ mol/L) of (1) flavone (2) chrysin (3) quercetin (4) rutin.

4.1.2.4 Effect of Phenolics Concentration on Egg White Proteins and Egg Yolk Proteins Fluorescence Spectra

The effect of phenolics concentrations on the fluorescence intensity of egg white proteins is shown in Figure 4.13. A decrease of the fluorescence intensity was observed when increasing concentration of phenolics solutions. The glycoside rutin (curve 4, Figure 4.13) quenched egg white proteins fluorescence greater than other phenolics studied. These results suggest that phenolics can bind egg white proteins, and the interaction of egg white proteins with phenolics is influenced by the concentration and structure of phenolic compounds (Xiao *et al.*, 2009).

Quenching effect of phenolics on egg yolk proteins is shown in Figure 4.14. Flavone, chrysin, quercetin and rutin decreased the fluorescence spectra of egg yolk proteins. Rutin quenched egg yolk proteins fluorescence greater than other phenolics followed by quercetin, chrysin and flavone. These results suggest that phenolics can bind egg yolk proteins, and the formation of complexes is influenced by the concentration and structure of phenolic compounds (Cao *et al.*, 2009).
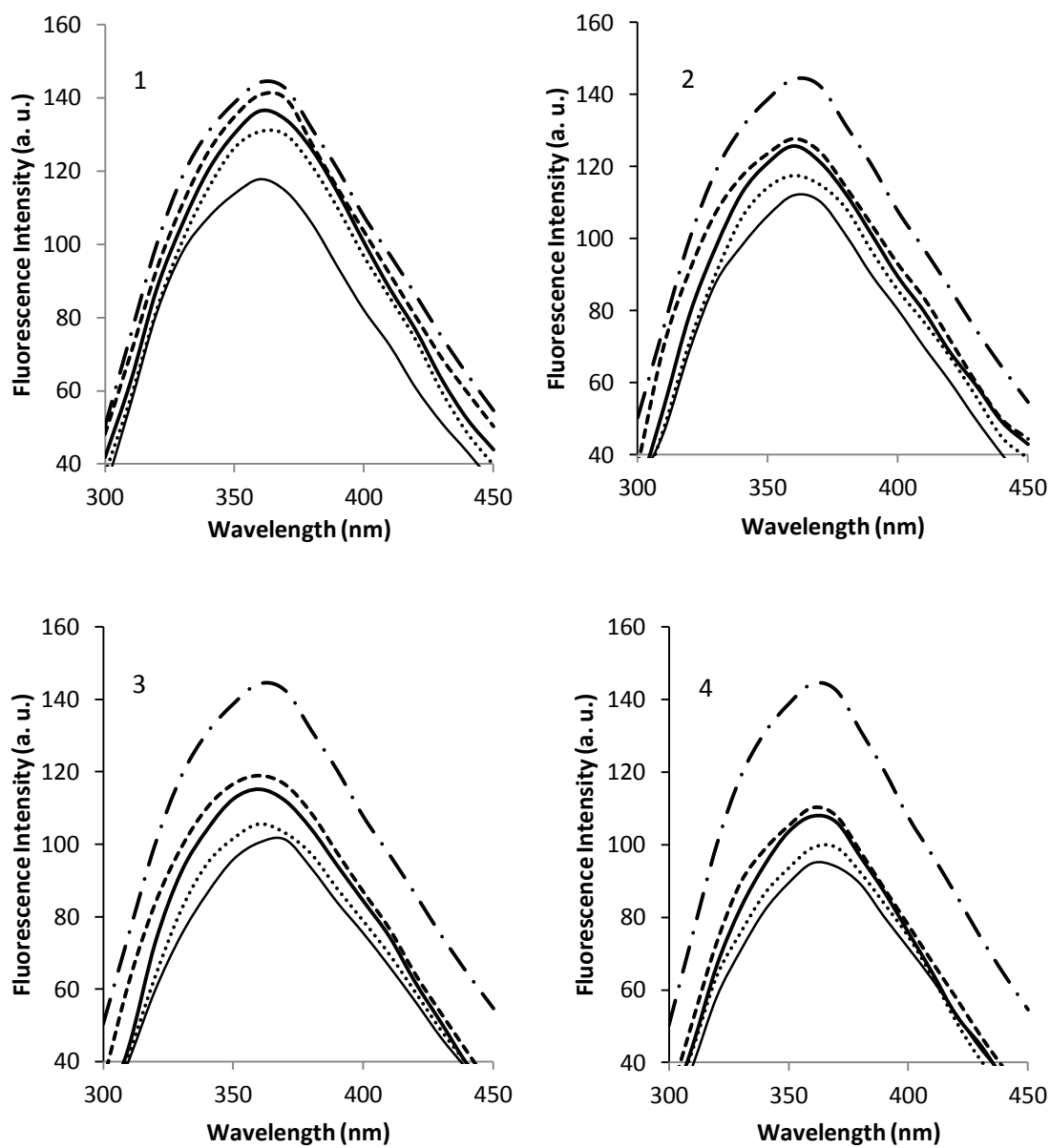
Figure 4.13 Effect of increasing concentration of phenolics on fluorescence spectra of egg white proteins; (**—·—·**) 0.00, (**----**) 2.5, (**——**) 5, (**......**) 7.5, (**——**)10 (× 10^{-4} mol/L) of (1) flavone (2) chrysin (3) quercetin (4) rutin.
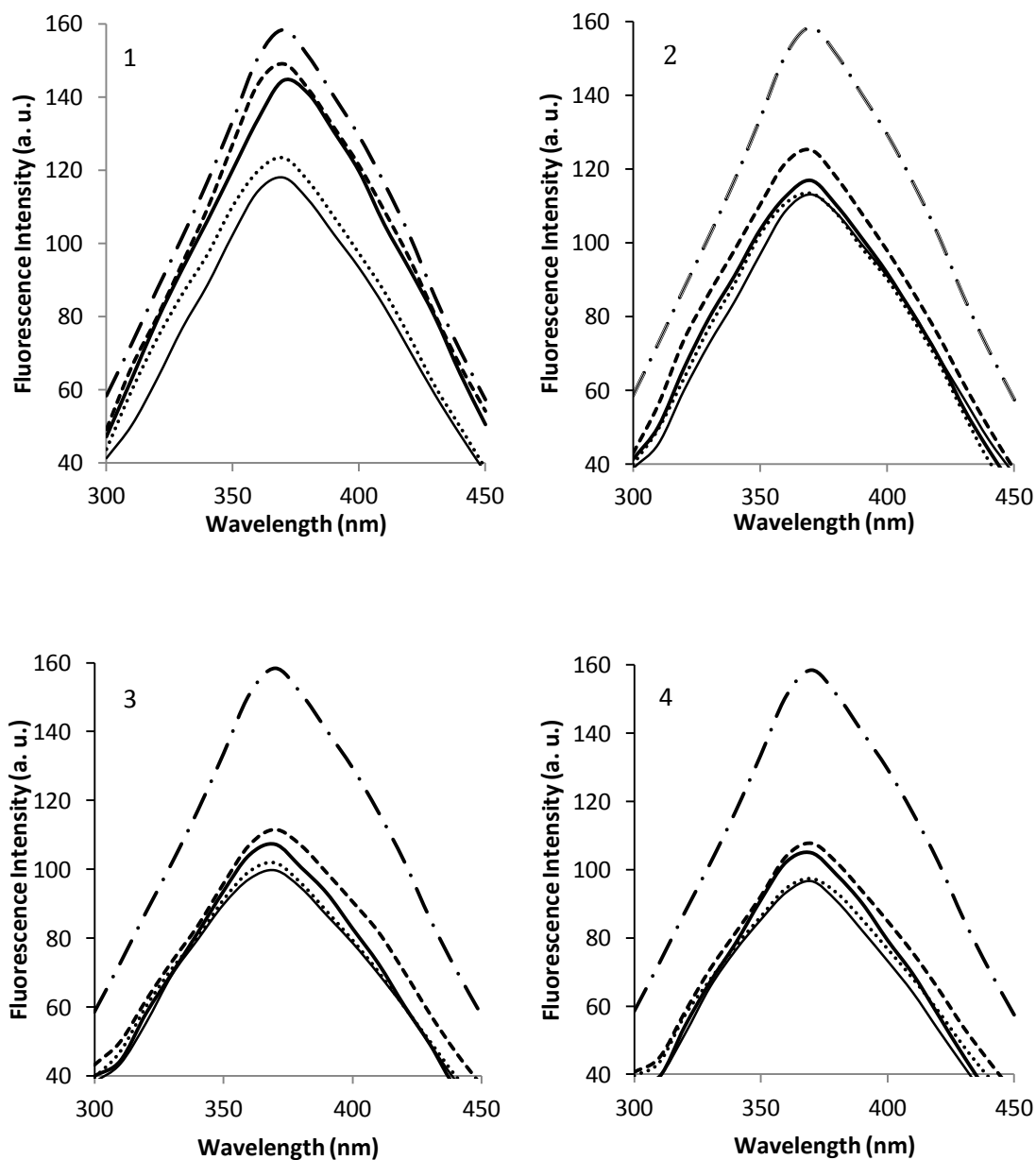
Figure 4.14 Effect of increasing concentration of phenolics on fluorescence spectra of egg yolk proteins; (**— · — · —**) 0.00, (**- - - -**) 2.5, (**———**) 5, (**......**) 7.5, (**———**)10 ($\times$ 10^{-4} mol/L) of (1) flavone (2) chrysin (3) quercetin (4) rutin.

4.1.3 Digestibility of Egg Protein-Phenolic Products *in vitro*

4.1.3.1 Enzymatic Hydrolysis of Egg Protein-Phenolic Products by Bacterial Protease

The effect of phenolic compounds on egg protein hydrolysis was investigated. Figure 4.15 and Table 4.3 show the degree of hydrolysis (DH) of non-heat treated (undenatured) egg proteins by bacterial protease. The DH of undenatured ovalbumin-phenolic reaction products decreased compared with non-denatured ovalbumin alone; with chrysin having the greatest effect on reducing DH of ovalbumin compared with the other phenolics. The effect of phenolics on DH of undenatured egg white proteins was reduced by all phenolic compounds; egg white protein-quercetin reaction product showed the lowest DH compared to the other phenolic compounds. The DH of conalbumin-phenolic reaction products and egg yolk protein-phenolic reaction products were affected to a lesser extent by the phenolic compounds.

After heat-denaturing egg protein-phenolic reaction products at 100 ℃ for 10 min, there was a decrease in DH of denatured egg proteins by bacterial protease (Figure 4.16 and Table 4.4). The results show that DH of denatured egg proteins was higher than that of undenatured egg proteins; in the presence of the phenolic compounds, the DH of egg proteins decreased. The phenolic compounds reduced the DH of denatured ovalbumin and egg white protein to a greater extent than that of denatured conalbumin and egg yolk protein. The reaction of denatured ovalbumin with quercetin and of denatured egg white protein with flavone showed the lowest DH compared to the other phenolic compounds. These results are similar to those obtained using other enzymes. Kroll *et al.*, (2003) reported that the derivitization of myoglobin by phenolic compounds flavone, apigenin, kaempferol, quercetin and myricetin affect negatively the chymotryptic hydrolysis of myoglobin. Lysozyme-chlorogenic acid derivatives were reported to be resistant to enzymatic hydrolysis using trypsin, chymotrypsin and pepsin enzymes (Rawel *et al.*, 2000).
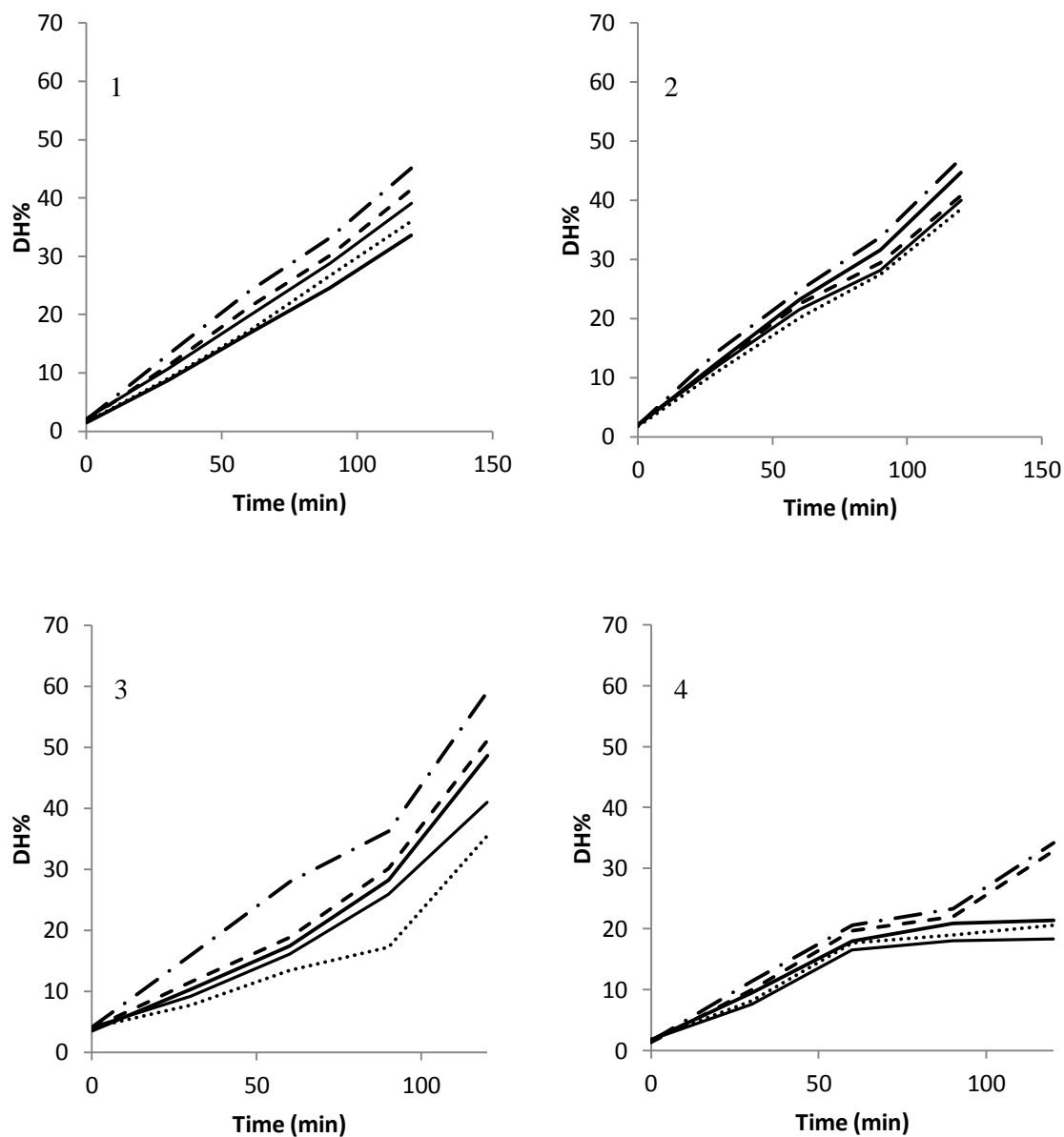
Figure 4.15 Enzymatic hydrolysis of phenolic reaction products of undenatured (1) ovalbumin, (2) conalbumin, (3) egg white proteins and (4) egg yolk proteins with (—·—·) control, (----) flavone, (——) chrysin, (.......) quercetin and (——) rutin by bacterial protease.
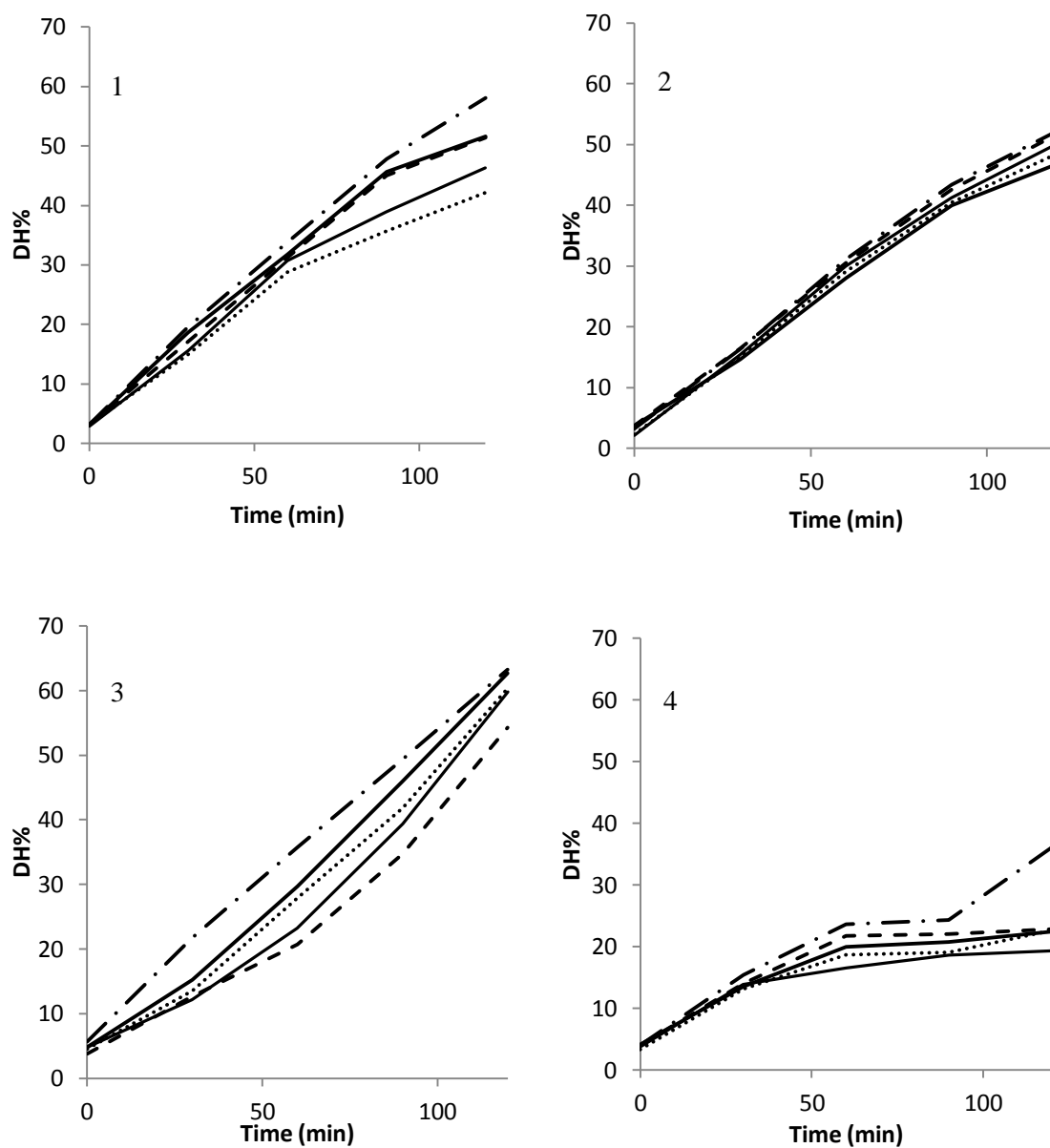
Figure 4.16 Enzymatic hydrolysis of phenolic reaction products of denatured (1) ovalbumin, (2) conalbumin, (3) egg white proteins and (4) egg yolk proteins with (▬ · ▬ · ▬ ·) control**, (- - - -)** flavone, (▬▬) chrysin, **(.......)** quercetin and (▬▬) rutin by bacterial protease.

Table 4.3 %DH of undenatured egg protein-phenolic products using bacterial protease

Egg protein-phenolic products	DH%				
	0min	30min	60min	90min	120min
Ovalbumin (control)	2	13.1	24	33.2	45.1
Ovalbumin + flavone	1.9	11.2	21.3	30.1	41.4
Ovalbumin + chrysin	1.5	8.7	16.8	24.6	33.6
Ovalbumin + quercetin	1.6	9	17.2	26.7	28.8
Ovalbumin + rutin	2.2	10.6	19.7	28.8	39.1
Conalbumin (control)	2.1	14.6	24.8	33.8	47.1
Conalbumin + flavone	2.3	12.5	22.5	29.4	40.8
Conalbumin + chrysin	2	12.7	23.2	31.6	44.7
Conalbumin + quercetin	1.8	11.2	20.1	27.5	38.5
Conalbumin + rutin	2	12.1	21.5	28.1	40
Egg white proteins (control)	4.1	15.9	27.9	36.2	59.1
Egg white proteins + flavone	3.9	11.5	18.8	30.1	51.1
Egg white proteins + chrysin	3.5	10.3	17.4	28.2	48.6
Egg white proteins + quercetin	4	7.7	13.4	17.2	35.5
Egg white proteins + rutin	4.1	9.2	16.1	25.9	41
Egg yolk proteins (control)	1.5	11.4	20.6	23.3	34.1
Egg yolk proteins + flavone	1.3	10	19.7	22	32.8
Egg yolk proteins + chrysin	1.7	9.5	18	20.9	21.4
Egg yolk proteins + quercetin	1.8	8.1	17.7	19	20.6
Egg yolk proteins + rutin	1.8	7.6	16.5	18	18.3

Table 4.4 %DH of denatured egg protein-phenolic products using bacterial protease

Egg protein-phenolic products	DH%				
	0min	30min	60min	90min	120min
Ovalbumin (control)	3.1	19.6	33.8	47.8	58.1
Ovalbumin + flavone	3.1	17.1	31.3	45	51.4
Ovalbumin + chrysin	3	18.7	31.8	45.6	51.6
Ovalbumin + quercetin	2.3	15	28.8	35.7	42
Ovalbumin + rutin	2.9	15.6	30.7	38.9	46.3
Conalbumin (control)	3.2	16.4	31.1	43.4	52.2
Conalbumin + flavone	3.7	16.5	30.5	42.6	51.7
Conalbumin + chrysin	3.7	14.6	28	40	46.8
Conalbumin + quercetin	2.3	15.1	29.1	40.5	48.5
Conalbumin + rutin	1.2	15.4	29.8	41.2	50.1
Egg white proteins (control)	5.7	21.8	35.7	49.3	63.2
Egg white proteins + flavone	3.8	12.7	20.5	34.6	54.3
Egg white proteins + chrysin	4.8	15.2	29.7	45.9	62.7
Egg white proteins + quercetin	4.6	13.5	27.9	41.8	60.3
Egg white proteins + rutin	4.8	12.2	23.1	39.4	59.8
Egg yolk proteins (control)	4	15.4	23.6	24.3	36.1
Egg yolk proteins + flavone	4.2	14	21.7	22	22.8
Egg yolk proteins + chrysin	4.1	13.5	20	20.7	22.4
Egg yolk proteins + quercetin	3.3	13.1	18.7	19.1	22.6
Egg yolk proteins + rutin	3.8	12	16.5	18.6	19.3

4.1.3.2 Enzymatic Hydrolysis of Egg Protein-Phenolic Products by Trypsin-Chymotrypsin Mixture

The enzymatic hydrolysis of undenatured egg protein-phenolic products *in vitro* by trypsin-chymotrypsin mixture is shown in Figure 4.17 and Table 4.5. In all cases, phenolic compounds reduced the degree of hydrolysis (DH) of egg proteins. The DH of undenatured ovalbumin-phenolic products decreased compared with undenatured control ovalbumin; chrysin caused the greatest effect on reducing DH of ovalbumin compared with the other phenolics. Egg white protein-flavone products shows DH values lower than the other phenolics. The lowest DH of conalbumin and egg yolk proteins was by quercetin and rutin, respectively.

The adverse effect of phenolic compounds on the enzymatic hydrolysis of egg proteins was observed after heating egg protein-phenolic products at 100 ℃ for 10 min (Figure 4.18 and Table 4.6). In all cases, the DH of denatured egg proteins was higher than that of undenatured egg proteins; in the presence of the phenolic compounds, the DH of egg proteins decreased. The phenolic compounds reduced the DH of denatured egg white proteins to a great extent than that of the other tested proteins. Denatured ovalbumin-chrysin reaction products showed the lowest DH compared to the other phenolics. Denatured conalbumin-quercetin reaction products and denatured egg yolk protein-chrysin reaction products showed the lowest DH compared to the other phenolics. The results obtained are in agreement with that suggested by other authors. Rawel *et al.*, (2002) suggested that phenolic compounds negatively affect the enzymatic hydrolysis of soy proteins, while the adverse effect of phenolic compounds on the tryptic and chemotryptic hydrolysis of whey proteins was documented by Rawel *et al.*, (2003).

It is known that trypsin enzyme breaks peptide bonds that contain the amino acids lysine or arginine in the protein sequence, while chymotrypsin breaks peptide bonds that contain tryptophan, tyrosine and phenylalanine; therefore, it can be suggested that the interaction of egg proteins with phenolic compounds may lead to changes in protein structure that can prevent the access to those peptide bonds targeted by trypsin and chyotrypsin enzymes (Rawel *et al.*, 2001; Rawel *et al.*, 2003)..
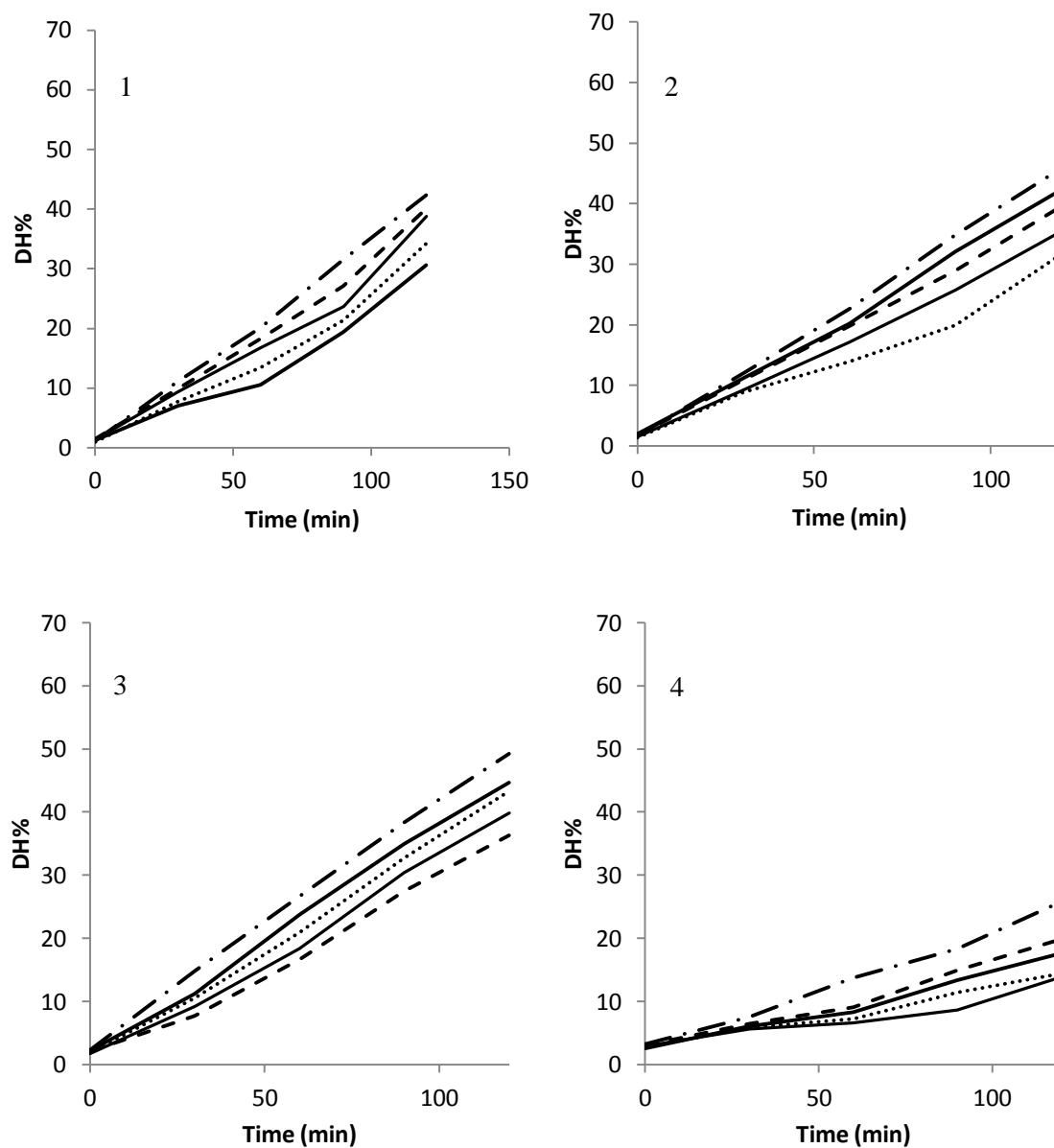
Figure 4.17 Enzymatic hydrolysis of phenolic reaction products of undenatured (1) ovalbumin, (2) conalbumin, (3) egg white proteins and (4) egg yolk proteins with (▬ · ▬ ·) control, (- - - -) flavone, (▬▬) chrysin, (…….) quercetin and (▬▬) rutin by trypsin-chymotrypsin mixture.
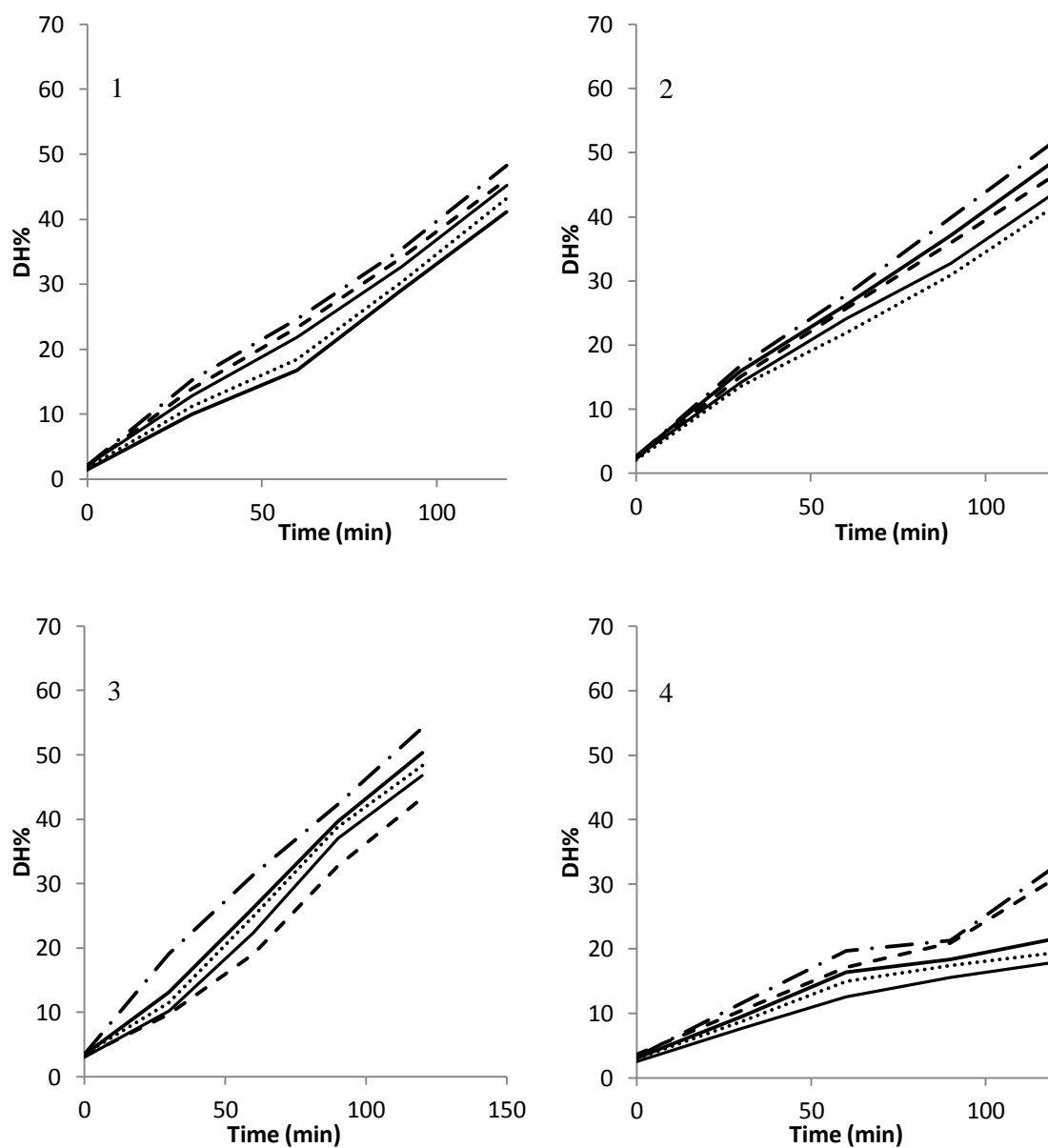
Figure 4.18 Enzymatic hydrolysis of phenolic reaction products of denatured (1) ovalbumin, (2) conalbumin, (3) egg white proteins and (4) egg yolk proteins with (– ∙ – ∙) control, (- - - -) flavone, (——) chrysin, (.......) quercetin and (——) rutin by trypsin-chymotrypsin mixture.

Table 4.5 %DH of undenatured egg protein-phenolic products using trypsin-chymotrypsin mixture

Egg protein-phenolic products	DH%				
	0min	30min	60min	90min	120min
Ovalbumin (control)	1.2	11.2	20.1	31.5	42.3
Ovalbumin + flavone	1.5	9.9	18.3	27.1	40.1
Ovalbumin + chrysin	1.3	7	10.5	19.4	30
Ovalbumin + quercetin	1.1	7.8	13.4	21.3	34.8
Ovalbumin + rutin	1.3	9.4	16.8	23.7	38.8
Conalbumin (control)	1.7	12	22.6	34.8	45.9
Conalbumin + flavone	1.8	10.9	19.8	28.9	39.6
Conalbumin + chrysin	2	11.2	20.1	32.1	42.3
Conalbumin + quercetin	1.4	8.9	13.9	19.8	31.7
Conalbumin + rutin	1.5	9.2	17.1	25.7	35.5
Egg white proteins (control)	2.3	14.8	26.7	38.3	49.2
Egg white proteins + flavone	2.1	7.7	16.7	27.4	36.3
Egg white proteins + chrysin	2.2	11.2	23.8	34.9	44.7
Egg white proteins + quercetin	1.9	10.5	20.9	32.7	43.3
Egg white proteins + rutin	1.7	9.2	18.4	30.4	39.8
Egg yolk proteins (control)	3.2	7.5	13.7	18.3	25.8
Egg yolk proteins + flavone	3	6.4	9.1	14.9	19.9
Egg yolk proteins + chrysin	2.6	6.1	8.3	13.3	17.6
Egg yolk proteins + quercetin	2.7	5.9	7.2	11.4	14.4
Egg yolk proteins + rutin	2.9	5.6	6.9	8.7	13.9

Table 4.6 %DH of denatured egg protein-phenolic products using trypsin-chymotrypsin mixture

Egg protein-phenolic products	DH%				
	0min	30min	60min	90min	120min
Ovalbumin (control)	2.1	15.2	24.6	35.4	48.3
Ovalbumin + flavone	2	13.9	23.3	34.1	46.1
Ovalbumin + chrysin	1.4	10	16.7	29.1	41.1
Ovalbumin + quercetin	1.7	11.2	18.4	30.3	43.2
Ovalbumin + rutin	2.1	12.8	21.8	32.7	45.2
Conalbumin (control)	2.6	16.8	27.7	39.8	51.9
Conalbumin + flavones	2.3	15.1	25.7	35.9	46.4
Conalbumin + chrysin	2.7	16	26.2	37.1	48.8
Conalbumin + quercetin	2.1	13.6	21.9	30.7	41.3
Conalbumin + rutin	2.5	14.2	24.1	32.7	43.9
Egg white proteins (control)	3.5	19.1	31.4	42.3	54
Egg white proteins + flavone	3.2	9.7	19.1	32.7	43.3
Egg white proteins + chrysin	3.4	13.1	26.3	39.6	50.3
Egg white proteins + quercetin	3.5	11.5	24.9	38.8	48.3
Egg white proteins + rutin	3	10.2	22.3	37	46.8
Egg yolk proteins (control)	3.2	11.5	19.7	21.3	32.8
Egg yolk proteins + flavone	3.6	10.4	17.1	20.9	30.9
Egg yolk proteins + chrysin	3	9.5	16.4	18.3	21.6
Egg yolk proteins + quercetin	2.9	8.7	14.9	17.4	19.4
Egg yolk proteins + rutin	2.5	7.6	12.5	15.6	17.9

4.2 Egg Protein-Phenolic Interactions in Muffin Model

4.2.1 Electrophoresis of Muffin Proteins

SDS-PAGE and native-PAGE analysis of muffin protein-phenolic reaction products at different phenolic concentrations are shown in Figure 4.19 and Figure 4.20, respectively. SDS-PAGE shows five bands as follows: band a with molecular weight 66kDa, band b (60.5kDa), band c (45kDa) which is ovalbumin, band d (35.7kDa) and band e (31.5kDa). At 100 mg phenolics concentration/Kg muffin, ovalbumin-phenolic reaction products analyzed by SDS-PAGE showed differences in their electrophoretic behavior with chrysin (lane 3), quercetin (lane 4); a slight change was observed with flavone (lane 2) and rutin (lane 5) compared to control muffin proteins (lane 1) (Figure 4.19A). Similar results were observed after increasing phenolics concentration to 400 and 1000 mg/Kg muffin (Figure 4.19B and 4.19C). The other bands did not show any change in their electrophoretic behaviour.

Native-PAGE results of muffin proteins with different concentration of phenolics: 100 mg/Kg muffin (Figure 4.20A), 400 mg/Kg muffin (Figure 4.20B) and 1000 mg/Kg muffin (Figure 4.20C) showed no differences in electrophoretic patterns of ovalbumin-phenolic products bands indicated by the rectangles. These results are in contrast with studies reported for other food products by other researchers; Jongberg *et al*., (2011) reported that electrophoresis investigations (SDS-PAGE) showed that cross-linkages formation decreased in myosin extracted from beef patties treated with white grape extract for beef patties containing 12% w/w low molecular weight phenolic compounds.
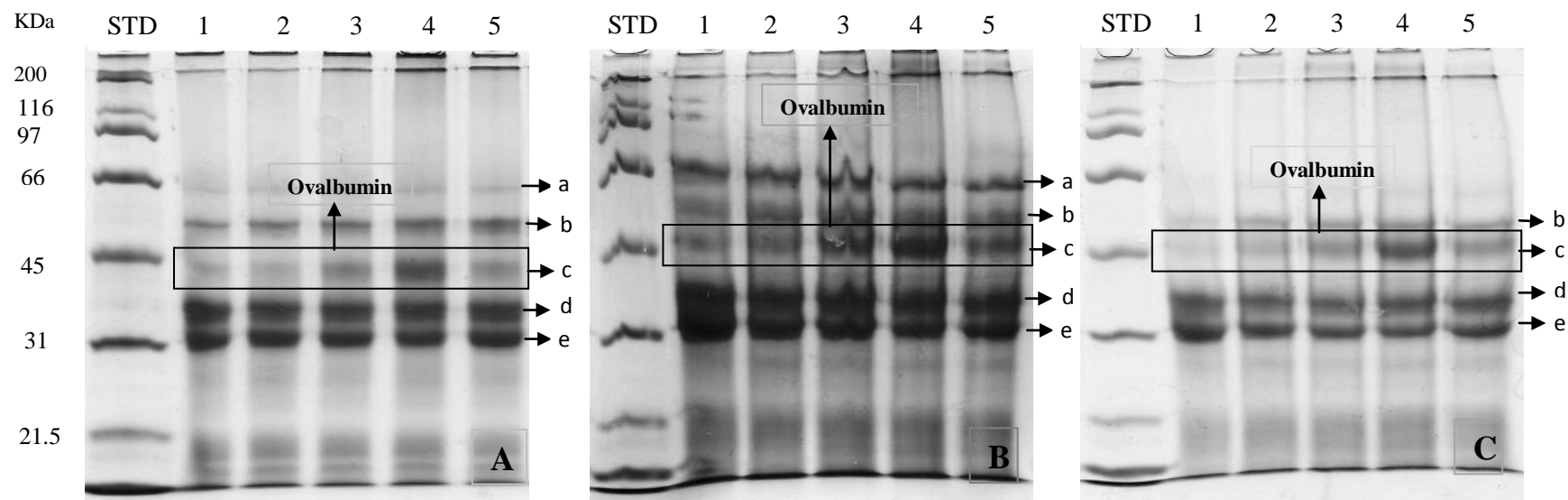
Figure 4.19 SDS-PAGE of muffin protein (MP)-phenolic products. STD= standard proteins; lanes: 1= muffin control; 2= MP-flavone products; 3= MP-chrysin products; 4= MP-quercetin products; 5= MP-rutin products; concentration of phenolics is (A) 100 mg/Kg muffin; (B) 400 mg/Kg muffin; (C) 1000 mg/Kg muffin.
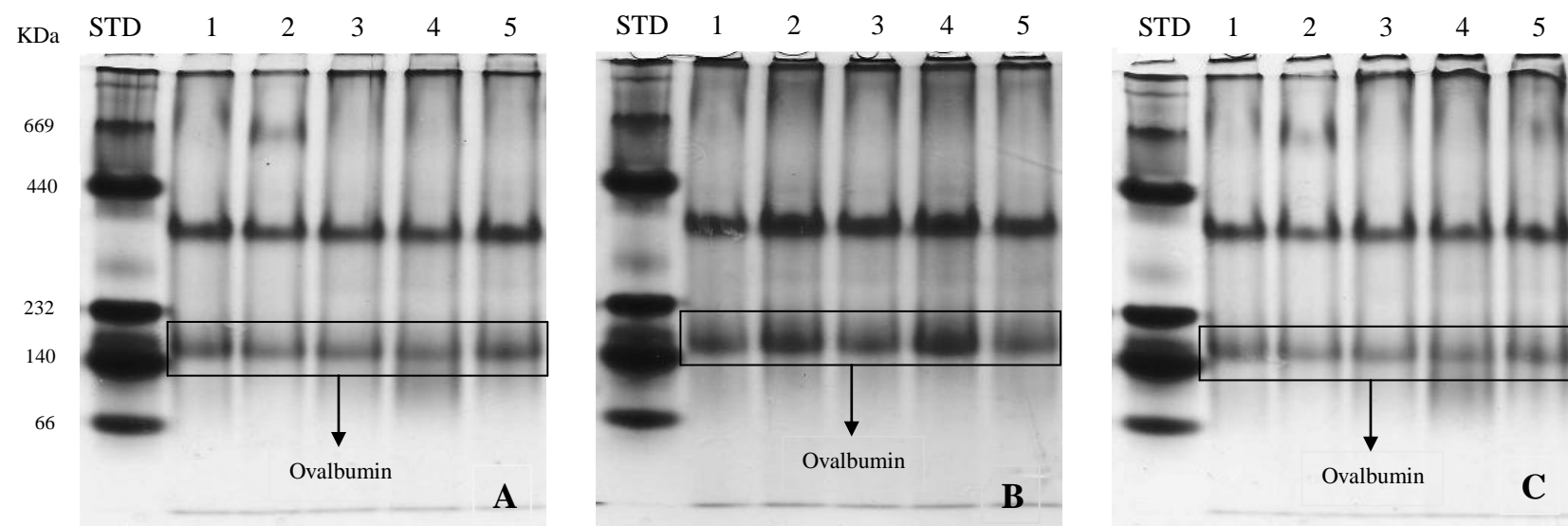
Figure 4.20 Native-PAGE of muffin protein (MP)-phenolic products. STD= standard proteins; lanes: 1= muffin control; 2= MP-flavone products; 3= MP-chrysin products; 4= MP-quercetin products; 5= MP-rutin products; concentration of phenolics is (A) 100 mg/Kg muffin; (B) 400 mg/Kg muffin; (C) 1000 mg/Kg muffin.

4.2.2 Digestibility of Muffin Protein-Phenolic Products *in vitro*

4.2.2.1 Enzymatic Hydrolysis of Muffin Protein-Phenolic Products by Bacterial Protease

The degree of hydrolysis (DH) of muffin proteins with 100 mg phenolic compounds by bacterial protease is given in Figure 4.21 and Table 4.7. The DH of muffin protein-phenolic products decreased compared with control muffin proteins. The muffin protein-chrysin reaction products showed the lowest DH. After 120 min of enzymatic hydrolysis, the DH of muffin protein-flavone products was 38.6%, rutin 36%, quercetin 34.4% and chrysin 33.4% compared to control muffin protein 46.6%.

After increasing the concentration of selected phenolic compounds to 10-fold, the DH of muffin protein-phenolic products was much lower compared with muffin control as shown in Figure 4.22 and Table 4.8. In addition, the enzymatic hydrolysis of muffin proteins treated with 1000 mg phenolics was inhibited much greater than that inhibited by 100 mg phenolics; the inhibitory activity of the selected phenolic compounds against bacterial protease was as follows: chrysin > rutin > quercetin > flavone.
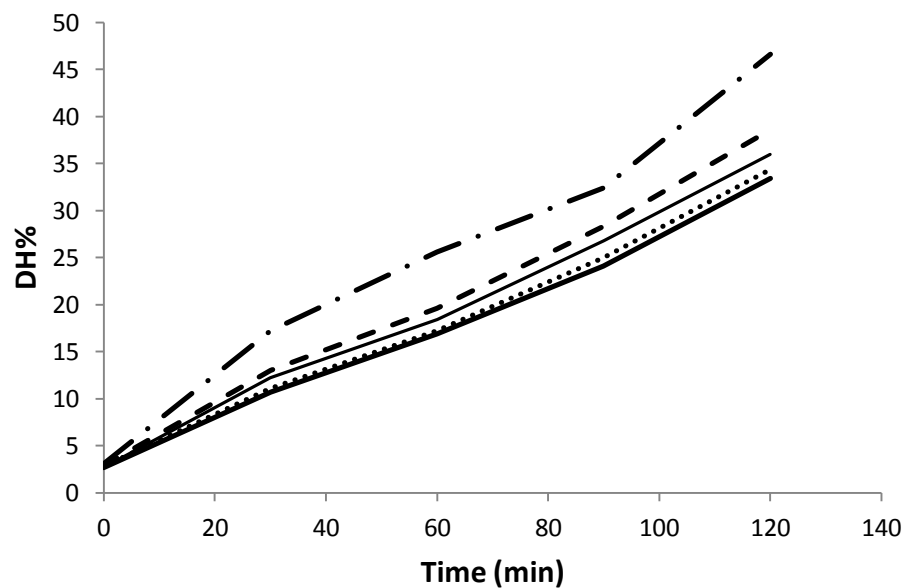
Figure 4.21 Enzymatic hydrolysis of proteins extracted from muffin containing; (–·–·–·) muffin control; (–·–·) 100 mg flavone; (——) 100 mg chrysin; (.......) 100 mg quercetin; and (——) 100 mg rutin by using bacterial protease.
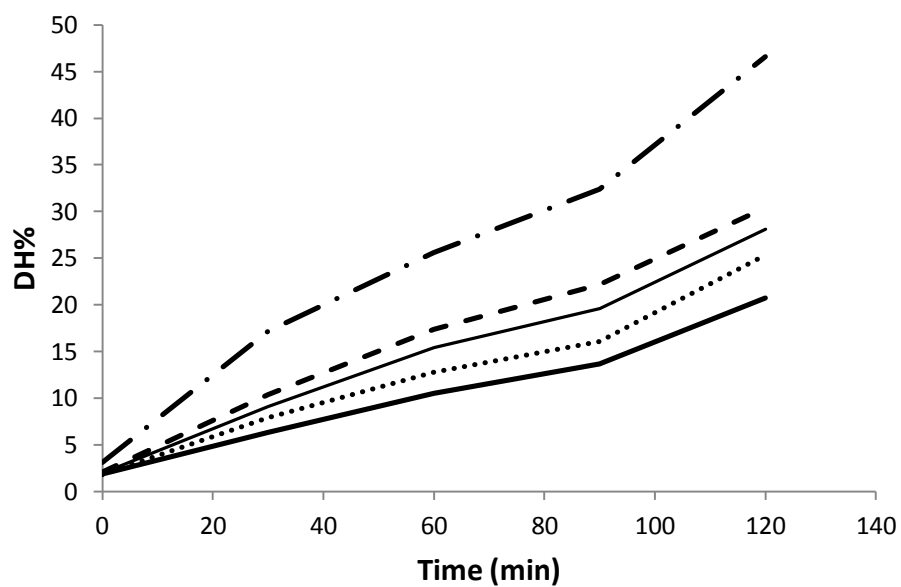
Figure 4.22 Enzymatic hydrolysis of proteins extracted from muffin containing; (–·–·–·) muffin control; (–·–·) 1000 mg flavone; (——) 1000 mg chrysin; (.......) 1000 mg quercetin; and (——) 1000 mg rutin by using bacterial protease.

Table 4.7 %DH of proteins extracted from muffin treated with 100 mg/Kg phenolics using bacterial protease

| Time | %DH | | | | |
(min)	MP (control)	MP-flavone	MP-chrysin	MP-quercetin	MP-rutin
0	3.1	2.9	2.7	2.8	2.7
30	17.2	13	10.7	11.1	12.2
60	25.6	19.6	16.9	17.2	18.4
90	32.4	28.3	24.1	25	26.8
120	46.6	38.6	33.4	34.4	36

MP = Muffin Protein

Table 4.8 %DH of proteins extracted from muffin treated with 1000 mg/Kg phenolics using bacterial protease

| Time (min) | %DH | | | | |
	MP-control	MP-flavone	MP-chrysin	MP-quercetin	MP-rutin
0	3.1	2.1	1.9	1.8	2
30	17.2	10.4	6.3	7.9	9.1
60	25.6	17.4	10.5	12.8	15.4
90	32.4	22.2	13.7	16.1	19.6
120	46.6	30.4	20.7	25.3	28.1

MP = Muffin Protein

4.2.2.2 Enzymatic Hydrolysis of Muffin Protein-Phenolic Products by Trypsin-Chemotrypsin Mixture

The degree of hydrolysis (DH) of muffin proteins with 100 mg phenolic compounds by trypsin-chymotrypsin mixture is shown in Figure 4.23 and Table 4.9. Muffin protein-phenolic reaction products showed a decrease in their DH compared to control muffin proteins. After 120 min, The DH of muffin protein-flavone reaction products showed the lowest DH with 22.8%, quercetin 24.6%, rutin 26% and chrysin 27.3%, respectively compared with control muffin which its DH was 33.8%.

Figure 4.24 and Table 4.10 show the degree of hydrolysis (DH) of muffin proteins with 1000 mg phenolic compounds by trypsin-chymotrypsin mixture. The DH of muffin protein-phenolic reaction products was much lower compared to that of 100 mg phenolics. After 120 min of enzymatic hydrolysis, the DH of muffin protein-flavone products was 13.3% compared with the other phenolics. It can be suggested that tryptic-chemotryptic hydrolysis was inhibited by the interaction of muffin proteins with phenolic compounds.
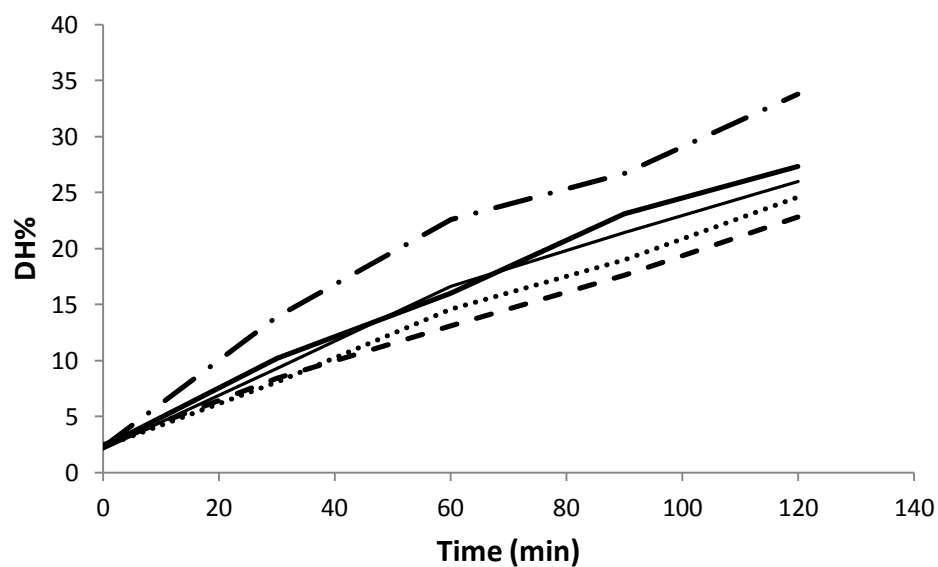
Figure 4.23 Enzymatic hydrolysis of proteins extracted from muffin containing; (‒‒·‒‒·) muffin control; (‒‒‒‒) 100 mg flavone; (——) 100 mg chrysin; (.......) 100 mg quercetin; and (——) 100 mg rutin by using trypsin-chymotrypsin mixture.
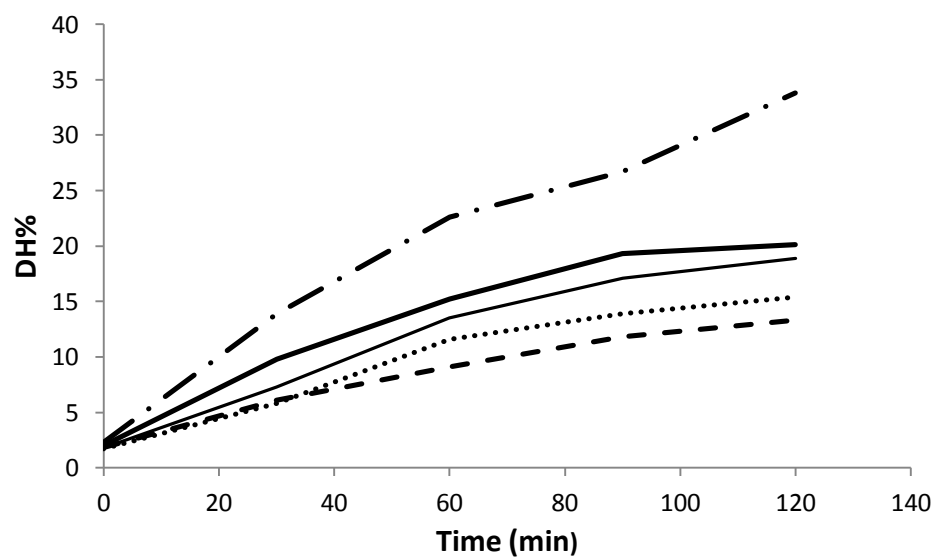
Figure 4.24 Enzymatic hydrolysis of proteins extracted from muffin containing; (‒‒·‒‒·) muffin control; (‒‒‒‒) 1000 mg flavone; (——) 1000 mg chrysin; (.......) 1000 mg quercetin; and (——) 1000 mg rutin by using trypsin-chymotrypsin mixture.

Table 4.9 %DH of proteins extracted from muffin treated with 100 mg/Kg phenolics using trypsin-chymotrypsin mixture

Time (min)	%DH				
	MP-control	**MP-flavone**	**MP-chrysin**	**MP-quercetin**	**MP-rutin**
0	2.3	2.5	2.3	2.7	2.1
30	13.9	8.4	10.2	8.1	9.3
60	22.6	13.1	16	14.6	16.6
90	26.7	17.6	23.1	19	21.4
120	33.8	22.8	27.3	24.5	26

MP = Muffin Proteins

Table 4.10 %DH of proteins extracted from muffin treated with 1000 mg/Kg phenolics using trypsin-chymotrypsin mixture

Time (min)	%DH				
	MP-control	**MP-flavone**	**MP-chrysin**	**MP-quercetin**	**MP-rutin**
0	2.3	1.8	2	1.7	1.8
30	13.9	6.1	9.8	5.8	7.3
60	22.6	9.1	15.2	11.6	13.5
90	26.7	11.8	19.3	13.9	17.1
120	33.8	13.3	20.1	15.4	18.9

MP = Muffin Proteins

GENERAL CONCLUSION

The interaction between egg proteins and phenolic compounds can lead to significant effects on egg protein properties. Egg protein-quercetin reaction products analyzed by SDS-PAGE showed effects on the electrophoretic behavior of egg proteins, while native-PAGE showed that phenolic compounds influenced electrophoretic pattern of conalbumin; these results suggest effects of quercetin on egg proteins structure. Phenolic compounds can affect the fluorescence spectra of egg proteins; in this study it was observed that increasing phenolics concentrations resulted in a decrease in fluorescence spectra of egg proteins with rutin showing the highest quenching followed by quercetin, chrysin and flavone; the fluorescence quenching results also suggest the effect of phenolics on egg proteins structure.

Data from Stern-Volmer constant values (Ksv) and the quenching rate constant (Kq) obtained indicated that the quenching type of egg protein fluorescence by the phenolic compounds investigated is the static quenching with the formation of stable non-fluorescent complexes. The binding data analysis also suggests that glycosylation (sugare moiety in C-ring of rutin) and hydroxylation (hydroxyl group number in quercetin and chrysin) of phenolic compounds lower the affinity for egg proteins, and suggests that hydrophobic interaction is involved.

Phenolic compounds can bind egg proteins and affect in-vitro enzymatic hydrolysis. The DH of denatured and non-denatured egg protein-phenolic products decreased compared to the control. In vitro protein digestion with bacterial protease, trypsin and chymotrypsin enzymes was adversely affected by egg protein-phenolic interactions, likely due to structural changes in egg proteins.

Muffin protein-phenolic reaction products analyzed by SDS-PAGE showed changes in their electrophoretic behaviour as a result of the phenolics; ovalbumin-phenolic products showed changes in their electrophoretic behaviour with chrysin and quercetin, while flavone and rutin affected slightly the electrophoretic pattern of ovalbumin. A decrease in DH of denatured and non-denatured muffin protein-phenolic products was observed compared to the control, suggesting that enzymatic hydrolysis of muffin proteins is influenced by phenolic compounds.

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